International
Vascular Biology Meeting
June 2–5, 2012
Wiesbaden, Germany

Wiesbaden, June 2-5, 2012

17th International Vascular Biology Meeting 2012

Abstracts

hosted by the „Verein für wissenschaftliche Fachtagungen in der Biomedizin e.V." (VWFB)

SPEAKERS' ABSTRACTS

UNDERLINED NAME = SPEAKER

Blood-brain barrier induction, maintenance and modulation

N Joan Abbott. Institute of Pharmaceutical Science, King's College London, London SE1 9NH, United Kingdom

The neurons of the brain and spinal cord (central nervous system, CNS) require precise control of their bathing microenvironment for optimal function, and an important element in this control is the blood-brain barrier (BBB). The BBB is formed by the endothelial cells of the brain microvasculature, with tight junctions responsible for the 'physical barrier', membrane carriers (solute transporters) and vesicular mechanisms contributing the 'transport barrier', and surface and intracellular enzymes adding an 'enzymatic' or 'metabolic' barrier function (1). The endothelial cells are supported by a number of closely-associated cells and extracellular structures: pericytes (and smooth muscle cells in larger vessels), the end feet of astrocytic glial cells, microglia, and the extracellular matrix/basal laminae. A small group of these cells together with the 'client' neurons they supply form a 'Neurovascular Unit' (NVU) (1). Coordinated interactions within NVU modules form the basis for local control of blood flow and metabolic support of the neurons; self-repair of minor damage is probably also organised at this local level. Recent studies from several groups have emphasised the role of pericytes and astrocytes in inducing barrier properties in the endothelial cells during brain development and in maintenance in the adult (2,3).

Many CNS and systemic pathologies involve disturbed BBB function, and for several of these changes in the NVU are documented (1,4). In vitro models can shed light on some of the underlying processes, in BBB induction, maintenance and modulation, both physiological and pathological. I will present recent work from our group using three in vitro models with advantageous properties: the immortalised rat brain endothelial cell line RBE4, and two primary cultured cell models, from rat (5) and porcine brain endothelial cells (6). We have examined cell:cell interactions between endothelium and astrocytes (7), astrocytic induction of BBB properties in endothelium, changes in induction in the presence of glioma-derived astrocytes, the modulation of barrier function by inflammatory mediators, and the role of reactive oxygen species (ROS) in this modulation.

References:

1) Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010; 37:13-25.

2) Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature 2010; 468:562-6.

3) Winkler EA, Bell RD, Zlokovic BV. Central nervous system pericytes in health and disease. Nat Neurosci. 2011; 14:1398-405.

4) Stanimirovic DB, Friedman A. Pathophysiology of the neurovascular unit: disease cause or consequence? J Cereb Blood Flow Metab. 2012; Mar 7. doi: 10.1038/jcbfm.2012.25.

5) Abbott NJ, Dolman DE, Drndarski S, Fredriksson SM. An improved in vitro blood-brain barrier model: rat brain endothelial cells co-cultured with astrocytes. Methods Mol Biol. 2012; 814:415-30.

6) Patabendige AK, Skinner R, Abbott NJ (submitted)

7) Abbott NJ, Rönnbäck L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006 7:41-53.

Molecular regulation endothelial sprouting and angiogenic blood vessel growth

Ralf H. Adams and colleagues. Max Planck Institute for Molecular Biomedicine, Department Tissue Morphogenesis, and University of Muenster, Faculty of Medicine, Muenster, Germany

Angiogenesis is the main process mediating the expansion of the blood vessel network during development, tissue regeneration or in pathological conditions such as cancer. The formation of new endothelial sprouts, a key step in the angiogenic growth program, involves the selection of endothelial tip cells, which lack a lumen, are highly motile, extend numerous filopodia, and lead new sprouts. Angiogenic sprouting is induced by tissue–derived, pro–angiogenic signals such as vascular endothelial growth factor (VEGF), which activates and triggers signaling by cognate receptor tyrosine kinases in the endothelium. However, this response is strongly modulated by intrinsic signaling interactions between endothelial cells (ECs). For example, expression of the ligand Delta-like 4 (Dll4) in tip cells activates Notch receptors in adjacent (stalk) ECs and is thought to downregulate VEGF receptor expression in these cells. Thus, the tip cell phenotype is suppressed in stalk cells and a balance between sprouting and the necessary preservation of existing endothelial tubes is established.
Our work is providing further insight into the regulation of sprouting angiogenesis. The Notch ligand Jagged1 is a potent pro-angiogenic regulator with the opposite role as Dll4. In contrast to current models, we found that Notch controls VEGFR2 only moderately whereas VEGFR3 is strongly regulated. Moreover, blocking of Notch enables angiogenic growth even in mutant animals lacking endothelial VEGFR2 expression.
We also found that endothelial sprouting and proliferation extension depend on VEGF receptor endocytosis. Ephrin-B2, a ligand for Eph family receptor tyrosine kinases, is required for endothelial cell motility, VEGF receptor endocytosis and the activation of downstream signal transduction cascades. More recently, we have identified Disabled 2, a clathrin-associated sorting protein, and the cell polarity protein PAR-3 as novel interaction partners of ephrin-B2 and VEGF receptors. These results establish that regional VEGF receptor endocytosis, which is controlled by a complex containing Dab2, PAR-3 and ephrin-B2, play a key role in the spatial organization of angiogenic growth.

Acknowledgement: This study has been supported by the Max Planck Society, the University of Muenster and the German Research Foundation.

ADAM10/Notch signaling controls the development of specialized vascular niches

Carl P. Blobel. Hospital for Special Surgery at Weill Medical College of Cornell University New York, NY, 10021, USA

Branching morphogenesis of the developing retinal vascular tree is regulated by components of the Notch signaling pathway, including Dll4 and presenilin/γ-secretase. Notch receptors are membrane-anchored transcription factors that are activated through sequential proteolytic processing steps, first by an ADAM (a disintegrin and metalloproteinase), usually ADAM10, and then by the intramembrane proteinase presenilin/γ-secretase. We have shown that targeted deletion of ADAM10 in endothelial cells (*Adam10*Δ*EC*) results in increased vascular density in the developing retinal vascular tree, presumably because Notch signaling is blocked, and in increased pathological neovascularization in the oxygen-induced retinopathy model. Moreover, we identified several defects in organ-specific vascular niches in *Adam10*Δ*EC* mice, including enlarged subcapsular hepatic veins, enlarged glomeruli, intestinal polyps containing endothelial cell masses, and abnormal endochondral ossification, leading to stunted long bone growth. These findings raised questions about the role of Notch1 in the development of these specialized vascular niches. However, little is currently known about the contribution of Notch1 to postnatal angiogenesis and retinal vascular development since mice lacking Notch1 in endothelial cells (*Notch1*Δ*EC*) die early in embryogenesis, so we generated and analyzed *Notch1*Δ*EC* mice that frequently live up to a few weeks after birth. These animals display several of the vascular defects that were first observed in *Adam10*Δ*EC* mice, including enlarged veins under the liver capsule and epicardium, enlarged glomeruli and a slight retardation of long bone growth. However, unlike *Adam10*Δ*EC* mice, *Notch1*Δ*EC* mice had no significant increase in retinal vascular branching or vessel density at postnatal day 5, considered a hallmark of Notch signaling, and no intestinal polyps caused by endothelial cell masses. This constellation of vascular defects defines novel requirements for Notch1 in endothelial cells in the development of organ-specific vascular niches, and raises questions about the role of other components of the Notch signaling pathways in the development of organ specific vascular structures that were affected in *Adam10*Δ*EC* mice, but not in *Notch1*Δ*EC* mice.

Microparticles, vascular function and atherothrombosis

Chantal M. Boulanger, Pierre-Emmanuel Rautou, Sophie Pégorier, Anne-Clémence Vion, Yin Min, Nicolas Amabile, Gilles Chironi, Alain Simon, Alain Tedgui. INSERM, U970, Paris Cardiovascular Research Center PARCC, Paris, France and Université Paris Descartes, Sorbonne Paris Cité, Paris, France

Membrane-shed submicron microparticles (MPs) are released following cell activation or apoptosis. High levels of MPs circulate in the blood of patients with atherothrombotic diseases, where they could serve as a useful biomarker of vascular injury and a potential predictor of cardiovascular mortality and major adverse cardiovascular events. Atherosclerotic lesions also accumulate large numbers of MPs of leukocyte, smooth muscle cell, endothelial and erythrocyte origin. A large body of evidence supports the role of MPs at different steps of atherosclerosis development, progression and complications. First, circulating MPs impair the atheroprotective function of the vascular endothelium, at least partly by decreasing nitric oxide synthesis. In addition, plaque MPs stimulate angiogenesis, a key event in the transition from stable to unstable lesions. Plaque MPs also favor local inflammation by augmenting the expression of adhesion molecule such as ICAM-1 at the surface of endothelial cell, monocyte recruitment within the lesion and their transmigration. MPs isolated from human plaque also stimulate macrophage proliferation in vitro and the formation of foam-cell. Finally, MPs may promote local cell apoptosis, leading to the release and accumulation of new MPs. Thus, accumulation of MPs in atherosclerotic plaques might create a vicious circle towards inflammation, plaque growth and instability. Finally, highly thrombogenic plaque MPs could increase thrombus formation at the time of rupture, together with circulating MPs released in this context by activated platelets and leukocytes. Later, MPs could participate in repairing the consequences of arterial occlusion and tissue ischemia by stimulating endothelial progenitor differenciation and promoting post-ischemic neovascularization.

Cellular oxygen sensors control tumor growth, angiogenesis, and vessel maturation

Anne Klotzsche-von Ameln[1], Antje Kettelhake[1], Ina Prade[1], Alexander Kuzmanov[1], Maryam Rezaei[1], Joanna Kalucka[1], Antje Muschter[1], David Poitz[2], Konstantinos Anastassiadis[3], Francis Stewart[3,4], Ben Wielockx[1], and Georg Breier[1,3]. [1]Dept. of Pathology, [2]Dept. of Cardiology, [3]DFG Research Center and Cluster of Excellence for Regenerative Therapies Dresden (CRTD), and [4]Biotec, TU Dresden, Germany; [5]Max Planck Institute of Molecular Biomedicine, Münster, Germany

All higher organisms possess mechanisms to maintain oxygen homeostasis. Low oxygen levels stimulate a variety of adaptive reactions, including angiogenesis. The cellular response to hypoxia is mediated by transcriptional regulators called hypoxia-inducible factors (HIFs) which, in turn, are regulated by prolyl and asparaginyl hydroxylases. These enzymes require oxygen for their activity and thus act as cellular oxygen sensors. - We are studying the function of the HIF hydroxylases in physiological and pathological angiogenesis by gain-of-function and loss-of-function approaches, with specific focus on their role in tumor angiogenesis. Silencing of PHD2 in mouse LM-8 osteosarcoma or Lewis Lung Carcinoma (LLC) cells stimulated tumor angiogenesis. Unexpectedly, however, these tumors grew hardly or even completely disappeared over time. This was accompanied by reduced tumor cell proliferation, resulting from enhanced TGF-beta signaling. Because TGF-beta stimulates the growth of control LM-8 tumors *in vivo*, we conclude that its growth promoting activity can be reverted by PHD2 silencing. The growth of transplanted LLC tumors was not affected by Flk1Cre-mediated endothelial cell-specific ablation of the PHD2 gene in mice, yet vessel number and morphology were altered. - Silencing of PHD3 in LM-8 osteosarcoma cells significantly enhanced tumor growth *in vivo* in comparison to control tumors. This was accompanied by dramatic alterations of the tumor vasculature: while vessel density was decreased, the vessels were enlarged and showed coverage by a layer of alpha-smooth muscle actin positive cells. This phenotype was accompanied by elevated levels of PDGF-C. Silencing of PDGF-C in the PHD3-knockdown tumors led to reversion of the vessel phenotype and slowed down tumor growth. Thus it appears that PHD3 regulates the recruitment of perivascular cells and influences vessel architecture via PDGF-C signaling. – PDGF-C was also upregulated in tumors that overexpress the asparaginyl hydroxylase, factor inhibiting HIF (FIH). This led to increased recruitment of mural cells, enhanced vessel maturation, and rapid tumor growth. - Taken together, our data show that prolyl and aspariginyl hydroxylases that function as cellular oxygen sensors control tumor growth and influence both vessel growth and maturation.

Neutrophil extracellular traps: From host defence to autoimmunity

Brinkmann, V. Max Planck Institute for Infection Biology, Berlin, Germany

Neutrophil granulocytes are the most abundant group of leukocytes in the peripheral blood. As professional phagocytes, they engulf bacteria and kill them intracellularly when their antimicrobial granules fuse with the phagosome. We found that neutrophils have an additional way of killing microorganisms: upon activation, they release granule proteins and chromatin that together form extracellular fibers that bind Gram-positive and -negative bacteria as well as fungi. These novel structures, or Neutrophil Extracellular Traps (NETs), degrade virulence factors and kill bacteria, fungi and parasites [1, 2].
Using correlative microscopy combining TEM, SEM, immunofluorescence and live cell imaging techniques, we could show that upon stimulation, the nuclei of neutrophils lose their shape and the eu- and heterochromatin homogenize. Later, the nuclear envelope and the granule membranes disintegrate allowing the mixing of NET components. Finally, the NETs are released as the cell membrane breaks. This cell death program is distinct from apoptosis and necrosis and depends on the generation of Reactive Oxygen Species by NADPH oxidase [3].
Neutrophil extracellular traps are abundant at sites of acute inflammation. NETs appear to be a form of innate immune response that bind microorganisms, prevent them from spreading, and ensure a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria thus allowing neutrophils to fulfill their antimicrobial function even beyond their life span. There is increasing evidence, however, that NETs are also involved in diseases that range from infertility to auto-immune syndromes [4, 5].

Literature:

[1] V. Brinkmann et al, *Science* **2004**, *303*, 1532-1535. [2] V. Brinkmann, A. Zychlinsky, *Nat. Rev Microbiol.* **2007**, *5*, 577-584. [3] T.A. Fuchs et al, *J. Cell Biol.* **2007**, *176*, 231-241 [4] K. Kessenbrock et al., *Nat. Med.* **2009**, *15*, 623-625. [5] A. Hakkim et al., *Proc. Natl. Acad. Sci. USA.* **2010**, *107*, 9813-9818.

Metabolic and homeostatic functions of the endothelium

Peter Carmeliet. Leuven, Belgium

Angiogenesis, the growth of new blood vessels, plays a crucial role in numerous diseases, including cancer. Anti-angiogenesis therapies have been developed to deprive the tumor of nutrients. Clinically approved anti-angiogenic drugs offered prolonged survival to numerous cancer patients. However, the success of anti-angiogenic VEGF-targeted therapy is limited in certain cases by intrinsic refractoriness and acquired resistance. New strategies are needed to block tumor angiogenesis via alternative mechanisms. We are therefore exploring whether targeting endothelial metabolism can be a possible alternative therapeutic strategy for anti-angiogenic therapy.

DEL-1, an endogenous anti-inflammatory agent

Triantafyllos Chavakis[1], Kyoung-Jin Chung[1], Jindrich Chmelar[1], Jong-Hyung Lim[1], Eun Young Choi[1], George Hajishengallis[2]. [1]Division of Vascular Inflammation, Diabetes and Kidney, Department of Internal Medicine, University Dresden, Germany; [2]Dept. of Microbiology, School of Dental Medicine, University of Pennsylvania, USA

The leukocyte adhesion cascade is a major paradigm in innate and adaptive immunity. In order to extravasate, leukocytes have to undergo a well-coordinated series of adhesive interactions with the endothelium, including the selectin-mediated rolling, the chemokine-induced activation and the integrin-dependent firm arrest and transendothelial migration. Whereas multiple adhesion and chemokine receptors have been described to promote the leukocyte adhesion cascade, little is known about endogenous inhibitors of the cascade. Recently, a new field of endogenous inhibitors of the leukocyte adhesion cascade has emerged, as pentraxin-3 was shown to block P-selectin-dependent rolling (Deban et al., Nat Immunol, 2010), GDF-15 was found to inhibit chemokine-induced leukocyte integrin activation (Kempf et al., Nat Med 2011) and we have identified endothelial-derived Developmental endothelial locus-1 (Del-1) as an inhibitor of LFA-1 integrin-mediated neutrophil adhesion and recruitment (Choi et al., Science 2008) and more recently of IL-17-dependent inflammation (Eskan et al., Nat Immunol 2012). Aging-associated loss of Del-1 expression in the tooth-supporting tissue, gingiva, resulted in local upregulation of IL-17-dependent neutrophil recruitment, mediated by the integrin LFA-1 and thereby in periodontitis with inflammatory bone loss. The phenotype of accelerated inflammatory bone loss in Del-1-deficient mice was reversed in mice with concomitant deficiencies of either LFA-1 or IL-17-receptor. In turn, IL-17-mediated downregulation of endothelial Del-1 expression promotes LFA-1-dependent neutrophil accumulation in inflammation. Together, Del-1 as an endogenous inhibitor of inflammatory cell recruitment and IL-17-dependent inflammation; loss of Del-1 expression is associated with development of inflammatory disease.

Protease-activated receptors

Coughlin, SR. Cardiovascular Research Institute, University of California, San Francisco, USA

Protease-activated receptors (PARs) are G protein-coupled receptors that provide a mechanism by which thrombin and related proteases can regulate the behavior of platelets and other cells. Together, the coagulation cascade and PARs link mechanical information in the form of disruption of blood vessel integrity to appropriate cellular responses involved in hemostasis, inflammation, repair, and even pain perception. This talk will focus on 1) lessons from ex vivo studies of human platelets and mouse and zebrafish models that speak to the roles of PARs in platelet activation and in hemostasis and thrombosis, 2) how these lessons might relate to the possible utility of PAR1 as a target for novel antithrombotics and 3) roles for PARs in endothelium and epithelia in embryonic development.

Perivascular mesenchymal stem cells in human normal organs and disease

Mihaela Crisan. Erasmus MC – Medical Faculty, Dept. of Cell Biology, Erasmus Stem Cell Institute (ESI), Rotterdam, The Netherlands

Mesenchymal stem/stromal cells (MSC) are currently the best candidate therapeutic cells for regenerative medicine related to osteoarticular, muscular, vascular and inflammatory diseases, although these cells remain heterogeneous and necessitate a better biologic characterization. We and others recently described that MSC originate from two types of perivascular cells namely pericytes and adventitial cells. The perivascular cell compartment contains the *in situ* counterparts of MSC in developing and adult human organs including skeletal muscle, pancreas, adipose tissue, and placenta, which can be prospectively purified using well defined cell surface markers. Pericytes closely encircle endothelial cells of capillaries and microvessels and express the adhesion molecule CD146 and PDGFRβ, but lack endothelial and hematopoietic cell markers such as CD34, CD31, vWF (von Willebrand factor), the ligand for Ulex europaeus 1 (UEA1), VE cadherin and CD45 respectively. The proteoglycan NG2 is a pericyte marker exclusively associated with the arterial system. Besides its expression in smooth muscle cells, smooth muscle actin (αSMA) is also detected in subsets of pericytes. Adventitial cells surround the largest vessels and, opposite to pericytes, are not closely associated to endothelial cells. Adventitial cells express CD34 and lack αSMA and all endothelial and hematopoietic cell markers, as for pericytes. Altogether, pericytes and adventitial perivascular cells express *in situ* and in culture markers of MSC such CD44, CD90, CD73 and CD105 and display capacities to differentiate toward osteogenic, adipogenic and chondrogenic cell lineages. Moreover, perivascular cells purified from skeletal muscle or non muscle tissues were myogenic in culture and *in vivo* when transplanted into injured mouse muscle. Importantly, adventitial cells can differentiate into pericyte-like cells under inductive conditions *in vitro*. Thus, blood vessel walls harbor a reserve of progenitor cells that may be integral to the origin of the elusive MSCs and other related adult stem cells. Importantly, perivascular like cells isolated from human cancer prostate are mutated and support tumor formation *in vivo.*

Cytokine interplays controlling endothelial cell-pericyte tube coassembly and stabilization events

George E. Davis and Amber N. Stratman. Department of Medical Pharmacology and Physiology, University of Missouri School of Medicine, Columbia, MO 65212, USA

We have established conditions whereby human endothelial cells (ECs) and pericytes co-assemble into tubes under serum-free defined conditions in 3D extracellular matrices. This system depends on the presence of the hematopoietic cytokines, stem cell factor (SCF), interleukin-3 (IL-3) and stromal-derived factor (SDF)-1 alpha which allows for this co-assembly process. By contrast, VEGF and FGF-2 primarily function as priming agents in that they prepare EC responses to the hematopoietic factors by inducing upregulation of EC receptors for these hematopoietic factors. These cytokines function to regulate EC tubulogenesis in the presence or absence of added pericytes. EC-lined tubes, which are induced by hematopoietic cytokines, recruit pericytes along their abluminal surface in a manner dependent on EC-derived PDGF-BB and HB-EGF. During this process, EC-pericyte interactions lead to vascular basement membrane matrix assembly, a critical step in vessel maturation. Of great interest is that pericyte motility and proliferative responses in 3D matrices depends on the co-presence of ECs and the EC-derived factors, PDGF-BB as well as HB-EGF, together play key roles in these responses. Additional work demonstrates an important role for EC-derived TGF-beta1 and activin B which act in an autocrine and paracrine manner on ECs and pericytes to regulate tube assembly and maturation events including vascular basement membrane matrix deposition in 3D matrices.

Pathological development of brain microvasculature

Dejana E. IFOM-IEO-Campus and Milan University, Milan, Italy

Brain microvasculature constitute a highly specialized and selective vascular barrier between blood and the central nervous system, called blood brain barrier (BBB). In these vessels endothelial cells present a highly developed system of tight junctions (TJs), absence of fenestration and low pinocytotic activity. Cells of the brain parenchyma, the astrocytes, contribute to the BBB-coverage with their foot processes, which constitute about the eighty percent of the basal aspect of the vessels. As a consequence, circulating solutes do not readily enter the brain parenchyma unless through specific endothelial “transporters”. Thus, BBB also limits the passage of anti-cancer drugs from the blood to the brain. Therefore, it would be therapeutically useful to develop systems to modulate BBB permeability. To this aim it is important to define the molecular mechanisms that regulate the establishment and maintenance of BBB properties.

Data from our laboratory suggest a key role of the Wnt/β-catenin signaling pathways in the induction, regulation and maintenance of the BBB characteristics during embryonic and post-natal development. In endothelial cells, Wnt signaling induces barrier differentiation by increasing the stabilization and the transcriptional activity of β-catenin. On the contrary, inactivation of β-catenin causes significant downregulation of junctional proteins, and consequent BBB breakdown.

Besides β-catenin, other three proteins, CCM1, CCM2 and CCM3, expressed by brain endothelial cells, are emerging as key modulators of the organization and function of the BBB. Indeed, mutations occurring in any of the genes encoding these proteins, leads to Cerebral Cavernous Malformation (CCM), a pathology characterized by brain vascular malformations. The endothelium in the lesions presents very few tight junctions and gaps are observed between endothelial cells. In addition, the vascular basal lamina is disorganized and the astrocytes do not take contact with the endothelial wall. The structural alterations of the BBB observed in CCM lesions are associated with severe clinical manifestations, such as cerebral haemorrhages and stroke. Additional data point to a possible link between the β-catenin and TGF βpathways and the functions of CCM proteins in the regulation of BBB stability. These data open new therapeutic opportunities for this so far incurable disease.

Control of lymphatic maturation and function in health and disease

Michael Detmar. Institute of Pharmaceutical Sciences, ETH Zurich, Switzerland

We previously identified tumor lymphangiogenesis as a novel paradigm in cancer progression. More recently, we found that sentinel lymph node lymphangiogenesis is an important mediator and a very early indicator of cancer metastasis to distal lymph nodes and beyond. Based on these findings, we have developed in vivo imaging methods to image tumor-induced lymph node lymphangiogenesis as the earliest sign of metastasis, and methods for the non-invasive imaging and quantification of lymphatic vessel function in development, inflammation and tumor progression, using near-infrared dyes. In a quest to identify inhibitors of tumor lymphangiogenesis, we have applied chemical genetics screens, using a three-dimensional sprouting assay and several chemical and natural extract libraries, as well as ligand-based virtual screens. These studies identified novel classes of lymphangiogenic inhibitors. Additional transcriptional profiling studies of ex vivo isolated lymphatic and blood vascular endothelium revealed new pathways involved in lymphatic vessel development and maturation. In particular, interactions between semaphorin 3A and neuropilin-1 play an essential role in the patterning of the lymphatic vascular system and in the maturation of valves in collecting lymphatic vessels. Lymphatic endothelium also plays an important role in chronic inflammatory diseases, and recent work from our laboratory indicates that activation of lymphatic endothelium via vascular endothelial growth factor-C (VEGF-C) reduces acute, immune- or UVB-induced inflammation, and also inhibits the development of chronic, psoriasis-like skin inflammation in experimental mouse models.

Ubiquitin and autophagy networks

Ivan Dikic. Buchmann Institute for Molecular Life Sciences and Institute of Biochemistry, Goethe University, Theodor-Stern-Kai 7, 60590 Frankfurt/Main, Germany

Removal of harmful protein aggregates, damaged organelles and microbes is mediated by autophagy, a process by which the cell sequesters cytosolic cargo and delivers it for degradation by the lysosome. Characterization of the molecular mechanisms, which govern the specificity, has been made possible by the identification of ATG proteins that regulate nucleation and maturation of the autophagosome in yeast and mammals. The central question of selective autophagy is how a particular substrate can engage the autophagosomal machinery to mediate phagophore assembly at a specific cellular location?
We have recently identified Optineurin as a new autophagy receptor, which together with p62 and NDP52, mediates selective autophagy of ubiquitin-coated cytosolic Salmonella enterica. The protein kinase TBK1 (TANK binding kinase 1) was shown to bind to and phosphorylates Optineurin on serine 177 enhancing LC3 binding affinity and autophagic clearance of cytosolic Salmonella. TBK1 also phosphorylates the UBAN domain (Ub binding domain present in NEMO and ABIN) of Optineurin resulting in an inducible increase in the binding affinity toward distinct ubiquitin chains. Conversely, ubiquitin- or LC3-binding Optineurin mutants and silencing of Optineurin or TBK1 impaired Salmonella autophagy resulting in increased intracellular bacterial proliferation. TBK1-mediated phosphorylation of both LC3- and Ub-binding domains ensure a rapid engulfment of cytosolic bacteria and efficient autophagic clearance thus representing a general mechanism for regulation of cargo-selective autophagy.
Ubiquitin chains modify major parts of the proteome but information regarding localization and dynamics of these signals remains sparse due to the absence of appropriate tools to study them. We have employed fluorescent sensors based on selective ubiquitin-binding domains to monitor linear and K63-linked ubiquitin chains in vitro and in cells. GFP-tagged UBAN sensors selectively recognize linear chains, whereas the UIM (Ubiquitin-interacting motif) of Rap80 and the NZF (Npl4 zinc finger) domain of TAB2 act as selective K63-ubiquitin sensors. Linear sensors labeled the ubiquitin coat surrounding cytosolic Salmonella during bacterial autophagy, whereas K63-sensors were used for selective monitoring of DNA double strand breaks and Parkin-induced mitophagy. Both linear and K63 sensors differentially regulated TNF-α-induced NF-κB pathways. Biosensors based on specific UB- or UBL-binding domains can thus be used to monitor localization and function of ubiquitin chains and ubiquitin-like signals in vivo.

Molecular circuits in thrombosis and inflammation

Charles T. Esmon. Coagulation Biology Laboratory, Oklahoma Medical Research Foundation, Howard Hughes Medical Institute, Oklahoma City, OK 73104 USA

Inflammatory cytokines promote the activation of coagulation through the induction of tissue factor, down regulation of thrombomodulin and upregulation of plasminogen activator inhibitor. In addition to these mechanisms, infections can trigger the release of extracellular traps from leukocytes consisting of DNA and histones. Tissue injury results in release of nucleosomes. Either of these histone containing structures activate platelets and form a potent procoagulant surface on polyphosphates secreted from the platelets, thereby augmenting thrombus formation. In addition, the histones can inhibit thrombomodulin function. The combination of augmenting the platelet procoagulant activity and impairing thrombomodulin activity probably explains the microvascular thrombotic problems observed when histones are infused into mice. Of the histones, H4 is the most potent in all of these activities. DNAase or blocking histone H4 can decrease the thrombotic response initiated by either the extracellular traps or nucleosomes. In addition to the direct prothrombotic activity of histone-DNA complexes, the complexes trigger activation of the toll like receptors 2, 4 and 9 thereby increasing inflammatory cytokine formation and fostering thrombotic responses through the mechanisms mentioned previously. Furthermore, these cytokines are likely to increase cell necrosis and apoptosis releasing nucleosomes and further augmenting the activation of leukocytes with the subsequent release of extracellular traps. Blocking this histone-mediated cascade has the potential to impact a variety of clinical conditions including sepsis, trauma, chemical toxicity, transplant injury and reperfusion injury.

Role of the microenvironment in VEGF-dependent and -independent angiogenesis

Napoleone Ferrara. Genentech, Inc, 1 DNA Way, South San Francisco, CA, 94080, USA

Vascular endothelial growth factor (VEGF)-A is a key regulator of growth of blood vessels during embryonic development and in a variety of physiological processes, such as skeletal growth and reproductive functions. Multiple VEGF inhibitors have been shown to block tumor growth and neovascularization in numerous preclinical models, consistent with an important role of VEGF-A in tumor angiogenesis. A humanized anti-VEGF-A monoclonal antibody and several small molecule VEGF receptor kinase inhibitors have been approved for therapy of multiple tumor types. Furthermore, blocking VEGF-A prevented vision loss and had a major impact on the progression of neovascular age-related macular degeneration and ischemic retinal disorders. We have been recently investigating the mechanisms of refractoriness/resistance to anti-VEGF therapies in tumor models. These studies indicate that, depending on the model, different pro-angiogenic mechanisms may be implicated. Factors produced by tumor-infiltrating myeloid cells or by fibroblasts were identified as key mediators of VEGF-independent angiogenesis. Efforts are ongoing to determine the therapeutical significance of such findings.

CCM1 and Notch signaling in the endothelium and the formation of vascular malformations

Andreas Fischer. German Cancer Research Center (DKFZ) Heidelberg, Vascular Signaling and Cancer, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Vascular malformation is a collective term for different disorders of the vasculature. Our laboratory is interested in the molecular mechanisms underlying the slow-flow venous cerebral cavernous malformations (CCM) and the fast-flow arteriovenous malformations (AVM). The hereditary form of CCM is caused by mutations in CCM1, CCM2 or CCM3. We have shown that CCM1 (KRIT1) inhibits angiogenesis, endothelial migration and proliferation. These functions are mediated by activation of PI3K/AKT and Notch signaling and repression of Rho kinase and MAPK/ERK activity. Thus, CCM1 is needed to keep the vasculature quiescent. The CCM1 interacting protein ICAP1 (ITGB1BP) promotes CCM1 activity by stabilizing the CCM1 protein and may play a role in the formation of venous vascular malformations.
It remains unclear if Notch signaling is needed for the prevention of vascular malformations. Our recent data indicate that endothelial Notch signaling is essential for vascular functions in the adult. Loss of the Notch transducer protein RBPSUH (Rbpjk) in the endothelium of adult mice leads to severe cardiovascular abnormalities. In turn, the
endothelial-specific overexpression of active NOTCH1 in mice causes the formation of arterio-venous shunts and arterialization of the venous bed. We are analyzing how Notch signaling affects endothelial functions, maintenance of barrier functions and the interaction with mural cells.
In an approach to identify genes that modify Notch signaling we isolated several proteins that interact with Notch ligands. While SYNJ2BP (ARIP2) stabilizes DLL1 and DLL4 proteins, the serine protease HTRA1 leads to degradation of JAG1. However, both binding proteins enforce Notch signaling in endothelial cells implicating that JAG1 has opposing functions compared to DLL4. This is insofar interesting since JAG1 mutations cause the degenerative small artery disease CADASIL while HTRA1 mutation is linked to the recessive form of this disease (CARASIL).

Macrophage phenotypic diversity and the resolution of inflammation in atherosclerotic plaques

Edward A. Fisher. New York University School of Medicine, New York, NY, USA

Macrophages are the central cell of atheroma formation, and the persistence of activated ones is a hallmark of the failure of resolution of inflammation thought to maintain the pathological state. It is well appreciated that the phenotypic state of macrophages is exquisitely sensitive to their microenvironment. In the progression of atherosclerosis in mouse models, most data indicate that as the plaque advances, the growth is by the recruitment of the Ly6Chi subset of circulating monocytes, which subsequently become M1-type macrophages in the plaque. Using 4 different mouse models, we have recently observed that the macrophage population changes during plaque regression in 2 ways- the number of macrophages decrease and for those remaining, their phenotype shifts from a predominantly M1 to a M2 profile, with increased expression of arginase I, mannose receptor, and IL10, and decreased expression of NF-kB targets. In the 4 models, the plaque environment is dramatically altered by the changes in plasma lipoprotein levels, but in different ways: 1) Transplantation of aortae from apoE-/- mice into wild-type recipients results in lower non-HDL cholesterol (C) and higher HDL-C levels; 2) Conditionally inactivating hepatic MTP in LDL-/- mice lowers non-HDL-C levels; 3) Treatment of LDL-/- mice with an antagamir to miR-33 selectively raises HDL-C; and, 4) Transplantation of aortae from apoE-/- mice into apoAI-transgenic/apoE-/- mice also selectively raises HDL-C. Thus, it appears that lipoprotein changes known (LDL-C lowering) or thought to be (HDL-C raising) clinically beneficial not only promotes the resolution of inflammation in plaques by decreasing the number of macrophages in plaques, but also by promoting the acquisition of anti-inflammatory features. Whether the phenotypic changes represent alterations in an individual macrophage or its replacement by a newly recruited monocyte that becomes M2-like is the subject of ongoing studies. Grant support: NIH HL084312 and HL098055

Regulation of dynamic endothelial cell rearrangements during angiogenic sprouting and remodelling

Holger Gerhardt. Vascular Biology Laboratory, London Research Institute – Cancer Research United Kingdom

Angiogenic sprouting and anastomosis create an immature vascular plexus which subsequently remodels into a hierarchically branched network. Experimentation and computational modelling have established a mechanistic concept of the regulation of angiogenic sprouting, involving VEGF mediated activation and Dll/Notch mediated selection of distinct endothelial cell responses, the coordination of which critically influences initial patterning.
Our recent mosaic analysis illustrated that individual cells compete for the leading tip cell position, a process that is regulated by differential VEGF receptor levels under Notch-signaling control. Live imaging highlighted an extraordinary level of cell rearrangements in the sprout. We now established a new computational model to incorporate the dynamic cell arrangements and to investigate the underlying principles. I will be presenting new insights from this model.
In addition, our mosaic in vivo studies in the mouse retina indicated that rearrangements are not restricted the sprout but might control regression. Systematic analysis of the cellular principles and molecular mechanisms regulating vessel regression identifies that blood vessel lumen constriction is an initiating step in vessel regression followed by extensive endothelial cell migration and rearrangements with endothelial cells incorporating in neighbouring branches, without signs of endothelial cell death. Single cell analysis showes that regressing endothelial cells are highly active, extending filopodia away from the regressing branch. Studying lumen, junctional profiles, endothelial cell shape and endothelial cell polarity, we propose that regression involves at least four discrete steps: 1) selection of the regressing branch; 2) physical lumen constriction; 3) junction remodelling during endothelial cell retraction; and 4) resolution of the vessel segment regression, which leaves behind a pericyte covered basement membrane sleeve. Studying the underlying molecular regulation, we find that loss of endothelial Wnt ligand-secretion leads to excessive vessel regression, indicating that endothelial cells themselves secrete Wnt ligands relevant for stabilizing the immature vessel branch. We propose that Wnt is involved in the initial selection step of the remodelling branch and affects coordinated and polarized endothelial cell migration.

Developmental clues in vascular maturation and repair

Adriana C Gittenberger- de Groot[1,2], Beerend P Hierck[2], Marie-Jose Goumans[3], Robert Poelmann[2] and Marco C DeRuiter[2]. Dept. of Cardiology[1], Anatomy and Embryology[2], Molecular Cell Biology[3], Leiden University Medical Center, The Netherlands

Study of human specimens with tricuspid and bicuspid aortic valves and ascending aortic aneurysm formation triggered a renewed study of development of the cellular components of the vascular wall with special focus on the process of epithelial-mesenchymal-transition (EMT) and endothelial-mesenchymal-transition (endoMT). It is clear that the TGFbeta pathway is essential for these processes but the emerging data are paradoxal. For the process of endoMT we have shown in vitro specific differences between the adult endoMT in which shear stress induced Klf2 inhibits TGFbeta signalling through activation of the inhibitor SMAD7. In the embryo, however, high flow activates TGFbeta/Alk5 signalling upstream of klf2. A primary cilium on endothelial cells aids in sensing fluid flow. Exclusively in the absence of a cilium embryonic endothelial cells show flow-induced endoMT. These observations need correlation with the convex and concave sites of the aneurysmatic aortic wall. Endothelial cells are in close interaction with the underlying vascular smooth muscle cells (VSMCs) of the media of the aorta and a binary status of the medial VSMCs has been reported for TGFbeta signalling which might possibly link to VSMCs derived in the embryo from the mesoderm of the second heart field or neural crest cells.The differences in vascular wall pathology between cases with tricuspid and bicuspid aortic valves directed our studies to a new contributing component being the arterial epicardium forming the layer that borders the fluid filled pericardial cavity and the vascular adventitia. We studied the embryonic EMT of this layer using the Wilms tumor suppressor antibody (WT-1) as an epicardial marker and showed a cellular contribution of these epicardium derived cells (EPDCs) to the developing vascular wall and the semilunar valves. In parallel with the recent reports of the cardiac epicardium for its repair potential with redeployment of the embryonic EMT process after myocardial infarction, we could show that this renewed EMT also applied to the aneurysmatic aortic wall. The dependency of both endoMT and EMT on TGFbeta signalling adds a new element to the study of the aortic wall histopathology.

Organotypic endothelial differentiation and functions: The liver paradigm

Sergij Goerdt. Department of Dermatology, Venereology, and Allergology, University Medical Center and Medical Faculty Mannheim, University of Heidelberg, and Center of Excellence in Dermatology, Mannheim, Germany

Currently, it is increasingly acknowledged that the organ-specific blood vasculature is of utmost importance in embryonic organ development and in organ-specific physiological functions as well as in organ-specific disease development such as tumor growth and metastasis and in regeneration and artificial organ construction. Liver sinusoidal endothelium is a prime example for organ-specific microvascular differentiation characterized by numerous fenestrations and lack of a well-formed basal lamina. The major functions of these specialized endothelial cells comprise clearance of macromolecular waste molecules form the circulation via endocytosis and transendothelial transport, maintenance of low portal blood pressure and induction of immunological tolerance. We have identified a novel hyaluronan receptor-like gene family including stabilin-1 and stabilin-2 proteins that function as major scavenger receptors preferentially in liver sinusoidal endothelium. Stab1/2 double deficient mice develop severe glomerulofibrosis and albuminuria; in these mice, glomerulofibrotic nephropathy is caused by impaired hepatic clearance of a noxious factor from the peripheral blood. Hence, organotypic blood vessels in the liver, but also in other organs may serve important homeostatic functions for distant organs as well as for the whole organism.
In maintaining the functions of hepatic sinusoidal endothelium in homeostasis, several signalling pathways are involved. Vascular endothelial growth factor (VEGF) has been described to be crucial in this respect. We have shown that wnt2 acts as an autocrine growth and differentiation factor for hepatic endothelium that cross-stimulates the VEGF pathway by enhancing expression of VEGFR2. Others have shown the importance of NO, PDGF and shh signalling for proper differentiation and function of LSEC as well as their impact on hepatic diseases. Using comparative microvascular gene expression analysis, a hepatic microenvironment-dependent liver sinusoidal endothelial cell (LSEC)-specific differentiation program was identified by us. This program included growth and transcription factors, endocytosis-related and cytoskeleton-associated molecules as well as the novel junctional molecule Leda-1 that preferentially localizes to the abluminal cell surface and is sorted basolaterally suggesting functional involvement in cell adhesion and polarity. Upon cultivation, specific LSEC features such as fenestrations as well as stabilin-1/2 expression are rapidly lost. In hepatic disease, hepatic sinusoidal endothelium undergoes capillary transdifferentiation with similar changes. In both instances, hepatic sinusoidal endothelial cells are dysfunctional. In this context, the molecular and cellular determinants that regulate maintenance and remodelling of well-differentiated and functional LSEC in health and disease will be discussed and LSEC-directed targeting strategies to correct defective signalling pathways and to rescue LSEC from dysfunction will be developed.

Transient receptor potential channels and cardiovascular function

Thomas Gudermann. Walther-Straub-Institute of Pharmacology and Toxicology, University of Munich, Goethestr. 33, 80336 Munich, Germany

Over the past decade transient receptor potential (TRP) cation channels gained broad recognition as versatile cellular sensors. More than 50 trp genes have been cloned from different species amounting to 20% of known genes coding for ion channels. In contrast to other ion channel families, TRP channels have so far been defined on the basis of primary sequence homology as opposed to common ligands or distinct ion permeability profiles. Upon heterologous overexpression TRP channels can be activated by strikingly diverse chemical and physical stimuli and there is no doubt that some TRP family members serve as bona fide sensory proteins. However, very little is known about the activation mechanisms of TRP channels under physiological conditions in vivo.

At the cellular level, TRP channels are intimately involved in the regulation of calcium influx, the release of calcium from internal compartments and cell depolarization. However, TRP proteins do not function in isolation, but are organized as structural or functional protein modules. At present, the molecular make-up of TRP channel-containing physical protein complexes in the plasma membrane has not been systematically investigated.

At the organismic level, TRP proteins control a variety of essential autonomous physiological functions such as maintenance of body temperature, blood vessel tone, transmitter release from neurons, mineral and trace element homeostasis and reproduction. Thus, it did not come as a surprise that more than 10 hereditary human diseases caused by defects in TRP channels have already been described. In most instances, however, a detailed understanding of the underlying pathophysiology is still missing, thereby obviating targeted, specific therapeutic strategies.

This presentation will summarize the role of TRPC channels for myogenic responsiveness, hypoxic pulmonary vasoconstriction, and vascular endothelial cell function.

Immune activation in atherosclerosis

Göran K. Hansson. Karolinska Institute, Stockholm, Sweden

Lesions of atherosclerosis contain macrophages, T cells and other cells of the immune response, together with cholesterol derived from low-density lipoprotein (LDL) particles retained in the intima. Targeted deletion of genes encoding costimulatory factors and proinflammatory cytokines results in less disease in mouse models, whereas interference with regulatory immunity accelerates it. Innate as well as adaptive immune responses have been identified in atherosclerosis, with components of cholesterol carrying low LDL triggering inflammation, T cell activation and antibody production during the course of disease.

By using T cell cloning strategies, we have identified peptide fragments of the LDL protein, apoB100, as antigenic epitopes eliciting T cell activation. Certain isotypes of T cell receptors (TCR) mediate these responses. Blocking the immunological synapse involved in T cell recognition of ApoB100 not only inhibits T effector responses and secretion of proinflammatory cytokines, it also profoundly reduces atherosclerosis. Transgenic mice overexpressing such a TCR display strong cellular immune reactions towards LDL and develop anti-lipoprotein antibodies.

ApoB100 also contains a peptide sequence that can trigger activation of macrophages, leading to secretion of inflammatory mediators such as interleukin-8, interleukin-6, and MCP-1. This peptide, termed ApoB danger associated signal-1 (ApoBDS-1), is released upon degradation of LDL particles in the atherosclerotic lesion.

Together, these findings reveal that protein components of LDL can elicit innate as well as adaptive immune responses that promote vascular inflammation and the development of atherosclerosis.

Capturing pathogens by factor XIII - a novel defense mechanism?

Heiko Herwald. Lund University, Lund, Sweden

During the past decade, evolutionary conservation of pathways in innate immunity has laid the fundament for a series of groundbreaking discoveries, which originated from studies on insects (for example TOLL). It is now generally accepted that coagulation and innate immunity have coevolved from a common ancestral substrate early in eukaryotic development, and that these systems continue to function as a highly integrated unit for survival and defense following tissue injury. Notably, insect models have shown a functional equivalent of blood clotting, namely the coagulation of hemolymph, as an integral part of the immune system. Comparison of invertebrate and vertebrate coagulation systems reveals that transglutaminase and coagulation factor XIII (F XIII) share substantial sequence homology, suggesting that the two enzymes also have similar physiologic or even patho-physiologic properties. While Drosophila transglutaminase has been reported to sequester bacteria in the clot matrix, little is known about the role of coagulation factor XIII in the host response to infection.
Upon endothelial injury pathogenic bacteria may enter the blood stream and cause systemic complications. Thus, an immediate immobilization of the invading pathogen within a formed clot at the damaged site, appears to be a plausible strategy to prevent blood-born infections. Our findings show that the induction of coagulation by Streptococcus pyogenes leads to immobilization and killing of the bacteria inside the clot. The entrapment is mediated via cross-linking of bacteria to fibrin fibers by the action of F XIII. In a streptococcal skin infection model, F XIII(-/-) mice developed locally severe signs of pathologic inflammation, and F XIII treatment of wild-type animals dampened bacterial dissemination during early infection. Bacterial killing and cross-linking to fibrin networks was also detected in tissue biopsies from patients with streptococcal necrotizing fasciitis, supporting the concept that bacterial entrapment by F XIII is part of the early innate immune system.

Sphingosine 1-phosphate signaling via its G protein-coupled receptors

Timothy Hla. Weill Cornell Medical College, Cornell University, New York, NY 10065, USA

In vertebrates, metabolism of sphingomylein by the sphingomyelinase pathway results in the formation of sphingosine 1-phosphate (S1P) which is secreted by its transporters to the extracellular milieu. S1P in extracellular fluids is bound by chaperones such as Apolipoprotein M of HDL and serum albumin. Vertebrates also have five G protein-coupled receptors for S1P which signal via intracellular effectors to regulate fundamental cellular behavior, for example, cell migration, proliferation, morphogenesis and survival. In mammals, S1P is highly concentrated in the circulatory system whereas its concentration in the interstitial fluids is much lower, thereby forming a gradient. The receptors for S1P are highly expressed in vascular and immune cells. An S1P receptor inhibitor, FTY720 aka Fingolimod (Gilenya) has entered the clinic as the first oral medication in multiple sclerosis. It is known that S1P signaling in the extracellular environment via its G protein-coupled S1P1 receptor regulate immune cell egress and interference with this mechanism leads to suppression of autoimmune neural inflammation. However, vascular expression of S1P receptors is critical for vascular development and homeostasis. Using an inducible knockout system for S1P1 in the mouse, we show that S1P1 is a fundamental regulator of early steps of angiogenesis. It regulates flow-dependent stability of new angiogenic vessels and sustains angiogenesis. In its absence, hypersprouting and mispatterning of vessels ensues. In addition, mature vascular beds require S1P1 signaling to maintain the barrier function in organs such as the lung. These studies suggest that S1P signaling via its receptors is essential for vascular development and homeostasis.

Systems biology of mechanotransduction

AJG Horrevoets. Department of Molecular Cell Biology and Immunology, Medical Faculty Room A222, VU University Medical Center, Amsterdam, The Netherlands

Changes in blood flow characteristics as sensed by endothelial cells are the driving force for vascular remodeling, e.g. collateral artery remodeling and atherosclerosis. Atherosclerosis only develops in certain pre-disposed areas of the vasculature where endothelial cells experience low or oscillatory shear stress, which activates the pro-inflammatory and pro-coagulant transcription factors activator protein 1 (AP-1) and nuclear factor κB (NFκB), thus inducing a pro-inflammatory, pro-coagulant surface. In contrast, healthy endothelial cells that are exposed to prolonged high laminar shear stress, express anti-inflammatory and anti-coagulant genes. Increased sustained levels of shear stress lead to outward arterial remodeling, a process that depends on recruitment of monocytes to initiate this process. Genomic databases and technologies have revolutionized our understanding of vascular biology. Our integrated genomics approach has identified key regulatory proteins that drive endothelial and monocytic gene expression and phenotype in response to flow-driven changes of the vascular wall. The key shear stress-induced transcription factors that govern the flow-induced healthy endothelial phenotype are Krüppel-like factor 2 (KLF2) and nuclear factor erythroid 2-like 2 (Nrf2). Together they govern ~70% of the shear stress-elicited gene sets, including anti-inflammatory, antioxidant and anti-coagulant proteins, either directly or by inhibiting the pro-inflammatory transcription factors AP-1 and NFκB. Based on genomic analysis of patient-derived monocytes combined with image analysis and clinical parameters, we identified several molecules that determine the extent of beneficial outward remodeling of coronary arteries. Most notably Interferon-beta and Galectin-2 act as a natural break on these processes and thus constitute attractive targets for intervention related to myocardial infarction. Given the underlying genetic cause for interindividual differences, personalized approaches to stratification and targeted modulation become feasible. The wide-spread beneficial effect of key biomechanical transcription factors holds the promise that their targeted modulation might lead to a new class of cardiovascular drugs.

JAM-C in inflammation and vascular disease

Beat A. Imhof. Department of Pathology and Immunology, Medical Faculty, Geneva, Switzerland

We investigate the pathology of leukocyte migration, inflammatory diseases and angiogenesis. Simultaneously with other laboratories we discovered the vascular adhesion molecules JAM-B and JAM-C. Expression and production of recombinant JAMs enabled the development of a large panel and monoclonal and polyclonal antibodies, we produced transgenic mice over-expressing JAM-C in the vasculature by insertion of the vascular promoter Tie2 and we have JAM-C and JAM-B deficient mice. Using these reagents and the gene modified animals we showed JAM-C involved in immune responses against microorganisms preventing pneumonia. Furthermore, antibodies against JAM-C reduced the severity of acute and chronic inflammatory pathologies such as pancreatitis, peritonitis, rheumatoid arthritis. These effects of JAM-C seem to be due to its role in leukocyte transendotheial migration. The molecules form a vascular barrier for tissue leukocytes returning back to the blood i.e. it contributes to the one-way traffic observed during leukocyte homing. In collaboration with Sussan Nourshargh, London we visualized neutrophils and monocytes reverse, polarised transendothelial migration. These events were detected by blocking JAM-C in vitro and in vivo using 3D intravital microscopy of inflammation following ischemia/reperfusion injury.
(JAM-C) is expressed by vascular endothelium and also by human but not mouse B lymphocytes. Together with Thomas Matthes, Geneva we recently described that the level of JAM-C expression defines B cell differentiation stages and allows the classification of marginal zone (JAM-C positive) and germinal center (JAM-C negative) B cell lymphomas. Now we found a role for this lymphocyte JAM-C in migration of human B cells, using a xenogeneic NOD/SCID human/mouse model. Treatment with anti-JAM-C antibodies reduced homing of normal and malignant human, JAM-C expressing B cells to bone marrow, lymph nodes and spleen. Blocking spleen homing is remarkable as most other anti-adhesion antibodies reduced homing of B cells only to bone marrow and lymph nodes but not to spleen. Plasmon resonance studies identified JAM-B as the major vascular ligand for JAM-C while homotypic JAM-C interactions remained at background levels. Accordingly, anti-JAM-C antibodies blocked adhesion of JAM-C expressing B cells to blood vessels in human and mouse lymphoid organs. In particular, JAM-B is highly expressed by the spleen vasculature.

Endothelial heterogeneity: The basis and molecular support to organ-specific physiological needs

M. Luisa Iruela-Arispe, Lauren Goddard, and Josephine Enciso. Department of Molecular, Cell and Developmental Biology, University of California at Los Angeles, Los Angeles, CA 90095, USA

Endothelial cells constitute the inner lining of the entire vascular system and while apparently homogeneous, they can offer a wide array of organ-specific functions through unique molecular distinctions. Understanding the nature of these signatures and their specific endothelial functions is particularly critical to the homeostatic function of most organs. In addition to its role in the physiology of the female reproductive tract, progesterone also impacts the cardiovascular system. Nonetheless, the specific effects of this steroid hormone in blood vessels remain unclear. The recent generation of mouse models that lack progesterone receptor (PR) have confirmed that vascular function is affected by progesterone, yet a deeper understanding is still lagging and urgently needed considering the questionable effect of hormonal replacement therapy in the amelioration of cardiovascular disease. Using an animal that expresses beta-galactosidase in the PR locus (PRknock-in beta gal) we found that this gene is expressed in both smooth and endothelial cells providing a rationale to pursue a detailed evaluation of PR function in blood vessels. To gain further insight on the role of PR signaling in the vasculature, we generated a transgenic mouse that over and missexpresses PR in the endothelium of several vascular beds. Systemic treatment with progesterone resulted in pathological vascular permeability in the subset of organs that expressed the transgene. This unexpected finding was in accordance with some phenotypic aspects of the PRKO mouse, which failed to mount an endometrial decidual response upon stimulation with hormones. The decidual response includes concrete alterations in endometrial glands, stromal differentiation, and vascular permeability. Further investigation using endothelial cultures revealed that PR promotes fundamental changes in inter- endothelial junctional complexes. Furthermore, endothelial PR had profound effects in the trafficking of inflammatory cells within the endometrium. In an attempt to better understand progesterone signaling within endothelial cells, our lab has generated a mouse with endothelial specific deletion of progesterone receptor (PR^{ECKO}) by using the endothelial specific VE-Cadherin Cre transgenic mouse. Although reproductively similar to controls, aged PR^{ECKO} uteri contain increased numbers of Mac-1+, Gr-1+ polymorphonuclear cells and decreased numbers of CD3e+ T cells in the uterus compared to controls. These results suggest that progesterone signaling within endothelial cells may play a role in the trafficking of leukocytes within the murine uterus. We believe that further elucidation of the role of PR on blood vessels is long overdue and required to gain a concrete understanding of the physiological and pathological effects of this hormone in the cardiovascular system.

New insights into the thrombo-inflammatory function of platelets

Shaun P. Jackson, Zane Kaplan, Mehran Ghasemzadeh, Yuping Yuan, Imala Alwis, Simone M. Schoenwaelder, Warwick S. Nesbitt, Erik Westein, and Zaverio Ruggeri. Australian Centre for Blood Diseases, 6th Floor, Burnet Tower, The Alfred Hospital, Melbourne, Australia

The recruitment of leukocytes to sites of infection or inflammation has been well characterized and is dependent on a unidirectional multi-step adhesion mechanism with the surface of endothelial cells or through 'secondary capture' by endothelial-bound platelets. Platelet thrombi are also efficient at capturing leukocytes from flowing blood and promoting leukocyte infiltration into ischaemic tissues, however the mechanisms underlying this remain poorly defined. Platelet thrombi provide specific challenges for leukocyte recruitment. In particular, the 3-dimensional growth of thrombi provides a physical barrier to the recruitment of leukocytes to the site of tissue injury. Furthermore, it is unclear whether leukocytes migrate through, or around the margins of thrombi, or how thrombi provide directional cues for leukocyte guidance to the sites of vascular injury. We have established two murine models of endothelial injury, involving localized trauma or ischemia-reperfusion injury, and utilized confocal or brightfield/epifluorescence intravital imaging techniques to visualize platelet and leukocyte adhesive interactions with the injured vessel wall. Using these models, we have demonstrated the existence of a distinct form of leukocyte recruitment that is specifically mediated by platelet thrombi and leads to rapid and extensive leukocyte recruitment to sites of vascular injury. Three distinct, sequential phases regulate leukocyte-thrombus interactions in vivo; (i) a leukocyte recruitment step mediated by platelet P-selectin; (ii) a rheology-dependent leukocyte accumulation step mediated by platelet GPIb and the leukocyte counter-receptor Mac-1; and (iii) a directed intravascular migration step mediated by the platelet chemokine NAP-2. The extensive recruitment and intraluminal migration of leukocytes is dependent on the 3-dimensional size of thrombi as well as the graded activation status of platelets. In this talk I will discuss the distinct features of platelet thrombi that lead to the highly efficient recruitment of leukocytes to sites of endothelial injury.

VEGF-mediated vascular perturbations in the developing and adult lung

Eli Keshet and Alon Lazarus. Dept. of Dev Biol & Cancer Res, the Hebrew University – Hadassah Medical School, Jerusalem 91120, Israel

There is growing evidence that VEGF and the organ vasculature play multiple homeostatic roles in mature organs, in addition to the traditional role in tissue perfusion. To uncover such functions, we employ conditional transgenic systems based on a reversible VEGF gain- or loss of function to expand or eliminate, respectively, the lung vasculature during successive stages of lung development and in the adult lung. We show that branching morphogenesis of the vasculature and airways are tightly coordinated and that in the absence of vessels the highly stereotypic pattern of airway branching is lost. Notably, this novel role of the vasculature in controlling branching stereotypy (but not airway branching per se) is perfusion-independent. Inhibition of VEGF signaling in the adult lung via conditional induction of a VEGF decoy receptor leads to a significant vascular loss, reflecting a need for ongoing VEGF signaling to maintain normal microvascular density. Indeed, terminating the VEGF blockade results in rapid restoration of normal MVD. Importantly, vascular restoration also leads to regeration of lost epithelium and reversal of the emphysema-like phenotype that develops during chronic VEGF inhibition.

Novel ways to noninvasively study antiangiogenic therapy effects

Fabian Kiessling. University of Aachen (RWTH), Pauwelsstraße 20, D-52074 Aachen, Germany

Noninvasive imaging plays an important role in both preclinical and clinical research and has potential to facilitate clinical translation. This talk summarizes our experiences in microstructural, functional, and molecular imaging of tumor vessels.
The micro-architecture of vessels can longitudinally be studied in tumor bearing animals using high resolution µCT. DCE MRI and HF-US are introduced as favorable tools to characterize perfusion, vessel permeability and vessel maturation during antiangiogenic treatments. Using targeted probes it is shown that “Fluorescence Molecular Tomography (FMT)”, MRI and US are capable to estimate the density of angiogenic marker molecules on tumor vessels and the activity of matrix-associated enzymes in the interstitial space during vascular remodeling. Some examples are also given for nano-sized theranostics and it is shown how US can aid in better accumulating them at the target site by inducing vascular permeation.
Many of these novel imaging concepts and tools can relatively easy be translated into clinics. Thus, one can expect them to play a major role in the clinical management of tumor treatments soon.

Therapeutic targeting of the chemokine system in atherosclerosis

Rory R. Koenen. Institute for Preventive Cardiology, Ludwig-Maximilians-University Munich
Cardiovascular Research Institute Maastricht, Maastricht University, Maasstricht, The Netherlands

Chemokines are small chemotactic cytokines that are important for the trafficking of leukocytes from the blood to target tissues and vice versa. They can be classified in four subclasses, of which the CC- and CXC-classes are the largest. The monomeric units of chemokines all share common structural characteristics, such as a flexible N-terminus, an antiparallel beta-sheet and a C-terminal alpha-helix. On a quaternary structural level however, chemokines from different classes have notable differences. It is believed that the globular part of a chemokine interacts with its corresponding G protein-coupled 7-transmembrane receptor, while binding of the flexible N-terminus to a defined pocket between the helices leads to activation of the receptor. In addition, chemokines have particular patches of charged residues that are important for binding to glycosaminoglycans (GAGs) on the surface of e.g. endothelial cells and for the formation of higher-order multimeric structures. These features enable the correct presentation of chemokines to the vessel wall, making them “visible” for passing leukocytes. In a particular (patho-)physiologic context, the expression of a specific palette of GAGs leads to the retention of a particular group of chemokines, which in turn attracts a specific subset of leukocytes that carry the correct set of chemokine receptors, eventually serving to generate a “tailor-made” immune reaction.Needless to say, the chemokine system has posed an attractive target for treatment and prevention of inflammatory diseases. However despite major efforts by the pharmaceutical industry, the development of chemokine receptor antagonists was hampered by efficacy problems and the occurrence of unexpected side effects. To date only 2 compounds, a CCR5 and a CXCR4 antagonist, achieved FDA approval. Thus, alternative strategies for the pharmacologic intervention in the chemokine system are required. The potential benefits of interfering with the heterophilic interaction of chemokines with other chemokines or with the GAGs of the vessel wall were explored. First, synthetic peptides were implemented to inhibit the interaction of the platelet-derived chemokines CCL5 and CXCL4 and this resulted in a loss of their synergistic enhancement to attact monocytes to the vessel wall, which is a crucial process in the development of atherosclerosis. Administration of these peptides in hyperlipidemic mice significantly reduced atherosclerotic lesion formation, which could be attributed by a decreased macrophage content in the plaques. Notably, the inhibition of the interaction between CCL5 and CXCL4 did not lead to adverse immunologic side effects. Secondly, the interference of chemokine binding to the vessel wall was investigated. Here, a modified variant of CCL2 was used, which did not activate CCR2, but showed a strongly increased affinity for the GAGs, such as present on endothelial cells. This compound was able to compete with endogenous CCL2 for binding to the vessel wall and effectively inhibited inflammatory monocyte recruitment in vitro and in vivo. Administration of this novel chemokine competitor in mice reduced neointima formation after wire-injury and tissue damage after myocardial infarction. Taken together, alternative strategies for manipulation of the chemokine system have proven effective in mice and might be amenable for the treatment and prevention of human cardiovascular diseases.

Organ specific angiogenesis and vascular remodeling

Gou Young Koh and LVBSC members. Laboratory of Vascular Biology and Stem Cells, Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, 305-701, Korea

Angiogenesis is a fundamental and dynamic process that is essential not only for the development and maintenance of every organ, but also for the regeneration of failing organs. In addition, angiogenesis is a versatile process, which actively and differently responds to the micro-environment of each organ. Here, we report three instances of organ specific angiogenesis and vascular remodeling. First, uterine angiogenesis is an essential process not only for uterine growth but also for embryonic growth during pregnancy. We found that blood vessels in the uterus were dynamically and markedly remodeled during post-implantation, pregnancy, and after delivery. We observed that VEGF-VEGFR2 and uNK cells, rather than Ang-Tie2 and uDCs, actively participate in uterine angiogenesis during post-implantation period. Most importantly, progesterone rather than estrogen significantly influence the decidual angiogenesis in uterus by regulating the expression of VEGF-VEGFR2 system during post-implantation period. Thus, progesterone secreted from ovary governs decidual angiogenesis in uterus during pregnancy. Second, ischemic retinopathies including retinopathy of prematurity and proliferative diabetic retinopathy involve vascular pathologies in the retina, such as avascular region formation, subsequent aberrant angiogenesis, and vascular leakage that eventually lead to blindness. Here we show that intravitreal supplementation of angiopoietin-1 (Ang1) induced ordered retinal angiogenesis into central avascular retina presumably mediated through the integrin $\alpha v\beta 5$ signaling pathway, leading to reduced avascular regions, hypoxia and neovascular tuft formation by improving blood perfusion in the mouse oxygen-induced retinopathy model. Third, the thyroid gland vasculature has distinguishable characteristics such as endothelial fenestrations, and it also serves as a conduit between thyroid gland microenvironment and systemic circulation. Here, we show that high follicular workload of thyroid gland as induced by thyroid-stimulating hormone (TSH) actively regulates thyroid gland vasculature through vascular endothelial growth factor (VEGF) during adult physiological state. We observed that daily administration of recombinant TSH increased the level of mature thyroid hormones such as T3 and T4 while inducing remodeling of follicular architecture, suggesting a paracrine role of VEGF in thyroid follicle.

Angiogenesis and heart failure

Issei Komuro. Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan

Cardiac hypertrophy is developed as an adaptive response to increased workload. Although cardiac function first remains normal, prolonged cardiac hypertrophy causes heart failure. We have recently demonstrated that DNA damage of cardiomyocytes induces p53 accumulation and thus inhibition of angiogenesis, resulting in the transition from cardiac hypertrophy to heart failure. This result suggests that angiogenic therapy might be effective for heart failure. We have treated over 100 "no-option" patients with critical limb ischemia by implantation of peripheral blood mononuclear cells. Cell therapy using peripheral blood mononuclear cells was very effective for ~70% of patients with limb ischemia, and its efficacy was associated with increases in plasma levels of angiogenic factors. Implantation of the cells induced regeneration of skeletal muscle and activated satellite cells produced various kinds of angiogenic growth factors, thereby promoting neovascularization in ischemic tissues. Interestingly implantation of peripheral blood mononuclear cells into ischemic limbs improved cardiac ischemia, suggesting that this therapy is growth factor-induced angiogenic therapy. We have recently found that implantation of peripheral blood mononuclear cells into heart is also effective to improve cardiac ischemia. Quite recently, we have found novel mechanisms of coronary vascular formation and I will talk about it.

Minamino T, Komuro I et al. Peripheral-blood or bone-marrow mononuclear cells for therapeutic angiogenesis? Lancet 360:2083-2084, 2002.
Tateno K, Komuro I et al. Critical Roles of Muscle-Secreted Angiogenic Factors in Therapeutic Neovascularization. Circ Res 98:1194-1202, 2006.
Sano M, Komuro I et al. p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. Nature 446:444-448, 2007
Moriya J, Komuro I et al. Long-term outcome of therapeutic neovascularization using peripheral blood mononuclear cells for limb ischemia. Circulation Cardiovasc Interv. 2:245-254, 2009

Roles of Wnt antagonists, Dickkopf1 and Dickkopf2, in regulating angiogenesis

Young-Guen Kwon. Department of Biochemistry, Yonsei University, Seoul, Korea

Patterning of new vascular structure is a coordinated multi-step process that involves sprouting and morphogenesis of endothelial cells (ECs) and requires the formation of asymmetric EC phenotypes and their dynamic interconversion. These processes are precisely controlled by a large number of genes and a specific gene expression within developing vessel is indispensable for establishing functional vascular network. Recently, we have identified several genes, which are potentially involved in regulating angiogenesis and vascular patterning, by employing in vitro endothelial cell differentiation model and microarray-based gene expression analysis. Interestingly, biochemical and genetic studies revealed that Wnt regulators, Dickkopf-1 and -2 (DKK-1 and -2), play distinct roles in neovascularization, with their temporal and reciprocal expression during EC proliferation and morphogenesis. DKK1 significantly inhibited EC proliferation and tube formation in vitro. EC-specific DKK1 transgenic mice showed delayed vessel development and reduced vessel density compared to the wild type littermates. On the contrary, DKK-2 promoted angiogenesis in vitro and in vivo. Importantly, local injection of DKK-2 protein significantly improved tissue repair, with enhanced neovascularization in animal models of both hind limb ischemia and myocardial infarction. We further showed that DKK-2 stimulated filopodial dynamics and angiogenic sprouting of ECs via a signaling cascade involving LRP6-mediated APC/Asef2/Cdc42 activation. In mice xenograft model, DKK-1, and -2 inhibited and enhanced tumor angiogenesis, respectively. Thus, our findings provide new insight for controlling neovessel formation and offer a new opportunity for treating angiogenesis-dependent vascular diseases.

Mechanoinduction of lymph vessel expansion

Eckhard Lammert. Institute of Metabolic Physiology, Heinrich-Heine-University of Duesseldorf Universitätsstr. 1, D-40225 Duesseldorf, Germany

Lymphatic vessels expand in size during physiologic processes, such as embryonic growth and adult regeneration as well as during some pathological processes, such as inflammation, allergies, lymph edema and some cancer diseases. Therefore, it is key to understand the molecular mechanisms of lymph vessel expansion.
Here we introduce experimental approaches, which are useful to uncover the molecular mechanisms of lymph vessel growth in the developing mouse embryo. We provide data showing that the beta1-integrin is strictly required for lymph vessel expansion and that mechanoinduced activation of integrins results in tyrosine phosphorylation and activation of the vascular endothelial growth factor receptor-3 (VEGFR3). We argue that both mechanical stimuli (e.g. interstitial fluid accumulation or lymphatic endothelial cell stretching) and the vascular endothelial growth factor-C (VEGF-C) are needed for a full expansion of the developing or regenerating lymphatic vasculature.

Signaling events regulating collateral arterial network development in ischemic cardiovascular disease

Ferdinand le Noble. Max Delbrueck Center for Molecular Medicine, Berlin, Germany

In ischemic cardiovascular diseases, the progressive closing of arterial vessels by atherosclerosis results in a mismatch between the arterial blood flow – oxygen supply and the metabolic demands of the tissue supplied by the feed vessel. In severe cases this will lead to heart infarct and stroke. Strategies to preserve tissue function in this setting are aimed at restoring blood flow involving formation of functional vascular networks to bypass the arterial stenosis via a process called arteriogenesis. Arteriogenesis, the formation of a collateral arterial network to bypass a stenosis, is triggered by hemodynamic factors, involves activation of inflammatory pathways, and recruitment of blood borne cells into the wall of developing collaterals, collectively augmenting arterial growth. We recently addressed the nature of the hemodynamic signals relevant for arterial differentiation and found that pulsatile shear forces induce and maintain arterial identity gene expression, including the gap junction protein connexin-40 (Gja5) in arteries. Using a (conditional) loss of function approach in mice, we showed that connexin-40 controls number of native collateral vessels, shear stress induced outward remodeling of collateral arterioles, and functional recovery in arterial occlusion models. Furthermore, increased shear stress activates kininogen-bradykinin signaling in growing collateral arteries, and bradykinin-1 receptor (B1R) expressing circulating myeloid cells, are determinants of arteriogenic efficiency in ischemic cardiovascular disease models. Bone marrow chimeric mice generated by reconstituting wild-type mice with B1R mutant bone marrow showed reduced arteriogenesis, whereas transplantation of wild-type bone marrow cells into irradiated B1R mutant mice restored arteriogenesis, after femoral artery occlusion. The bradykinin signaling pathway may thus act as a molecular link between changes in hemodynamic conditions with activation of inflammatory pathways relevant for stimulating collateral arterial network growth.

How antigen-specific interactions between CD4 T cells and antigen-presenting cells in the aortic wall influence atherosclerosis in Apoe-/- mice

Klaus Ley. La Jolla Institute for Allergy and Immunology, La Jolla, USA

Multiphoton imaging of the aortic adventitia and media of CD11cYFP mice shows vigorously moving fluorescent antigen presenting cells (APCs), most of which express CD11b and CD11c. Fluorescently labeled TCR transgenic OTII CD4 T cells undergo long-lasting interactions with DCs in the presence, but not absence of their cognate antigen (ovalbumin). The OTII cells proliferate and produce the cytokines interferon-γ, TNF-α and IL-17A in an antigen-dependent manner. In CD11cYFPApoe-/- mice fed western diet for 12-15 weeks, YFP+ APCs are found both in the adventitia and in the atherosclerotic neointima, where show a larger, rounded shape reminiscent of foam cells. Effector memory T cells (CD4+CD44hiCD62L-) isolated from spleens of Apoe-/- mice, but not from C57BL/6 controls interact with DCs in aortic walls of CD11cYFPApoe-/- mice and also produce cytokines. These findings suggest that endogenous antigens are being presented to effector memory T cells in the aortic wall of atherosclerotic mice, resulting in antigen-specific production of cytokines. Interferon-γ results in enhanced uptake of oxidized or minimally modified low density lipoprotein (LDL) in mouse bone-marrow derived macrophages and in F4/80+CD11b+ macrophages in aortas of Apoe-/- mice, supporting foam cell formation. This suggests that autoimmune processes in atherosclerosis significantly contribute to chronic vascular inflammation. This is supported by our finding that the clinically used immunosuppressant mycophenolate (MMF) reduces atherosclerosis in mice, mainly by suppressing Th17 cells. The MMF effect is reversed by supplementing IL-17A. Th17 cells are also regulated by IL-27 receptor, a heterodimeric receptor consisting of WSX1 and the common gp130 subunits. Ldlr-/- mice receiving bone marrow from WSX1 deficient mice show enhanced atherosclerosis and a very large increase in aortic IL-17 production. Taken together, our findings suggest that CD4 T cell interactions with APCs are mostly pro-inflammatory, producing IFN-γ and IL-17A. These effects overwhelm the regulatory effects of Tregs and tolerogenic APCs.

New facets of inflammation in atherosclerosis

Peter Libby. Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA

According to the classical view, atherosclerotic plaques consist of a bland deposit of lipid trapped in a maze of smooth muscle cells and extracellular matrix. Multiple lines of evidence now implicate inflammation in to all stages of atherosclerosis. Innate immune responses provide a central transducer of various risk factors and altered artery wall biology. Chemoattractant cytokines promote migration of monocytes into the arterial intima, which then mature into macrophages when stimulated by other cytokines. These phagocytic cells drive many aspects of subsequent atherosclerotic progression, and also contribute to the propensity of plaques to rupture by production of proteases that weaken the fibrous plaque cap, and to thrombosis by generation of tissue factor. Recent work has highlighted the importance of monocyte/macrophage subsets in the biology of atherosclerosis. A pro-inflammatory population of moncytes prevails in peripheral blood in hypercholesterolemic mice, and may provide precursors for plaque phagocytes that promote atherogenesis and lesion complication. Hypercholesterolemic mice develop a reservoir of pro-inflammatory monocytes in the spleen due to extramedullary hematopoiesis. This population of proinflammatory monocytes mobilizes rapidly to sites of acute tissue injury such as myocardial infarction. Spleen-derived monocytes also migrate to chronic inflammatory lesions such as atherosclerotic plaques. Other innate immune cells including mast cells may also contribute to plaque formation and evolution. Adaptive immune cells, including T lymphocyte subsets, can modulate atherosclerosis as well, although the antigenic stimuli remain incompletely understood. Thrombosis provoked by disrupted atheromatous plaques causes most acute coronary events. Leukocyte-derived mediators including matrix-degrading proteinases and procoagulants such as tissue factor can drive this process. Blood biomarkers of the inflammatory response such as C-reactive protein (CRP) correlate consistently with cardiovascular events. Inflammation furnishes a new therapeutic target in atherosclerosis. Targeting the prominent inflammatory mediator interleukin-1 (IL-1) beta has emerged as an attractive candidate for anti-inflammatory therapy based on substantial preclinical data and recent biological observations. A large-scale clinical trial now in progress will test whether administration of an antibody that neutralizes IL-1 beta, can reduce recurrent events in survivors of acute myocardial infarction who have evidence of residual inflammation (CRP>2 mg/L) despite standard-of-care treatment including statin administration. Thus, the growing recognition of the importance for atherogenesis of innate immunity in particular, and of inflammation in general, has both theoretical and practical clinical implications.

Molecular Regulation of endothelial Blood-Brain Barrier function in health and disease

Stefan Liebner. Institute of Neurology (Edinger-Institute), Johann Wolfgang Goethe-University Frankfurt Medical School, Heinrich-Hoffmann-Straße 7, 60528 Frankfurt, Germany

Endothelial Wnt/β-catenin signaling is necessary for developmental angiogenesis of the central nervous system and differentiation of the blood-brain barrier (BBB), but it seems to be inoperable in the adult. In particular, its relevance for BBB maintenance, as well as for vascularization and barrier alterations in brain glioma is largely unknown.
To investigate the effect of Wnt/β-catenin signaling for brain tumor angiogenesis, we generated mouse GL261 glioma cell lines expressing either Wnt1 or the Wnt signaling inhibitor dickkopf-1 (Dkk1) in a doxycycline-dependent manner.
We show that in subcutaneous and intracranial glioma, endothelial β-catenin stabilization by Wnt1 resulted in a more quiescent vessel phenotype and induced the attachment of mural cells. Accordingly, tumor vessels of Wnt1 expressing glioma were less permeable and showed distinct junctional staining of the tight junction marker claudin-3, claudin-5 and ZO-1. Conversely, Dkk1 increased vessel density and promoted tumor growth. The vascular phenotype observed in Wnt1-expressing glioma was corroborated in unmodified GL261 glioma cells, transplanted intracranially into mice with endothelial-specific, dominant-active β-catenin signaling.
β-Catenin transcription activated the Dll4/Notch pathway in tumor endothelia, inhibiting an angiogenic and favoring a quiescent endothelial phenotype.
Furthermore, we identified the platelet-derived growth factor B (PDGF-B) as a novel target of the Wnt/β-catenin pathway, involved in the mural cell/pericyte recruitment to glioma vessels.
How the regulation of PDGF-B downstream of Wnt/β-catenin relates to BBB maintenance is subject of current investigation.
In conclusion, physiological levels of Wnt/β-catenin signaling promote angiogenesis, whereas sustained and reinforced signaling leads to inhibition of angiogenesis and vessel stabilization, which might proof to be a valuable therapeutic target for anti-angiogenic cancer therapy.

Signaling cascade of endostatin: From cell surface binding to nucleus internalization

Yongzhang Luo. National Engineering Laboratory for Anti-tumor Protein Therapeutics, Beijing Key Laboratory for Protein Therapeutics, Cancer Biology Laboratory, School of Life Sciences, Tsinghua University, Beijing, P. R. China

Angiogenesis and lymphangiogenesis play a fundamental role in tumor growth and metastasis. Endostatin is a potent endogenous angiogenesis inhibitor. We have discovered that cell surface nucleolin is a novel receptor for endostatin, which is specifically expressed on the angiogenic endothelial cell surface in tumor tissues. Vascular endothelial growth factor and extracellular matrix can mobilize nucleolin from nucleus to cell surface via nonmuscle myosin heavy chain 9 and heat shock cognate 70. Although it has been verified that endostatin can be translocated from endothelial cell surface to the nucleus via nucleolin thus regulate gene expression, the mechanism by which this occurs is still partially understood. Recently, we further revealed that endostatin can induce the complex formation of nucleolin with integrin α5β1 and urokinase-type plasminogen activator receptor (uPAR) on cell surface. Upon binding to this complex, endostatin undergoes internalization via both caveolae and clathrin pathways and nuclear translocation via importin α1β1, and thus inhibits endothelial cell migration and proliferation by suppressing the transcription of a series of genes related to hypoxia-inducible factor 1α. In addition, we found that nucleolin is also selectively expressed on the cell surface of lymphangiogenic endothelial cells both in vitro and in vivo. The effect of endostatin on lymphangiogenesis and lymph node metastasis has also been discovered. The phase II clinical trial with long lasting endostatin molecule M2ES, guided by nucleolin, will also be presented.

Leukocyte CD47 functions as an important modulator of the innate and adoptive immune system during inflammation

Veronica Azcutia[1], Matthew Routledge[1], Marcie R. Williams[1], Gail Newton[1], Andre Manica[2], Kevin Croce[2], and Francis W. Luscinskas[1]. [1]Center for Excellence in Vascular Biology, Department of Pathology, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA. 02115; [2]Department of Cardiology, Brigham and Women's Hospital, Boston, MA. 02115, USA

CD47 is broadly expressed and participates in leukocyte recruitment and in multiple immune functions through its interactions with leukocyte expressed receptors SIgnal Regulatory Proteins (SIRPα, SIRPγ). We previously have reported that endothelial cell CD47 interacting with T-cell ligands, such as SIRPγ, play an important role in T-cell transendothelial migration in vivo and in vitro, and that CD47 signaling in the endothelium occurs during leukocyte transmigration. CD47 was previously known as Integrin Associated Protein (IAP) because it associates with multiple integrins in a variety of cell types. Few studies, however, have evaluated whether CD47 regulates leukocyte β1 or β2 integrin adhesive functions in vivo or in vitro in models of inflammation. Our current project tested the hypothesis that CD47 regulates αLβ2 (LFA-1) and α4β1 (VLA-4) integrin adhesive functions during leukocyte recruitment. Intravital microscopy studies revealed that adoptively transferred in vitro generated CD47-/- Th1 cells had reduced adhesion/rolling interactions with wild type (WT) TNF-α inflamed cremaster microvessels versus WT Th1 cells. Studies in an in vitro flow model showed that CD47-/- Th1 cells, as compared to WT cells, also had reduced adhesion and transendothelial migration (TEM) across TNF-activated murine heart endothelium. Furthermore CD47-/- bind less to immobilized mICAM-1 and mVCAM-1 but have normal interactions with mE-selectin. Similarly, human Jurkat T-cells lacking CD47 adhered less to TNF-activated human endothelial monolayers and to immobilized ICAM-1 and VCAM-1. Finally, Jurkat CD47 null cells have a reduction in Mn+2 -induced binding to soluble ICAM-1 chimera and binding of mAb 24 (67%) and KIM127 (51%), which are mAb that detect activated conformations of β2 integrins. However, the absence of CD47 does not affect Jurkat β2 integrin dependent adhesion strengthening to immobilized ICAM-1 as determined by a cell detachment assay. Taken together, our results indicate CD47 primarily affects T-cell LFA-1 and VLA-4 affinity regulation, and this function is necessary for T cell adhesion and diapedesis.

Neutrophils as target cells to reduce atherosclerosis-related myocardial injury

François Mach. Division of Cardiology, Faculty of Medicine, Geneva University Hospital, avenue de la Roseraie 64, 1211 Geneva 4, Switzerland

More than 50 years ago, the concept of risk assessment was introduced by the Framingham Heart Study, with the identification of major cardiovascular risk factors. However, in the following decades, clinical studies showed that these traditional factors had a quite low specificity and sensitivity to predict acute cardiovascular events. Since inflammation has been shown as a key pathogenetic process in atherosclerosis, inflammatory cells and soluble mediators have been investigated to potentially improve the assessment of the cardiovascular risk. Since these factors are detectable in the blood stream, in atherosclerotic plaques as well as in peripheral ischemic tissues, a “new” concept of global “patient vulnerability” (taking into account of all these parameters) has been recently proposed. In particular, this global cardiovascular risk has been suggested to better predict acute cardiovascular events. Among different immune cells (such as monocytes/macrophages, T lymphocytes, dendritic cells, foam cells and mast cells), we recently focused on neutrophils. These leukocytes are circulating cells and might infiltrate both atherosclerotic plaques and injured ischemic tissues. Therefore, they might be promising candidates playing a crucial role in the global vulnerability. In my presentation, I will update evidence on different neutrophil-mediated mechanisms of cardiovascular vulnerability in atherosclerotic lesions, in the blood stream and in the infarcted myocardial tissue. The activities of novel neutrophilic mediators (cannabinoid system, RANKL axis and Nampt pathway, respectively) will be also discussed in both human and animals models.

Roles of thromboxane receptor signaling in enhancement of angiogenesis

Masataka Majima. Department of Pharmacology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 252-0374, Japan

Thromboxane A2 (TXA2) receptor, TP signaling induces platelet adhesion. It is previously reported that platelets promote angiogenesis. Yet the cellular and molecular mechanisms of platelet-dependent angiogenesis especially recovery from ischemic conditions, and the involvement of TP-signaling are not fully elucidated. This study was aimed at investigating whether TP-dependent platelet adhesion contributes to angiogenesis in mice hind limb ischemia model. TP knockout mice (TP-/-) delayed blood-flow recovery from ischemia and impaired angiogenesis compared with wild-type mice (WT). In vivo microscopic studies revealed that selective platelet adhesion to the ischemic endothelial cells via P-selectin was identified in WT but not in TP-/-mice. A P-selectin ligand, PSGL-1 was upregulated in ischemic muscles, and was suppressed in TP-/- mice not in WT mice. Expressions of PSGL-1 on cultured HUVEC were enhanced by a throboxane analogue, U-46619 and CoCl2. Plasma levels in stromal derived factor-1 (SDF-1) and vascular endothelial growth factor (VEGF) were increased after ischemia, and antibodies against CXCR4 and VEGF blocked angiogenesis in WT but not in TP-/-. These suggested that TP-signaling facilitates angiogenesis by P-selectin-mediated platelet adhesion bound to PSGL-1 on the vasculature at the ischemic sites, and that the adhered platelets supply proangiogenic factors to enhance angiogenesis.

Morphogenesis of the lymphatic vascular system

Taija Makinen. Lymphatic Development Laboratory, Cancer Research UK London Research Institute, London, United Kingdom

The lymphatic system is comprised of a network of lymphatic capillaries that collect protein rich fluid from the interstitial space and drain it via collecting vessels first into lymph nodes and then to larger lymphatic ducts, which connect to the venous system. Generation and maintenance of lymph flow relies on the contractions of the smooth muscle cells around the collecting vessels and on the action of luminal valves, which open and close in response to pressure changes and prevent lymph backflow. In humans, congenital malformation of the lymphatic system such as valve defects cause primary lymphoedema, which is usually a progressive and lifelong condition, characterized by gross swelling of the affected limb and accompanied by fibrosis and susceptibility to infections. The talk will focus on the cellular and molecular processes that form and maintain the collecting lymphatic vessels. In particular, a critical role for communication of endothelial cells with the extracellular matrix and the smooth muscle cells during valve morphogenesis and collecting vessel maturation is discussed.

Regulation of the (auto)immune response in atherosclerosis

Ziad Mallat. University of Cambridge, Cambridge, United Kingdom

Atherosclerosis is a chronic inflammatory disease of the arterial wall responsible for most ischaemic cardiovascular diseases. Circulating levels of several cytokines are associated with disease burden, and CRP levels predict the risk of future cardiovascular events. Furthermore, the incidence of cardiovascular disease (CVD) is increased in patients with chronic systemic inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).
We will briefly review the studies on the various pathogenic and regulatory immune subsets in atherosclerosis and the pathophysiological pathways involved in immune regulation. We will then focus on indoleamine 2,3-dioxygenase, a rate-limiting enzyme that catalizes the degradation of tryptophan along the kynurenine pathway, and known to be a major mediator of immune tolerance. We will present data detailing the potential role of IDO in the regulation of the immune response in atherosclerosis.

Plasticity of endothelial cell junctions in health and disease

Donald M. McDonald. Comprehensive Cancer Center, Cardiovascular Research Institute, and Department of Anatomy, University of California- San Francisco, San Francisco, California USA

Blood vessels and lymphatic vessels undergo conspicuous changes in structure and function during development and in disease. Changes in the junctions between endothelial cells are among these. The identification of protein components of adherens junctions and tight junctions between endothelial cells led to advances in understanding of endothelial barrier function in blood vessels and in cell and fluid entry in lymphatics. All endothelial cells express junctional proteins, but the distribution, functions, and pathophysiology of the junctions vary in different types of vessels. Endothelial cell junctions in the venular segment of blood vessels have the distinctive property of responding to inflammatory mediators that result in plasma leakage due to rapid formation of intercellular gaps at sites of focal loss of junctional integrity. Loss of junctional integrity in endothelial cells also occurs in chronic inflammation and tumors, where blood vessels undergo prominent remodeling, leakage is sustained, and cell trafficking has great importance.

Endothelial junctions are also specialized in the initial part of lymphatics, where fluid and cells enter. The cellular mechanisms underlying this entry are important to lymphatic function because they govern the clearance of edema fluid from tissues, transport antigen-presenting cells, and serve as routes for tumor cell spread to lymph nodes and distant sites. The discovery of button-like junctions (“buttons”) between endothelial cells of initial lymphatics brought additional understanding to the cellular basis of fluid and cell entry. Discontinuous button junctions are unique to initial lymphatics and distinctly different from continuous zipper-like junctions (“zippers”) found in other regions of lymphatics and in blood vessels. Buttons first appear in lymphatics around the time of birth as a consequence of transformation of zippers, which are present when lymphatics initially form in the embryo. However, buttons can revert back to zippers at sites of inflammation or high VEGF-C expression, which are accompanied by growth of new lymphatics. Although the proliferation of new lymphatics would be expected to improve lymphatic function, this does not necessarily occur when lymphatic function is impaired by transformation of buttons into zippers. Recognition of the plasticity of endothelial cell junctions, as blood vessels and lymphatic vessels change in disease, helps explain accompanying functional changes and identifies new therapeutic targets for the treatment of conditions where vascular growth and remodeling contribute to the pathophysiology.

The essential role for sphingosine-1-phosphate transporter Spns2 of vascular endothelial cells in lymphocyte egress from lymphoid organs

Shigetomo Fukuhara[1], Szandor Simmons[2], Asuka Inoue[3], Junken Aoki[3], Masaru Ishii[2], and Naoki Mochizuki[1]. [1]Dept. of Cell Biol.,Natl. Cerebr. & Cardiovasc. Ctr. Res. Inst. Japan, [2]Lab. of Cellular Dyn. WPI-Immunology Front. Res. Ctr. Osaka Univ. Japan, [3]Lab. of Mol. Cell. Biochem. Grad. Sch. of Pharma. Sci. Tohoku Univ. Japan

Sphingosine-1-phosphate (S1P) is not only a vaso-active sphingolipid but also stimulates lymphocytes expressing S1P1 receptor to induce the egress of T lymphocytes from thymus and B lymphocytes from bone marrow. To promote the egress of lymphocytes, S1P gradient between blood vessels and lymphocytes is produced. However, it has remained unclear where and how S1P is released to activate S1P1 receptors on lymphocytes essential for the egress. We previously identified a S1P transporter, Spns2, by a forward genetic screening using zebrafish. To clarify the function of Spns2 in mammals and to examine the involvement of Spns2 in lymphocyte egress, we developed global and conditional (endothelial-specific) Spns2 knockout mice. Here, we show that Spns2, a S1P transporter, is responsible for the egress of mature T lymphocytes and immature B lymphocytes from thymus and bone marrow, respectively.

Global Spns2 knockout mice exhibit marked accumulation of mature T lymphocytes in thymi and a decrease of peripheral T cells in blood and secondary lymphoid organs. Mature recirculating B lymphocytes were reduced in the bone marrow as well as in blood and secondary lymphoid organs. These data suggest a crucial role of Spns2 in egress of T and B lymphocytes from the primary lymphoid organs. In addition, bone marrow reconstitution studies revealed that Spns2 is not involved in S1P release from blood cells and suggested the significance of Spns2 in other cells. Consistently, endothelial-specific deletion of Spns2 resulted in the defects of lymphocyte egress similar to those observed in global Spns2 KO mice, indicating an essential role for endothelial cells in releasing S1P responsible for lymphocyte egress from primary lymphoid organs.

How the endothelial cell lateral border recycling compartment regulates transmigration

William A. Muller. Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA

The lateral border recycling compartment (LBRC) is a parajunctional reticulum of interconnected tubulovesicular membrane composed of a subset of membrane at the intercellular borders. Molecules important for transmigration (e.g. PECAM, CD99, JAM-A) are in this compartment while the structural components of the adherens junctions (e.g. VE-cadherin, β-catenin, p120) are not. During transendothelial migration (TEM) membrane from this compartment moves along microtubules in a kinesin-dependent manner to the site of transmigration. Membrane from the LBRC surrounds the transmigrating leukocyte providing membrane surface area and possibly unligated adhesion/signaling molecules to interact with the leukocyte. Trafficking of LBRC to the site of transmigration is required for TEM of neutrophils, monocytes, and lymphocytes whether the transmigration is paracellular or transcellular. Anything that blocks targeted recycling blocks TEM.

Interactions between molecules on the leukocyte and the endothelial cell borders regulate diapedesis. Homophilic interactions between PECAM (CD31) on the leukocyte and PECAM at the endothelial cell border initiate diapedesis by recruiting the LBRC. Homophilic interactions between CD99 on the leukocyte and CD99 at the endothelial cell border are important for completion of diapedesis. We now show that for monocytes interaction between monocyte DNAM-1 and poliovirus receptor (PVR, CD155) on the endothelial cell regulate TEM at a step that is functionally between those regulated by PECAM and CD99. Similar to PECAM and CD99, PVR is a component of the LBRC and the PECAM, PVR, and CD99 molecules that are in the LBRC (not those molecules at the surface of the junction) are required for TEM.

Leukocyte PECAM-endothelial cell PECAM interactions are believed to be a signal that recruits the LBRC to initiate TEM. When these interactions are blocked, LBRC is not recruited and leukocytes migrate along the endothelial borders unable to penetrate. Artificially stimulating an increase in cytosolic free calcium ion concentration overcomes this blockade of PECAM function and stimulates recruitment of LBRC allowing transmigration to proceed in the face of an ongoing PECAM block. This suggests that a downstream consequence of PECAM-PECAM interactions is to stimulate this increase in intracellular free calcium concentration. Beyond the requirement for intact microtubules and kinesin activity, the mechanics of recruitment of the LBRC are also largely unknown. We recently found that the scaffolding molecule IQGAP1 is specifically associated with the cytoplasmic side of the LBRC and is recruited to the site of TEM along with LBRC. Knockdown of IQGAP1 by siRNA inhibits targeted trafficking of LBRC and blocks TEM. The mechanisms underlying calcium signaling and IQGAP1 regulation of LBRC recruitment are under active investigation.

Cardiac phenotyping of Akt1/PKBα deficiency during development and after myocardial infarction

Katrien Vandoorne[1], Brian Hemmings[2] and Michal Neeman[1]. [1]Biological Regulation, Weizmann Institute, Rehovot Israel and [2]Friedrich Miescher Institute for Biomedical Research, Basel CH-4058, Switzerland

Akt1, also known as PKB alpha, is an intracellular signaling molecule that coordinates metabolism and survival. In vascular endothelial cells, Akt1 acts downstream of vascular endothelial growth factor (VEGF) to induce vascular permeability and angiogenesis. Akt1 was implicated in cardio-protection, angiogenesis and recovery from ischemia. Previous studies demonstrated that acute Akt1 overexpression is cardio-protective with enhanced angiogenesis and reduced dysfunction after infarction while chronic Akt1 overexpression seemed in other models to have opposite effects. Mice with disruption of the Akt1 gene have 30% neonatal mortality and are smaller than their wild type littermates with the heterozygous mice in the middle. This was recently also associated with a thin myocardium and ventricular septum defects. However, the role of Akt1 during cardiac development and after myocardial infarction is still unclear. Thus, the aim of our study was to non-invasively characterize cardiac structure and function of Akt1 knockout and heterozygous relative to wildtype mice, during cardiac development and after myocardial infarction. In utero echocardiography, was done on pregnant heterozygous mice mated with heterozygous males at E16.5 and E18.5. Adult hearts were imaged by MRI using 9.4T Bruker Biospec at 10 to 15 weeks old. Mice were also imaged by MRI on days 1, 8, 15 and 29 or 30 after myocardial infarction (MI). In utero ultrasound showed reduced heart function in Akt1-/- and partly also in Akt1+/-. Remarkably, adult Akt1-/- mice showed increased EF and reduced LV remodeling at 15 and 29 days post MI relative to Akt1+/+ mice. Thus, long term disruption of Akt1 gene could be an avenue for future therapeutic treatment of heart disease.

Targeting the angiogenic extracellular matrix with armed antibodies: From the bench to the clinic

Dario Neri. Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH Zürich, Switzerland)

Antibodies can be used to deliver bioactive molecules (drugs, cytokines, photosensitizers, radionuclides, etc.) to the tumor environment, thus sparing normal tissues. The targeting of modified sub-endothelial extracellular matrix components using armed antibodies is particularly attractive, because of:
(i) the abundance and stability of some of these antigens (e.g., splice isoforms of fibronectin and tenascin-C); (ii) the dependence of cancer on new blood vessels; (iii) accessibility of these structures from the blood-stream; (iv) the fact that some of these antigens are very abundant in many different cancer types, while being virtually undetectable in most normal adult tissues [Refs. 1-3]. Vascular targeting approaches can be used beyond oncology, for the treatment of other serious conditions which are characterized by the over-exuberant proliferation of new blood vessels. While cytokines can be conveniently delivered at site of disease by the construction of fusion proteins with antibody vehicles ("immunocytokines"), the targeted delivery of cytotoxic drugs requires more sophisticated chemical strategies. We have recently explored the development of linkerless strategies for the coupling of potent cytotoxic drugs to tumor-targeting antibodies [Refs. 4-5]. Advanced preclinical and clinical data on armed antibodies will be presented in this lecture. In addition, we have recently started to explore whether small organic ligands, specific to tumor-associated antigens, can be used for pharmacodelivery applications in vivo. Selective ligands can be conveniently isolated from large DNA-encoded chemical libraries [for a recent review, see Ref. 6].

References:

1. Neri, D. & Bicknell, R.. Nature Rev. Cancer. 2005, 5, 436-446
2. Neri, D. & Supuran, C. Nature Rev. Drug Discov., 2011, 10, 767-777
3. Neri, D. & Pasche N. Drug Discov. Today, 2012, in press
4. Bernardes, G. et al. Angew. Chemie Int. Ed. Engl., 2012, 51, 941-944
5. Casi, G. et al. J. Am. Chem. Soc., 2012, 134, 5887-5892
6. Mannocci, L. et al., Chem. Commun., 2011, 47, 12747-12753

Plaque microenvironments, metalloproteinases and macrophage phenotypes

Andrew C. Newby, Jason L. Johnson, Graciela B. Sala-Newby. University of Bristol, Bristol Heart Institute, Bristol BS2 8HW, United Kingdom

Plaque rupture accounts for three quarters of all hearts attacks. It occurs when the tensile haemodynamic forces overwhelm the resistance of a thin and collagen-depleted fibrous cap. Such highly-inflamed plaque caps are one example of a crucial microenvironment within the plaque. The lipid core represents another microenvironment, which is also poor in collagen and contains dead or dying cells. Leaky intraplaque microvessels and the haemorrhage that results from them lead to another interesting microenvironment. The sub-intimal space is also specialised, as are areas dominated by smooth muscle cells and connective tissues. Over the past few years it has become clear that these different microenviroments are associated with distinct macrophage phenotypes that not only respond to but also play a major part in creating and maintaining these distinct areas of the plaque by secreting and expressing proteins on their surfaces. We have focussed on the metalloproteinase system because it has the potential to regulate extracellular matrix integrity as well as the proliferation, migration and apoptosis of vascular cells. Here, we present evidence for the association of distinct metalloproteinase and inhibitor profiles with different plaque microenvironments and macrophage phenotypes, with emphasis on areas of pro-inflammatory activation and hypoxia. We also report results from animal experiments and human tissue biobanks that demonstrate the pathophysiological potential of metalloproteinases and the possibilities for new diagnostics and treatments.

Thrombo-inflammation in akute stroke

Bernhard Nieswandt. Chair of Vascular Medicine, University Hospital Würzburg and Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine, University of Würzburg, 97080 Würzburg, Germany

Ischaemic stroke is a leading cause of death and disability worldwide. The complex cellular interactions leading from thromboembolic vessel occlusion to infarct development within the brain parenchyma in acute stroke are poorly understood, which translates into only one approved effective treatment, thrombolysis. Importantly, however, patients can develop progressive stroke despite reperfusion of previously occluded major intracranial arteries, a process referred to as “reperfusion injury” which can be reproduced in the mouse model of transient middle cerebral artery occlusion (tMCAO). Although pathological platelet and coagulant activity have long been recognised to be involved in the initiation of ischaemic stroke, their contribution to infarct maturation remained elusive. Experimental evidence now suggests that early platelet adhesion/activation mechanisms involving the von Willebrand factor (vWF) receptor glycoprotein (GP) Ib, its ligand vWF, and the collagen receptor GPVI are critical pathogenic factors in infarct development following tMCAO, whereas platelet aggregation through GPIIb/IIIa is not. Further experimental work indicates that these pathways in conjunction with coagulation factor XII (FXII)-driven processes orchestrate a "thrombo-inflammatory" cascade in acute stroke that results in infarct growth. On the other hand, T cells have recently been identified as important mediators of ischaemic brain damage but the underlying mechanisms and, in particular, their interaction with platelets and the coagulation system in this setting have remained unclear.
This presentation will summarise recent developments in understanding the contribution of platelet adhesion and activation mechanisms to the development of arterial thrombosis and discuss possible mechanisms underlying thrombo-inflammatory neurodegeneration in the setting of ischaemic stroke.

Integrin trafficking during cell migration and invasion

Jim C Norman. Beatson Institute for Cancer Research, Glasgow, Scotland, United Kingdom

Integrins are cell adhesion receptors involved in controlling cell growth and invasive migration during processes such as cancer metastasis and angiogenesis. We have established that Rab-regulated integrin trafficking contributes to processes involving cell migratory events – such as the invasive migration of cancer cells through three-dimensional matrices and the angiogenesis of tumours. In this talk, I will present data describing two novel aspects of integrin trafficking in cell migration. Firstly, I will describe how αvβ3 integrin negatively regulates the trafficking of other receptors (such as VEGFR2 and EGFR1) to dictate the invasiveness and vascularisation of tumours. This will also include the role played by mutant p53 in influencing metastasis via the control of integrin trafficking. Secondly, I will describe how the Chloride Intracellular Channel Protein 3 (CLIC3) acts to recycle 'active' integrins that have been targetted to late endosomes/lysosomes by the action of Rab25, and present recent clinical findings that this new pathway likely contributes to the invasion and metastasis of pancreatic ductal adenocarcinoma.

Leukocyte motility through venular walls in vivo

Sussan Nourshargh. William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, United Kingdom

Migration of leukocytes to sites of injury or inflammation is a crucial component of both innate and adaptive immunity. To penetrate venular walls, leukocytes must migrate through multiple barriers, endothelial cells (EC), the pericyte sheath and the venular basement membrane (BM)1 . Although there is increasing interest and understanding of the mechanisms that mediate transendothelial cell migration (TEM), the relative contribution of individual molecules requires further clarification1;2. Within our group we aim to investigate the mechanisms of leukocyte transmigration through imaging of inflamed tissues by multiple methods, including the application of high resolution confocal intravital microscopy. With this approach we have characterised the profile and dynamics of different modes of leukocyte transmigration through venular walls and have obtained evidence for differential roles of different EC junctional molecules in this process, including a role for JAM-C in regulation of polarised luminal to abluminal neutrophil TEM3;4. Our studies have also shed light on the mechanisms through which leukocytes breach the venular basement membrane and the pericyte sheath5-7. Collectively, through rigorous analysis of leukocyte-vessel wall interactions by direct real-time imaging of inflamed tissues, our findings provide novel insights into the cellular and molecular mechanism via which leukocytes cross the venular walls in vivo.

Funded by the Wellcome Trust

Reference List

1. Nourshargh S, Hordijk PL, Sixt M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. *Nat Rev Mol Cell Biol* (2010), 11:366-378.
2. Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology* (2007), 7:678-689.
3. Woodfin A, Voisin MB, Beyrau M et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat Immunol (2*011), 12:761-769.
4. Woodfin A, Voisin MB, Imhof BA et al. Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A and PECAM-1. *Blood* (2009), 113: 6246-6257.
5. Wang S, Voisin M-B, Larbi KY et al. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating PMN. *J Exp Med (*2006), 203:1519-1532.
6. Voisin MB, Probstl D, Nourshargh S. Venular basement membranes ubiquitously express matrix protein low-expression regions: Characterization in multiple tissues and remodeling during inflammation. *Am J Pathol* (2010), 176:482-495.
7. Proebstl D., Voisin M-B., Woodfin A., Whiteford J., D'Acquisto F., Jones G., Rowe D. & Nourshargh S. Pericytes support neutrophil sub-endothelial cell crawling and breaching of venular walls *in vivo. J Exp Med*., (2012), in press.

Control of vascular growth and homeostasis through Foxo and Sirtuin pathways

Michael Potente. Vascular Epigenetics Group, Institute of Cardiovascular Regeneration, Center for Molecular Medicine, and Department of Cardiology, Internal Medicine III, Goethe University, Frankfurt, Germany

Blood vessels form the first organ in the developing embryo and build extensive networks that supply all cells with nutrients and oxygen throughout life. As blood vessels get older, they often become abnormal in structure and function, thereby contributing to numerous age-associated diseases including ischemic heart and brain disease, neurodegeneration, or cancer. First described as regulators of the aging process in invertebrate model organisms, Forkhead box “O” (FOXO) transcription factors and sirtuin deacetylases are now emerging as key regulators of mammalian vascular development and disease. This presentation will illustrate recent insights into the biology of FOXOs and sirtuins in the vascular endothelium and discuss their relevance for disease-related changes of the vasculature. The integration of individual FOXO and sirtuin family members into various aspects of vessel growth, maintenance, and function provides new perspectives on disease mechanisms of aging, the most important risk factor for medical maladies of the vascular system.

Hypoxia, metabolism and cancer – novel anticancer approaches

Jacques Pouysségur. Institute of Research on Cancer and Aging (IRCAN), University of Nice, CNRS, INSERM, Centre A. Lacassagne, 33, Av Valombrose, 06189, Nice, France

In metazoan, sensing the availability of oxygen and key nutrients is integrated with growth factor signaling. This nutrient check-point control is essential for cells to receive the order to progress through the division cycle. Therefore, rapidly growing cells have developed sophisticated regulatory systems to rapidly respond to oxygen and nutrient fluctuations in the microenvironment.
Early on in evolution, oxygen sensing emerged, as a central control mechanism of energy metabolism and vasculogenesis. At the heart of this regulatory system is the Hypoxia-Inducible Factor, HIF-1, which controls the expression of, among other gene products, VEGF-A, Angiopoïetin-2 and Notch-ligand, three key angiogenic factors in vertebrates. This finding has placed the hypoxia-signaling pathway at the forefront of nutritional control. HIF can induce a vast array of gene products controlling glycolysis, intracellular pH (pHi), angiogenesis, cell migration and invasion, and so has become recognized as a strong promoter of tumor growth. The pro-invasion feature of HIF-1, measured by stimulation of Epithelial-Mesenchyme-Transition, could be seen as an integrated program 'designed' for migration-induced nutrient-search, as in microorganisms. It is therefore not surprising that HIF-1 also promotes access to another source of nutrients by inducing macro-autophagy. In this presentation, we will highlight some of the HIF1-induced gene products - carbonic anhydrases IX and XII (CAs) and monocarboxylate transporters (MCTs) – which regulate pHi by controlling export of metabolically-generated acids (carbonic and lactic acids). We report that targeting pHi-regulated processes severely restricts tumor growth, a process that compromises glycolysis-generated ATP levels. We propose that membrane-bound carbonic anhydrases (CAIX, CAXII), monocarboxylate transporters (MCT1 and MCT4) as well as their chaperon Basigin/EMMPRIN/CD147), which are associated with exacerbated tumor metabolism represent new potential targets for anticancer therapy.

Angiogenic factors and glomerular barrier function

Susan Quaggin. Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Joseph & Wolf Lebovic Health Complex, Toronto, Ontario, Canada

The glomerular filtration barrier is a highly specialized microvasculature bed and the site of formation of 180L of primary urinary filtrate each day in the adult human. It is comprised of a fenestrated vascular endothelium supported by perivascular cells known as podocytes and an intervening glomerular basement membrane. Podocytes produce angiogeneic factors including VEGF-A and Angiopoietin-1 (Angpt1) that are essential for development and maintenance of the glomerular microvasculature. Loss of VEGF-A from podocytes results in thrombotic microangiopathy in adult mice and in a subset of patients treated with VEGF inhibitors, whereas embryonic loss of VEGF-A results in profound developmental defects and early postnatal death due to kidney failure. In contrast, embryonic deletion of Angpt1 from podocytes or more globally from the entire vasculature, results in milder, capillary remodelling defects but predisposes the microvasculature to severe injury following an endothelial challenge, such as diabetes or malaria. Strikingly, loss of either the Flk1/KDR or Flt1 receptors from glomerular endothelium or podocytes, produce dramatic but different effects. Taken together, the results demonstrate a requirement of tight regulation of multiple angiogenic signaling pathways in the glomerular microvasculature to maintain renal function.

Vascular niche-derived angiocrine signals in the regulation of stem cell homeostasis

Shahin Rafii, Bisen Ding, Dan Nolan, Sina Rabbany, Daylon James and Jason M Butler.
Weill Cornell Medical College, New York, NY 10021, USA

Interaction of stem cells with their niche cells is essential for organ regeneration. We have found that within each organ, specialized capillary endothelial cells (ECs) are not just passive conduits to deliver oxygen and nutrients, but also establish an instructive vascular niche, which by elaboration of paracrine trophogens, known as angiocrine factors, directly balance the rate of stem cell self-renewal and differentiation. Specifically, sinusoidal ECs (SECs) within the hematopoietic tissues (1,2) and liver (3) compose of specialized vascular niche cells that by production of angiocrine factors support hematopoietic reconstitution after myeloablation. Activation of Akt-mTOR pathway in the SECs within the bone marrow stimulates expression of angiocrine factors, including Notch-ligands, IGFBPs, and FGFs that induce expansion of authentic long-term repopulating hematopoietic stem cells. While MAPkinase co-activation induced expression of angiocrine factors, including colony stimulating factors that supported differentiation of the hematopoietic stem cells into lineage-committed progenitors. After partial hepatectomy, SECs within the liver stimulated liver regeneration by angiocrine expression of Wnt2 and hepatocyte growth factor (HGF). Transplantation of liver SECs restored liver regeneration in mice with defect in hepatic proliferation. We have recently shown that pulmonary capillary endothelial cells (PCECs) also compose of unique vasculature that after left pneumonectomy, by production of specific angiocrine factors, including MMP14 and liberation of epidermal growth factor (EGF)-ligands initiates and sustains lung regeneration (4). Notably, transplantation of PCECs into mice with defect in lung regeneration restored the capacity of the lung epithelial progenitors to proliferate thereby restoring alveolar regeneration and respiratory function. Therefore, the capillary ECs within each organ are heterogeneous and are programmed to express defined set of angiocrine factors to support stem cell self-renewal and organ regeneration. Tissue-specific modulation of angiocrine factors or transplantation of primed organ-specific ECs provide for a therapeutically effective means to initiate and sustain self-renewal and differentiation of stem and progenitor cells orchestrating organ regeneration. Ongoing studies will also address the question as to how the tissue-specific angiocrine heterogeneity is specified in various organs at steady state conditions and during regeneration.

1) Kobayashi H, et al. Angiocrine factors derived from Akt-activated endothelium balance self-renewal and differentiation of hematopoietic stem cells. Nature Cell Biology, 11:1046-56, 2010.
2) Butler JM,et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell Stem Cell, 3:251-64. 2010.
3) Ding BS, et al. Inductive signals from sinusoidal endothelial cells initiate and sustain physiological liver regeneration. Nature, 468:310-5, 2010.
4) Ding BS, et al. Endothelial-derived inductive angiocrine signals initiate and sustain regenerative lung alveolarization. Cell, 47:539-53, 2011.

The Factor XII-driven contact system: A proinflammatory and procoagulant protease cascade

Thomas Renné. Division of Clinical Chemistry, Department of Molecular Medicine and Surgery and Center of Molecular Medicine, Karolinska Institute, Stockholm, Sweden

Coagulation and inflammation have long been considered as two distinct pathologies. More recently, their close interaction has been recognized as the unifying principle for a variety of disorders primarily of the cardiovascular system and infectious entities.
Factor XII (FXII, Hageman factor) is a plasma protease that initiates the contact system. This FXII-driven protease cascade starts a series of procoagulant and proinflammatory reactions via the intrinsic pathway of coagulation, and the bradykinin-producing kallikrein-kinin system, respectively. The biochemistry of the contact system in vitro is well understood, however its in vivo functions are just beginning to emerge.
Studies in genetically modified animals revealed that FXII is essential for thrombus formation while being dispensable for hemostatic processes that terminate blood loss. Challenging the dogma of a coagulation balance, targeting FXII protected from cerebral ischemia without interfering with hemostasis. In contrast, excess FXII activity is associated with a life threatening inflammatory disorder, Hereditary angioedema. We recently have identified platelet polyphosphate (an inorganic polymer) and mast cell heparin as in vivo FXII activators with implications on the initiation of thrombosis and edema.
The FXII-driven contact system serves as a paradigm to identify common principles, interactions and cross-talk between coagulation and inflammation, to identify novel therapeutic targets. Elucidating the FXII-driven contact system offers the exciting opportunity to develop strategies for safe interference with both thrombotic and inflammatory diseases.

More information: www.renne.net

Tissue factor in inflammation and thrombosis

Wolfram Ruf, The Scripps Research Institute, La Jolla, CA 92037, USA.

The hemostatic system is an integral part of physiological repair mechanisms and innate host defense against infection. The initiation of coagulation by tissue factor (TF) is crucial for orchestrating multi-cellular interactions through fibrin deposition and platelet activation. In addition, the TF pathway mediates coagulation protease cell signaling through protease activated receptor (PAR) 1 and 2. TF procoagulant and signaling activities are tightly controlled by TF targeting to cellular micro-domains, receptor recycling, and the association with co-receptors. Cell damage and release of extracellular ATP is crucial for switching on TF's procoagulant function. ATP activation of the purinergic P2X7 receptor initiates thiol exchange- and protein disulfide isomerase-dependent microparticle release and thrombosis. Thus, P2X7-induced TF procoagulant activation and interleukin 1 release leads to a coordinated activation of thrombo-inflammatory circuits in vascular inflammation. The upstream coagulation proteases TF-VIIa and Xa are crucial for the regulation of innate immune cell function through PAR signaling and TF-VIIa signaling promotes obesity and sustains adipose tissue macrophage inflammation and insulin resistance. The paradigm is emerging that the TF pathway not only serves to initiate inflammation, but plays pivotal roles in the control and resolution of chronic inflammation.

Neuropilin in angiogenesis and permeability

Christiana Ruhrberg. London, United Kingdom

Neuropilin (NRP) 1 is a receptor for the vascular endothelial growth factor VEGF-A that is essential for normal angiogenesis, but also regulates vascular permeability. I will describe new findings that shed light on the role of NRP1 in VEGF-A induced angiogenesis versus vascular permeability. Firstly, NRP1 promotes developmental and pathological angiogenesis independently of its cytoplasmic tail by promoting endothelial tip cell function in vivo. Secondly, the NRP1 cytoplasmic tail is essential for VEGF-A induced permeability in several vessel beds in a mechanism that does not rely on the known cytoplasmic tail interactor synectin.

The role of vasohibin family in tumor angiogenesis

Yasufumi Sato. Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Japan

The local balance between angiogenesis stimulators and inhibitors determines the occurrence and progress of angiogenesis. We isolated vasohibin-1 (VASH1) as a negative-feedback regulator of angiogenesis. VASH1 is induced in ECs by angiogenesis stimulators such as VEGF and FGF-2. During the subsequent database search, we found 1 gene homologous to VASH1 and named it vasohibin-2 (VASH2). The amino acid sequence of the human VASH2 protein is 52.5% homologous to that of human VASH1. The gene for human VASH1 gene is located on chromosome 14q24.3, whereas the gene for human VASH2 is located on chromosome 1q32.3. It reveals that see squirt posses one common ancestry vasohibin gene, and homology between this ancestry gene and human VASH1 or human VASH2 is almost same. Every vertebrates have VASH1 and VASH2, and amino acid sequences of those VASH1 and VASH2 are well conserved. Thus, a common ancestry gene seems to be divided into VASH1 and VASH2 during the evolution from vertebrate.
Because of the similarity between VASH1 and VASH2, we examined their expression and function by the use of hypoxia-induced angiogenesis model in mice. Our analysis revealed that VASH1 is mainly expressed in ECs in the termination zone to halt angiogenesis, whereas VASH2 is mainly expressed in mononuclear cells mobilized from the bone marrow in the sprouting front to stimulate angiogenesis. Thus, these 2 VASH family members regulate angiogenesis perhaps in a contradictory manner. We then investigated the expression of VASHs under conditions accompanied by conducive to pathological angiogenesis. VASH1 was shown in ECs of various human cancers. As tumor angiogenesis and metastasis were augmented in VASH1 (-/-) mice, endogenous VASH1 plays an inhibitory role. We then examined the expression of VASH2. Interestingly, VASH2 is detected preferentially in cancer cells. Moreover, both loss-of-function and gain-of-function analyses indicated that VASH2 in cancer cells promoted tumor growth via the stimulation of angiogenesis. We thus propose that these 2 factors play a role of yin and yang in tumors.

Pulmonary hypertension: Therapies beyond vasodilatation

Ralph Schermuly. Universities of Giessen and Marburg Lung Center (UGMLC), Justus-Liebig University of Giessen, Germany

Pulmonary Hypertension is a severe lung disease, which is characterized by vasoconstriction and remodelling of the vessel wall. By targeting the increased vascular tone, prostacyclin and its analogues, endothelin-receptor antagonists and phosphodiesterase type 5 inhibitors have been approved for treatment of PAH and represent the current therapeutic options. Beside the development of new vasodilators, current research focuses on the development of causal treatment regimens aiming a normalization of the vessel structure. Mechanistically, increased proliferation, migration and a resistance to apoptosis of vascular cells represent key events in disease progression. In this context, tyrosine kinase inhibitors have been shown to possess reverse remodelling potential in preclinical models of pulmonary hypertension by inducing apoptosis and blocking proliferation. Further, one class of endogenously expressed small noncoding RNAs, the so-called microRNAs (miRs), have gained considerable attention since they are able to regulate gene expression on the posttranscriptional level by binding to the mRNAs of their target genes, thereby inducing translational inhibition or mRNA degradation. Moreover, it is well known that miR expression is altered in various diseases and that deregulated miR levels can substantially contribute to disease progression. Suppression of pathological upregulated miRs by antagomirs may offer a new treatment area of PH. We have recently demonstrated that Antagomir-17 improves lung and heart function in experimental pulmonary hypertension by p21 mediated suppression of proliferation. Taken together, there is advanced research in the field of pulmonary vascular diseases and the efficacy of several new drugs is currently addressed in clinical trials.

Platelet concentrate extracellular vesicle (PLC-EV) subclasses are carriers of biomarkers involved in vascular/metabolic- and neurological disease

G. Schmitz, A. Pienimäki-Römer, K. Rübsaamen, A. Böttcher, M. Scherer, G. Liebisch.
Institute for Laboratory and Transfusion Medicine, University of Regensburg, Franz-Josef-Strauß-Allee 11, D-93053 Regensburg, Germany

Introduction: Concentration and composition of circulating extracellular vesicles (EVs) are critical in vascular/metabolic disease. EVs are heterogenous and either derived from exosomes, multivesicular bodies (MVBs), autophagic vesicles or surface membrane blebs. 70-90% of circulating EVs are derived from senescent or activated platelets. Methods: We established an *in vitro* model to monitor platelet senescence in stored human PLCs aimed for transfusion to analyze platelets, released EVs and surrounding autologous plasma over 5 days with multi-omics platforms. We isolated platelet-free EVs by differential centrifugation, filtration and purification of 5 EV-subclasses by density-gradient ultracentrifugation. Size characterization of EV subfractions was carried out in a NanoSight analyzer. Characterization of composition, compared to platelets and plasma, was carried out by lipid mass spectrometry, Western blot, protein mass spectrometry, microarray technology and deep sequencing. Results: Senescent platelets double EV-release, antagonized by Apo A-I/HDL. Platelets showed 4-fold elevation of the major lecithin-cholesterol-acyltransferase (LCAT) esterification product in plasma (cholesteryl ester, CE 18:2), due to uptake of plasma lipids and lipoproteins into the platelet open canalicular system, comprising 30% of the platelet volume. Ceramide (Cer) increased significantly in platelets, due to metabolic shift of the "Cer-sphingosine-1-phosphate rheostat" towards Cer, which may affect the platelet storage lesion, mimicking *in vivo* platelet dysfunction in vascular and metabolic disease. Compared to platelets and plasma, PLC-EVs enriched free cholesterol, sphingomyelin, dihydrosphingomyelin, plasmalogen-, Cer-, lysophosphatidylcholine (LPC)- and lysophosphatidic acid (LPA)-species. PLC-EV subfractions differentially accumulated, nucleic acid binding proteins, caveolin-1 and apolipoproteins I, J and E, predominantly expressed in subfractions 3-5, whereas mitochondrial marker proteins and cardiolipin are enriched in subfractions 4-5. The neurodegeneration-related proteins amyloid β precursor protein (APP) and α-synuclein are enriched in PLC-EV subfractions 3-4 and 1-2, respectively. MiRNA analysis of stored PLCs showed significant up-regulation of several miRNAs in platelets, of notice the autophagy marker mir-630 and angiogenesis-related mir-483-5p. Corresponding miRNAs were significantly increased in released EVs, which additionally enriched the sepsis marker mir-150 and coronary artery disease related mir-135a. Treatment of macrophages with PLC-EVs induced inflammatory M2 marker expression (e.g. CD163). Conclusion: PLC-EVs are carriers of lipid ligands for G-protein coupled signaling (LPC-, LPA-, Cer-species), precursors for inflammatory eicosanoid biosynthesis (plasmalogen species), apolipoproteins and miRNA, involved in vascular health, as well as regulate macrophage phenotype to orchestrate cellular processes involved in vascular/metabolic disease. As carriers of neurodegeneration-related cargo, PLC-EVs might be involved the regulation of neurological disorders. A population of PLC-EVs might be released as autophagic vesicles.

Lymphangiogenesis in fish and mice - similarities and discrepancies

Stefan Schulte-Merker. Hubrecht Institute, Utrecht, The Netherlands

The lymphatic vasculature serves key functions in fluid homeostasis within the body. It absorbs fluid and macromolecules from the interstitium, it takes up dietary lipids from the small intestine, and it provides a trafficking route for cells of the immune system. The architecture of the lymphatic vessel system is quite different from the one of the blood vasculature: it is blind-ending with uni-directional flow, and its endothelium is specialized to allow uptake of macromolecules and other substances. Perturbations in the function of the lymphatic system can be primary (hereditary) or secondary (acquired through parasite infection or surgery), and lead to disfigurating lymphedema formation.
The molecular cues that govern the development and the physiological functioning of the lymphatic vessels are very poorly understood, particularly when compared to the knowledge we have of blood vessels within the circulatory system.
Our work in zebrafish has identified the ontogeny of lymphatic endothelial cells in this model organism. Comparative studies with murine lymphatic development enables us now to compare the molecular and cellular events that govern lymphangiogenesis in both species, and recent insights concerning this process will be discussed.

Hypoxia and cancer cell adaptation to metabolic stress

M. Celeste Simon. University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA 19104-6160, USA

Solid tumors exhibit heterogeneous microenvironments, often characterized by limiting concentrations of oxygen (O2), glucose, and other nutrients. How oncogenic mutations alter stress response pathways, metabolism, and cell survival in the face of these challenges is incompletely understood. Here we report that constitutive mTORC1 activity renders hypoxic cells dependent on exogenous desaturated lipids, as levels of de novo synthesized unsaturated fatty acids are reduced under low O2. Specifically, we demonstrate that hypoxic Tsc2-/- cells deprived of serum lipids exhibit a magnified UPR response, but fail to appropriately expand their ER, leading to IRE1-dependent cell death that can be reversed by the addition of unsaturated lipids. UPR activation and apoptosis were also observed in Tsc2-deficient kidney tumors. Importantly, we observe this phenotype in multiple human cancer lines and suggest that cells committed to unregulated growth within ischemic tumor microenvironments are unable to balance lipid synthesis due to a critical limitation in desaturated lipids.

Integrative signaling in arteriogenesis

Michael Simons. RW Berliner Professor of Medicine and Cell Biology Chief, Section of Cardiovascular Medicine Yale University School of Medicine New Haven, CT, 06510, USA

Formation of arterial vasculature, during development and in adult tissues, requires multiple steps that all need to be integrated with each other. A number of signaling pathways coordinate this process including VEGF/VEGF-R and VEGF-Neuropilin signaling, FGF, PDGF, Notch/Delta, and many others signaling pathways. Recent studies have begun shedding light on how these various signaling inputs are integrated and coordinated. This will be the subject of the presentation.

Vascular basement membranes and their contribution to microvessel integrity

Lydia Sorokin. Physiological Chemistry and Pathobiochemistry; Muenster University, Germany.

The biochemical compositional of basement membranes (BM) varies with both blood vessel and with tissue type. Of all BM components, the laminin family shows the greatest variability and represents the biological active component of BMs, interacting with a wide repertoire of integrin and non-integrin receptors to control functions such as vessel integrity and permeability. Focus will be on the central nervous system (CNS) microvessels, which have a unique composition of cellular and extracellular matrix layers that collectively constitute the blood-brain barrier. In addition to the endothelial cell monolayer and its underlying BM, cerebral microvessels are ensheathed by astrocyte endfeet and leptomeningeal cells, which contribute to a second BM, the so-called parenchymal BM as it delineates the border to the brain parenchyma. At the level of capillaries these two BMs fuse to form a single structure, which shares characteristics of both endothelial and parenchymal BMs. While considerable information is available on the cellular constitutents of the CNS microvessels and their contribution to the BBB, little is known about the BM layers. Our work has shown that endothelial and parenchymal BMs of CNS vessels are structurally and functionally distinct, and has highlighted their importance in the restricted permeability characteristic of the CNS microvessels. In particular, laminin isoforms are heterogeneously localized along the length of CNS microvessels and play an important role in defining sites of high and low penetrability by infiltrating cells, such as extravasating leukocytes during inflammation1. Data will be presented on the biochemical differences of BMs of CNS microvessels, and how vascular laminins provide cues that determine mechanisms of leukocyte penetration of CNS postcapillary venules1.

1. Wu, C., F. Ivars, P. Anderson, R. Hallmann, D. Vestweber, P. Nilsson, H. Robenek, K. Tryggvason, J. Song, E. Korpos, K. Loser, S. Beissert, E. Georges-Labouesse, & L.M. Sorokin. 2009. Nat Med. 15, 519-27

Sphingosine Kinase 1 and sphingosine-1-Phosphate involvement in Angiogenesis and Lymphangiogenesis during breast cancer progression

Masayuki Nagahashi, Akimitsu Yamada, Sheldon Milstien, Kazuaki Takabe and Sarah Spiegel. Division of Surgical Oncology, and Department of Biochemistry and Molecular Biology, and the Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, Virginia USA

Sphingosine-1-phosphate (S1P) is a potent bioactive lipid mediator that promotes breast cancer progression by diverse mechanisms that remain somewhat unclear. In this lecture I will present our work showing a critical role for sphingosine kinase 1 (SphK1), one of the isoenzymes producing S1P in tumor-induced hemangiogenesis and lymphangiogenesis. In a murine model of breast cancer metastasis, we found that S1P levels increased both in the tumor and the circulation. Interestingly, serum S1P levels were significantly elevated in stage IIIA human breast cancer patients, compared with age/ethnicity-matched healthy volunteers. We also found that a specific SphK1 inhibitor, SK1-I, suppressed S1P levels, reduced metastases to lymph nodes and lungs, and decreased overall tumor burden. Both S1P and angiopoietin 2 stimulated hemangiogenesis and lymphangiogenesis in vitro, and SK1-I inhibited both. We quantified both processes in vivo from the same specimen by combining directed in vivo angiogenesis assays with fluorescence-activated cell sorting, thereby confirming the results obtained in vitro. Notably, SK1-I decreased hemangiogenesis and lymphangiogenesis not only at the primary tumor but also in lymph nodes, with peritumoral lymphatic vessel density reduced in SK1-I–treated animals. Our findings indicate that SphK1-produced S1P is a crucial mediator of breast cancer–induced hemangiogenesis and lymphangiogenesis and implicate SphK1 and S1P as therapeutic targets in breast cancer.

This work was supported by NCI grant R01CA61774 to S. Spiegel and NIH grant K12HD055881 and Susan G. Komen for the Cure Research Foundation Career Catalyst Research Grant KG090510 to K. Takabe

Development of form and function of the zebrafish heart

Didier Stainier. UCSF, San Francisco, CA, USA

Our work is concerned with the formation, function and homeostasis of organs during vertebrate development. We are interested in understanding the cellular and molecular events that underlie cellular differentiation, tissue morphogenesis and organ function during the formation of the cardiovascular system (the heart and the blood vessels) as well as the liver and pancreas. One approach consists of screening for mutations that affect these processes in zebrafish, a vertebrate model system that allows forward genetics as well as embryological studies. We also utilize the tools of chemical genetics to identify pathways that regulate these processes, taking advantage of the high-throughput methods available in zebrafish.
This talk will focus on cardiac development and function, specifically on the differentiation and behavior of myocardial cells during the formation of the trabeculae, sheet-like muscular structures within the ventricles of the heart. Despite the important role of trabeculae in the development and physiology of the heart, little is known about their mechanism of formation. Live imaging of zebrafish hearts and lineage-tracing data suggest that 1) individual myocytes form the trabecular layer via directed delamination, and 2) this delamination occurs via apical constriction coupled to the extension of membrane protrusions luminally.

Hematopoietic stem cells in the hypoxic niche

Toshio Suda. Keio University School of Medicine, Tokyo, Japan

Hematopoietic stem cells (HSCs) are sustained in a specific microenvironment known as the stem cell niche. Adult HSCs are kept quiescent during the cell cycle in the endosteal niche of the bone marrow. Normal HSCs maintain intracellular hypoxia, stabilize the hypoxia-inducible factor-1α (HIF-1α) protein and generate ATP by anaerobic metabolism. In HIF-1α-deficiency, HSCs became metabolically aerobic, lost cell cycle quiescence, and finally exhausted. An increased dose of HIF-1α protein in VHL mutated HSCs and their progenitors induced cell cycle quiescence and accumulation of HSCs in the BM (Cell Stem Cell, 2010). Restored glycolysis by pyruvate dehydrogenase kinases (PDKs) ameliorated cell cycle quiescence and stem cell capacity. HSCs directly utilize the hypoxic microenvironment to maintain their cell cycle by HIF-1α-dependent metabolism by down-regulating reactive oxygen species (ROS). The abnormal hematopoiesis was also detected in PDK2 and PDK4-double KO mice. In this presentation, we will discuss the importance of oxygen homeostasis and energy metabolism for maintenance of HSC function and long-term self-renewal.

Requirement for endothelial stem-like cells and involvement of the Tie2-APJ axis for mature blood vessel formation

Nobuyuki Takakura. Department of Signal Transduction, Research Institute of Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan, JST, CREST, K's Gobancho, 7, Gobancho, Chiyoda-ku, Tokyo, 102-0076 Japan

The regulation of blood vessel maturation is very important in many vascular diseases including both vascular proliferative diseases and ischemic diseases. For example, well-organized permeability or improvement of hypoxia by the normalization/maturation of the tumor vasculature permits cancer drugs to penetrate better into the tumor parenchyma or increases sensitivity to radiation therapy, respectively. Moreover, without induction of maturation, newly-developed blood vessels in therapeutic angiogenesis will regress. Postnatal neovascularization is mainly accomplished by angiogenesis, i.e., new blood vessel formation from pre-existing vessels. Accumulating evidence suggests that cells from many sources such as bone marrow and mesenchymal stem cells might be involved in such new vessel formation. However, cells that are responsible for constructing mature blood vessels in angiogenesis have not yet been identified. Here, we report the presence of stem/progenitor-like ECs in preexisting blood vessels using a method in which stem cell populations are identified as side populations that efflux the DNA dye, Hoechst 33342. These EC stem/progenitor cells are included within the population already committed to the EC lineage, are quiescent in the steady state but self-renew under tissue hypoxia, are not derived from bone marrow and are not identical to EPCs. They produce large numbers of mature ECs to generate long-term surviving mature blood vessels when injected into ischemic limbs. Focusing on cellular mechanisms of mature blood vessel formation, here we discuss the role of the angiopoietin/Tie2 and apelin/APJ axes in mature blood vessel formation and the usefulness of apelin for tissue regeneration in therapeutic angiogenesis as well as for normalization of the tumor vasculature in cancer treatments.

Novel functions of HDL relevant to atherogenesis

Alan R. Tall. Department of Medicine, Columbia University, New York, USA

Increased levels of leukocytes and platelets are associated with a greater incidence of athero-thrombotic diseases. Low HDL levels are also associated with accelerated atherosclerosis. Recent findings suggest that HDL-mediated cholesterol efflux may be linked to control of the production of leukocytes and platelets in the bone marrow. HDL and apoA-1 mediate cholesterol efflux via the activity of specific ATP binding cassette transporters. ABCA1 promotes cholesterol efflux to lipid poor apoA-1 while ABCG1 and ABCG4 promote cholesterol efflux to HDL particles (1). ABCA1 and ABCG1 are highly expressed in macrophage foam cells where they play an athero-protective role. These transporters are also highly expressed in hematopoietic stem and multipotential progenitor cells (HSPCs) in the bone marrow where they act to control HSPC proliferation (2). Mice with knockouts of ABCA1/G1 develop a myeloproliferative disorder with dramatic monocytosis and neutrophilia and accelerated atherosclerosis. In contrast, ABCG4 is highly expressed only in megakaryocyte progenitor cells and controls their proliferation by promoting cholesterol efflux to HDL. Thus ABCG4 deficient mice develop increased numbers of megakaryocytes, thrombocytosis and accelerated atherosclerosis. Treatments that lead to increased levels of HDL, such as by infusions of reconstituted HDL, suppress WBC and platelet production in these models (3), suggestive a novel anti-atherogenic action of HDL treatments.

Refs: (1) Wang, N et al, PNAS 2004; (2) Yvan-Charvet L et al, Science 2010; (3). Murphy, A et al, JCI 2011.

Semaphorin signals controlling tumor angiogenesis and metastatic spreading

Luca Tamagnone. University of Torino – Institute for Cancer Research, Candiolo, Italy

Semaphorins and their receptors, Neuropilins and Plexins, were originally implicated as developmental guidance signals for neuronal processes. Later on it was shown that they can regulate neural crest migration in the embryo and developmental angiogenesis. Importantly, a growing body of evidence indicates that semaphorins may furthermore regulate tumor progression, by promoting or inhibiting angiogenesis, cancer cell migration, invasiveness, and metastatic spreading. For example, we demonstrated that Semaphorin 3A (SEMA3A) inhibits primary tumor growth and metastatic dissemination by disrupting tumor vasculature. In addition, Sema3A directly targets cancer cells, via neuropilin-1, and inhibits their migration and metastatic dissemination in vivo.

We found that another secreted semaphorin known to regulate angiogenesis, SEMA3E, is abundantly expressed in metastatic human tumors, where it promotes cancer cell migration and invasion, and extravasation in vivo (in mouse models), leading to metastatic spreading. Notably, these biological activities of SEMA3E depend on its proteolytic conversion by furin-like convertases, generating the active fragment “p61”-SEMA3E. In cancer cells, p61-SEMA3E induces the association of the receptor PlexinD1 with the oncogenic tyrosine-kinase receptor ErbB2, leading to ErbB2 activation and enhanced tumor invasion and metastasis. Notably, a mutated uncleavable variant of SEMA3E (Uncl-SEMA3E) binds the receptor PlexinD1 but lacks any pro-metastatic activity, thereby acting as a dominant negative ligand in cancer cells. On the other hand, Uncl-SEMA3E is able to trigger PlexinD1 signaling in endothelial cells leading to strong anti-angiogenic effects in vitro and in vivo, including in tumor models refractory to drugs blocking VEGF signaling. Thus Uncl-Sema3E delivered in mice bearing spontaneous or transplanted tumor models concomitantly achieves inhibition of tumor growth and metastatic spreading, by independently targeting cancer and endothelial cells

Regulation of endothelial junctions

Dietmar Vestweber. Max-Planck-institute of Molecular Biomedicine, Muenster, Germany

The formation of endothelial junctions and their regulation in patho-physiological processes is regulated by complex mechanisms that modulate the actomyosin based cytoskeleton and cell adhesion activities at endothelial contacts. The stability of endothelial junctions is centrally dependent on the adhesive function of VE-cadherin. We have recently shown that knock in mice expressing a fusion protein consisting of VE-cadherin and α-catenin instead of wt VE-cadherin are strongly impaired in VEGF- or histamine-induced vascular permeability and in cytokine-induced recruitment of leukocytes into various inflamed tissues. VE-cadherin-α-catenin associated more efficiently with the actin cytoskeleton than VE-cadherin. These results demonstrate that it is in vivo necessary to interfere with the adhesive function of VE-cadherin in order to enhance vascular permeability or to enable leukocytes to extravasate.
In vitro studies have suggested that the phosphorylation of certain tyrosine residues in VE-cadherin is involved in the induction of transendothelial permeability and transmigration of leukocytes through cultured endothelial cell layers. In agreement with this, we found that the endothelial specific receptor type protein tyrosine phosphatase, VE-PTP, associates with VE-cadherin and supports its adhesive function. Induction of permeability and transmigration of leukocytes was accompanied by the dissociation of VE-PTP from VE-cadherin.
In vivo we showed recently that preventing the dissociation of VE-PTP from VE-cadherin strongly impaired induction of permeability and leukocyte extravasation, demonstrating that tyrosine phosphorylation of VE-cadherin and/or other associated proteins is involved in the regulation of both processes in vivo.
To analyze the relevance of tyrosine phosphorylation of VE-cadherin for the regulation of endothelial junctions in vivo we have generated knock in mice where certain tyrosine residues in VE-cadherin were replaced by phenylalanines. In addition, we generated specific antibodies for each of these different phosphorylated tyrosines within VE-cadherin. Surprisingly, we found differential regulation of the phosphorylation of these residues in vivo. Furthermore, analysis of the induction of vascular permeability and leukocyte recruitment in the different knock in mice revealed distinct roles for different tyrosine residues.

Vascular malformations and lymphedema: Etiopathogenic mechanisms

Miikka Vikkula. Laboratory of Human Moleculer Genetics, de Duve Institute, Université catholique de Louvain, Brussels, Belgium

Vascular malformations are localized errors of vascular development, whereas primary lymphedema is a more generalized swelling, commonly encountered on lower extremities. Both can be congenital, i.e. present at birth, or develop soon after birth. They usually develop slowly with the child. Vascular malformations often affect the skin, but also other organs, such as the liver, intestine and the brain. Most of them occur sporadically, but some as part of a syndrome or as an inherited disorder.

Venous malformations are the most commonly seen vascular malformations in specialized multidisciplinary centers. Two familial forms exist: VMCM, for mucocutaneous venous malformation, and GVM, for glomuvenous malformation. We unraveled that these forms are caused by dominant activating TIE2 mutations (VMCM) or loss-of-function glomulin mutations (GVM). We have now also identified the cause for sporadically occurring Blue Rubber Bleb Nevus syndrome (BRBN).

Primary lymphedema is also a chronic disease, with lifelong morbidity. Many familial types have been reported. For eight of them, the causative mutated gene has now been unraveled. Yet, they only explain about 40% of primary hereditary lymphedema, and thus other factors must be involved.

The large clinical variability regarding the location, number and size of inherited vascular lesions, made us hypothesize that a somatic second-hit is needed for lesions to develop, as suggested by Knudson for retinoblastoma. We have now extensive proof to support this. Moreover, our extensive tissular screens have identified local, somatic genetic defects that cause 50% of the most common sporadic form of venous malformations. These mutations differ from the inherited ones in regard to downstream transcriptomic profiles. These data highlight the importance of assessing for tissue-based acquired genetic changes, as possible pathophysiological causes, which have been largely overlooked in developmental disorders. Large-scale somatic screens are likely essential in uncovering the nature and prevalence of such changes, and their downstream effects.

(miikka.vikkula@uclouvain.be; http://www.deDuveInstitute.be/vikkula)

The C-type lectin receptor CLEC-2 regulates development of the lymphatics and is required for integrity of the blood – cerebrospinal fluid barrier

Steve Watson. Centre for Cardiovascualar Sciences, The College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, United Kingdom

The platelet C-type lectin receptor CLEC-2 signals through a pathway that is critically dependent on the tyrosine kinase Syk following clustering induced by its endogenous ligand, podoplanin, or by a specific antibody or the snake venom toxin, rhodocytin. Clustering of CLEC-2 leads to Src kinase- and Syk-dependent phosphorylation of a conserved tyrosine residue in the cytosolic chain of the C-type lectin receptor. Binding of the tandem SH2 domains of Syk to the phosphorylated hemITAM sequence in two CLEC-2 receptors leads to its activation and initiation of a downstream cascade that culminates in activation of PLC□2 and powerful platelet stimulation. The functional role of CLEC-2 and Syk in this pathway is revealed through the generation of mutant mice (Finney et al., 2012). Homozygous loss of CLEC-2 or Syk results in defects in brain vascular and lymphatic development, lung inflation and perinatal lethality. Furthermore, deletion of Syk in the haematopoietic lineage, or conditional deletion of CLEC-2 or Syk in the megakaryocyte/platelet lineage, also causes defects in brain vascular and lymphatic development, although the mice are viable. A similar pattern of defects is seen in mice containing a point mutation in the first of the two SH2 domains of Syk thereby preventing clustering of CLEC-2. To address the mechanism of this defect, we show that platelets modulate the migration and intercellular adhesion of lymphatic endothelial cells through a pathway that is dependent on CLEC-2 and Syk. These studies demonstrate that megakaryocyte/platelet expression of CLEC-2 and Syk is required for normal brain vasculature and lymphatic development and that platelet CLEC-2 and Syk modulates lymphatic endothelial cell behaviour in vitro.

Finney, B.A., Schweighoffer, E., Navarro-Núñez, L., Bénézech, C., Barone, F., Hughes, C.E., Langhan, S., Lowe, K.L., Pollitt, A.Y., Mauro-Sa, D., Sheardown, S., Nash, G.B., Smither, N., Reis e Sousa, C., Tybulewicz, V.L.J. and Watson, S.P. (2012) CLEC-2 and Syk in the megakaryocytic/platelet lineage are essential for development. Blood 119:1747-1756

This work was supported by the Wellcome Trust and British Heart Foundation

Role of dendritic cells in atherosclerosis

Alma Zernecke. Department of Vascular Surgery, Klinikum rechts der Isar, Technical University Munich, Munich, and Rudolf-Virchow-Center/DFG Research Center for Experimental Biomedicine, University of Wuerzburg, Würzburg, Germany

Chronic inflammation drives the development of atherosclerosis. Dendritic cells (DCs) are known as central mediators of adaptive immune responses and the development of immunological memory and tolerance. DCs are present in non-diseased arteries, and accumulate within atherosclerotic lesions where they can be localized in close vicinity to T cells. However, the exact role of DCs in atherosclerosis remains elusive.

We have shown that the DC-derived chemokine CCL17 is present in advanced human and mouse atherosclerosis and that CCL17-expressing DCs accumulate in atherosclerotic lesions. In atherosclerosis-prone mice, Ccl17 deficiency entailed a reduction of atherosclerosis, which was dependent on Tregs. Expression of CCL17 by DCs limited the expansion of Tregs by restricting their maintenance and precipitated atherosclerosis in a mechanism conferred by T cells. Conversely, a blocking antibody specific for CCL17 expanded Tregs and reduced atheroprogression. These data identified DC-derived CCL17 as a central regulator of Treg homeostasis, and implicated effector functions of DCs as important mediators in atherogenesis.

But also phenotypically distinct plasmacytoid dendritic cells (pDCs) have been identified within atherosclerotic lesions. pDCs are specialized to produce type-I interferons in response to pathogenic single-stranded nucleic acids but can also sense self-DNA released from dying cells or in neutrophil extracellular traps (NETs) complexed to the antimicrobial peptide Cramp/LL37 in autoimmune disease. We have shown that self-DNA, *e.g*. released from dying cells or in NETs, and an increased expression of the antimicrobial peptide Cramp in atherosclerotic lesions can stimulate a pDC-driven pathway of autoimmune activation and the generation of anti-ds-DNA antibodies, critically aggravating atherosclerosis lesion formation.

A complex equilibrium and interplay between immune-cell subpopulations and DC effector functions contribute to the process of atherosclerosis. CCL17 but also targeting pDC activation may offer novel therapeutic approaches to limit atherosclerotic lesion development.

ORAL PRESENTATION ABSTRACTS

UNDERLINED NAME = PRESENTER; * = POSTER PRESENTER

Functional human endothelium for personalized cell based assays

William J. Adams[1,2,3], Yuzhi Zhang[1,2], Jennifer Cloutier[1,2], Pranati Kuchimanchi[1,2], Gail Newton[2], Seema Sehrawat[2], Tanya N. Mayadas[2], F. William Luscinskas[2], Guillermo García-Cardeña[1,2]. [1]Program in Developmental and Regenerative Biology, Harvard Medical School, Boston, MA 02115 USA, [2]Center for Excellence in Vascular Biology, Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115 USA, [3]School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, 02138 USA

The endothelium is a diverse, multifunctional interface that displays dynamic cellular plasticity critical for vascular homeostasis. Dysfunction of the endothelium plays a significant pathological role in cardiovascular disease, in the vascular complications common in patients with diabetes mellitus and chronic kidney disease and has been associated with the pathogenesis of systemic lupus erythematous, Kawasaki's disease and several hereditary vascular malformations. Notably, there is limited availability of relevant functional endothelia for patient, anatomical location, and disease-specific research or therapeutic discovery. Recently, however, induced pluripotent stem cells (iPSC) have been differentiated into a variety of cell types offering a new source of personalized tissue. But, before their scientific and therapeutic potential can be realized, the fidelity and functionality of stem cell-derived tissues must be carefully assessed. To this end, we have reproducibly derived endothelial cells from human iPSC and investigated whether this endothelium is able to perform the complex functions of adult endothelium using cell-based assays translatable to high throughput technologies. We documented temporal expression profiles of genes critical for pluripotency, mesoderm and vascular commitment throughout differentiation from iPSC to endothelium within embryoid bodies. An emerging endothelial population resided in vessel-like structures, which we isolated documenting that it had typical endothelial morphology and molecular markers. This endothelium possessed Weibel-Palade bodies containing von Willibrand Factor and rapidly expressed P-selectin on the cell surface in response to histamine. Pro-inflammatory stimuli, such as LPS, TNF-alpha, and IL-1beta, induced E-selectin, ICAM1 and VCAM1 surface expression, secretion of pro-inflammatory cytokines IL8, MCP1, and RANTES, and caused human neutrophils and T lymphocytes to roll, arrest, and transmigrate. The endothelium maintained a dynamic barrier responsive to VEGF, prostaglandin-E2, histamine, and sphingosine-1-phosphate, measured by transendothelial electrical resistance. Biomechanical stimulation with atheroprotective shear stress induced an anti-inflammatory response, increasing expression of KLF2 and its downstream targets. In contrast, atheroprone shear stress increased pro-inflammatory gene expression. Statin treatment induced expression of atheroprotective genes associated with the pleiotropic effects of statins, including KLF2, and eNOS, while decreasing Ang-2 and ET-1. Importantly, the iPSC-derived endothelium is unique for its lack of anatomical origin. Therefore, to investigate whether this endothelium can be differentiated into specific endothelial subtypes, we exposed these endothelial cells to shear stress waveforms derived from a human abdominal aorta or saphenous vein. Arterial shear stress upregulated expression of several markers of arterial identity including, EphrinB2, Hey1/2, KDR, Notch4, and VEGF. Moreover, VEGFC treatment was able to upregulate expression of markers of lymphatic endothelium, LYVE1 and Sox18. These data suggest that iPSC-derived endothelium displays plasticity for the acquisition of distinct endothelial identities. Collectively, we have shown that human iPSC-derived endothelium is capable of complex dynamic functionality. Establishing iPSC as a new source of endothelium, able to be generated from specific patients and genetic backgrounds into several endothelial subtypes, is a critical step towards research on genetic disorders and the vasculature, the personalization of cardiovascular medicine and future therapeutic discovery. Ongoing studies are coupling functional assays with endothelium derived from specific genetic disorders with the ultimate goal of elucidating new mechanisms of disease.

Procontractile G-protein-mediated signaling pathways antagonistically regulate smooth muscle differentiation in vascular remodeling

Till F. Althoff[1], Julián Albarrán-Juárez[1], Shengpeng Wang[1], Angela Wirth[1,2], Mikito Takefuji[1], Nina Wettschureck[1], Stefan Offermanns[1,3]. [1]Department of Pharmacology, Max-Planck-Institute for Heart and Lung Research, Ludwigstr. 43, 61231 Bad Nauheim, Germany, [2]Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld INF366, 60120 Heidelberg, Germany, [3]Medical Faculty, Goethe University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

Vascular smooth muscle cells (VSMC) can switch between differentiated and proliferative phenotypes. Their differentiation state can be regulated through serum response factor (SRF) which activates genes involved in smooth muscle differentiation and proliferation by recruiting cofactors, such as members of the myocardin family and ternary complex factors (TCF), respectively. However, the extracellular cues and upstream signaling mechanisms regulating SRF-dependent VSMC differentiation under in vivo conditions are poorly understood. The receptors of most vasocontractile stimuli acting on VSMCs, including angiotensin-II, sphingosine-1-phosphate, thrombin, thromboxane A2 or endothelin-1, are dually coupled to the G-proteins Gq/G11 and G12/G13, that synergistically induce VSMC contraction through distinct signaling pathways. Here we show that these pro-contractile signaling pathways mediated by G12/G13 and Gq/G11, antagonistically regulate VSMC plasticity in different models of vascular remodeling. In the media of arterial vessels from smooth muscle specific Gα12/Gα13-deficient mice, we detected a decreased expression of smooth muscle marker genes, as well as of the smooth muscle-enriched microRNAs 143 and 145. In addition, we observed an exaggerated downregulation of smooth muscle marker genes in Gα12/Gα13-deficient VSMCs, in response to carotid artery ligation or femoral artery injury. This was accompanied by a dramatically enhanced vascular remodeling. Moreover, smooth muscle specific Gα12/Gα13-deficiency promoted VSMC dedifferentiation, as well as plaque-progression in the ApoE-/--model of atherosclerosis. In contrast, vessels from mice lacking Gαq/Gα11 specifically in VSMCs, displayed a normal basal expression of smooth marker genes and an attenuated downregulation in response to carotid artery ligation or femoral artery injury. Of note, SMC-specific Gαq/Gα11-deficiency protected mice from neointimal hyperplasia in both models. We found that the activity of RhoA in the arterial media increases upon carotid artery ligation. However, this increase was absent in mice lacking Gα12/Gα13 or their effector, the RhoGEF protein LARG, specifically in SMCs. Interestingly, LARG-deficient mice also showed exaggerated SMC dedifferentiation as well as excessive neointimal and medial hyperplasia, indicating that G12/G13-mediated differentiation of VSMCs in vivo involves LARG-dependent activation of RhoA. In line with cell based studies demonstrating that recruitment of cofactors of the myocardin family by SRF is regulated by RhoA, we found that G12/G13-mediated signaling is required for transcriptional activation of myocardin as well as nuclear translocation of myocardin related transcription factor A. On the other hand we found that, upon carotid artery ligation, Gq/G11- but not G12/G13-mediated signaling induces the consecutive phosphorylation of the MAP-kinase ERK1/2 and its substrate, the TCF Elk-1, resulting in transcriptional activation of the early response genes c-fos, ets-1 and egr-1, thereby repressing VSMC differentiation. This data indicate that Gq/G11 and G12/G13 antagonistically regulate VSMC differentiation by controlling the recruitment of transcriptional cofactors by SRF. Our in vivo studies in different models of vascular disorders demonstrate that the balanced activity of both pathways in VSMCs controls the remodeling response of the vessel in vascular diseases. The opposite regulation of VSMC gene expression by the two pathways may allow for modulation of VSMC differentiation under pathological conditions by biased GPCR ligands or by inhibitors of G-protein-mediated signaling processes.

Effective treatment of edema and endothelial barrier dysfunction with imatinib

Jurjan Aman[1], Jan van Bezu[1], Stephen M. Vogel[2], Stephan Huveneers[3], Etto Eringa[1], Anton Vonk Noordegraaf[4], A.B. Johan Groeneveld[5], Victor W.M. van Hinsbergh[1], Geerten P. van Nieuw Amerongen[1]. [1]Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands, [2]Department of Pharmacology, University of Illinois at Chicago, Chicago, Il, United States, [3]Hubrecht Institute, University Medical Center Utrecht, Utrecht, The Netherlands, [4]Department of Pulmonary Diseases, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands, [5]Department of Intensive Care, Erasmus Medical Center, Rotterdam, The Netherlands

Introduction Endothelial barrier dysfunction and vascular leak are significant pathogenic phenomena. Although contributing to life-threatening conditions like sepsis, they currently lack specific therapy. In a recent case-report we reported fast resolution of pulmonary edema upon treatment with the tyrosine kinase inhibitor imatinib (Overbeek, Eur Respir J, 2008). We hypothesized that imatinib protects against vascular leak and edema formation by enhancing endothelial barrier integrity. Methods & Results The direct effect of imatinib on endothelial barrier function was assessed by macromolecule passage and electrical resistance over human endothelial monolayers during endothelial activation with thrombin or histamine. Pretreatment with imatinib dose-dependently (optimal concentration 10μM) attenuated the thrombin-induced macromolecule passage over endothelial monolayers (40% reduction, $P<0.001$, n=4), and reduced the maximal drop in electrical resistance during thrombin and histamine stimulation (24% reduction, $P<0.001$, n=5, and 37% reduction, $P=0.01$, n=4, respectively) in macro- and microvascular endothelial cells. To find out via which kinase imatinib exerts its protective effect, we performed a systematic siRNA knock-down of the imatinib-sensitive kinases (c-Abl, Abl-related gene [Arg], PDGFR, DDR-1 and c-KIT). Only Arg knock-down mimicked the barrier-protective effects of imatinib during thrombin stimulation (22% reduction of macromolecule passage, $P<0.01$, n=4; 22% reduction of the maximal drop in electrical resistance, $P=0.01$, n=4). Because c-Abl, PDGFR, DDR-1 and c-KIT depletion did not affect the thrombin response, and because imatinib had no additive protective effect in Arg depleted cells, imatinib exerts its protective effects predominantly via inhibition of Arg. Measuring Arg activity by phosphorylation of Crk-L at Tyr207 (a surrogate marker of Arg activity) revealed that thrombin stimulation led to fast (within 2min) and robust Arg activition. Similarly, endothelial stimulation with the permeability factors VEGF and histamine enhanced Arg activity. Imatinib limited Arg-mediated endothelial barrier dysfunction by enhancing Rac1 activity and enforcing adhesion of endothelial cells to the extra-cellular matrix, demonstrated by enhanced formation of paxillin-containing focal adhesions in particular at the cell periphery. The protective effect of imatinib was independent of calcium- and RhoA/Rho kinase-mediated signaling pathways, as inhibition of these pathways had additive protective effects to imatinib treatment alone. In vivo, imatinib attenuated VEGF-induced vascular leakage in murine skin as measured by Evans Blue extravasation (50% reduction, $P<0.001$, n=4), and inhibited edema formation in the lung (66% reduction, $P<0.01$, n=6), as measured in the isolated perfused mouse lung model. Conclusion These data demonstrate that imatinib protects against endothelial barrier dysfunction and vascular leakage at clinically relevant concentrations and under various conditions. Furthermore, the tyrosine kinase Arg was identified as an entirely novel mediator of endothelial barrier dysfunction. Altogether, this study indicates imatinib as a potential candidate for future treatment of vascular leakage and edema. Supported by NHF grants #2003T3201 (JA)

SR-BI expressed by lymphatic vessels is required for removal of cholesterol from peripheral tissues

Hwee Ying Lim[1]*, Chung Hwee Thiam, Kim Pin Yeo, Chung Shii Hii, Kristine CY McGrath[2,3], Alison Heather, J. Steven Alexander[4], and Véronique Angeli[1]. [1]Dept. of Microbiology, Immunology Programme, Yoon Loo Lin School of Medicine, National University of Singapore, Singapore, [2]School of Medical and Molecular Biosciences, University of Technology, Sydney, Australia, [3]Heart Research Institute, Sydney, Australia, [4]Department of Molecular and Cellular Phyisology, LSU Health Sciences Center-Shreveport, USA

The lymphatic and blood vessels function to maintain tissue fluid homeostasis. With the exception of immune cell entry, the prevailing view is that the lymphatic vasculature plays a passive role in the uptake of fluid and macromolecules from the periphery. Of interest, high-density lipoprotein (HDL) macromolecules transport excess cholesterol from the peripheral tissues back to the bloodstream, a process referred to as reverse cholesterol transport (RCT). Although several lines of evidence support the role of lymphatics in RCT and less so via the venous capillaries, it has not been directly demonstrated. Here we sought to determine whether lymphatic vessels are essential in RCT and the mechanisms underlying cholesterol transport by lymphatics. Using fluorescent-labelling methods, we show that cholesterol and its major vehicle, HDL are normally transported by peripheral lymphatic vessels and, disruption of lymphatic drainage results in lipoprotein accumulation in tissues and reduced RCT. Lymphatic endothelial cells (LECs) express HDL transporters including adenosine tri-phosphate binding cassette receptor A1 (ABCA1) and scavenger receptor class B type I (SR-BI). Silencing RNA interference against SR-BI but not ABCA1 potently abrogated HDL uptake by LECs. Blocking SR-BI with neutralizing antibody prevented in vitro HDL uptake by LECs and reduced HDL transport by lymphatic vessels in vivo. Previously, using apolipoprotein-E deficient (apoE-/-) mice as a model of dyslipidemia, we reported that hypercholesterolemia is associated with impaired lymphatic drainage and increased lipid accumulation in peripheral tissues. We now demonstrate that restoring lymphatic drainage in these mice significantly improved lipid clearance. Collectively, this study challenges the current view that lymphatic endothelium is a passive exchange barrier for cholesterol clearance and provide further evidence for its interplay with lipid biology in health and disease.

Angiopoietin-2 differentially regulates angiogenesis through Tie2 and integrin signaling

Felcht M[1,2], Schering A[1*], Seidel P[1], Srivastava K[1], Hu J[1], Bartol A[1,3], Kienast Y[4], Vettel C[5], Loos EK[1], Kutschera S[1,3], Bartels S[1], Appak S[1,3], Besemfelder E[1], Terhardt D[1], Chavakis E[6], Wieland T[5], Klein C[4], Thomas M[4], Uemura A[7], Goerdt S[2], Augustin HG[1,3]. [1]Division of Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Heidelberg, Germany, [2]Department of Dermatology, Venerology, and Allergology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [3]Department of Vascular Biology and Tumor Angiogenesis (CBTM), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [4]Roche Diagnostics GmbH, Penzberg, Germany, [5]Institute of Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [6]Department of Internal Medicine, Cardiology and Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, Goethe University, Frankfurt am Main, Germany, [7]Division of Vascular Biology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan

The Tie2 ligand Angiopoietin-2 (Ang-2) exerts context-dependent effects on endothelial cells (EC). It acts as a negative regulator of Ang-1/Tie2 signaling during angiogenesis and vessel maturation thereby controlling the responsiveness of EC to exogenous cytokines. Ang-2 may under certain conditions also act as pro-angiogenic molecule. The molecular mechanisms of differential Ang-2 functions are poorly understood which poses a critical limitation in the rational exploitation of Ang-2 as a target of anti-angiogenic therapy. We show here that the activated endothelium during angiogenesis harbors a subpopulation of Tie2-negative EC (Tie2low). As such, Tie2 and angiogenic EC integrins are differentially expressed by sprouting tip cells and remodeling stalk cells. Ang-2 binds to αvβ3, αvβ5 and α5β1 integrins in Tie2low EC, subsequently inducing in a Tie2-independent manner FAK phosphorylation at Tyr397, Rac1 activation, migration and sprouting angiogenesis. Correspondingly, Ang-2 blockade in vivo interferes with integrin signaling and inhibits FAK[Tyr397] phosphorylation and sprouting angiogenesis of Tie2low EC. The data establish a contextual model of pro-angiogenic and vessel-destabilizing functions of Ang-2 which is controlled by differential Tie2 vs. integrin expression, binding and activation.

Von Willebrand factor mediates platelet binding and microthrombus formation upon melanoma cell-induced endothelial cell activation

Alexander T. Bauer[1], Anna Desch[1], Elwira A. Strozyk[2], Jan Suckau[1], Volker Huck[1] , Verena Niemeyer[1] , Thomas Wieland[3], Viktor Umansky[4] and Stefan W. Schneider[1].
[1]Department of Dermatology, Venereology and Allergology, Medical Faculty Mannheim, Heidelberg Ruprecht-Karls-University, Mannheim, Germany, [2]HelmholtzCentreMunich, German Research Center for Environmental Health, Institute of Molecular Immunology, Munich, Germany, [3]Institute of Pharmacology and Toxicology, Medical Faculty Mannheim, Heidelberg Ruprecht-Karls-University, Mannheim, Germany, [4]German Cancer Research Center, University Hospital Mannheim, Heidelberg Ruprecht-Karls-University, Mannheim, Germany

Malignant melanoma is a cancer associated with poor prognosis and limited therapeutic success. Melanoma spreading leads to multiple metastatic tumors and is the major reason for patient death. To metastasize to distinct organs, circulating cancer cells have to interact with vascular endothelial cells (ECs) and migrate through the vessel wall. We recently showed that melanoma cells activate ECs by a tissue factor (TF)-mediated thrombin generation, followed by Weibel Palade body (WPB) exocytosis and thereby the release of the procoagulatory protein von Willebrand factor (VWF). Next to this indirect, thrombin-dependent pathway recent data demonstrated a second direct pathway of melanoma-induced EC activation. Using Proteome Profile assays, knockdown studies and specific inhibitors, we identified melanoma-derived vascular endothelial growth factor-A (VEGF-A) as the only molecule of direct (thrombin-independent) EC activation. Although many tumor-derived cytokines have been hypothesized to activate ECs, our data represent the first direct experimental evidence for such a mechanism. Furthermore, our data demonstrate that MMP-2, activated on the cell surface of melanoma cells, regulates VEGF-A expression via an integrin αvβ5/phosphoinositide-3-kinase-dependent (PI3K) pathway. Both the thrombin- and the VEGF-dependent pathways induce an acute exocytosis of WPBs and the release of inflammatory cytokines, P-selectin, Angiopoietin-2 and VWF transforming the vascular endothelium into a prothrombotic surface. Luminally released VWF is stretched under high shear flow conditions and forms ultra large VWF (ULVWF) fibers mediating the adhesion of platelets and circulating cells. This process may result in microthrombus formation and a recruitment of tumor cells to the vessel wall. In order to proof the relevance of our in vitro findings in vivo, we evaluated the effects of the low-molecular-weight heparin (LMWH) tinzaparin, known to inhibit P-selectin, thrombin generation and VEGF-A, in a ret-transgenic mouse model developing spontaneously melanoma. Interestingly, treatment with the clinically approved anti-coagulant resulted in a strong tendency of survival benefit and a reduced total tumor weight. Immunofluorescence studies demonstrated the formation of intraluminal ULVWF fibers in the tumor vasculature mediating platelet aggregation, indicative for acute EC activation in the microenvironment of the tumor. Presence of tinzaparin attenuated EC activation in the tumor vessels, quantified by a clearly reduced VWF fiber formation and platelet aggregation. Finally, we identified EC- derived ULVWF fibers in the vasculature of human malignant melanoma, whereas tissue of basal cell carcinoma as control showed a typical localization of VWF in ECs without fiber formation, concluding that melanoma progression is associated with activation of ECs. In summary we present a sound molecular explanation for the mode of action of tumor-mediated EC activation confirmed by ex vivo analysis of tumor vessels of mice and humans. This will not only identify new therapeutic targets, but further forster investigations using anti-coagulants for cancer treatment.

Hyaluronan oligosaccharides induce lymphangiogenesis through LYVE-1

Bauer, J.[1], Schmaus, M.[1], Rothley, M.[1,2], Quagliata, L.[2], Dimmler, A.[3], Sipos, B.[4], Faller, G.[3], Thiele, W.[1,2], Allgayer, A.[2], Hohenberger, P.[2], Post, S.[2],Jackson, D.[5] and Sleeman, J.P.[1,2]. [1]Karlsruhe Institute of Technology (KIT), Institut für Toxikologie und Genetik, Postfach 3640, 76021 Karlsruhe, Germany, [2]Universitätsmedizin Mannheim, Medical Faculty Mannheim, University of Heidelberg Ludolf-Krehl-Str. 13 - 17, 68167 Mannheim, Germany, [3]Institut und Gemeinschaftspraxis für Pathologie an den St. Vincentiuskliniken Karlsruhe, Südendstrasse 37, 76137 Karlsruhe, Germany, [4]Universitätsklinikum Tübingen, Department of Pathology, Liebermeisterstraße 8, 72076 Tübingen, Germany,[5]MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS, United Kingdom

Hyaluronic acid (HA) is composed of repeating disaccharide units of D-glucuronate and N-acetylglucosamine, and is a major component of the extracellular matrix. Within tissues it normally exists as a high molecular weight polymer and is synthesized and accumulated by most cells, particularly during proliferation. HA turnover occurs constantly, but is enhanced in tumors and in areas of wounding and inflammation through the activation of endogenous hyaluronidases and also by reactive oxygen species, processes that degrade high molecular weight HA into small fragments. The small HA oligosaccharides (sHA) so produced are highly bioactive and proinflammatory. A major role for sHA, particularly in the context of tumors, is in the induction of angiogenesis. Thus, while high molecular weight HA is anti-angiogenic, sHA ranging in size from 3 to 20 disaccharides in length is a potent inducer of angiogenesis. Here we report that sHA also promotes lymphangiogenesis, as at concentrations of 1-10 ï□-g/ml it stimulates the proliferation of primary lymphatic endothelial cells (LECs) and induces outgrowth of lymphatic capillaries in ex vivo thoracic duct ring assays. Lymphatic vessel density in lymph nodes draining skin into which sHA has been intradermally injected is also increased. VEGF-C and sHA act additively to induce lymphangiogenesis. Loss of function analysis indicates that LYVE-1 is the receptor that mediates the pro-lymphangiogenic role of sHA. Tumor interstitial fluid can contain up to 6 µg/ml sHA, indicating that sHA accumulation in tumors may contribute to tumor-induced lymphangiogenesis.

Notch-dependent VEGFR3 upregulation enables angiogenesis without VEGF/VEGFR2 signalling

Rui Benedito, Susana F. Rocha, Marina Woeste, Martin Zamykal and Ralf H. Adams. Max-Planck-Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, and University of Muenster, Faculty of Medicine, Muenster, Germany

During angiogenesis, endothelial cells sense different extracellular molecular cues that change their behaviour. The vascular endothelial growth factor (VEGF) family of secreted ligands and receptors are known to influence endothelial cell sprouting, proliferation and survival in a variety of organs and pathological processes. Besides being influenced by external factors, endothelial cells have also endogenous signalling mechanisms that modulate their response to the surrounding environment. One such mechanism is the Notch signalling pathway that upon cell-to-cell activation leads to a reduction in angiogenesis. Mechanistically, it was suggested that VEGF-A besides inducing pro-angiogenic signals, it also induces the expression of the Notch ligand Dll4, which strongly activates Notch receptors, leading to a decrease in the expression of VEGF receptors and angiogenesis. Impairment of this Notch-dependent negative feedback mechanism leads to excessive angiogenesis. By using inducible loss-of-function genetics in combination with inhibitors in vivo, we found that Dll4 protein expression in retinal tip cells is only weakly modulated by VEGFR2 signalling. Surprisingly, Notch inhibition also had no significant impact on VEGFR2 expression and induced deregulated endothelial sprouting and proliferation even in the absence of VEGFR2, which is the most important VEGF-A receptor and considered to be indispensable for these processes. In contrast, VEGFR3, the main receptor for VEGF-C, was strongly modulated by Notch. VEGFR3 kinase inhibition but not ligand-blocking antibodies suppressed the sprouting of endothelial cells with low Notch signalling. Our results establish that VEGFR2 and VEGFR3 are regulated in a highly differential fashion by Notch. We propose that successful anti-angiogenic targeting of these receptors and their ligands will strongly depend on the status of endothelial Notch signalling.

Local endotoxemia causes remote organ injury through formation of leukocyte aggregates

Martina Beyrau[1], Abigail Woodfin[1], Mathieu-Benoit Voisin[1], Bartomeu Colom[1], Jennifer Bodkin[1], Beat A Imhof[2], Nancy Hogg[3] & Sussan Nourshargh[1]. [1]William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, United Kingdom, [2]Centre Médical Universitaire, Geneva, Switzerland, [3]Leukocyte Adhesion Laboratory, Cancer Research UK London Research Institute, London, United Kingdom

Neutrophil migration into sites of inflammation and injury is a vital part of the innate immune response (1). We have recently shown that the luminal to abluminal movement of neutrophils through the endothelial cell (EC) layer is finely regulated and that under certain inflammatory conditions (e.g. following ischemia-reperfusion [I-R] injury) neutrophils can exhibit reverse transendothelial migration (TEM) in vivo, i.e. move in an abluminal-to-luminal direction (2). This reverse TEM response was associated with the presence of a subset of ICAM-1high neutrophils within the pulmonary vasculature of mice subjected to localised I-R injury and the development of lung damage (2). We now report that neutrophil reverse TEM can also occur in a model of local endotoxemia. Specifically, intrascrotal injection of lipopolysaccharide (LPS; 100-1000 ng) caused a dose-dependent increase in neutrophil reverse TEM through mouse cremasteric venules as analysed by real-time confocal intravital microscopy. Furthermore, this reaction induced lung inflammation (quantified by measurement of total neutrophil infiltration in the lung tissue), a response that was associated with the occurrence of ICAM-1high neutrophils in the pulmonary vasculature of the mice. To obtain a better understanding of the potential pathogenic ability of the ICAM-1high neutrophils we conducted a detailed analysis of their phenotype by flow cytometry. The findings showed that a significant proportion of the ICAM-1high neutrophils were aggregates of two or more cells composed of predominantly neutrophils but also of monocytes. Of interest, ICAM-1high neutrophil aggregates could not be detected in chimeric mice deficient in leukocyte ICAM-1, indicating that the source of ICAM-1 on neutrophil aggregates was leukocyte derived. Collectively, the present study provides additional evidence for the occurrence of neutrophil reverse TEM in vivo and further indicates an association between this response and the development of second organ inflammation as mediated by the generation of a sub-set of activated neutrophils with an ICAM-1high phenotype. 1. Nourshargh, S., Hordijk, P.L. & Sixt, M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. Nat. Rev. Mol. Cell Biol. 11, 366-78 (2010). 2. Woodfin, A. et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat. Immunol. 12, 761-9 (2011). This work was supported by funds from The Wellcome Trust and a Marie Curie Fellowship awarded to MB.

The Rab27/Rab3 effector Synaptotagmin-like protein 4-a (Slp4-a) positively regulates hormone-evoked Weibel-Palade Body exocytosis

Ruben Bierings[1]*, Nicola Hellen[1], Athinoula Meli[2], Robert J. Rowlands[1], Bijal Patel[1], Lindsay Hewlett[1], Laura Knipe[1], Charles McKenna[3], Ambrosius Snijders[4], Marlen Rose[2], Matthew J. Hannah[1] and Tom Carter[1]. [1]MRC National Institute for Medical Research, London, United Kingdom, [2]Imperial College, London, United Kingdom, [3]University of Southern California, Los Angeles, USA, 4MRC Clinical Sciences Centre, London, United Kingdom

Weibel Palade body (WPB) exocytosis underlies hormone-evoked VWF secretion from endothelial cells. The identities of components that positively regulate hormone-evoked WPB exocytosis remain unclear. Here we identify two new components of the WPB; Rab3B and the Rab27\Rab3 effector Slp4-a. Using multiple biochemical and live cell imaging approaches we show that Rab3B is abundantly expressed on WPBs, that Rab3B and Rab27a contribute to recruitment of Slp4-a, and that Slp4-a positively regulates hormone-evoked WPB exocytosis and VWF secretion. siRNA knockdown of Slp4-a strongly inhibited VWF secretion, while over expression of EGFP-Slp4-a increased WPB exocytosis monitored directly in living cells. Single or double knockdown (KD) of Rab3B and Rab27a suggest that Slp4-a-Rab27a is the functionally dominant complex mediating positive regulation of VWF secretion. KD of the Rab27a-specific effector MyRIP resulted in a small but significant increase in VWF secretion while over expression of EGFP-MyRIP strongly inhibited WPB exocytosis. Together, these data show that WPBs recruit multiple Rab effectors and that the balance of regulation is tilted in favour of a positive drive towards WPB exocytosis and VWF secretion through recruitment of Slp4-a.

Netrin-1 guides sympathetic arterial innervation

Isabelle Brunet[1], Karine Bouvrée[1], Raquel del Toro[1], Maria Machado[1], Chun Liu[2], Brunella Cristofaro[1], Bruno Larrivée[2], Luc Pardanaud[1], Thomas Mathivet[1], Dong Broqueres-You[3], Timothy E. Kennedy[4], Michael Simons[2], Bernard I. Levy[3] and Anne Eichmann[1,2]. [1]CIRB Collège de France, Inserm U1050/ CNRS UMR7241, 11 place Marcelin Berthelot, 75005 Paris, France, [2]Department of Cardiology, Yale University School of Medicine, 300 George Street, New Haven, CT 06510-3221, USA, [3]Paris Cardiovascular Research Center (PARCC), INSERM U970, 56 rue Leblanc, 75015 Paris, France, [4]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec, Canada

Innervation of peripheral resistance arteries by autonomic sympathetic nerves controls blood supply to organs by regulating vascular tone. During development, arteries guide outgrowth of sympathetic nerves towards their targets, but also eventually become targets for innervation. Here, we identify the axon guidance cue Netrin-1 as the first known factor required for arterial innervation, a process that we also show to occur in mice one week after vascular alignment with sympathetic nerves. Netrin-1 is produced by arteries at the onset of innervation and signals via its receptor, Deleted in Colorectal Cancer (DCC), expressed on sympathetic growth cones. Function-blocking approaches indicate that Netrin-1 and DCC are specifically required for arterial innervation. Strikingly, adult Ntn-1+/- mice show severe and selective reduction of sympathetic innervation in resistance arteries, leading to defective vasoconstriction and thermoregulation. These findings reveal a novel role for Netrin-1 as a guidance molecule critical for arterial innervation and blood flow regulation.

Transfer of H-Ras protein from endothelium to interacting leukocytes

Elisabeth Buchberger[1], Oded Rechavi[2], Yoel Kloog[2], and Christine Brostjan[1]. [1]Department of Surgery - Medical University of Vienna, Austria, [2]Department of Neurobiology - Tel Aviv University, Israel

Interacting cells are capable of exchanging entire membrane patches. Thus, functional receptor molecules as well as membrane-associated components of the signaling cascade may transfer from one cell to the other and intracellular signals initiated in the donor can be passed onto the recipient cell. These effects have mainly been observed for leukocyte-leukocyte interactions. In this study we investigated the transfer of the membrane-associated signal transducer H-Ras from activated endothelial cells (ECs) to interacting leukocytes. EGFP fusion proteins of wildtype H-Ras or the constitutively active G12V mutant as well as EGFP control protein were introduced into ECs by transient transfection. While EGFP was predominantly expressed in the cytosol and the perinuclear region, the chimeric EGFP-H-Ras proteins showed distinct membrane localization. Protein transfer was assessed using an in vitro model in which human peripheral blood mononuclear cells migrated through a confluent monolayer of transfected ECs. Flow cytometry and confocal imaging of CD45+CD146- leukocytes demonstrated that lymphocytes as well as monocytes acquired EGFP-H-Ras from CD45-CD146+ ECs. While protein transfer was low for the EGFP control (1% for lymphocytes and 10% for monocytes) about 7% of lymphocytes and 65% of monocytes presented positive for EGFP-H-Ras. There was no difference in uptake efficiency between wildtype and mutant H-Ras. The functional impact of H-Ras transfer was further investigated in terms of ERK activation in recipient leukocytes. To elucidate the mechanism accounting for H-Ras exchange, ECs were labeled with the membrane dye DiR. Co-transfer of EGFP-H-Ras and DiR was partly observed indicating that H-Ras is likely to be passed from ECs onto leukocytes via general membrane exchange. In conclusion, transfer of H-Ras signaling molecules proceeds rapidly from activated endothelial cells to interacting leukocytes and may represent an efficient mode of exchanging cell activation signals during diapedesis in inflammation.

RNase1 protects against cardiac ischemia/reperfusion injury

Cabrera-Fuentes H.A.[1,4]*, Sanchez J.A.[2], Fischer S[1], Ilinskaya O.N.[4], Schlüter K.D.[3], Preissner K.T.[1]. [1]Justus-Liebig University, Institute for Biochemistry, Medical Faculty, Giessen, Germany, [2]Hospital Universitari Vall d'Hebron, Servicio de Cardiologia, Barcelona, Spain, [3]Justus-Liebig University, Institute of Physiology, Medical Faculty, Giessen, Germany, [4]Kazan Federal University, Department of Microbiology, Kazan, Russian Federation

Extracellular RNA (eRNA), exposed after tissue trauma, ischemia or damage, has been shown to exert prothrombotic and hyperpermeability-inducing functions, which are prevented by Ribonuclease1 (RNase1) treatment in vivo. Following ischemia and myocardial necrosis during reperfusion, the presence of eRNA (as cofactor for cytokines and coagulation proteases) might potentiate the development of rigor contracture. Here, the contribution of the eRNA/RNase1 system in ischemia/reperfusion (I/R) injury was investigated in isolated rat hearts in a Langendorff system. Lactate dehydrogenase (LDH) release, a marker of cell damage/necrosis, as well as eRNA and RNase-activity were determined in the perfusate before and during reperfusion (120 min) following 45 min of ischemia. To study the influence of RNase1 on physiological parameters, left ventricle (LV) pressure was continuously recorded. RNase1 was added in different concentrations to the perfusion buffer, starting 3 min before the ischemic phase and maintained for the whole duration of the experiment. In the initial period of reperfusion (following the ischemia phase) there was a sharp increase in LDH release (32.83 ± 0.3 U/g dry tissue), a prominent initial peak of eRNA (52.9 ± 3.3 ng/ml) followed by a prolonged high level of eRNA between 15 and 60 min of reperfusion. Only very low endogenous RNase1-activity was found in the perfusate. Treatment with RNase1 in a concentration-dependent manner induced a lower and delayed increase in diastolic pressure during ischemia, indicating a less severe rigor contracture. In addition, functional recovery of heart tissue after 30 min reperfusion was preserved as indicated by elevated increase of LV developed pressure (I/R: 53 ± 5 % vs. baseline; RNase1-treatment: 83 ± 13 % vs. baseline; p=0.03). Finally, RNase1 reduced the severity of the maximal hypercontracture (I/R: 67 ± 8 mmHg; RNase1-treatment: 19 ± 3 mmHg; p=0.05) during the initial reperfusion phase and prevented the initial LDH release (16.31 ± 2 U/g dry tissue, 30 min after reperfusion), indicating less myocardial damage and protection against necrosis. Together, eRNA is released from the rat heart during I/R and may contribute to the outcome of cardiac injury. RNase1 intervention appears be a new potential therapeutic regimen against cardiac I/R injury, whereby the underlying mechanisms deserve further investigation.

Brag2, An Arf6 activator, mediates endocytosis of beta1-integrins In endothelial cells and angiogenesis

Yosif Manavski, Guillaume Carmona, Jens Kroll, Stefanie Dimmeler, Emmanouil Chavakis. Institute for Cardiovascular Regeneration, Goethe University of Frankfurt, Germany III. Department of Internal Medicine, Goethe University of Frankfurt, Germany Vascular Biology, Center for Biomedicine and Medical Technology (CBTM), Medical Faculty Mannheim, Heidelberg, Germany

The molecular mechanisms mediating angiogenesis are not completely understood. ADP-ribosylation factor 6 (ARF6) is a small GTPase activated by distinct guanine nucleotide exchange factors (GEF) and is involved in traffic and cytoskeletal organization in the cell periphery thereby regulating cell motility. Interestingly, the function of Arf6 is dependent on the upstream GEF activating it. Angiogenic factors such as VEGF have been shown to promote Arf6 activity. However, the regulation of Arf6 activity in endothelial cells (EC) is unclear. Recent evidence suggests that Brag2, a GEF for Arf6 GTPase, mediates receptor tyrosine kinase-induced Arf6 activity. In the present work we studied the role of Brag2 in angiogenesis. Human umbilical vein endothelial cells (HUVEC) express Brag2 as assessed by western blot. Silencing of Brag2 with siRNA significantly blocked angiogenic sprouting of HUVEC under basal conditions and after VEGF stimulation (by 40 ± 7 %) in a 3-dimensional spheroidal EC culture system and tube formation in a matrigel assay in comparison to scrambled siRNA-transfected EC. Moreover, silencing of Brag2 with siRNA significantly reduced the VEGF-induced 3-dimensional migration of EC (49 % inhibition), while not affecting proliferation. EC migration is dependent on adhesion to matrix proteins. Interestingly, knock down of Brag2 with siRNA significantly increased EC adhesion on the alpha5beta1-integrin ligand, fibronectin. In line with these results, silencing of Brag2 reduced the endocytosis of beta1-integrins and as consequence increased surface expression of the alpha5beta1-integrin in EC as assessed by FACS. In order to address the role of Brag2 in in vivo angiogenesis, we studied vessel formation in developing zebrafish embryos by employing the morpholino technology. Strikingly, in vivo silencing of the Brag2 homologue in zebrafish with a morpholino disturbed the formation of the parachordal vessels and led to defects in the dorsal longitudinal anastomotic vessel and in the intersomitic vessels. These data reveal that the Arf6 GEF, Brag2 is essential for in vivo developmental angiogenesis probably by mediating EC migration and angiogenic sprouting through regulation of adhesion by promoting integrin internalization.

miR-24 modulates MMP-14 protein expression in macrophages suggesting a novel regulatory mechanism associated with atherosclerotic plaque progression

Karina Di Gregoli, Rebecca Salter, Stephen White & Jason L Johnson. Bristol Heart Institute, Clinical Sciences at South Bristol, University of Bristol, United Kingdom

Purpose and Methods. Our recent studies have highlighted membrane type-1 MMP (MMP-14) as a selective marker for an invasive subset of macrophages related to atherosclerotic plaque progression. Moreover, colony stimulating factors (CSF) may exert divergent effects on macrophage MMP expression. We therefore investigated the mRNA and protein expression pattern of MMP-14 in macrophage (M)-CSF and granulocyte-macrophage (GM)-CSF differentiated macrophages and their subsequent invasion in vitro. Results. Mouse macrophages differentiated with M-CSF for 7 days showed a significant up-regulation (5-fold) of MMP-14 mRNA ($p \leq 0.005$) and a significant down regulation of MMP-14 protein (80% by western blotting $p \leq 0.005$, 90% by ICC $p \leq 0.001$) compared to adherent monocytes. Contrastingly, GM-CSF differentiation resulted in a significant down-regulation of MMP-14 mRNA level (2-fold $p \leq 0.05$), whereas protein was increased (2.1-fold, $p \leq 0.05$) compared to M-CSF differentiated macrophages. Accordingly, compared to M-CSF macrophages, GM-CSF macrophages exhibited increased in vitro invasion (2.9-fold $p \leq 0.05$) which was significantly reduced by a MMP-14 neutralising antibody (2.2 fold $p=0.05$). Expression of the micro-RNA (miR)-24, which we have validated in-house to target MMP-14 mRNA, was significantly increased during M-CSF macrophage maturation (85%, $p<0.05$). Silencing miR-24 in M-CSF macrophages significantly increased MMP-14 protein expression (1.4-fold, $p \leq 0.05$) and enhanced their invasive capacity (5.1-fold, $p \leq 0.01$) compared to a scrambled control. Concomitantly miR-24 expression was significantly lower in GM-CSF matured macrophages compared to M-CSF macrophages (24%, $p \leq 0.01$) and in the presence of exogenous miR-24, MMP-14 protein expression was reduced by 25% ($p \leq 0.05$) suggesting that GM-CSF can modulate MMP-14 protein expression and subsequent invasion of mouse macrophages in a miR-24 dependent manner. To corroborate our findings in vivo, we have utilised a mouse model of granuloma formation using matrigel-infused subcutaneous polyurethane sponges. Accordingly, macrophage recruitment was significantly reduced in the presence of exogenous miR-24 (65%, $p \leq 0.05$) and increased in the presence of a miR-24 inhibitor (134%, $p \leq 0.05$) compared to control. Consequently, the number of MMP-14 positive macrophages present within the sponges was decreased in the exogenous miR-24 treated mice as assessed by flow cytometry (56%, $p \leq 0.05$) and IHC (49%, $p \leq 0.05$) confirming the essential role of miR-24 in mediating macrophage recruitment/invasion in areas of inflammation by modulating MMP-14 protein expression in vivo. Subsequently, to translate our findings to the human disease, we demonstrated increased MMP-14 protein expression in foam-cell macrophages is associated with human carotid and coronary atherosclerotic plaque progression and predicts future clinical outcomes. Furthermore, miR-24 expression in atherosclerotic plaques is inversely related with MMP-14 protein expression. Moreover, stable plaques contained higher miR-24 levels (as assessed by Q-PCR) than unstable plaques ($p \leq 0.05$), and by in situ hybridisation, co-localised with foam-cell macrophages which exhibited low MMP-14 protein expression. Conclusions. Taken together our data suggest an essential role for MMP-14 in promoting macrophage proteolysis in a subset of macrophages and subsequent plaque progression and that the microRNA miR-24 provides a novel regulatory mechanism for MMP-14 protein levels and therefore plaque stability, highlighting its therapeutic potential.

CCM1/ICAP-1 complex controls beta1 integrin-dependent extracellular matrix remodeling and vascular integrity

Eva Faurobert[1,2,3]*, Claire Rome[1,3], Justyna Lisowska[1,2,3], Sandra Manet-Dupé[1,2,3], Gwénola Boulday[4], Marilyne Malbouyres[7], Martial Balland[3,8], Michelle Kéramidas[1,3], Daniel Bouvard[1,2,3], Jean-Luc Coll[1,3], Florence Ruggiero[7], Elisabeth Tournier[1]. [1]INSERM U823, Institut Albert Bonniot, Equipe1 DySAD, Grenoble, France, [2]ERL CNRS 5284, Grenoble, France, [3]Université Joseph Fourier, Grenoble, France, [4]INSERM, UMR-S 740, Université Paris Diderot, 10 Avenue de Verdun, 75010 Paris, France, [5]Université Paris 7-Denis Diderot, Faculté de Médecine, Site Lariboisière, Paris, F-75010, France, [6]AP-HP, Groupe hospitalier Saint-Louis Lariboisiere-Fernand-Widal, Paris, F-75010, France, [7]UMR CNRS 5242 - ENS Lyon, France, [8]Laboratoire interdisciplinaire de Physique UMR CNRS 5588, Saint Martin d'Hères, France

Human Cerebral Cavernous Malformations (CCM) are clusters of hemorrhagic dilated blood vessels composed of fragile endothelium lacking mural cells and with altered sub-endothelial extracellular matrix (ECM). Familial forms result from loss-of-function mutations of CCM1, CCM2 or CCM3 genes which encode a multifunctional protein complex involved in cell-cell junction, cell polarity and acto-myosin contractility. The association of ICAP-1, a beta1 integrin negative regulator, with CCM proteins prompted us to investigate the role of ICAP-1 in vascular integrity and to address for the first time the role of CCM proteins in the interaction of the endothelial cell with the ECM. ICAP-1 deficient mouse showed a chaotic network of dilated, branched, tortuous and more permeable blood vessels. Their basal lamina and surrounding ECM presented ultrastructural defects as reported for CCM lesions. These defects correlated with abnormal fibronectin fibrils deposition as around CCM1 or CCM2 mouse lesions. We showed that beta1 integrin mediated fibronectin fibrillogenesis by endothelial cells was altered upon CCM1 or CCM2 silencing concomitantly with a strong destabilization of ICAP-1 protein. Impaired fibronectin fibrillogenesis resulted from increased beta1 integrin activation, excessive acto-myosin contractility and redistribution of cellular traction forces. We provide evidence that CCM proteins form a node regulating beta1 integrin activation and ECM remodelling in addition to controlling cell-cell junctions. Our study adds another dimension to mechanotransduction in CCM pathology by proposing that biomechanical remodeling of the microenvironment might favor expansion of the lesions.

Inflamatory activities of extracellular RNA in the vascular system are connected with cytokine release from monocytes

Silvia Fischer[1], Tobias Grantzow[2], Judith I. Pagel[2], Markus Sperandio[2], Klaus T. Preissner[1], Elisabeth Deindl[2]. [1]Department of Biochemistry, Medical School, Justus-Liebig-University, D-35392 Giessen, Germany, [2]Walter-Brendel-Centre of Experimental Medicine, Ludwig-Maximilians-University, D-81377 Munich, Germany

Extracellular RNA (eRNA), released from cells under conditions of tissue injury or during vascular disease, acts as potent prothrombotic factor and promotes vascular hyper-permeability related to eema formation *in vivo*. Exogenously administered RNase1 (but not DNase) neutralized these activities and conferred vessel-protective functions. In this study, we aimed to investigate the mechanisms by which eRNA triggers inflammatory processes, particularly associated with leukocyte recruitment. Using intravital microscopy of murine cremaster muscle venules, eRNA (but not DNA or hydrolyzed RNA) significantly induced leukocyte adhesion and transmigration *in vivo*, which was comparable in its effect to the function of tumor-necrosis-factor-α (TNF-α). *In vitro*, eRNA promoted adhesion and transmigration of monocytic cells on and across endothelial cell monolayers, albeit to a weaker extent as *in vivo*. eRNA-induced monocyte adhesion *in vitro* was mediated by activation of the vascular endothelial growth factor (VEGF)/VEGF-receptor-2 system and was abolished by neutralizing antibodies against intercellular adhesion molecule-1 or the β2-integrin Mac-1. Additionally, eRNA induced the release of TNF-α from monocytes in a concentration- and time-dependent manner reaching maximal values after two to four hours of RNA treatment from 271±24 to 334±12 pg TNF- α/mg protein. RNA-mediated TNF-α release involved activation of TNF-α-converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase also known as ADAM17. Specific inhibitors of TACE inhibited RNA-induced TNF-α release completely but not that one of other cytokines like IL-6. RNA-induced TNF-α release involved signaling via the NF-κB pathway and further activation of the p38 MAPkinase. According to these results, infusion of mice with eRNA significantly enhanced TNF-α expression in cremaster muscle tissue. Our findings present evidence that eRNA in connection with tissue/vascular damage or remodeling provokes a potent inflammatory response by inducing leukocyte recruitment directly and indirectly by mobilizing proinflammatory cytokines from monocytes.

Control of vascular inflammation by a microRNA feedback loop

Henry Cheng and Jason E. Fish. Toronto General Research Institute, University Health Network

Vascular inflammation plays a key role in the initiation and progression of atherosclerosis. Identifying negative regulators of inflammation could therefore aid in the development of therapies to prevent and/or treat this disease. We have found that exposure of endothelial cells to inflammatory stimuli induces the expression of the miR-146 microRNA family (composed of miR-146a and miR-146b). Interestingly, the increase in miR-146a/b levels is delayed compared to other inflammatory mRNAs. This delay in expression is mediated by a delay in the processing of the miR-146a/b precursors into mature microRNA, since the transcription of the miR-146a/b loci are in fact rapidly induced by inflammation. miR-146 acts as a negative feedback regulator of inflammation since over-expression of this microRNA blunts the endothelial inflammatory response. This is mediated in part by inhibition of the activation of the MAP kinase pathway and the downstream Egr transcription factor pathway. In addition, we have found that the transcriptional induction of miR-146a/b is mediated by Egr proteins. We have therefore uncovered a negative feedback regulatory loop that controls endothelial inflammatory pathways and may impact on the development of atherosclerosis.

Shear-induced binding of platelets to von Willebrand factor is enhanced by clustering of Glycoprotein Ibα

Eelo Gitz[1], Dave J. van den Heuvel[2], Hans Deckmyn[3], Hans C. Gerritsen[2], Jan-Willem N. Akkerman[1] and Rolf T. Urbanus[1]. [1]Thrombosis and Haemostasis Laboratory, Department of Clinical Chemistry and Haematology, UMC-Utrecht, [2]Department of Molecular Biophysics, Utrecht University, The Netherlands and [3]Laboratory for Thrombosis Research, KU Leuven campus Kortrijk, Belgium

Background: Initial platelet arrest at the exposed arterial vessel wall is mediated through Glycoprotein (GP) Ibαbinding to the immobilized A1 domain of von Willebrand factor (VWF). This interaction occurs at sites of elevated shear force, and strengthens upon increasing hydrodynamic drag. The increased interaction is suggested to be caused by a conformational change in the A1 domain, but the exact mechanism remains ill defined. We have previously found that GPIbα clusters upon platelet cooling and hypothesized that a similar mechanism enhances binding to VWF under (patho)physiological conditions. Methods: We analyzed clustering of GPIbα in platelets exposed to VWF or shear force with Förster Resonance Energy Transfer (FRET) using time-gated Fluorescence Lifetime Imaging Microscopy (FLIM). Platelet reactivity towards VWF was analyzed with light transmission aggregometry and real-time video microscopy. Results: Fresh platelets showed a FRET efficiency of 1.9±0.8% (n=5), indicating that resting platelets have dispersed GPIbαreceptors. Both a pathological shear rate (10,000 s-1) and perfusion over a vWF surface (1600 s-1) induced GPIbα clustering, as indicated by increased FRET efficiency (9.9±0.5% and 12.2±0.9%, respectively). Platelets with pre-clustered GPIbα showed an enhanced reactivity towards VWF, as demonstrated by a 2.5 fold increased ristocetin induced agglutination and a 15% decreased rolling velocity when perfused over VWF. Inhibition of GPIbαclustering by N-acetyl-D-glucosamine fully blocked both responses. Conclusions: Pathologic shear force and platelet rolling over a VWF surface induce clustering of GPIbα, which potentiates its interaction with the multimeric protein. This newly identified mechanism emphasizes the role of GPIbαas a sensitive mechanoreceptor and inhibition of clustering may potentially be deployed as a novel strategy to prevent atherothrombosis.

VEGF dose negatively regulates the stabilization of newly induced vessels by inhibiting the Sema3A/Nrp1+CD11b+ monocytes/TGF-β1 axis

E. Groppa[1]*, S. Reginato[1], R. Gianni-Barrera[1], M.G. Muraro[2], M. Heberer[1,2] and A. Banfi[1].
[1]Cell and Gene Therapy, Departments of Biomedicine and of Surgery, Basel University Hospital, Switzerland, [2]Oncology, Departments of Biomedicine and of Surgery, Basel University Hospital, Switzerland

Rapid stabilization, i.e. the persistence of newly induced vessels independently of further growth factor expression, is required to achieve functional benefit in therapeutic angiogenesis with short-term gene therapy vectors. VEGF induces normal or aberrant angiogenesis depending on its expression level in the microenvironment around each cell in vivo. Further, four weeks of sustained expression are required for stabilization of normal angiogenesis, whereas aberrant vessels remain VEGF-dependent (Ozawa, JCI 2004). Here we took advantage of a highly controlled myoblast-mediated gene delivery platform to rigorously investigate how VEGF dose regulates stabilization of newly induced vessels in skeletal muscles. Clonal populations of retrovirally transduced myoblasts were implanted in the ear or limb muscles of SCID mice to homogeneously produce specific levels of VEGF164: low and medium VEGF caused normal angiogenesis and high VEGF induced aberrant angioma growth. VEGF signaling was abrogated at defined time-points by systemic treatment with recombinant VEGF-Trap. We found that VEGF impaired vascular stabilization in a dose-dependent fashion. In fact, 35% and 50% of normal vessels induced by low VEGF levels already stabilized by 2 and 3 weeks, respectively. Instead, similarly normal angiogenesis induced by medium VEGF levels completely regressed by 2 weeks and only 10% stabilized by 3 weeks. Aberrant structures caused by high VEGF levels never stabilized. Intravascular staining with fluorescent L. esculentum lectin showed that all vessels induced by all VEGF levels were normally perfused. Further, all normal capillaries induced by low and medium VEGF doses displayed similar normal pericyte coverage, despite different stabilization rates. Consistently, the expression of the endogenous Pdgfb gene was also similar among all conditions. Only aberrant vessels induced by high VEGF, which never became VEGF-independent, were surrounded by α-SMA-positive mural cells and not by pericytes. Therefore, pericyte recruitment is necessary but not sufficient to achieve rapid vascular stabilization. However, gene expression analysis on the injected muscles, revealed a correlation between decreasing stabilization rates at the different VEGF doses and tissue expression of TGF-β1 and Sema3A, whereas endogenous VEGF, Ang1, and EphrinB2 did not change. Sema3A has been recently described to promote vessel maturation through the recruitment of Nrp1+CD11b+ monocytes (NEM). To elucidate the mechanisms of VEGF-dependent vascular stabilization, we investigated the relation between VEGF dose and NEM recruitment. We found that: 1) VEGF164 down-regulated Sema3A expression by endothelial cells in vitro; 2) NEM recruitment in injected muscles was reduced with increasing VEGF doses; 3) Sema3A expression in endothelial cells FACS-purified ex vivo from injected muscles was down-regulated with increasing VEGF doses; 4) FACS-purified NEM expressed TGF-β1, but its level per cell was not affected by VEGF dose; 5) however, in vitro stimulation of endothelial cells with TGF-β1 up-regulated Sema3A expression dose-dependently, providing the basis for a positive feedback loop between Sema3A, NEM recruitment, TGF-β1, and further Sema3A expression, in conditions of low VEGF. Taken together, these data suggest a model in which VEGF164, expressed within a range of doses that induce only normal angiogenesis, negatively regulates vascular stabilization by inhibiting the Sema3A/NEM/TGF-β1 axis, rather than directly acting on the endothelium-pericyte crosstalk.

Cyclic strain modulates IPS cell differentiation to VSMC: Role of Notch1

Shaunta Guha[1], Caitriona Lally[2], Qingbo Xu[1]. [1]King's College London, [2]Dublin City University

BACKGROUND: Stem cell-based therapeutic vascular regeneration may offer broad potential in treatment of cardiovascular diseases. Recent studies have looked at blood vessel tissue engineering using stem-cell technology to generate engineered blood vessels for transplantation. Induced pluripotent stem cells (IPSC) are generated through reprogramming of adult somatic cells, with specific transcription factors. Reprogramming of the somatic cells allows them, as with embryonic stem cells (ESC) to be differentiated under specific conditions, to a given cell type, while avoiding the ethical and immunogenicity issues of ESC. Cell differentiation from both ESCs and IPSCs, is a complicated process and is regulated by various molecular signal pathways through mechanisms which are poorly defined. To date, the response of IPS cells to strain, and the mechanisms involved, has been poorly investigated. METHODS and RESULTS: IPSC derived from murine fibroblasts were seeded onto flexible-bottomed 6-well plates and after 5 days culture on collagen IV, were exposed to equibiaxial strain (5% strain, 60 cycles/min, 0-24h) using a Flexcell Tension Plus Unit. mRNA levels for the smooth muscle markers ï¡-SM actin, SM22ï¡ and calponin were analysed by quantitative real-time PCR and were found to increase in a temporal manner following strain (4h, 8h, 12h, 24h), while mRNA levels of endothelial markers Flk1, CD31 and VE-cadherin remained unaffected. Western blot analysis was performed and protein levels for alpha-SM actin, SM22alpha and calponin were found to be significantly increased following strain (12h, 24h). Pharmacological inhibition of Notch Signalling by DAPT (10uM) attenuated the strain effect on VSMC marker, while inhibition of either TGFβ signalling (LY36497, 10μM) or PDGF signalling (AG1296, 15μM) did not attenuate the upregulation of SMC markers by strain. mRNA and protein levels for Notch1, and it's downstream targets Hes1 and Hey2, were also significantly increased in a temporal manner following exposure to cyclic strain. In parallel experiments, infection of IPSC under static conditions with Notch1 adenovirus significantly increased the expression of SMC markers. Similarly, stimulation with the Notch ligand Jag 1 significantly increased both mRNA and protein levels of Notch1 and SMC markers, while knockdown of Notch 1 signalling by Notch1 siRNA significantly decreased the mRNA and protein expression of SMC markers in IPSC cultured under static conditions. CONCLUSIONS: Our findings demonstrate that cyclic strain promotes differentiation of mouse IPSC to vascular smooth muscle cells, via activation of Notch1 in vitro. Cyclic strain may prove to be a suitable stimulus for differentiation of IPSC to VSMCS and may provide a valuable source for efficient SMC generation for vascular regenerative therapies.

ROCK mediates dendritic cell migration within lymphatic capillaries under steady state and inflammation

M. Nitschké[1], D. Aebischer[1], M. Abadier[2], M. Lucic[1], H. Luche[3], J. Fehling[3], O. Biehlmaier[4], R. Lyck[2] and C. Halin[1]. [1]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, Switzerland, [2]Theodor Kocher Institute, University of Bern, Bern, Switzerland, [3]Institute of Immunology, University Clinics Ulm, Ulm, Germany, [4]Biozentrum University of Basel, Basel, Switzerland

Dendritic cell (DC) migration via afferent lymphatic vessels to draining lymph nodes (dLNs) is an important event in the induction of effective adaptive immune responses. However, to date, the migration of DCs into and within lymphatic vessels has only been marginally investigated by means of intravital imaging technologies. In this study, we performed confocal intravital microscopy (IVM) in the ear skin of transgenic mice with red-fluorescent vasculature to further characterize the mode of DC movement within afferent lymphatic capillaries. IVM revealed that both adoptively transferred as well as endogenous yellow fluorescent protein (YFP)-expressing DCs actively migrated within small lymphatic capillaries. Only rarely were round-shaped DCs observed that were rapidly flushed through lymphatic vessels. To further characterize interstitial and intra-lymphatic DC migration, we pharmacologically blocked the Rho-associated protein kinase (ROCK), which is essential for acto-myosin-mediated nuclear contraction and uropod de-adhesion from integrin ligands. ROCK blockade with the small molecule inhibitor Y27632 significantly reduced DC migration from the ear to dLNs in FITC painting experiments. IVM revealed that ROCK inhibition profoundly reduced the speed of interstitial DC migration in the ear dermis and also significantly slowed down DCs that migrated within lymphatic vessels. Interestingly, ROCK blockade more effectively reduced intra-lymphatic DC migration in the context of inflammation than under steady state conditions. Since inflammation coincided with a strong upregulation of ICAM-1 in lymphatic endothelial cells (LECs) the latter findings suggest that ROCK mediates DC de-adhesion from LEC-expressed ICAM-1. In support of this hypothesis, flow chamber assays performed on LEC monolayers revealed an involvement of ICAM-1 in DC crawling. Furthermore, in agreement with our IVM experiments, pharmacologic blockade of ROCK significantly reduced DC crawling velocity on LEC monolayers in flow chamber assays. Overall our findings show that DC movement within lymphatic capillaries involves active, ROCK-dependent cell migration and provide first mechanistic insights into this process.

Nuclear receptor Nur77 is anti-inflammatory in macrophages and reduces atherosclerosis

Anouk A.J. Hamers, Menno P.J. de Winther, Marion J.J. Gijbels, Vivian de Waard, Carlie J.M. de Vries. Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, The Netherlands

Rationale: Nuclear receptor Nur77 is also known as NR4A1, TR3 or NGFI-B and is expressed in human atherosclerotic lesions in macrophages, endothelial cells, T cells and smooth muscle cells. Nur77-knockout (Nur77-KO) mice lack Ly6C-minus monocytes, but have normal LyC6-positive numbers. Macrophages play a critical role in atherosclerosis and we have demonstrated that Nur77 has an anti-inflammatory function in THP-1 macrophages. The aim of the current study is to assess the function of Nur77 in myeloid cells in atherosclerosis and study the effect of bone marrow-specific deficiency of Nur77 on atherosclerosis. Objective: This study aims to delineate the function of Nuclear receptor Nur77 in macrophages in atherosclerosis. Methods and results: Bone marrow-derived macrophages (BMM) from wild-type and Nur77-KO mice were cultured and classically activated with LPS or alternatively activated with IL-4. Nur77-KO BMM exhibit changed expression of M2-specific markers and a pro-inflammatory polraziation in response to LPS with enhanced expression of IL12, IFNγ, and SDF-1α. NO synthesis is also strongly induced in (non)-stimulated Nur77-KO BMM. SDF-1αis a potent chemotactic factor for B cells and monocytes. The chemoattractive activity of Nur77-KO BMM is abolished by SDF-1αinhibiting antibodies as well as by overexpression of Nur77 in the Nur77-deficient cells. In line with these data, Nur77-KO mice show enhanced thioglycollate-elicited migration of macrophages and B cells. The effect of bone marrow-specific deficiency of Nur77 on atherosclerosis was studied in low density lipoprotein receptor-deficient (Ldlr-KO) mice. Ldlr-KO mice with a Nur77-deficient bone marrow transplant develop 2.1-fold larger atherosclerotic lesions than wild-type bone marrow transplanted mice. These lesions contain more macrophages, T cells, smooth muscle cells and larger necrotic cores. SDF-1αexpression is higher in lesions of Nur77-KO transplanted mice, which may explain the observed aggravation of lesion formation, since SDF-1αis not only a chemoattractant for monocytes but also enhances smooth muscle cell proliferation. Conclusion: In macrophages the nuclear receptor Nur77 has an anti-inflammatory function, represses SDF-1αexpression and, most importantly, bone-marrow transplantation studies in Ldlr-KO mice revealed that Nur77 inhibits atherosclerosis.

Endothelial cells act as a rheostat of liver regeneration through an autocrine Angiopoietin-2 loop

Junhao Hu[1]*, Kshitij Srivastava[1], Hellmut Augustin[1,2]. [1]Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), [2]Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim, Heidelberg University (CBTM), Germany

Liver regeneration requires precisely coordinated proliferation between different cellular compartments of the liver, most notably hepatocytes and liver sinusoidal endothelial cells (LSEC), to reconstitute liver structure and physiological function. Recent evidence has shown that LSEC, beyond their role as structural determinants of liver architecture, may play an active role during liver damage and liver regeneration by secreting angiocrine factors that promote hepatocyte proliferation. This was evidenced by landmark studies showing that the disruption of LSEC function by VEGFR2 deletion or VEGFR2 blockade severely impaired liver regeneration. In order to identify other positively or negatively acting endothelial cell-derived angiocrine factors, we performed gene expression analyses of partially hepatectomized and sham operated livers and assessed differential gene expression. LSEC-derived Angiopoietin-2 (Ang-2) expression was rapidly downregulated upon 2/3 partial hepatectomy. Correspondingly, partial hepatectomy experiments in Ang-2-deficient mice revealed that loss of Ang-2, which is exclusively expressed by LSEC, enhanced hepatocyte proliferation during the inductive phase of liver regeneration (day 0-4). The mechanistic analysis of this angiocrine crosstalk between LSEC and hepatocytes identified autocrine acting Ang-2 as regulator of LSEC TGFß1 expression, which is known to act as a negative regulator of hepatocyte proliferation. While LSEC-derived negative Ang-2 regulation served as an enhancer of hepatocyte proliferation, liver regeneration was surprisingly found to be slowed in Ang-2-deficient mice during the angiogenic phase of liver regeneration (day 4-8). Correspondingly, Ang-2 was in wildtype mice prominently upregulated during the angiogenic phase of liver regeneration suggesting that the balance of direct vascular vs. indirect parenchymal Ang-2 effects may be shifted towards direct direct angiogenesis regulating effects during the later stages of liver regeneration. Mechanistically, this interpretation was supported by the finding that Ang-2 during the angiogenic phase served as positive regulator of VEGFR2 expression and the established endothelial Wnt pathway regulator Wnt2. Collectively, the experiments identify Ang-2 as an endothelial rheostat of liver regeneration whose expression is dynamically regulated during the different stages of liver regeneration to differentially control endothelial cell function (direct effects) and hepatocyte proliferation (indirect effects through EC-derived angiocrine growth factors).

Activated protein C unexpectedly binds directly to the endothelial receptor, TIE-2, to rapidly enhance endothelial barrier integrity

Nikita Minhas[1], Pradeep Naik[2], Meilang Xue[1], Christopher J Jackson[1]. [1]Sutton Arthritis Research Laboratory, Institute for Bone and Joint Research, Kolling Institute of Medical Research, University of Sydney at Royal North Shore Hospital, Sydney, Australia, 2065, [2]Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, India

Activated protein C (APC) is a natural anticoagulant with strong cyto-protective and anti-inflammatory properties and promotes chronic wounds to heal at least partly by stimulating angiogenesis. APC is thought to exert most of these effects by binding to its receptor, endothelial protein C receptor (EPCR) and cleaving protease activated receptor(PAR)-1 or PAR-2. The aim of this study was to investigate the mechanism of action of APC's favourable effect on endothelial barrier integrity. Results showed that APC did not require EPCR or PARs to enhance endothelial barrier integrity in the short term (< 1 hr). Instead, APC rapidly activated Tie2, the endothelial receptor which has recognised ligands of angiopoietin(Ang)-1 and -2. The ratio of phosphorylated(P)-Tie2:total(T)-Tie2 increased by ~ 2-fold after 15 minutes and ~4.5-fold after 60 minutes of APC treatment. Docking studies revealed intermolecular H-bonds between APC and Tie2 indicating binding between the two proteins. Protein binding assay using Biacore confirmed strong binding affinity (Kd = 0.003 nM) between APC and Tie2, which was only slightly less than Ang1-Tie2 (Kd=0.002) and remarkably, ~ 11 times higher than Ang2-Tie2 (Kd = 0.032 nM). In a mouse model of induced vascular leakage, APC significantly reduced lipopolysaccharide-induced vascular permeability in lungs and kidneys of C57BL6 mice. Inhibition of the Tie2 receptor blocked this effect of APC. In summary, this study reveals that APC is a new ligand for Tie2. By binding to and activating Tie2 APC rapidly enhances endothelial barrier function and prevents vascular leakage. Thus, APC may utilize Tie2 to stimulate the formation of „normal" blood vessels, the type required for wound healing.

Tissue Inhibitor of Metalloproteinase (TIMP)-2 regulates macrophage invasion by modulating MMP-14 Expression and retards plaque progression

Karina Di Gregoli, Rebecca Salter, Sarah J George, Christopher L Jackson, Andrew C Newby, Jason L Johnson. School of Clinical Sciences, University of Bristol, Bristol, United Kingdom

Rationale: Matrix metalloproteinases (MMP) are proposed to precipitate atherosclerotic plaque progression and instability. However, the role of their endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMP) remains unclear. Objective: Our objective is to elucidate the role of TIMP-2 in atherosclerotic plaque progression in apolipoprotein E-deficient (ApoE-/-) mice Methods and Results: Circulating monocytes were isolated from mice with a single deficiency of ApoE and either TIMP-1 or TIMP-2. TIMP-2 deficient monocytes and monocyte-derived macrophages demonstrated significantly ($p<0.05$) increased MMP-14 protein expression. Add back of recombinant TIMP-2 to TIMP-2 deficient macrophages decreased MMP-14 expression by 52% ($p<0.05$) but had no effect in TIMP-1 deficient macrophages. Conversely, siRNA for TIMP-2 but not TIMP-1 in wild-type macrophages increased MMP-14 expression 3-fold ($p<0.05$). Accordingly, monocyte/macrophage invasion was increased both in vitro (94%, $p<0.01$) and in vivo (77%, $p<0.001$) in TIMP-2 deficient mice compared to wild-type controls. Concomitantly, addition of exogenous TIMP-2 to TIMP-2 knockout monocyte/macrophages reduced invasion both in vitro (81%, $p<0.01$) and in vivo (70%, $p<0.001$) and also decreased macrophage MMP-14 expression by 69% ($p<0.05$). Analysis of brachiocephalic artery plaques revealed that loss of TIMP-1 had no effect on lesion area, compared with age-matched, strain-matched apoE knockout controls after 8 weeks of high-fat feeding. Similarly, lesions from apoE/TIMP-2 double knockout animals exhibited no change in cross-sectional area but composition and characteristics associated with predisposition to rupture, were greatly augmented. For example, a greater number ($p<0.001$) of plaques with a layered phenotype (a marker of plaque progression and complexity), compared to apoE single knockout controls. Moreover the plaque vulnerability index calculated as the macrophage and lipid content divided by VSMC and collagen content, was significantly increased ($p<0.001$) in TIMP-2 deficient animals compared to controls. Furthermore, intra-plaque macrophage MMP-14 expression was increased by 74% ($p<0.05$) in TIMP-2 deficient mice compared to wild-type controls, however no such change was observed in plaques between TIMP-1 wild-type and deficient mice. Despite a significant reduction in SMC content ($p<0.01$), lesions from apoE/TIMP-1 double knockouts did not differ in any other parameters compared to control animals. Conclusions: These data suggest that TIMP-2 retards monocyte/macrophage accumulation in atherosclerotic lesions and thus promotes plaque stability possibly through modulating both MMP-14 protein expression and activity. These findings support our ancillary studies in human plaques demonstrating that increased macrophage MMP-14 expression is associated with rupture-prone atherosclerotic lesions. Taken together, our results highlight the therapeutic potential of targeting MMP-14, possibly through elevating TIMP-2 levels, for the stabilisation of unstable atherosclerotic lesions.

Misfolded/partly unfolded extracellular proteins activate platelets by interaction with the stress response cell surface chaperones, glucose-regulated protein (GRP) 78/BIP, and protein disulfide isome

Anne Bertling[1], Kerstin Jurk[1], Martin F. Brodde[1], Christopher Stavermann[1], Meike Horn[1], Muzaffar Hussain[4], Christina Stürzel[1], Joachim Roth[5], Anja Müller[1], Jerzy-Roch Nofer[3], Judith Lahav[2], Hugo Van Aken[1], Christine Heilmann[4] and Beate E. Kehrel[1]. [1]Department of Anaesthesiology, Intensive Care and Pain Therapy, Experimental and Clinical Haemostasis, University Hospital, Muenster, Germany, [2] Department of Human Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv; _The Haemostasis Laboratory, Rabin Medical Center - Beilinson Hospital, Petah Tikva, Israel, [3]Center for Laboratory Medicine, University Hospital Muenster, Muenster, [4]Institute of Medical Microbiology, University of Muenster, [5]Department of Veterinary Physiology, Faculty of Veterinary Medicine, Justus-Liebig-University Giessen, Giessen, Germany

Introduction: Protein modifications such as oxidation, glycation, high shear conditions, cause proteins to lose their correct fold. In addition misfolded/partly unfolded proteins act as a functional coat for microorganisms. Acquired protein misfolding seems to be a much more widespread process than previously believed and might play an important role in vascular diseases. Herczenik et al. have shown, that a number of misfolded proteins were able to activate platelets. Molecular chaperones, previously believed to be restricted to the endoplasmic reticulum inside cells, can be exposed on the surface of cells and have been shown to act as endogenous modulators of the innate immune response. As platelets present the chaperones GRP78, and thiol isomerases on their surface, we studied the role of these chaperones in platelet activation by different misfolded proteins, HOCl oxidised LDL, Eap from S. aureus, human neutrophil alpha defensin modified plasma proteins, thrombospondin-1 in its amyloid like state and von Willebrand factor under high shear conditions. Methods: The effect of the completely different misfolded proteins on platelet adhesion, activation, secretion, aggregation, and thrombus formation was investigated by aggregometry, flow cytometry, thrombus formation in in vitro perfusion chambers and confocal laser scanning microscopy. Results: The chaperones GRP78, and protein disulfide isomerase showed prominent, punctuate staining, with striking co-localization at the platelet surface. All tested misfolded proteins induced platelet adhesion, GPIIb/IIIa activation, granule secretion, platelet aggregation and in vitro thrombus formation. All used agonistic proteins exposed aggregation-prone hydrophobic regions, a hallmark of misfolding. Soluble GRP78 as well as soluble PDI, inhibitors of the PDI and the hydrophobic probe 4,4'-bis(1-anilinonaphthalene 8-sulfonate) (bis-ANS) inhibited platelet activation in a dose dependent manner. Conclusions: Human platelets present the chaperones GRP78 and PDI on their surface. These chaperones are known to sense and bind misfolded proteins that are exposing hydrophobic patches. As misfolded proteins activate platelets and as soluble chaperones as well as a hydrophobic probe inhibited this activation, GRP78 and the PDI are very likely to be involved in thrombus formation under environmental conditions that induce the presentation of misfolded proteins to platelets, like infection, inflammation, shear stress, oxidative stress and glycation. As cell surface chaperones can be found on leukocytes, endothelial cells and smooth muscle cells as well, we believe that the observed interaction of cell surface chaperones with misfolded extracellular proteins is a more general one. The new mechanism contributes to the understanding of the intertwined processes thrombosis and inflammation.

Bench to bedside development of novel “molecular assassin” strategies targeting immediate-early genes

Levon M. Khachigian. Centre for Vascular Research, University of New South Wales, Sydney Australia

Immediate early genes (IEGs) are genes that are activated rapidly and transiently in response to a wide range of stimuli without new protein synthesis. We used DNA-based enzymes (DNAzymes) to probe the function of the IEG and bZIP transcription factor c-jun. DNAzymes are single-stranded catalytic all-DNA molecules with phosphodiester interbase linkages that bind and cleave target RNA. Dz13 is a DNAzyme with 9+9 nt hybridization arms targeting c-jun mRNA. Dz13 inhibits the pathogenesis of a range of experimental models of pathologic angiogenesis and inflammation including rheumatoid arthritis, tumor growth and lung sepsis. We recently used Dz13 as therapeutic tools for eye disease and skin cancer, and successfully completed the world-first clinical trial of a DNAzyme of any kind. Retinal neovascularization is a critical component in the pathogenesis of common ocular disorders that cause blindness, and treatment options are limited. In a novel model of pre-existing retinal neovascularization, a single injection of Dz13 in a lipid formulation inhibited c-Jun expression and reduced retinal microvascular density. The DNAzyme inhibited retinal microvascular density as effectively as VEGF-A antibodies. Comparative microarray and gene expression analysis determined that Dz13 suppressed not only c-jun but a range of growth factors and matrix-degrading enzymes that lie downstream of c-jun. Moreover, animals treated with Dz13 sensed the top of the cage in a modified forepaw reach model suggesting the DNAzyme may improve eyesight. We have also recently demonstrated the capacity of Dz13 to inhibit growth of two common skin cancer types, basal cell carcinoma and squamous cell carcinoma, in a therapeutic setting after the tumors became established. Dz13 inhibits tumor growth in both immunodeficient and immunocompetent syngeneic mice, and reduced metastasis. It suppressed neovascularisation in tumor-bearing mice and tumor-bearing zebrafish, and increased apoptosis. Dz13 inhibition of tumor growth, which requires an intact catalytic domain, is due in part to the induction of tumor immunity. Dz13 reduces the expression of c-jun and numerous MMPs and growth factors in the tumors. Dz13 also increased tumor apoptosis and activated numerous caspases and p53 expression. In a series of good laboratory practice (GLP)-compliant toxicology studies we commissioned in cynomolgus monkeys, minipigs and rodents, the DNAzyme is safe and well-tolerated. It also does not interfere in over 70 physiologically-relevant bioassays. We conducted a Phase I/IIa “first-in-human” safety and tolerability dose-escalation trial of Dz13 in skin cancer patients (the “DISCoverY“ trial) that have recently concluded. Our findings thus demonstrate the key regulatory role of c-jun in pathologic angiogenesis and the therapeutic potential of DNAzymes in a range of vascular disorders.

Visualization of the initial steps of lymphatic vessel development and the role of CCBE1 in this process using ultramicroscopy

R. Hägerling[1]*, C. Pollmann[1], M. Andreas[1], L. Kremer[1], Uwe Schröer[3], Volker Andresen[3], S. Schulte-Merker[2] and F. Kiefer[1]. [1]Cell Signaling Laboratory, Department of Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, Röntgenstraße 20, D-48149 Muenster, Germany, [2]Developmental Biology, Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, [3]LaVision BioTec GmbH, Astastraße 14, D-33617 Bielefeld, Germany

The lymphatic system, the second vascular systems of vertebrates, plays an essential role in fluid homeostasis, the initiation of immune reactions and fat absorption, but also contributes to pathological conditions. After the initial formation of blood vessels during embryonic development, a subpopulation of endothelial cells in the cardinal vein in response to an as yet unknown signal express the transcription factor prospero-related homeobox 1 (PROX1), subsequently followed by vascular endothelial growth factor receptor 3 (VEGFR-3) and other lymphatic-specific markers. Lymphatic endothelial progenitor cells then leave the cardinal vein (CV) and follow a gradient of VEGF-C to form the first lymphatic structures and the thoracic duct. The primary superficial lymphatic plexus finally is formed by radial sprouting of lymphatic endothelial cells (LECs). Several competing models for the process of initial lymphangiogenesis exist. To resolve the mechanisms of the initial steps of lymph vessel formation, we generated high resolution 3-dimensional renderings of mouse developmental stages between E9.5 and E12.5. We analyzed immunofluorescence stained and PROX1-driven mOrange2-genetically-labeled mouse embryos, using the novel light sheet imaging modality ultramicroscopy. Our analysis revealed that initially LECs migrate away from the CV as streams of non-luminized, loosely connected cells, forming a mesh network of LECs. The LECs stream coalesces into a first peripheral longitudinal lymphatic vessel (PLLV), located lateral to the somites and subsequently forms a large luminized structure, we refer to as the primary thoracic duct (pTD) near the cardinal vein. Collectively, these first lymphatics structures have been referred to as lymph sacs. An area of highest level PROX1 expression demarks the site of closest juxtaposition between the CV and pTD and likely gives rise to the first venous-lymphatic valve. LECs sprouting from the PLLV form superficial lymphatics. In CCBE-1-deficient embryos, Prox-1-positive LECs arose normally in the CV and between E10 and E10.5 the initial migration of LECs away from the CV was unperturbed. Unexpectedly, the PROX1 expression domain in the CV was broadened in CCBE1-deficient embryos, which displayed increased VEGFR-3 expression and sprouting of venous endothelium. Concomitantly in CCBE1 KO embryos, we observed a distinct loss of PROX1-positive cardiomyocytes. Our study suggests that ultramicroscopy is an extremely powerful tool for the analysis of developmental processes and allowed us to develop a novel model for the initial steps of blood lymphatic separation and initial lymphangiogenesis. Our future goals include investigation of the mechanisms controlling LEC migration and identification the molecules regulating the special control of this process.

The Notch pathway regulates the expression of pro-angiogenic transcription factor Sox17

Sungsu Lee, Hanseul Yang, Gou Young Koh, and Injune Kim. Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea

Angiogenic behaviors of endothelial cells are influenced by various environmental regulations through surface receptors. However, little is known about the transcriptional regulation in endothelial cells during vascular development while the Notch pathway is involved in the formation of vascular network. We found the transcription factor Sox17 is expressed in endothelial cells and studied the role of Sox17 in vascular development and vascular differentiation by using Sox17 loss-of-function mouse models and Sox17-null embryonic stem cells. While endothelial cells emerged successfully without Sox17 in development and differentiation, developmental angiogenesis was arrested with the reduced sprouting of endothelial cells by Sox17 deletion. Sox17-null endothelial cells had a tighter cell-cell interaction leading to the reduced migratory behavior. These demonstrate that endothelial Sox17 is a pro-angiogenic regulator required for proper vascular development. Interestingly, we found that the expression of endothelial Sox17 is regulated by the Notch pathway which is important for the specification of tip and stalk cells. We also observed Sox17 is preferentially expressed in tip cells compared to stalk cells. Enforced Sox17 expression induced more tip cells in postnatal retinal angiogenesis. These findings suggest that Sox17 is a downstream player in tip and stalk cell determination mediated by the Notch pathway. Collectively, Sox17 is a pro-angiogenic regulator by converting environmental cues into transcriptional regulation in endothelial cells.

The AMP-activated protein kinase regulates endothelial angiotensin-converting enzyme (ACE) expression via p53 and microRNA-143/145

Karin Kohlstedt, Caroline Trouvain, Ingrid Fleming. Institute for Vascular Signalling Centre of Molecular Medicine Goethe-University Theodor Stern Kai 7 60590 Frankfurt am Main Germany

The angiotensin converting enzyme (ACE) plays a major role in blood pressure regulation and cardiovascular disease development, but relatively little is known about the molecular mechanisms regulating its expression. Recently, ACE levels were found to be regulated by the miR143/145 cluster in murine vascular smooth muscle cells and by the AMP-activated protein kinase (AMPK) in monocytes. Since shear stress activates AMPK in endothelial cells, we determined whether or not flow changes endothelial ACE expression and whether this involves AMPK and/or miR143/145. Human umbilical vein endothelial cells exposed to shear stress (12 dyn/cm2) demonstrated a time-dependent decrease in ACE mRNA and protein expression. Silencing of the catalytic AMPKα2 subunit (siRNA), but not the AMPKα1, increased ACE expression under static conditions and attenuated the decrease in ACE protein and mRNA induced by shear stress. The suppressive effect of AMPK on ACE expression was confirmed by pharmacological activation of AMPK (AICAR, Metformin). In vivo, significantly higher ACE levels were detected in situ in AMPKα2-/- endothelial cells (aorta, aortic arch, small mesenteric arteries, femoral arteries and kidney) than their wild-type littermates. There was no difference in ACE expression in wild-type and AMPKα1-/- mice. Moreover, while blood pressure was comparable in AMPKα2+/+ and AMPKα2-/- mice under basal conditions, the ACE inhibitor ramipril (5 days, 5mg/kg/d, in the drinking water) caused a more pronounced decrease in blood pressure in AMPKα2-/- mice. The latter animals also demonstrated a significantly impaired bradykinin-induced vasodilatation of the hindlimb vasculature that could be restored by ACE inhibition with ramiprilat. We found no difference in the acetylcholine-induced vasodilatation in AMPKα2-/- and AMPKα2+/+ animals and no differences in the bradykinin- or acetycholine-induced vasodilatation in AMPKα1-/- and AMPKα1+/+ animals. Overexpression of miR-143/145 decreased ACE expression in human endothelial cells and silencing AMPKα2 decreased endothelial miR143/145 expression. Fluid shear stress increased levels of mature miR-143/145, but failed to increase pri-miR143/145 levels. The latter indicates a regulation of miR143/145 expression at the posttranscriptional rather than transcriptional level. We therefore assessed the involvement of the AMPK target p53 in this response as it is known to enhance the posttranscriptional processing of several miRNAs. Indeed, p53 expression and phosphorylation (on Ser15) were increased by shear stress, an effect blocked by AMPKα2 siRNA (but not AMPKα1 siRNA). These data demonstrate that endothelial ACE expression is suppressed by AMPKα2 via mechanism involving the activation of p53 activation and upregulation of miR143/145. Since dysregulation of the AMPK as well as p53 plays a major role in the development of several diseased (e.g. diabetes, cancer), their effect on the regulation of miRNA143/145 and thus ACE levels might contribute to the development of disease-associated cardiovascular disorders.

Micro RNA, collagen at RISC. A story of miR21

Felix F. Kolk, Sanne Verbruggen, Daniel G.M. Molin, Mark J. Post. Dept. of Physiology, CARIM, MUMC+, Maastricht, The Netherlands

Collagen production in vascular tissue engineered grafts (VTEG) is pivotal since collagen contributes to the extracellular matrix (ECM) and ensures vessel strength. Collagens are regulated by growth factors, and micro RNAs (miRs). Previous studies showed that miR21 stimulates collagen production in fibroblasts by lowering SMAD7 expression. In this study we investigate the interplay of miR21 and TGFβ signaling in Human Coronary smooth muscle cells (CASMCs) and Human Aorta smooth muscle cells (AoSMCs) to establish whether miR intervention can be used to improve collagen production. CASMCs and AoSMCs were seeded, grown to full confluence and, subsequently, transfected with either mimic-, antagomiR-21 or scrambled control, and cultured in starvation medium (Promocell SMC medium, 0.5% FBS or Optimem, 0.1% FBS), with or without TGFβ (10ng/mL) and/or ALK5 inhibitor. Next, cells were lyzed and total mRNAs, miRNAs or protein was isolated. mRNA expression levels of Collagen 1α1, 1α2, 3α1, SMAD3 and 7 and miRLet7d, miR29a and miR21 were determined by RT-QPCR and collagen protein content by Sirius red assay. All conditions were normalized to control (unstimulated condition). Cells stimulated with TGFβ gave highest collagen 1α1 and 1α2 mRNA expression after both 2h and 48h of culture. Also collagen protein levels were increased after 72h of stimulation. Expression patterns of miR Let7d, miR29a and miR21 differed between conditions and time points. miR21 was already increased after 2h of stimulation, and increased even further during culturing. Expression of collagen repressing miRLet7d and miR29a was lowered at 2h, however increased both during culturing, yet expression was much less than miR21 (100-1,000 fold). Cells stimulated with miR21 mimic showed significant higher collagen mRNA, miR21 and protein expression. AntagomiR 21 had no effect on mRNA collagen expression, whereasmiR21 expression was decreased along with total collagen protein levels. Our data show that TGFβ¬ stimulation greatly affects collagen mRNA, miR21 and collagen protein expression. A positive correlation between miR21 and collagen mRNA expression was apparent for all time points analyzed. Stimulation with mimic miR21 can increase collagen protein expression. From our data we conclude that miR21 regulates collagen expression.

RGS-5 attenuates smooth muscle cell contraction during arteriogenesis

Anja Feldner, Larissa Pfisterer, Thomas Wieland, Marina Schorpp-Kistner, Peter Angel, Markus Hecker and Thomas Korff. Institute of Physiology and Pathophysiology, Division of Cardiovascular Physiology, University of Heidelberg; Department of Internal Medicine III - Cardiology, University of Heidelberg; Division of Signal Transduction and Growth Control (A100), German Cancer Research Center (DKFZ), Heidelberg, Germany

Arteriogenesis is an important adaptive remodeling process which involves the enlargement and thickening of arterioles running in parallel to an occluded conduit artery, and is capable of partially compensating the consequences of peripheral artery disease by creating natural bypasses. While arteriolar remodeling is orchestrated by changes in the biomechanical forces to which the collateral blood vessels are exposed to, the impact of this process on their functional properties is still elusive. To this end, we observed that arteriogenesis is accompanied by a robust increase in the abundance of the regulator of G-protein signaling 5 (RGS-5) in vascular smooth muscle cells (SMC). Subsequent in vitro experiments revealed that RGS-5 expression is augmented by activation of cGMP-dependent signaling through nitric oxide - a pivotal determinant of arteriogenesis. Based on previous findings in genetically altered mice indicating that a failure in arterial contractile capacity entails an increase in RGS5 protein expression, we hypothesized that RGS5 alters contractile responses of arterioles during arteriogenesis. Subsequent studies employing RGS5-over-expressing SMCs suggested that this regulatory protein blunts both Gq/11- and G12/13-mediated intracellular calcium mobilization evoked by prototypic vasoconstrictor agents such as sphingosine-1-phosphate or angiotensin II. Further analyses of changes in the respective signaling pathways showed that RGS5 shifts vasoconstrictor-induced responses from calcium-mediated contraction to Rho-kinase dependent stress fiber formation. Collectively, these findings suggest that shear stress-mediated release of nitric oxide triggers the increase in RGS5 expression in vascular SMCs of arteriogenic collaterals which promotes stress fiber formation and attenuates their responsiveness to prototypic vasoconstrictor agents. This mechanism may improve the mechanical stability of the vessel wall and may play a decisive role in controlling the contractile capacity of SMCs during arteriogenic remodeling processes.

microRNA family-23 causes endothelial dysfunction and is dysregulated during cardiac fibrosis

Guido Krenning, Erwin Berkema, Jan-Renier A.J. Moonen, Arjen H. Petersen, Marja G.L. Brinker and Martin C. Harmsen. Cardiovascular Regenerative Medicine Research Group (CAVAREM), Dept. Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1 (EA11), 9713GZ Groningen, The Netherlands

Development of cardiac fibrosis is a common trait in patients with cardiac failure, regardless of the etiology of the cardiomyopathy. Cardiac fibrosis is characterized by endothelial dysfunction, vascular rarefaction and disruption of the normal structure of the heart by accumulating myofibroblasts that deposit extracellular matrix between the cardiomyocytes. Recently, we have shown that myofibroblasts may originate from endothelial cells, through a process termed 'endothelial-mesenchymal-transition', and that this transition coincides with specific changes in microRNA expression. In this study, we show that expression of microRNA family-23 members (i.e. miR-23b, -24 and -27b) is increased during cardiac fibrosis and decided to investigate the effect of microRNA family-23 expression of endothelial cell phenotype and function. We lentivirally transduced endothelial cells with miR-23 family members which resulted in spontaneous activation (i.e. E-selectin/VCAM1 expression, IL1b/TNFa expression). microRNA target analysis revealed that the miR-23 family targets various members of the PPARg signaling pathway(i.e. PPARG, PGC1 and the RXRs), and their repression by miR-23 family members resulted in increased NFkB expression and consequential endothelial activation. Furthermore, decreased expression of PGC1a resulted in hampered mitochondrial biogenesis, distorted mitochondrial ultrastructural morphology, and increased ROS production. Overexpression of the microRNA-23 family did not however, result in endothelial-mesenchymal transition. In conclusion, we show that distinct microRNAs are dysregulated during cardiac fibrosis. The microRNA Family 23 causes endothelial dysfunction by targeting PPARg signaling, resulting in endothelial activation and ROS production. Therefore, we propose that the microRNA Family-23 may pose a novel therapeutic target in preventing endothelial dysfunction during cardiac disease.

Flt1 controls vascular branching morphogenesis and modulates neurogenesis in the zebrafish embryo

Janna Krueger[1,2], Stefan Kunert[1], Ferdinand le Noble[1,2]. [1]Dept. of Angiogenesis and Cardiovascular Pathology, Max Delbrueck Center for Molecular Medicine, D13125, Berlin, Germany, [2]Center for Stroke Research Berlin, Charité, D10117 Berlin, Germany

During embryonic development, blood vessels and nerves grow in parallel to each other. They furthermore share a similar functional organization and genetic pathways underlying their network formation. VEGF has initially been characterized as an endothelial cell-specific growth factor, but recent studies indicate that VEGF is also important for neuronal function. The VEGF bioavailability is mainly dependent on the presence of soluble VEGFR-1 (sFlt1). We therefore investigated the role of VEGFR-1 (Flt1) during embryonic development in zebrafish embryos. Here, we present genetic data showing that flt1 acts as negative regulator of tip cell formation, and arterial branching morphogenesis. Moreover, we obtained evidence for a potential role of flt1 during neurogenesis. Zebrafish embryos expressed soluble Flt1 (sFlt1) and membrane bound Flt1 (mFlt1). In Tg(flt1BAC:yfp) x Tg(kdrl:ras-cherry) embryos, flt1:yfp was expressed in tip, stalk and base cells of segmental artery sprouts, and overlapped with kdrl:cherry expression in these domains. flt1 morphants showed increased tip cell numbers, enhanced angiogenic behavior, and hyperbranching of segmental artery sprouts. The additional arterial branches developed into functional vessels carrying blood flow. In support of a functional role for the extracellular VEGF binding domain of Flt1, both overexpression of sflt1 or mflt1 rescued aberrant branching in flt1 morphants, and overexpression of sflt1 or mflt1 resulted in short arterial sprouts, with reduced numbers of filopodia. flt1 morphants showed reduced expression of Notch receptors, the Notch downstream target efnb2a, and ectopic expression of flt4 in arteries, consistent with loss of Notch signaling. Conditional overexpression of notch1a-intra cellular cleaved domain (NICD) in flt1 morphants restored segmental artery patterning. Macrophages had no impact on the hypersprouting observed in flt1 mophants. Interestingly, vascular specific overexpression of sflt1 resulted in ermergence of sFlt1 protein in the neural tube. Therefore the developing nervous system of the trunk may contribute to the distribution of Flt1, relevant for vessel guidance. In addition to the vascular expression of flt1, we detected prominent flt1:yfp expression in interneurons of Tg(flt1BAC:yfp) x Tg(kdrl:ras-cherry) embryos. Loss of flt1 associated with reduced neuronal cell number, indicating that flt1 might be relevant for neurogenesis. Thus, Flt1 acts, in a Notch dependent manner, as a negative regulator of tip cell differentiation and branching morphogenesis. Flt1 distribution may be fine-tuned involving interactions with the developing nervous system. Additionally to flt1´s role during branching morphogenesis, flt1 might exert a function during neurogenesis in zebrafish embryos.

TSAd controls VEGF induced angiogenic sprouting via Src activation downstream of VEGFR2

Simone Kutschera, Zuyue Sun, Laurens van Meeteren, Narendra Padhan, Xiujuan Li, and Lena Claesson-Welsh. Uppsala University, Rudbeck Laboratory, Dept. Immunology, Genetics and Pathology, Uppsala, Sweden

T-cell specific adaptor protein (TSAd) is expressed in the cytoplasm of endothelial cells and binds to VEGFR2 at Y949. The TSAd/VEGFR2 signaling axis is involved in endothelial cell migration and tumor angiogenesis (Matsumoto et al., 2005). Here we show the role of TSAd in VEGF-induced Src activation and the contribution to endothelial cell function in vitro and in vivo. In vivo, phosphorylation of Src is decreased in VEGF-injected tsad-/- mice compared to wild type mice whereas phosphorylation of VEGFR2 (Y-949 and Y-1173) is not affected by TSAd-deficiency. This defect in activating Src leads to vessel stabilization and specific attenuation of VEGF-induced tracer extravasation while agents such as histamine still induce permeability (Sun et al, in revision). Embryonic stem (ES) cells established from tsad-/- mice show reduced sprouting capacity in 3D embryoid bodies (EB); the deficiency can be rescued by lentiviral transduction of wild type TSAd. In contrast, transduction of wild type ES cells with a TSAd mutant lacking a functional SH2 domain, required for binding to VEGFR2, or transduction with a TSAd mutant lacking the C-terminal proline-rich region disrupts VEGF-induced angiogenic sprouting. In accordance with the decreased sprouting in tsad-/- EBs and the reduced tumor angiogenesis reported on earlier (Matsumoto et al. 2005), sprouting angiogenesis is reduced in vivo in TSAd-deficient mice. Subcutaneous matrigel plugs loaded with VEGF are densely and homogenously vascularized in wild type mice whereas plugs in tsad-/- mice yield few vessels at the rim of the plug. This sprouting defect can be rescued by FGF2 which points to a compensation mechanism for the defective VEGFR2/TSAd/Src signaling axis. Taken together our data identify TSAd as an important regulator of VEGF-induced angiogenic signaling regulating Src activation in endothelial cells, angiogenic sprouting and vessel integrity. References: Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, Wang L, Wikner C, Qi JH, Wernstedt C, Wu J, Bruheim S, Mugishima H, Mukhopadhyay D, Spurkland A, Claesson-Welsh L. VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J. 2005 Jul 6;24(13):2342-53. Sun Z, Li X, Massena S, Kutschera S, Padhan N, Gualandi L, Sundvold-Gjerstad V, Gustafsson K, Choy W, Zang G, Quach M, Jansson L, Phillipson M, Abid MR, Spurkland A, Claesson-Welsh L. VEGFR2 -TSAd -Src signaling regulates vascular permeability in vivo. In revision.

Dynamic cell rearrangements shape the vascular network of the developing zebrafish embryo

Anna Lenard, Lukas Herwig, Elin Ellertsdottir, Heinz-Georg Belting and Markus Affolter.
Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

To form an interconnected network of endothelial tubes, a number of vessels of the developing vasculature have to interact and connect to each other. While much is known about vessel sprouting, little is known about the vessel fusion at morphogenetic and molecular levels. To describe the dynamic of endothelial cell behaviors that lead to formation of this complex tubular network we have generated transgenic fish lines expressing EGFP-fused versions of the junctional proteins ZO1 and VE-cadherin, which mark the presence and the de novo formation of AJ/TJ and the outline of endothelial cells (ECs). Using these novel tools, we have characterized the process of vessel fusion in various regions of the zebrafish embryo vasculature. Previously, we described the fusion of intersegmental vessels (ISVs) in the tail of the fish (Herwig et al., Curr.Biol. 2011). Since these vessels arise very early, they develop in the absence of stable heart beat and are not subjected to blood/plasma pressure during the initial fusion steps. We would like to understand how these forces influence vascular development; therefore we are currently analysing vessel fusion in the head region of older embryos where stable plasma pressure is present. We have observed that the initial steps of the new contact formation are similar to the ones characterized before for the ISVs. They include new junctional material deposition on the contact site in the form of a spot that is subsequently elaborated into a ring accompanied by de novo apical membrane deposition, as shown by the localization of the apical marker podocalyxin2. After the establishment of the new connection, the process of lumen formation is highly influenced by the presence of plasma pressure and therefore differs from the ISVs. The vessel sprouts are lumenized already as they establish the new contact. The membrane of the tip cell invaginates towards the sprouting tip, as shown by antibody staining and confocal time lapse imaging. Perfusion of the new vessel involves further tip cell membrane invagination and finally membrane fission at the contact site with another cell to form a unicellular tube with continuous transcellular lumen. This unicellular conformation is transient and endothelial cells engage in a series of dynamic cell movements within the existing vessel to finally form a multicellular tube. We characterized in detail the cellular rearrangements involved in this process and have observed, that the cells that initially had transcellular lumen split on one side of the tube to allow the neighbouring cells to move closer together and establish a new contact. We have observed this process in several head vessels of 2.5-4-day-old fish embryos and we could show that cell splitting and new contact formation within the existing tube are crucial for unicellular to multicellular vessel transformation. The described sequence of events is recurrent and seems to be a default mechanism for vessel fusion.

EC4-Fc, a ~50KDa fragment of N-cadherin, reduces apoptosis and promotes atherosclerotic plaque stability

Cressida A Lyon, Jason L Johnson, Steve White, Graciela M Sala-Newby, Sarah J George. University of Bristol, United Kingdom

Introduction: Apoptosis of vascular smooth muscle cells (VSMCs) is detrimental to atherosclerotic plaque stability, potentially resulting in plaque rupture and clinical events, such as heart attack or stroke. Therefore, agents that reduce VSMC apoptosis are of great clinical interest. We have previously shown that soluble N-cadherin (SNC) conjugated to the Fc immunoglobulin G domain (120kDa) reduces VSMC apoptosis and also increases markers of atherosclerotic plaque stability1. We also demonstrated that it mediated this effect via activation of the fibroblast growth factor receptor (FGF-R). In this study, we aimed to identify the active site of SNC which would enable us to produce a smaller protein that has similar beneficial effects on plaque stability. A smaller molecule would have greater clinical translation potential Results: . Firstly, to identify whether it is the FGF-R binding site or the N-cadherin binding site within SNC that is important for the anti-apoptotic effect, we mutated these sites. Treatment with SNC containing the mutated N-cadherin binding site reduced human VSMC apoptosis (as determined by cleaved caspase-3 immunocytochemistry) compared with VSMC treated with Fc control (4.9 +/- 1.0% vs. 15.7 +/- 2.0%, n=3, $p<0.05$). However, SNC with a mutated FGF-R binding site did not reduce apoptosis (15.2 +/- 2.8% vs. 15.7 +/- 2.0%, n=3, $p<0.05$), suggesting that FGF-R binding, rather than N-cadherin binding, is vital for the anti-apoptotic effect. The FGF-R binding site is located in the fourth (of 5) extracellular (EC) domain of N-cadherin, known as EC4. We cloned EC4-Fc and produced the ~50kDa protein. EC4-Fc had the same significant anti-apoptotic effect in VSMCs as SNC-Fc (3.9 +/- 1.1% (EC4-Fc) or 4.1 +/- 1.4% (SNC-Fc) vs. 15.1 +/- 2.3%, n=3, $p<0.05$). EC4-Fc increased phospho-Akt (163 +/- 21% of control, n=4, $p<0.05$) and phospho-FGF-R (142 +/- 13% of control, n=5, $p<0.05$) to a similar extent to SNC-Fc. EC4-Fc also significantly reduced apoptosis in mouse blood derived macrophages (4.4 +/- 3.2% vs. 25.7 +/- 2.9%, n=3, $p<0.05$) and human umbilical vein endothelial cells (8.0 +/- 1.3% vs. 22.8 +/- 4.8%, n=3, $p<0.05$). Elevation of plasma levels of EC4-Fc (n=13), SNC-Fc (n=11) or Fc (n=15) in male Apolipoprotein E deficient mice with existing atherosclerosis was achieved by tail-vein delivery of adenoviruses overexpressing EC4-Fc, SNC-Fc or Fc. Plasma elevation of EC4-Fc significantly reduced apoptosis in brachiocephalic artery plaques by ~65% compared to the Fc control, as determined by cleaved caspase-3 immunohistochemistry ($p<0.05$). Additionally EC4-Fc significantly reduced the size of the plaques by ~35% ($p<0.05$) and the incidence of buried fibrous caps (a surrogate plaque rupture marker) by ~50% ($p<0.05$). Conclusion: In summary, this study demonstrates that EC4 contains the active site and that EC4-Fc alone has almost identical effects to SNC, reducing apoptosis and stabilising atherosclerotic plaques. Therefore, EC4-FC or peptides from this region could be clinically useful to retard plaque instability.

Endothelial-mesenchymal transition promotes the natural regression of infantile hemangiomas

Logan A. Walsh[1,2], John B. Mulliken[3] and Damian Medici[1,2]. [1]Division of Matrix Biology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA, [2]Department of Developmental Biology, Harvard School of Dental Medicine, Harvard Medical School, Boston, MA 02115, USA, [3]Department of Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA

Hemangiomas are the most common tumors of infancy, formed by clonal expansion of vascular endothelial cells. These tumors rapidly proliferate over the first year of life then undergo a slow period of regression and completely disappear before adolescence. Although some insights have been gained into the proliferating phase of the disease, little is know about how these tumors naturally regress. We show that endothelial-mesenchymal transition (EndMT) is a critical mechanism of hemangioma regression by mediating the differentiation of hemangioma endothelial cells into adipocytes. Adipogenesis is a hallmark of hemangioma regression, as the endothelial tumor is replaced by fat tissue over time. We show that the adipocytes found in regressing hemangiomas express endothelial-specific biomarkers, whereas normal subcutaneous adipocytes do not. Furthermore, X-chromosome inactivation studies demonstrate that adipocytes expressing endothelial markers in regressing hemangiomas are clonal, suggesting that they are derived from the tumor cells. An influx of macrophages into the tumors over time promotes EndMT by secreting Transforming Growth Factor-beta2 (TGF-β2). These newly formed mesenchymal cells demonstrate a stem cell phenotype by expressing mesenchymal stem cell markers and acquiring multipotent differentiation capabilities. Insulin-like Growth Factor-1 (IGF-1), also secreted by macrophages and systemically elevated with age in response to human growth hormone, is essential for differentiating these endothelial-derived mesenchymal stem-like cells into adipocytes. These data identify a critical role for EndMT-dependent differentiation of vascular endothelial cells into fat cells during hemangioma regression and provide the first evidence that EndMT can resolve human disease.

Retinoic acid induces blood-brain barrier development

Mark R. Mizee[1]*, Desiree Wooldrik[1], Kim A.M. Lakeman[1], Bert van het Hof[1], Joost A.R. Drexhage[1], Dirk Geerts[2], Marianna Bugiani[3], Eleonora Aronica[4], Reina E. Mebius[1], Alexandre Prat[5], Helga E. de Vries[1], and Arie Reijerkerk[1]. [1]Blood-Brain Barrier Research Group, Department of Molecular Cell Biology and Immunology, VU university medical center, Amsterdam, The Netherlands, [2]Department of Pediatric Oncology/Hematology, Sophia Children's Hospital, Erasmus University Medical Center, Rotterdam, The Netherlands, [3]Department of Pathology, VU university medical center, Amsterdam, The Netherlands, [4]Department of Neuropathology, Academic Medical Center, University of Amsterdam, The Netherlands, [5]Neuroimmunology Research Laboratory, CHUM Notre-Dame Hospital, Faculty of Medicine, Université de Montréal, Montréal, Quebec, Canada

The blood-brain barrier (BBB) is crucial in the maintenance of a controlled environment within the brain to safeguard optimal neuronal function. The endothelial cells (ECs) of the BBB possess specific properties which restrict the entry of cells and metabolites into the central nervous system (CNS). The specialized BBB endothelial phenotype is induced during neurovascular development by surrounding cells of the CNS. However, the molecular differentiation of the BBB endothelium remains poorly understood. Retinoic acid (RA) plays a crucial role in the brain during embryogenesis. Since radial glial cells supply the brain with RA during the developmental cascade and associate closely with the developing vasculature, we hypothesize that RA is important for the induction of BBB properties in brain ECs. Analysis of human post-mortem fetal brain tissue shows that the enzyme mainly responsible for RA-synthesis, retinaldehyde dehydrogenase, is expressed by radial glial cells. Moreover, pharmacologic inhibition of RAR activation during the differentiation of the murine BBB resulted in leakage of serum proteins into the developing brain and reduced the expression levels of important BBB determinants. In addition, the most important receptor for RA-driven signaling in the CNS, RA-receptor beta (RARbeta), is markedly expressed by the developing brain vasculature and not detectable in post-natal stages. Our findings have been further corroborated by in vitro experiments showing RA and RARbeta dependent induction of different aspects of the brain endothelial cell barrier. Together, our results point to an important role for RA in the induction of the BBB during human and mouse development.

1,12 EET contributes to acute hypoxic pulmonary vasoconstriction by stimulating the association of α and β1 subunits of mitochondrial BK channels

Isabelle Moneke[1], Annemarieke E. Loot[1], Matthias Oelze[2], Andreas Daiber[2], Ingrid Fleming[1]. [1]Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University, Frankfurt am Main, [2]2nd Medical Clinic, Department of Cardiology, Johannes Gutenberg University, Mainz, Germany

In the systemic circulation, 11,12-epoxyeicosatrienoic acid (11,12-EET) has been shown to elicit vascular relaxation, partially through the activation of large conductance Ca2+-activated potassium (BK) channels. However, in the pulmonary circulation, 11,12-EET can enhance hypoxia-induced pulmonary vasoconstriction. Since the β1 subunit plays an important role in the regulation of BK channel function we assessed hypoxia- and 11,12-EET-induced pulmonary vasoconstriction following BKβ1 subunit deletion.In buffer-perfused mouse lungs, acute exposure to hypoxia (1% O2) increased pulmonary artery pressure and this was significantly enhanced in the presence of nitric oxide synthase and cyclooxygenase inhibitors. Under these conditions the elevation of tissue EET levels using an inhibitor of the soluble epoxide hydrolase (sEH-I), further increased the hypoxic contraction. Direct administration of 11,12-EET also increased pulmonary artery pressure, and both the sEH-I- and 11,12-EET-induced effects were prevented by the BK inhibitor, iberiotoxin, and absent in BKβ1-/- mice. As vascular contraction is more usually associated with depolarization than hyperpolarization, we next determined the effect of 11,12-EET on the potential of cultured murine pulmonary artery smooth muscle cells loaded with the potential sensitive indicator, Di-8-ANNEPS. We found that, whereas cells from wild-type and BKβ1-/- mice responded similarly to KCl, 11,12-EET induced a membrane depolarization in cells from wild-type but not BKβ1-/- mice. In pulmonary artery smooth muscle cells a subpopulation of BK channels is localized in the mitochondria where opening is associated with a loss of the mitochondrial membrane potential. In intact pulmonary artery smooth muscle cells, 11,12-EET caused an iberiotoxin-sensitive loss of mitochondrial membrane potential (JC-1 fluorescence) in wild-type, but not in BKβ1-/- cells. Mechanistically, this could be linked to the 11,12-EET-induced association (co-immunoprecipitation) of the α and β1 subunits of the BK channel. Taken together, the results of the present investigation indicate that 11,12-EET contributes to pulmonary vasoconstriction by stimulating the association of the α and β1 subunits of mitochondrial BK channels. The 11,12-EET-induced activation of BK channels results in loss of the mitochondrial membrane potential and subsequent depolarization of the pulmonary artery smooth muscle cell membrane.

A new therapeutic modality for ischemia-reperfusion injury: Manoparticle-mediated delivery of pitavastatin into reperfused myocardium reduces ischemia/reperfusion injury in rats

Kazuhiro Nagaoka, Tetsuya Matoba, Kaku Nakano, Kenji Sunagawa, Kensuke Egashira.
Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Background: In ST-elevation acute myocardial infarction, early reperfusion is the most effective therapeutic strategy. Therapeutic effect of myocardial reperfusion is limited by ischemia-reperfusion (IR) injury. Pretreatment with statins at high doses (a regimen that could lead to serious adverse side effects in a clinical setting) before the occurrence of ischemia is known to reduce IR injury by activating reperfusion injury salvage kinase (RISK) pathway, whereas treatment with statins at the time of reperfusion is reported to elicit no therapeutic effects. Nanoparticle (NP)-mediated drug delivery system may be promising for targeting reperfused myocardium where vascular permeability is enhanced. Hence we tested the hypothesis that NP-mediated site-specific delivery of pitavastatin into ischemic myocardium ameliorates IR injury through activation of RISK pathway. Methods and Results: In the rat model of 30-min myocardial ischemia and subsequent reperfusion, we intravenously (IV) injected 0.1 mg FITC solution or NP containing 0.1 mg FITC at the time of reperfusion. Three hours after reperfusion, significant FITC fluorescence signals were detected in viable cardiomyocytes within ischemic myocardium (area at risk) in FITC-NP treated rats, whereas no significant FITC signals were observed after IV injection of FITC only. IV treatment of pitavastatin-NP containing 1.0 mg/kg pitavastatin, but not pitavastatin solution (1.0, 10 mg/kg), significantly reduced infarct size (64.7 ± 1.2 % in vehicle group vs 41.5 ± 3.0 % in pitavastatin-NP group, $P<0.001$) and number of TUNEL positive nuclei in peri-infarct area (3.5 ± 0.4 % in vehicle group vs 1.4 ± 0.2 % in pitavastatin-NP group, $P<0.01$) 24 hours after reperfusion. Pretreatment of wortmannin, a PI3K inhibitor, blunted the therapeutic effects of pitavastatin-NP on infarct size. Westernblot analysis revealed that pitavastatin-NP activated RISK pathway such as Akt signals (enhanced phosphorylation at Ser 473) in the reperfused myocardium. Immunohistochemistry showed that infiltration of myeloperoxidase-positive leukocytes into ischemic myocardium was significantly attenuated in pitavastatin-NP group than in vehicle group (1285 ± 157 /mm2 in vehicle group vs 384 ± 63 /mm2 in pitavastatin-NP group, $P<0.001$). Tissue concentartions of pitavastatin at 3 hours of reperfusion were greater in pitavastatin-NP group than pitavastatin solution group (48 ± 8 ng/g tissue vs 23 ± 3 ng/g tissue, $P< 0.05$). Conclusions: NP-mediated delivery of pitavastatin into ischemic myocardium at the time of reperfusion reduced myocardial infarct size by activating RISK pathway in this IR injury model. This NP-based technology can be an innovative therapeutic modality for cardioprotection from IR injury in clinical settings.

Therapeutic benefit of urocortin in intracerebral hemorrhage

Hock-Kean Liew[1,2], Chih-Wei Hsu[3,4], Mei-Jen Wang[2], Jon-Son Kuo[5], Jia-Yi Wang[6], Cheng-Yoong Pang[2,7]. [1]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, [2]Department of Medical Research, Tzu Chi General Hospital, Hualien, [3]Department of Emergency Medicine, Tzu Chi General Hospital, Hualien, [4]School of Medicine, Tzu Chi University, Hualien, [5]Neuro-Medical Scientific Center, Tzu Chi General Hospital, Hualien, [6]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, [7]Institute of Medical Sciences, Tzu Chi University, Hualien

Intracerebral hemorrhage (ICH) accounts for about 15% of all deaths from stroke. It frequently causes brain edema, leading to an expansion of brain volume that exerts a negative impact on ICH outcomes. ICH induced secondary brain injury which involves inflammatory mechanism. In vitro studies have shown that urocortin (UCN), a 40-amino-acid neuropeptide, exhibits anti-inflammatory and neuroprotective effects. The anti-inflammatory and neuroprotective effects of UCN on ICH were further investigated in experimental ICH rats. ICH was induced by an infusion of bacteria collagenase VII-S or autologous blood into unilateral striatum of anesthetized rats. At one hour after the induction of ICH, UCN was given (0.05, 0.5 and 5 micrograms via intracerebroventricular injection; 2.5 and 25 micrograms/kg via intraperitoneal injection). We examined the distribution of injected UCN, hematoma volume, sizes of injury area, brain water content, disruption of blood-brain barrier (BBB), neurological deficits, microglial activation, neuronal loss, and profiles of pro-inflammatory cytokines after ICH. UCN, administered in the ipsilateral lateral ventricle, was able to penetrate into the injured striatum. The UCN post-treated ICH rats have reduced injury area, brain edema, BBB disruption, and shown improved neurological function. UCN, given systemically at a very low dose (2.5 micrograms/kg), also penetrated into the injured striatum. It also reduced neurological deficits post-ICH (days 1-7), brain edema, BBB disruption, microglial activation and neuronal loss. Pro-inflammatory cytokines (TNF-alpha, IL-1beta, and IL-6) reduced on 1, 3 and 7 days post-ICH as compared to the non-treated ICH rats. Our result suggests UCN is a potent anti-inflammatory agent that reduces brain injury after ICH, and may have a therapeutic potential for acute ICH patients. Ref:

Peroxisome proliferator activated receptor γ: A regulator of endothelial progenitor cell fate

Kate A. Parham[1,2,3], Stuart M. Pitson[1,3,4], Gabor Tigyi[5], Claudine S.Bonder[1,2,3,4]. [1]Centre for Cancer Biology, SA Pathology, Adelaide, South Australia, [2]School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, South Australia, [3]Co-operative Research Centre for Biomarker Translation, La Trobe University, Melbourne, Victoria, [4]School of Molecular and Biomedical Sciences, University of Adelaide, Adelaide, South Australia, [5]University of Tennessee Health Science Center Memphis, Memphis, USA

Endothelial progenitor cells (EPCs) reside in the bone marrow and upon stimulation are mobilized, enter the circulation and travel to sites in need of new blood vessel development. Circulating EPCs can contribute to vasculogenesis in a paracrine manner or by differentiating into mature endothelial cells (ECs) to form the inner lining of the vasculature. Because EPCs are regulators of both physiological and pathological vasculogenesis these cells have become the target of close to 200 clinical trials aiming to control the biggest killers worldwide, cancer and cardiovascular disease. To this end, elucidation of factors involved in regulation of EPC differentiation is necessary. Transcription factors have been widely described as master switches for the determination of cell fate. Peroxisome proliferator-activated receptor-γ (PPARγ), a transcriptional member of the nuclear receptor super family, has been implicated in a number of vascular processes such as, vasoprotection, EPC proliferation, EPC migration and EPC survival. This study aims to implicate PPARγ as a master regulator of EPC differentiation by assessing a correlation between PPARγ activity and EPC phenotype and function. Comparison of both endogenous PPARγ protein and mRNA revealed significantly elevated levels in human EPCs when compared to ECs. Importantly, PPARγ protein was shown to be primarily localised to the nucleus in EPCs, suggesting the presence of transcriptionally active PPARγ in the cell. In addition, pharmacological manipulation of PPARγ alters cell phenotype and cell function in Matrigel, a tube formation assay that mimics in vivo vasculogenesis. Herein, we also reveal a previously undescribed PPARγ regulatory complex in EPCs, with data suggesting that the bioactive second messenger, sphingosine-1-phosphate (S1P) plays a role in PPARγ gene regulation by binding to and activating PPARγ. Taken together, this new regulatory system controlling EPC phenotype and function may provide a new target for controlling aberrant vasculogenesis in disease.

Anti-inflammatory vascular effects of resveratrol are mediated by the RNA binding protein KSRP

Julia Art[1], Verena Besche[2], Matthias Bros[2], Huige Li[1], Norbert Handler[3], Florian Bauer[3], Thomas Erker[3], Felix Behnke[5], Bettina Mönch[6], Ulrich Förstermann[1], Verena M. Dirsch[4], Oliver Werz[6], Hartmut Kleinert[1], Andrea Pautz[1]*. [1]Department of Pharmacology, Johannes Gutenberg-University Medical Center, Mainz, Germany, [2]Department of Dermatology, Johannes Gutenberg-University Medical Center, Mainz, Germany, [3]Department of Pharmaceutical/Medicinal Chemistry,University of Vienna, Vienna, Austria, [4]Department of Pharmacognosy, University of Vienna, Vienna, Austria, [5]Pharmaceutical Institute, University Tuebingen, Tuebingen, Germany, [6]Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University, Jena, Germany

Atherosclerosis, the most common cause of cardiovascular diseases, is a chronic inflammatory disease. A number of different risk factors, such as hypercholesterolemia, smoking or high blood pressure, contribute to the onset of atherosclerosis. It has been demonstrated that the polyphenolic compound resveratrol, found in fresh grapes and wine, has several cardiac protective effects mediated by reduction of vascular inflammation, inhibition of low-density lipoprotein oxidation, inhibition of platelet aggregation and cell proliferation, as well as vasorelaxation. Moreover in different animal models resveratrol treatment reduced the size and density of atherosclerotic lesions. Despite intensive research, only a few direct resveratrol target proteins have been identified but their role in resveratrol-mediated cardio protection is still elusive. In target fishing experiments using immobilized resveratrol and extracts of human peripheral blood mononuclear cells we identified the „KH-type splicing regulatory protein“ KSRP as a direct and high affinity binding target of resveratrol. KSRP is an RNA-binding protein involved in the regulation of pro-inflammatory gene expression and miRNA biogenesis. KSRP initiates the decay of mRNAs encoding for pro-inflammatory mediators by binding to AU-rich elements in the 3'-untranslated region of those mRNAs. Thereby it recruits the exosome, a multi protein complex of 3'-5' exoribonucleases, which degrades the mRNA. In human DLD-1 cells we could demonstrate that resveratrol treatment reduced the mRNA expression of IL-8, TNF-αand iNOS, which are all known to be implicated in atherosclerotic processes and which are targets of KSRP-mediated mRNA degradation. We provided evidence that these resveratrol effects rely on an increase of KSRP dependent destabilization of those mRNAs rather than on modulation of their promoter activity. Moreover, we detected in resveratrol treated DLD-1 cells an increased expression and activity of hsa-miR155, a miRNA whose biogenesis is promoted by KSRP. It has been assumed that many of the cardio protective effects of resveratrol are mediated by the histone/protein deacetylase sirtuin 1 (SIRT-1). In our experiments inhibition of SIRT-1 activity had no effect on resveratrol-mediated mRNA destabilization. Therefore we can exclude an involvement of SIRT-1. Rather, our data indicate that resveratrol promotes the decay of pro-inflammatory mRNAs by enhancing the activity of KSRP. So we demonstrated that resveratrol prevented a p38 MAPK dependent inhibitory phosphorylation at threonine residues of the protein, without blocking p38 MAPK activation or activity. Experiments in apolipoprotein E deficient mice confirmed our cell culture data. In mice orally treated with resveratrol we detected also reduced phosphorylation of KSRP and consecutively decreased expression of KSRP-regulated pro-inflammatory mRNAs such as TNF-αor IL-12 in different tissues. This implicates that resveratrol directly binds to KSRP in-vivo and thereby induces KSRP activity. Therefore KSRP may be an important denominator of the anti-inflammatory effects of resveratrol in the vasculature.

Endothelial cell specific deletion of tissue factor attenuates inflammation but not coagulation in mouse model of sickle cell disease

Pichika Chantrathammachart, Nigel Mackman, Erica Sparkenbaugh, Nigel S. Key and Rafal Pawlinski. Division of Hematology /Oncology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Sickle cell disease (SCD) is a hematologic disorder caused by a single nucleotide mutation of the beta-globin gene. This mutation results in the formation of sickled red blood cells that are prone to hemolysis and adhere to the endothelium. These primary events result in obstruction of the microvasculature, leading to ischemic injury in multiple organs. Furthermore, SCD is associated with increased tissue factor (TF) expression, activation of coagulation and chronic vascular inflammation. Using a mouse model of SCD (BERK mice), we have previously demonstrated that inhibition of TF with a rat-anti mouse TF (1H1) antibody not only abolishes activation of coagulation (measured by plasma levels of thrombin anti-thrombin (TAT) complexes) but also reduces inflammation and endothelial cell (EC) injury, indicated by attenuation of plasma levels of IL-6 and sVCAM-1, respectively. Interestingly, SCD is one of the few diseases where TF expression has been demonstrated on ECs in vivo. Therefore, in this study we investigated the effect of EC-specific deletion of TF gene on activation of coagulation, inflammation and EC injury in a mouse model of SCD. Sickle cell (SS) mice that expressed normal levels of TF or have EC-specific deletion of TF gene (TFfl/flEC) were generated by transplanting TFfl/fl (normal expression of TF) and TFfl/fl Tie2 Cre+ mice (lacking TF gene in both hematopoietic cells and ECs) with bone marrow from SS mice. Since bone marrow transplantation resulted in reconstitution of TF expression in all hematopoietic cells, TF gene deletion in TFfl/fl Tie2 Cre+ mice was now restricted only to the ECs. Five months after bone marrow transplantation mice were sacrificed and activation of coagulation, inflammation and EC injury were analyzed. We found that activation of coagulation (TAT) and EC injury (sVCAM-1) were not affected by EC-specific deletion of TF gene. In contrast, plasma levels of IL-6 were significantly reduced in TFfl/flEC SS mice compared to SS mice with normal levels of TF expression (mean+/-SEM; 6.9+/-1.8 vs. 14.9+/-3.0 pg/ml; $p<0.05$). Interestingly, similar results were observed in SS mice lacking protease activated receptor-2 (PAR-2) expression in all non-hematopoietic cells compared to SS mice expressing normal levels of PAR-2 (no difference in plasma levels of TAT and sVCAM-1; reduction of IL-6, 9.4+/-0.9 vs 18.9+/-4.5 pg/ml; $p<0.05$). Our data indicate that increased expression of EC TF contributes to inflammation in this mouse model of SCD. This effect could be mediated via activation of PAR-2 on ECs. Furthermore, EC-specific deletion of TF had no significant effect on the activation of coagulation and EC injury. We are currently investigating the mechanism by which of TF expressed by other cell types (monocytes, perivascular cells) contributes to these processes.

Imbalance between apoptosis and autophagy mediates protective effect of high-density lipoproteins (HDLs) in experimental abdominal aneurysms development

Grisha Pirianov, Evelyn Torsney and Gillian Cockerill. Division of Clinical Sciences, St George's University of London, London, United Kingdom

Cellular attrition in the aortic wall results weakened and rupture in abdominal aneurysms (AA). Cellularity of the vessels is determined by dynamic balance of processes of cellular regeneration and death. Recently we found that elevation of plasma concentration of HDLs inhibits experimental AA formation. To investigate mechanism by which HDLs inhibit AA formation, we examined whether HDLs affect Angiotensin II-induced cell death in Apo- E deficient mouse model. We found that HDLs blocked caspase-3/7 and -9 activation and PARP cleavage processing at the site of lesions formation. Whilst HDLs protected against Angiotensin II-triggered apoptosis they significantly enhanced autophagy in this model. This imbalance was associated with down regulation of JNK and ERK activation. Intriguingly, Angiotensin II-driven inflammation results induction of apoptosis and reduction of autophagy in this model. In conclusion, we found differential effect of HDLs on two modes of cell death. Further understanding of the mechanisms which regulate balance between apoptosis and autophagy during inflammatory environment may explain therapeutic action of HDLs on regression of AA.

Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodeling

Christoph Reinhardt[1,2,3], Mattias Bergentall[1,2], Thomas U. Greiner[1,2], Florence Schaffner[4], Gunnel östergren-Lundén[1,2], Lars Christian Petersen[5,6], Wolfram Ruf[4], Fredrik Bäckhed[1,2].
[1]Sahlgrenska Center for Cardiovascular and Metabolic Research/Wallenberg Laboratory, [2]Department of Molecular and Clinical Medicine, University of Gothenburg, Gothenburg, Sweden, [3]Center for Thrombosis and Hemostasis (CTH), University Medical Center Mainz, Experimental Research, Mainz, Germany, [4]Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA, USA, [5]Haemostasis Biology, Novo Nordisk A/S, Maalov, [6]Novo Nordisk Foundation Center for Basic Metabolic Research, Section for Metabolic Receptology and Enteroendocrinology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

The gut microbiota is among the most densely colonized ecosystems with more than 1000 microbial species identified. These gut microbial communities have coevolved with their habitat. In agreement with previous results we found that colonization of germ-free (GF) mice with a gut microbiota promotes increased vessel density in the small intestine. We revealed that expression levels of angiopoietin-1 (Ang-1) and phosphorylation of its receptor Tie-2 was increased in mice that were colonized form birth with a gut microbiota (CONV-R) compared with GF controls. Treatment of ex-GF mice that were colonized for 14 days with a cecal microbiota from a CONV-R donor mouse (conventional-derived; CONV-D) with the angiopoietin-1 (Ang-1) inhibiting peptibody mL4-3 resulted in decreased vascularization of the small intestinal mucosa and in decreased Tie-2 phosphorylation. Since angiogenesis is linked to the cellular initiation of coagulation and since coagulation factor signaling has been shown to modulate angiogenesis we set out to test whether microbial colonization of the intestinal mucosa impacts coagulation factor signaling and vascular remodeling in the host. Treatment of CONV-D mice with a functional inhibitory antibody directed against the coagulation initiator tissue factor (TF) reduced vascularization and Ang-1 expression levels in the small intestine mucosa. We observed increased TF-dependent initiation of coagulation in conventionally-raised (CONV-R) mice that are colonized from birth with a complex microbiota in contrast to GF control mice that were reared in sterile plastic isolators. Both, factor Xa formation and levels of thrombin-antithrombin complexes (TAT) were elevated in CONV-R compared with GF mice. Western blot analyses revealed that small intestinal tissue lysates and primary epithelial cells from CONV-R mice contain increased levels of an N-glycosylated 42 kDa TF form compared with GF controls. This band was also significantly increased in CONV-D mice. In the small intestine TF was mainly expressed in the epithelium. Moreover, isolated enterocytes from CONV-R mice showed increased surface localization of N-glycosylated TF antigen compared with their GF counterparts. Accordingly, the interaction of TF with the cell surface marker integrin β1 was increased in CONV-R mice. The difference in expression levels of the 42 kDa TF form was due to increased N-glycosylation of TF as shown by treatment of intestinal tissue lysates with PNGase F and by tunicamycin-treatment of primary enterocytes from CONV-R mice. Treatment of CONV-R enterocytes with the N-glycosylation inhibitor tunicamycin diminished the interaction between TF and integrin β1. Small intestinal expression levels of PAR1 and phosphorylation of the TF cytoplasmic domain was increased in CONV-R mice compared with GF controls. Moreover, mice deficient in the thrombin-receptor PAR1 (F2r-/-) showed reduced TF phosphorylation and reduced vascularization in the small intestine. Hirudin-treatment of CONV-D mice decreased TF phosphorylation and stimulation of primary enterocytes from CONV-R mice with thrombin resulted in increased TF phosphorylation. Our results suggest that the gut microbiota augments N-glycosylation and surface localization of TF in enterocytes and thus leads to PAR1-dependent phosphorylation of the cytoplasmic TF domain which is linked to Ang-1 dependent vascular remodeling.

Identification of a novel KRIT1 interactor involved in the control of actin cytoskeleton dynamics and cell resistance to oxidative stress

Paolo Guazzi[1], Luca Goitre[2], Elisa Ferro[1], Valentina Cutano[2], Chiara Martino[2], Lorenza Trabalzini[1], and Saverio Francesco Retta[2]. [1]Department of Biotechnology, University of Siena, 53100 Siena, Italy; [2]Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano (TO), Italy

Loss-of-function mutations of the KRIT1 gene (CCM1) have been associated with the Cerebral Cavernous Malformations disease, which is characterized by serious alterations of brain capillary architecture. The KRIT1 protein contains multiple interaction domains and motifs, suggesting that it might act as a scaffold for the assembly of functional protein complexes involved in signalling networks. In previous work, we defined structure-function relationships underlying KRIT1 intramolecular and intermolecular interactions and nucleocytoplasmic shuttling, and found that KRIT1 plays an important role in molecular mechanisms involved in the maintenance of the intracellular Reactive Oxygen Species homeostasis to prevent oxidative cellular damage. Here we report the identification of the Kelch family protein Nd1-L as a novel molecular interactor of KRIT1. This interaction was discovered through yeast two-hybrid screening of a mouse embryo cDNA library, and confirmed by pull-down and co-immunoprecipitation assays of recombinant proteins, as well as by co-immunoprecipitation of endogenous proteins in human endothelial cells. Furthermore, using distinct KRIT1 isoforms and mutants, we defined the role of KRIT1 domains in the Nd1-L/KRIT1 interaction. Finally, fluorescence microscopy studies showed that overexpression of Nd1-L induces a nucleus-to-cytoplasm translocation of KRIT1, indicating that the novel identified interacting protein may contribute to the regulation of KRIT1 nucleocytoplasmic shuttling. As both KRIT1 and Nd1-L have been involved in the regulation of Rho GTPases, cytoskeleton dynamics and cellular responses to oxidative stress, our findings provide a novel and important piece of the molecular puzzle involving KRIT1, thus expanding the knowledge of molecular complexes and mechanisms that may underlie CCM disease.

Sources and targets of oxidative stress in vascular diseases

Harald HHW Schmidt S Altenhöfer, K Radermacher, P Kleikers, R Hermans, K Wingler, C. Kleinschnitz. Dept. of Pharmacology, CARIM, University of Maastricht, the Netherlands
Dept. Neurology, University Würzburg, Germany

Oxidative stress has been postulated for many years as a major cause and driver of vascular disease states such as coronary artery disease, peripheral artery disease, hypertension and stroke. However, all therapeutic applications have focused on antioxidants with little or no success and no clinical implications. Here we show that identifying and specifically targeting the molecular sources of vascular oxidative stress and its target proteins is much more promising and provides specific, biomarker-assisted therapeutic avenues. One of the major functional targets of oxidative stress is the nitric oxide-cyclic GMP signalling pathway (NO-cGMP). Relevant sources include the innate immune response enzyme, gp91phox, a NADPH oxidase, now termed NOX2. Related NOX2-like enzymes are expressed on many surface cells (endothelium, epithelium) as an extended innate immune or stress response system. NOX deficient mice were subjected to ischemia, ischemia-reperfusion and metabolic stress models. NOX1 promotes atherosclerosis and is associated with a hypertensive phenotype. NOX4 is upregulated in ischemia-reperfusion and causes neurodegeneration after stroke. Impaired NO-cGMP signalling is found in heart failure and coronary artery disease and can be targeted in stroke, hypertension and diabetes. eNOS recoupling appears to be efficient in peripheral artery disease. Biomarkers indicate both oxidative stress as well dysfunctional NO-cGMP signalling. These data suggest that oxidative stress can be efficiently targeted using inhibitors of NOX or activators/stimulators of cGMP formation. Together with recent genetic evidence, the NO-cGMP pathway in conjunction with NOX-derived oxidative stress appears to be one of the first mechanism-based vascular therapies.

AMP-activated protein kinase (AMPK) induces vasodilation of microvessels by two different mechanisms

Schubert KM[1], Kreutz C[1], Mederos y Schnitzler M[2], Gudermann T[2] and Pohl U[1]. [1]Walter Brendel Centre for Experimental Medicine, Ludwig-Maximilians-University of Munich, Germany, [2]Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians University of Munich, Germany

Question: Balanced organ function requires the continuous matching of blood supply with metabolism which occurs through changes of microvascular arterial tone. The energy-sensing enzyme AMP-activated protein kinase (AMPK) plays a key role in regulating cellular and tissue energy metabolism and may therefore be a potential regulator of smooth muscle tone. Thus we studied the potential vasomotor function of vascular smooth muscle AMPK in isolated small arteries. Methods: Studies in intact cannulated segments as well as in freshly isolated smooth muscle cells, were conducted in skeletal muscle resistance arteries (hamster and mouse; n=150). In the isolated vessels, pre-treated with the COX-inhibitor indomethacin (30µM) and the NOS-inhibitor L-NAME (30µM), we measured simultaneously smooth muscle intracellular calcium levels [Ca2+]i (FURA2-AM) and vascular diameters. Conventional perforated patch-clamp recordings were carried out in the isolated cells. Results: In vessels pre-constricted with norepinephrine (0.3µM) the AMPK-stimulator A769662 (A76) induced a dose-dependent and endothelium-independent vasodilation which reached its maximum at 100µM and was associated with a decrease in [Ca2+]i. Metformin (0.1-3mM) and a second AMPK-activator, PT-1 (300nM-30µM), had qualitatively similar effects. Partial knock down of the AMPK by siRNA induced a significant rightward shift in the dose-response-curves of A76. In vessels pre-constricted by high extracellular potassium (60mM) the vasodilation was diminished and the [Ca2+]i-decrease abolished completely. The remaining dilator effect suggesting a change in calcium sensitivity could be completely blocked by the myosin-light-chain-phosphatase (MLCP) inhibitor calyculinA (100nM). Patch-clamp studies revealed activation of BKchannels by A76, which could be blocked by the specific inhibitors paxilline (500nM), iberiotoxin (100nM) and the AMPK inhibitor compound C (100µm). Accordingly, in microvessels pre-treated with norepinephrine and paxilline (100nM) or iberiotoxin (100nM) the A76 dilator effect decreased by about 20% for lower A 76 concentrations (1-10µM). Furthermore, paxilline and iberiotoxin counteracted the PT-1 induced dilation by about 30%. Conclusion: AMPK augments BKchannel current and reduces calcium sensitivity of the contractile machinery of arterial microvessels. These two mechanisms may contribute to microvascular vasodilation under conditions of smooth muscle AMPK stimulation.

Integrin alpha6 controls endothelial podosome rosettes formation in tumor angiogenesis

Giorgio Seano[1,2]*, Laura di Blasio[1,2], Roberto Sessa[1,2], Paolo Armando Gagliardi[1,2], Federico Bussolino[1,2], Guido Serini[1,2], and Luca Primo[2,3]. [1]Department of Oncological Sciences, University of Torino, Turin, Italy, [2]Institute for Cancer Research and Treatment, Candiolo, Turin, Italy, [3]Departments of Clinical and Biological Sciences, University of Torino, Turin, Italy

The mechanisms, by which angiogenic endothelial cells (EC) break the physical barrier of vascular basement membrane (vBM) and consequently sprout in a new vessel, are not clear. It is known that cultured EC show specialized plasma-membrane microdomains, called podosome, and in EC they are organized in rosettes; the latter combine adhesive and proteolytic activities to spatially restricted sites of matrix degradation. We demonstrate that angiogenic EC and tumor vessels show a significant increase of podosome rosettes in comparison to quiescent EC. The increment of the endothelial podosome rosettes corresponds to an increased ability to degrade extracellular matrices. Integrins are the principal adhesion regulator of podosome-type structures. We show that integrin alpha6, whose expression is up-regulated in angiogenic EC, is recruited in podosome-rosettes both in cultured EC and in tumor vessels. Moreover, down-regulation and functional blocking of integrin alpha6 impairs podosome formation and maturation. Similarly, high level of laminin - the major component of vBM and the ligand of integrin alpha6 - blocks podosome formation. We demonstrate that podosome rosettes do not form in EC when integrin alpha6 is not available for trafficking into nascent podosomes due to the binding to laminin in focal adhesions. Only over-expression of integrin alpha6 - simulation of angiogenic factors stimulation effects - is able to overcome the anti-podosome effects of laminin. To understand the physiological relevance of the involvement of integrin alpha6 in endothelial podosome rosettes, we treat a genetic tumor mice model with anti-alpha6 integrin neutralizing antibody. The functional blocking of integrin alpha6 impairs podosome rosettes formation in vivo, by impairing tumor angiogenesis. Taken together, these results suggest that laminin in vBM stabilize mature vessels by recruiting integrin alpha6 in focal adhesions. The endothelial increase of integrin alpha6 expression, induced by angiogenic growth factors stimulation, overcomes the anti-angiogenic effect of vBM, by increasing integrin alpha6 availability to form podosome rosettes, necessary to degradate vBM and form new vessels. Moreover, these studies provide evidence that endothelial podosome rosettes play a crucial role in angiogenic in vivo models and that their inhibition impairs tumor angiogenesis.

Micro RNA-146a and its role in vascular smooth muscle cells during vascular remodeling processes

Daniel Sedding, Wiebke Bielenberg, Jan-Marcus Daniel, Christian Hamm, Rebecca Widmer-Teske. Dept. of Cardiology/Angiology Giessen University, Giessen, Germany

Background: Micro RNAs (miRNAs) are implicated to regulate mRNA levels of up to 30% of mammalian genes, comprising key regulators for cellular function including proliferation, differentiation and apoptosis in human diseases. Up to now, the role of miRNAs for vascular smooth muscle cell function remains elusive. Thus, the aim of this study was to evaluate the regulation and impact of miR 146a for smooth muscle cell function during the development of neointimal lesions in vitro and in vivo and to screen miRNA expression in this setting. Methods/Results: Using microarray based expression analysis, we screened for regulated miRNAs during the development of restenosis. Neointima formation was induced in C57BL6/N by dilation of the femoral artery and miRNA was isolated 10 and 21 days after injury. About 59% of all known miRNAs was found to be aberrantly regulated after 10 days what was even enhanced to 88% after 21 days. Noticeably, miR-146a appeared to be one of the most regulated miRNAs during restenosis. Further expression analysis in isolated primary vascular cell types revealed that miR-146a, besides in monocytes/macrophages and in endothelial cells, was highly expressed in vascular smooth muscle cells. In vitro, the upregulation of miR-146a could be attributed to the inflammatory stimulus Il-1β. In the following, computational miRNA target prediction, the „TargetScan database“, was used to find potential target genes for 146a. Quantitative Real-Time-RT-PCR tests were performed after overexpression using precursor forms of miR-146a. The transcript for IRAK1, a key adapter molecule in TLR- and IL-1 receptor signaling cascades, was significantly downregulated and hence represents a molecular target for 146a. To further assess the functional role of miR-146a, smooth muscle cells were transfected with miR-146a inhibitors using 2'-O methylated RNA targeting 146a resulting in a significantly decreased total cell count and migration of smooth muscle cells. In complementing in vivo experiments, the inhibition of miR-146a in injured murine femoral arteries significantly reduced the proliferation of neointimal smooth muscle cells as assessed by Ki67 staining. Data of the morphometric analysis showed a significantly decreased neointima formation following 146a inhibition. Conclusion: These findings reveal a pivotal role of miR-146a for function in vascular smooth muscle cells, especially under conditions of pathological vascular remodeling processes. Thus, modulating miR-146a expression may represent a novel approach for the prevention and treatment of vascular proliferative diseases.

Anti-angiogenic properties of protease nexin-1

Sonia Selbonne, Feriel Azibani, Soria Iatmanen, Yacine Boulaftali, Benjamin Richard, Martine Jandrot-Perrus, Marie-Christine Bouton, Véronique Arocas. INSERM, Paris, France

The serpin protease nexin-1 (PN-1) is expressed by vascular cells including fibroblasts, endothelial cells and smooth muscle cells, and is secreted by platelets upon activation. PN-1 is also known to interact with several modulators of angiogenesis, such as proteases (thrombin, plasmin and plasminogen activators), matrix proteins (vitronectin), and glycosaminoglycans (heparan and chondroitin sulfate proteoglycans). We therefore investigated the impact of PN-1 in vitro and ex vivo on endothelial cell angiogenic responses, and in vivo in PN-1-deficient mice. Recombinant PN-1 variants with loss-of-function mutations were expressed to analyze the role of the heparin binding site and of the anti- protease activity of the serpin. We found that PN-1 is anti-angiogenic in vitro: it inhibits vascular endothelial growth factor (VEGF)-induced endothelial cell responses, including proliferation, migration and capillary tube formation, and it decreased cell spreading on vitronectin. These effects are related to the interaction of PN-1 with endothelial cells glycosaminoglycans but do not require the anti-protease activity of the serpin. In addition, our results indicate that PN-1 does not act by blocking VEGF binding to its heparan sulfate proteoglycan coreceptors. The results obtained in vitro were supported ex vivo in PN-1-deficient mice, where the microvascular network sprouting from aortic rings is significantly enhanced. Moreover, in vivo, neovessel formation is promoted in the Matrigel plug assay in PN-1-deficient mice compared to wild-type mice, and these effects are reversed by the addition of recombinant PN-1. In agreement, PN-1-deficient retina displayed increased retinal vascularization in the postnatal period, with a rise in capillary thickness and density, and an increased number of veins and arteries, compared to wild-type mice. Retinal PN-1 expression, analyzed in wild-type whole retina lysates by immunoblotting, was elevated in the first postnatal days and progressively decreased to a very low level in adult retina. We did not observe any difference in the kinetics of retinal vasculature development, in VEGF expression, or in the overall retinal structure. Taken together, our data provide evidence that PN-1 exerts a direct anti-angiogenic activity and is a yet-unrecognized player in the angiogenic balance.

Notch signaling and flow control blood vessel pruning in zebrafish embryos

Eva Kochhan, Arndt F. Siekmann. Max-Planck-Institute for Molecular Biomedicine Roentgenstr. 20, 48149 Muenster, Germany

The vasculature forms a ramified network of interconnected tubes. Angiogenesis, or the sprouting of new blood vessels from pre-existing ones, has received increasing attention over the recent years. Many of the genetic players involved in controlling angiogenesis, such as Vascular Endothelial Growth Factor (VEGF) and Notch have been identified. In contrast to this, the mechanisms underlying pruning of pre-existing blood vessels are less well understood. Here, we show that the cranial division of the internal carotid artery (CrDI) in the eyes of zebrafish embryos undergoes stereotypical pruning between 32 and 48 hours post fertilization (hpf). Prior to pruning, a new blood vessel, the nasal ciliary artery (NCA) connects to the CrDI, thereby forming a transient „Y“ shaped branch structure. Gene expression analysis revealed that the Notch ligand Dll4 is differentially expressed in endothelial cells of the NCA and CrDI. Accordingly, interfering with Notch signaling by blocking dll4 function either via morpholino knockdown or in zebrafish mutants for dll4 impairs CrDI pruning. Activation of Notch signaling in endothelial cells via Notch intracellular domain overexpression similarly affected CrDI pruning, suggesting that differences in Notch signaling between CrDI and NCA are necessary for CrDI pruning to occur. Likewise, changes in CrDI blood flow influenced pruning. Lack of blood flow caused the formation of persistent „Y“ shaped branch structures that were resolved upon reestablishment of blood flow. Together, these studies reveal the importance of blood flow and the Notch signaling pathway, which was previously recognized for its role in angiogenic sprouting, during blood vessel pruning.

KIAA1109 is differentially expressed post myocardial infarction and is required for vascular integrity in zebrafish

Sara Solaymani-Kohal[1,2], Marta Milo[2], TJA Chico[1,2]. [1]University of Sheffield MRC Centre for Developmental and Biomedical Genetics, [2]University of Sheffield NIHR Cardiovascular Biomedical Research Unit, United Kingdom

Background: We aimed to identify genes that are differentially expressed (DE) in whole blood in patients following myocardial infarction (MI), and assess these functionally in zebrafish. Methods: Patients admitted with acute coronary syndromes (myocardial infarction or unstable angina) were recruited from our hospital. We performed whole genome microarrays on RNA extracted from whole blood at 1, 3, 7, 30 and 90d post admission, comparing the transcriptome of patients with MI (evidence of myocardial necrosis) with unstable angina (no myocardial necrosis). We then functionally assessed one DE candidate; KIAA1109. Results: From >54000 genes assessed, 39 genes were significantly DE 1d post MI, returning to baseline thereafter. 28 have clear zebrafish homologues, 14 with protein similarity >50%. 9 were upregulated in MI, while 4 genes were downregulated, including a gene of completely unknown function; KIAA1109. We therefore used a start blocking MO to knockdown KIAA1109 in developing zebrafish and assessed its effect on vascular development. We also performed a microarray comparing whole embryo RNA from 36h post fertilization KIAA1109 morphants with control to assess the transcriptional effect of loss of function of KIAA 1109. KIAA1109 Fli1:GFP/Gata1:dsRED transgenic morphant zebrafish (GFP in endothelium, dsRED in erythrocytes) were examined by confocal microscopy for evidence of vascular abnormalities. KIAA1109 morphants developed normally but developed cerebral haemorrhage between 2-4dpf (2dpf control 10%, vs KIAA1109 morphants 43% $p<0.05$). Although control morphants occasionally develop cerebral haemorrhage, mean haemorrhage volume was far larger in KIAA1109 morphants (2dpf control 1.2um3 vs KIAA1109 morphants 11.3 um3 $p<0.05$). Further investigating developmental factors affected we found that there is a significant decrease in the number of endothelial cell nuclei in the forebrain of KIAA1109 morphants (47±4 vs 73±6 in control) as well as in the hindbrain (53±4 morphants vs 95±7 in control). There was a significant decrease in the number of endothelial cell nuclei in the vein of KIAA1109 morphants (68±6 vs 101±8 in control). The microarray of KIAA1109 morphants at 36hpf determined DE genes that were significantly down regulated such as protocadherin 1 gamma 2, involved in cell to cell signaling and the cadherin-signaling pathway as well as nodal related 2, which involved in cell communication and the TGFbeta pathway. Significant genes that were down regulated include ligand of numb-protein X1, involved in the NOTCH pathway as well as calcium/calmodulin-dependent protein kinase, involved in the immune system response. Conclusion: KIAA1109 is significantly down regulated in human whole blood 1d post MI. Knockdown of KIAA1109 induces severe cerebral hemorrhaging and significant DE of important genes such as protocadherins, CaM kinase and nodal related 2. Functional and transcriptomic assessment in zebrafish suggests KIAA1109 is required for vascular integrity and may be a therapeutic target post MI.

Characterization of lung microvascular endothelium from pulmonary arterial hypertension patients under high shear stress

Robert Szulcek[1], HJ Bogaard[1], TA Leyen[2], R Fontijn[2], VWM van Hinsbergh[3], A Vonk-Noordegraaf[1], and GP van Nieuw Amerongen[3]. [1]Department of Pulmonology, [2]Molecular Cell Biology and Immunology, and [3]Physiology, Institute for Cardiovascular Research (ICar-VU), VU University Medical Center, Amsterdam, The Netherlands

Background: During development of pulmonary arterial hypertension (PAH), blood flow velocity in small pulmonary blood vessels increases. The healthy endothelium counteracts the resulting increase in forces by functional and structural adaptations. Aim: We hypothesize that severe pulmonary vascular remodeling in PAH results from sustained endothelial injury caused by increased shear stress (SS). Therefore we tested whether microvascular endothelial cells from PAH-patients are capable to adapt to high levels of shear stress (HSS). Methods: Human pulmonary microvascular endothelial cells (PMVEC) were isolated from PAH-patients and controls and cultured in-vitro. Electrical resistance of the cultured PMVEC monolayers was recorded by ECIS (electrical cell-substrate impedance sensing) and analyzed as a measure of cell proliferation, barrier function and cell-cell/cell-matrix adhesion. Physiological (2.8 dyn/cm2) and high (15 dyn/cm2) levels of pulsatile, unidirectional SS were successively applied. Results: Both, in-vitro cultured PAH-PMVEC and controls formed similar characteristic barriers with a baseline resistance of 7767 vs. 8102 ohm, and responded to challenge with the hyper-permeability inducer thrombin (1 U/mL) by a specific maximal drop in resistance (2109 vs. 2083 ohm). Nevertheless, PAH-PMVEC markedly differed in their proliferation rate and reached confluence twice as fast as their control counterparts, consistent with hyper-proliferative endothelial cell growth seen in PAH-patients. However, most striking are the differences in structural adaptation to HSS. Control PMVEC re-aligned in flow direction, whereas PMVEC from PAH-patients failed to align. Specifically, 86 % of the total number of control PMVEC elongated, whereas 70 % of those cells re-aligned within an angel of less then 20 degrees relative to the flow direction. Opposing only 35 % of PAH cells showed a stretched morphology and failed to align by forming angles above 40 degrees even after five days HSS challenge. Expression of none of the tested genes, known to play a role in shear sensing and adaptation (e.g. KLF2 and TGF-β), were affected. Modeling of the electrical data indicated changes in cell-cell and cell-matrix adhesion to be involved in the failing adaptation. Conclusion: In conclusion, PMVEC from PAH-patients maintain their disease-specific characteristics in-vitro, but moreover fail to adapt to HSS, supporting the role of HSS in disease progression of PAH. The functional consequences of the failing adaptation are under testing and point towards a reduced wound healing capacity and an overall decreased barrier integrity.

A single transcription factor, Tcf21, is required for cell fate determination of cardiac fibroblasts

Asha Acharya[1], Seung Tae Baek[1], Guo Huang[1], Banu Eskiocak[1], Sean Goetsch[2], Caroline Y. Sung[1], Serena Banfi[1], Marion F. Sauer[1], Gregory S. Olsen[1], Jeremy S Duffield[3], Eric N. Olson[1], and Michelle D. Tallquist[1]. [1]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas TX-75390, USA, [2]Department of Cardiology, University of Texas Southwestern Medical Center, Dallas TX-75390, USA, [3]Division of Nephrology, University of Washington, Seattle WA-98019, USA

The basic helix-loop-helix (bHLH) family of transcription factors orchestrates cell fate specification, commitment, and differentiation in multiple cell lineages during development. Here, we describe the role of a bHLH transcription factor, Tcf21 (Epicardin/Pod1/Capsulin), in specification of the cardiac fibroblast lineage. In the developing heart, the epicardium constitutes the primary source of progenitor cells that form two cell lineages, coronary vascular smooth muscle cells (cVSMCs) and cardiac fibroblasts. These fibroblasts constitute two types, those that are scattered throughout the ventricular wall (interstitial) and those that deposit matrix around blood vessels (adventitial). Currently, there is a debate regarding if the specification of the smooth muscle cells and the fibroblasts occurs early in the formation of the epicardium or later after the cells have entered the myocardium. Lineage tracing using a tamoxifen-inducible Cre expressed from the Tcf21 locus demonstrated that the majority of Tcf21 expressing epicardial cells are committed to the cardiac fibroblast lineage prior to initiation of epicardial epithelial-to-mesenchymal transition (EMT). Furthermore, Tcf21 null hearts fail to form cardiac fibroblasts, and lineage tracing of the null cells showed their inability to undergo EMT. This is the first report of a transcription factor essential for the development of interstitial and adventitial fibroblasts of the heart. We show a unique role for Tcf21 in multipotent epicardial progenitors prior to the process of EMT that is essential for cardiac fibroblast development.

Natural IgM antibodies protect from atherosclerosis by binding circulating microparticles resulting in neutralization of their proinflammatory activities and regulation of splenic B cell maturation

Dimitrios Tsiantoulas[1,2], Karsten Hartvigsen[1,2], Thomas Perkmann2 , Vesna Krajina[1,2], Christoph J. Binder[1,2]. [1]CeMM, Research Center for Molecular Medicine, Medical University of Vienna, Austria, [2]Department of Laboratory Medicine, Medical University of Vienna, Austria

Background/Aim: Atherosclerosis is characterized by the accumulation of oxidized lipoproteins and apoptotic cells. There is substantial evidence that oxidation-specific epitopes present on apoptotic cells and oxidized LDL (OxLDL) are dominant targets of natural IgM antibodies (NAbs). Mice deficient in serum IgM (sIgM-/-) have been shown to develop accelerated atherosclerosis. The aim of our study was to characterize the underlying mechanisms by which NAbs protect from atherosclerosis. Methods: Female LDLR-/-sIgM-/- (n=15) and LDLR-/- (n=14) mice were fed an atherogenic diet for 16 weeks. Lesion formation was evaluated in the entire aorta and the effect of NAbs in the clearance and pro-inflammatory effects of microparticles was evaluated ex vivo and in in vitro assays, respectively. In addition potential effects on immune cell homeostasis were analyzed. Results: LDLR-/-sIgM-/- mice developed more lesions in the aorta than control LDLR-/- mice (10.5% vs.7.2%; p<0.001). Unexpectedly, the numbers of circulating microparticles (MPs) were reduced in LDLR-/-sIgM-/- mice (26±5 x103 /μL vs. 56±10 x103; P<0.01), suggesting that MPs are maintained in circulation by NAbs. Indeed, a large percentage of circulating microparticles isolated from human plasma was found to have IgM antibodies to their surface. Because we have previously shown that a large part of NAbs binds oxidation specific epitopes, we analyzed them for the presence of oxidation specific epitopes using specific monoclonal antibodies by flow cytometry. Consistent with the proinflammatory capacity of oxidation specific epitopes we could show that circulating MPs have the capacity to induce IL-8 production in THP-1 cells, which could be inhibited by addition of an OxLDL-specific monoclonal IgM antibody. Because our data indicated microparticles as major source of self-antigens that are critically involved in immune cell maturation, we investigated whether LDLR-/-sIgM-/- mice display a disturbed immune homeostasis. While T cells were unaltered in these mice we found robust changes in the B cell compartments, which have recently demonstrated to play a key role in atherogenesis. Detailed B cell analysis revealed increased frequencies of CD21lowCD23- and CD21intCD23low B2 cells and a decreased frequency of follicular B2 cells (CD21intCD23+), which exhibited a decreased B cell receptor kappa/lambda light chain ratio, indicative of an autoimmune-like phenotype. Conclusions: LDLR-/-sIgM-/- mice develop accelerated atherosclerosis. Circulating MPs carry oxidation specific epitopes that are recognized by NAbs. Deficiency of NAbs in LDLR-/- mice results in increased MP-mediated inflammation and the generation of potentially pro-atherogenic B2 cells.

Microglia cells home to the perivascular niche and produce pro-angiogenic factors in glioblastoma

Kati Turkowski[1], Susan Brandenburg[1], Yordan Todorov Radev[1], Sergej Rot[1], Andreas Hippe[2], Bernhard Homey[2], Peter Vajkoczy[1]. [1]Charité-Universitätsmedizin Berlin, Department of Neurosurgery, Institute of Experimental Neurosurgery, [2]Universitätsklinikum Düsseldorf, Department of Dermatology, Germany

Glioblastoma multiforme is one of the most malignant brain tumors. This cancer is characterized by high vascularisation, strong invasiveness and the occurrence of a prominent number of microglia cells. Until now, the function of these cells in the tumor region is not completely understood. The aim of our research is to investigate the microglia-tumor-interaction, especially the influence of microglia on tumor angiogenesis. We implanted intracranially GL261 tumor cells into syngeneic mice and studied the phenotype and contribution of microglia cells in the tumor area by immunofluorescence stainings. Furthermore, we purified the microglia of naive and tumor bearing mice by MACS technology. These isolated cells were used for RNA extraction following Realtime-PCR focused on pro-angiogenic factors. In addition, the primary microglia cells were used for in vitro cultivation. We found an increased number of microglia cells in the tumor area, expressing Iba-1, CD11b and CD68. These Iba-1+ cells showed a preference for the perivascular niche and they are especially associated with endothelial cells of the tumor blood vessels. Furthermore, we isolated CD11b+ cells from mice brains with purity up to 95%. The expression analysis of these cells from tumor bearing mice showed an upregulation of pro-angiogenic factors (e.g. VEGF) and in particular chemokines (e.g. CXCL2, CCL2, CCL5) on mRNA level as well as protein level. Moreover, we established the cultivation as well as activation of primary microglia and co-cultures with endothelial cells for in vitro investigation of their cell interaction. Our results imply a role for microglia-derived factors on tumor vascularisation. According to our model, tumor cells activate microglia cells which then localize to the perivascular niche and stimulate blood vessel growth.

Arhgef15 promotes angiogenesis by mediating VEGF-induced Cdc42 activation and potentiating RhoJ inactivation in endothelial cells

Akiyoshi Uemura and Yoko Fukushima. Division of Vascular Biology, Kobe University Graduate School of Medicine, Japan

Signal transduction via Rho family small GTPases regulates cell motility by modulating actin cytoskeletons, cell-matrix adhesion, and membrane trafficking. However, it remains elusive what distinct Rho molecules are critically involved in regulating endothelial cell motility. Here, by exploiting FACS-array transcriptome analyses in postnatal mouse retinas, we identified that the small GTPase RhoJ is up-regulated in endothelial cells of angiogenic blood vessels. While RhoJ mediates Sema3E-induced endothelial cell contraction by facilitating actin depolymerization and PlexinD1 endocytosis, hemi- or homozygous RhoJ deficiency resulted in variable morphogenetic defects in retinal vasculature. We further show that Arhgef15 is an endothelial-specific RhoGEF in mouse retinas, and mediates VEGF-induced Cdc42 activation and RhoJ inactivation, thereby promoting the formation of actin stress fibers. Disruption of the Arhgef15 gene led to delayed extension of the growing retinal vasculature, which was equivalent to that caused by endothelial RhoJ overexpression. Our study uncovers novel molecular targets to manipulate EC motility in various disease settings such as cancer and neovascular eye diseases.

The Rho-GEF Trio controls leukocyte transendothelial migration by promoting docking structure formation

Jos van Rijssel, Jeffrey Kroon, Mark Hoogenboezem, Floris P.J. van Alphen, Elena Kostadinova, Dirk Geerts, Peter L. Hordijk and Jaap D. van Buul. Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands. Department of Pediatric Oncology/Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands

Leukocyte transendothelial migration involves the active participation of the endothelium through the formation of apical membrane protrusions that embrace adherent leukocytes, termed docking structures. Using live-cell imaging, we find that prior to transmigration, docking structures form around 80% of all neutrophils. Previously, we showed that endothelial RhoG and SGEF control leukocyte transmigration. Here, our data reveal that both full-length Trio and the Rac1/RhoG-exchanging DH-PH (TrioD1) domain of Trio interact with ICAM-1 and are recruited to leukocyte adhesion sites. Moreover, upon clustering of ICAM-1, the Rho-GEF Trio activates Rac1, prior to activating RhoG, in a filamin-dependent fashion. We further show that docking structure formation is initiated by ICAM-1 clustering into ring-like structures, which are followed by membrane protrusion. Interestingly, Rac1 is required for ICAM-1 clustering, whereas RhoG controls membrane protrusion formation. Finally, silencing endothelial Trio expression or reducing TrioD1 activity without affecting SGEF impairs both docking structure formation and leukocyte transmigration. We conclude that Trio promotes leukocyte transendothelial migration by inducing endothelial docking structure formation in a filamin-dependent fashion through the activation of Rac1 and RhoG.

Medial elastin fragmentation leads to intraplaque neovascularization, acute plaque rupture, atheroembolic stroke and sudden death in atherosclerotic mice

Carole Van der Donckt[1]*, Dorien M. Schrijvers[1], Greetje Vanhoutte[2], Annemie Van der Linden[2], Marleen Verhoye[2], Wim Martinet[1], Hidde Bult[1], Arnold G. Herman[1] and Guido R.Y. De Meyer[1]. [1]Laboratory of Physiopharmacology, [2]Bio-Imaging Lab, University of Antwerp, Belgium

Atherosclerotic plaque rupture remains the leading cause of acute cardio- and cerebrovascular events. Current animal models flaw because they merely generate an unstable plaque phenotype without spontaneous rupture and clinical sequels. We have previously shown that elastin fragmentation of the vessel wall - as seen in humans during aging - leads to increased vascular stiffness, vessel dilation and exacerbated atherogenesis in fat fed apolipoprotein E deficient mice (apoE-/-) that have a mutation (C1039G+/-) in the fibrillin-1 (fbn1) gene. Female apoE-/- and apoE-/- fbn1C1039G+/- mice were fed a Western type diet for 35 weeks (n=20 mice in each group). In contrast to standard apoE-/- mice, atherosclerotic plaques of apoE-/- fbn1C1039G+/- mice were 2-fold larger, highly unstable (as shown by a large necrotic core and a decreased amount of smooth muscle cells and collagen fibers) and showed prominent neovascularization and hemorrhage, important features of vulnerable human plaques. Neovessels were commonly observed in the smaller vessels such as the brachiocephalic and left common carotid artery. Microvessel density was related to the degree of medial elastin fragmentation and stenosis of the vessel, indicating that both permeability of the media and size of the plaque (hypoxia) contribute to intraplaque neovascularization. Moreover, these neovessels appeared to be leaky, as seen by the extravasation of red blood cells into the plaque. Acute plaque rupture was observed in 70% of apoE-/- fbn1C1039G+/- mice, but never in apoE-/- mice. Furthermore, apoE-/- fbn1C1039G+/- mice showed neurological symptoms such as head tilt, disorientation and motor disturbances, and died suddenly (15 out of 20 mice, of which 50% after 20 weeks). In all apoE-/- fbn1C1039G+/- mice, magnetic resonance imaging (MRI) showed brain lesions that contained cholesterol clefts, macrophages and smooth muscle cells, indicating embolization of plaque debris after rupture. ApoE-/- fbn1C1039G+/- mice represent the first mouse model developing spontaneous plaque rupture with relevant clinical endpoints as seen in humans, including most of the features of vulnerable human plaques. The presence of abundant intraplaque neovascularization and the occurrence of acute plaque rupture in apoE-/- fbn1C1039G+/- mice point out a pivotal role for neovascularization in intraplaque hemorrhage, lipid core expansion, and plaque rupture. This mouse model is a unique tool to explore and validate novel plaque stabilizing therapies.

The influence of flow on vascular endothelial cell senescence

Christina M. Warboys[1], Amalia de Luca[1], Narges Amini[1], Paul C. Evans[2]. [1]British Heart Foundation Cardiovascular Sciences Unit, National Heart and Lung Institute, Imperial College London, [2]Department of Cardiovascular Science, Faculty of Medicine, Dentistry & Health, University of Sheffield, United Kingdom

Blood flow influences atherosclerosis by modulating multiple physiological processes in endothelial cells (EC) including inflammatory activation, viability and proliferation. Here we studied the influence of flow on cellular senescence, which is a state of replicative arrest. Senescent-like endothelial cells (EC) have been identified overlying human atherosclerotic plaques, however their potential role in atherogenesis is uncertain. Human umbilical vein endothelial cells (HUVEC), at low passage were seeded onto 6-well plates and placed on an orbital shaker for 3 days, which exposed cells in the centre of the well to low/disturbed flow and cells in the periphery to uniform, laminar flow. Rates of endothelial senescence were determined using a chromogenic assay for senescence-associated β-galactosidase (SA-β-Gal) activity, a biomarker of senescence, combined with analysis of cellular morphology. Senescent EC were detected at a significantly higher frequency in areas of low, disturbed flow compared to regions of uniform flow (P=0.028). To identify the molecular mechanism we measured the expression of the cell cycle regulators p53, p21WAF1/CIP1 and p16INK4A. Senescent EC expressed high levels of p53 and the cell cycle inhibitor, p21, compared to surrounding cells (P=0.0016 and P=0.0018 respectively). By contrast, the expression of the cell cycle inhibitor p16 was not increased in senescent EC (P=0.247). Activation of the p53-p21 pathway is required for flow-induced senescence since knockdown of p53 or p21 using small interfering RNA reduced the induction of senescent EC in response to low/disturbed flow (P=0.048 and P = 0.0002 respectively). The activation of p21 was dependent on p53 since p21 expression was significantly reduced in HUVEC treated with small interfering RNA targeting p53 (P = 0.0004). Given that senescent EC have been associated with vascular dysfunction, we examined whether resveratrol (an anti-atherogenic polyphenol found in wine, nuts) can exert protective effects. Treatment of HUVEC with resveratrol significantly reduced the induction of EC senescence by disturbed flow (P=0.0017). We concluded that the protective effects of resveratrol were mediated via activation of Sirt1 (an NAD+-dependent deacetylase which deacetylates and deactivates p53) since inactivation of Sirt1 (using Sirtinol) restored flow-induced senescence in resveratrol-treated EC (P=0.0081). Finally, we performed en face staining to measure the frequency of SA-β-Gal-positive EC in aortae from pigs (aged 6-12 months) or hypercholesterolemic mice (LDLR-/-; aged 12 weeks). SA-β-Gal-positive EC were more frequent at the inner curvature of the aortic arch (low shear, atheroprone site) compared to the outer curvature (high shear, protected site). We conclude that disturbed flow promotes EC senescence by activating p53-p21 signaling. Thus flow and possibly shear stress may influence the focal nature of atherosclerosis, in part, by regulating EC senescence. Resveratrol may protect arteries by preventing the induction of EC senescence by disturbed flow.

Intracellular transport of full functional lipid loaded lysosomes from macrophages into vascular smooth muscle cells during cell-cell-contacts and their subsequent phonotypical alteration

Weinert Sönke[1], Poitz David M.[2], Medunjanin Senad[1], Herold Jörg[1], Schmeisser Aleander[1], Strasser Ruth H.[2], Braun-Dullaeus Rüdiger C.[1]. [1]Otto-von-Guericke-Universität, Klinik für Kardiologie, Angiologie und Pneumologie, [2]Herzzentrum Dresden, TU-Dresden, Germany

The local interaction of macrophages [MP] with vascular smooth muscle cells [VSMC] is known to play a key role in atherosclerotic plaque development and destabilization. An in-vitro co-culture model was established to study how Ma interact with and modulate the behavior of VSMC. Monocyte-derived macrophages were exposed to fluorescence-labelled acetylated LDL [FL-LDL] prior to co-culture. Semi-confluent VSMC were treated with mitomycin C to stop proliferation and co-cultured with the FL-LDL-tagged MP in a ratio of 1:3, respectively. Immune cytochemistry demonstrated that, within 4 weeks of co-culture, > 20% of all sm-actin positive cells (VSMC) were positive for Fl-LDL. Time-lapse microscopy revealed that the VSMC acquired the FL-LDL from the MP in co-culture by cell-cell-contact with in a timescale of hours and that MP apoptosis was not involved. The separation in a trans-well co-culture system resulted in complete absence of double-labelled cells. High resolution wide-field fluorescent microscopy and subsequent deconvolution of the microscopic volumes visualized first transport of FL-LDL within 6h after co-culture implementation. 24h after co-cultivation the entire cytosol of single VSMC was filled with FL-LDL particles. When MP had been fed with FL-LDL in complex with fluorescence-labeled cholesterol [FL-Chol] prior co-culture these complexes were also transferred during co-culture and resulted in cholesterol positive lipid droplet formation in VSMC. The sub cellular localisation under the usage of the acidotropic fluorescence dye LysoTracker™ demonstrated that some, but not all Fl-LDL reached the VSMC acidic lysosomes. In contrast, the expression of a fusion protein of LAMP1 and fluorescent protein reporter (FP) marking all lysosomes in VSMC demonstrated that the remaining FL-LDL was located in non acidic lysosomes. In a next step, VSMC were infected with a virus coding for a Rab5a-FP fusion protein to mark all early endosomes. In opposition to endothelial cells, which were used as control and were able to phagocytose FL-LDL from the culture medium, no co-localisation between Rab5a-FP and the transported Fl-LDL in co-cultured VSMC could be found, implying a phagocytosis independent mechanism. When the MP were infected with a virus encoding for LAMP1-FP prior co-culture it could be demonstrate that beside the FL-LDL intact fluorescence-marked lysosomes were transported into the VSMC. The rescue of the VSMC from co-culture and subsequent analysis of FL-LDL positive VSMC showed an increase in phagocytotic activity. Xenogenic cell composure (rat VSMC, human MP) and subsequent quantitative RT-PCR with rat specific primers demonstrated induction of genes typical for MP (CD68 and Mac2) and expectable alteration of cholersterol sensitive genes (HMG-CoA reductase down) in VSMC. Our results demonstrate that whole organelles are transported from MP to VSMC in-vitro. The lysosomal transfer of atherogenic molecules such as LDL and cholesterol is able to alter the phenotype of the VSMC. Our data have new implications on the pathogenesis of atherosclerosis.

Diverse roles of protein kinase A signaling in early stage endothelial cell differentiation

Jun K. Yamashita[1,2] Kohei Yamamizu[1,2]. [1]Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, [2]Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan

We have been investigating cardiovascular cell differentiation and regeneration using embryonic stem (ES) and induced pluripotent stem (iPS) cells. We established a mouse ES/iPS cell differentiation system for cardiovascular cells using Flk1+ cells as common progenitors (Nature, 2000; Circulation, 2008). In this system, cardiomyocytes, blood cells, three endothelial cell (EC) types (arterial, venous, and lymphatic) and vascular mural cells, can be systematically induced (Trends Cardiovasc Med, 2007). With the use of this system, we previously showed that activation of cAMP signaling in Flk1+ progenitors enhanced EC differentiation as well as arterial EC appearance (Arterioscler Thromb Vasc Biol, 2006). Subsequently, we succeeded in demonstrating molecular functions of cAMP and protein kinase A (PKA) signaling in EC differentiation and arterial EC diversification. That is, Notch and beta-catenin signaling were simultaneously activated by non-PKA-mediated cAMP signaling, and cleaved notch intracellular domain and beta-catenin formed a protein complex on various arterial gene promoters, which induced arterial EC fates (J Cell Biol, 2010). On the other hand, PKA activation induced increase in expression of VEGF receptors, Flk1 and neuropilin1, in Flk1+ cells, which enhanced common EC differentiation from vascular progenitors (Blood, 2009). Recently, we reported kapper opioid receptor signaling can work as an endogenous humoral factor system to negatively regulate PKA signaling thereby inhibiting EC differentiation (Blood, 2011). Thus, we have been elucidating pivotal functions of cAMP/PKA signaling in EC differentiation. In the present study, we further investigated cAMP/PKA roles in earlier stages of EC differentiation and succeeded in elucidating two novel distinct functions. Ets-family protein Etv2 (also called ER71 or Etsrp) is a key factor for vascular and blood development from mesodermal cells. We found Etv2 was markedly upregulated by PKA activation in undifferentiated ES cells, preceding EC differentiation. We identified two cAMP response element (CRE) sequences in Etv2 promoter region, and confirmed that CRE-binding protein (CREB) directly binds to the CRE sites. Expression of dominant negative form of CREB completely inhibited PKA-elicited Etv2 expression and induction of ECs from ES cells. Moreover, PKA/CREB activation induced expressions of vascular growth factor receptors, Flt1, Flk1, Flt4, Neuropilin1, Tie1, and Tie2 through Etv2 induction. PKA/CREB pathway is, thus, a critical regulator for the initiation of EC differentiation through Etv2 transcription (Stem Cells, in press). When PKA was activated in undifferentiated ES cells, not only mesodermal cells but also endoderm and ectoderm cells appeared approximately two times earlier. PKA activation increased protein expression of G9a, a H3K9 methyltransferase, along with earlier H3K9 dimethylation and DNA methylation in Oct3/4 and Nanog gene promoters. Deletion of G9a completely abolished the PKA effects. PKA activation increased G9a protein expression post-translationally through inhibition of an ubiquitin ligase for G9a, APC/C-cdh1 complex. G9a knockout mice showed prolonged expressions of Oct3/4 and Nanog at embryonic day-7.5 and delayed development, indicating that PKA regulates the differentiation timing in early ES cell differentiation to mesoderm through epigenetic inhibition of pluripotent gene expressions by G9a (Cell Stem Cell, in press). PKA, thus, enhances early EC differentiation processes through differentiation stage-specific function with transcriptional and epigenetic regulation.

CD40L deficiency ameliorates diet-induced adipose tissue inflammation - but does not protect from insulin resistance and hepatic steatosis in mice

Dennis Wolf[1], Felix Jehle[1,2], Ingo Hilgendorf[1], Peter Stachon[1], Florian Willecke[1], Natalie Hoppe[1], Bianca Dufner[1], Alexandra Ortiz-Rodriguez[1], Nora Bukosza[1], Christoph Bode[1], Karlheinz Peter[2], Andreas Zirlik[1]. [1]Atherogenesis Research Group, Department of Cardiology, University of Freiburg, Freiburg, Germany, [2]Baker IDI Heart and Diabetes Institute, Melbourne, Australia, [3]Department of Laboratory Medicine, Medical University of Vienna and Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria

Background: Adipose tissue inflammation fuels the metabolic syndrome. We recently reported that CD40L - an established marker and mediator of cardiovascular disease - induces inflammatory cytokine production in adipose cells in vitro. Here, we tested the hypothesis that CD40L deficiency modulates adipose tissue inflammation in vivo. Methods and Results: WT or CD40L-/- mice consumed a high fat diet (HFD) for 20 weeks (n≥15 per group). Inflammatory cell recruitment was impaired in mice lacking CD40L as shown by a decrease of adipose tissue macrophages, B-cells, and an increase in protective T-regulatory cells. Mechanistically, CD40L-deficient mice expressed significantly lower levels of the pro-inflammatory chemokine MCP-1 both, locally in adipose tissue and systemically in plasma. Moreover, levels of pro-inflammatory IgG-antibodies against oxidized lipids were reduced in CD40L-/- mice. Accordingly, CD40L deficiency partially protected from weight gain and fat deposition in the early stages of diet-induced obesity (DIO). Also, circulating low-density lipoproteins and insulin levels were lower in CD40L-/- mice. However, CD40L-/- mice consuming HFD were not protected from the onset of insulin resistance and hepatic steatosis, suggesting that CD40L selectively limits the inflammatory features of diet-induced obesity rather than its metabolic phenotype. Interestingly, CD40L-/- mice consuming a low fat diet (LFD) showed both, a favorable inflammatory and metabolic phenotype characterized by diminished weight gain, improved insulin tolerance, and attenuated plasma adipokine levels. Conclusion: We present the novel finding that CD40L deficiency limits adipose tissue inflammation in vivo. These findings identify CD40L as a potential mediator at the interface of cardiovascular and the metabolic disease.

Lymphadenectomy promotes tumor growth and cancer cell dissemination

Yeo Kim Pin*, Karen Khoo, Tan Karwai, Anne-Laure Puaux, Jean-Pierre Abastado, and Véronique Angeli. National University of Singapore, Singapore

Cancer cells disseminate through the hematogenous or the lymphatic route to colonize its tumor-draining lymph nodes (TDLNs) or region beyond. TDLNs can act as filters preventing cancer cell dissemination or it can serve as cancer cell reservoirs for further dissemination and metastasis though the primary tumor has been removed. In spite of limited convincing clinical benefits from randomized prospective trials, resection of TDLNs is a standard practice in several types of cancers including breast cancer and cutaneous melanoma. Hence, the role of TDLNs is complex and the effect of LN resection on cancer progression needs further evaluation. Using a spontaneous model of uveal melanoma, RET/AAD mouse, we found that mandibular and superficial parotid LNs drain the eyes. Growth of the primary eye tumor was accompanied by increased lymphangiogenesis in the mandibular lymph nodes while only peritumoral lymphatic vessels were observed in the tumor-bearing eyes. In addition, colonization of cancer cells in the draining lymph nodes is associated with the primary tumor size. We went on to remove both mandibular and superficial parotid LNs. Unexpectedly, early resection of the TDLNs increased tumor growth and cancer cell dissemination to the lungs and skin. Close examination of the primary tumor revealed increased primary tumor nodules as well as intratumoral blood vessel density. Here, using the spontaneous melanoma mouse model that closely mimic human cancer cases, we provide evidence that resection of TDLNs promotes cancer cell dissemination, tumor growth and metastasis.

Stalk cell phenotype depends on integration of Notch and Smad1/5 signaling cascades

Lieve Umans, Ivan M. Moya, Elke Maas, Paulo N.G. Pereira, Karen Beets, Annick Francis, Ward Sents, Elizabeth J. Robertson, Christine L. Mummery, Danny Huylebroeck, and An Zwijsen. VIB11 Center for Biology of Disease, Laboratory of Developmental Signaling VIB11 Center for Biology of Disease, Laboratory of Developmental Signaling and Department of Molecular and Developmental Genetics (VIB11), Laboratory of Molecular Biology (Celgen) VIB and Center for Human Genetics, KU Leuven, 3000 Leuven, Belgium

Gradients of vascular endothelial growth factor (VEGF) induce single endothelial cells to become leading tip cells of emerging angiogenic sprouts. Tip cells then suppress tip-cell features in adjacent stalk cells via Dll4/Notch-mediated lateral inhibition. We report here that Smad1/Smad5-mediated BMP signaling synergizes with Notch signaling during selection of tip and stalk cells. Endothelium-specific inactivation of Smad1/Smad5 in mouse embryos results in impaired Dll4/Notch signaling and increased numbers of tip-cell-like cells at the expense of stalk cells. Smad1/5 downregulation in cultured endothelial cells reduced the expression of several target genes of Notch and of other stalk-cell-enriched transcripts (Hes1, Hey1, Jagged1, VEGFR1, and Id1-3). Moreover, Id proteins act as competence factors for stalk cells and form complexes with Hes1, which augment Hes1 levels in the endothelium. Our findings provide in vivo evidence for a regulatory loop between BMP/TGFb-Smad1/5 and Notch signaling that orchestrates tip- versus stalk-cell selection and vessel plasticity.

POSTER ABSTRACTS

* = POSTER PRESENTER

Beneficial effects of endothelial 'oxidative stress' on coronary vascular functions

Jun Feng, Vinicius Schoeps, Cesario Bianchi, Frank Sellke, and Ruhul Abid*. Brown University Alpert Medical School, Cardiovascular Research Center, Rhode Island Hospital, 1 Hoppin St, Coro West 5.237, Providence, RI 02903, USA

Ischemic heart disease (IHD) or myocardial ischemia, is a disease characterized by tissue hypoxia due to reduced blood supply to the heart muscle, usually caused by coronary artery disease (CAD). IHD is the leading cause of death and morbidity in the USA and western world. Increase in coronary vessel diameter by vasodilatation (acute response), and increase in vessel density (delayed response) are two major defenses of myocardium from ischemic insults. Coronary vasodilatation is primarily dependent on endothelium-generated nitric oxide (NO). Increased levels of reactive oxygen species (ROS) are often observed in many cardiovascular diseases, including IHD, giving rise to the notion that ROS cause endothelial dysfunction. However, recent major interventional clinical trials using antioxidants (e.g. HOPE, ATBC), have largely produced negative results in reducing primary endpoints of cardiovascular death and morbidity (with some implication of potential harm). We will present data demonstrating that reduced ROS levels inhibit signal transduction events that are essential for NO generation in the vascular endothelium and for vasodilatation in intact coronary vessels. Our data will also show that c-Src responds to changes in endothelial redox levels and promotes downstream PI3K-Akt signaling, which in turn activates eNOS and inhibits the growth inhibitory transcription factor, FOXO1, in human coronary vascular endothelial cells (EC). Together, these findings reveal a disconnect between the current notions about the causal effects of ROS on vascular pathologies and the reality (the clinical outcomes, animal models), that necessitates careful studies to re-examine the prevailing dogma. Our ongoing studies aim at examining a novel hypothesis that temporal increase in EC-specific ROS will activate c-Src-PI3K-Akt-eNOS pathway to enhance coronary vasodilatation and will inhibit FOXO1 to increase vessel density in a myocardial ischemia model in vivo. Results will be presented using a novel binary transgenic animal model that can induce conditional increase in ROS in a vascular endothelium-specific manner. The main objective of our study is to examine whether conditional increase in EC-ROS improves coronary vascular tone and/or collateral vessel formation in the ischemic myocardium. The long-term goals are to develop redox-based novel therapeutic modalities that will 'pre-condition' coronary endothelium to improve coronary circulation in myocardial ischemia/infarction.

The RhoG-DOCK4-Rac1 signalling axis controls angiogenesis

Sabu Abraham[1]*, Margherita Scarcia[2], Tracey Harvey[2], Maggie Yeo[1], Christopher J. Marshall[1]and Georgia Mavria[2]. [1]Institute of Cancer Research, Cancer Research UK Centre for Cell and Molecular Biology, 237 Fulham Road, London SW3 6JB, [2]Leeds Institute of Molecular Medicine, Wellcome Trust Brenner Building, St Jame's University Hospital, Leeds LS9 7TF, UK. *Current address: Institute of Ophthalmology, University College London, London, EC1V 9EL, United Kingdom

During angiogenesis endothelial cells migrate, branch, contract to form cell-cell associations and develop lumens to make functional vessels. As Rho family GTPases are key regulators of actin dynamics we conducted siRNA-based screens of Rho family GTPases and their regulators in order to delineate signalling in different phases of vessel development. We have identified DOCK4 as a Rac1guanine nucleotide exchange factor (GEF), acting downstream of VEGF signalling. DOCK4 is required in endothelial cells for side-to-side cell-cell adhesion and sprouting through regulation of filopodia formation. We show that DOCK4 and Rac1 operate downstream of RhoG and that RhoG is required for tube formation in an organotypic angiogenesis assay. Knockdown of DOCK4 inhibits lumen formation in the tissue culture model and in tumours in vivo. This study therefore highlights the novel key role of the Rac1 GEF DOCK4 in angiogenesis.

ARP2/3 complex controls endothelial junction integrity

Abdallah Abu Taha*, Jochen Seebach, Muna Taha and Hans Schnittler. Dept. of Anatomy and Vascular Biology Westfälische Wilhelms-Universität Muenster Vesaliusweg 2-4 48149 Muenster, Germany

The vascular endothelial (VE)-cadherin-catenin complex comprises the backbone of endothelial cell junctions and is functionally linked to the actin filament cytoskeleton. The dynamics of these two structures is essential in junction regulation, but is poorly understood. Dynamic analysis of mCherry-tagged VE-cadherin by fast spinning disc microscopy uncovered a continuous remodelling of VE-cadherin that appeared mostly in two forms: as small individual clusters along the junctions that form a rope ladder like pattern (RLLP) and as a continuous linear pattern (CLP). In less confluent cells the RLLP predominates, while with increasing cell density the CLP becomes prominent. Contemporary dynamic analyses of EGFP tagged P20, a subunit of the actin-related protein-2/3 complex (ARP2/3 complex), uncovered the formation of junction associated intermittent lamellipodia (JAIL) of various sizes that appeared mostly between VE-cadherin clusters in less confluent cells. JAIL's size and frequency of occurrence depend on cell density and junction maturation. The functional importance of JAILs is shown by inhibition of the ARP2/3 complex-mediated actin polymerization using the specific inhibitor (CK-548) that brakes down the para-endothelial barrier function, resulting in loss of the monolayer integrity. The patho-physiological importance of this mechanism was further shown by stimulation of endothelial cell cultures with thrombin that transiently blocked the ARP2/3 activity and thus caused break down in para-endothelial barrier function by remodelling of the CLP of VE-cadherin into a RLLP. Restoring the barrier function was due to reappearance of the ARP2/3 activity, leading to formation of CLP of VE-cadherin. Dynamic quantitative analysis of the junctional ARP2/3 complex activity revealed that the kinetics of the break down and restoration of the barrier function concisely fits with the kinetics of ARP2/3 complex disappearance and restoring. These data highlight the critical role of the ARP2/3 complex in maintaining, opening and resealing of endothelial cell junctions in close interaction with actin- and VE-cadherin to allow remodelling and maintenance of junction integrity.

Synaptojanin-2 binding protein interacts with the Notch ligands DLL1 and DLL4 and inhibits sprouting angiogenesis

M. Gordian Adam[1,2,3]*, Caroline Berger[1,2,3], Joycelyn Wüstehube[1,2], Stefanie E. Herberich[1,2,3], Marcel Pinder[1], Sabine Gesierich[2], Hellmut G. Augustin[1,2] and Andreas Fischer[1,2,3]. [1]Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim (CBTM), Heidelberg University, D-68167 Mannheim, Germany, [2]Vascular Oncology and Metastasis, [3] Vascular Signaling and Cancer, German Cancer Research Center (DKFZ-ZMBH Alliance), D 69120 Heidelberg, Germany

The formation of novel blood vessels is initiated by vascular endothelial growth factor (VEGF) and counterbalanced by Delta-Notch signaling. Notch loss-of-function disrupts proper angiogenesis leading to the formation of a hyperdense vascular network with disturbed blood flow. Notch signals are also essential to promote arterial differentiation of endothelial cells. The present study was aimed at identifying novel modifiers of Notch signaling which interact with the Notch ligands DLL1 and DLL4. SYNJ2BP (ARIP2) was identified as a novel Delta interaction partner interacting physically with the carboxyterminal PDZ binding motif of DLL1 and DLL4. SYNJ2BP enhanced DLL4 protein stability, strongly extended its half-life time and promoted Notch signaling in endothelial cells. SYNJ2BP induced the arterial-specific Notch target genes HEY1 and ephrin-B2, reduced phosphorylation of ERK1/2 and decreased expression of the angiogenic factors Angiopoietin-2 and VEGF-C. Consistently, SYNJ2BP inhibited endothelial migration, proliferation and VEGF-induced angiogenesis. This could be rescued by blockade of Notch signaling. Implantation of SYNJ2BP-silenced human endothelial cells into immunocompromised mice led to the formation of a functional blood vessel network with significantly increased vascular density. These data identify SYNJ2BP as a novel inhibitor of angiogenesis executing its functions predominately by promoting Delta-Notch signaling.

Permissive role of miR-663 in induction of VEGF and activation of the ATF4 branch of unfolded protein response in endothelial cells by oxidized phospholipids

Taras Afonyushkin*, Olga V. Oskolkova, Valery N. Bochkov. Department of Vascular Biology and Thrombosis Research, Centre for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria

Previously we showed that oxidized palmitoyl-arachidonoyl-phosphatidylcholine (OxPAPC) stimulated production of VEGF and angiogenic switch, and characterized the ATF4 arm of unfolded protein response (UPR) as a key transcription mechanism mediating OxPAPC-induced upregulation of VEGF. In order to get further insights into the mechanisms of cellular stress-induced angiogenesis we studied the role of a specific micro RNA (miR-663) in the mechanisms of VEGF induction by oxidized phospholipids and inducers of unfolded protein response. OxPAPC upregulated in human endothelial cells (ECs) miR-663. Suppression of miR-663 resulted in inhibition of OxPAPC-induced upregulation of VEGF mRNA in two types of human ECs. In addition, knockdown of miR-663 suppressed upregulation by OxPAPC of ATF4 mRNA and protein, as well as a downstream gene TRB3. Similarly to the inhibition of OxPAPC effects, knockdown of miR-663 suppressed elevation of ATF4, VEGF and TRIB stimulated by another inducer of UPR, tunicamycin. Overexpression of miR-663 did not elevate VEGF, but completely reversed the inhibition of VEGF induction by miR-663 inhibitor. Thus, we conclude that miR-663 is critically important for 2 key events induced in ECs by stress agents and oxidized lipids, namely induction of transcription factor ATF4 and its downstream gene VEGF.

In vitro and in vivo analyses of mesenchymal cells originated by endothelial-mesenchymal transition (EndMT)

Yuichi Akatsu*, Hajime Mihira, Yasuhiro Yoshimatsu, Kohei Miyazono, and Tetsuro Watabe. Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Endothelial cells undergo mesenchymal transition characterized by increased expression of various mesenchymal markers such as α-smooth muscle actin (α-SMA) and decreased expression of endothelial markers such as VE-cadherin. This biological process is called endothelial-mesenchymal transition (EndMT), and plays significant roles not only in various physiological events including heart valve formation but also in pathological conditions such as cancer and organ fibrosis. Since EndMT has been implicated in the formation of a part of cancer associated fibroblasts (CAFs) which contributes to the progression of tumor, understanding the molecular mechanisms underlying it is crucial for developing novel therapeutic strategies for cancer. We previously reported that transforming growth factor (TGF)-ï□¢ï€ induces the mesenchymal transition of mouse embryonic stem cell-derived endothelial cells (Kokudo et al., 2008) and MS-1 mouse pancreatic microvascular endothelial cells (Mihira et al., 2012). However, molecular mechanisms how EndMT is regulated need to be further elucidated in vitro and in vivo. In the present study, we examined the characteristics of mesenchymal cells derived from EndMT using in vitro MS1 culture system and in vivo human B16 melanoma xenograft system. When MS1 endothelial cells are treated by TGF-ï□¢2, they exhibit the features of EndMT including loss of cell-cell contact and acquisition of spindle-shaped morphology. We examined which mesenchymal markers are induced by TGF-β2 in MS-1 cells. TGF-β2 induced the expression of various mesenchymal markers including α-SMA, SM22α, fibronectin 1 and matrix metalloproteinase 2, but not of some fibroblast markers such as FSP-1. These results suggest that the mesenchymal cells that are derived from MS1 cells via EndMT have characteristics of mural cells. We next examined the features of the mesenchymal cells that are derived from endothelial cells in a mouse model of B16F10 melanoma. In order to genetically mark the Tie2-expressing endothelial cells, we used transgenic mice carrying Tie2-Cre and R26Rosa-lox-Stop-lox-LacZ transgenes. In these transgenic mice, Cre recombinase activity is preferentially present in Tie2 expressing endothelial cells, and irreversibly marks the cells to induce the expression of LacZ (β-galactosidase) gene. We studied the characteristics of mesenchymal cells derived from Tie2 positive cells by staining the B16F10 mouse melanoma subcutaneously transplanted into the transgenic mice for various mesenchymal markers. We found that more than 30% of FSP-1 positive fibroblast cells in B16F10 melanoma also express LacZ, suggesting that these fibroblasts are derived from mesenchymal transition of Tie2 positive cells. In contrast, we were hardly able to detect α-SMA positive cells expressing LacZ. These results suggest that the mesenchymal cells that are derived from in vitro cultured MS1 cells and those from Tie2 positive cells in mouse model of B16 melanoma may have different characteristics.

The vascular endothelial growth factor receptor neuropilin-1 modulates endothelial apoptosis in arteriosclerosis

Nadia Al-Fakhri[1]*, Lilli Mey[1], Zeina Kharip[1], Andreas Hildenberg[1], Christa Löwer[1], Mareike Hörmann[2], Tibo Gerriets[3]. [1]Institute for Laboratory Medicine and Pathobiochemistry, Molecular Diagnostics; Philipps-University, Marburg; Germany, [2]Clinic for Vascular Surgery, University Clinic Cologne, Germany, [3]Clinic for Neurology, Justus-Liebig-University Giessen and Kerckhoff-Institute Bad Nauheim, Germany

Introduction: Vascular endothelial growth factor (VEGF) inhibits apoptosis of endothelial cells via VEGFR-2, JNK and Akt by inducing poly(ADP-ribose)-polymerase (PARP) expression, as previously demonstrated. In the present study, we investigated the involvement of neuropilin-1 (NP-1) in the regulation of PARP and its modulatory effect on VEGF-dependent apoptosis inhibition in cell culture, in human arteriosclerosis and in the animal model. Methods: HUVEC and the human cell line EA.hy.926 were incubated with VEGF-A(165) or VEGF-A(121) that display receptor-specific binding and with VEGF-receptor inhibitors SU5416 and A7R. Apoptosis was induced by adhesion inhibition with cRGDfV. Apoptosis was analysed by annexin V-flow cytometry. Signal transduction and PARP expression were investigated by Western blot and real-time RT-PCR. VEGFR-2 and NP-1 were detected by immunofluorescence microscopy. Immunohistochemistry was applied to human arteriosclerotic and normal arteries, to a vascular organ culture model and to rat brains derived from a cerebral ischemia-reperfusion model to investigate apoptosis, VEGF, PARP, NP-1 and VEGFR-2 expression. In ApoE-deficient and wildtype mice (+/- high-fat diet), NP-1 protein and mRNA expression was demonstrated. Results: NP-1 acted as a coreceptor to VEGFR-2 in VEGF-dependent inhibition of endothelial apoptosis in an isoform-specific manner. NP-1 contributed to the regulation of PARP expression by VEGF via Akt and JNK. NP-1 augmented the cellular response to VEGF-A(165), but incubation with VEGF-A(121) that shows little binding to NP-1 only induced a slight increase in PARP expression; this was reproduced by incubation with receptor inhibitors. An increased expression of NP-1 was demonstrated in human arteriosclerotic arteries and in the organ culture model compared to controls. In the ApoE-knockout mouse, NP-1 expression was increased compared to wildtype mice. In regions of cerebral ischemia, an increase in endothelial NP-1 was detected in the rat brain in the vicinity of endothelial apoptosis. Conclusion: NP-1 acts as an important coreceptor for VEGFR-2 and as a modulator of apoptosis inhibition and PARP regulation by VEGF in the endothelium. NP-1 adapts the situative response of endothelial cells to VEGF. The increased NP-1 expression in human arteriosclerosis and in the animal model point to a role for NP-1 in the modulation and intensification of VEGF effects aimed at maintaining endothelial integrity in arteriosclerosis.

A Cdc42-MRCK-myosin II signaling axis potentiates endothelial barrier function through formation of circumferential actin bundles in Rap1 signal-activated endothelial cells

Koji Ando[1,2]*, Shigetomo Fukuhara[1], Kazuomi Noda[1], Takahiro Moriya[2], Naoki Mochizuki[1]. [1]Dept. Cell Biol., Natl. Cerebr. & Cardiovasc. Ctr. Res. Inst, [2]Dept. Cell. Signaling, Grad. Sch. Pharmaceut Sci., The Univ. of Tohoku, Osaka, Japan

Endothelial cells lining blood vessels regulate endothelial barrier function, which restricts the passage of plasma proteins and circulating cells across the endothelium. Compromising barrier function of endothelial cells leads to an increase in vascular permeability, which is associated with chronic inflammation, edema, and tumor angiogenesis. Endothelial barrier function is dynamically controlled by reorganization of actin cytoskeleton. Previously, we have reported that cyclic AMP (cAMP)-Epac-Rap1 pathway induces formation of circumferential actin bundles (CAB) along the cell-cell junctions, thereby potentiating vascular endothelial (VE)-cadherin-mediated cell-cell adhesion. VE-cadherin stabilizes at cell-cell junctions by being anchored to CAB through alpha- and beta-catenins. However, the mechanism how cAMP-Epac-Rap1 pathway regulates actin dynamics remains unclear. In this study, we tried to address its underlying molecular mechanism by focusing on non-muscle myosin II (NM-II), a key regulator of actin cytoskeleton. In confluent human umbilical vein endothelial cells (HUVECs), NM-II was activated on the CAB along the cell-cell junctions upon stimulation with forskolin and 007, an adenylyl cyclase activator and a cAMP analogue specific for Epac, respectively. Inhibition of NM-II by blebbistatin and its knockdown by siRNA prevented forskolin- and 007-induced CAB formation. These results indicate the essential role of NM-II in CAB formation by a cAMP-Epac-Rap1 pathway. Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) is known to act downstream of Cdc42 to induce NM-II activation. Thus, we investigated the involvement of MRCK in Rap1-induced NM-II activation at cell-cell contacts and CAB formation. Knockdown of either MRCK or Cdc42 by siRNA inhibited NM-II activation at cell-cell contacts and subsequent CAB formation by forskolin and 007. Both MRCK and Cdc42 were recruited to cell-cell junctions by stimulation with forskolin and 007. However, MRCK failed to localize at cell-cell junctions in the absence of Cdc42 expression. In addition, a MRCK mutant lacking Cdc42-binding domain was not recruited to cell-cell contacts in HUVECs stimulated with forskolin and 007. These results indicate that activation of a cAMP-Epac-Rap1 pathway induces recruitment of MRCK to cell-cell junctions in a Cdc42-dependent manner, thereby inducing CAB formation through activation of NM-II at cell-cell contacts. Furthermore, we explored the biological significance of Rap1-induced CAB formation through a Cdc42-MRCK-NM-II signaling axis. Fluorescence recovery after photobleaching analysis revealed that knockdown of MRCK inhibits Rap1-induced stabilization of VE-cadherin at cell-cell contacts. By using Electric Cell-substrate Impedance Sensing or by performing in vitro permeability assay, we found that Rap1 activation by forskolin and 007 potentiates endothelial cell-cell junctions through a Cdc42-MRCK-NM-II pathway. Finally, we showed that intravenous injection of 007 in mice dramatically suppresses mustard oil-induced vascular leakage by performing modified Miles assay. These results indicate that Rap1-induced CAB formation through a Cdc42-MRCK-NM-II pathway potentiates endothelial barrier functions. Collectively, these results indicate that Rap1 induces NM-II activation at cell-cell contacts by inducing junctional localization of MRCK in a Cdc42-dependent manner, thereby promoting formation of CAB leading to potentiation of endothelial barrier function.

Lymphatic vessel endot helial hyaluronic acid receptor-1 expressing macrophages as gatekeepers of blood vessel homeostasis

Hwee Ying Lim[1], Lihui Lim[1], Chern Goh[1], Yeo Kim Pin[1], Ling Li[2], Xiao Nong Wang[4], Stanley Richard[3], Matthew Collin[4], Florent Ginhoux[5] and Veronique Angeli[1]*. [1] Department of Microbiology, National University of Singapore, [2]Institute for Molecular Biology, Singapore, [3]Albert Einstein College, NYC, NY, USA, [4]New Castle University, [5]Singapore Immunology Network, Singapore

As components of the circulatory system, lymphatic and blood vessels play a critical role in tissue/organ homeostasis and immunity. Dysregulation of their function often contributes to pathogenic processes including chronic inflammation, cancer, vascular and infectious diseases. In contrast to our knowledge on the response of vessels to inflammation, little information is available on the mechanisms that support vessel homeostasis. Although lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) is a marker for lymphatic vessels it has been reported on macrophages. Here, we demonstrate that this population of macrophage expressing LYVE-1+ is essential for blood vessel homeostasis by maintaining smooth muscle cells. Unexpectedly, tissue whole-mount immunostaining for LYVE-1+ and CD68, a marker for macrophage, revealed that LYVE-1+ macrophages were lining the outer layer of blood vessels but only those covered by smooth muscles and not pericytes namely: veins, arteries and aorta. Since we found that deficiency in mice of macrophage colony stimulating factor (M-CSF) or its receptor and the inhibition of M-CSFR activity by small molecule resulted in a severe depletion in LYVE-1+ macrophages in tissues, we employed these strategies to assess the consequences of the loss of these cells on blood vessel structure and function. Loss of LYVE-1+ macrophages resulted in the thinning of the media of arteries, veins and aorta. In the aorta, media thinning led to vascular leakage. These results prompted us to investigate whether atherosclerotic arteries, which exhibit thinning of the media, could be associated with any alterations in LYVE-1+ macrophages. Aorta from atherosclerotic apoE-/- and Ldlr-/- mice showed a marked reduction in LYVE-1+ macrophages in the adventitia, which preceded the loss of smooth muscles in the media. Conversely, regression of atherosclerosis induced by lowering-cholesterol drug, restored the population of LYVE-1+ macrophages and improved media thinning. Finally, we went on elucidate the possible mechanisms that which these LYVE-1+ macrophages could maintain smooth muscle cells and thus blood vessel integrity. We found that this function may be in part mediated by the expression of TGF-beta by LYVE-1+ macrophages, a growth factor known to be indispensable for smooth muscle maintenance. These findings demonstrating a novel function for this population of macrophages expressing LYVE-1 not only shed some light on the control of vasculature structure and function but also provide means to improve or design therapeutic strategies for revascularization.

Comparison of endothelin and nitric oxide synthase blockade on hemorheological parameters in endotoxaemic rats

Okan Arihan[1]*, Neslihan H. Dikmenoglu[1], Alper B. Iskit[2]. [1]Hacettepe University, Faculty of Medicine, Department of Physiology, Ankara, Turkey, [2]Hacettepe University, Faculty of Medicine, Department of Pharmacology, Ankara, Turkey

Septic shock alters cardiovascular and hemodynamic status vastly and constitutes a major health problem. Increase in the production of endothelium-derived substances such as nitric oxide (NO) and endothelin is a counterpart of this endotoxaemic state. This study was planned to test the hypothesis that nitric oxide synthesis blockers (L-NAME and L-Canavanine) or endothelin receptor antagonist (Bosentan) will reverse the action of septic shock on hemorheological parameters. Sprague-Dawley rats (250-300 g) were administered intraperitoneally bosentan (30 mg kg-1), L-NAME (3 mg kg-1) or L-Canavanine (100 mg kg-1) 2 hours after they have received saline or E.coli endotoxin (4 mg kg-1). After the withdrawal of blood into heparinized tubes three main parameters contributing to blood viscosity were measured at the 4th hour of endotoxaemic state. Erythrocyte deformability and erythrocyte aggregation were measured with laser-assisted optical rotational cell analyzer (LORCA). Plasma viscosity was measured by a cone-plate viscometer. Data were analyzed with ANOVA. Plasma viscosity and erythrocyte deformability were not changed significantly during endotoxaemia. Endotoxin application significantly increased aggregation half time (t1/2) (inversely related with aggregation speed), lowered erythrocyte aggregation amplitude (AMP) and aggregation index (AI - a combination of the other two parameters) compared to the control group, indicating a slower and weaker aggregation. AMP, AI and t1/2 values of the endotoxin and control groups were: (5.1±1.3 and 15.0±0.9 au n=6, $P<0.05$; 9.1±2.0 and 37.3±2.0 n=6 $P<0.05$, 43.3±7.0 and 7.0±0. 8 n=6, $P<0.01$ respectively). Non-selective nitric oxide synthase inhibition by L-NAME and inducible nitric oxide synthase inhibition by selective inhibitor L-Canavanine abolished all of the effects of endotoxin without altering the values in control animals whereas endothelin receptor antagonist Bosentan did not perform such a restoration. AMP, AI and t1/2 values of the Endotoxin+L-NAME, endotoxin+L-canavanine and endotoxin+bosentan groups were: AMP 8.8±2.5, 7.4±1.5 and 7.1±0.6 au; AI 33.9±4.7, 31.7±4.1 and 16.1±3.5; t1/2 9.2±1.6, 9.5±1.4 and 23.1±4.0 sec respectively. The prolonged time in t1/2, lower levels of AMP and AI in endotoxin-treated group suggest a depletion of factors inducing aggregation of erythrocytes. Increased coagulation tendency in septic shock can lead to overuse of plasma proteins such as fibrinogen which contribute to the observed attenuation of aggregation parameters in this experimental setup. An increase in blood viscosity due to deterioration in any of the parameters that contribute to it, such as erythrocyte aggregation, hinders blood flow. On the other hand a certain level of blood viscosity is believed to be essential for normal circulation. Whether the decrease in erythrocyte aggregation in septic shock is an unwanted effect and its reversal by nitric oxide synthase blockade is a wanted effect needs to be determined.

Angiopoietin-1 is critical for coronary venogenesis in the developing heart

Yoh Arita[1]*, Yoshikazu Nakaoka[1], Taichi Matsunaga[2], Kohei Yamamizu[2], Hiroyasu Kidoya[3], Nobuyuki Takakura[3], Naoki Mochizuki[4], Gou Young Koh[5], Jun K. Yamashita[2], Issei Komuro[1]. [1]Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan, [2]Laboratory of Stem Cell Differentiation, Institute for Frontier Medical Sciences/ Department of Cell Growth & Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan, [3]Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Suita, Japan, [4]Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Suita, Japan, [5]Biomedical Research Center and Department of Biological Sciences, Korea

Coronary artery disease is the leading cause of mortality all over the world. Elucidating the developmental program of coronary vessels would help to understand the disease and lead to novel therapeutic options. Recently, Red-Horse et al. have revealed that coronary vessels arise from angiogenic sprouts of the sinus venosus, and that both vessel sprouting and outgrowth depend on unknown signals derived from the ventricle and epicardial tissues of the heart (Nature 2010). Here, we show that the Tie2 ligand, angiopoietin-1 (Ang1), secreted from myocardium, is critical for the formation of coronary veins, namely „coronary venogenesis“. We created cardiomyocyte-specific Ang1 knockout (Ang1CKO) mice using Cre-loxP system. Ang1CKO mice showed embryonic lethality between embryonic day 12.5 and 14.5, with various abnormalities in embryonic hearts such as impaired adhesion between endocardium and myocardium, poor trabeculation and thin compact layer. Whole-mount CD31-immunostaining analysis revealed that Ang1CKO embryos have severe defects in the formation of superficial coronary vessels which recapitulate the venous lineages. Expression levels of venous marker genes such as Eph receptor B4 (Ephb4) and chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) in the hearts of Ang1CKO embryos were significantly decreased compared with those of control littermates. Consistent with these findings, immunohistochemical analyses revealed that coronary veins positive for EphB4 were lost in the hearts of Ang1CKO mice, but not in those of control littermates. On the other hand, the formation of coronary arteries in the heart was almost comparable between control and Ang1CKO embryos. Consistently, the expression levels of arterial marker genes such as EphrinB2, neuropillin-1, Delta-like 4 were not significantly decreased in the hearts of Ang1CKO embryos compared with those of control embryos. Furthermore, EphrinB2-positive coronary arteries were detected in the hearts of Ang1CKO mice similarly in those of control embryos. The above findings suggest that Ang1 secreted from myocardium is critical for the formation of coronary veins, but not for that of coronary arteries. To reveal the involvement of Ang1 in the differentiation of premature endothelial cells, we examined the effect of cartilage oligomeric matrix protein (COMP)-Ang1, a potent variant of Ang1, on the arterial lineage recapitulation by the treatment with VEGF and cAMP in Flk1-positive embryonic stem (ES) cells. Surprisingly, co-application of Ang1 inhibited the induction of the arterial marker EphrinB2 after treatment with VEGF and cAMP, and upregulated COUP-TFII expression. Moreover, we found that co-application of Ang1 with VEGF promoted migration of Flk1-positive ES cells much more strongly compared with single application of VEGF or Ang1. Taken together, Ang1 secreted from myocardium is critical for coronary venogenesis in the developing heart.

CSN5, a novel player in atherosclerosis, confers atheroprotection

Yaw Asare[1]*, Erdenechimeg Shagdarsuren[2], Johannes A. Schmid[3], Jochen Grommes[2,4], Omar El Bounkari[1], André Gessner[5], Martina Panattoni[6], Ruggero Pardi[6], Christian Weber[7,8,9], Menno P.J. de Winther[9], Heidi Noels[2], Jürgen Bernhagen[1]. [1]Institute of Biochemistry and Molecular Cell Biology, and [2]Institute of Molecular Cardiovascular Research (IMCAR), RWTH Aachen University, Aachen, Germany, [3]Center for Physiology and Pharmacology, Department of Vascular Biology and Thrombosis Research, Medical University Vienna, Vienna, Austria; [4]European Vascular Center Aachen-Maastricht, Department of Vascular Surgery, RWTH Aachen University, Aachen, Germany; [5]Institute for Medical Microbiology and Hygiene, University of Regensburg, Regensburg, Germany; [6]School of Medicine and Scientific Institute, Vita-Salute San Raffaele University, San Raffaele, 20132 Milan, Italy; [7]Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Munich, Germany, [8]Munich Heart Alliance, Munich, Germany, [9]Cardiovascular Research Institute Maastricht, Maastricht University, The Netherlands

Inflammatory processes play a crucial role in all stages of atherogenesis. As a key trigger, the activation of the NF-κB signaling pathway drives the expression of pro-inflammatory and pro-atherosclerotic genes. The COP9 signalosome (CSN), a multifunctional protein complex involved in the regulation of Cullin-RING-E3 ubiquitin ligases (CRLs), has emerged as a regulator of NF-κB signaling. This function has been assigned to the deneddylase activity of its subunit CSN5. However, the effect of the CSN and in particular CSN5 on NF-κB-mediated pro-atherogenic responses in endothelial cells and macrophages and in atherogenesis in vivo is still elusive. This study sought to identify a role of the CSN complex and in particular CSN5 in IKK/NF-κB signaling in atherogenic endothelial cells in vitro and in murine atherosclerosis invivo. Co-immunoprecipitation in human umbilical vein endothelial cells (HUVECs) revealed the presence of a supercomplex between IKK and CSN, which dissociates upon TNF-αstimulation. Furthermore, transient overexpression of CSN5 protected IκBαfrom degradation and reduced NF-κB activation upon TNF-αstimulation, whereas CSN5 silencing enhanced TNF-α-induced IκB-αdegradation and NF-κB activity in luciferase reporter assays. This was paralleled by NF-κB-driven upregulation of atherogenic chemokines and adhesion molecules, as measured by real-time PCR and flow cytometry and translated into an increased arrest of perfused monocytes on TNF-α- stimulated, CSN5-depleted HUVECs. Importantly, double-immunostaining for CSN and CD31 confirmed the expression of CSN subunits in the endothelial layer of human atherosclerotic lesions, and revealed an increased expression of CSN5 which correlated with atheroprogression. Employing a macrophage-specific deletion of Csn5 in a murine atherosclerosis model, this study identifies Csn5 as a novel player in atherosclerosis. Ablation of Csn5 was found to exacerbate murine atherosclerosis, an observation that confirms the atheroprotective role of CSN5 demonstrated for the first time in this study. Taken together, CSN5 attenuates NF-κB-dependent pro-inflammatory gene expression and monocyte arrest on atherogenically stimulated endothelial cells, and negatively regulate murine atherosclerotic lesion formation.

The unique lymphatic vascular phenotype of the Schlemm's canal develops via a VEGF-C/VEGFR-3 independent mechanism

Aleksanteri Aspelund[1]*, Tuomas Tammela[1] Ilkka Immonen[2] Kari Alitalo[1].
[1]Molecular/Cancer Biology Program, Research Programs Unit, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland, [2]Department of Ophthalmology, Helsinki University Central Hospital, Haartmaninkatu 4 C, 00029 Helsinki, Finland

Raised intraocular pressure (IOP) is the major risk factor for the development of glaucoma, the second leading cause of blindness worldwide today. IOP is generated and regulated by a delicate balance between the constant secretion of aqueous humor (AqH) into the posterior chamber of the eye and its absorption from the anterior chamber through a high resistance drainage system. An essential component in this drainage system is the Schlemm's canal (SC), a ring-shaped vessel that encircles the cornea. The SC is the final barrier for the AqH and antigen presenting cells of the uveal tract to cross before returning to systemic circulation. In this sense, the SC functions like lymphatic vessels; nevertheless, it has been considered a component of the blood vascular system. The investigation of the lymphatic phenotype of the SC has been limited by the lack of specific lymphatic markers until recently. In the present study, we provide description of the expression of lymphatic endothelial markers in the developing and adult SC; these include the prospero-related homeobox 1 (Prox1) transcription factor, the lymphangiogenic receptor tyrosine kinase Vegfr3 and the chemokine CCL21. Like the lymphatic vascular system, the developing SC was found to have a venous origin and its initial stages of development involved the induction of Prox1, the master regulator of the lymphatic phenotype, followed by the induction of Vegfr3 and further maturation. However, using heterozygous knockout mice and adenoviral expression of VEGF-C/-D trapping proteins, we demonstrate that VEGFR-3 signaling, and it ligands VEGF-C or VEGF-D, are not critical for development and maintenance of the SC, unlike in the lymphatic vascular tree. Furthermore, unlike in the lymphatic vascular tree, the SC maintains connections to the blood vascular system at even intervals though aqueous veins, which drain the SC directly into the blood vascular system. In conclusion, the SC has both functional and structural features of the lymphatic vascular system, its development follows a similar molecular program as the development of the lymphatic vascular tree, and it is shown to express many of the same lymphatic endothelial cell markers, yet its development is distinctly regulated.

New insights into JAM-C/JAM-B function in lymph node and bone marrow homeostasis

ML. Arcangeli, V. Frontera, A. Zarubica, F. Bardin, S. Adams, JC. Lissitzky, R.H. Adams, M. Aurrand-Lions*. [1]INSERM ; Centre de Recherche en Cancérologie de Marseille, Aix-Marseille University, Institut Paoli-Calmettes, F-13009 Marseille, France, [2]Max Planck Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, and University of Muenster, Faculty of Medicine, Muenster, Germany

Over the last ten years, the junctional adhesion molecule, JAM-C, and its counter receptor JAM-B, have been extensively studied in the context of vascular function. Their role in the regulation of inflammation, angiogenesis and leukocyte trans-endothelial migration is well established. In contrast, little is known about the function of JAM-B and JAM-C expression on stromal cells of hematopoietic organs and their contribution to the establishment and maintenance of tissue homeostasis. We addressed these issues through the study of JAM-C function in lymph node stromal cells and in hematopoietic stem cells (HSC). In bone marrow as well as in lymph nodes, we found that JAM-C is essential for the maintenance of tissue homeostasis. In the bone marrow, the interaction of JAM-C expressed by HSC, with JAM-B expressed on stromal cells, is essential to the maintenance of HSC quiescence and retention in the bone marrow as demonstrated by of the hematopoietic defect observed in Jam-B deficient mice. In lymph nodes, we found that JAM-C expressed by stromal cells contributes to tissue homeostasis through the control of constitutive chemokine secretion by Fibroblastic Reticular Cells. These results will be discussed in the context of previously reported vascular functions of JAM-B and JAM-C and in the context of inflammatory regulation.

S1P3 regulates implant arteriogenesis by recruitment and localization of anti-inflammatory monocytes to surrounding microvessels

Anthony O. Awojoodu[1]*, Lauren S. Sefcik[2], Kyle Martin[3], Shayn M. Peirce-Cottler[3], Edward A. Botchwey[1]. [1]Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, Georgia, [2]Department of Chemical and Biomolecular Engineering, Lafayette University, Easton, Pennsylvania, [3]Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia, USA

Biocompatible implants have been widely used to deliver therapeutic cells and molecules but a significant hurdle to their success is surmounting host responses to foreign materials. Implantation is followed by an immediate recruitment of inflammatory cells driven by cytokines and chemokines that often leads to fibrous encapsulation: preventing effective delivery of products. There are two distinct sets of monocytes that are known to contribute to the inflammatory response: inflammatory (IM) monocytes and anti-inflammatory (AM) monocytes. AM contribute to tissue remodeling and regeneration. Creating implants that regulate inflammation, promote microvascular remodeling and augment tissue regeneration can dramatically enhance healing therapies. The objective of this study was to harness sphingosine 1-phosphate (S1P) receptor signaling to regulate implant arteriogenesis through recruitment of AM during wound healing. S1P is a pleiotropic, autocrine and paracrine signaling molecule that binds to a family of 5 high affinity G-coupled receptors (S1P1-S1P5) to direct a wide range of biological processes. Because strong coordination exists between recruitment and surveillance of blood cells, modulation of S1P receptor signaling may play an important role in recruiting regenerative AM. FTY720 (S1P1/S1P3 agonist) was encapsulated in 50:50 poly(lactic-co-glycolic acid) (PLAGA) and delivered in the murine dorsal skinfold window chamber (backpack) model to C57Bl/6 mice. MCP-1 was significantly increased in the tissue 1 day after surgery while SDF-1 was significantly increased 7 days after. When FTY720 was delivered 7 days after surgery (delayed) there was a robust increase in microvascular growth (154%) as opposed to immediately (acute) after surgery (43%) and also over PLAGA controls (-47% and 13%) and sham implants (-23%). These results were dependent on S1P3 on marrow-derived and local cells as S1P3-/- bone marrow mice (17%) and S1P3-/- mice with wild type marrow (28%) show attenuated remodeling with delayed FTY720. This also suggests a coordination between S1P3 and SDF-1. S1P3 activation decreased the recruitment of CD11b+ monocytes to vessel lumens as well as the proportion of IM in tissue (12%) compared to PLAGA (19%) but increased the proportion of AM (10% compared to 7%). In FTY720-treated backpack tissue from CX3CR1-eGFP mice, which express a high level of CX3CR1 on AM, there was a significant increase in rolling and adhering cells that expressed high levels of CX3CR1 immediately and one day after surgery. FTY720 significantly enhanced hydrophillicity of, encouraged monocyte adhesion to and prevented monocyte blasting and activation on PLAGA films. In vivo, FTY720 significantly decreased the expression of inflammatory chemokines (TNF-a, MIP-1, MCP-1, IL-6, IL-1B) known to attract IM. Spinotrapezius muscles on CX3CR1-eGFP mice were ligated and FTY720 enhanced tortuosity of vessels in the ischemic watershed, promoted recruitment and co-localization of CX3CR1-high cells with remodeling vessels and significantly enhanced the number of vessel associated CX3CR1+ cells (38) in the downstream watershed compared to unloaded PLAGA(11). We have shown that implant-induced arteriogenesis can be enhanced by S1P3 activation. FTY720 decreases the secretion of inflammatory molecules, attenuates monocyte activation and enhances AM cell recruitment to remodeling vessels. Strategies to promote regeneration through regulating inflammation can enhance healing outcomes mediated by biomaterial implants.

Mechanism of acute lung injury induced by transfusion of antibody against endothelial cells

Behnaz Bayat[1]*, Yudy Tjahjono[1], Sydykov Akylbe[2], Stephan Hippenstiel[3], Nobert Weissman[2], Ulrich J Sachs[1], Sentot Santoso[1]. [1]Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig University, Giessen, Germany, [2]University of Giessen Lung Center, Giessen, Germany, [3]Department of Infectious Diseases and Respiratory Medicine of the Charité Medical University, Berlin, Germany

Transfusion related acute lung injury (TRALI) has been described as a severe side-effect of blood transfusion closely related to pathogenesis of acute lung injury (ALI) and turned out to be the leading cause of transfusion related fatalities. Recent studies showed that antibodies against the allelic isoform of choline transporter like protein 2 (also known as HNA-3a) are associated with high TRALI mortality. The mechanism underlying this fatal reaction, however, is not clear. In this study, we aimed to identify the mechanism of fatal lung injury induced by HNA-3a antibody under in vitro as well as in vivo conditions. Total mRNA analysis of different blood and endothelial cells (EC) by real-time PCR revealed the existence of most abundant copies of CTL-2 transcripts in ECs in comparison to other blood cells. This result was confirmed by immunochemical analysis using rabbit antibody specific for CTL-2 as well as human antibodies against HNA-3a. Treatment of EC with HNA-3a antibodies leads to significant production of reactive oxygen species (ROS). In transwell system, a significant increase in albumin-FITC influx was observed when HNA-3a positive EC monolayer were treated with human anti-HNA-3a antibodies. In line with this observation, a significant drop in transendothelial electrical resistance and gap formation were detected. Immunoblotting analysis of ECs treated with anti-HNA-3a antibodies showed a significant increase of VE-cadherin phosphorylation. This observation indicates that anti-HNA-3a antibodies induce EC barrier disturbance via ROS-mediated destabilization of VE-cadherin in cell junctions. Accordingly, this antibody-mediated barrier leakage can be ameliorated by the vasoactive peptide adrenomedulin (ADM), which prevents cell destruction in response to oxidative stress. In an in vivo murine model of TRALI, injection of anti-HNA-3a antibodies in lipopolysaccharide primed C57BL/6 resulted in significant increase of mice lung weight, elevated concentration of albumin and number of neutrophils in the bronchoalveolar lavage, confirmed expected lung edema formation in these mice. Treatment of mice with ADM after induction of TRALI with anti-HNA-3a alleviated the destructive effects of antibody and remedies the TRALI symptoms in mice. Surprisingly, neutrophil depletion in these mice failed to prevent TRALI reaction. In NOX2 deficient mice no TRALI reaction was observed when mice were challenged with anti-HNA-3a antibodies. Taken together, our data demonstrate that anti-HNA-3a antibodies can impair endothelial barrier integrity in vitro and in vivo via ROS mediated endothelial junction instability. The deleterious effect of antibody can be inhibited by the treatment with ADM. This knowledge may help us to described the importance antibody directed against endothelial cells in development lung injury and may open a new era therapeutic approach preventing endothelial dysfunction.

Intermediate filament breakdown controls activation of the angiogenic switch

Hyeong-Il Kwak, Hojin Kang, Jui M. Dave, E. Adriana Mendoza, Shih-Chi Su, Roland Kaunas, Steve A. Maxwell, and Kayla J. Bayless*. Department of Molecular & Cellular Medicine, Texas A&M Health Science Center, College Station, TX, 77843-1114, USA

Endothelial cells normally line the vasculature and remain quiescent. However, these cells can be rapidly stimulated to undergo morphogenesis and initiate new blood vessel formation given the proper cues. This study uses two models to stimulate endothelial sprouting responses in three-dimensional collagen matrices, specifically sphingosine 1-phosphate (S1P) combined with angiogenic growth factors (GF) and wall shear stress (WSS). We find here a new mechanism for initiating angiogenic sprout formation that involves vimentin, the major intermediate filament protein in endothelial cells. Initial studies confirmed vimentin was required for S1P/GF- and S1P/WSS-induced endothelial cell invasion. In both systems, vimentin was cleaved by calpains during invasion. Calpains were predominantly activated by GF and were required for sprout initiation. Because others have reported membrane type 1 - matrix metalloproteinase (MT1-MMP) is required for endothelial sprouting responses, we tested whether vimentin and calpain acted upstream of MT1-MMP. Both calpain and vimentin were required for successful MT1-MMP membrane translocation, which was stimulated by S1P. In addition, vimentin complexed with MT1-MMP in a manner that required both the cytoplasmic domain of MT1-MMP and calpain activation, which increased the soluble pool of vimentin in endothelial cells. Altogether, these data indicate that pro-angiogenic signals converge to activate calpain-dependent vimentin cleavage and increase vimentin solubility, which act upstream to facilitate MT1-MMP membrane translocation, resulting in successful endothelial sprout formation in three-dimensional collagen matrices. These findings help explain why S1P and GF or S1P and WSS synergize to stimulate robust sprouting in 3D collagen matrices.

The new lymphangiogenesis inhibitor esVEGFR2 is down-regulated in advanced stage neuroblastoma and induced upon differentiation.

Jürgen Becker* and Jörg Wilting. University Medicine Göttingen Anatomy and Cell Biology Kreuzbergring 36 37075 Göttingen, Germany

Tumor metastasis is facilitated by hemangiogensis and lymphangiogenesis. Key regulators of angiogenesis are the members of the vascular endothelial growth factor (VEGF) family and their receptors (VEGFR). Recently, an endogenous soluble VEGFR-2 splice variant (esVEGFR-2) was described (Albuquerque et al. 2009). It does not bind VEGF-A, the hemangiogenic ligand of the membrane-bound variant, but rather VEGF-C, the key inducer of lymphangiogenesis. We investigated esVEGFR2 in neuroblastoma (NB), the most common solid extracranial malignancy of childhood. NB is an embryonic tumour, derived from neural crest descending sympatho-adrenal progenitor cells. In clinical NB staging, infestation of distant lymph nodes is a critical sign, demanding classification into the most advanced stage 4. We present that expression of esVEGFR-2 is lower in the progressed stages 3 and 4. We also found that MYCN amplification, the most adverse prognostic marker in NB, correlates with lower esVEGFR-2 expression. In human embryonic tissue we demonstrate expression in sympathetic ganglia and the adrenal medulla, indicating that esVEGFR-2 contributes to normal development of sympatho-adrenal organs. Examination of esVEGFR-2 in NB cells after treatment with the differentiating agent all-trans retinoic acid (ATRA) reveals that ATRA-induced differentiation enhances esVEGFR-2 expression. Tumor angiogenesis is not only achieved by the up-regulation of pro-angiogenic molecules (VEGF-A, VEGF-C) but also by the down-regulation of inhibitory molecules like esVEGFR-2. Additionally, esVEGFR-2 is associated with normal development of sympatho-adrenal tissues and can be induced in NB cell lines by differentiating ATRA treatment. Therefore sVEGFR-2 may be a potent regulator of lymphangiogensis in both normal development and tumors.

Evidence for a role of hypoxia inducible factor 1 (HIF-1) regulated lysyl oxidase in Staphylococcus aureus abscess-formation

Beerlage, C.[1]*, Greb, J.[1], Kretschmer, D.[2], Trackman P.C.[3], Lieberz, R.[4] , Bonin, M.[5] , Brüne, B.[6] , Peschel, A. [2], Kempf, V.A.J.[1]. [1]Institut für Medizinische Mikrobiologie und Krankenhaushygiene; Universitätsklinikum Frankfurt am Main; Paul Ehrlich Str. 40; 60596 Frankfurt am Main; Germany, [2]Institut für Medizinische Mikrobiologie und Hygiene; Universitätsklinikum Tübingen; Elfriede Aulhorn Str. 6; 72076 Tübingen; Germany, [3]Henry M. Goldman School of Dental Medicine, Boston University; 700 Albany street; MA 02188 Boston; USA, [4]Senckenbergisches Institut für Pathologie; Universitätsklinikum Frankfurt am Main; Theodor-Stern-Kai 7; 60590 Frankfurt am Main; Germany, [5]Institut für Medizinische Genetik; Universitätsklinikum Tübingen; Calwerstr. 7; 72076 Tübingen; Germany, [6]Institut für Biochemie I; Universitätsklinikum Frankfurt am Main;Frankfurt, Germany

HIF-1 is the key transcriptional factor involved in the adaption process of mammals to hypoxia and plays a crucial role in, e.g., cancer angiogenesis. Recent evidence suggests a leading role of HIF-1 in inflammatory and infectious diseases. We demonstrated previously that human pathogenic S. aureus leads to a HIF-1 activation in vitro, ex vivo and in vivo and that this HIF-1 activation is detrimental in a S. aureus peritonitis model. To analyze the role of HIF-1 in S. aureus infections in more detail, we investigated the HIF-1 dependent host cell response upon a S. aureus infection. For this purpose, control and HIF-1 -/- HepG2 cells were infected with S. aureus and gene expression was analyzed four hours later using Affymetrix WT 1.0 gene arrays. Data revealed that expression of in total 228 genes was influenced of which 22 turned out to be regulated via HIF-1. These 22 genes include genes involved in cell metabolism and genes known to affect tumour growth. One of the upregulated genes was lysyl oxidase, (lox), a copper-dependent amine oxidase, which catalyzes the cross linking of collagen and elastin molecules in the extracellular matrix and might be involved in the development of a fibrosis after S. aureus infection. Further experiments revealed that lox is upregulated in vitro and in vivo using HepG2 infection models, abscess-harbouring kidneys of intravenously infected NMRI-mice and tissue samples from patients suffering from microbiological proven S. aureus infections. The role of lox in abscess formation and, moreover, in chronic infections known to result in fibrous scarring of tissues needs to be further elucidated.

A cellular choreography of vascular network formation: Distinct cell activities are coordinated during blood vessel fusion

Lukas Herwig, Anna Lenard, Yannick Blum, Alice Krudewig, Heinz-Georg Belting*, Markus Affolter. Dept. of Cell Biology, Biozentrum/Uni Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

Organ formation requires the coordination of behaviors of individual or groups of cells to organize themselves into proper three-dimensional structures. The vertebrate vascular system consists of a highly ramified and stereotyped network of blood vessels that is formed is by a variety of morphogenetic mechanisms. To dissect the cellular activities that underlie the formation and the connection of blood vessels, we are using transgenic zebrafish reporters that allow us to follow the dynamics of cell junctions of endothelial cells in vivo. By observing the formation of the dorsal longitudinal anastomotic vessel (DLAV) we find, that during initial steps of anastomosis, small junctional complexes, which have formed at the initial cell contact sites, expand into rings. These junctional rings contain luminal pockets that are surrounded by apical membrane compartments, as defined by the localization of the apical protein Podocalyxin-2 (PDXL2). The luminal pockets are then fused by either of two mechanisms, cell membrane invagination or cord hollowing, to generate different types of vascular tubes. (1) During the cord hollowing process, the luminal pockets are brought together via cell rearrangements, resulting in a multicellular tube. (2) Vessel fusion by membrane invagination occurs adjacent to a preexisting lumen in a proximal to distal direction. Here, the invaginating inner (apical) cell membrane extends a „transcellular lumen“ through the endothelial cell, which thus generates a unicellular tube. To test, which parameters determine morphogenetic pathway choice, we have examined the role of blood pressure and found that the transcellular pathway depends on blood flow, while cell rearrangements occur normally. We further analyzed the role of blood flow, by imaging anastomosis events at later stages of development, when angiogenic sprouts are lumenized and exposed to higher levels of blood pressure. In these blood vessels, endothelial cells follow a strict choreography, in which contact formation is followed by transcellular (unicellular) lumen formation. Subsequent cell rearrangements together with a novel cellular mechanism, we term „cell splitting“Â□, converts the unicellular into a multicellular tube. Taken together, these experiments show that endothelial cells can use variable mechanisms during anastomosis. Exposure of the angiogenic sprout to blood pressure, however, generates an environmental constraint, which forces endothelial cells into a certain sequence of morphogenetic activities.

Instrumental role of tenascin-C matrix as scaffold and niche in tumor angiogenesis and metastasis

B. Langlois*, I. Gasser, F. Saupe, Huschluk R.&, C. Spenle, M. van der Heyden, P. Simon-Assmann, V. Djonov, Klaus-Peter Janssen, G. Christofori, G. Orend. INSERM U682, 3 Av. Moliére, Strasbourg; & Department of Medicine, Gross Anatomy and Vascular Biology, University of Fribourg, Fribourg Switzerland, Institute of Biochemistry and Genetics, Department of Biomedicine, University Basel, Switzerland, Department of Surgery, Technische Universität München, Germany

The extracellular matrix molecule tenascin-C (TNC) is a major component of the cancer specific matrix, is prominently expressed in the tumor microenvironment of several human cancer types and plays a promoting role in malignant tumor progression. However, the impact of TNC organization on tumor angiogenesis is poorly understood. Here we show that tumor angiogenesis is largely aberrant upon ectopic TNC expression. By using tumor mice with defined TNC expression levels (knockout, transgenic) in a stochastic neuroendocrine tumor model and in an orthotopic immune-compromised colon cancer xenograft model we analyzed TNC matrix organization. In addition TNC matrix organization was determined in human insulinomas and human colorectal carcinomas. Both in the murine tumor models and in human tumors we observed that TNC rich matrix structures are surrounding blood vessels. Moreover, TNC is also organized into tracks devoid of endothelial cells that eventually form conduits together with laminins, collagens IV and V, fibronectin and other ECM molecules. By FITC-dextran perfusion, immunofluorescence analysis and transmission electron microscopy we provide evidence that TNC conduits are connected to the circulation. They also are enriched by carcinoma associated fibroblasts (CAF) and tumor associated macrophages (TAM) which suggests that TNC matrix conduits might potentially serve as niche and guiding cue for these cells. Our data support a model in which tumor angiogenesis is initiated at multiple sites with a crucial involvement of the ECM and in particular of TNC. The TNC conduits appear to represent a scaffold for angiogenesis and a short distance transport system for cancer cells, CAF and TAM. An alternative matrix-based trafficking route is most likely unaffected by anti-angiogenic drugs and thus potentially could have an impact on anti-angiogenic treatment failure and subsequent enhanced metastasis.

An endogenous vitamin K-dependent mechanism regulates cell proliferation in the brain subventricular stem cell niche. Interplay between the anticoagulant factor, protein S and its homolog Gas6.

Aurore Gely[1,6], Valérie Coronas[1,6], Thomas Harnois[1], Laetitia Prestoz[1], Nathalie Mandairon[2], Anne Didier[2], Jean Marc Berjeaud[3], Arnaud Monvoisin[1], Nicolas Bourmeyster[1,4], Pablo Garciá de Frutos[5], Michel Philippe[1] and Omar Benzakour[1]*. [1]Institut de Physiologie et Biologie Cellulaires - FRE3511 CNRS, Université de Poitiers - 86022 Poitiers Cedex - France, [2]Université Claude Bernard Lyon 1, CNRS - UMR5020 50, LYON cedex 07, France., [3]Equipe de Microbiologie, CNRS-UMR 6008, Université de Poitiers, Poitiers, France., [4]CHU de Poitiers, BP 576, Rue de la Miletrie, 86021 Poitiers, France, [5]Instituto de Investigaciones Biomédicas de Barcelona, IIBB-IDIBAPS-CSIC, Barcelona, Spain. [6]equal contribution

Neural stem cells (NSC) persist in the adult mammalian brain, within the subventricular zone (SVZ). The endogenous mechanisms underpinning SVZ stem and progenitor cell proliferation are not fully elucidated. Vitamin K-dependent proteins (VKDP) are mainly secreted factors that were initially discovered as major regulators of blood coagulation. Warfarin, a widespread anticoagulant, is a vitamin K antagonist that inhibits the production of functional VKDP. We demonstrate that the suppression of functional VKDP production, in vitro, by exposure of SVZ cell cultures to warfarin or in vivo by its intracerebroventricular injection to mice, leads to a substantial increase in SVZ cell proliferation. We identify the anti-coagulant factor, protein S and its structural homolog Gas6, as the two only VKDP produced by SVZ cells and describe the expression and activation pattern of their tyrosine kinase receptors. Both in vitro and in vivo loss of function studies consisting in either Gas6 gene invalidation or in endogenous protein S neutralization, provided evidence for an important novel regulatory role of these two VKDP in the SVZ neurogenic niche. Our study opens up new perspectives for investigating further the role of vitamin K, VKDP and anticoagulants in neural stem cell biology in health and disease. References 1. Gely-Pernot A, Coronas V, Harnois T, Prestoz L, Mandairon N, Didier A, Berjeaud JM, Monvoisin A, Bourmeyster N, de Frutos PG, Philippe M, Benzakour O. Stem Cells. 2012 Jan 30 (in press). 2. Benzakour Omar. Thromb Haemost. 2008 Oct;100(4):527-9.

Aerobic exercise slows breast cancer growth and normalizes tumor vasculature

Allison S. Betof[1]*, Christopher D. Lascola[2], Lee W. Jones[3], Mark W. Dewhirst[1,3]. Departments of [1]Pathology, [2]Radiology, [3]Radiation Oncology Duke University Medical Center Durham, NC, USA

The beneficial effects of exercise on systemic vasculature are well known, including improved endothelial function and increased perfusion. Additionally, recent epidemiological studies suggest that exercise imparts a survival benefit in cancer patients, but the mechanisms by which physical activity affect tumor physiology are poorly understood. In this work, we evaluate the hypothesis that exercise improves the structure and function of tumor blood vessels, resulting in decreased hypoxia, slower tumor growth, and increased therapeutic effectiveness. To investigate the effects of exercise on tumor microenvironment, we injected syngeneic 4T1 breast tumor cells into the mammary fat pad of immune-competent BALB/c mice. The exercise intervention was designed to mimic four clinically-relevant scenarios: 1- patients who are sedentary before and after diagnosis, 2- previously sedentary patients who begin exercising after diagnosis, 3- previously active patients who stop exercising after diagnosis, and 4- previously active patients who continue to exercise after diagnosis. Voluntary wheel running was selected as the most relevant, least stressful model of aerobic exercise. Accordingly, animals in Groups 3 and 4 exercised for nine weeks before tumor cell transplant, and animals in Groups 1 and 2 were sedentary during that time. Immediately after transplant, animals in Groups 2 and 4 were given access to a voluntary running wheel, and animals in Groups 1 and 3 were sedentary. Tumors were allowed to progress for three weeks, and then tumor perfusion was mapped using MRI, followed by removal of the tumor for analysis. In a follow-up experiment, BALB/c mice were immediately implanted with tumor cells and then randomized to running or sedentary conditions with or without cyclophosphamide chemotherapy given one week after tumor transplant (three 100 mg/kg doses, every other day). Tumors were again allowed to progress for three weeks, and MRI was performed prior to tumor removal. Animals voluntarily ran 5-6 km per day prior to transplant and 4-5 km per day after transplant. Body weight was unaffected by exercise. Voluntary wheel running reduced tumor growth rate nearly twofold ($p<0.004$) and significantly increased tumor cell apoptosis ($p<0.05$). Additionally, running after tumor implantation caused significant increases in microvessel density (CD31, $p<0.003$) and vessel maturity (co-localization of CD31 with NG2 and desmin, $p<0.003$). Hypoxia (EF5, $p<0.002$) was significantly reduced in the exercising animals, and MRI showed that tumors were more uniformly perfused in the running groups. To our knowledge, this the first study showing that tumor vascular normalization can be achieved non-pharmacologically. Furthermore, exercise significantly enhanced the effectiveness of cyclophosphamide in slowing tumor growth ($p<0.01$). Taken together, these results suggest that aerobic exercise slows breast tumor growth, improves tumor vessel structure and function, and augments the effectiveness of chemotherapy. This work was supported by the following grants: NIH CA40355, CA142566, CA138634, CA133895, CA125458, and DOD BC093532.

The Rab27/Rab3 effector Synaptotagmin-like protein 4-a (Slp4-a) positively regulates hormone-evoked Weibel-Palade Body exocytosis

Ruben Bierings[1]*, Nicola Hellen[1], Athinoula Meli[2], Robert J. Rowlands[1], Bijal Patel[1], Lindsay Hewlett[1], Laura Knipe[1], Charles McKenna[3], Ambrosius Snijders[4], Marlen Rose[2], Matthew J. Hannah[1] and Tom Carter[1]. [1]MRC National Institute for Medical Research, London, United Kingdom, [2]Imperial College, London, United Kingdom, [3]University of Southern California, Los Angeles, USA, 4MRC Clinical Sciences Centre, London, United Kingdom

Weibel Palade body (WPB) exocytosis underlies hormone-evoked VWF secretion from endothelial cells. The identities of components that positively regulate hormone-evoked WPB exocytosis remain unclear. Here we identify two new components of the WPB; Rab3B and the Rab27\Rab3 effector Slp4-a. Using multiple biochemical and live cell imaging approaches we show that Rab3B is abundantly expressed on WPBs, that Rab3B and Rab27a contribute to recruitment of Slp4-a, and that Slp4-a positively regulates hormone-evoked WPB exocytosis and VWF secretion. siRNA knockdown of Slp4-a strongly inhibited VWF secretion, while over expression of EGFP-Slp4-a increased WPB exocytosis monitored directly in living cells. Single or double knockdown (KD) of Rab3B and Rab27a suggest that Slp4-a-Rab27a is the functionally dominant complex mediating positive regulation of VWF secretion. KD of the Rab27a-specific effector MyRIP resulted in a small but significant increase in VWF secretion while over expression of EGFP-MyRIP strongly inhibited WPB exocytosis. Together, these data show that WPBs recruit multiple Rab effectors and that the balance of regulation is tilted in favour of a positive drive towards WPB exocytosis and VWF secretion through recruitment of Slp4-a.

A novel cell population from peripheral blood as surrogate marker for monitoring active tumor angiogenesis

Fagiani E.[1], Bill R.[1]*, Laurent J.[2], Ruegg C.[3], Christofori G.[1]. [1]Department of Biomedicine, University of Basel, Basel, Switzerland, [2]Division of Experimental Oncology (DEO), Centre Pluridisciplinaire d'Oncologie (CePO), Centre Hospitalier Universitaire Vaudois (CHUV) and Faculty of Biology and Medicine, University of Lausanne (UNIL), Lausanne, [3]Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland

New strategies to suppress early invasion and metastasis and to identify reliable quantitative surrogate markers of tumor angiogenesis are to be developed to monitor the response to anti-angiogenic therapies. Towards these goals, we employed mouse models of tumor angiogenesis, such as Rip1Tag2 (RT2), MMTV-PyMT and injection of syngeneic TRAMPC1 cells in C57Bl/6 mice in order to identify a potential correlation between the extent and activity of tumor angiogenesis and the levels of circulating bone marrow-derived cell populations. Flow cytometry analyses of different combinations of cell surface markers known to define certain subsets of bone marrow-derived cells, were performed on mononuclear peripheral blood cells (PBMNCs) from above mentioned tumor mice and healthy C57Bl/6 mice. We identified the following cell populations that were increased in their levels by the presence of active tumor angiogenesis: CD45dim/VR1-, CD45dim/VR1-/CD31low. To investigate whether these cell populations are indeed good candidates as surrogate markers for tumor angiogenesis, tumor and healthy mice were treated with anti-angiogenic drugs blocking VEGF. The treatments did not affect levels of cell populations in healthy mice. In all tumor mice, we could observe a decreased microvessel density and a consistent reduction of CD45dim/VR1- and CD45dim/VR1-/CD31low cell population levels after short-term (five days) treatments. In contrast to these results, in the long-term (ten days or three weeks) treated RT2 mice, the levels of the cell populations investigated remained unchanged or rather increased. However, tumor volume and microvessel density were significantly reduced. In literature, it has been shown that repression of VEGF/VEGF receptor signaling leads to the activation of signaling pathways induced by angiogenic factors other than VEGFs, therefore, we also interfered with the function of VEGF, FGF, PDGF-mediated signaling. A reasonable decrease in tumor volume and in microvessel density was obtained after all treatments as well as a reduction in CD45dim/VR1- and CD45dim/VR1-/CD31low cell population levels. In order to characterize the cell population identified, CD45dim/VR1-/CD31low cells were FACS sorted and a gene expression array was performed, which is validated by RT-qPCR. In conclusion VEGF, FGF and PDGF could play a role in the mobilization of CD45dim/VR1- and CD45dim/VR1-/CD31low from bone marrow during active tumor angiogenesis. CD45dim/VR1-/CD31low is a sub-population of CD45dim/VR1- and is currently classified based on the results of the gene expression array performed on this cell subpopulation.

The transcription factor Erg is essential for embryonic vascular development and postnatal retinal angiogenesis

Graeme M. Birdsey[1]*, Irene Aspalter[2], Aarti V. Shah[1], Holger Gerhardt[2], Ralf H. Adams[3], and Anna M. Randi[1]. [1]NHLI Vascular Sciences, Imperial College London, London, UK; [2]London Research Institute, Cancer Research UK, London, UK, [3]Max Planck Institute for Molecular Biomedicine, Muenster, Germany

The endothelial transcription factor Erg plays an important role in endothelial homeostasis and angiogenesis by regulating many endothelial cell functions such as survival, junction stability and migration. In order to further define a role for Erg in endothelial homeostasis and angiogenesis, we have used Cre/loxP technology to generate endothelial-specific Erg deletion mouse transgenic models. Mice carrying the Erg gene flanked by loxP sites (Ergfl/fl) were crossed with mice expressing constitutively active Cre recombinase under the control of the Tie2 promoter (Tie2Cre). Tie2Cre-Ergfl/fl mice are embryonic lethal at mid gestation, whilst the Tie2Cre-Ergfl/+ mice are viable and survive into adulthood. Examination of the yolk sac from Tie2Cre-Ergfl/fl mice at embryonic age (E)10.5 reveals an absence of blood flow and decreased vascularisation, suggesting disruption of yolk sac angiogenesis. Endomucin staining of the endothelium in Tie2Cre-Ergfl/fl embryos shows a generalised decrease in vascular complexity accompanied by premature termination of vessel branches. In order to investigate the role of Erg in postnatal angiogenesis, we studied retinal vascularisation using inducible endothelial-specific deletion of Erg by crossing mice carrying the Erg floxed gene with mice expressing tamoxifen-inducible Cre recombinase under the control of the VE-cadherin promoter (Cdh5(PAC)-CreERT2). The ability of this Cre line to recombine the Erg floxed allele leading to deletion of Erg in the endothelium was confirmed by western blotting. Following tamoxifen treatment, the resulting EC-specific (ErgiECKO) mutant mice showed a decrease in the extent of the retinal vascular plexus, decreased numbers of tip cells at the angiogenic front and a decrease in branch complexity at postnatal day 6 (P6). Staining for isolectin-B4 and collagen IV in mutant retinas revealed an increase in the number of empty collagen IV sleeves, indicating an increase in vascular remodelling following deletion of Erg. Thus, we show for the first time in vivo that endothelial deletion of Erg is embryonic lethal due to defects in vascular development and angiogenesis, and that inducible deletion of Erg inhibits angiogenesis during postnatal retinal vascularisation.

HDL antagonizes the defects in the lymphatic system that contribute to the pathogenesis of Rheumatoid Arthritis

Radjesh J. Bisoendial[1,2]*, Wolfgang Weninger[3,4], Francine Petrides[1], Fatiha Tabet[1], Paul-Peter Tak[2,5], Kerry Anne Rye[1]. [1]Lipid Research Group, Heart Research Institute, Newtown, NSW, Australia, [2]Department of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands, [3]Depts. of Dermatology, Royal Prince Alfred Hospital, Camperdown, NSW, Australlia, [4]The Centenary Institute, Newtown, NSW, Australia, [5]Glaxo-Smith Kline, Brentford, London, United Kingdom

Rheumatoid arthritis (RA) is a common inflammatory erosive arthropathy that increases cardiovascular (CV) risk two-fold. Lymphatic dysfunction, a hallmark of chronic inflammation, is considered an attractive target to resolve synovial inflammation. Given the strong vascular anti-inflammatory properties of high-density lipoprotein (HDL) and its key apolipoprotein (apo)A-I, we hypothesized that they may promote inflammation resolution in RA by improving lymphatic endothelial function via up-regulation of the homeobox-containing transcription factor, Prox-1,a key regulator of inflammation-induced lymphangiogenesis (IIL). Primary human dermal lymphatic endothelial cells (LEC) were treated for 3 days with TNFα(10 ng/mL) or pre-incubated with apoA-I (1.2 mg/mL) for 24 h, followed by TNFαexposure for 3 days. Prox1 mRNA levels were quantified by qPCR. For the tube formation assay, LECs were seeded into a pre-coated Matrigel 24-well plate, and treated with TNFαfor 6 h or pre-incubated with apoA-I for 24 h, followed by TNFαexposure for 6 h. Mouse thoracic ducts (TD) were isolated from 2-4 month old C57Bl/6 mice for studying lymphatic vessel (LV) sprouting. TD rings (1 mm) were implanted into a 3-D culture system containing Matrigel under hypoxic conditions and either treated with TNFα, or pre-incubated for 24 h with apoA-I then exposed to TNFα. Incubation with TNFαdecreased LEC Prox1 mRNA levels by about 56% ($P<0.05$). Prior exposure of LECs to apoA-I blocked TNFα-mediated Prox-1 suppression ($P<0.05$). TNFαalso reduced LEC tube formation by about 55% ($p<0.05$) and completely inhibited LV sprouting from TD rings ($p<0.05$). ApoA-I protected against TNFα-mediated inhibition of LEC tube formation (44.7±8.1 versus 81.9±15.0 % of control; $P<0.05$) and the inhibitory effect of TNFαon LV sprouting ($P<0.05$). These results suggest that apoA-I directly regulates IIL by upregulating Prox1 and protecting against TNFα-mediated restriction of lymphatic sprouting and growth in vitro. Hence, raising HDL may provide dual therapeutic benefits in RA by targeting inflammation and reducing cardiovascular risk. New classes of HDL-raising drugs will allow us to study the long term effects of increasing HDL levels on RA progression and CV risk.

Effects of ezetimibe on alpha linolenic fatty acid absorption in cardiac patients

DP Blackwood[1,2]*, RK La Vallée[1], A Al-Busaidi[2], DS Jassal[1,2], GN Pierce[1,2]. [1]Institute of Cardiovascular Science, St. Boniface Research Centre, Winnipeg, Canada, [2]Faculty of Medicine, University of Manitoba, Winnipeg, Canada

Vascular disease is a leading cause of mortality worldwide. Atherosclerosis is a progressive inflammatory disease which greatly contributes to the development of severe vascular disease. Alpha linolenic acid (ALA), an essential omega-3 polyunsaturated fatty acid, possesses anti-atherogenic effects. The presence of elevated ALA in circulation may be an important benefit to overall cardiovascular health. Past studies have demonstrated that the bioavailability of essential fatty acids is enhanced in the presence of a cholesterol supplemented diet. It follows, therefore, that cholesterol-lowering drugs may inhibit fatty acid absorption. The purpose of the study was to determine if the pharmaceutical ezetimibe, a cholesterol absorption inhibitor, will decrease circulating levels of ALA. Ezetimibe selectively inhibits the Niemann-Pick C1-Like 1 receptor which is responsible for sterol transport across the intestinal brush border. Human subjects between 44-80 years old, requiring statin therapy to regulate blood cholesterol levels, were randomly assigned to one of four groups for a 6 week trial: 1) control; 2) ezetimibe therapy; 3) a supplement of flaxseed oil (containing 1.0g ALA); or 4) ezetimibe and flaxseed oil supplementation. There were no significant differences amongst the groups in terms of circulating total cholesterol, LDL, HDL, triglyceride, DHA or EPA levels. Immunoassay data was also analyzed for hs-CRP and other cardiovascular biomarkers. A significant increase in circulating ALA levels was observed in patients ingesting flaxseed oil as compared to control and all other treatment arms containing ezetimibe. Patients treated with ezetimibe did not increase their circulating ALA levels even in the presence of flax oil supplementation. Our data demonstrates that ezetimbe therapy attenuates the absorption of cardioprotective ALA. Patients undergoing ezetimibe therapy, therefore, will not benefit from omega-3 fatty acid supplementation. This study identifies a potentially serious negative drug-diet interaction. Supported by the Canadian Institutes for Health Research and St Boniface Hospital Foundation.

Nuclear targeting of apelin in vascular smooth muscle cells: Implications in cell proliferation and atherosclerotic plaque formation?

Chiraz Chaabane[1], Cécile Brun[2], Angela Roatti[2], Stéphane König[2], Yves Audigier[3], Alex J Baertschi[2], Marie-Luce Bochaton-Piallat[1]*. [1]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [2]Department of Neuroscience, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [3]Unité U-589 INSERM, I2MR, Cancer Department, Toulouse, France. C. Chaabane and C. Brun contributed equally to this work as first author. AJ Baertschi and ML Bochaton-Piallat contributed equally to this work as last author

Background: The main circulatory form of Apelin is a highly conserved 13 amino acid hypotensive peptide derived from a 77 amino acid prepropeptide. The very low plasma levels suggest autocrine, paracrine and possibly intracrine functions of Apelin. It is synthesized in smooth muscle cells (SMCs), and we found a nuclear expression in rhomboid type (R), poorly differentiated SMCs. The biological features of R-SMCs (i.e. enhanced proliferative and migratory activities) explain their capacity to accumulate in the intima. Objectives: Investigate the role of intracrine Apelin in SMC phenotypic change, a process characteristic of atherosclerotic plaque formation Methods and Results: As shown in our previous work, a 3'-shift in translation can lead to N-truncated peptides with new address sequences (FASEB J. 20(6): 732-4, 2006). P-SORT software analysis of preproApelin sequence suggests that N-terminal truncated Apelin may target the nucleus, and we confirm this in many cell types by overexpression of mutated preproApelin-EGFP and preproApelin-His-tag. Transfection of mutated preproApelin-His-tag encoding plasmid in differentiated spindle-shaped (S) SMCs induces a transition towards a R-phenotype associated with increased proliferative activity, as well as downregulation of SMC differentiation markers (i.e. ï□¡-smooth muscle actin) and increased nuclear expression of S100A4 (a marker typical of R-SMCs). In contrast, transfection of S-SMCs with wild type preproApelin-His-tag encoding plasmid does not induce nuclear targeting of Apelin or S100A4, and does not change the S-phenotype. Stimulation of S-SMCs with PDGF-BB also induces nuclear targeting of both Apelin and S100A4 and transition to the R-phenotype. In vivo, Apelin is expressed in SMC nuclei of stent-induced intimal thickening, while its expression in SMCs of the media is mainly cytoplasmic. Conclusions: Altogether, theses preliminary studies provide evidence for nuclear targeting of Apelin in SMCs and suggest actions on gene expression, proliferation, and differentiation. The pathophysiological consequences of this retargeting could be of importance in the understanding of artherosclerosis.

Oxidized phospholipids (OxPLs) induce production of stem cell factor (SCF) and activation of its receptor c-KIT in endothelial cells (ECs)

Taras Afonyushkin, Markus Binder, Olga V. Oskolkova, Valery N. Bochkov*. Department of Vascular Biology and Thrombosis Research, Centre for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria

OxPLs that accumulate in atherosclerotic vessels are increasingly recognized as pathogenic factors in cardiovascular disease. We found that OxPLs stimulated in cultured venous and arterial endothelial cells (ECs) expression of mRNA encoding for SCF. SCF is a pleiotropic cytokine inducing a variety of biological effects, and in particular those related to endothelial homeostasis, e.g. stimulation of ECs migration and proliferation, protection of ECs from apoptosis, and recruitment of circulating progenitor cells. OxPLs upregulated two mRNA isoforms encoding for soluble and membrane-bound SCF. Treatment of ECs with OxPLs upregulated levels of soluble SCF in cell culture medium. Furthermore, conditioned medium from OxPLs-treated ECs stimulated phosphorylation of SCF receptor, c-KIT. In addition to OxPLs, SCF was induced by electrophilic agents such as OxLDL and iso-prostaglandin A2. Electrophilic stress response played mechanistic role in SCF upregulation since siRNA against NRF2 suppressed induction of SCF by OxPLs. We found that OxPLs treatment promoted survival of ECs under conditions of serum starvation; this protective effect was blocked by c-KIT inhibitor imatinib. Our data allow hypothesizing that OxPL-induced SCF may promote ECs survival in the lesion under conditions of atherogenic cellular stress and induce endothelial repair via mobilization of circulating progenitor cells. These responses may compensate for toxic action of OxPLs and other deleterious agents present in the plaque.

A chronic inflammation - the main trigger for cardiovascular diseases?

Franziska Bollmann[1]*, Zhixiong Wu[2], Matthias Oelze[3], Daniel Siuda[1], Andreas Daiber[3], Hartmut Kleinert[2], Huige Li[2], Andrea Pautz[2]. [1]Center of Thrombosis and Hemostasis (CTH), University Medical Center Mainz, Germany, [2]Department of Pharmacology, Medical Center of the Johannes Gutenberg University, Mainz, Germany, [3]2nd Medical Clinic, Department of Cardiology, Medical Center of the Johannes Gutenberg University, Mainz, Germany

Enhanced atherosclerotic and thrombotic events during the cause of chronic inflammatory autoimmune diseases like rheumatoid arthritis (RA) result in increased cardiovascular disorders and higher mortality rates. It has been assumed that abnormal expression of pro-inflammatory factors in RA patients is one of the major reasons leading to this augmented cardiovascular risk. Tristetraprolin (TTP) is a RNA-binding protein, known to be an important regulator of pro-inflammatory gene expression. It binds to the 3'-untranslated region of the mRNA of many pro-inflammatory genes and thereby initiates the degradation of the transcript. Well known TTP targets are Mip1-a or TNF-a. Mice deficient in the TTP gene develop severe chronic inflammation and arthritis due to the overexpression of many inflammatory genes. In this report we analysed the relationship between chronic inflammation and the development of cardiovascular diseases, using the TTP knock out model. In this model we detected the upregulation of „classical“ TTP targets as well as increased mRNA levels of atherosclerotic and thrombotic marker genes, such as osteopontin, cathepsin S, tissue factor, and von Willebrand factor. Those results suggest the relationship between a chronic inflammatory status, due to a TTP-deficiency, and cardiovascular diseases like atherosclerosis. The deficiency in the TTP gene led to a massive, cholesterol-independent endothelial dysfunction with increased formation of reactive oxygen and nitrogen species in the heart and blood of the mice. Levels of asymmetric dimethylarginine, considered to be a cardiovascular risk marker gene, were not increased in mice lacking TTP. In addition to this atherosclerotic phenotype the TTP knock out mice expand other cardiovascular disorders. In tail vein bleeding assays we observed significantly longer bleeding times of TTP deficient mice, likely triggered by a stronger granulopoeisis due to the TTP knock out. Due to our results, we hypothesize that the chronic inflammation in RA patients has a higher importance for the development of cardiovascular diseases than traditional risk factors.

Characterization of a distinct population of circulating human colony forming endothelial progenitor cells

Sarah L Appleby[1,2], Jyotsna Pippal[1,2], Michaelia P Cockshell[1,2], Emma Thompson[1,2,3], Gert Talbo[2], Angel Lopez[1,2,3] and Claudine S Bonder[1,2,3]*. [1]Centre for Cancer Biology, SA Pathology, Adelaide, South AUSTRALIA, [2]Co-operative Research Centre for Biomarker Translation, LaTrobe University, Melbourne, Victoria, Australia, [3]School of Medicine, University of Adelaide, Adelaide, South Australia

The identification of progenitor cells with pro-angiogenic function in adult peripheral blood has significant clinical implications for the treatment of cancer whilst on the other hand offer much promise in therapeutic revascularization in post-occlusion intervention in cardiovascular disease. However, the effects of these progenitors in humans have been less robust and much more variable than in preclinical rodent studies. Considering the necessity to better understand circulating colony forming endothelial progenitor cells (CF-EPCs), we isolated a human umbilical cord blood non-adherent CD133+ progenitor cell population, enriched for CF-EPCs over 4 days and compared their genomic and proteomic profile against donor matched human umbilical vein endothelial cells (HUVEC). CF-EPCs expressed the hematopoietic progenitor cell markers (CD133, CD34, CD117, CD90 and CD38) together with mature endothelial cell markers (VEGFR2, CD144 and CD31). These cells also expressed low levels of CD45 but did not express the lymphoid markers (CD3, CD4, CD8) or myeloid markers (CD11b and CD14). Functional studies demonstrated the key features to be expected from CF-EPCs , that is (i) bound Ulex europaeus lectin, (ii) demonstrated acetylated-low density lipoprotein uptake, (iii) increased vascular cell adhesion molecule (VCAM-1) surface expression in response to TNF, and (iv) in co-culture with mature endothelial cells increased the total number of tubes formed, the number of branches and the number of loops in a 3-dimensional matrix. More importantly, when placed in a Matrigel plug in NOD-SCID mice, these CF-EPCs incorporated into the vasculature and expressed CD144. Proteomic profiling using tandem mass spectrometry identified intercellular adhesion molecule (ICAM)-3 on the CF-EPCs as well as freshly isolated CD133+CD117+ progenitor cells but not HUVEC. Moreover, functional analysis demonstrated that ICAM-3 mediated the rolling and adhesive events of CF-EPCs under shear stress. We suggest that the distinct population of CF-EPCs identified and characterized represents a new valuable therapeutic target to control aberrant vasculogenesis during disease.

Notch signaling induces extracellular matrix protein expression in endothelial cells through repression of microRNA-29b

Reinier A. Boon*, Guillaume Carmona, Katherina R. Keller, Susanne Heydt, Virginia Guarani, Michael Potente, Stefanie Dimmeler. Institute of Cardiovascular Regeneration, Center for Molecular Medicine, Goethe University, Frankfurt, Germany

Notch signaling plays a critical role in the development, homeostasis and maturation of the vascular system. MicroRNAs (miRs) are non-coding RNAs of about 22 nucleotides that regulate gene expression in many biological processes such as angiogenesis. However, the contribution of miRs to Notch signaling in blood vessels is unclear. To test whether Notch signaling alters the expression of miRs, miR expression patterns were determined by microarray in endothelial cells (EC) stimulated with immobilized Dll4 proteins to activate Notch signaling. Among 116 individual miRs reliably detected by microarray, 25 miRs were significantly regulated by Notch signaling. Specifically, 15 miRs were upregulated (e.g. miR-874, miR-20b and miR-23b) and 10 were down-regulated (e.g. miR-29b, miR-494, miR-29a and miR-27a) after 36 hours culture of EC on recombinant Dll4. We focused our attention on miR-29b, which was rapidly and profoundly down-regulated by Notch activation (6 hours: 34±26%, 36 hours: 58±19%) in a gamma secretase-dependent manner. Of the other miR-29 family members, miR-29a was also down-regulated, whereas miR-29c was only slightly decreased upon Notch activation. Consistently, overexpression of a constitutively active form of Notch1 (NICD1) decreased the expression of mir-29b in endothelial cells (by 49±11%). Since Notch is a critical regulator of endothelial proliferation, matrix remodeling and vessel maturation, we investigated the effect of miR-29 on EC proliferation and expression of matrix proteins. Whereas overexpression of miR-29 did not affect endothelial proliferation, miR-29 significantly downregulated the extracellular matrix proteins elastin, fibrillin-1 and collagen I and collagen IV. This finding is consistent with the previously reported regulation of extracellular matrix proteins in the heart and vasculature. Moreover, miR-29 overexpression diminished the increase of extracellular matrix proteins induced by Notch signaling (e.g. Elastin expression: Dll4+miR-Control: 7.2±0.07-fold, Dll4+miR-29: 3.1±0.12-fold compared to PBS treated EC). Taken together, our data indicate that several miRs are regulated by Notch signaling in endothelial cells. The Notch-dependent down-regulation of miR-29b thereby contributes to increased matrix protein expression in EC. These data indicate that miR-29 plays a crucial role in regulating matrix composition in response to Notch signaling.

Pericytes from infantile hemangioma display pro-angiogenic properties and dysregulated angiopoietin-dependent vascular maturation

Elisa Boscolo[1]*, John B. Mulliken[2], and Joyce Bischoff[1]. [1]Vascular Biology Program and Department of Surgery, Children's Hospital Boston, Harvard Medical School, MA, USA, [2]Department of Plastic and Oral Surgery, Children's Hospital Boston, MA, USA

Infantile hemangioma (IH) is a rapidly growing tumor affecting newborns. IH is mainly composed of immature endothelial cells and pericytes that proliferate into a disorganized mass of blood vessels that, with the years, spontaneously regress. In this study we characterized the abundant perivascular cells during the proliferating phase of IH. We hypothesized that pericytes in proliferating IH are unable to drive blood vessel maturation. We isolated pericytes from proliferating and involuting hemangioma specimens to understand their differences. We first confirmed the pericytic phenotype with comparison to human normal retinal and placental pericytes. We then injected proliferating and involuting IH pericytes in vivo, in combination with endothelial colony forming cells (ECFC) and analyzed whether those pericytes are involved in the pro-vasculogenic phenotype. Proliferating IH pericytes induced a very high microvascular density compared to involuting IH and retinal pericytes. We thereby analyzed IH pericytes pro-angiogenic characteristics: they are fast proliferative cells, they express high VEGF-A levels, they do not stabilize ECFC growth and migration in vitro. We then measured contractile ability with a silicon substrata-based 'wrinkle assay' and detected lower contractility in proliferating IH pericytes. With an angiogenesis antibody array we detected strong downregulation of Angiopoietin 1 (ANGPT1) in the supernatant of proliferating IH pericytes compared to retina. Expression of ANGPT1 was slightly higher in involuting versus proliferative IH pericytes suggesting the phenotype of these cells is less pro-angiogenic and resembles a normal pericyte.

Adenosine A2B receptor agonism inhibits vascular smooth muscle cell proliferation and intimal hyperplasia in ApoE deficient mice

Ilze Bot[1]*, Henk de Vries[2], Martine Bot[1], Theo J.C. Van Berkel[1], Adriaan P. IJzerman[2], Johan Kuiper[1]. [1]Division of Biopharmaceutics, [2]Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands

The A2B adenosine receptor (A2BR) is highly expressed in macrophages and vascular smooth muscle cells (vSMC) and has been established as an important regulator of inflammation and vascular adhesion. Recently, it has been demonstrated that A2BR deficiency enhances neointimal lesion formation after injury. Therefore, we hypothesize that A2BR agonism may protect against injury-induced intimal hyperplasia. To test this hypothesis, nine week old female ApoE deficient mice were fed a Western-type diet for 1 week, after which the left common carotid artery was denuded using a guide wire. Mice (n=10 per group) were treated daily with either vehicle control or the A2BR agonist BAY 60-6583 (50 µg/mouse), leading to peak plasma concentrations of approximately 1 µg/mL at 2 hours after injection. After 18 days, mice were sacrificed and lesions analyzed. Interestingly, lumen stenosis as defined by the neointima/lumen ratio was inhibited by treatment with the A2BR agonist from 2.1 ± 0.3 in the controls to 1.3 ± 0.2 ($P<0.05$) in treated mice. Total vessel area remained unaffected (controls: 140 ± 8*103 µm2 versus BAY 60-6583: 131 ± 8*103 µm2, P=NS), demonstrating absence of outward remodeling. Collagen content was increased from 10.7 ± 10.9% in the control animals to 17.0 ± 2.0% in the BAY 60-6583 treated mice ($P<0.05$), while macrophage content was unchanged. To determine whether A2BR agonism affects vSMC proliferation, cultured murine vSMCs were stimulated with BAY 60-6583 for 24 hours, after which [3H]Thymidine incorporation was measured. We observed a dose-dependent reduction in vSMC proliferation from 240 ± 11*103 dpm to 111 ± 13*103 dpm at 1 µg/mL BAY 60-6583 ($P<0.001$) and even up to 5 ± 2*103 dpm at a concentration of 10 µg/mL. Within this concentration range BAY 60-6583 did not induce cell death. Furthermore, collagen production by cultured vSMCs as determined using a picrosirius red staining was increased by 20% and 50% at increasing BAY 60-6583 concentrations ($P<0.05$). In conclusion, our data show that activation of the adenosine A2B receptor protects against vascular injury, while also it also enhances plaque stability as indicated by increased collagen content. These outcomes thus point to A2B receptor agonism as a new therapeutic approach in the prevention of restenosis.

Tie2-dependent knockout of alpha6 integrin subunit in mouse reduces angiogenesis

Bouvard, Claire[1]*, De Arcangelis Adèle[2], Dizier Blandine[1], Galy-Fauroux Isabelle[1], Fischer Anne Marie[1], Georges-Labouesse Elisabeth[2], Helley Dominique[1]. [1]UMR S765, INSERM - Université Paris Descartes, Paris Sorbonne Cité, Faculté des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, 75006 Paris, France, [2]IGBMC, 1 rue Laurent Fries, 67404 Illkirch, France

Integrins α6β1 and α6β4 are receptors for laminin, the major component of the basement membrane underlying endothelial cells. In vitro, alpha6 integrin subunit (α6) expression at the surface of endothelial cells and their progenitors (EPCs) is upregulated by proangiogenic growth factors and is crucial for adhesion, migration and pseudotube formation. We thus wanted to study the role of α6 in angiogenesis and vasculogenesis in vivo. Since mice lacking α6 die at birth, we generated a mouse line in which the gene coding for α6 was deleted specifically in Tie2 expressing cells (endothelial cells and subsets of monocytes/macrophages and hematopoietic stem cells), using the cre-lox system. In a model of hindlimb ischemia, Tie2-dependent deletion of α6 reduced the reperfusion of the ischemic limb by 35% ($p<0.01$) and neovessel formation by 30% ($p<0.05$). Concerning the role of α6 in post-ischemic vasculogenesis, we previously showed that α6 was required for the recruitment of EPCs at the site of ischemia. We now found that the deletion of α6 also reduced EPC mobilization from the bone marrow to the bloodstream after ischemia ($p<0.05$). Histological analysis of the ischemic muscles showed that the recruitment of proangiogenic Tie2-expressing macrophages (TEMs) was reduced by 40% ($p<0.001$) when α6 was deleted. Tie2-dependent deletion of α6 also reduced tumor growth by 60% ($p<0.001$) and tumor vascularization in a B16F10 melanoma model. In the Matrigel plug assay, fibroblast growth factor-2 induced vascularization was reduced by 45% in mice lacking endothelial α6 ($p<0.001$). To especially investigate the role of α6 in angiogenesis, aortic rings were embedded in Matrigel or in collagen and cultured ex vivo. In Matrigel, neovessel outgrowth from rings lacking α6 was strongly reduced ($p<0.001$), whereas there was no genotype-dependent difference in collagen. These results suggest that α6 is crucial for post-ischemic revascularization, as it supports the mobilization of EPCs from the bone marrow, the recruitment of Tie2-expressing macrophages at the site of ischemia, and the sprouting of blood vessels from the preexisting mature vasculature. We also demonstrated that α6 is involved in tumor vascularization.

Therapeutic angiogenesis in prevention of chronic heart failure

Sébastien Banquet[1], Elodie Gomez[1], Lionel Nicol[1], Florence Edwards-Lévy[2], Jean-Paul Henry[1], Renhai Cao[3], Damien Schapman[4], Brigitte Dautreaux[1], Francoise Lallemand[1], Fabrice Bauer[1], Yihai Cao[3], Christian Thuillez[1], Paul Mulder[1], Vincent Richard[1], Ebba Brakenhielm[1]*. [1]Inserm U644, Institute for Biomedical Research, Rouen University, France, [2]CNRS UMR 6229, Faculty of Pharmacy, University of Reims-Champagne-Ardenne, France, [3]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, [4]Regional Platform for Cell Imaging (PRIMACEN), European Institute for Peptide Research (IFRMP 23), Rouen University, France

Therapeutic angiogenesis is a promising approach for the treatment of cardiovascular diseases including myocardial infarction and chronic heart failure (CHF). However, current strategies of therapeutic angiogenesis or arteriogenesis in cardiovascular diseases have proven insufficient. We aimed to improve pro-angiogenic therapies by: i) identifying novel arteriogenic growth factor combinations; ii) developing injectable delivery systems for spatiotemporally-controlled growth factor release; and iii) evaluating functional consequences of targeted, intramyocardial growth factor delivery in CHF. First, we observed that Fibroblast Growth Factor (FGF)-2 and Hepatocyte Growth Factor (HGF) synergistically stimulated vascular cell migration and proliferation in vitro. Using two in vivo angiogenesis assays, the mouse corneal model and the mouse matrigel model, we found that the growth factor combination of FGF-2 and HGF resulted in a more potent and durable angiogenic response than either growth factor used alone. Further, we determined that the molecular mechanisms involved potentiation of Akt and MAPK signal transduction pathways, as well as upregulation of the corresponding angiogenic growth factor receptors. Next, we developed injectable crosslinked albumin-alginate microcapsules that sequentially release FGF-2 and HGF for up to six weeks. Finally, we investigated the effects of growth factors used alone or in combination in a rat model of CHF induced by coronary ligation. We found that intramyocardial slow-release of FGF-2 together with HGF, but not growth factors used alone, potently stimulated angiogenesis and arteriogenesis at 1 and 3 months post myocardial infarction, leading to improved cardiac perfusion after 3 months, as shown by MRI. Further, the combined treatment, as well as HGF used alone, prevented cardiac hypertrophy and fibrosis, as determined by immunohistochemistry. These multiple beneficial effects resulted in reduced adverse cardiac remodeling and improved left ventricular function, as revealed by echocardiography. In conclusion, our study provide proof-of-principle that localized sustained delivery of low doses of a specific combination of angiogenic growth factors is sufficient to generate stable and functional blood vessels in the heart. Specifically, our data, showing the selective advantage of dual-delivery of FGF-2 together with HGF, suggests that this growth factor combination may constitute an efficient novel treatment for the treatment of cardiovascular diseases including CHF.

The dual role of the transcription factor Junb in tumor angiogenesis

Jennifer Braun[1]*, Karin Strittmatter[1], Kristin Kiesow[1], Dorde Komljenovic[2], Tobias Bäuerle[2], Peter Angel[1], Marina Schorpp-Kistner[1]. [1]Division of Signal Transduction and Growth Control, German Cancer Research Center (DKFZ), [2]Division of Radiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

The transcription factor AP-1 subunit Junb has been shown to play a pivotal role in angiogenesis. It positively regulates angiogenesis by regulating VEGF in a HIF-independent way as well as MMP-13 through CBF-b. Here we show that ablation of junb in all stromal cells including endothelial cells, vascular smooth muscle cells (vSMC) and fibroblasts lead to a reduction in tumor growth in two different syngenic murine tumor models (B16-F1 melanoma and the lewis lung carcinoma (LLC)). Parallel in vitro analyses revealed that Junb-deficient endothelial cells have a decreased angiogenic activity as shown by a reduced sprouting capacity in a spheroid sprouting assay compared to Junb-positive control cells. On the other hand, Junb-negative mural cells, vSMCs and fibroblast, show a pro-angiogenic phenotype. Spheroid cultures of HUVECs incubated with supernatant of Junb-deficient fibroblasts show an increase in sprouting capacity compared to HUVEC spheroids incubated with supernatant from wild-type cells. Moreover, co-culture of Junb-null endothelial cell spheroids with Junb-deficient vSMCs rescued the angiogenic phenotype of the endothelial cells in the sprouting assay, whereas co-culture with Junb-positive vSMCs failed to do so. Subsequent analysis of the fibroblast supernatant revealed hepatocyte growth factor, HGF, as one pro-angiogenic factor being highly abundant in the supernatant of Junb-negative cells compared to control cells. Taken together, these data confirm that the transcription factor Junb is a key regulator of angiogenesis. Dependent of the cell type Junb acts as a positive or as a negative regulator of angiogenesis. These results imply that we need to unravel the context-dependent role of Junb in different cell types in order to be able to understand its function in tumor angiogenesis and cancer.

Hydrogen peroxide signaling in laminar shear stress.

Rosa Breton-Romero*, Fernando Rodriguez-Pascual and Santiago Lamas. Laboratorio Mixto CSIC-FRIAT de Fisiopatología Vascular y Renal Centro de Biología Molecular "Severo Ochoa" (CBMSO-CSIC) Madrid, Spain

Endothelial cells in the vascular system are constantly subjected to the frictional force of shear stress due to the pulsatile nature of blood flow. Laminar Shear stress (LSS) is considered to be a protective hemodynamic stimulus for cells, being critical in limiting the development of different inflammatory diseases related to the redox state. Several lines of evidence and previous work in our group have shown that laminar flow regulates the production of vascular endothelial reactive oxygen species (ROS). Using the cone/plate model we found that LSS (12dyn/cm2) rapidly promotes an increase in superoxide radical anion and hydrogen peroxide (H2O2). Although high levels of ROS have been described to be toxic and promote atherogenesis, substantial evidence suggests that a transient production of H2O2 behaves as an intracellular messenger. We demonstrated that LSS-mediated H2O2-generation is partially necessary for the sequential activation of p38 MAPK, which leads to an increase in the activity of the endothelial nitric oxide synthase (eNOS), the subsequent nitric oxide production and the protection of endothelial function. LSS is able to modulate the expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPX-1), balancing ROS production. Nevertheless the mechanism by which it restricts oxidative stress under short periods of pulsatile shear stress is still unclear. We have focused our attention on peroxiredoxins (PRXs), which are broadly distributed peroxide scavengers with different subcellular topology. PRXs are exquisitely sensitive to low levels of hydrogen peroxide and use redox-sensitive cysteines to reduce peroxides. Preliminary results in our lab have shown that PRX3 (mainly located in the mitochondria) is potentially regulated by fluid flow, since LSS is able to produce a transient dimerization of this enzyme. These data point to mitochondrial PRX3 as a primordial candidate capable of regulating H2O2 signaling in endothelial cells subjected to short periods of LSS.

Role of the transcriptional repressors Bcl-6/BCoR in angiogenic sprouting and endothelial cell cycle progression

Elisabeth Buchberger, Miriam El Harchi, Dietmar Payrhuber, Anna Zommer, and Christine Brostjan*. Department of Surgery - Medical University of Vienna, Austria

Based on an in vitro model of endothelial - tumor cell interactions, we performed microarray analyses of endothelial cell transcripts following stimulation with colon carcinoma culture supernatant. Real-time PCR analysis confirmed a profound upregulation of the transcriptional repressors Bcl-6 (B-cell lymphoma 6) and BCoR (Bcl-6 interacting co-repressor) in human microvascular endothelial cells (HMEC). While Bcl-6 and BCoR had been described to interact in chromatin remodeling and transcriptional repression of Bcl-6 target genes in lymphocytes and monocytes, their role in endothelial cells was largely unknown. Thus, we set out to elucidate the functional role of Bcl-6/BCoR in endothelial cells, with a particular focus on angiogenesis. We performed a selective gene knock down with siRNAs and studied the effect on HMEC sprouting and proliferation. Angiogenic sprouting was investigated in a three-dimensional in vitro assay with HMEC spheroids embedded in a collagen matrix. Of interest, Bcl-6/BCoR silencing resulted in a pronounced induction of HMEC sprouting. The effect was observed without the addition of pro-angiogenic factors and was comparable in efficiency to stimulation by VEGF. Furthermore, we found Notch target genes to be altered at the transcript level upon Bcl-6/BCoR silencing, indicating a possible involvement of the Notch pathway in Bcl-6/BCoR regulation of endothelial sprouting. With respect to cell proliferation, Bcl-6/BCoR silencing had a negative effect on endothelial cell cycle progression: HMECs were arrested in G0/G1 phase which correlated with an increase in the cell cycle inhibitor p21 and a concomitant decrease of cyclin A, cyclin B1 and cyclin D2 mRNA. In summary, the transcriptional repressors Bcl-6/BCoR were found to regulate EC function by blocking angiogenic sprouting while promoting cell proliferation. Further investigations on the intracellular pathways involved are currently ongoing.

Mutations in eight lymphangiogenic genes explain 20% of primary lymphedema

Pascal Brouillard[1]*, Matthieu Schlögel[1], Antonella Mendola[1], Mustapha Amyere[1], Laurence Boon[1,2], Nicole Revencu[2,3], Miikka Vikkula[1,2,3,4]. [1]Laboratory of Human Molecular Genetics, de Duve Institute, Université catholique de Louvain, Brussels, Belgium, [2]Center for Vascular Anomalies, Cliniques Universitaires St-Luc, Université catholique de Louvain, Brussels, Belgium, [3]Center for Human Genetics, Cliniques Universitaires St-Luc, Université catholique de Louvain, Brussels, Belgium, [4]Walloon Excellence in Lifesciences and Biotechnology (WELBIO), Université catholique de Louvain, Brussels, Belgium

Primary lymphedema can be inherited as an autosomal dominant or recessive trait, with reduced penetrance and variable expression. Both syndromic and non-syndromic cases have been linked to mutations in eight genes with major role in lymphangiogenesis. FLT4 (encoding VEGFR3) and GJC2 (Cx47) are mutated in patients with autosomal dominant, and rarely autosomal recessive, primary congenital lymphedema, and in hydrops fetalis. Mutations in the latter were also recently shown to predispose to secondary lymphedema following breast cancer therapy. Mutations in FOXC2 and SOX18 cause lymphedema-distichiasis and hypotrichosis-lymphedema-telangiectasia syndromes, respectively, which can be inherited or sporadic, and can also be expressed as hydrops fetalis. Mutations in the CCBE1 were identified only in patients with Hennekam syndrome, and PTPN14 was found mutated in rare cases of lymphedema associated with choanal atresia. A few months ago, mutations in GATA2 were shown to cause Emberger syndrome (lymphedema associated with myelodysplasia), and early this year, KIF11 (EG5) mutations were discovered in patients with autosomal dominant or sporadic lymphedema associated with microcephaly and/or chorioretinopathy. We have screened these genes in a large series of sporadic and familial index patients with primary lymphedema (n=270), and available family members. We discovered mutations in about 20% of the patients, including dominant, recessive or de novo mutations. These findings allow precise molecular diagnosis for these primary lymphedema patients, and extend the molecular classification of primary lymphedemas. It also allows the development of more precise follow-up and treatment, needed steps towards personalized medicine. Unraveling these genes also provides clues for the pathogenic mechanisms behind primary lymphedema, eventually elucidating genetic predisposition to secondary lymphedema. Finally, the samples without a mutation in the known genes are extremely valuable for our ongoing genome-wide strategies using massive parallel sequencing to identify novel genes causing lymphedema that are likely to play an important role in development and/or stability of the lymphatic vascular system.

Tie1 controls Ang-1/Tie2 signaling by regulating endothelial cell surface Tie2 presentation

Soniya Savant[1,2]* , Annika Budnik[1,2]*, Andrew Benest[1], Dorothee Terhardt[1], Hellmut Augustin[1,2]. [1]Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), [2]Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim, Heidelberg University (CBTM), Germany

Endothelial cell survival and vascular maturation are controlled by Angiopoietin-Tie signaling. The endothelial cell-specific receptor tyrosine kinase Tie1 is an orphan receptor with minimal kinase activity. As such, it does not activate signaling pathways on its own, but rather appears to modulate the kinase activity of the related receptor Tie2. Tie2 plays a key role in vascular remodeling and maintenance. We and others have observed that shedding of the Tie1 ectodomain upon VEGF stimulation enhances Tie2 signaling. Recent studies have demonstrated dynamic cell surface Tie1-Tie2 complex formation. Furthermore, Tie2 signaling has also been shown to be dependent on its cell surface localization (cell-cell vs. cell-substratum contacts). To shed further light on the contribution of Tie1 to Ang/Tie2 signaling, we spatiotemporally analyzed endothelial cell Tie1 surface presentation and trafficking from and into intracellular pools at high resolution in relation to Tie2 surface presentation. Quantitative flow cytometry, surface turnover and vesicular localization studies of internalized receptors revealed a counterregulatory mechanism of Tie1 vs. Tie2 expression with Tie1 acting as regulator of Tie2 cell surface presentation. At steady state, Tie1 was primarily stored in intracellular pools, whereas Tie2 was preferentially expressed on the cell surface. We consequently silenced Tie1 and analyzed the effects on Tie2 surface presentation and signaling. Silencing of Tie1 had only minimal effects on steady-state Tie2 cell surface expression. Yet, it altered Tie2 surface turnover after ligand stimulation. Upon Ang-1 stimulation, Tie2 was more rapidly endocytosed and Ang-1 induced Tie2 phosphorylation was faster downregulated. To examine if Tie1 is similarly affecting Tie2 surface trafficking and signaling *in vivo*, we next analyzed Tie2 expression in heterozygously Tie1-deficient mice. Corresponding to the cellular data, increased Tie2 protein expression was detectable in embryos and newborn Tie1 heterozygous mouse pups. Taken together, these data suggest that the orphan receptor Tie1 contributes, in a dosage-dependent manner, indirectly to Tie2 signal transduction by controlling its cell surface presentation.

Angiogenesis is challenged in experimental brain metastases

Edina Bugyik*, Vanessza Szabó, Katalin Dezső, Péter Nagy, Sándor Paku. First Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary

It is widely accepted that angiogenesis plays an important role in the maintenance of tumor growth both in primary and secondary brain tumors. Tumors can acquire their vasculature e.g. by vessel sprouting or inducing intussusceptive microvascular growth. These processes require fibronectin and fibrin containing collagenous matrix. Since collagen I is absent in the brain parenchyma with the exception of the wall of arteries, the significance of these types of angiogenesis during vascularization of brain metastases is questionable. In the present study our aim was to analyze the vascularization of experimental metastases. Five different tumor lines were used. Experimental brain metastases were produced by direct injection of the tumor cells to the brain parenchyma of mice. Morphometric analysis was performed on methanol fixed frozen sections following various immunofluorescent labeling. After BrdU and laminin staining, labeling index of the blood vessels within the tumor and within 200μm distance from the periphery of the lesion was determined. No angiogenesis was observed in the peritumoral zone of these lesions. The tumors acquired their vasculature merely by incorporating the host vessels. Vessel density was lower but vessel diameter and vascular cell proliferation was higher within all tumors compared to peritumoral tissue. A negative correlation was found between the number of incorporated vessels and vascular cell proliferation. Tumors of epithelial origin showing pushing growth pattern had lower vessel density and elevated vascular cell proliferation, compared to tumors showing invasive growth. Incorporated vessels retained their normal structure with the exception of astrocyte foot processes replaced by the tumor cells. Attachment to the vascular basement membrane led to the differentiation of the invading breast cancer cells. A process remarkably similar to intussusceptive angiogenesis was observed in the brain metastases derived from fibrosarcoma cell line. Tumor cells attached to the vessel caused the vessel lumen to split, and the pillars formed were filled by tumor cells. However, branching angiogenesis was observed neither in the tumorous lesions nor in the control cerebral wounds. These data suggest that under experimental conditions no sprouting angiogenesis is needed for the incipient growth of metastatic cerebral tumors. According to our observations metastases acquire their vasculature exclusively by vessel incorporation in the mouse brain. This phenomenon may also be valid for small metastases in the human brain. Under these conditions the use of antiangiogenic agents may need rethinking.

The effect of smooth muscle cell-monocyte interactions on inflammatory molecules and oxidative stress production

Elena Butoi (Dragomir)[1]*, Ana Maria Gan[1], Ileana Manduteanu[1], Daniela Stan[1], Manuela Calin[1], Monica Pirvulescu[1], Rory Ryan Koenen[2], Christian Weber[2], Maya Simionescu[1].
[1]Institute of Cellular Biology and Pathology "N. Simionescu" of the Romanian Academy, Bucharest, Romania, [2]Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Munich, Germany

Objective: The initiation and early progression of atherosclerosis results from complex interactions of circulating factors and various cell types in the vessel wall, including endothelial cells, lymphocytes, monocytes and smooth muscle cells (SMC). Although endothelial cells are thought to be the major cell type responsible for interacting with macrophages, increasing evidence suggests that adhesive interactions between migrated monocytes and vascular SMC may contribute to monocyte-macrophage retention within the vasculature. We questioned whether the interaction of monocytes with SMC contributes to production of inflammatory molecules and oxidative stress. Methods and results: SMC were co-cultured with monocytes or LPS-activated monocytes (18 h) and then the cells were separated and individually investigated for the gene and protein expression of TNFα, IL-1β, IL-6, CX3CR1 and metalloproteinases (MMP-2, MMP-9). We found that SMC-monocyte interaction induced, in each cell type, an increased mRNA and protein expression of TNFα, IL-1β, IL-6, CX3CR1, MMP-2 andMMP-9. Blocking the binding of fractalkine to CX3CR1 (by pre-incubation of monocytes with anti-CX3CR1 or by CX3CR1 siRNA transfection) before cell co-culture decreased the production of TNFα, CX3CR1 and MMP-9. Monocyte-SMC interaction induced the phosphorylation of p38MAPK and activation of AP-1 transcription factor. Silencing the p65 (NF-kB subunit) inhibited the IL-1β and IL-6 and silencing c-jun inhibited the TNFα, CX3CR1 and MMP-9 induced by SMC-monocyte interaction. The monocyte-SMC interaction increases the reactive oxygen species in both cell types. Moreover, the cell co-culture increased the NOX1 (in SMC) and NOX2 (in monocytes), by a mechanism dependent by STAT3 transcription factor. Conclusions: The cross-talk between SMC and monocytes/LPS-activated monocytes augments the inflammatory response in both cell types as revealed by the increased expression of TNFα, IL-1β, IL-6, CX3CR1 and MMPs. The oxidative stress is also induced upon SMC-monocyte interaction. Up-regulation of TNFα, CX3CR1 and MMP-9 induced by interaction of SMC with monocytes is dependent on fractalkine/CXRCR1 pair. Inflammatory molecules and reactive oxygen species produced locally as a result of the monocyte-SMC adhesive interactions may have an important part in the pathogenesis of atherosclerosis.

Imaging the angiogenic activity in very small breast cancer xenografts by ultrasound molecular imaging of VEGFR-2 with BR55 microbubbles

Jessica Bzyl[1]*, Moritz Palmowski[1,2], Anne Rix[1], Susanne Arns[1], Isabelle Tardy[3], Sibylle Pochon[3], Josef Ehling[1,4], Simone Schrading[5], Fabian Kiessling[1], Wiltrud Lederle[1].
[1]Institute for Experimental Molecular Imaging, RWTH-Aachen University, Aachen, Germany, [2]Department of Nuclear Medicine, RWTH-Aachen University, Aachen, Germany, [3]Bracco Research SA, Geneva, Switzerland, [4]Institute of Pathology, RWTH-Aachen University, Aachen, Germany. [5]Department of Diagnostic and Interventional Radiology, RWTH-Aachen University, Aachen, Germany

Rationale: Molecular ultrasound with microbubbles targeting molecular markers expressed on the activated endothelium is a well established method for imaging angiogenic activity of tumours of well discernable size in animals. In this study, we utilized molecular ultrasound targeting the VEGFR-2 in differently small sized breast tumours to determine down to which tumour size there is a high VEGFR-2 expression, which generally accompanies the onset of angiogenesis. This would show if molecular ultrasound could possibly be utilised for the detection and characterisation of micro-lesions in breast cancer patients, being of great advantage for the clinical routine because the detection of small lesions or micro-metastases still poses many problems. Methods: MCF-7 breast cancer xenografts were orthotopically implanted in mice. Tumours of sizes as small as 4 mm³ (2 mm in diameter), 14 mm³, 34 mm³ and 65 mm³ (5 mm in diameter) were examined with high-frequency 3D molecular ultrasound using clinically translatable VEGFR-2 targeted microbubbles (BR55). Additionally, the relative tumour blood volume (rBV) was assessed with non-targeted microbubbles (BR38). Data validation included quantitative immunofluorescence staining for vessel density (CD31), VEGFR-2 expression, vessel maturation (α-SMA) and the determination of the vessels' lumina size. Results: This tumour model has a high VEGFR-2 expression at a very small size (4 mm³), which was evident by a high binding of VEGFR-2 -targeted BR55 microbubbles. Significantly less microbubble binding was already detected in 14 mm³ sized tumours ($p<0.05$). In tumours of larger sizes than 14 mm3, the amount of VEGFR-2 bound microbubbles remained at the same lower level. The rBV was comparable at all tumour sizes. Both findings were confirmed by immunofluorescence staining for the vessel density and the VEGFR-2 expression. The MCF-7 tumours of 4 mm³ have most likely just undergone the onset of angiogenesis, leading to a strong up regulation of VEGFR-2 and a strong angiogenic response. With ongoing tumour growth, the VEGFR-2 expression is obviously down regulated. This could be explained by a highly significantly enhanced number of enlarged and more mature vessels, that was histologically detected with increasing tumour size ($p<0.01$). Thus, by supplementing the ultrasound data with further quantitative immunohistological analyses we could validate and explain the observations made by ultrasound and confirm the great value and accuracy of molecular ultrasound targeting the VEGFR-2 for imaging the angiogenic activity also in very small lesions. Conclusion: 3D molecular ultrasound utilising BR55 microbubbles accurately depicts the early angiogenic response of very small breast lesions and the angiogenic activity during tumour growth. Thus, molecular ultrasound targeting the VEGFR-2 may be a good candidate for the detection and characterisation of micro-lesions in patients.

RNase1 protects against cardiac ischemia/reperfusion injury

Cabrera-Fuentes H.A.[1,4]*, Sanchez J.A.[2], Fischer S[1], Ilinskaya O.N.[4], Schlüter K.D.[3], Preissner K.T.[1]. [1]Justus-Liebig University, Institute for Biochemistry, Medical Faculty, Giessen, Germany, [2]Hospital Universitari Vall d'Hebron, Servicio de Cardiologia, Barcelona, Spain, [3]Justus-Liebig University, Institute of Physiology, Medical Faculty, Giessen, Germany, [4]Kazan Federal University, Department of Microbiology, Kazan, Russian Federation

Extracellular RNA (eRNA), exposed after tissue trauma, ischemia or damage, has been shown to exert prothrombotic and hyperpermeability-inducing functions, which are prevented by Ribonuclease1 (RNase1) treatment in vivo. Following ischemia and myocardial necrosis during reperfusion, the presence of eRNA (as cofactor for cytokines and coagulation proteases) might potentiate the development of rigor contracture. Here, the contribution of the eRNA/RNase1 system in ischemia/reperfusion (I/R) injury was investigated in isolated rat hearts in a Langendorff system. Lactate dehydrogenase (LDH) release, a marker of cell damage/necrosis, as well as eRNA and RNase-activity were determined in the perfusate before and during reperfusion (120 min) following 45 min of ischemia. To study the influence of RNase1 on physiological parameters, left ventricle (LV) pressure was continuously recorded. RNase1 was added in different concentrations to the perfusion buffer, starting 3 min before the ischemic phase and maintained for the whole duration of the experiment. In the initial period of reperfusion (following the ischemia phase) there was a sharp increase in LDH release (32.83 ± 0.3 U/g dry tissue), a prominent initial peak of eRNA (52.9 ± 3.3 ng/ml) followed by a prolonged high level of eRNA between 15 and 60 min of reperfusion. Only very low endogenous RNase1-activity was found in the perfusate. Treatment with RNase1 in a concentration-dependent manner induced a lower and delayed increase in diastolic pressure during ischemia, indicating a less severe rigor contracture. In addition, functional recovery of heart tissue after 30 min reperfusion was preserved as indicated by elevated increase of LV developed pressure (I/R: 53 ± 5 % vs. baseline; RNase1-treatment: 83 ± 13 % vs. baseline; p=0.03). Finally, RNase1 reduced the severity of the maximal hypercontracture (I/R: 67 ± 8 mmHg; RNase1-treatment: 19 ± 3 mmHg; p=0.05) during the initial reperfusion phase and prevented the initial LDH release (16.31 ± 2 U/g dry tissue, 30 min after reperfusion), indicating less myocardial damage and protection against necrosis. Together, eRNA is released from the rat heart during I/R and may contribute to the outcome of cardiac injury. RNase1 intervention appears be a new potential therapeutic regimen against cardiac I/R injury, whereby the underlying mechanisms deserve further investigation.

Extracellular RNA-mediated induction of inflammatory cytokines in macrophages during atherogenesis: The contribution of sialoadhesin-1

Hector A. Cabrera-Fuentes[1]*, Svenja Meiler[2], Silvia Fischer[1], Yvonne Baumer[2], Klaus T. Preissner[1], William A. Boisvert[2]. [1]Institute for Biochemistry, Medical School, Justus-Liebig University, Giessen, Germany, [2]Center for Cardiovascular Research, John A. Burns School of Medicine, University of Hawaii, Honolulu, USA

Extracellular RNA was previously shown by our group to exert prothrombotic and inflammatory properties in the vasculature, involving a cofactor function in protease auto-activation and cytokine mobilization. Administration of RNase1 in different in vivo models of thrombosis and stroke indicated a potent antithrombotic and anti-inflammatory property of this nuclease, which (besides its exocrine pancreatic origin) is produced and secreted mainly by vascular endothelial cells. Here, the role of the extracellular RNA/RNase system in the progression of atherogenesis was studied in LDL-receptor knockout mice as well as in bone-marrow-derived macrophages (BMDM) from wild-type and sialoadhesion-1-deficient mice. Sialoadhesin-1 is known to mediate cell-cell interactions in the context of atherogenesis through the recognition of sialylated glycoconjugates and may contribute to the progression of atherothrombosis. Using an RNA-binding fluorescence dye the presence of extracellular RNA in atherosclerotic lesions from LDL-receptor knockout mice was demonstrated by confocal microscopy. During the development of atherosclerosis between weeks 4 and 36 of high fat diet (HFD) feeding, extracellular RNA was found in association with macrophages and other damaged cells within the lesions in a time-progressive fashion. Concomitantly, RNase activity in plasma samples from these mice indicated a biphasic characteristic with a temporary increase during the first two weeks, followed by a significant decrease to about 20% of control after 8 weeks of HFD regimen which correlated well with the presence of extracellular RNA in the necrotic core of the atherosclerotic lesions. The response of BMDM towards extracellular RNA was studied in vitro using wild-type and sialoadhesin-1 knockout cells, whereby a 24 h exposure revealed a concentration-dependent upregulation of pro-inflammatory mediators such as arginase-2 (ARG-2), interleukin (IL)-6, Interferon-gamma (IFN-γ) and sialoadhesin-1 but only moderate increase of IL-10 in wild-type cells. In contrast, quantitative gene expression analysis in extracellular RNA-treated sialoadhesin-1 knockout BMDM showed a prominent down-regulation of the pro-inflammatory mediators ARG-2, IL-6 and IFN-γ, while the anti-inflammatory IL-10 was significantly raised. Overall, these results point to a prominent role of extracellular RNA as pro-atherogenic factor, whereby the expression of the pro-inflammatory sialoadhesin-1 on macrophages might play an important role as putative recognition site for ribonucleic acids. These data identify the RNA/RNase1 system as a new pathophysiological contributor in atherogenesis.

Altered expression of calcification regulatory factors in osteoprotegerin deficient mouse aortas and vascular smooth muscle cells

Andrea Callegari*, Kate Deuell, Michael E Rosenfeld and Marta Scatena. Departments of Bioengineering and Pathology, University of Washington, Seattle WA, USA

Osteoprotegerin (OPG) is a secreted TNF receptor-like decoy receptor regulating bone homeostasis and more recently postulated to modulate vascular calcification. We have shown that inactivation of OPG in ApoE-/- mice accelerates the progression of atherosclerotic plaque by increasing lesion size and plaque calcification. OPG may thus act as an athero-protective factor; however the molecular mechanisms mediating OPG effects are unknown. To better understand the mechanisms underlying OPG regulation of vascular calcification, we performed expression analyses of calcification promoters and inhibitors in whole mouse aorta and cultured vascular smooth muscle cells (VSMCs) isolated from ApoE-/-OPG-/- and ApoE-/-OPG+/+, and in response to OPG ligands RANKL and TRAIL. Using quantitative PCR, aortas from ApoE-/-OPG-/- mice expressed higher levels of osteopontin (OPN) and lower levels of matrix gla protein (MGP) when compared to ApoE-/-OPG+/+ aortic tissue. VSMCs derived from aortas of ApoE-/-OPG-/- mice and cultured in vitro showed a similar pattern when compared to VSMCs isolated from ApoE-/-OPG+/+ aortas. When treated with calcification medium containing high phosphate and low serum, ApoE-/-OPG-/- VSMCs calcified more than ApoE-/-OPG+/+ cells. qPCR gene analysis 4 days after treatment with calcification medium resulted again in increased expression of OPN and decreased MGP in ApoE-/-OPG-/- VSMCs. Western blot protein analysis also revealed that VSMCs lacking OPG expressed less of the smooth muscle specific marker, smooth muscle alpha-actin than VSMCs wild type for OPG. This correlated with increased ERK1/2 phosphorylation in VSMCs lacking OPG, suggesting that OPG may regulate SMC differentiation state. To further explain the mechanism that leads to this phenotypic change in VSMCs, we also analyzed the effect of the addition of the OPG ligands RANKL and TRAIL to VSMCs in culture. Addition of either RANKL or TRAIL to ApoE-/-OPG-/- VSMCs in calcification medium further enhanced mineralization. However, addition of RANKL or TRAIL to ApoE-/-OPG+/+ VSMCs did not affect the calcification process. In response to RANKL and TRAIL ApoE-/-OPG-/- VSMCs showed increased expression of OPN, and alkaline phosphatase and reduced expression of MGP. Furthermore, RANKL treatment of ApoE-/-OPG-/- VSMCs resulted in increased Runx2 activity compared to ApoE-/-OPG+/+ VSMCs as measured by luciferase reporter assay. The addition of the anti-apoptotic protein zVAD to RANKL and TRAIL treated ApoE-/-OPG-/- VSMCs indicated that TRAIL induction of calcification might also be due to increased cell apoptosis. In contrast, the effect of RANKL on calcification was not dependent on cell death. We, thus, suggest that in the absence of OPG the RANK/RANKL pathway may activate pro-mineralization processes. Further studies will determine whether re-expression of OPG in ApoE-/-OPG-/- VSMCs is able to normalize gene expression and rescue calcification. This work is supported by funding from NIH RO1HL093469-01

Tumor lymphangiogenesis and immunological tolerance: A role for CD4+ T cells?

Francesca Capotosti[1]*, A.W. Lund[2], S. Hirosue[1], P. Corthesy Henrioud[1], C. Halin[3], S. Hugues[2], and Melody Swartz[1]. [1]Institute of Bioengineering (IBI) and Swiss Institute for Experimental Cancer Research (ISREC), Faculty of Life Sciences, EPFL, Lausanne, [2]Department of Pathology and Immunology, University of Geneva Medical School, Geneva [3]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland

Beside the well-established hypothesis that solid tumors can induce lymphatic growth (lymphangiogenesis) to form an “escape route” for dissemination, it is becoming more and more attractive the hypothesis that these tumor-associated lymphatic vessels may also play an active role in modulating the tumor microenvironment and favor tumor growth. We are now further exploring the hypothesis that tumor lymphangiogenesis may correlate with the development of a tolerogenic microenvironment that is able to suppress the anti-tumor immune response and promote tumor growth. In particular we are focusing on the possibility that lymphatic endothelial cells (LECs) might express in a functional way class II molecules and modulate the CD4+ T cell repertoire to contribute to the tumor-induced immune tolerance. Our data indicate that LECs can express class II molecules and that expression can be modulated under specific conditions. Moreover LECs seem to be able to functionally present a model antigen on class II molecules and to induce CD4+ T cell proliferation. The next goal of this project is the dissection of the mechanisms that underline these processes to, hopefully, identify new potential targets against the tumor-induced immune tolerance.

RIP-1Tag2 mice lacking HRG display impaired vascular function and increased metastasis

Jessica Cedervall[1]*, Yanyu Zhang[1], Åsa Thulin[3], Maria Ringvall[1], Wilhelm Jahnen-Dechent[2], Agneta Siegbahn[3] and Anna-Karin Olsson[1]. [1]Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden, [2]Department of Biomedical Engineering, RWTH Aachen University, Germany, [3]Department of Medical Sciences, Uppsala University, Sweden

The heparin binding plasma protein HRG (histidine-rich glycoprotein) is an endogenous inhibitor of angiogenesis, capable of reducing vascularisation and tumor growth in mice. Mice lacking the HRG gene are viable and fertile, but have an enhanced coagulation resulting in decreased bleeding times1. By crossing the HRG ko mouse with the Rip1Tag2 mouse model for insulinoma, we have previously shown that mice with HRG deficiency display an elevated angiogenic switch which subsequently leads to increased tumor volumes2,3. However, this effect can be suppressed by platelet depletion, showing that the altered platelet phenotype is of importance for the elevated angiogenic switch. Despite the enhanced angiogenesis during early tumor progression, no difference in vascular density could be observed in later stage tumors2, 3. We can now show that the function of the tumor vasculature is affected by the lack of HRG. HRG ko mice exhibit significantly impaired vessel perfusion, paralleled with lower pericyte coverage and enhanced vascular leakage. In addition, we have observed a significant increase in metastasis in mice lacking HRG, as compared to HRG wt mice. We are now further investigating the mechanisms by which HRG is affecting the various steps of tumor progression, specifically focusing on the connection between HRG and platelet activation.

Extracellular S100A4 mediates phenotypic transition of arterial smooth muscle cells: An important phenomenon in atherosclerosis and restenosis

Chiraz Chaabane[1]*, Claus W. Heizmann[2], Marie-Luce Bochaton-Piallat[1]. [1]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [2]Department of Pediatrics, Division of Clinical Chemistry and Biochemistry, University of Zürich, Zürich, Switzerland

Background: It has been proposed that smooth muscle cells (SMCs) from the arterial wall are heterogeneous and that only a subset of medial SMCs are prone to accumulate into the intima leading to atheromatous plaque formation. We isolated 2 distinct SMC phenotypes from porcine coronary artery: spindle-shaped (S) and rhomboid (R). Biological features of R-SMCs (i.e. enhanced proliferative and migratory activities as well as poor level of differentiation) explain their capacity to accumulate into the intima. We identified S100A4 as being a marker of the R-SMCs in vitro and of intimal SMCs, both in pig and human coronary arteries. S100A4 is an intracellular Ca2+ signalling protein that can also be secreted; it has extracellular functions probably via the receptor for advanced glycation end products (RAGE). Objective: Investigate the role of S100A4 in arterial SMC phenotypic transition, a process characteristic of atherosclerotic plaque formation. Methods and Results: Transfection of a human S100A4-containing plasmid in S-SMCs (practically devoid of S100A4) led to overexpression of S100A4 in approximately 10% of SMCs, release of S100A4, and a transition towards a R-phenotype of the whole SMC population. In contrast, silencing of S100A4 mRNA in R-SMCs and during platelet-derived growth factor-BB-induced S-to-R-phenotypic change did not affect SMC morphology in spite of decreased proliferative activity. However, treatment of S-SMCs with S100A4-rich conditioned medium collected from S100A4-transfected S-SMCs (48 hours post-transfection) induced a transition towards a R-phenotype, which was associated with increased proliferative and migratory activities as well as downregulation of SMC differentiation markers (i.e. alpha-smooth muscle actin and smooth muscle myosin heavy chains), whereas conditioned medium collected from empty vector-transfected S-SMCs had no effect. Conversely, blockade of extracellular S100A4 in R-SMCs with commercially available polyclonal S100A4 neutralizing antibody induced a transition from R- to S-phenotype, decreased proliferative activity and upregulated SMC differentiation markers. This suggests a pivotal role of extracellular S100A4 in SMC phenotypic changes. Moreover, treatment of S-SMCs with S100A4-rich conditioned medium yielded activation of the transcription factor NF-kappa B without changing RAGE mRNA baseline level detected by means of real time PCR. Preliminary results show that RAGE is barely detectable in normal porcine coronary artery media and mainly localized in the cytoplasm of SMCs. Interestingly; it has nucleus localization in intimal SMCs after stent-induced intimal thickening. Conclusions: Extracellular S100A4 is a key modulator of arterial SMC phenotypic transition. It mediates activation of the transcription factor NF-kappa B and possibly nuclear translocation of RAGE. We hope to further clarify the function of extracellular S100A4 on intimal SMC accumulation. We think that a better understanding of the role of extracellular S100A4 in SMC phenotypic transition will help to shed light on the mechanisms of SMC accumulation in the intima leading to the atheromatous plaque formation. Extracellular S100A4 could be a new target to prevent SMC accumulation during atherosclerosis and restenosis.

The helix-loop-helix transcription factor nPAS4 induces sprouting angiogenesis and regulates tip cell formation.

Mei Schmidt, Anne Charlet*, Sophia Heck, Jennifer Heinke, Daniel Epting, Albrecht Kramer-Zucker, Bianca Engert, Cam Patterson, Christoph Bode, Martin Moser. University Hospital Freiburg, Cardiology, Freiburg, Germany

Introduction: The helix-loop-helix-PAS (HLH-PAS) transcription factor neuronal PAS4 (nPAS4) - formerly known as LE-PAS- has recently been cloned as the vertebrate homologue of the drosophila protein dysfusion (dys). In drosophila nPAS4 is indispensable for tracheal migration, adhesion and fusion. Enhanced expression of dys leads to aberrant sprouting of tracheal tubes, whereas the lack of dys results in impaired midline fusion of tracheal tubes. nPAS4 is furthermore strongly expressed in neurons, where it plays a key role in the development of GABA-releasing synapses. Based on the morphological similarity between tracheal tube formation, neurogenesis and angiogenesis, we aimed to investigate the role of nPAS4 in angiogenesis in vertebrates. Methods and Results: As shown by RT-PCR and Western blotting analyses nPAS4 is expressed in various endothelial cell lines at low levels. The expression of nPAS4 is not upregulated by angiogenic growth factors but is induced by hypoxia. Moreover, membrane depolarization using potassium chloride results in a calcium-dependent upregulation of nPAS4. Functionally, overexpression of nPAS4 in endothelial cells results in enhanced sprouting and branching in two- and three-dimensional models. Moreover, when nPAS4 expression is blocked via siRNA, endothelial cell sprouting is significantly reduced. In the endothelial cell spheroid sprouting model the formation of tip cells with their typical features such as the location at the forefront of vessel branches and the highly polarized nature is dependent on the presence of nPAS4. When nPAS4 is overexpressed, tip cell formation is strongly enhanced. Furthermore, the number of filopodia -also a typical feature of tip cells- is increased in nPAS4 overexpressing cells, as visualized by actin staining in immunocytochemistry. In a Transwell Migration Assay, endothelial cell migration was found to be inhibited. Further experiments traced this back to a stronger adhesion of the cells to different extracellular matrices. Preliminary data suggest an upregulation of nPAS4 in a blood vessel injury model supporting a role of nPAS4 in blood vessel reparation. Using fli-GFP transgenic zebrafish as an in vivo model, we found that a knock-down of nPAS4 causes disturbed intersomitic blood vessel formation. Conclusions: In conclusion, these data demonstrate that the HLH-PAS transcription factor nPAS4 is a novel regulator of endothelial tip cell formation as well as of endothelial cell sprouting.

Distinct roles of CD40 and costimulatory molecules B7.1 and B7.2 in leukocyte-adipocyte interactions

Antonios Chatzigeorgiou* and Triantafyllos Chavakis. Division of Vascular Inflammation, Diabetes and Kidney, Department of Medicine III, Technical University Dresden, Germany

Macrophages and lymphocytes are implicated in obesity-related adipose tissue inflammation and may interact with adipocytes. The costimulatory systems CD40-CD40L and B7.1/B7.2-CD28 are essential for T cell activation and inflammatory reactions, yet the contribution of these pathways in the intercellular communications between inflammatory cells and adipocytes during adipose tissue inflammation remains unclear. Here, we assessed expression and function of these costimulatory systems in the interactions between adipocytes and lymphocytes and macrophages. TNF but not palmitate upregulated the expression of CD40 and B7.2 in differentiated 3T3-L1 adipocytes, whereas palmitate increased the expression of CD40, B7.1 and B7.2 on mouse bone marrow derived macrophages (BMDM), indicating that these costimulatory pathways may be relevant in the adipose tissue microenvironment. Stimulation of adipocytes with CD40L increased the expression of chemokines, such as MCP-1, CCL4 and CCL5. Consistently, conditioned media of CD40L-treated adipocytes increased the migration of bone marrow derived mononuclear cells, as compared to control conditioned media. In contrast, the expression of CD40, B7.1 and B7.2 on adipocytes did not contribute to the adhesion of T cells to adipocytes. Thus, the inflammation-dependent upregulation of CD40 on adipocytes in the adipose tissue and the recruitment of CD40L-expressing T cells can stimulate chemokine expression in adipocytes, thereby mediating macrophage accumulation in the adipose tissue and perpetuating adipose tissue inflammation.

Src-like adapter proteins are negative regulators of GPVI/ITAM-signaling in platelets

Deya Cherpokova[1#*], Markus Bender[1#], Martina Morowski[1], Peter Kraft[2], Guido Stoll[2], Craig E. Hughes[3], Leonard L. Dragone[4], Steve P. Watson[3], Michael G. Tomlinson[5], and Bernhard Nieswandt[1]. [1]Chair of Vascular Medicine, University Hospital Würzburg and Rudolf Virchow Center, DFG-Research Center for Experimental Biomedicine, Würzburg, Germany; [2]Department of Neurology, University of Würzburg, Würzburg, Germany; [3]School of Clinical and Experimental Medicine, Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom; [4]National Jewish Health, Denver; University of Colorado, Denver, CO and Colorado Denver Children's Hospital, Aurora, United States; [5]School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom [#]These authors contributed equally

At sites of vascular injury, platelets come into contact with exposed components of the subendothelial extracellular matrix (ECM) which triggers their adhesion and activation. This activation is mainly mediated through interaction of glycoprotein (GP) VI with collagens, resulting in a cascade of signaling events downstream of the FcR gamma-chain associated immunoreceptor tyrosine-based activation motif (ITAM).The Src-like adapter protein (SLAP) and the closely related SLAP2 are expressed in a variety of human and mouse tissues and cell types, including peripheral blood cells. Both adapter proteins are involved in the regulation of T- and B-cell receptor surface expression levels and signaling. The function of SLAP and SLAP2 in platelet biology is largely unknown. Here we show that SLAP/SLAP2-deficient mouse platelets displayed significantly elevated GPVI expression levels and markedly increased integrin activation, granule release and aggregation upon stimulation with GPVI-specific agonists. Furthermore, GPVI-mediated activation of the mutant platelets resulted in enhanced and sustained tyrosine phosphorylation of different proteins involved in the GPVI/ITAM-signaling cascade. Importantly, analysis of Slap/Slap2-/-Gp6+/- mice, in which GPVI surface expression was reduced to 50% of the GPVI expression levels in Slap/Slap2-/- mice, revealed that the enhanced GPVI activation cannot be ascribed to the increased GPVI expression, but rather indicates specific GPVI-signaling defects. These results demonstrate that the adapter proteins SLAP and SLAP2 function as negative regulators of the GPVI/ITAM-signaling pathway in platelets thereby limiting pathological activation of the cells.

Mast cells in arteriogenesis - a pre-clinical study employing a murine hindlimb model of collateral artery growth.

Omary Chillo*, Judith Irina Pagel, Teresa Trenkwalder, Christopher A. Reichel, Elisabeth Deindl. Walter Brendel-Centre of Experimental Medicine, Marchionini Street 15, Munich, Germany

The central role of leukocytes for arteriogenesis is quite well characterised. The role of mast cells remains to be elucidated, which was the aim of our study. To evaluate the role of mast cells, wild type mice (WT) were subjected to right femoral artery ligation (fal) in order to induce arteriogenesis; the left side was sham operated. WT (6) mice received different treatments; 1. The mast cell stabilizer cromolyn, 2. Diprotin A, increasing mast cell recruitment from the bone marrow, 3. Stem Cell Factor (SCF), enhancing mast cell maturation and differentiation, 4. The mast cell degranulator compound (C) 48/80, 5. A combination of Diprotin A and C 48/80, 6. A combination of cromolyn and Diprotin A. Controls were treated with 0.9% saline. 3 days after ligation, adductor and calf muscles were isolated for immunohistochemical analyses. Relative perfusion recovery of hindlimbs was monitored by Laser Doppler Imaging, before, post ligation and at day 3, 7, 14 and 21. Administration of Diprotin A as well as C 48/80 significantly improved perfusion recovery after fal. However, the combinatory treatment with Diprotin A and C48/80 enhanced arteriogenesis even further and at an earlier time point. Treatment with cromolyn was associated with poor perfusion recovery, and the positive effect of Diprotin A on arteriogenesis was abolished after application of cromolyn. In contrast to saline and cromolyn treated WT mice, the C48/80 and Diprotin A combinatory treated group evidenced enhanced leukocyte recruitment and extravasation. Taken together, our results show that mast cells play a significant role for arteriogenesis. Promoting their recruitment and degranulation enhances collateral artery growth. Treatment with Diprotin A and/or compound 48/80 might serve as a new therapeutic approach for the treatment of arterial occlusive diseases.

The role of Pten during zebrafish development and tumor progression

S.Choorapoikayil[1]*, B. Weijts[2], A.de Bruin[2], S. Schulte-Merker[1], J. den Hertog[1]. [1]Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, [2]Pathobiology Department, Faculty of Veterinary Medicine, Utrecht University,Yalelaan 1, 3508 TD Utrecht, The Netherlands

PTEN is one of the most frequently mutated tumor suppressor genes in progression of cancer. In general it is known that PTEN counteracts the PI3Kinase/Akt pathway, a key player in cell growth, proliferation and survival. Somatic deletion of PTEN induces tumor formation in various organs, whereas germ line mutation causes diseases sharing similar pathological features, like formation of multiple benign tumors with an increased susceptibility to malignant cancers. Our group uses zebrafish to reveal the cellular response in a multi cellular organism after Pten loss of function. The zebrafish genome encodes two pten genes, ptena and ptenb, which have a partially redundant role during early development. Mutant zebrafish lacking either Ptena or Ptenb do not display embryonic phenotypes and even mutants with only a single wild type pten allele are viable and fertile. These adult fish develop a specific tumor type with relatively high incidence, classified as hemangiosarcoma. Homozygous loss of both pten genes is embryonically lethal. From 3 dpf onwards the embryos displayed developmental defects, in particular hyperbranching of intersegmental vessels caused by overproliferation of endothelial cells. Our results demonstrate that both embryos and adult zebrafish with impaired PTEN function display defects that are associated with overproliferation of endothelial cells.

HIV-1 Nef is a wanderer between blood and endothelial cells and induces vascular dysfunction

Matthias Clauss*, Tina Wang and Linden Green. IU School of Medicine, Indianapolis, USA

With the prevalence of antiviral therapy in the developed world, many HIV-1-infected people die of diseases other than AIDS. One of the emerging major causes is cardiovascular disease, leading to the prediction that the majority of HIV-1 patients are expected to develop cardiovascular complications. Endothelial dysfunction is thought to be a key event in the development of cardiovascular diseases, particularly atherosclerosis. When testing the effect of HIV on endothelial activation, we noticed that direct contact with HIV-1 infected T cells induced far more endothelial cell activation than HIV-1 virus alone, suggesting an intracellular HIV protein responsible for endothelial activation. The HIV-1 viral protein Nef, which is responsible for T cell activation and maintenance of high viral loads in vivo, has been shown to mediate its own transfer to bystander cells. We demonstrate here for the first time that Nef induces nanotube-like conduits connecting T cells and endothelial cells. Nef is indeed transferred from T cells to endothelial cells via these nanotubes and is necessary and to induce HIV-1 dependent endothelial cell activation. Moreover, we found that Nef protein, but not nef gene, is in endothelial cells from patients with HIV-1 and in coronary arteries of SIV-infected macaques, indicating that Nef transfer to the endothelium occurs in vivo. HIV-1 Nef expression in endothelial cells causes endothelial apoptosis, ROS and MCP-1 production. Interestingly, a Nef SH3 binding site mutant abolishes Nef-induced apoptosis and ROS formation and reduces MCP-1 production in endothelial cells, suggesting that the Nef SH3 binding site is critical for Nef effects on endothelial cells. Nef induces apoptosis of endothelial cells through an NADPH oxidase- and ROS-dependent mechanism, while Nef-induced MCP-1 production is NF-kB dependent. Together, these data suggest that HIV-1 Nef can mediate its transfer from T cells to endothelial cells through nanotubes to induce endothelial dysfunction in vivo. Importantly, this is also of relevance for patient treated with highly active anti-retroviral therapy (HAART), as Nef is constitutively produced even in quiescent a-viremic cells. Thus, Nef is a promising new therapeutic target for reducing the risk for cardiovascular disease in the treated and untreated HIV-1 positive population.

EMAP II is necessary for inflammation and stenosis in a wire-induced vascular injury model

Matthias Clauss[1]*, Linden Green[1], Dietlind Zohlnhöfer[2] and Karsten Peppel[3]. [1]IU School of Medicine, [2]I. Medizinische Klinik und Deutsches Herzzentrum der Technischen Universität München; [3]Jefferson Medical College Philadelphia, USA

Endothelial Monocyte-Activating Polypeptide II (EMAP II) is a pro-inflammatory mediator, which selectively can induce apoptosis in endothelial cells and is upregulated in the lungs of cigarette smoke exposed mice and men. Previously, we have shown that EMAP II is an essential mediator of cigarette smoke-induced lung emphysema, which links endothelial cell apoptosis with inflammation. In particular we could demonstrate that EMAP II is part of a feed forward loop in cigarette smoke-induced emphysema based on its ability to be secreted from apoptotic cells and to induce apoptosis in pulmonary microvascular endothelial cells. Here we address the role of EMAP II in vascular arterial injury. This is also based on our previous reports that EMAP II protein is strongly increased in smooth muscle cells during the first 2-4 weeks of arterial injury and downregulated by rapamycin leading to decreased inflammation and stenosis. Using wire-induced denudation of carotid and femoral arteries, we found that EMAP II neutralizing antibodies strongly suppressed recruitment of inflammatory cells and almost completely abrogated stenosis when applied for 4 weeks by bi-weekly Ip injections. In addition to dramatically decreased number of monocytes and neo-intima formation, re-endothelization was improved based on staining with VE-cadherin antibodies. Interestingly, Iv injected recombinant EMAP II worsened inflammation and ne-intima formation leading to the presence of many occluded arteries 4 weeks after wire injury. Because vascular injury coincided with endothelial CXCR3 upregulation, we tested in tissue culture experiments whether endothelial CXCR3 upregulation, which we induced by lowering serum concentrations, would increase sensitivity to EMAP II-induced endothelial caspase-3 activation and DNA fragmentation, two hallmarks of cellular apoptosis. Indeed, EMAP II treatment was up to 10 fold enhanced with increased CXCR3 receptor surface expression.In conclusion, concerted upregulation of EMAP II and CXCR3 by injury could reduce repair of the endothelium and promote inflammation to promote neo-intima formation and stenosis.

Calmodulin is a marker of a distinct smooth muscle cell population recruited by plaque-derived macrophages from the human carotid artery media

Matteo Coen[1]*, Giovanna Marchetti[2], Carlotta Zerbinati[2], Giuseppe Guastella[2], Teresa Gagliano[2], Francesco Bernardi[2], Patricia M. Palagi[3], Francesco Mascoli[4], Marie-Luce Bochaton-Piallat[1]. [1]Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland, [2]Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy, [3]Proteome Informatics Group, Swiss Institute of Bioinformatics, Geneva, Switzerland, [4]Operative Unit of Vascular and Endovascular Surgery, S. Anna University-Hospital, Ferrara, Italy

Background: Different experimental observations suggest the expansion of a distinct medial smooth muscle cell (SMC) subset during atheromatous plaque establishment and restenosis. Objectives: We investigated the presence and features of such SMC subset in cell cultures derived from human carotid endarterectomy (CEA) specimens; moreover, we aimed at finding markers that could play a role in SMC behavior. Methods and Results: CEA specimens comprised a grossly undiseased (UP) and a diseased portion (DP). The UP contained a thin intimal thickening with the underlying media and the DP an atherosclerotic plaque with the underlying media. Cell cultures were initiated by tissue explantation. From plaque tissues only macrophage-derived foam cells were retrieved. From medial tissues 2 distinct SMC phenotypes were identified: 1) large SMCs, flat with a monolayered growth pattern, isolated from the UP media; 2) small SMCs, fusiform and growing in multilayers, isolated from both the UP and DP media only when cocultured with plaque-derived macrophages. Small SMCs displayed higher proliferative and migratory activities and a poor level of differentiation compared with large SMCs. Two-dimensional polyacrylamide gel electrophoresis followed by tandem mass spectrometry showed that calmodulin (CaM), a calcium-binding protein involved in cell-cycle regulation, was mainly expressed in small SMCs. This result was confirmed using a specific CaM antibody. Coculture of large SMCs with plaque-derived macrophages induced a switch to the small phenotype and was associated with increased CaM expression. Consistently, the specific CaM inhibitor W-7 hampered the large-to-small switch and predominantly inhibited the proliferation of small SMCs. In vivo, CaM was markedly expressed in atherosclerotic plaques whereas it was barely detectable in the media. Conclusions: Plaque-derived macrophages promote the selective migration of a distinct SMC subpopulation exhibiting features of an „atheroma-prone“ phenotype. CaM is a marker of small SMCs in vitro and of plaque SMCs in vivo, and plays a role in SMC phenotypic modulation. Further studies on these SMC populations can be instrumental in understanding and influencing the evolution of atherosclerosis.

An investigation into the expression and regulation of expression of endothelial cell JAM-C in inflammation in vivo

Bartomeu Colom[1]*, David A Leinster[1], Mathieu-Benoit Voisin[1], Triantafyllos Chavakis[2], Beat A. Imhof[3] & Sussan Nourshargh[1]. [1]William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, [2]Dresden University of Technology, Dresden, Germany, [3]Centre Médical Universitaire, Geneva, Switzerland

JAM-C is a member of the Ig-superfamily that localizes to cell-cell contacts and is specifically enriched at tight junctions. Originally detected on endothelial cells (ECs), JAM-C has since been reported to be expressed on numerous other cell types such as spermatids, epithelial cells, smooth muscle cells, fibroblasts and Schwann cells. As such JAM-C has been associated with numerous biological functions but most notably it has been studied in the context of vascular and inflammatory responses. Despite a growing interest in this complex molecule there remain many unanswered questions with respect to the expression and functions of JAM-C. In the present study we sought to investigate the expression and regulation of expression of EC JAM-C under inflammatory scenarios. The expression of EC JAM-C was investigated in mouse ear skin and cremaster muscle by immunofluorescent staining and confocal microscopy using whole-mount tissues. JAM-C was found to be strongly expressed at junctions of ECs in capillaries and venules, but not arterioles. The venular expression of JAM-C was unaltered in ears stimulated with the chemokines KC and MIP-2 (both at 500ng) but was significantly reduced in LTB4-stimulated tissues (300ng). The latter occurred in a time-dependent manner with reduced expression of JAM-C being noted at 30 mins, peaking at 4 h and returning back to normal levels by 24 h post LTB4 administration. Whilst full details of the mechanism associated with the reduced expression of JAM-C are at present unclear, studies with neutrophil-depleted mice and neutrophil elastase (NE) KO mice suggested a role for neutrophil-derived NE in LTB4-induced regulation of expression of JAM-C. On-going works aim to address the functional significance of loss of EC JAM-C on regulation of neutrophil transmigration. Collectively our data indicate that the expression and regulation of expression of EC JAM-C is vessel type and stimuli specific, and that JAM-C maybe shed from ECs after LTB4 stimulation in a leukocyte and NE dependent manner. This work was supported by funds from The Wellcome Trust.

The vascular supply of zebrafish teeth, and its effect on tooth replacement

Jeroen Crucke*, Ann Huysseune. Evolutionary Developmental Biology, Ghent University, Belgium

Over the last few decades, there has been an enormous progress of knowledge with regard to the genetic and molecular mechanisms that regulate tooth development. However, certain aspects have remained rather poorly studied. The role of angiogenesis and neurogenesis in natural tooth development is one of them. This is remarkable considering the importance of innervation in organ formation, maturation and homeostasis, and given that vascularization, apart from having an essential nutritive function, also plays a vital role in providing developmental signals to promote organ morphogenesis. The connection between vascular and neural development, maintenance and functioning is termed the 'neurovascular link' and has received renewed interest over the last few years. Our aim is to test the hypothesis that tooth replacement depends on a properly functioning neurovascular link using a model with natural in situ tooth replacement, the zebrafish. Prior to unravelling the role of vascular and neural elements during the process of tooth development and replacement, it is important to obtain a profound understanding of the mutual (spatial and temporal) relationships between teeth, blood vessels and nerves. Light microscopical analysis has revealed that the arterial supply of the paired pharyngeal jaws is secured by branches of the unpaired hypobranchial artery, which anastomoses around the bases of the functional teeth. This blood vessel develops from the first aortic arch at 3 to 3.5 dpf (Isogai et al. 2001), i.e. exactly at the time of first replacement tooth formation at approx. 80 hpf. Capillaries branching off from the anastomosing network supply the developing replacement teeth. Apart from understanding the spatial relationship between developing teeth and neural/vascular elements, a functional approach is used to study the role of angiogenic factors in tooth development and replacement. With regard to the neurovascular link, we focus on factors involved in linking both angiogenic and neurogenic events, so called angioneurins. A highly likely candidate is vascular endothelial growth factor (VEGF), an angiogenic factor that also appears to be involved in several neurobiological processes. Its role during zebrafish tooth development and replacement is studied by interfering with its signalling function through application of the pharmaceutical compound SU5416, a potent and selective inhibitor of the VEGF receptor Flk-1/KDR. Preliminary data show that this has no effect on the development of replacement teeth. However, a slight delay can be observed in the developmental stage of the tooth when compared to non treated-larvae, indicating a solely nutritive function for the blood vessels, which allow the teeth to properly grow and develop. In addition, we will also study these angioneurins both at the mRNA level and at the protein level, using in situ hybridization and immunocytochemistry respectively. This research was funded by a grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) to JC. Isogai S, Horiguchi M, Weinstein BM, 2001. Dev Biol 230: 278-301

ERK 1/2 is involved in anti-angiogenic effect of polyphenols-enriched fraction from chilean propolis.

Cuevas, Alejandro[1,2]*, Saavedra Nicolás[1,2], Rudnicki Martina[2], Abdalla, Dulcineia SP[2], Salazar LA[1]. [1]Center of Molecular Biology and Pharmacogenetics, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Temuco, Chile, [2]Laboratory of Clinical Chemistry, Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, Sao Paulo, Brazil

Background: Compelling evidence have shown that polyphenol-enriched fraction from propolis can modulate angiogenesis in both in vitro and in vivo models. However, the molecular mechanisms are poorly understood. Thus, the aim of this study was to investigate the impact of an ethanolic-extract of chilean propolis (EEP) on in vitro angiogenesis and evaluate their effect on hypoxia-inducible factor 1aï€ (HIF-1a) protein expression and on extracellular signal-regulated kinases (ERK 1/2) signaling pathway. Methods: The effects of the EEP on endothelial cell migration and sprouting were assessed in human umbilical vein endothelial cell (HUVEC) using the in vitro scratch wound assay and matrigel angiogenesis and in the ex vivo rat aortic ring model. Western blot analysis was performed to evaluate HIF1-αprotein expression and ERK 1/2 phosphorylation. In addition, the mRNA expression of miRNA-126 and SPRED1 was determined by real time-PCR. Results: At 10 µg/mL, EEP inhibited in vitro cell migration and the formation of capillary-like structures in matrigel (large of sprouting, sprouting number and nodes number) was attenuated, but total cell area had no differences. In addition, EEP significantly suppress the ex vivo sprouting of endothelial cells from the rat aorta fragment. In addition, the treatment of EEP showed no effect on HIF1-αprotein accumulation in hypoxia- or dimethyloxallylglycine-stimulated endothelial cells. In contrast, the key step on MAPK/ERK signal pathway, ERK 1/2 phosphorylation, was inhibited. Finally, we studied the expression of miR-126, a miRNA that regulates vascular integrity by suppressing SPRED1, a negative regulator of Ras/MAPK signaling. Contrary to the hypothesized, in stimulated HUVEC (VEGF 10 ng/mL) treated with EEP, the miR-126 was up-regulated and SPRED1 lightly down-regulated. A polyphenol-enriched extract is a complex mixture that can act on different target and may even have opposite effects on the same test. Up-regulation of miR-126 is related to activation of RAS/MAPK pathway, suggesting a pro-angiogenic activity of EEP. However, the inhibitory effect on the phosphorylation of ERK 1/2 is correlates with the anti-angiogenic effect described above and was independent of the effect of miR-126 upstream. Conclusion: In summary, these results suggesting that the inhibition of ERK1/2 phosphorylation, is a critical mechanism of angiogenesis suppression by EEP, but more studies are needed to corroborate these findings.

Elucidating the role of astrocyte-derived Wnt growth factors for BBB maintenance

Cathrin J. Czupalla[1]*, Claudio A. Franco[2], Holger Gerhardt[2], Karl-Heinz Plate[1], Stefan Liebner[1]. [1]Institute of Neurology (Edinger-Institute), Goethe University, Heinrich-Hoffmann-Str. 7, 60528 Frankfurt, Germany, [2]Vascular Biology Laboratory, London Research Institute, 44 Lincoln's Inn Fields, WC2A 3LY London, United Kingdom

Tight control of vascular permeability is essential for the homeostasis and functionality of the central nervous system (CNS) microenvironment. The blood-brain barrier (BBB) proper is constituted of endothelial cells (ECs) interconnected by tight junctions (TJs). In the past astrocytes (ACs) of the neuro-vascular unit (NVU), were suspected to be the source of BBB phenotype inducing growth factors. In general it is well known that during developmental brain angiogenesis and also in the adult, the specific brain milieu is responsible for the induction and maintenance of BBB characteristics in ECs. However, it has never been shown which inductive cues play a role in BBB maintenance. In the last couple of years the canonical Wnt pathway was identified as a brain-specific angiogenic pathway, that is key for the early induction of the BBB phenotype in ECs. In in vitro systems high electrical resistance reflects the integrity of the mature BBB, which is considered to be due to complex tight junctions between ECs of an intact monolayer. Previously, it has been shown that AC conditioned medium (ACM) or AC and EC co-cultivating conditions, control local tight junction biogenesis in brain capillary ECs, suggesting the importance of astrocyte-produced factors (Arthur et al. 1987; Dehouck et al. 1990). Initially, the isolation of murine WT primary ACs was established and the transcription of Wnt genes was determined over different passages using RT-PCR. Almost all Wnt growth factors were expressed at mRNA level with only little variations over passages P0 to P3 in vitro. In order to characterize the BBB maintaining factors, we isolated primary mouse brain ECs and treated them with primary ACM. To particularly determine the role of Wnt growth factors as a BBB maintenance cue, we used a mouse line for AC isolation that exhibits an AC-specific knockout (KO) for Evi („Gpr177"/"wntless"), a protein, which is essential for Wnt growth factor transport through the Golgi to the plasma membrane, and thus regulating the release of the growth factors. To address changes in BBB integrity in vitro, the trans-endothelial electrical resistance (TER) was measured in primary WT ECs treated with Evi-deficient or WT ACM. Since we initially did not observe any differences in TER maintenance between the Evi WT and KO ACs treated ECs, we determined again the genetic background of the primary ACs and found that during passaging the KO was lost. Repeatedly, recombination was induced by treatment of Evilox/lox ACs with TAT-Cre in vitro and new ACM was produced. Preliminary TER measurements indicate a shortened maintenance effect of Evilox/lox-Cre+ ACs in comparison to their WT controls in an AC/EC-co-culture paradigm. Roughly half of the GFAP-Cre-Evilox/lox mice analyzed so far, showed intracerebral hemorrhages indicating a partial breakdown of BBB structures that however did not lead to a lethal phenotype. Differential expression of TJ molecules, vessel morphology and extend of vessel leakiness need to be addressed. Thus, we conclude that Wnt growth factors released by ACs might play a major role in the maintenance of the BBB phenotype.

Loss of endothelial surface glycocalyx in the glomerular filtration barrier as a first step towards the development of proteinuria

Martijn Dane[1,2]*, Dan Potter[2], Cristina Avramut[3], Hans Vink[2], Johan van der Vlag[4], Anton Jan van Zonneveld[1], Ton Rabelink[1], Bernard van den Berg[1]. [1]Department of Nephrology and the van Einthoven Institute of Vascular Medicine, LUMC, Leiden, [2]Department of Physiology, MUMC, Maastricht, [3]Molecular Cell Biology, Electron Microscopy Section, LUMC, Leiden, [4]Nephrology Research Laboratory, Department of Nephrology, Nijmegen Centre for Molecular Life Sciences, RUNMC, Nijmegen, The Netherlands

Background: The glomerular filtration barrier consists of an endothelial layer together with its surface bound glycocalyx, podocytes and a glomerular basement membrane. Although the sequence of events that can result in proteinuria is not known for cardiovascular conditions such as T2D, the endothelial glycocalyx has been shown to be damaged in proteinuric T2D patients. We therefore hypothesize that damage of this glycocalyx can result in proteinuria. Methods: In 12 weeks old C57BL/6J mice, one of the major glycocalyx components, hyaluronan, is enzymatically degraded through continuous infusion of hyaluronidase for up to 4 weeks using an osmotic mini-pump with a cannula in the right jugular vein. Systemic vascular glycocalyx volume, urine protein/creatinin- and dex500/dex40 ratios, as well as heart rate and blood pressure were determined. After 4 weeks of hyaluronidase treatment and after 4 weeks of subsequent recovery, perturbation of the endothelial glycocalyx was analyzed with electron microscopy using cupromeronic blue staining, leakage of endogenous albumin and exogenous ferritin (tail vein injection) and with confocal microscopy using lectins LEA, WGA and BSI (binding N-acetyl-β-D-glucosamine, N-acetyl-β-D-glucosaminyl and N-acetyl-α-D-galactosaminyl, respectively). Results: Systemic effects of chronic hyaluronidase infusion were tested first. After two weeks of infusion a +/- 50% decrease in systemic glycocalyx volume, together with increased dextran 500/40 release in urine was observed, without changes in blood pressure. Four weeks of hyaluronidase infusion resulted in decreased luminal staining of both glucosamine and galactosamine binding lectins (LEA: 0.73 vs. -0,14 μm; BSI: 0,89 vs. 0,34 μm, resp., $P<0,001$). WGA lectin, staining the outside of the CD31 perimeter, was not affected by hyaluronidase. Four weeks after removal of the osmotic pump, both LEA and BSI staining restored to control thickness. Without evidence for albuminuria, electron microscopy revealed that endogenous albumin had passed the glomerular filtration barrier in the hyaluronidase treated group and was present on the membranes of podocytes and parietal epithelial cells as well as inside vesicles in these cells. No albumin was found past the barrier in control mice or after 4 weeks of recovery. Conclusion: Chronic hyaluronidase treatment results in a reversible reduced glomerular endothelial glycocalyx leading to a perturbed glomerular filtration barrier. This insult was without obvious changes in extravascular carbohydrate content, or overt macroscopic changes of the glomerulus (i.e. micro-aneurisms, GBM thickening or podocyte flattening). Changing these barrier properties resulted in leakage of endogenous albumin. However, treatment did not result in albuminuria which indicates an efficient albumin uptake by downstream epithelial cells. Altogether, loss of endothelial surface glycocalyx in the glomerular filtration barrier might be the first step towards development of proteinuria in cardiovascular conditions like T2D.

Two-chain high molecular weight kininogen inhibits neointimal lesion formation by preventing leukocyte recruitment

J.-M. Daniel*, F. Reich, J. Dutzmann, K. T. Preissner, C. W. Hamm, D. G. Sedding. Molecular Cardiology Section (UGMC), Dept. of Cardiology, Justus-Liebig-University, Giessen, Germany

Purpose: The cleavage of single-chain high molecular weight kininogen (HK) results in the release of bradykinin and two-chain HK (HKa). HKa and in particular its peptide domain 5 (D5) exert anti-adhesive properties during inflammatory cell recruitment via binding to extracellular matrix proteins and impeding the function of the β2-integrin molecule Mac-1 (CD11b/ CD18). In this study, we investigated the effects of HKa and D5 on the accumulation of circulating cells and the function of resident vascular cells in a mouse model of neointima formation. Methods: After lethal irradiation C57BL/6 mice were transplanted with bone marrow from transgenic mice expressing enhanced green fluorescence protein (EGFP). Wire induced injury of the femoral artery was performed on chimeric mice with local application of HKa, D5, or control to the dilated artery in a thermosensitive pleuronic gel. Vessels were harvested 1 day after injury to test the sustained release of the substances (n=3) and at 3 weeks after injury for morphometric analysis and immunohistochemistry (n=6). Results: Neointima formation was significantly reduced after treatment with HKa and even more prominent after D5 application (HKa: 0.981 ± 0.174; D5: 0.549 ± 0.076 vs. 1.54 ± 0.150; $P<0.05$). The attenuation of the neointimal lesion was accompanied by a reduced accumulation of EGFP+-cells and monocytes/ macrophages in the treatment groups. Confocal microscopy revealed that EGFP+-cells did not co-express smooth muscle myosin heavy chain or calponin, indicating no trans-differentiation of BM-derived cells into smooth muscle cells. Importantly, HKa and D5 significantly reduced the number of proliferating resident smooth muscle cells in the vascular wall ($P<0.05$). In contrast, the ratio of apoptotic cells/ all neointimal cells was increased in the treatment groups, although the absolute numbers of apoptotic vascular cells as well as the process of re-endothelialization were not different. Conclusion: Endogenous HKa decreases the inflammatory response to vascular injury due to its anti-adhesive properties and thus reduces proliferation of local vascular cells. Therefore, application of HKa or D5 points towards the importance of inhibiting leukocyte accumulation after vascular injury and may provide a novel therapeutic strategy for attenuating atherosclerosis or neointimal lesion development.

Effect of tellurium compounds on the interaction between retinal pigment epithelium (RPE) cells and endothelial cells (EC) in co-culture

Rima Dardik[1,2]*, Tami Livnat[1,2], Yael Nisgav[2], Shirley Azar-Avivi[2], Benjamin Sredni[3], Dov Weinberger[2]. [1]Institute of Thrombosis and Hemostasis, Sheba Medical Center, Tel Hashomer, [2]Department of Ophthalmology, Rabin Medical Center and Tel-Aviv University School of Medicine, Tel Aviv; Israel, [3]The Mina & Everard Goodman Faculty of Life Sciences, CAIR Institute, Bar-Ilan University, Ramat-Gan, Israel

Aim: Choroidal neovascularization (CNV) is the leading cause of vision loss in various pathological conditions. CNV tends to develop under conditions where the RPE and EC are no longer separated by the Bruch's membrane, resulting in exposure of the two cell types one to the other. Enhanced angiogenesis and inflammation are the major processes underlying the pathophysiological manifestations of CNV. We developed a co-culture model simulating pathological contact between EC and RPE cells, which leads to CNV. We have recently reported that the contact between EC and RPE cells stimulates cellular signals enhancing the angiogenic potential of EC and activity of matrix metalloproteinases (MMPs) (1). The non toxic, immunomodulators compounds: ammonium trichloro (dioxyethylene -0-0') tellurate (AS101), and octa-O-bis-(R,R)-Tartarate Ditellurane (SAS) have been shown to exert beneficial anti-inflammatory and anti-angiogenic effects in diverse preclinical and clinical studies. In the present study, we examined the effects of SAS on the interaction between RPE and EC in co-culture. Methods: RPE and EC grown for 7 days in co-culture (1) were exposed to SAS (1 micorgram/ml over the last 24 hours of co-culture), followed by separation between the two cell types using antibody-coated magnetic beads. Gene expression was studied by quantitative real-time PCR. MMPs activity was examined by zymography. PEDF levels were examined by ELISA. Results: RPE cells grown in co-culture with EC demonstrated significantly increased mRNA expression of COX-1 (X 7000) and IFN-gamma (x8), both of which are involved in inflammation, and of MMP2 (X 3) which plays an important role in angiogenesis. Addition of SAS abolished the elevations in COX-1, IFN-gamma and MMP2 expression at the mRNA level. Furthermore, addition of SAS resulted in a significantly increased expression of PEDF, which is a potent antiangiogenic factor, at both mRNA and protein levels (X 4.2 and X 2.7, respectively). Finally, addition of SAS significantly suppressed the activity of MMP2 (X 3) secreted by the co-cultured cells to the conditioned medium, as demonstrated by zymography. Conclusion: In our in-vitro model of CNV, the tellurium compound SAS exhibited antiangiogenic and anti-inflammatory properties We suggest that SAS may exert beneficial effects by preventing, or even reversing, the pathological processes involved in CNV. (1)Dardik R, Livnat T, Nisgav Y, Weinberger D. Enhancement of angiogenic potential of endothelial cells by contact with retinal pigment epithelial cells in a model simulating pathological conditions. Invest Ophthalmol Vis Sci. 2010 ;51:6188-95

VE-cadherin - α- catenin homozygous knock-in mice die of developmental defects during midgestation

Nina Dartsch[1]*, Dörte Schulte[2] and Dietmar Vestweber[1]. [1]Max-Planck-Institute of Molecular Biomedicine, Muenster, Germany, [2]Hubrecht Institute, Utrecht, The Netherlands

The endothelial-specific adhesion molecule VE-cadherin is essential for the formation and stability of interendothelial cell contacts. To determine whether leukocytes need to open these endothelial cell contacts during extravasation, mice with strongly stabilized endothelial junctions were generated. For this purpose, VE-cadherin was genetically replaced by a VE-cadherin-α-catenin (VEC-α-cat) fusion construct through application of the RMCE technology. Such mice were completely resistant to the induction of vascular leaks by VEGF or histamine. Neutrophil or lymphocyte recruitment into inflamed cremaster, lung and skin, respectively, were strongly inhibited in these mice. This establishes the importance of the junctional route as the main pathway for leukocyte extravasation in these tissues. Whereas about 50% of homozygous VEC-α-cat mice were healthy and fertile on a mixed genetic background (129Sv/C57Bl6), about 50% died during embryonic development. On a strict C57Bl6 background all embryos died. Investigating the reason for lethality, we found that homozygous VE-cadherin-α-catenin embryos exhibited developmental defects leading to lethality from E13.5 onwards. We observed two different abnormalities: embryos suffered from a lack of hematopoiesis in the fetal liver and from severe edema formation. The latter seems to be due to defects in the development of the lymphatics. Analyzing the hematopoiesis defect we discovered that the formation of clusters of hematopoietic progenitors from the hemogenic endothelium of the aorta in the aorta-gonad-mesonephros (AGM) region was normal. In addition, the number of lin-ckit+ progenitor cells in the fetal circulation of VEC-α-cat embryos was elevated compared to wt embryos. However, about 40% of homozygous VEC-α-cat embryos lacked any hematopoietic cells in their fetal liver. To investigate a possible role of VE-cadherin for the development of hematopoietic progenitors in the fetal liver, VE-cadherin was deleted in these progenitor cells by breeding mice which carry floxed VE-cadherin alleles and express Vav driven Cre. The hematopoiesis in the fetal liver of these mice was not affected arguing that leukocyte progenitors do not require VE-cadherin for development within the fetal liver. It follows that VEC-α-cat hinders the entry of hematopoietic progenitors into the fetal liver tissue.

Sphingosine-1-phosphate receptor 3 promotes neointimal hyperplasia in mouse iliac-femoral arteries

Takuya Shimizu[1], Allison De Wispelaere[1,2], Martin Winkler[2,3], Travis D'Souza[2], Jacob Caylor[2], Lihua Chen[1,2], Frank Dastvan[1,2], Jessie Deou[1,2], Aesim Cho[1,2], Axel Larena-Avellaneda[3], Michael Reidy[1], and Guenter Daum[1]*. [1]University of Washington, Department of Pathology and [2]University of Washington, Department of Surgery, Seattle, Washington, USA, [3]University of Hamburg, University Hospital Hamburg-Eppendorf, Department of Vascular Medicine, University Heart Center, Hamburg, Germany

Restenosis is a significant complication of surgical and percutaneous procedures to restore arterial blood flow. Arterial lesions are characterized by intimal hyperplasia and negative remodeling of the artery, both causing luminal narrowing. Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that plays a role in vascular pathologies including coronary artery disease, atherosclerosis, and intimal lesion formation after arterial injury. In diseased arteries or upon arterial injury, S1P may be released from platelets and also locally produced by growth factor or cytokine-mediated induction of sphingosine kinase, which phosphorylates sphingosine to generate S1P. S1P binds to five G protein-coupled receptors, S1PR1-S1PR5. S1PR1, S1PR2 and S1PR3 are ubiquitously expressed, whereas S1PR4 and S1PR5 are mainly expressed in immune cells and brain, respectively. The role for S1P in arterial lesion formation may be complex since cellular responses to S1P depend on which receptor is expressed, given that they couple to different G-proteins. The objective of this study is to define a role for S1PR3 in the formation of neointimal lesions following denudation of the iliac-femoral artery in mice. This model was chosen because we found that in mice and humans, S1PR3 expression in iliac arteries is considerably high when compared to other arteries, e.g. the carotid artery. At 28 days after surgery, wild-type arteries form significantly larger lesions than S1PR3-null arteries. BrdU labeling experiments demonstrate that upon injury, wild-type arteries exhibit higher medial as well as intimal proliferation than S1PR3-null arteries. Because S1PR3 expression in vitro is low, we expressed S1PR3 in S1PR3-null SMCs using retroviral-mediated gene transfer to study S1PR3 effects on cell functions and signaling. SMCs expressing S1PR3, but not vector-transfected controls, respond to S1P stimulation with activation of Rac, Erk and Akt. SMCs expressing S1PR3 also grow and migrate more. From these data, we conclude that S1PR3 promotes neointimal hyperplasia upon denudation of iliac-femoral arteries in mice, likely by stimulating cell migration and proliferation through activation of signaling pathways involving Erk, Akt and Rac.

Angiopoietin-1 requires IQ domain GTPase-activating protein 1 to activate Rac1 and promote endothelial barrier defense

David S*, Ghosh CC, Mukherjee A, Parikh SM. Medical School Hannover, Department of Nephrology Beth Israel Deaconess Deaconess Medical Center and Harvard Medical School, Center for Vascular Biology Research

IQ domain GTPase-activating protein 1 (IQGAP1) contributes to cytoskeletal network regulation in epithelial cells by its scaffolding properties and by binding the Rho GTPase Rac1 to maintain its activity. The functions of IQGAP1 in endothelial cells beyond angiogenesis remain unclear. We hypothesized that IQGAP1 participates in the regulation of endothelial barrier function. Silencing IQGAP1 in human microvascular endothelial cells resulted in a disruption of adherens junctions, formation of interendothelial gaps, and a reduction in barrier function. Furthermore, silencing of IQGAP1 abrogated the barrier enhancement effect of angiopoietin-1 (Angpt-1) and abolished the barrier-stabilizing effect of Angpt-1 on thrombin-stimulated cells. Coimmunoprecipitation detected binding of endogenous IQGAP1 with Rac1 at baseline that was stronger when Rac1 was activated and weaker when it was deactivated. Measurement of GTP-bound Rac1 revealed that Angpt-1 failed to activate Rac1 not only if IQGAP1 was silenced but also if cells were transfected with a mutant disabled in Rac1 binding (T1050AX2). Furthermore, a dominant-active Rac1 was sufficient to completely reverse the morphological and functional changes induced by reduction in IQGAP1. These experiments are the first demonstration of IQGAP1 regulating barrier function in any cell type. Further, our data show that Angpt-1 requires IQGAP1 as an indispensable activator of Rac1.

Complement factor C5a as mast cell activator mediates vascular remodeling in vein graft disease

Margreet R. de Vries[1,2]*, Anouk Wezel[2,3], Abbey Schepers[2], Johan Kuiper[3], Ilze Bot[3], Paul HA Quax[1,2]. [1]Einthoven Laboratory for Experimental Vascular Medicine, [2]Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands, [3]Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Introduction We previously showed that innate immunity factors complement factor C3 and mast cells are involved in atherosclerosis and vein graft disease (VGD). The role of complement-factor C5a in these processes is unknown. Mast cells express C5a-Receptors, and can be activated by complement-factor C5a. We here studied the effect of C5a on mast cell activation in VGD and subsequent accelerated atherosclerosis in ApoE-KO mice. Methods and Results In murine vein grafts (n=3-4/time point) perivascular mast cell numbers decreased after surgery (6h, 1 and 3d) and than increased from 7 to 28d. C5a and C5aR levels (RNA and protein) increased after surgery due to influx of inflammatory cells, than decreased and stabilized further on. In vitro C5a-induced mast cell-activation resulted in an increase of tryptase release by 13% and an increase MCP-1 release by 9%. To study the effect of mast cells on VGD, vein grafts were placed in mast cell deficient Kit(W-sh/W-sh) mice, in ApoE-KO mice with systemic treatment with Cromolyn (a mast cell-stabilizer) or local application of dinitrofluorobenzene (DNP, a mast cell-activator) and control mice (n=10/group). Mast cell deficiency and Cromolyn stabilization resulted, after 28d, in a decrease in vein graft-thickening (VGT) of 45% and 22% resp. DNP stimulation showed an increase of VGT of 36% moreover much more plaque erosions were seen in the DNP treated group which was accompanied by extensive fibrin depositions. Local C5a application resulted in an increase of 79% of VGT accompanied by an increase in perivascular mast cells and macrophage influx of 60%. Systemic application of a C5aR-antagonist resulted in decreased VGT (40%), and a reduction in number of MC by 40% and macrophage content of 33% To assess the direct activation of mast cells by C5a, mice were treated with C5a and Cromolyn, VGT was decreased by 54% compared to C5a-treated mice, to the level of Cromolyn treated mice. This was accompanied by a decrease in macrophage content of 13%. Conclusion These data provide evidence that complement factor C5a induced mast cell activation is highly involved in vein graft disease and may form targets to prevent cardiovascular diseases.

Cancer predisposes peripheral neutrophils to generate extracellular DNA traps: A new link to cancer-associated thrombosis

Mélanie Demers[1,2]*, Daniela S. Krause[3], Daphne Schatzberg[1], Kimberly Martinod[1,4], Jaymie R. Voorhees[1], Tobias A. Fuchs[1,2], David T. Scadden[3], and Denisa D. Wagner[1,2].
[1]From the Immune Disease Institute, Boston, MA, USA, the Program in Cellular and Molecular Medicine, Children's Hospital Boston, Boston, MA, USA, [2]Department of Pediatrics, Harvard Medical School, Boston, MA, USA, [3]The Center for Regenerative Medicine, Massachusetts General Hospital, Boston, MA, USA, [4]Graduate Program in Immunology, Division of Medical Sciences, Harvard Medical School, Boston, MA, USA

Thrombosis is the second most common cause of death in cancer patients. Even in the absence of obvious thrombosis, cancer patients have a hypercoagulable condition without a clear etiology. Recently, our group has shown that the generation of neutrophil extracellular traps (NETs) provides a scaffold and stimulus for thrombus formation. Since a myeloid leukocytosis is often associated with the development of malignancies, we hypothesized that cancer could create a systemic environment that would predispose neutrophils to the generation of extracellular DNA traps. Using murine models of chronic myelogenous leukemia (CML), mammary and lung carcinoma, we show that peripheral blood neutrophils from leukemic mice and tumor-bearing mice are more prone to NET formation in vitro. At late stages of the disease, cancer-associated neutrophilia leads to spontaneous NET formation that correlates with presence of DNA in the plasma. Moreover, our results show that simulation of a minor systemic infection in tumor-bearing mice, but not control mice, results in the release of large quantities of chromatin which induces an early prothrombotic state, as assessed by tail bleeding time. Our results show that by activation of the systemic environment cancer predisposes the host to an exacerbated innate immune response that results in a pro-coagulant state. Taken together, our data identify extracellular chromatin released through NET formation as an important cause for cancer-associated thrombosis and unveil a new target in the effort to decrease the incidence of thrombosis in cancer patients.

Overexpression of heat shock protein 60 leads to vascular smooth muscle cell proliferation through alterations in nuclear protein import

JF Deniset[1,2]*, MN Chahine[1], TE Hedley[1] E Dibrov[1], and GN Pierce[1,2]. [1]Institute of Cardiovascular Sciences, St Boniface Research Centre , Winnipeg, Canada, [2]Department of Physiology, University of Manitoba, Winnipeg, Canada

Background-Vascular smooth muscle cell (VSMC) proliferation plays an important role in pathological vascular remodelling processes such as atherosclerosis and restenosis. Heat shock protein 60 (HSP60), an upregulated stress protein in atherosclerotic plaques, is thought to be involved in stress-induced VSMC proliferation. However, the mechanism by which HSP60 contributes to proliferation remains unknown. This study aims to determine whether HSP60 can directly initiate proliferation through modifications in nuclear protein import (NPI). Methods-Rabbit primary VSMCs were transfected with an adenoviral overexpressing system encoding endogenous HSP60. NPI was measured at 48 hours by microinjecting a fluorescent import substrate into the cell cytoplasm and tracking its migration into the nucleus. NPI machinery expression, MAPKs activation, and proliferation in transfected VSMCs were examined at 48 hours via western blot analysis. Results-PCNA, an indicator of cell proliferation, was upregulated at 48hrs in HSP60 overexpressing VSMCs compared to control and adenoviral control. This increase in proliferation in HSP60 overexpressing VSMCs was accompanied with increased ERK 1/2 phosphorylation. However, no significant changes in p38 phosphorylation were observed between groups. Overexpression of HSP60 in VSMCs caused a significant upregulation in NPI machinery including NUP62, NUP153, importin-alpha, importin-beta and Ran compared to control and adenoviral control. These observed changes in NPI machinery coincide with an increased rate of NPI in VSMCs overexpressing HSP60. In addition, the increase in proliferation and NPI could be normalized with ERK1/2 inhibitor (PD 98059) administration. Conclusion- These results indicate that overexpression of HSP60 can directly cause VSMC proliferation through alterations in NPI via a ERK 1/2 dependent mechanism and an increase in the expression of transport chaperones. (This study was supported by a grant from CIHR. JFD holds a CIHR Canada Graduate Scholarship)

The role of Wnt signaling in glucose transport at the blood-brain barrier

Kavi Devraj*, Marco Reis, Jenny Zinke, Ian Simpson, Richard Hawkins, Stefan Liebner
[1]Edinger Institute of Neurology, Goethe University Medical School, Frankfurt, Hessen, Germany, [2]Department of Neural and Behavioral Sciences, College of Medicine, Pennsylvania State University, Hershey, Pennsylvania, USA, [3]Department of Biophysics and Physiology, The Chicago Medical School, Rosalind Franklin University of Medicine and Science, Chicago, Illinois, USA

Glucose is the primary metabolic fuel for the mammalian brain with about 20% in circulation utilized by brain. Glucose enters the brain via the facilitative transporters namely GLUT-1 present at the blood-brain barrier (BBB), an interface made up of microvascular endothelial cells supported by pericytes and astrocytes. The BBB functions to maintain the CNS homeostaisis by preventing access to circulating toxins by the presence of tight junctions between the endothelial cells and efflux transporters such as P-glycoprotein expressed on their plasma membranes. Prior studies indicated that GLUT-1 exists in different conformations in the luminal and abluminal plasma membranes of the BBB. We extended these studies by using a combination of 2D-PAGE/Western blotting and immunogold electron microscopy, and determined that these different conformations are exhibited in vivo and arise from differential phosphorylation of GLUT-1 and not from alternative splicing or altered O- or N-linked glycosylation (Devraj K et al, 2011). While glucose transport is not rate-limiting in physiological conditions it can become one under situations of heightened demand such as hypoxia, hypoglycemia. Malignant brain tumors such as glioblastoma become hypoxic, thus turning towards glycolytic metabolism for tumor growth and progression. It was reported by Liebner et al. (2008), Stenman et al. (2008), and Daneman et al. (2009) that Wnt signaling is critically involved in induction and maturation of the BBB, which includes GLUT-1 as an important target that is regulated by this pathway. We are currently studying the role of Wnt signaling on glucose transport at the BBB in healthy and in tumor-transplanted mice. Our preliminary data using vessels isolated from control and Wnt-1 expressing tumors surprisingly indicate no change in GLUT-1 levels, however the activity of the transporters is yet to be investigated.

The influence of chylomicron remnants on macrophage phenotype: modulation by fatty acid composition and oxidative state

Paola Di Maggio* Charlotte Lawson Caroline P. D. Wheeler-Jones Kathleen M. Botham.
The Royal Veterinary College, London, United Kingdom

Previous work has demonstrated that chylomicron remnants (CMR), lipoproteins carrying dietary lipids, are pro-atherogenic since they induce macrophage foam cell formation. In contrast, they have also been shown to exert anti-inflammatory effects, including down-regulation of nuclear factor-kB activity. Macrophages are known to exhibit both pro- and anti-inflammatory features depending on various environmental stimuli. In response to activating signals, they can be polarized towards differentiated phenotypes, ranging from the pro-inflammatory classically-activated M1, to the deactivated M2c, passing through the alternatively-activated M2a. Recent studies have also identified a novel macrophage phenotype Mox, with a distinct gene expression pattern that develops in response to oxidative tissue damage, and promotes transcription of antioxidant genes involved in the regulation of the cell redox status. The dual activity of CMR on macrophages suggests that these lipoproteins may positively influence macrophage polarization from a pro-inflammatory (M1) to an alternative (M2-like) phenotype, thus switching the expression of mediators from a pro-atherogenic to a protective, anti-inflammatory state. In this study we investigated the effects of CMR on macrophage phenotypes, and how they are influenced by the fatty acid composition and the oxidative state of the particles. Macrophages derived from the human monocyte cell line THP-1 were incubated with CMR-like particles (CRLPs) containing saturated fatty acids (p-CRLPs), n-3 polyunsaturated fatty acids (d-CRLPs), n-6 polyunsaturated fatty acids (normal, t-CRLPs; oxidized, o-CRLPs; or protected from oxidation, a-CRLPs) and M1-, M2a-, M2c-activating stimuli (LPS/IFN-γ , IL-4/IL-13 and IL-10, respectively). Expression of phenotype markers and the secretion of cytokines were evaluated after 6h and 24h using qPCR and ELISA. mRNA levels for heme oxygenase-1, sulforedoxin-1 and thioredoxin reductase, redox-regulated genes which are elevated in cells exposed to oxidative stress, were strongly up-regulated in d-CRLP-treated cells, and increased, to a progressively lesser extent, by exposure to o-, t- and p-CRLPs. In contrast, expression of Mox markers was blocked by a-CRLPs treatment. All CRLPs types suppressed transcription of the M1-marker interleukin-1ß, and reduced secretion of the pro-inflammatory cytokines monocyte chemoattractant protein-1 and tumor necrosis factor-αcompared to untreated cells. Interleukin-6 release by CRLP-treated cells was also reduced after 24h incubation. However, a-CRLPs showed weaker inhibitory effects on M1-marker expression as compared with the other particles. mRNAs for arginase-1 and mannose receptor (M2-markers) showed a modest increase or remained unchanged in p-, t-, d-, a-CRLP-treated as compared to untreated cells. These findings provide evidence that CMR suppress pro-inflammatory pathways and/or activate antioxidant protective mechanisms in THP-1 macrophages, depending upon the oxidative state and fatty acid composition of the particles. Collectively, our data support a key role for dietary lipids carried in CMR in regulating macrophage phenotype and have relevance for the understanding macrophage function in atherosclerosis.

The Role of basement membrane molecule, laminin α 5, in arterial endothelial cell physiology

Jacopo Di Russo[1]*, Verena Drüppel[2], Erik NTP Bakker[3], Hans Oberleithner[2], Ed vanBavel[3] and Lydia Sorokin[1]. [1]Institute of Physiological Chemistry and Pathobiochemistry, University of Muenster, Germany, [2]Institute of Physiology II, University of Muenster, Germany, [3]Department of Biomedical Engineering and Physics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

The extracellular matrix (ECM) plays a critical role in several aspects of the circulatory system. The most specialized ECM network is the basement membrane (BM) composed of laminins, type IV collagen, heparan sulphate proteoglycans and nidogens. In the vasculature, BMs underline endothelial cells (EC) and encase smooth muscle cells (SMC) and pericytes. Laminins are a family of glycoprotein heterotrimers, each composed of an α, β, and γ chain, and are considered to represent the biologically active component of BMs. The laminin αchains interact with a wide repertoire of integrin and non-integrin receptors, conveying a broad range of different intracellular signals. The vascular endothelium expresses two main laminin isoforms, laminin 511 (composed of α5, β1, and γ1 chains) and laminin 411 (composed of α4, β1, and γ1 chains). One of the important roles of the vascular endothelium is sensing and transducing the blood flow frictional force (shear stress), a critical mechanism for many blood flow depend processes. Several studies have suggested a role for laminins in sensing and transduction of shear stress by the endothelial cells via integrin mediated interactions that trigger specific intracellular signalling pathways1-2. To better understand the role of laminin in the endothelial cell BM, we have generated an endothelial cell specific laminin α5 KO mouse3. The mice are viable and fertile and immunofluorescence and electron microscope analyses show the absence of an overt defect in the endothelial BM. Preliminary analyses of ex-vivo mesenteric arteries suggest an enhanced dilation to shear in arteries lacking the endothelial laminin α5, without defects in smooth muscle function, including pressure induced contraction (myogenic response) and dose response to vasoconstrictors (phenylephrine and U46619) and vasodilators (methacholine and sodium nitroprusside). Atomic force microscopy of KO excised vessels revealed reduced flexibility of the endothelial surface, while in vitro studies, utilizing endothelial cells plated on purified laminin 511, show a concentration dependent enhancement of cortical stiffness compared to endothelial cells plated on purified laminin 411 or uncoated glass. Our data suggest that laminin α5 in the endothelial cell BM affects the mechanical properties of endothelial cells and their response to changes in blood flow and shear. Receptors responsible for this interaction and molecular mechanisms involved are currently being investigated. References: 1. Gloe T., Pohl U. (2002). News Physiol Sci. 17, 166-169 2. Jalali S. et al. (2001). Proc Natl Acad Sci USA 98, 1042-1046 3. Kisanuki et al., 2001. Development 230, 230-242.

Krüppel-like factor 2 reduces endothelial metabolic activity

Anuradha Doddaballapur[1]*, Eduard Hergenreider[1], Riekelt H. Houtkooper[2,3], Johan Auwerx[2], Stefanie Dimmeler[1], Reinier A. Boon[1]. [1]Institute for Cardiovascular Regeneration, Centre of Molecular Medicine, Goethe University, Frankfurt, Germany. [2]Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne, Switzerland. [3]Current address: Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam, The Netherlands

The flow responsive transcription factor Krüppel-like Factor 2 (KLF2) is a pivotal mediator of the quiescent state of endothelial cells. However, the precise mechanism by which KLF2 drives endothelial cells towards a quiescent state is unclear. We hypothesize that KLF2 achieves this by regulating endothelial metabolism. First, we assessed whether KLF2 reprograms endothelial metabolism at the level of the mitochondria, which are highly dynamic organelles that play a vital role in redox regulation, signal transduction and metabolism. To investigate the role of KLF2 in mitochondrial energy metabolism of endothelial cells, we overexpressed human KLF2 in HUVECs by stable lentiviral integration, to levels similar to that of prolonged pulsatile flow exposure. Bioenergetic measurements on Seahorse XF analyzer revealed that cells overexpressing KLF2 have lower oxygen consumption rate (-1.49 fold +/- 0.01, $p<0.05$), when compared to mock transduced control cells, indicative for low mitochondrial respiration. Moreover, real time PCR for mitochondrial DNA quantification confirmed that KLF2 decreases endothelial mitochondrial mass (-1.61 fold +/- 0.07, $p<0.05$). On the other hand, KLF2 overexpression resulted in a slight increase in mitochondrial membrane potential (1.2 fold +/- 0.05, $p<0.05$), as measured by JC-1 staining, indicating that mitochondrial integrity is not perturbed. Interestingly, KLF2 did not induce acidification, a measure for glycolytic lactate production, indicating that lower oxygen consumption is not compensated by increased glycolysis. In line with this finding, we also observed that KLF2 reduces glucose uptake (-1.23 fold +/- 0.05, $p<0.05$), as assessed by flow cytometry. In summary, metabolic and mitochondrial function analyses showed that overexpression of KLF2 in endothelial cells reduces metabolic activity, and thus contributes to maintaining an atheroprotective and quiescent state of the endothelium.

Homing of normal and malignant human B cells to lymphoid organs is controlled by Junctional adhesion molecule C (JAM-C)

C. Donate[1]*, C. Ody[2], T. Mckee[3], S. Ruault-Jungblut[1], N. Fischer[4], P. Ropraz[2], B.A. Imhof[2], T. Matthes[1]. [1]Hematology Service, University Hospital Geneva, Switzerland, [2]Department of Pathology, University Medical Center, Geneva, Switzerland, [3]Clinical Pathology Service, University Hospital Geneva, Switzerland, [4]Novimmune SA, Plan-les Ouates, Geneva, Switzerland

Homing of malignant B cells to bone marrow and secondary lymphoid organs is of critical importance in disease progression of lymphoproliferative syndromes. Junctional adhesion molecule C (JAM-C) is expressed by vascular endothelium and human, but not mouse B lymphocytes. The level of JAM-C expression defines B cell differentiation stages and allows the classification of marginal zone derived (JAM-C positive) and germinal center derived (JAM-C negative) B cell lymphomas. In the current study, we investigated the role of JAM-C in the migration of normal and malignant B cells. Human B cells were isolated from peripheral blood of healthy donors and lymphoma patients. To study the role of JAM-C in B cell migration, B cells were injected i.v. into NOD/SCID mice and homing of cells to lymphoid organs (bone marrow, spleen, lymph nodes) was analyzed one hour later by flow cytometry and immunohistochemistry. In parallel experiments, B cells were incubated with anti-JAM-C, anti-alpha-4 integrin or combinations of both antibodies prior to injection into mice. To identify the interactions of JAM-C on B cells, adhesion and surface plasmon resonance assays were performed. In vivo studies demonstrated that JAM-C pos B cells from mantle cell lymphoma and marginal zone lymphoma have a reduced capacity to home to lymph nodes (decrease of 77% in number of cells compared to normal B cells). Furthermore, treatment with anti-JAM-C antibodies reduced the homing of normal and JAM-C pos lymphoma B cells to lymph nodes, bone marrow and spleen by 50-60%. This contrasted to the reduced homing of B cells into bone marrow and lymph nodes, but not into spleen, when the cells were incubated with anti-alpha-4 antibodies prior to injection. Interestingly, combination of both antibodies resulted in inhibited homing into the three lymphoid organs. Plasmon resonance studies identified JAM-B as the major ligand for JAM-C while homotypic JAM-C interactions remained at backround levels. Accordingly, anti-JAM-C antibodies blocked adhesion of JAM-C expressing B cells to its ligand JAM-B, and immunofluorescence analysis demonstrated the expression of JAM-B on murine and human lymphatic endothelial cells. Our results show for the first time a functional role of JAM-C in B cell homing to lymphoid organs. Targeting JAM-C could thus constitute a new therapeutic strategy to prevent lymphoma cells from reaching supportive microenvironments not only in the bone marrow and lymph nodes but also in the spleen.

Patients with peripheral arterial disease show an ameliorated proinflammatory status over 6 months parallel to an increased walking distance

Joern F. Dopheide[1]*, Vivane Obst[1], Christopher Doppler[1], Martin Scheer[1], Marie-Christine Radmacher[1], Markus Radsak[2], Tommaso Gori[1], Ascan Warnholtz[1], Christian Fottner[3], Thomas Münzel[1], Andreas Daiber[1] and Christine Espino[1]. [1]2nd Medical Department, Universitätsmedizin Mainz, Johannes-Gutenberg-University, Mainz, Germany, [2]3rd Medical Department, Universitätsmedizin Mainz, Johannes-Gutenberg-University, Mainz, Germany, [3]1st Medical Department, Universitätsmedizin Mainz, Johannes-Gutenberg-University, Mainz, Germany

Background- Atherosclerosis is a disease triggered by diverse exogenous stimuli and sustained by chronic inflammatory processes as well as reactive oxygen species (ROS). Monocytes and Dendritic cells (DC) play a crucial role in regulating chronic inflammatory process. Methods- Peripheral blood leucocytes of 60 patients with peripheral arterial disease (PAD) with intermittent claudicatio (IC: Fontaine State II a and b) and 30 healthy controls were analysed from whole blood by flow cytometry (FACS-Canto, bd-biosciences). Monocytes and DC were identified by different gating strategies in relation to size and granulation (FSC/ SSC) and surface molecules (CD14/CD45 for monocytes; HLA-DR/ CD11c/CD123 for DC) and analysed for CD14/ CD16 and M-DC8 (Slan) on monocytes, and for CD86, CD80, CD40 and CD83 on DC. ROS formation from leukocytes was determined by chemiluminescence (L-012 ECL) assays in whole blood after addition of phorbol-12,13-dibutyrate (PDBu). Clinical data including CRP, fibrinogen, ankle-brachial-index (ABI) and walking distance were analysed after standard protocol. Follow-up was performed after 6 months. Results- PAD patients show a significant increased proportion of proinflammatory CD14-CD16++ and M-DC8+ monocytes in comparison to healthy controls (all $p<0.001$). Myeloid and plasmacytoid DC (mDC, pDC) did not differ between patients and controls. After 6 months, we observed a shift in PAD patients towards a significant increased proportion of antiinflammtory CD14++CD16+ monocytes ($p<0.01$), and parallely a decrease in proinflammatory CD14-CD16++ and M-DC8+ monocytes (both $p<0.05$) compared to the beginning of the study. Similarly, mDC of PAD patients had a significant decreased expression of CD86, CD80, CD40 and CD83 (all $p<0.05$), whereas pDC showed no changes after 6 months. ROS production by PDBu decreased similarly after 6 months ($p<0.001$). CRP, fibrinogen and ABI did not differ significantly after 6 months. However, the pain free walking distance increased about 250 m ($p<0.05$). Conclusions- PAD patients show a decreased proinflammatory phenotype on peripheral blood monocytes and DC, as well as a decreased ROS production of after 6 months parallel to an increased walking distance. Here, we show in vivo insights in the process of atherogenesis of patients with PAD in the state of intermittent claudication indicating an amelioration of the disease through anti-inflammatory strategies like intensive exercise training.

Fzd7 regulates endothelial cell junctions

Nancy Ferreira Tojais, Claire Peghaire, Fréderic Larrieu, Catherine Moreau , Thierry Couffinhal, Cécile Dupla, Pascale Dufourcq*. Univ. De Bordeaux, Adaptation cardiovasculaire à l'ischémie U1034, F-33600 Pessac, France. INSERM, Adaptation cardiovasculaire à l'ischémie, U1034, F-33600 Pessac, France

Recent reports highlight the important role of the Wnt/Frizzled (Fzd) pathway in vascular morphogenesis and angiogenesis, through the control of endothelial cell (EC) differentiation, proliferation, cell polarity and survival. Previously, we evidenced that frizzled7 (Fzd7) interacted with a Wnt pathway modulator sFRP1 and was involved on endothelial cell morphogenesis. Here, using a murine embryonic stem cell model, we demonstrated that fzd7 expression increases during embryoid body commitment into an EC lineage. We showed that Fzd7 was expressed in CD31+ cells and in vascular tubes during the kinetic of EB differentiation. Fzd7 expression was observed at the cell membrane and accumulated at points of endothelial cell-cell contact. Fzd7 colocalized with VE-cadherin and beta catenin-positive junctions. To examine potential interactions between Fzd7 and VE-cadherin, HEK-293 Tg cells were co-transfected with full length Fzd7 (Fzd7-myc or Fzd7-GFP tagged) and with a VE-cadherin encoding plasmid (VE-cadherin-DsRed). Immunoprecipitation experiments demonstrated an interaction between Fzd7 and VE-Cadherin. To analyze the role of Fzd7 in endothelial cell function, effect of fzd7 knock down (KD) by siRNAs was investigated in HUVEC. KD of fzd7 had no effect on endothelial cell proliferation. In contrast KD of fzd7 results in impairment in EC orientation, organization of cell-cell junctions, and a strong increase in inter-endothelial permeability. In order to study the role of fzd7 in vivo, fzd7 was deleted specifically in EC using CreLox strategy. fzd7ECKO mice were viable. We showed that genetic fzd7 inhibition in the endothelium increased basal and VEGF-stimulated permeability in vivo. Our findings establish a novel role for Fzd7 in vascular biology, and demonstrate crosstalk between the Fzd7-induced pathway and cell-cell adhesion in EC, which is required for the maintenance of endothelium integrity.

New insights into the pro-inflammatory activities of angiopoietin-1 (Ang1) on neutrophils; induction of MIP-1beta synthesis and release

Dumas, Elizabeth[1,2]* Martel, Karine[1,2] Neagoe, Paul-Eduard[1] and Sirois, Martin G[1,2]. [1]Research Center, Institut de cardiologie de Montréal, Montréal, Canada, [2]Pharmacology department, Medicine faculty, Université de Montréal, Montréal, Canada

We previously reported that both angiopoietins (Ang1 and Ang2) exert pro-inflammatory activities on endothelial cells (ECs) by their capacity to promote platelet activating factor (PAF) synthesis and P-selectin translocation. In addition, we reported the expression of angiopoietin receptor Tie2 on human neutrophils and the capacity of both angiopoietins to induce neutrophil PAF synthesis, CD11b/CD18 activation and migration. More recently, we observed differential effects between Ang1 and Ang2 on the induction of two specific pro-inflammatory activities in neutrophils. For instance, Ang1, but not Ang2, does: 1) induce interleukin-8 (IL-8) synthesis and release, and 2) prolong neutrophil viability through IL-8 release, which delays the entry of the cells into apoptotic and necrotic phases. Since Ang1 has the capacity to promote IL-8 synthesis and release by the neutrophils, we addressed whether Ang1 and/or Ang2 could modulate the synthesis and release of other pro-inflammatory cytokines. Human neutrophils were isolated from venous blood of healthy volunteers, and quantification of protein synthesis and release of elected biomarkers was assessed by ELISA. We observed that Ang1, but not Ang2, is capable to mediate macrophage inflammatory protein-1 beta (MIP-1β; CCL4) synthesis and release by the neutrophils. Basal neutrophil synthesis and release of MIP-1β was undetectable from 0 to 6 hours post-isolation, whereas at 24-hour post-stimulation, the basal synthesis and release was approximately 95 and 35 pg/million neutrophils, respectively. Treatment with Ang1 (up to 10 nM) and IL-8 (positive control; 25 nM) increased neutrophil MIP-1β synthesis by 310 and 307% (raising it from 94.6 to 293.4 and 290.8 pg/million neutrophils) and its release by 388 and 413% (raising it from 34.4 to 133.3 and 142.0 pg/million neutrophils) respectively. Neutrophils pretreatment with blocking anti-Tie2 antibodies did not inhibit the capacity of Ang1 to induce MIP-1β synthesis and release by the neutrophils, suggesting that this effect of Ang1 is Tie2-independent and could rather be mediated upon integrins interactions. Furthermore, pretreatment with p38MAPK and p42/44MAPK inhibitors (SB203580 and U0126, respectively) completely abrogated Ang1 effects on MIP-1β synthesis and release, while pretreatment with Akt inhibitors (triciribine, wortmannin and LY294002) did not block Ang1-induced MIP-1β synthesis and release from neutrophils. In addition, pretreatment with NF-κB inhibitors (Bay7085, Bay7082 and IKK inhibitor VII) also completely abrogated the capacity of Ang1 to induce MIP-1β synthesis and release. Finally, to assess the capacity of Ang1 to promote MIP-1β DNA to mRNA transcription and protein synthesis, neutrophils were pretreated with a transcription inhibitor (actinomycin D), and a protein synthesis inhibitor (cycloheximide). We observed that Ang1-induced MIP-1β synthesis and release was completely blocked by both inhibitors, suggesting that Ang1-induced MIP-1β synthesis and release is dependent on its DNA transcription and mRNA translation. Our study is the first one to report Ang1 capacity to induce MIP-1β synthesis and release from the neutrophils, and that these effects are mediated through the activation of p38MAPK, p42/44MAPK and NF-κB signaling pathways. Since MIP-1β is a chemoattractant and/or an activator of monocytes, macrophages, natural killer cells and Th1 lymphocytes, these findings suggest that Ang1 could activate several leukocytes through neutrophil-MIP-1β release.

Activation of SIRT1 by resveratrol maintains a contractile phenotype and prevents proliferation in vascular smooth muscle cells

Jochen Dutzmann*, Jan-Marcus Daniel, Fabian Krämer, Sabina Vogel, Christian W. Hamm, Daniel G. Sedding. Institute for Molecular Cardiology (UGMC), Justus-Liebig-University, Giessen, Germany

Purpose: De-differentiation and the subsequent proliferation of vascular smooth muscle cells (VSMCs) are pivotal steps in the progression of atherosclerosis and neointima formation. Resveratrol is a grape polyphenol and a potent activator of the class III histone deacetylase SIRT1. The effects of resveratrol and SIRT1 on de-differentiation of activated VSMCs remain unknown. Methods: Resveratrol was administered at different concentrations (10 µM and 20µM) to human VSMCs. Knock-down of SIRT1 was achieved using specific siRNA, and over-expresion of SIRT1 was performed using an adenoviral vector system for wild-type SIRT1 or a deacetylase-inactive SIRT1 mutant. VSMCs were analyzed using qPCR and Western blotting, BrdU and TUNEL assays, as well as MALDI-TOF analysis of picked spots obtained by 2D gelelectrophoresis. Results: Treatment of activated VSMCs with resveratrol accounted for a dose-dependent reduction in the proliferation rate. In addition, the decreased proliferation was associated with a more differentiated phenotype of VSMCs, as determined by higher expression levels of smooth muscle-myosin heavy chain or calponin. The characterization of differentially regulated proteins by MALDI-TOF analysis revealed that resveratrol induced a highly significant up-regulation of 37 proteins. The most prominent cluster identified consisted of cytoskeletal and contractile proteins including actin, caldesmon, vinculin, tubulin, alpha-actinin, vimentin, tropomyosin. In contrast, the largest cluster of the 11 significantly down-regulated proteins accounted for proteins controlling cellular metabolism and energy regulation, including pyrovate kinase isoenzymes, fructose-bisphospate aldolase A, 3-hydroxyisobutyryl-CoA hydrolase, pyrovate dehydrogenase and Galectin-1. In VSMCs transfected with specific SIRT1-siRNA, the effects of resveratrol on inhibiting VSMC proliferation were significantly reduced. Moreover, over-expression of SIRT1 largely reproduced the effects of resveratrol on VSMCs, indicating that activation of SIRT1 by resveratrol represents a key mechanism for maintaining a differentiated and quiescent VSMC phenotype. Conclusion: Activation of SIRT1 by resveratrol induces a contractile phenotype in VSMCs and prevents proliferation in response to stimuli occurring during vascular proliferative diseases. Therefore, activating SIRT1 reflects an interesting therapeutic strategy to prevent the development of atherosclerosis or neointima formation.

Sphingosine kinase-1 regulates adhesion molecule expression for and neutrophil trafficking during the early phase of allergic inflammation

Wai Yan Sun[1,2,3], Latasha Abeynaike[4], Lisa Ebert[1,3]*, Samantha Escarbe[1], Stuart Pitson[3,5], Michael Hickey[4] and Claudine Bonder[1,2,3]. [1]Centre for Cancer Biology, SA Pathology, Adelaide, South Australia, [2]School of Medicine, University of Adelaide, Adelaide, South Australia, [3]CRC for Biomarker Translation, La Trobe University, Victoria, Australia, [4]Centre for Inflammatory Diseases, Monash University, Victoria, Australia, [5]School of Biochemistry, University of Adelaide, Adelaide, South Australia

Rapid recruitment of neutrophils to a site of inflammation is associated with allergic diseases, such as asthma and anaphylaxis. Although anti-histamines and steroids are the mainstay of treatment for symptomatic relief, their effectiveness is varied and the current health costs for allergy are still more than $7 billion yearly. Thus, a better understanding of acute allergic reactions is required. The classical paradigm for cell recruitment suggests that selectins, integrins and cellular adhesion molecules stabilize the rolling of neutrophils on the endothelium, enabling adhesion and emigration from the vasculature into inflamed tissues. P-selectin is stored in Weibel Palade bodies of vascular endothelial cells (ECs) and is rapidly exocytosed to the cell surface upon histamine stimulation for the recruitment of neutrophils. Understanding the mechanisms by which P-selectin regulates neutrophil recruitment may reveal novel approaches to controlling acute inflammation. This study examines the role of sphingosine kinase (SK)-mediated P-selectin expression on ECs for the rapid recruitment of neutrophils. SK is a highly conserved lipid kinase that catalyses the phosphorylation of sphingosine to form sphingosine-1-phosphate. SK is ubiquitously expressed but stored at varying levels in different cell types. Our results demonstrate that histamine-induced P-selectin expression on human umbilical vein ECs requires SK activity. Moreover, activation of ERK1/2 is integral to histamine-induced P-selectin expression but not exogenous S1P and the S1P receptors. We have shown that histamine-induced neutrophil rolling along the vasculature in vitro and in vivo is SK dependent. In addition, administration of FTY720 (an approved pro-drug for the treatment of multiple sclerosis) attenuates histamine-induced neutrophil recruitment. Overall, this study proposes that SK maybe a target for developing new therapeutics for the prevention of excessive neutrophil recruitment during inflammation which results in acute and fatal anaphylaxis.

Integrin α7β1 is a redoxregulated target of hydrogen peroxide in vascular smooth muscle cell adhesion

Flávia Figueiredo de Rezende[1], Augusto Martins Lima[1], Stephan Niland[1], Ilka Wittig[2], Heinrich Heide[2], Katrin Schröder[3], Johannes A. Eble[1]*. [1]Excellence Cluster Cardio-Pulmonary System, Vascular Matrix Biology, Center for Molecular Medicine, [2]Molecular Bioenergetics Group, Faculty of Medicine, Goethe-University Frankfurt, [3]Center for Cardiovascular Physiology, Frankfurt University Hospital, Frankfurt am Main, Germany

Upon adhesion to laminin-111, aorta smooth muscle cells initially form membrane protrusions with an average diameter of 2.9 µm. We identified these protrusions also as subcellular areas of increased redox potential. Hence, we termed these areas oxidative hotspots. They are spatially and temporally transient during the early stage of adhesion and depend on the activity of the H2O2-generating NAPDH oxidase4 (Nox4). Within these oxidative hotspots, cysteine residues of proteins were oxidized to sulfenic acid (-SOH), a reversible modification, which can be detected with dimedone. Presumably located close to or within these oxidative hotspots, integrin α7β1 mediates adhesion and migration of vascular smooth muscle cells to laminins, which are characteristic components of the pericellular basement membrane. Using protein chemistry and mass spectrometry, two sites within the integrin α7 subunit, comprising 6 cysteine residues, are selectively oxidized upon H2O2 treatment: one located in its genu region and another one within its calf 2 domain. The genu region is a hinge, around which the integrin domains pivot between a bent/inactive and an upright/active conformation. Also, cysteine oxidation within the calf 2 domain permits conformational changes related to integrin activation. H2O2 treatment of α7β1 integrin in concentrations of up to 100 µM increases integrin binding activity to laminin-111, suggesting a physiological redoxregulation of α7β1 integrin.

Role of endothelial Notch signalling in arteriogenesis

Manuel Ehling[1]*, Rui Benedito[1] and Ralf H. Adams[1,2]. [1]Max-Planck-Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, [2]University of Muenster, Faculty of Medicine Muenster, Germany

Notch receptors and their Delta-like and Jagged ligands play important roles in the regulation of angiogenic blood vessel growth. In addition to their expression in growing capillary beds, high levels of Notch pathway activity have been reported for the endothelium of developing and adult arteries.To analyze the function of Notch in developing arteries without interfering with the process of sprouting and angiogenesis, we made use of the transgenic mouse line Bmx(PAC)-CreERT2, which expresses a tamoxifen-inducible Cre fusion protein only in arterial endothelial cells.We found that arterial deletion of the gene encoding Rbp-Jκ, a transcriptional regulator that is indispensable for signalling downstream of activated Notch, led to defects in the coverage by smooth muscle cells (SMCs). In contrast, no overt differences were detected in the organization and branching pattern of arteries. Furthermore, we found that the Notch ligand Delta-like 4 (Dll4) is the only endothelial Notch ligand involved in this process. In contrast, the arterial EC-specific deletion of Dll1 nor Jagged1 had no effect on smooth muscle cell coverage. Using tamoxifen administration at different stages of development, we found that endothelial Notch signalling is only required at certain stages of arterial maturation. Our work suggests that Dll4-mediated Notch signaling is important for proper arterial wall assembly, but becomes dispensable once arteries are fully developed.

Plasma proteomics: An insight into the pathological activity of Abdominal Aortic Aneurysms (AAA)

Saima Ehsan[1]*, David Boocock[2], Graham Ball[2], Karl Herbert[1], Robert Sayers[1], Matthew Bown[1]. [1]University of Leicester, Leicester, United Kingdom, [2]Nottingham Trent University, Nottingham, United Kingdom

Background: Plasma proteomics for biomarker discovery is increasingly applied for improving clinical diagnostic and prognostic tools. However such an approach is equally applicable to study the pathological behaviour and host response of a disease in vivo. Such information may guide future therapy by identification of specific pharmacological targets. We studied the differential plasma protein expression of patients with AAA compared to that of controls. Our aim was to discover proteins which are associated with the disease activity and can be used as clinical biomarkers. Methods: This is a prospective case-control study. Venous plasma was collected from patients with AAA (n=13) and ultrasound screened matched controls (n=13) with clean phenotypes using standardized sample collection protocols. The workflow comprised of highly abundant plasma protein depletion (Seppro® IgY 14 Spin Columns, Sigma-Aldrich Co), sample clean-up using C18 reverse phase chromatography (ZipTip® Pipette Tips, Millipore MA USA) and tryptic digestion followed by mass spectrometric analysis (MALDI ToF MS). MS data thus obtained was analysed using artificial neural networks (ANN) to build a classifier to discriminate patients with AAA from controls. Once a statistically strong classifier was discovered tandem MS (LC MALDI) was carried out to discover protein identities of the classifier ions. Results: A classifier of AAA comprising of a combination of 4 ions achieved sensitivity and specificity of 93% and validation performance of 87% for discriminating patients with AAA from controls. The component ions were mapped to Kininogen-1, Inter-alpha-trypsin inhibitor heavy chain four and an immunoglobulin fragment (Ig mu chain C region) within the mass tolerance limit of 0.4 Da. Retinol-binding protein was traced to the classifier; however this protein was outside the cut off (1.04 Da) of the tolerance limit. Discussion: AAA classically has been associated with pathological inflammation, thrombosis and extracellular remodelling of the aortic wall. The proteins which show significant differential expression in patients with AAA in this study support this hypothesis. The over expression of Kininogen-1 and inter Inter-alpha-trypsin inhibitor heavy chain four reflect inflammation and thrombogenic tendency. A higher expression of Ig mu chain C region indicates host response by activation of immunogenic pathways. However all of these proteins are associated with host response and are not disease specific. Also whether this host response is a driver for AAA growth and rupture or not requires further investigations. Conclusion: The proteins identified in this study are biologically plausible markers of AAA. The classifier may have the potential for clinical translation as biomarker for AAA. Verification of these results using Enzyme-linked Immunosorbent Assay (ELISA) is currently being carried out.

Regulation of vascular remodeling during angiogenesis by pericytes

Hanna M. Eilken[1]*, Ann L. B. Seynhaeve[2], Susanne Adams[1], Inga Schmidt[1], Ralf H. Adams[1,3]. [1]Max Planck Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, and [3]University of Muenster, Faculty of Medicine, Muenster, Germany [2]Laboratory Experimental Surgical Oncology, Department of Surgery, Erasmus MC, Rotterdam, The Netherlands

The blood vessel system consists of endothelial cells and perivascular cells, namely vascular smooth muscle cells and pericytes. While it is well appreciated that pericytes stabilize endothelial tubes, support the blood brain barrier and share many characteristics with mesenchymal stem cells, their precise function during angiogenesis remains poorly understood. To study the role of pericytes during mammalian angiogenesis and the involved molecular mechanisms in vivo, we have generated a novel transgenic mouse line by BAC recombineering in which the expression of Tamoxifen-inducible Cre recombinase is restricted to PDGFRβ+ cells. Combining these Pdgfrb(BAC)-CreERT2 transgenic mice with suitable fluorescent Cre reporter mouse lines (e.g. Rosa26-YFP) and perinatal injection of Tamoxifen induced the expression of YFP in perivascular cells of the central nervous system, the heart, the skin and in experimental tumor models. To address the role of pericytes during blood vessel growth, we depleted PDGFRβ+ cells in mice shortly after birth using a Diphtheria Toxin A driven cellular suicide mechanism. Analysis of the postnatal retinal vasculature showed that pericyte depletion led to strong alterations in the growth, expansion and architecture of the endothelial network. While the phenotypic characterization of these mutants is still ongoing, our work has established a powerful genetic model for the characterization of pericyte function under physiological and pathological conditions.

Protein C inhibitor on microparticles - what are the functional roles?

Einfinger K.* and Geiger M. Institute of Vascular Biology and Thromboses Research/Medical University of Vienna, Austria

Protein C inhibitor (PCI, SERPINA5, PAI3) is a secreted, non-specific serine protease inhibitor (serpin) with a plasma concentration of 5 µg/mL (90 nM) (Silverman 2001; Geiger 2007). PCI inhibits several proteases in blood coagulation, fibrinolyses, and reproduction. During the PCI-mediated inhibition of its target proteases, PCI forms an acyl-bond enzymeserpin complex (Suzuki 1984). This complex formation is enhanced by heparin or negatively charged dextran sulfates (Suzuki 1983; Suzuki 1984). PCI also binds to specific negatively charged phospholipids such as phosphatidylserine (PS), oxidized PS and oxidized phosphatidylethanolamine (PE). Phospholipid-bound PCI efficiently stimulate the inhibitory activity towards aPC and thrombin in a heparin-like manner (Nishioka 1998; Malleier 2007). Thus, binding of PCI to oxidized or unoxidized phospholipids could lead to the accumulation of PCI at sites where these phospholipids are surface exposed. PE and PS are normally localized to the inner leaflet of the cell membrane, but are exposed on microparticles (MPs) (Hugel 2005). MPs are cell membrane-derived vesicles (size: 0.1-1 µm) with important roles in coagulation, inflammation, and endothelial function. The type of stimulus that causes their release determines number, functional characteristics, and biochemical composition of circulating MPs (Freyssinet 2010; Leroyer 2008). In this study we investigated whether PCI can be found on the surface of MPs. In order to determine whether PCI is incorporated into MPs during membrane blebbing various cell lines were serum-starved or stimulated with staurosporine to generate MPs. First data show that MPs shed from apoptotic/necrotic cells incorporate endogenous PCI during membrane blebbing. Furthermore, we could show by flow cytometry the presence of PCI on MPs isolated from the blood of healthy donors. These MPs expose PS on their surface as judged by Annexin V-binding and are mainly derived from platelets and/or megakaryocytes. To evaluate the inhibitory activity of MP-bound PCI towards aPC or thrombin the remaining amidolytic activity was determined from the cleavage of a chromogenic substrate. We could show that MP-bound PCI did not inhibit aPC or thrombin. These MPs also did not stimulate the inhibition of aPC or thrombin by exogenously added PCI. Furthermore, MPs were already saturated with plasma PCI since they did not bind exogenously added labelled PCI as seen by flow cytometry analyses. Immunoprecipitation of MP-lysates with anti-PCI-IgG was performed to identify the binding partners of PCI on/in MPs. Mass spectrometry revealed the co-precipitation of complement proteins with PCI. Thus, we investigated the role of PCI in the complement cascade. Western blot analyses showed cleavage of PCI by purified C1s, a serine protease of the classical complement pathway. However, PCI did not inhibit C1s amidolytic activity. We therefore propose that PCI is a substrate for C1s. In vivo this may interfere with complement activation as well as lead to the inactivation of PCI at site of complement activation.

Unique cell type-specific junctional complexes in vascular endothelium of human and rat liver sinusoids

Cyrill Géraud[1], Konstantin Evdokimov[1]*, Beate K. Straub[2], Wiebke K. Peitsch[1], Alexandra Demory[1], Yvette Dörflinger[1], Kai Schledzewski[1], Astrid Schmieder[1], Peter Schemmer[3], Hellmut G. Augustin[4], Peter Schirmacher[2], Sergij Goerdt[1]. [1]Department of Dermatology, Venerology and Allergology, University Medical Center and Medical Faculty Mannheim, University of Heidelberg, and Center of Excellence in Dermatology, Mannheim, Germany, [2]Department of Pathology, University Clinic Heidelberg, University of Heidelberg, Germany; [3]Department of General and Transplantation Surgery, Ruprecht-Karls-University, Heidelberg, Germany, [4]Joint Research Division Vascular Biology, German Cancer Research Center, Heidelberg, and Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

Liver sinusoidal endothelium is strategically positioned to control access of fluids, macromolecules and cells to the liver parenchyma and to serve clearance functions upstream of the hepatocytes. While clearance of macromolecular debris from the blood is performed by liver sinusoidal endothelial cells (LSECs) using a delicate endocytic receptor system, vascular permeability and cell trafficking are controlled by transcellular pores, i.e. the fenestrae, and by intercellular junctional complexes. In contrast to blood vascular and lymphatic endothelial cells in other organs, the junctional complexes of LSECs have not yet been consistently characterized in molecular terms. In a comprehensive analysis, we have shown that LSECs express the typical proteins found in endothelial adherens junctions, i.e. VE-cadherin as well as α-, β-, p120-catenin and plakoglobin. Claudin-5 and occludin, transmembrane proteins typical of endothelial tight junction, were not expressed by rat LSECs while heterogenous immunreactivity for claudin-5 was detected in human LSECs. In contrast, junctional molecules preferentially associating with tight junctions such as JAM-A, B and C and zonula occludens proteins ZO-1 and ZO-2 were readily detected in LSECs. Remarkably, among the JAMs JAM-C was considerably over-expressed in LSECs as compared to lung microvascular endothelial cells. In conclusion, our data prove the existence of organ-specific intercellular junctions between endothelial cells of the liver sinusoids characterized by co-occurrence of endothelial adherens junctions proteins, and of ZO-1and -2, and JAMs. The comprehensive molecular characterization of the specialized intercellular junctions between LSECs corroborates previous ultrastructural findings and provides a framework for further functional investigations of the transendothelial barrier of liver sinusoids in numerous pathological conditions ranging from hepatic inflammation to formation of liver metastasis.

Attenuated, or inversed dicrotic wave of toe photoplethysmography is predictive of hypotension: Observation and application in a patient with severe hypoxic-ischemic brain injury

Jin Fan*, ZhiQing Fu, Jian Li, Qing Zhu, XiuJin Zhang, QuanJin Si, YuTang Wang. First department of Geriatric Cardiology of 301 Hospital, Beijing, China

The call for early detection of hypotension creates a heavy demand for new method that can be used for beat-by-beat continuous non-invasive blood pressure changing trend monitoring in clinical practice. An emerging type of BP monitors using PPG waves has drawn great attentions for this application because it is comfortable and capable of providing continuous readings. We assessed the feasibility of using toe PPG in the blood pressure change trend monitoring by comparing blood pressure measurements acquired by the conventional intermittent NIBP equipped on the same monitor in a patient suffered with severe hypoxic-ischemic brain injury. In conclusion: When changing of blood pressure is more important than absolute values, beat-by-beat noninvasive toe PPG waveforms to predict low blood pressure seems reliable in critically ill patient. We demonstrated that variation of the dicrotic waves amplitude of toe PPG are associated with SBP measured by NIBP and attenuated and inversed dicrotic waves of toe PPG predict acute hypotension.

MicroRNA 10a and 92a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro

Yun Fang* and Peter F. Davies. Institute for Medicine & Engineering Department of Pathology and Laboratory Medicine University of Pennsylvania, USA

A chronic proinflammatory state precedes pathological change in arterial endothelial cells located within athero-susceptible regions. The contributions of regulatory microRNAs to this disequilibrium were investigated by artery site-specific profiling in normal adult swine. Differential expression analysis identified 34 modulated miRNAs in athero-susceptible aortic arch endothelia when compared with athero-protected descending aorta. Endothelial miR-10a was lower while miR-92a was higher in the athero-susceptible aortic arch. Endothelial transcriptome analysis identified IkB/NF-kB-mediated inflammation as the top up-regulated biological process in miR-10a knockdown cells. Phosphorylation of IκBα, a prerequisite for IκBαproteolysis and NF-kB activation, was significantly up-regulated in miR-10a knockdown HAEC and was accompanied by increased nuclear expression of NF-kB p65. The inflammatory biomarkers were elevated following miR-10a knockdown. Two key regulators of IκBαdegradation, - mitogen-activated kinase kinase kinase 7 (MAP3K7) and beta-transducin repeat-containing gene (betaTRC) - contain a highly conserved miR-10a binding site in the 3' UTR. Both molecules were regulated by miR-10a manipulations. Conversely, parallel computational prediction, in vitro cell manipulation, and luciferase assay identified endothelial miR-92a suppression of transcription factors KLF4 and KLF2. Knockdown of miR-92a in vitro resulted in partial rescue from cytokine-induced proinflammatory marker expression (monocyte chemotactic protein 1, vascular cell adhesion molecule-1, E-selectin, and endothelial nitric oxide synthase) that was attributable to enhanced KLF4 expression. Leukocyte-human arterial endothelial cell adhesion experiments supported this conclusion. Comparative expression studies of endothelium located in athero-susceptible aortic arch and athero-protected descending thoracic aorta identified significantly up-regulated MAP3K7, betaTRC as well as lower expression of KLF4 and KLF2 suggesting that the differential expressions of miR-10a and miR-92a contribute to the regulation of proinflammatory endothelial phenotypes in athero-susceptible regions in vivo.

Cyclo-oxygenase enzymes regulate the angiogenic functions of human lung microvascular endothelial cells

C E Farrar*, A C McSloy, E Garonna, T Warner, J Mitchell and CPD Wheeler-Jones. The Royal Veterinary College, London United Kingdom

There is emerging evidence that protease-activated receptors (PARs), novel G-protein coupled receptors implicated in inflammatory signalling, are important in the reparative angiogenesis associated with inflammation. We have recently shown that PARs selectively couple to inducible cyclo-oxygenase-2 (COX-2) expression to influence the angiogenic functions of human umbilical vein endothelial cells (HUVEC). The relevance of these receptors for angiogenic control of human microvascular endothelial cell function and the significance of COX enzymes for these processes are not yet defined. In this study we have investigated the functional importance of PARs in human lung microvascular endothelial cells (HLMEC). Immunoblotting studies showed that HLMEC express PAR-2 and PAR-4. To begin to define roles for these receptors in HLMEC function the kinetics of microvascular EC and HUVEC differentiation on Matrigel were analysed using real-time imaging in cells exposed to thrombin (0.01 - 1 U/ml) (cleaves PAR-1 and PAR-4), to PAR-2-(2-furoyl(2f)-LIGKV-OH) (10 μM) and PAR-4 (AYPGKF) (50 μM)-selective agonist peptides (PAR2-AP; PAR4-AP) and to VEGF (25 ng/ml). In HUVEC, all agonists, including thrombin, increased the rate of capillary-like tube formation with a maximum effect evident after 4 hours exposure. PAR2-AP, PAR4-AP and VEGF also promoted HLMEC differentiation with similar kinetics to those observed in HUVEC. In contrast, incubation with thrombin markedly suppressed tube formation by HLMEC in a concentration-dependent manner. In keeping with these data, thrombin (0.01 U/ml - 1 U/ml) was highly mitogenic for HUVEC but exerted anti-proliferative effects on HLMEC as assessed by nuclear staining with propidium iodide. Treatment with hirudin (thrombin inhibitor) rescued the anti-angiogenic effect of thrombin on HLMEC and inhibited its pro-angiogenic actions on HUVEC, confirming that these effects require thrombin's proteolytic activity. Analysis of vessel growth from aortic rings from wild-type, COX-1-/- and COX-2-/- mice cultured in Matrigel demonstrated reduced sprouting from COX-2-/- aortas whereas COX-1-/- aortas exhibited increased sprouting compared to wild-type vessels. Stimulation with thrombin, PAR2-AP and VEGF, but not PAR4-AP, induced COX-2 expression in both HLMEC and HUVEC. Incubation with the selective COX-2 inhibitor celecoxib did not affect the rate of tube formation by HLMEC in response to PAR2-AP, PAR4-AP or VEGF but partially reversed thrombin's inhibitory effects on their differentiation. Preliminary data indicate that selective pharmacological blockade of COX-1 activity with sc-560 also partially rescued the suppressive effects of thrombin on HLMEC tube formation without affecting its pro-angiogenic actions on HUVEC. These findings provide new evidence to support the importance of COX isoforms in regulating endothelial cell functions relevant for angiogenesis and emphasise the likely agonist- and tissue-specific roles for COX enzymes as modulators of these processes.

CCM1/ICAP-1 complex controls beta1 integrin-dependent extracellular matrix remodeling and vascular integrity

Eva Faurobert[1,2,3]*, Claire Rome[1,3], Justyna Lisowska[1,2,3], Sandra Manet-Dupé[1,2,3], Gwénola Boulday[4], Marilyne Malbouyres[7], Martial Balland[3,8], Michelle Kéramidas[1,3], Daniel Bouvard[1,2,3], Jean-Luc Coll[1,3], Florence Ruggiero[7], Elisabeth Tournier[1]. [1]INSERM U823, Institut Albert Bonniot, Equipe1 DySAD, Grenoble, France, [2]ERL CNRS 5284, Grenoble, France, [3]Université Joseph Fourier, Grenoble, France, [4]INSERM, UMR-S 740, Université Paris Diderot, 10 Avenue de Verdun, 75010 Paris, France, [5]Université Paris 7-Denis Diderot, Faculté de Médecine, Site Lariboisière, Paris, F-75010, France, [6]AP-HP, Groupe hospitalier Saint-Louis Lariboisiere-Fernand-Widal, Paris, F-75010, France, [7]UMR CNRS 5242 - ENS Lyon, France, [8]Laboratoire interdisciplinaire de Physique UMR CNRS 5588, Saint Martin d'Hères, France

Human Cerebral Cavernous Malformations (CCM) are clusters of hemorrhagic dilated blood vessels composed of fragile endothelium lacking mural cells and with altered sub-endothelial extracellular matrix (ECM). Familial forms result from loss-of-function mutations of CCM1, CCM2 or CCM3 genes which encode a multifunctional protein complex involved in cell-cell junction, cell polarity and acto-myosin contractility. The association of ICAP-1, a beta1 integrin negative regulator, with CCM proteins prompted us to investigate the role of ICAP-1 in vascular integrity and to address for the first time the role of CCM proteins in the interaction of the endothelial cell with the ECM. ICAP-1 deficient mouse showed a chaotic network of dilated, branched, tortuous and more permeable blood vessels. Their basal lamina and surrounding ECM presented ultrastructural defects as reported for CCM lesions. These defects correlated with abnormal fibronectin fibrils deposition as around CCM1 or CCM2 mouse lesions. We showed that beta1 integrin mediated fibronectin fibrillogenesis by endothelial cells was altered upon CCM1 or CCM2 silencing concomitantly with a strong destabilization of ICAP-1 protein. Impaired fibronectin fibrillogenesis resulted from increased beta1 integrin activation, excessive acto-myosin contractility and redistribution of cellular traction forces. We provide evidence that CCM proteins form a node regulating beta1 integrin activation and ECM remodelling in addition to controlling cell-cell junctions. Our study adds another dimension to mechanotransduction in CCM pathology by proposing that biomechanical remodeling of the microenvironment might favor expansion of the lesions.

Palmitic acid promotes pro-oxidant adaptor protein p66Shc expression and affects vascularization factors in pro-angiogenic cells

Julie Favre*, Cansu Yildirim, Juan-Jesus Garcia-Vallejo, Ruud Fontijn, Tineke van der Pouw-Kraan, Anton Horrevoets. Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands

Type-2 diabetes is associated with impaired neovascularisation, which involves dysfunction of circulating pro-angiogenic mononuclear cells (CAC). Elevated plasma free fatty acid (FFA) levels such as palmitic acid (PA), and oxidative stress may directly affect CAC numbers and function. Interestingly, a recent study pointed out the lifespan regulator p66Shc, a pro-oxidant adaptor protein as a modulator of KLF2 (Krüppel-like factor 2), a beneficial actor of CAC and endothelial function. We thus aimed to follow the molecular impact of high levels of FFA in CACs in vitro. At day 6, adherent cells pretreated or not with 33µM resveratrol (which promotes endothelial KLF2) for 3 days, were submitted to a 24h treatment with PA (200 or 400 µM) or vehicle. Q-PCR analysis was performed. Cytoplasmic reactive oxygen species production was measured with the oxidative sensitive fluorescent probe CM-DCF. Protein levels of Tie2 and VEGFR2 were measured by FACS and Imagestream cytometer. We observed that while KLF2 expression tended to decrease, PA markedly and dose-dependently enhanced p66Shc mRNA expression (6 fold). PA did not markedly affect CAC number but a rise in oxidative stress was evidenced by higher number of CM-DCF positive cells. Resveratrol pretreatment reversed PA-induced oxidative stress and tended to normalize gene expression levels. Interestingly, PA-induced p66shc increase correlated with a large rise in mRNA expression of VEGF-A (10 fold). Conversely, surface membrane expression of VEGFR2 and Tie2 decreased after PA treatment. We show for the first time that high levels of FFA can directly impair CAC function, possibly via an increase in p66Shc-induced oxidative stress leading to a deregulated VEGF-A over-expression. The associated decrease in major receptors involved in neovascularization, such as VEGFR2 and Tie2, highlights the potent deleterious action of PA in this process.

HoxB5 as an important factor in angiogenesis

Anne Feßner, Christoph Bode and Martin Moser. Uniclinic Hospital Freiburg, Cardiology, Freiburg, Germany

Introduction: The homeobox gene HoxB5 is an important regulator of endothelial cell development. As a transcription factor HoxB5 binds to the VEGFR-2 gene. As a consequence, HoxB5 plays a key role in blood vessel formation. In previous studies we have demonstrated that Ang2 is upregulated upon HoxB5 overexpression and that the HoxB5 effect is reversed by the angiopoietin antagonist Tie-2. Now we aimed to investigate the mechanism of the HoxB5 effect during angiogenesis. Methods and Results: To assess the role of HoxB5 in angiogenesis in vivo femoral artery occlusion was performed in C57BL/6 mice and HoxB5 was overexpressed in vivo by concomitant intramuscular injections of an adenovirus coding for HoxB5 at sites downstream of the occlusion compared with control virus. The blood flow recovery rate was determined using Laser Doppler imaging and showed an improved perfusion rate for the HoxB5 treated mice. To identify possible target genes of HoxB5 that may be involved in angiogenesis, we overexpressed HoxB5 in cultured endothelial cells and performed a human angiogenesis proteome profiler array. Among the upregulated genes were the pro-inflammatory molecules Endothelin-1 (ET-1), Monocyte chemotactic protein-1 (MCP-1), Inteleukin-1β (IL-1β) and Interleukin-6 (IL-6). These findings were confirmed using real time PCR as well as by Western blot or ELISA. To investigate whether overexpression of HoxB5 also regulates these genes in vivo, we quantified RNA levels of the respective transcripts from muscle tissue of hindlimb ischemia operated mice. Indeed MCP-1, IL-1β and IL-6 were increased in HoxB5 treated ischemic tissue compared with control ischemic tissue in mice. To assess the functional consequences of HoxB5 overexpression in endothelial cells in vitro we transfected HUVEC cells with a plasmid coding for HoxB5 or control. Endothelial cell migration was enhanced after HoxB5 overexpression in a Boyden migration chamber. Moreover, HoxB5 overexpression leads to an increased endothelial cell sprouting. Conclusion: HoxB5 overexpression enhances blood flow recovery after hindlimb ischemia in mice. Our data support the hypothesis that this may take play by activation of inflammatory molecules such as MCP-1, IL-1β and IL-6 resulting in increased endothelial cell migration. Thus HoxB5 may be a novel therapeutic option for ischemic diseases.

LTB4 induces changes in vascular morphology

Michaela Finsterbusch*, Mathieu-Benoit Voisin & Sussan Nourshargh. William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, United Kingdom

Migration of circulating leukocytes through venular walls to sites of inflammation is a crucial component of host immune response against pathogens and tissue injury. Although there is at present much interest in deciphering the mechanisms of leukocyte transendothelial cell migration, little attention has been paid to analysis of the subsequent steps, i.e. leukocyte migration through the pericyte layer and the venular basement membrane (BM). In this context, we have previously demonstrated that neutrophils preferentially transmigrate through gaps between adjacent pericytes, regions that are associated with sites of low matrix protein expression (LERs; 1-3). The aim of the present study was to extend these findings by investigating the impact of the potent neutrophil chemoattractant LTB4 on pericyte morphology and BM deposition in relation to neutrophil transmigration in vivo. The model employed was that of the mouse cremaster muscle which through its thin and transparent nature enables high resolution analysis of changes in vascular morphology as observed and quantified by immunofluorescent staining and confocal microscopy (1-3). To induce inflammation LTB4 was injected locally by intrascrotal injection (30ng/300µl in PBS). After an in vivo test period of 2-72 hrs, tissues were analysed for neutrophil infiltration, pericyte shape-change and BM remodelling using specific markers. As expected LTB4 induced a time-dependent neutrophil transmigration response that peaked at 4 hrs, a response that was also associated with a time-dependent change in pericyte morphology (as quantified through enlargement of gaps between adjacent pericytes) and size of collagen IV and laminin LERs. Of interest we observed that LTB4-induced changes in pericyte morphology was absent in mice depleted of their circulating neutrophils and appeared to be mediated by endogenously generated TNFα, a cytokine that we have previously shown to directly induce pericyte shape change in vivo (Proebstl, D et al., manuscript in preparation). Collectively, the present results provide the first evidence for the ability of LTB4 to induce changes in vascular morphology in vivo. The noted effects may contribute to the pro-inflammatory properties of LTB4 through impacting the barrier function of venular walls for migrating cells and/or macromolecules. 1. Voisin, M.B., Woodfin, A., & Nourshargh, S. Monocytes and neutrophils exhibit both distinct and common mechanisms in penetrating the vascular basement membrane in vivo. Arterioscler. Thromb. Vasc. Biol. 29, 1193-1199 (2009). 2. Voisin, M.B., Probstl, D., & Nourshargh, S. Venular basement membranes ubiquitously express matrix protein low-expression regions: characterization in multiple tissues and remodelling during inflammation. Am. J. Pathol. 176, 482-495 (2010). 3. Wang, S. et al. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. J. Exp. Med. 203, 1519-1532 (2006). This work was supported by funds from the British Heart Foundation and The Wellcome Trust.

Investigation of the causal contribution of selective blood-brain barrier glucose transport processes to brain edema formation and functional deterioration after experimental focal brain ischemia and traumatic brain injury

Ellaine Salvador[1], Winfried Neuhaus[1], Herman Koepsell[2], Serge Thal[3] and Carola Förster[1]*. [1]Department of Anesthesia and Critical Care, University Wuerzburg, [2]Departement of Anatomy and Cell Biology I, University Wuerzburg, [3]Klinik für Anästhesiologie, University Mainz, Germany

Traumatic brain injury (TBI) is the leading cause of death in children and young adults globally. Malignant cerebral edema plays a major role in the pathophysiology which evolves after severe TBI. Added to this is the significant morbidity and mortality from cerebral edema associated with acute stroke, hypoxic ischemic coma, neurological cancers and brain infection. Therapeutic strategies to prevent cerebral edema are limited and if brain swelling persists beyond 24 h, the risks of permanent brain damage or mortality are greatly exacerbated. During traumatic brain injury (TBI) and stroke, both, oxygen and glucose deprivation (OGD) may be encountered. Due to a higher demand for glucose in the brain in these cases, enhanced levels of glucose transporters are expressed, facilitated glucose transporter-1 (GLUT1) and sodium-dependent glucose transporter-1 (SGLT1). Besides their function as energy supply, glucose may influence brain water homeostasis by a direct hygroscopic effect; a possible cause for early brain edema formation. The present study investigated the expression of sodium-dependent glucose transporters (SGLT) and the effect of their deficiency on brain edema formation after experimental TBI.

The vitamin K-dependent anti-coagulant factor, protein S, inhibits in a Mer- and SHP2- dependent manner, multiple VEGF-A- dependent angiogenesis related events

Sylvain Fraineau[1]*, Arnaud Monvoisin[1], Julie Talbot[1], Jonathan Clarhaut[2], Sandip M. Kanse[3], Michel Philippe[1] and Omar Benzakour[1]. [1]Institut de Physiologie et de Biologie Cellulaires, UMR FRE 3511, Université de Poitiers, Poitiers, France, [2]CIC 0802 INSERM, Centre Hospitalier Universitaire de Poitiers, Poitiers, France, [3]Institute for Biochemistry, Justus Liebig University, Giessen, Germany

Protein S (ProS), encoded by PROS1 gene, is a plasma vitamin K-dependent glycoprotein, mainly known as a negative regulator of blood coagulation. ProS heterozygous mice have been shown to display defects in vessel development and function (Burstyn-Cohen T. et al., 2009). Endothelial cells (ECs) produce ProS (Burstyn-Cohen T. et al., 2009) and express its putative tyrosine kinase receptors termed TAM for Tyro3, Axl and Mer (Stitt T.N. et al., 1995; Tijwa. et al., 2008; Zhu D. et al., 2010). However the interaction of ProS with the endothelium and in particular its role in angiogenesis has not been studied as yet. The major aim of our study is to investigate ProS implication in the angiogenic process both in vivo and in vitro. We performed matrigel plug assays in mice and assessed ProS effects on Human Umbilical Vein Endothelial Cells (HUVECs) morphogenesis, proliferation and signalling. ProS at its circulating concentration (20 µg/ml) inhibited Vascular Endothelial Growth Factor A (VEGF-A) and Fibroblast Growth Factor-2 (FGF-2)-induced angiogenesis in vivo in matrigel plug assays. In vitro, ProS inhibited HUVECs morphogenesis on matrigel in a dose-dependent manner. ProS also inhibited VEGF-A- induced HUVECs proliferation, migration, VEGFR-2 phosphorylation on SHP2 sensitive site, Erk 1/2 and Akt activation. These effects were abolished in the presence of NSC 87877 (Chen L. et al., 2006) , an inhibitor of SHP1/2. The use of neutralizing antibodies and siRNA revealed that Mer, which is activated by ProS, is the tyrosine kinase receptor responsible for ProS inhibition of HUVECs proliferation and SHP2 recruitment. Altogether, our data corroborate recent findings suggesting a major role of Mer in endothelial recruitment by cancer cells (Png KJ. et al., 2011) and suggest that ProS/Mer/SHP-2 axis, by inhibiting VEGFR-2 activation, may regulate ECs proliferation, migration and subsequently vasculature development remodelling and stability.

Proinflammatory cytokines induce nanoscale changes at the plasma membrane of endothelial cells

Jonas Franz* Moritz Beutel Christoph Riethmüller. Medical Faculty, University of Muenster Physical Faculty, University of Heidelberg Serend IP GmbH, Center for Nanotechnology, Heisenbergstrasse 11, Germany

Endothelial cells control the extravasation of leukocytes into tissues. Inflammatory cytokines are already known to activate the endothelium by upregulation of adhesion molecules, which promote leukocyte attachment. The firm adhesion of a leukocyte to the endothelium is preceeding the actual transmigration step, whose initializing mechanisms are not fully clarified yet. During firm adhesion, both leukocytes and endothelial cells form protrusions of their plasma membrane. The leukocyte forms invadopodia to palpate the endothelial cell for suitable entry sites and the endothelial cell engulfs the attached leukocyte with filopodia. Recently we have shown, that sole activation of endothelium is sufficient for maximal leukocyte transmigration rates (in vitro). The question is open, whether distinct morphological changes at the endothelial membrane correlate with pro-inflammatory activation in order to facilitate leukocyte capturing. For high-resolution analysis of endothelial cell surface, atomic force microscopy (AFM) under physiological buffer conditions is used. The cell model is human umbilical vein endothelial cells (HUVEC) stimulated with TNFalpha or interleukin1beta, respectively. Images are evaluated quantitatively using computer vision. A detailed morphometrical analysis revealed significant changes of nanostructures at the cell surface, that point to a pro-inflammatory phenotype of endothelial cells. The results are categorised and discussed.

Fibrin fragment E stimulates myofibroblast recruitment and differentiation

Peder Fredlund Fuchs[1]*, Johan Kreuger[1] and Pär Gerwins[1,2]. [1]Dept. of Medical Biochemistry and Microbiology, Uppsala University, Husargatan 3, P.O. Box 582, SE-751 23, Uppsala, Sweden, [2]Dept. of Oncology, Radiology, and Clinical Immunology, Section for Radiology, Uppsala University, Akademiska sjukhuset, 751 85 Uppsala, Sweden

Fibrin is a major constituent of the initial matrix formed after wounding and degradation of this provisional matrix is an important step during wound healing. Plasmin digestion of fibrin generates distinct degradation products and one of these, fibrin fragment E, have previously been shown to stimulate migration and proliferation of vascular endothelial and smooth muscle cells. As fibrin deposits are often found in association with tumors and with the emerging role of myofibroblast contribution to tumor progression and revascularization of wounds we investigated the involvement of fibrin degradation products in recruitment and differentiation of myofibroblasts. Fragment E was found to be a chemotactic factor for fibroblasts and generated a migratory response that was comparable to that induced by PDGF-BB. Fragment E alone did not affect basal expression of alpha smooth muscle actin (aSMA), a protein often upregulated during myofibroblast differentiation. However a low nano-molar concentration of fragment E potentiated TGF-beta induced aSMA expression with around two-fold higher levels of aSMA as compared to TGF-beta alone. Reducing integrin beta 3 expression by siRNA disturbed potentiation of aSMA expression, as did addition of cyclic-RGD peptide, suggesting integrin involvement. Toll-like receptor 4 (TLR-4) has previously been shown to interact with fibrinogen and is also implicated to play a role in renal fibrosis. Blocking TLR-4 signaling impaired the chemotactic potential of FnE. In this study we present evidence that fibrin fragment E is a chemotactic factor for fibroblasts and enhances TGF-beta mediated myofibroblast differentiation in vitro and may be important mechanisms in wound healing and revascularization.

VE-PTP antibodies enhance endothelial barrier function in vitro and in vivo

Maike Frye*, Mark Winderlich, Martina Dierkes and Dietmar Vestweber. Max-Planck-Institute of Molecular Biomedicine Department Vascular Biology Prof. Dr. Dietmar Vestweber Roentgenstraße 20, 48149 Muenster, Germany

Vascular endothelial protein tyrosine phosphatase (VE-PTP) is an endothelial-specific receptor-type tyrosine phosphatase that associates with the tyrosine kinase receptor Tie-2 and with VE-cadherin. VE-PTP is an essential regulator of the functions of each of these two endothelial membrane proteins. Ablation of the gene for VE-PTP in the mouse leads to embryonic lethality at midgestation, due to defects in vascular remodeling and dramatic vessel enlargement in the embryo and in extraembryonic tissues (1). Investigating the underlying mechanisms, we showed that an antibody against the extracellular part of VE-PTP could mimic the VE-PTP-deficiency defect when incubated with explant cultures of extraembryonic allantoides of wt mice. This effect was based on the ability of the anti VE-PTP antibodies to trigger the dissociation of VE-PTP from Tie-2, which lead to subsequent endocytosis of VE-PTP and activation of Tie-2 on the cell surface. This stimulated endothelial cell proliferation and caused in vivo an enlargement of vessel diameter in various tissues of juvenile mice upon injection of anti-VE-PTP antibodies. Our results established that VE-PTP balances Tie-2 activity during embryonic development and in newborn mice thereby determining blood vessel size (2). In addition to Tie-2, VE-PTP associates via extracellular domains with the endothelial adhesion molecule VE-cadherin. This interaction was found to enhance the adhesive function of VE-cadherin in transfected CHO cells and in endothelial cells (3). Recently, we could show that the dissociation of VE-PTP from VE-cadherin is a pre-requisite for the destabilization of endothelial cell junctions in vivo (4). Intriguingly, not only VE-cadherin but also Tie-2 supports the integrity of endothelial cell contacts. Stimulation of Tie-2 with its agonist Angiopoetin-1 or with recombinant multimeric COMP-Ang1 can inhibit VEGF-induced permeability in vivo. Furthermore, VE-PTP and Tie-2 are relocated to cell-cell contacts upon stimulation with Ang-1. Given that VE-PTP supports VE-cadherin function, but counteracts the activation of Tie-2 it is possible that VE-PTP enhances endothelial cell contact integrity via VE-cadherin, but may counteract junction stabilization via dampening of Tie-2 activation. To investigate this further, we tested whether anti-VE-PTP antibodies would influence vascular permeability in vitro and in vivo. Interestingly, we could indeed demonstrate that the antibodies counteracted the effect of permeability-increasing substances on a monolayer of primary HUVEC. This in vitro effect was confirmed in mice using the Miles assay, where vascular leak formation induced by intradermally injected VEGF was inhibited by systemic administration of the same antibodies. Since we have shown before that our anti-VE-PTP antibodies selectively displace VE-PTP from Tie-2 and trigger endocytosis of these VE-PTP molecules, whereas VE-PTP molecules associated with VE-cadherin stay unaffected, we conclude that anti-VE-PTP antibodies selectively stabilize endothelial junctions via the activation of Tie-2. We are currently investigating the mechanism whereby VE-PTP, Tie-2 and VE-cadherin regulate vascular permeability in concert using transgenic mouse models and siRNA approaches. (1) Bäumer et al. Blood, 2006, 107:475 (2) Winderlich et al., J. Cell Biol. 2009, 185:657 (3) Nottebaum et al., J. Exp. Med. 2008, 205:2929 (4) Broermann et al., J. Exp. Med. 2011, 208:2393

Visualizing cell-cycle progression of endothelial cells during vascular network formation in zebrafish

Shigetomo Fukuhara[1]*, Jianghui Zhang[1], Koji Ando[1], Yuki Wakayama[1], Hyouk-Bum Kwon1, Asako Sakaue-Sawano[2], Atsushi Miyawaki[2], Naoki Mochizuki[1]. [1]Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Fujishirodai 5-7-1, Suita, Osaka 565-8565, Japan, [2]Brain Science Institute, RIKEN, Wako, Saitama, Japan

Vascular network formation requires the strict regulation of endothelial cell proliferation. Although the molecular mechanism that regulates proliferation of endothelial cells has been extensively studied using cultured endothelial cells, it is still unclear „where“ and „when“ the proliferation of endothelial cells is promoted and halted during vascular network formation in vivo. To address these questions, we have developed transgenic zebrafish lines that express cell cycle biosensors, Fucci (Fluorescent, ubiquitination-based cell cycle indicator) specifically in endothelial cells. Fucci is a set of fluorescent proteins fused to the replication licensing factors, Cdt1 and Geminin, which are only present in the cells in G1- and S/G2/M phase of cell cycle, respectively. Thus, we have established transgenic zebrafish lines that express mCherry-Cdt1 and mVenus-Gemnin under the control of endothelial cell-specific flk1 promoter (Tg(flk1:mV-zGem);Tg(flk1:mC-Cdt1)). To visualize the cell cycle state of endothelial cells during developmental vascular network formation, we performed in vivo imaging of Tg(flk1:mV-zGeminin); Tg(flk1:mC-Cdt1) fish embryos at 24~48 hour post fertilization (hpf) using confocal microscope. We found that S/G2/M-phase endothelial cells were present mainly in sprouting and growing vessels and that most of them became arrested in G1-phase, once vascular network formation was completed. These results suggest that endothelial cells actively proliferate to form new blood vessels and immediately stop their proliferation by escaping from cell cycle when vascular networks are established. To further explore the significance of endothelial cell proliferation in vascular network formation, we focused on the formation of caudal vessels, since many S/G2/M-phase endothelial cells were observed in growing edge of caudal vessels. First, we performed time-lapse imaging of caudal vessel formation using Tg(flk1:mV-zGeminin);Tg(flk1:NLS-mCherry) fish embryo in which NLS-mCherry marks nuclei of each endothelial cell. At early stage of caudal vessel formation (approximately 24~30 hpf), NLS-mCherry-positive cells emerged just beyond the growing edge of caudal vessels. Subsequently, they proliferated and formed vascular cord structures, implying that angioblasts are responsible for early caudal vessel formation. On the other hand, during the late stage of caudal vessel formation (30~35 hpf), endothelial cells at growing edge of caudal vessels actively proliferated and migrated posteriorly to extend the caudal vessels. These findings suggest that the early and the late caudal vessel formation are formed by angioblasts and maturated endothelial cells, respectively. Vascular endothelial growth factor (VEGF) plays important roles in many aspects of vascular development. Thus, we next investigated the role of VEGF in caudal vessel formation. Zebrafish embryos depleted of two VEGF receptor-2 (VEGFR2) ortholog (kdr/kdrl) exhibited defective formation of caudal artery and intersegmental vessels. Whereas knockdown of kdr and kdrl also suppressed cell cycle progression of endothelial cells during late stage of caudal vessel formation, it did not affect angioblast differentiation and proliferation at the early stage. As previously reported, sonic hedgehog (Shh) was expressed in notochord, while VEGF expression was detected in the somite. Inhibition of Shh signaling by cyclopamine led to downregulation of VEGF expression in the somite and suppressed proliferation of endothelial cells at the late stage of caudal vessel formation. Collectively, these results indicate that Shh released from notochord induces VEGF expression in the somite, which in turn promotes endothelial cell proliferation responsible for caudal vessel outgrowth at the late stage.

Inflammatory stimuli down-regulate ribonuclease1 in human endothelial cells

Julia Gansler*, Klaus T. Preissner, Silvia Fischer. Department of Biochemistry, Medical School, Justus-Liebig-University, Giessen, Germany

Extracellular RNA released under pathological conditions was shown to serve as procoagulant, proinflammatory and permeability-increasing factor in vitro and in vivo. Thrombus and edema formation could be significantly reduced by administration of ribonuclease1 (RNase1) in vivo. RNase1 is expressed and secreted by endothelial cells as a naturally circulating component of the blood, and may thus serve as a counterpart of extracellular RNA with vessel-protective properties. The present study investigated the influence of several inflammatory stimuli on the expression of RNase1 as well as its inhibitor RNase inhibitor (RI) in endothelial cells. Quantitative PCR and Western Blot analysis revealed prominent mRNA and protein expression of RNase1 and RI by human umbilical vein endothelial cells (HUVEC) as well as an appreciable release of both factors. Long-term treatment of HUVEC with tumor necrosis factor-α(TNF-α), Interleukin-1β (IL-1β) or thrombin for 24 hours decreased the mRNA- and protein expression as well as the release of RNase1 up to 70% compared to untreated cells, while the expression and secretion of RI was not influenced by the stimuli. Accordingly, RNase1 activity was decreased in the cell supernatants as measured by zymography and a specific RNase activity test. Lipopolysaccharid (LPS) had no influence on the expression of RNase1 or RI. Analysis of the endothelial barrier function revealed that the down-regulation of RNase1 by TNF- α stimulation as well as RNase1-siRNA increased the permeability of the HUVEC monolayer as demonstrated by rearrangement of the VE-cadherin distribution and disintegration of cellular contacts. Mechanistically, the TNF-α-induced decrease of RNase1 was independent of the activation of NFkB, which was demonstrated by blocking experiments with the specific IkB-Inhibitor BAY 11-7082. Yet, inhibition of histone deacetylases by trichostatin A recovered RNase1 expression and secretion, indicating an acetylation-dependent regulation process. Furthermore, a depletion of the TNF- α stimulus induced a recovery of RNase1 expression and release in a time-dependent manner within 24 hours. These results demonstrate a down-regulation of RNase1 on mRNA and protein level by several inflammatory stimuli including TNF- α, which is mediated by changes of the acetylation status.

Uremia-induced nitric oxide resistance leads to decreased AV fistula function

I.L.A. Geenen[1,2]*, F.F. Kolk[1], D.G.M. Molin[1], A. Wagenaar[1], J.H.M. Tordoir[2], G.W.H. Schurink[2], J.G.R. de Mey[3], M.J. Post[1]. [1]Department of Physiology, CARIM, Maastricht University Medical Center, [2]Department of General Surgery, Maastricht University Medical Center, [3]Department of Pharmacology, Maastricht University Medical Center, The Netherlands

Introduction: Functional vascular acces is essential for end-stage renal disease (ESRD) patients who rely on hemodialysis. First choice is a native arteriovenous (AV) fistula, however, AV fistulas show a high risk of non-maturation due to insufficient vessel wall adaptation. AV fistula function depends on a sufficient flow rate and directly relates to fistula dilatation. The uremic status of ESRD patients induces oxidative stress, which can disturb nitric oxide (NO) signaling. NO is an important mediator of vessel relaxation, by activating its receptor soluble Guanylyl Cyclase (sGC) which induces smooth muscle cell (SMC) relaxation. The aim of this research was to investigate the influence of uremia on NO signaling and AV fistula function. Methods: Wistar rats were made uremic by 5/6th nephrectomy and control animals underwent a sham procedure. After 6 weeks, an AV fistula was created between the carotid artery and jugular vein. Vessel diameters were evaluated by ultrasound and flow was measured with a transit time flow probe directly after AV fistula creation and at 3 weeks before sacrifice. Serum parameters and histological characteristics of the AV fistula were obtained. Vascular reactivity of the carotid arteries was determined in a wired myograph after stimulation with acetylcholine (ACh) and an NO-donor (sodium nitroprusside, SNP). The effect of BAY60-2770, an activator of oxidized sGC, was evaluated as well. Results: Ureum and creatinine were significantly augmented in nephrectomized rats (n=10) compared to controls (n=6) (creatinine 35±6 vs. 85±40 µmol/L, ureum 8.7±1.4 vs. 18±9 mmol/L; $p<0.05$). AV fistula diameters increased during follow-up and were reduced at two weeks for uremic animals (1.58±0.26 vs. control group: 1.95±0.27 mm, $p<0.05$). Flow through the AV fistula was significantly lower in uremic rats (84.3 vs 112 mL/min; $p<0.05$). Intimal hyperplasia was observed in venous part of the AV fistulas but the amounts did not differ between groups. Endothelium dependent relaxation after ACh was comparable in uremic and control rats, indicating normal endothelial function. Meanwhile, vessel relaxation after SNP was decreased in the uremic group (LogEC 50: -7.1±0.1 vs -7.8±0.3M; $p<0.05$), indicating NO-resistance of sGC. BAY60-2770 induced stronger vessel relaxation in uremic vessel rings (LogEC 50: -7.6±0.3 vs. -8.4±0.2M; $p<0.0001$) compared to controls. Conclusion: Uremia disturbes vasomotoric function via oxidative stress induced NO-resistance of sGC. This could explain diminished AV fistula flow in uremic rats. BAY60-2770 activates oxidized sGC and could be a suitable therapy to enhance AV fistula flow in ESRD patients.

Tumor pericytes regulate malignancy by specifically controlling the transmigration of myeloid-derived suppressor cells (MDSC)

JongWook Hong, Hanyu Qin, Mirjana Poljakovic, Guillem Genové*. Karolinska Institute Dept of Medical Biochemistry and Biophysics Div of Vascular Biology Scheeles väg, 2 A:3-P:4 SE-171 77 Stockholm, Sweden

The interaction of different stromal cell types within the tumor provides a permissive environment for tumor development as well as for metastases to occur. Of all the different tumor stromal cells, the most obscure of them all are pericytes. We have previously shown that pericyte deficiency leads to increased tumor growth in a variety of subcutaneous tumor models (1). Here we report that pericyte depletion results in increased malignancy by specific recruitment of a subset of inflammatory cells. Upon pericyte deficiency, we found a significant upregulation of the cytokines Il-6, G-CSF, GM-CSF, Il-1b and COX2, all know to recruit and activate myeloid-derived suppressor cells (MDSC). In accordance, we found a significant increase in the amount of MDSC cells present in the pericyte-depleted tumors as compared to control. The recruitment of MDCS to the tumor is specific since the overall number of leukocytes (CD18+/CD45+) remained unchanged. Moreover, cytotoxic T-cell genes such as Granzyme 2and Perforin 1 were downregulated, indicating that MDSC were indeed activated and suppressing the cytotoxic T-cell activity in the pericyte-deficient tumors. The expression of the adhesion molecules E-Selectin and V-CAM was upregulated in pericyte-depleted tumors. Pericyte coverage of tumor blood vessels inversely correlated with the amount of MDSC present in the tumor. Interestingly, the amount of MDSC present in the tumor correlated with increased tumor cell dissemination in lungs and livers. The increased amount of metastasis observed in pericyte deficient tumors is in agreement of previously results linking pericyte coverage and tumor dissemination in preclinical studies as well as in human tumor patients. We propose that lower pericyte coverage of tumor vessels results in the specific recruitment and activation of MDSC cells, which suppress the cytotoxic T-cell response and promote malignancy and tumor cell dissemination. Our results advice caution in applying therapeutic approaches that target pericytes. (1) Nisancioglu et al, Cancer Res, 2010, 70(12), 5209-15 (2) Xian et al, JCI, 2006, 116, 642-51 (3) Yonenaga et al, Oncology, 2005, 69, 159-66

Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors

Kai Schledzewski[1], Cyrill Géraud[1]*, Bernd Arnold[2], Shijun Wang[3], Hermann-Josef Gröne[3], Tibor Kempf[4], Kai C. Wollert[4], Beate K. Straub[5], Peter Schirmacher[5], Alexandra Demory[1], Hiltrud Schönhaber[1], Alexei Gratchev[1], Lisa Dietz[1], Hermann-Josef Thierse[1], Julia Kzhyshkowska[1], Sergij Goerdt[1]. [1]Department of Dermatology, Venereology, and Allergology, University Medical Center and Medical Faculty Mannheim, University of Heidelberg, and Center of Excellence in Dermatology, Mannheim, Germany, [2]Department of Molecular Immunology and [3]Department of Cellular and Molecular Pathology, German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Department of Cardiology and Angiology, Hannover Medical School, Hannover, Germany. [5]Department of Pathology, University Clinic Heidelberg, University of Heidelberg, Heidelberg, Germany

Tissue homeostasis and remodeling are processes that involve high turnover of biological macromolecules. Many of the waste molecules that are by-products or degradation intermediates of biological macromolecule turnover enter the circulation and are subsequently cleared by liver sinusoidal endothelial cells (LSEC). Besides the mannose receptor, stabilin-1 and stabilin-2 are the major scavenger receptors expressed by LSEC. To more clearly elucidate the functions of stabilin-1 and -2, we have generated mice lacking stabilin-1, stabilin-2, or both stabilin-1 and -2 (Stab1-/-Stab2-/- mice). Mice lacking either stabilin-1 or stabilin-2 were phenotypically normal; however, Stab1-/-Stab2-/- mice exhibited premature mortality and developed severe glomerular fibrosis, while the liver showed only mild perisinusoidal fibrosis without dysfunction. Upon kidney transplantation into WT mice, progression of glomerular fibrosis was halted, indicating the presence of profibrotic factors in the circulation of Stab1-/-Stab2-/- mice. While plasma levels of known profibrotic cytokines were unaltered, clearance of the TGF-β family member growth differentiation factor 15 (GDF-15) was markedly impaired in Stab1-/-Stab2-/- mice but not in either Stab1-/- or Stab2-/- mice, indicating that it is a common ligand of both stabilin-1 and stabilin-2. These data lead us to conclude that stabilin-1 and -2 together guarantee proper hepatic clearance of potentially noxious agents in the blood and maintain tissue homeostasis not only in the liver but also distant organs.

Peroxisome proliferator-activated receptor gamma coactivator 1α deficiency accelerates endothelial dysfunction during chronic angiotensin II treatment by increasing mitochondrial oxidative stress and vascular aging

Jasmin Ghaemi Kerahrodi[1]*, Swenja Kröller-Schön[1,2], Thomas Jansen[1], Matthias Oelze[1], Philip Wenzel[1,2], Michael Hausding[1], Maike Beisele[3], Andreas Daiber[1], Thomas Münzel[1] and Eberhard Schulz[1]. [1]Department of Cardiology, II. Medizinische Klinik und Poliklinik, Universitätsmedizin Mainz, Germany, [2]Center for Thrombosis and Hemostasis (CTH), Universitätsmedizin Mainz, Germany, [3]Central Laboratory Animal Facility, Universitätsmedizin Mainz, Germany

Objective: Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) is an important mediator of mitochondrial biogenesis and function. We have previously shown that the AMP-activated protein kinase (AMPK) protects endothelial cells against oxidative stress by preservation of mitochondrial function in a PGC-1α dependent manner. Since dysfunctional mitochondria might be involved in the pathogenesis of vascular disease, the current study was designed to investigate the effects of in vivo PGC-1α deficiency during chronic angiotensin II (ATII) treatment. Methods and Results: Deletion of PGC-1α had no effect on endothelial function or mitochondrial mass in aortic tissue under basal conditions. However, chronic ATII infusion at subpressor doses (0.1mg/kg/d) resulted in mild endothelial dysfunction, which was significantly aggravated in PGC-1α knockout mice. In parallel, oxidative stress was increased in aortic rings from ATII-treated PGC-1α knockout mice while serum antioxidative capacity was decreased. By using the mitochondrial specific superoxide dye mitoSOX and complex I inhibitor rotenone, we identified the mitochondrial respiratory chain as the major PGC-1α dependent ROS source in vivo. In accordance with the prominent role of mitochondrial ROS for the signalling events leading to vascular aging and apoptosis, we found increased expression of p16INK4 and Chk-2 as markers of cell senescence as well as increased TUNELstaining accompanied by activation of the JNK pathway in aortas from ATII treated PGC-1α knockout mice. Conclusions: PGC-1α deficiency impairs endothelial function during chronic angiotensin II infusion by increasing mitochondrial ROS production, resulting in premature vascular aging and apoptosis.

EMMPRIN is a spatiotemporal switch for endothelial cell junction formation and vascular integrity

Vanessa Moreno[1]*, Pilar Gonzalo[1]*, Ángela Pollán [1], Fabrizio Orsenigo[2], Olga Barreiro[1], Kenji Kadomatsu[3], Elisabetta Dejana[2], Francisco Sánchez-Madrid[1], and Alicia G. Arroyo[1].
[1]CNIC, Madrid, Spain, [2]IFOM-IEO, Milan, Italy, [3]Nagoya University, Nagoya, Japan

EMMPRIN, a member of the Ig superfamily, is involved in heterophilic and homophilic protein interactions thus contributing to different pathophysiological processes. We have explored the function of EMMPRIN in endothelial cells (ECs). EMMPRIN expression and subcellular location are both regulated during endothelial monolayer formation; in particular, EMMPRIN gets highly glycosilated during this process and this correlates with a shift in its compartmentation from caveolin-rich microdomains (apical) to cell-cell junction domains (basolateral). Notably, endothelial morphology and VE-cadherin patterning at the junctions change in monolayers formed by EMMPRIN-interfered ECs pointing to an active role of EMMPRIN in junction formation; this is further supported by the increased permeability observed in EMMPRIN-interfered EC monolayers. In vivo, EMMPRIN heterozygous and null mice display an altered pattern of PECAM-1 and VE-cadherin at the junctions in microvessels together with an increase in vascular permeability indicating that EMMPRIN is essential for junction formation and function also in vivo. Mechanistically, EMMPRIN-interfered ECs show decreased peri junctional F-actin and increased stress fibers resulting in increased EC size; moreover, ECs in aortas from null mice are also larger indicating a role of EMMPRIN in actin polymerization also in vivo. EMMPRIN might contribute to local actin polymerization by interacting with cytoskeletal adaptors such as plakoglobin. In sum, we identify EMMPRIN as a key spatiotemporal switch for assembly of competent endothelial junctions and maintenance of vascular integrity in vivo.

Chronic therapy with isosorbide-5-mononitrate causes endothelial dysfunction, oxidative stress and a marked increase in vascular endothelin-1 expression

Anna Gottschlich[1]*, Matthias Oelze[1], Maike Knorr[1,2], Swenja Kröller-Schön[1,2], Sabine Kossmann[1], Robert Rümmler[1], Alexandra Schuff[1], Steffen Daub[1], Christopher Doppler[1], Hartmut Kleinert[3], Tommaso Gori[1], Andreas Daiber[1], Thomas Münzel[1]. [1]2nd Medical Clinic, Department of Cardiology, [2]Center of Thrombosis and Hemostasis (CTH) and [3]Department of Pharmacology, Medical Center of the Johannes Gutenberg University, Mainz, Germany

Aims: Isosorbide-5-mononitrate (ISMN) is one of the most frequently used compounds in the treatment of coronary artery disease predominantly in the US. However, ISMN was reported to induce endothelial dysfunction, which was corrected by vitamin C pointing to a crucial role of reactive oxygen species (ROS) in causing this phenomenon. We sought to elucidate the mechanism how ISMN causes endothelial dysfunction and oxidative stress in vascular tissue. Methods and results: Male wistar rats were treated with ISMN (75 mg/kg/d) for 7d. Endothelin expression was determined by immunohistochemistry in aortic sections. ISMN infusion caused a marked degree of endothelial dysfunction but no tolerance to ISMN itself, whereas ROS formation and NADPH oxidase activity in aorta, heart and whole blood were increased. ISMN upregulated the expression of NADPH subunits and caused uncoupling of the endothelial nitric oxide synthase likely due to a downregulation of the tetrahydrobiopterin synthesizing enzyme GTP-cyclohydrolase-1 and to S-glutathionylation of eNOS. The adverse effects of ISMN were improved in gp91phox knockout mice and normalized by bosentan in vivo/ex vivo treatment and suppressed by apocynin. In addition, a strong increase in the expression of endothelin within the endothelial cell layer and the adventitia was observed. Conclusion: Chronic treatment with ISMN causes endothelial dysfunction and oxidative stress, predominantly by an endothelin-dependent activation of the vascular and phagocytic NADPH oxidase activity and NOS uncoupling. These findings may explain at least in part results from a retrospective analysis indicating increased mortality in postinfarct patients in response long-term treatment with mononitrates.

Hyaluronan synthase 3 plays a key role in activation of smooth muscle cells in inflammatory regions of atherosclerotic plaques

Maria Grandoch*, Inga Kretschmer, Ariane Melchior-Becker, Berit Rabausch, Sören Twarock, Guang Dai, Jens W. Fischer. Institute for Pharmacology and Clinical Pharmacology, Düsseldorf University Hospital, Universitätsstr.1, 40225 Düsseldorf, Germany

Objective: Hyaluronan (HA) is synthesized by three HA-synthase isoforms (HAS1, -2, -3) in atherosclerotic lesions. HA functions as a provisional matrix that promotes the activated phenotype of smooth muscle cells and in addition possibly fulfills immunomodulatory functions. The aim of the present study was to identify the HAS isoenzyme that is associated with HA-matrix remodeling in inflammatory regions of atherosclerotic plaques, to determine underlying regulatory pathways and to elucidate functional aspects of this regulation for vascular smooth muscle cell (VSMC) phenotypes. Methods and Results: In symptomatic carotid artery plaques HAS3 was by far the most prominent HAS isoenzyme as determined by quantitative real-time RT-PCR. Furthermore, during murine atherosclerosis in ApoE deficient mice the onset of macrophage invasion precedes HA deposition and induction of HAS3 in aortic root plaques. In vitro, interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) specifically induced HAS3 via activation of NFκB as shown by ChIP assay and the NFκB inhibitor Bay 11-7082. HAS3 was also up-regulated in a co-culture system by activated macrophages via paracrine release of TNFα and IL-1β as shown by neutralizing antibodies. In human atherosclerotic lesions NFκB positive VSMC were frequently detected in close proximity with HA and F4/80 positive macrophages. To study the effects of HAS3 mediated HA synthesis in human coronary VSMC, lentiviral overexpression and knockdown of human HAS3 were employed. Overexpression and knock down of HAS3 revealed that HAS3 controls filopodial extensions of the plasma membrane of VSMC and focal adhesion kinase signaling. In turn, HAS3 induced migration and proliferation via CD44 and RHAMM and activation of PI3K and MAPK signaling. Conclusion: The present results strongly suggest that HAS3-dependent HA synthesis is induced in human VSMC by inflammatory cytokines released from activated macrophages in atherosclerotic lesions. Moreover, HAS3-mediated HA production induced phenotypic activation of VSMC.

Canonical Wnt-signaling via Lef-1 regulates the number of endothelial cells during zebrafish development, functionally opposing Notch signaling

Kathrin S. Grassme[1,2]*, Saulius Sumanas[3] and Wiebke Herzog[1,2]. [1]Westphalian Wilhelms-University Muenster, Germany, [2]Max-Planck-Institute for Molecular Biomedicine, Muenster, Germany, [3]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, USA

In vertebrate embryos endothelial and blood lineages develop in close association and are formed from mesodermal cells. Little is known about growth factor involved in cell fate decisions between endothelial and hematopoietic lineages. Contrary, growth factors that regulate angiogenesis have been studied more vigorously. Among these also the Wnt pathway has been implicated in promoting growth and differentiation of endothelial cells in vitro and in vivo during angiogenesis (Zerlin et al., 2008). However, it is still unclear whether Wnt signaling in involved in angioblast specification and vasculogenesis. When analyzing the role of canonical Wnt signaling for early endothelial cell differentiation, we found that zebrafish embryos with pharmacologically decreased levels of Wnt signaling, showed a significant decrease in the number of endothelial cells and a significant increase in the number of erythrocytes. We observed the same phenotype when we blocked Lef-1 translation through injection of Lef-1 antisense morpholino oligonucelotides. Our preliminary data indicate that the changes in endothelial cell numbers are not caused by alterations in cell proliferation. In contrast upregulation of canonical Wnt signaling using Bio or LiCl2 resulted in an increase in the number of endothelial cells and a decrease in erythrocytes. Recently, Notch signaling has been to shown to negatively regulate endothelial cell specification from mesodermal progenitors, acting as a cell fate switch between the hematopoietic and the endothelial lineages (Lee et al., 2009). Interestingly, when pharmacologically inhibiting Wnt- and Notch-signaling at the same time, we could restore wildtype numbers of the endothelial cells, indicating that canonical Wnt signaling might influence the endothelial-hematopoietic cell balance in the opposite direction of Notch signaling. By using transgenic inducible dominant negative TCF expression, we are going to investigate whether endothelial-hematopoietic precursors are directly affected by the Wnt-signal as well as at which level of differentiation Wnt is required.

How to modulate Tie2 activity from a phage display based approach to a new interacting protein: Neurexin

S.Graziano*, B. Assenzio, L.Mascia, M.Arese[#], F.Bussolino[#]. IRCC Institute for Cancer Research and Treatment, Candiolo-Torino, Italy. [#]These authors contributed equally to the project

Several studies suggest an association between tyrosine-kinase receptors and adhesion molecules and it is clear that these molecules reciprocally regulate one another's activities.A screening of a peptide library to identify binding partners for the endothelial cell specific tyrosine kinase receptor Tie2 has been carried out. Homology analysis of the interacting peptides led to the identification of the receptor-like transmembrane protein Neurexin. While Neurexins have been previously studied exclusively in the nervous system, we have recently demonstrated that they are expressed by cells of the blood vessels wall. Interestingly, Neurexins are physically associated with Tie2 in exogenously expressing cells and also in chick embryo arteries. To begin the analysis of the molecular/cellular mechanisms and the functions of this interaction in the vascular system we decided to use the Neurexin peptides isolated by phage display. These putative peptides, seven amino acids long, were fluoresceinated and tested by Elisa and immunofluorescence assays for their binding affinity on cells expressing Tie2. The ability of the peptides to modulate Tie2 function was also assessed in endothelial cell migration, tubulogenesis and sprouting. Since the Ang/Tie2 signaling is typically involved in the angiogenic remodeling process during development, we also evaluated the activity of these peptides in vivo in the chick chorioallantoic membrane (CAM) angiogenesys assay. The obtained results clearly show that Neurexin, originally identified in the nervous system, may have a function in the vascular system, probably during vessel assembly/maturation and/or differentiation of their cellular constituent. Moreover, generation of peptides based on sequence homology with Neurexins have been proved to be effective in modulating biochemical signals and biological activities of Tie2 related to the remodeling of the vascular system.

Release of extracellular RNA from vascular and tumor cells

Barbara Griemert*, Klaus T. Preissner, Silvia Fischer. Department of Biochemistry, Medical School, Justus Liebig University Giessen, Germany

Extracellular RNA (eRNA) is released under conditions of injury or tumor burden and has been shown to act as a procoagulant, permeability-increasing and proinflammatory factor in the vasculature. Different cell types were stimulated with apoptotic and necrotic agents to analyse the nature of released eRNA. Endothelial and tumor cells were treated with apoptosis-inducing agents such as tumor necrosis factor-α(TNFα), staurosporine or necrosis-inducing agents such as ethanol to induce RNA release. The MP originated from these cell types were isolated, quantified by using Trucount®-Tubes and were also used for subsequent stimulation in a concentration of about 105 MP/ml. MP were also verified by scatter and FACS analysis of Annexin V staining. After 24 h of stimulation cell culture supernatants were collected and centrifuged for 60 min at 21.000 g to isolate microparticles. This was followed by isolation and quantification of RNA from supernatants as well as from MP fractions. Tumor cell lines like HT29 released eRNA into their cell supernatant to a comparable degree as endothelial cells (human cerebral microvascular endothelial cells, HCMEC/D3) (~60 ng/mg protein within 24 h). Untreated monocytic cell lines like THP1 released only small amounts of eRNA (~20 ng/mg protein in 24 h). Released eRNA comprise partly of MP-associated RNA and consisted of rRNA, which was confirmed by PCR analysis. No mRNA was detectable using primers specific for β-Actin or Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH). The ratio of total RNA to MP-associated RNA was dependent on cell type and kind of stimulation used. Following treatment with apoptosis- or necrosis-inducing agents, the total amount of released eRNA as well as the ratio of the total RNA- to MP-associated RNA changed in comparison to untreated samples, dependent on the respective stimulus. Stimulation with HT29-derived MP for 8 h leads to an activation of HCMEC/D3, demonstrated by a slight increase of Intercellular adhesion molecule-1 (ICAM-1). eRNA is released from endothelial, tumor and monocytic cells and is partly associated with microparticles which themselves may activate endothelial cells. This indicates a possible function of MP in the exocytic process or protection of eRNA against degradation by enzymes like ribonucleases. The stimulus-dependent composition of released eRNA might be relevant for its functional activities. In addition, the release of eRNA under pathophysiological conditions may be used as a diagnostic marker for vascular diseases.

VEGF dose negatively regulates the stabilization of newly induced vessels by inhibiting the Sema3A/Nrp1+CD11b+ monocytes/TGF-β1 axis

E. Groppa[1]*, S. Reginato[1], R. Gianni-Barrera[1], M.G. Muraro[2], M. Heberer[1,2] and A. Banfi[1].
[1]Cell and Gene Therapy, Departments of Biomedicine and of Surgery, Basel University Hospital, Switzerland, [2]Oncology, Departments of Biomedicine and of Surgery, Basel University Hospital, Switzerland

Rapid stabilization, i.e. the persistence of newly induced vessels independently of further growth factor expression, is required to achieve functional benefit in therapeutic angiogenesis with short-term gene therapy vectors. VEGF induces normal or aberrant angiogenesis depending on its expression level in the microenvironment around each cell in vivo. Further, four weeks of sustained expression are required for stabilization of normal angiogenesis, whereas aberrant vessels remain VEGF-dependent (Ozawa, JCI 2004). Here we took advantage of a highly controlled myoblast-mediated gene delivery platform to rigorously investigate how VEGF dose regulates stabilization of newly induced vessels in skeletal muscles. Clonal populations of retrovirally transduced myoblasts were implanted in the ear or limb muscles of SCID mice to homogeneously produce specific levels of VEGF164: low and medium VEGF caused normal angiogenesis and high VEGF induced aberrant angioma growth. VEGF signaling was abrogated at defined time-points by systemic treatment with recombinant VEGF-Trap. We found that VEGF impaired vascular stabilization in a dose-dependent fashion. In fact, 35% and 50% of normal vessels induced by low VEGF levels already stabilized by 2 and 3 weeks, respectively. Instead, similarly normal angiogenesis induced by medium VEGF levels completely regressed by 2 weeks and only 10% stabilized by 3 weeks. Aberrant structures caused by high VEGF levels never stabilized. Intravascular staining with fluorescent L. esculentum lectin showed that all vessels induced by all VEGF levels were normally perfused. Further, all normal capillaries induced by low and medium VEGF doses displayed similar normal pericyte coverage, despite different stabilization rates. Consistently, the expression of the endogenous Pdgfb gene was also similar among all conditions. Only aberrant vessels induced by high VEGF, which never became VEGF-independent, were surrounded by α-SMA-positive mural cells and not by pericytes. Therefore, pericyte recruitment is necessary but not sufficient to achieve rapid vascular stabilization. However, gene expression analysis on the injected muscles, revealed a correlation between decreasing stabilization rates at the different VEGF doses and tissue expression of TGF-β1 and Sema3A, whereas endogenous VEGF, Ang1, and EphrinB2 did not change. Sema3A has been recently described to promote vessel maturation through the recruitment of Nrp1+CD11b+ monocytes (NEM). To elucidate the mechanisms of VEGF-dependent vascular stabilization, we investigated the relation between VEGF dose and NEM recruitment. We found that: 1) VEGF164 down-regulated Sema3A expression by endothelial cells in vitro; 2) NEM recruitment in injected muscles was reduced with increasing VEGF doses; 3) Sema3A expression in endothelial cells FACS-purified ex vivo from injected muscles was down-regulated with increasing VEGF doses; 4) FACS-purified NEM expressed TGF-β1, but its level per cell was not affected by VEGF dose; 5) however, in vitro stimulation of endothelial cells with TGF-β1 up-regulated Sema3A expression dose-dependently, providing the basis for a positive feedback loop between Sema3A, NEM recruitment, TGF-β1, and further Sema3A expression, in conditions of low VEGF. Taken together, these data suggest a model in which VEGF164, expressed within a range of doses that induce only normal angiogenesis, negatively regulates vascular stabilization by inhibiting the Sema3A/NEM/TGF-β1 axis, rather than directly acting on the endothelium-pericyte crosstalk.

Involvement of A2A adenosine receptors in insulin-increased L-arginine transport in human umbilical vein endothelium

Guzmán-Gutiérrez E, Westermeier F, Salomón C, Arroyo P, Pardo FN, Leiva A, Sobrevia L. Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynecology, Medical Research Centre (CIM), School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago, Chile

Adenosine causes vasodilatation of human placenta vasculature by increasing L-arginine transport via cationic amino acid transporters 1 (hCAT-1) involving A2A adenosine receptors (A2AAR) activation in human umbilical vein endothelial cells (HUVEC). hCAT-1 activity and expression is increased by insulin in HUVEC, and A2AAR stimulation increases insulin sensitivity in subjects with insulin resistance; however, a potential A2AAR involvement in L-arginine transport modulation by insulin in HUVEC is unknown. Our aim was to characterize whether insulin-stimulation of hCAT-1–mediated L-arginine transport involves A2AAR in HUVEC. Methods: Primary cultured HUVEC (passage 2) from full-term normal pregnancies were used. Insulin (1 nM, 8 hours) was assayed on hCAT1 expression (SLC7A1 promoter activity (firefly/renilla luciferase for pGL3-hCAT1-1606 and pGL3-hCAT1-650)), mRNA expression (quantitative real time PCR), protein abundance (Western blot)) and L-arginine transport (3 μCi/ml, 1 minute, 37ºC). Assays were done in absence or presence of ZM-241385 (10 nM, A2AAR antagonist), CGS-21680 (30 nM, A2AAR agonist) and/or NBTI (10 μM, adenosine transport inhibitor). Results: Insulin and NBTI increased extracellular adenosine concentration, the maximal velocity without altering the apparent Km for L-arginine transport, and hCAT-1 expression (protein and mRNA). These effects were blocked by 10 nmol/L ZM-241385 (A2AAR antagonist). ZM-241385–inhibited SLC7A1 reporter transcriptional activity was similar in cells transfected with pGL3-hCAT-1-1606 or pGL3-hCAT-1-650 constructs, and comparable to the activity determined for pGL3-hCAT-1-650 construct in presence of NBTI + insulin. However, reporter activity was increased by NBTI only in cells transfected with pGL3-hCAT-1-1606, and the ZM-241385 sensitive fraction of NBTI response was similar in absence or presence of insulin. Conclusion: hCAT-1 expression and activity is under regulation by insulin via a mechanism requiring functional A2AAR in HUVEC, which could be determinant in diseases associated with fetal insulin resistance, such as gestational diabetes. CONICYT (ACT-73 PIA, AT-24100210), FONDECYT (1110977, 1120928, 11110059), Faculty of Medicine, PUC (PMD 03/10). EG-G, FW and CS hold CONICYT-PhD fellowships. CS and PA hold Faculty of Medicine, Pontificia Universidad Católica de Chile-PhD fellowship.

Visualization of the initial steps of lymphatic vessel development and the role of CCBE1 in this process using ultramicroscopy

R. Hägerling[1]*, C. Pollmann[1], M. Andreas[1], L. Kremer[1], Uwe Schröer[3], Volker Andresen[3], S. Schulte-Merker[2] and F. Kiefer[1]. [1]Cell Signaling Laboratory, Department of Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, Röntgenstraße 20, D-48149 Muenster, Germany, [2]Developmental Biology, Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, [3]LaVision BioTec GmbH, Astastraße 14, D-33617 Bielefeld, Germany

The lymphatic system, the second vascular systems of vertebrates, plays an essential role in fluid homeostasis, the initiation of immune reactions and fat absorption, but also contributes to pathological conditions. After the initial formation of blood vessels during embryonic development, a subpopulation of endothelial cells in the cardinal vein in response to an as yet unknown signal express the transcription factor prospero-related homeobox 1 (PROX1), subsequently followed by vascular endothelial growth factor receptor 3 (VEGFR-3) and other lymphatic-specific markers. Lymphatic endothelial progenitor cells then leave the cardinal vein (CV) and follow a gradient of VEGF-C to form the first lymphatic structures and the thoracic duct. The primary superficial lymphatic plexus finally is formed by radial sprouting of lymphatic endothelial cells (LECs). Several competing models for the process of initial lymphangiogenesis exist. To resolve the mechanisms of the initial steps of lymph vessel formation, we generated high resolution 3-dimensional renderings of mouse developmental stages between E9.5 and E12.5. We analyzed immunofluorescence stained and PROX1-driven mOrange2-genetically-labeled mouse embryos, using the novel light sheet imaging modality ultramicroscopy. Our analysis revealed that initially LECs migrate away from the CV as streams of non-luminized, loosely connected cells, forming a mesh network of LECs. The LECs stream coalesces into a first peripheral longitudinal lymphatic vessel (PLLV), located lateral to the somites and subsequently forms a large luminized structure, we refer to as the primary thoracic duct (pTD) near the cardinal vein. Collectively, these first lymphatics structures have been referred to as lymph sacs. An area of highest level PROX1 expression demarks the site of closest juxtaposition between the CV and pTD and likely gives rise to the first venous-lymphatic valve. LECs sprouting from the PLLV form superficial lymphatics. In CCBE-1-deficient embryos, Prox-1-positive LECs arose normally in the CV and between E10 and E10.5 the initial migration of LECs away from the CV was unperturbed. Unexpectedly, the PROX1 expression domain in the CV was broadened in CCBE1-deficient embryos, which displayed increased VEGFR-3 expression and sprouting of venous endothelium. Concomitantly in CCBE1 KO embryos, we observed a distinct loss of PROX1-positive cardiomyocytes. Our study suggests that ultramicroscopy is an extremely powerful tool for the analysis of developmental processes and allowed us to develop a novel model for the initial steps of blood lymphatic separation and initial lymphangiogenesis. Our future goals include investigation of the mechanisms controlling LEC migration and identification the molecules regulating the special control of this process.

Investigating shear stress dependent changes in endothelial adhesion

Georgina Hazell*, Elaine Hayes, Jack Teasdale, Jason Johnson, Andrew Newby and Stephen White. Bristol Heart Institute, University of Bristol, United Kingdom

Background Endothelial cell behaviour is modified by shear stress, which therefore impacts on vascular pathology. Atherosclerotic plaques develop in regions where endothelial cells experience low and disturbed shear stress. Paradoxically, however, both plaque rupture and erosion localise to regions of stenosis that experience elevated shear stress. Endothelial erosion, which accounts for approximately a quarter of fatal myocardial infarctions results from a detachment of a large patch of endothelium precipitating thrombus formation. Therefore, understanding how endothelial adhesion is modified by shear stress could elucidate factors that contribute to acute coronary syndromes. Methods and Results We utilized a parallel plate flow apparatus to culture human umbilical vein endothelial cells (HUVECs) at either 'normal' laminar shear stress at 15 dynes/cm2 (LSS15) or 'elevated' shear stress at 75 dynes/cm2 (LSS75). No cell loss was observed when culturing HUVECs at LSS15 for 24 hours, although exposure to LSS75 caused a time-dependent loss of adhesion, with no loss of cells for the first 16 hours, after which cell number was progressively reduced. Loss of adhesion was significantly attenuated by the broad spectrum metalloprotease inhibitor GM6001, the overexpression of the tissue inhibitor of metalloproteinase TIMP-3, or by inclusion of the serine protease inhibitor Aprotinin. The specific loss of adhesion at LSS75 was also observed with human coronary artery endothelial cells. In HUVECs, LSS75 modified the expression of several proteases and protease receptors compared to LSS15. In particular, the protein expression of two metalloproteases MMP-10 and MMP-14 increased 6- and 5-fold, respectively. MMP-14 protein expression inversely correlated with its RNA expression, indicative of the involvement of microRNAs. A screen of candidate microRNAs predicted to bind to the 3'UTR of MMP-14, revealed that both miR-24 and miR-181 are highly expressed in HUVECs and their expression levels are reduced at LSS75. Overexpression and knockdown experiments are currently underway using adenoviral expression, or siRNA transfection, to identify whether MMP-10 and MMP-14 contribute to the reduction in adhesion caused by elevated shear stress. Conclusions Elevated shear stress modifies endothelial proteases expression and adhesion. This may have implications in the pathogenesis of endothelial erosion of plaques.

CD40 ligand-induced von Willebrand factor deposition on endothelial cells promotes platelet-monocyte interaction

Andreas H. Wagner[1], Kerstin Möller[1], Oliver Adolph[1], Manuel Schwarz[1], Chrysovalandis Schwale[2], Claus Bruehl[2], Rainer Nobiling[1], Thomas Wieland[3], Stefan W. Schneider[4] and Markus Hecker[1]*. Institute of Physiology and Pathophysiology, Divisions of [1]Cardiovascular Physiology and [2]Neuro- and Sensory Physiology, University of Heidelberg, Germany, [3]Institute for Experimental and Clinical Pharmacology and Toxicology and [4]Division of Experimental Dermatology, Medical Faculty Mannheim, University of Heidelberg, Germany

The co-stimulatory CD40-CD40 ligand (CD154) dyad has been implicated in atherogenesis mainly with regard to plaque destabilization. We hypothesized that CD40 ligation may also play a role in early lesion formation by promoting the deposition of ultralarge von Willebrand factor (vWF) multimers (ULVWF) on the luminal surface of an otherwise healthy endothelium that would enable the adhesion of platelets which in turn might fuel the recruitment of circulating monocytes to the atherosclerosis predilection site. To substantiate this hypothesis, human umbilical vein endothelial cells (HUVECs) were stimulated with soluble trimeric CD154 or mouse myeloma cells expressing human CD154. Release of vWF was quantified by a modified ELISA and visualized by immunofluorescence analysis. CD40 signaling in the cultured HUVECs was evaluated by a combination of voltage-clamp, calcium imaging and pharmacological inhibition. In addition, isolated mouse carotid artery segments were perfused at different levels of shear stress and then exposed to soluble CD154. ULVWF formation and fluorescence dye-labeled murine platelets and/or monocytes adhering to these multimers were visualized by confocal fluorescence microscopy. Upon CD40 ligation, the cultured HUVECs both released vWF and deposited it on their surface as ULVWF in the presence of shear stress; both of these effects were inhibited by the calcium chelator BAPTA. Monitoring CD154-stimulated calcium transients revealed a robust increase in intracellular calcium which was both delayed and prolonged as compared to a prototypic G-protein-coupled receptor (GPCR) agonist such as histamine. Voltage clamp recordings excluded an influx of extracellular calcium into the HUVECs. Further evaluation of CD40 signaling in these cells pointed towards stimulation of a Src-type tyrosine kinase followed by activation of phospholipase Cgamma1 and subsequent mobilization of intracellular calcium through inositol-1,4,5-trisphosphate. An involvement of both protein kinase A and C in CD154-stimulated vWF release could be excluded, and angiopoetin-1 (Ang-1), which blocks the stretch-induced release of Ang-2 from the endothelial cell Weibel Palade bodies (where it is stored together with vWF), had no effect on CD154-stimulated vWF release. Moreover, isolated carotid artery segments of wild type mice perfused at near physiological shear stress revealed a prominent ULVWF formation following exposure to soluble CD154 which was absent in segments derived from CD40-deficient animals. Subsequent perfusion with washed murine platelets resulted in their increased adherence to these multimers and activation, as judged by an increase in P-selectin immunoreactivity. Additional perfusion with isolated murine monocytes resulted in their firm adhesion to the luminal surface of the endothelium where they co-localized with both ULVWF and platelets. CD40 ligation thus elicits both the release of vWF from intact endothelial cells and its deposition on their surface as ULVWF. This vWF release is not brought about by classical GPCR-mediated signaling but likewise requires an increase in intracellular calcium. ULVWF deposition on the endothelial cell surface under flow in turn facilitates the adhesion and subsequent activation of circulating platelets which seem to amplify endothelial cell-monocyte interaction, thus possibly initiating or promoting early lesion formation in conduit arteries at the classical predisposition sites.

Seeking for the pro-angiogenic target of the metalloprotease meprin α

Jana Hedrich[1]*, Elisa Nitzsche[2], Tamara Jefferson[3], Philipp Arnold[4], Mario DeJung[4], Ulrich auf dem Keller[5], Judith S. Bond[6], Jürgen Brieger[7], Christoph Becker-Pauly[3].
[1]Institute of Physiology and Pathophysiolog, University Medicine Johannes Gutenberg-University, 55128 Mainz, Germany, [2]Cell and Matrix Biology, Johannes Gutenberg-University, 55128 Mainz, Germany, [3]Institute of Biochemistry, Unit for Degradomics of the Protease Web, Christian-Albrechts-University, Kie,l [4]Molecular Physiology, Johannes Gutenberg-University, 55128 Mainz, Germany, [5]Institute of Cell Biology, Swiss Federal Institute of Technology Zurich, ETH Hoenggerberg, HPM D24, CH-8093 Zuerich, Switzerland, [6]Department of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA, [7]University Hospital Mainz, Head and Neck Surgery, Langenbeckstrasse 1, Mainz, Germany

Recently, we could show that the specific knockdown of meprin αin zebrafish embryos revealed severe defects in the formation of the vascular system. A comparable phenotype was observed by Nasevicius et al. (2000) in a VEGF (vascular endothelial growth factor)-A morpholino fish. Interestingly, using the proteomics approach TAILS (terminal isotopic labeling of substrates) we found a cleavage side for meprin αin VEGF-A165. In this regard we could validate the cleavage of VEGF-A isoforms VEGF-A121 and VEGF-A189. So we hypothesized that meprin αmight be necessary for VEGF-A activation, due to a cleavage that might be responsible for the detachment of VEGF-A from the extracellular matrix. Indeed, immunogold staining in tissue sections of meprin αknockout mice revealed more VEGF-A clusters in the extracellular matrix compared to wildtype mice. However, in vitro and in vivo studies with human umbilical vene cells (Huvecs), the mouse aortic ring assay and the HET CAM assay did not approve an activation of VEGF-A by meprin α. By the TAILS-method CTGF (connective tissue growth factor) another regulator of VEGF-A activity was found to be a meprin αsubstrate. CTGF binds to VEGF-A and thereby inhibits the angiogenic effect of VEGF-A. Cleavage of CTGF in the complex releases active VEGF-A. We could identify a cleavage side for meprin αbetween the second and third domain of CTGF. By in vitro and in vivo assays we could show, that the processing of CTGF in complex with VEGF-A revives the angiogenic effect of VEGF-A.

In vitro differentiation of Hey deficient endothelia to delineate vascular Hey gene functions

Melanie Hein*, Andreas Fischer, Manfred Gessler. University of Wuerzburg, Biocenter, Developmental Biochemistry SFB 688, Wuerzburg, Germany

Hey basic helix-loop-helix transcription factors are direct targets of the Notch signaling pathway. As essential Notch transducers they are responsible for arterial fate specification of endothelia and suppression of the venous program. Combined loss of Hey1 and Hey2 in the mouse leads to impaired angiogenic remodeling and arterialization resulting in early lethality. As the molecular mechanisms how Hey proteins control cell differentiation are largely unknown, our goal is to determine the function of Hey genes in endothelia. To analyze these processes in vitro we have generated Hey1-/-/Hey2-/- deficient mouse embryonic stem cells from Hey1fl/fl/Hey2-/- mice. These cells are pluripotent as shown by alkaline phosphatase staining and strong expression of pluripotency marker like Nanog, Oct-4 and Sox-2 and they can be differentiated in vitro. To enrich for endothelial cells during in vitro differentiation we rely on biochemical selection markers. Cells were stably transfected with vectors containing endothelial specific promoters of VE-Cadherin or Tie-1 fused to a blasticidin resistance gene. Hey1-/-/Hey2-/- deficient ES cells are able to differentiate into endothelial cells in 2D cultures using a chemically defined medium. Antibiotic selection after activation of endothelial specific promoters strongly enriches for endothelial cells. These cells show positive immunofluorescent staining for CD31 (PECAM-1) and high expression of endothelial marker genes like VE-Cadherin, Tie-1 and Vegfr-2 by qRT-PCR when compared to their undifferentiated counterparts. In contrast, pluripotent marker genes are strongly down-regulated. With inducible Hey-transgenes we can now test the role of Hey genes in a dose- and time-dependent manner during differentiation of endothelial cells to detect possible differences in arterio-venous cell fate determination, concomitant gene expression changes and functional properties of the resulting cells.

Bone morphogenetic protein modulator BMPER induces a synthetic to contractile phenotype switch in vascular smooth muscle cells

Jennifer Heinke*, Franziska Bluhm, Thomas Helbing, Sebastian Grundmann, Christoph Bode, Martin Moser. University Medical Center Freiburg, Department for Cardiology, Albert-Ludwigs-University Freiburg, Germany

In several vascular disorders, such as atherosclerosis, restenosis, aneurysm and hypertension, dysregulated vascular smooth muscle cells (vSMC) contribute to the progression of the disease. VSMC are highly differentiated cells that exhibit contractile function and regulate systemic blood pressure through modulation of the vascular tone. A specific set of genes, including smooth muscle actin (SMA), calponin (CNN) or transgelin (TAGLN), is highly expressed in vSMC and marks the quiescent, contractile phenotype. However, upon vascular injury or induction of vascular remodeling, vSMC can switch from their contractile to a synthetic phenotype marked by increased migration, proliferation and decreased expression of contractile genes. Several growth factors are well known to modulate vSMC phenotypic plasticity: e.g. platelet-derived growth factor (PDGF) induces the synthetic phenotype, whereas members of the transforming growth factor β (TGF-β) and bone morphogenetic proteins (BMPs) shift the balance towards a contractile phenotype. Recently, we have shown that BMP endothelial cell precursor derived regulator (BMPER) is necessary for regular vascular sprouting of endothelial cells (ECs). Besides ECs, vSMCs are another important cell type to build a functional blood vessel. In this project, we now examine the role of BMPER in vSMC biology. To investigate the effect of BMPER on primary vSMCs, we analyzed the expression of several contractile markers (SMA, CNN and TAGLN) by quantitative realtime-pcr. After stimulation with recombinant BMPER protein, the expression of all contractile markers was increased. Along the same line, we detected enhanced SMA protein expression by using western blot analysis. Furthermore, vSMC were stimulated with recombinant BMPER protein to detect SMA stress fiber formation by immunocytochemistry staining. We subjected vSMCs to functional cell culture assays and observed decreased proliferation and migration capacity of vSMCs after BMPER stimulation, which is consistent with a contractile phenotype. For loss of BMPER experiments, siRNAs were transfected into vSMCs and the expression of contractile markers was analyzed on RNA and protein level along with the assessment of proliferation and migration capacity. Consistent with our previous results, BMPER knockdown vSMCs displayed decreased expression of SMA, CNN and TAGLN and increased proliferation and migration. In addition a collagen contraction assay was performed that displayed decreased contractibility of BMPER-depleted vSMCs indicating a synthetic SMC phenotype. Mechanistically, we discovered that in BMPER-depleted vSMCs BMP-responsive Smad1/5 signalling pathway activity is reduced compared to controls. Stimulation of vSMC with TGF-β, heparin or BMP4 lead to SMA stress fiber formation. Interestingly, in BMPER-depleted vSMC SMA stress fibers are not formed despite BMP4 stimulation. However, TGF-β or heparin still induced stress fibers even in BMPER depleted cells, suggesting a BMP-dependent mechanism for BMPER. In conclusion, our data demonstrate that BMPER causes a contractile phenotype in primary vSMC and consistently, loss of BMPER leads to a synthetic phenotype.

Follistatin as an inducer of lymph vessel formation in melanoma

Magdalena Heinz*, Heide Niederleithner, Peter Petzelbauer. Skin & Endothelium Research Division (SERD), Department of Dermatology, Medical University Vienna, Austria

Activin A proteins are homodimers of inhibin βA subunits and potently inhibit blood vessel angiogenesis in part via induction of p21. Follistatin serves as an antagonist, it binds activin with high affinity and neutralizes most but not all of its biological actions. To investigate the role of follistatin in lymph vessel angiogenesis in melanoma, we selected human A375 melanoma cells which express high levels of the inhibin βA subunit. We stably over-expressed control vectors or follistatin and injected them intradermally into SCID mice. The arising primary tumours were excised at a size of 400mm3 and animals were monitored for metastasis to sentinel lymph nodes (SLN). Blood and lymph vessel formation was quantified by immunohistochemistry and real time PCR. We found growth rates of primary tumors to be equal in controls and follistatin over-expressing melanoma, as well as percentages of Ki67 positive cells. In contrast, mean times to metastasis to SLN were reduced by follistatin and this inversely correlated with significantly increased numbers of lymph vessels in primary melanoma. In conclusion, our results point to a novel role of follistatin as an inducer of tumor lymph angiogenesis.

BMP activity controlled by BMPER modulates endothelial barrier function in acute lung injury

Thomas Helbing*, Elena Ketterer, Christoph Bode Martin Moser. Department of cardiology and angiology University hospital Freiburg, Germany

Introduction: Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome, are associated with high morbidity and mortality in patients. During the progression of ALI, the endothelial cell barrier of the pulmonary vasculature becomes compromised, leading to pulmonary edema, a characteristic feature of ALI. It is well-established that EC barrier dysfunction is initiated by cytoskeletal remodeling, which leads to disruption of cell-cell contacts and formation of paracellular gaps, allowing penetration of protein-rich fluid and inflammatory cells. Bone morphogenetic proteins (BMPs) are important players in endothelial dysfunction and inflammation but their effects on endothelial permeability in ALI have not been investigated until now. Methods and Results: As a first approach to assess the role of BMPs in acute lung injury we analysed BMP4 and BMPER expression in an infectious (LPS) and a non-infectious (bleomycin) mouse models of acute lung injury. In both models BMP4 and BMPER protein expression levels were reduced demonstrated by western blots, suggesting that BMPs are involved in progression ALI. To assess the role of BMPs on vascular leakage, a key feature of ALI, BMP activity in mice was inhibited by i.p. administration of LDN193189, a small molecule that blocks BMP signalling. After 3 days Evans blue dye (EVB) was administered i.v. and dye extravasation into the lungs was quantified as a marker for vascular leakage. Interestingly, LDN193189 significantly increased endothelial permeability compared to control lungs, indicating that BMP signaling is involved in maintenance of endothelial barrier function. To quantify effects of BMP inhibition on endothelial barrier function in vitro, HUVECs were seeded onto transwell filters and were exposed to LDN193189. After 3 days FITC-dextrane was added and passage into the lower chamber was quantified as a marker for endothelial barrier function. Thrombin served as a positive control. As expected from our in vivo experiments inhibition of BMP signaling by LDN193189 enhanced FITC-dextrane passage. To study specific effects of BMPs on endothelial barrier function, two protagonist of the BMP family, BMP2 and BMP4, or BMP modulator BMPER were tested in the transwell assay in vitro. Interestingly BMP4 and BMPER, but not BMP2, reduced FITC-dextrane passage demonstrating that BMP4 and BMPER improved endothelial barrier function. Vice versa, specific knock down of BMP4 or BMPER increased leakage in transwell assays. Im immuncytochemistry silencing of BMPER or BMP4 induced hyperpermeability as a consequence of a pro-inflammatory endothelial phenotype characterised by reduced cell-cell contacts and increased actin stress fiber formation. Additionally, the pro-inflammatory endothelial phenotype was confirmed by real-time revealing increased expression of adhesion molecules ICAM-1 or proinflammatory cytokines such as IL-6 and IL-8 in endothelial cells after BMPER or BMP4 knock down. Confirming these in vitro results BMPER +/- mice exhibit increased extravasation of EVB into the lungs, indicating that partial loss of BMPER impairs endothelial barrier function in vitro and in vivo. Conclusion: We identify BMPER and BMP4 as local regulators of vascular permeability. Both are protective for endothelial barrier function and may open new therapeutic avenues in the treatment of acute lung injury.

FGD5 is highly expressed by endothelial cells in vascular sprouts and modulates VEGFA signaling

Johan Heldin[1]*, Peder Fredlund Fuchs[1], Zsolt Kasza[1], Paul O'Callaghan[1], Pontus Aspenström[3], Anqi Xiong[2], Karin Forsberg-Nilsson[2], Pär Gerwins[1] and Johan Kreuger[1].
[1]Dept. of Medical Biochemistry and Microbiology, Science for Life Laboratory, Uppsala University, Husargatan 3, PO Box 582, SE-751 23, Uppsala, Sweden, [2]Dept. of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, 751 85 Uppsala, Sweden, [3]Dept. of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

Rho guanine nucleotide exchange factors (RhoGEFs) activate Rho GTPases that are important regulators of cell migration, proliferation and apoptosis. Here we present data showing that FGD5, belonging to the FGD-subfamily of RhoGEFs, is highly expressed by endothelial cells in growing vascular sprouts as well as by dormant vessels in the mouse brain. The subcellular localization of FGD5 was shown to be in the region close to the plasma membrane. siRNA-mediated knockdown of FGD5 expression in vitro had no apparent effects on endothelial cell proliferation or apoptosis, but suppressed VEGFA induced chemotaxis however without affecting the overall motility of the endothelial cells. Furthermore, FGD5 knockdown was shown to decrease VEGFA-induced phosphorylation of the Erk1/2 and p38 MAP kinases. Our data suggest that FGD5 modulates VEGFA signaling important for cell migration in endothelial cells.

Development of the common cardinal vein in zebrafish represents a new mode of vessel formation

Christian S. M. Helker[1], Terhi Kärpänen[2], Dagmar Zeuschner[3], Stefan Schulte-Merker[2] and Wiebke Herzog[1,3]*. [1]Westfälische Wilhelms-Universität, Muenster, Germany, [2]Hubrecht Institute, Utrecht, Netherlands, [3]Max-Planck Institut für Molekulare Biomedizin, Muenster, Germany

While it is clear that not all vessels are alike, currently there is little understanding of the differential regulation in formation of the complex vascular pattern. In this study we focused on the development of the common cardinal veins (CCVs) and found that these veins represent a novel and unusual way of vessel and lumen formation. To study the development of the CCVs, we took advantage of the optical clarity of the zebrafish embryo and used high resolution confocal time lapse imaging, enabeling us to observe vessel growth in vivo. The CCVs collect all blood from the embryo and transport it through the sinous venosus back to the heart. We analyzed CCV formation from angioblast specification in the lateral plate mesoderm to the finally completed vessel. By combining in vivo imaging with expression studies and photoconversion experiments, we can show that the angioblasts forming the CCV become specified at a later time point and as a separate population than the angioblasts forming the arteries in the trunk of the zebrafish embryo. Additionally we can show that they do not differ from future arterial cells in their expression profile, but instead venous identity is dependent on the time point of specification. After the future venous angioblasts are specified, the CCV endothelial cells first form an open ended lumen by aligning around a large luminal space, without ever passing through a cord hollowing step. This way of lumen formation has not been described before. Consecutively and after the onset of circulation the vessel grows around the flowing blood. Interestingly this growth is independent of the blood flow. We could show that Vegfc regulates the endothelial cell number in the CCVs. Surprisingly, we found that the source of Vegfc are hematopoietic cells, specifically erythrocytes. This is the first report of circulating blood cells regulating vessel formation. Therefore our findings suggest a new form of developmental crosstalk between the hematopoietic and endothelial lineages.

A novel mechanism for hypofibrinolysis in diabetes: The role of complement C3

K Hess[1]*, V Schroeder[2,5], S Alzahrani[2], M Mathai[3], G Howell[4], K Smith[2], AM Carter[2], N Marx[1], PJ Grant[2] and RA Ajjan[2]. [1]Department of Internal Medicine I, University Hospital RWTH Aachen, 52074 Aachen, Germany, [2]Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre (MCRC), University of Leeds, Leeds LS2 9JT, UK, [3]Department of Paediatrics, Bradford Royal Infirmary, Bradford BD9 6RJ, UK, [4]Faculty of Biological Sciences, University of Leeds, LS2 9JT, UK, [5]University Clinic of Hematology and Central Hematology Laboratory, Hemostasis Research Laboratory, University Hospital and University of Bern, Bern, Switzerland

Background: Impaired fibrin clot lysis is a key abnormality in diabetes and complement C3 is one protein identified in blood clots. We hypothesise that C3 has a role in hypofibrinolysis in this condition and investigate the relationship between C3 and fibrin clot lysis in type 1 diabetes (T1DM) both ex vivo and in vitro. Methods and Results: Fibrinolysis and C3 plasma levels were determined in T1DM subjects, and effects of glycemia investigated. C3 incorporation into the clot, modulation of fibrinolysis and C3-fibrinogen interactions were assessed by ELISA, immunoblot, turbidimetric assays, confocal microscopy and plasmon resonance. Clot lysis time was longer in 30 children with T1DM compared with 17 controls (599±18 and 516±12 sec, respectively; $p<0.01$) and C3 levels were higher (0.55±0.02 and 0.43±0.02 mg/ml, respectively; $p<0.05$). Improving glycemic control in young adults with T1DM (n=18) was associated with shorter lysis time compared with baseline (550±19 and 601±21 sec, respectively; $p<0.05$) and lower C3 levels (0.54±0.02 and 0.65±0.04 mg/ml, respectively; $p<0.05$). An interaction between C3 and fibrin was confirmed by lower protein levels in sera compared with corresponding plasma and C3 detection in plasma clots by immunoblot. In a purified system, C3 resulted in more prolongation in lysis time of clots made from diabetes compared with control fibrinogen (244±64 and 92±23 sec, respectively; $p<0.05$). Confocal microscopy showed 41% increased C3 incorporation into diabetes clots compared with controls ($p<0.05$), and fully formed clot lysis was prolonged by 764±76 and 428±105 sec, respectively ($p<0.05$). These differences in lysis, comparing diabetes and controls, were not related to altered plasmin generation or C3-fibrinogen binding. Conclusion: C3 incorporation into clots from diabetic fibrinogen is enhanced and adversely affects fibrinolysis. This may be one novel mechanism for compromised clot lysis in diabetes, potentially offering a new therapeutic target.

Tumor endothelial cells acquire drug resistance by MDR1 upregulation

Kyoko Hida[1]*, Kosuke Akiyama[1], Noritaka Ohga[1], Yasuhiro Hida[2], Taisuke Kawamoto[1], Yoshihiro Sadamoto[1], Nako Maishi[1], Tomoshige Akino[1,3], Miyako Kondoh[1], Masanobu Shindoh[4]. [1]Department of Vascular Biology, Graduate School of Dental Medicine, [2]Department of Cardiovascular and Thoracic Surgery, Graduate School of Medicine, [3]Department of Renal and Genitourinary Surgery, Graduate School of Medicine, [4]Department of Oral Pathology and Biology, Graduate School of Dental Medicine, University of Hokkaido, Japan

Tumor endothelial cells (TECs) are therapeutic targets in antiangiogenic therapy. Contrary to the traditional assumption, it has been reported that TECs can be genetically abnormal and might acquire drug resistance. In this study, mouse TECs and normal ECs (NECs) were isolated to investigate drug resistance of TECs and the mechanism by which it is acquired. TECs were more resistant to paclitaxel with upregulation of multidrug resistance 1 (MDR1) mRNA, which encodes the P-glycoprotein, compared to NECs. Normal human microvascular ECs (HMVECs) were cultured in tumor-conditioned medium (tumor CM) and were found to become more resistant to paclitaxel through MDR1 mRNA upregulation and nuclear translocation of Y-box-binding protein 1 (YB-1), which is an MDR1 transcription factor. Vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) and Akt were activated in HMVECs by tumor CM. We observed that tumor CM contained a significantly high level of VEGF. A VEGF receptor kinase inhibitor, Ki8751, and a PI3K/Akt inhibitor, LY294002, blocked tumor CM-induced MDR1 upregulation. MDR1 upregulation via the VEGF/VEGFR pathway in the tumor microenvironment is one of the mechanisms of drug resistance acquired by TECs. We observed that VEGF secreted from tumors upregulated MDR1 through VEGFR2 and Akt activation. This is a novel mechanism of acquisition of drug resistance by TECs in a tumor microenvironment.

Regulation of vascular smooth muscle cell proliferation rate by the inducible cAMP early repressor (ICER)

Hildebrandt I.[1]*, Seidl M. D.[1], Nunes F.[1], Endo S.[2], Kojima N.[2], Offermanns S.[3], Schmitz W.[1], Müller F. U.[1]. [1]Westfälische Wilhelms-Universität Institut für Pharmakologie und Toxikologie, Domagkstr 12, 48149 Muenster, Deutschland; [2]Tokyo Metropoli Institut für Pharmakologie und Toxikologie Westfälische Wilhelms-Universität Muenster Domagkstraße 12 48149 Muenster, Germany, [3]MPI Bad Nauheim Pharmakologie, Ludwigstr. 43, 61231 Bad Nauheim, Germany

The inducible cAMP early repressor (ICER), a prominent isoform of the transcription factor CREM (cAMP response element modulator), acts as a transcriptional repressor on cAMP responsive elements (CREs) in the promoter regions of its target genes. Previously, we observed that CREM/ICER is associated with anti-proliferative effects in vascular smooth muscle cells (VSMCs) in vivo. Accordingly, CREM-knockout mice, in which none of the multiple CREM isoforms can be expressed, exhibited an increased neointima formation after carotid ligation as well as an increased atherosclerotic plaque formation after high fat diet on an ApoE-deficient background. These observations were associated with an increased proliferation rate in isolated CREM deficient VSMCs. On this background we wanted to clarify the specific role of the ICER isoforms for the proliferative response of VSMCs. We examined the inducibility of Icer in smooth muscle cell lines after transient transfection of an Icer-promoter controlled luciferase reporter gene construct. The promoter activity was 14-fold increased after stimulation with forskolin (FSK), an activator of the adenylyl cyclase, in immortalized rat smooth muscle cells (luciferase activity, related to solvent control; 13.7 ± 0.9; n=3; 4h stimulation) and was 3-fold increased (3.2 ± 0.16; n=4 ; 2h stimulation) after FSK stimulation in A7r5 rat smooth muscle cells. The influence of different cAMP dependent transcription factors on the ICER promoter activity was tested with appropriate constructs for transient overexpression. ATF-1 (activating transcription factor 1; 1.1 ± 0.1; n=42 from 4 isolations) and caATF-1 (constitutive active ATF-1; 1.1 ± 0.1; n=4) had no significant influence on Icer promoter activity. In contrast, expression of caCREB (constitutive active cAMP response element binding protein) led to a maximum stimulation of 2.8 ± 0.1 (n=4) after 24 hours. In primary VSMC originating from ICER KO (IKO) mice the proportion of proliferating cells was higher (48.7 ± 2.2 %; $P<0.05$ vs. WT) as compared to WT VSMCs (38.9 ± 1.9 %). FSK stimulation of VSMCs inhibited the proliferation rate of both IKO and WT VSMCs and abolished the difference between the genotypes. Hence, stimulation of the cAMP-dependent signal transduction pathway by FSK led to the activation of the Icer promoter in VSMCs; ICER inactivation was linked to proliferation of VSMCs under non-stimulated conditions and FSK inhibited proliferation of VSMCs of both genotypes. We conclude that further cAMP dependent mechanisms - beyond Icer - contribute to the regulation of VSMCs proliferation. (supported by DFG)

HLX and FOXF1, two transcription factors involved in the regulation of sprouting angiogenesis

K. Lipnik, C. Sturtzel, J. Testori, R. Hofer-Warbinek and E. Hofer*. Department of Vascular Biology, Medical University of Vienna, A-1090 Vienna, Austria

Despite detailed knowledge on cellular mechanisms of sprouting angiogenesis, the knowledge on transcription factors controlling the differentiation state and sprouting capabilities of endothelial cells remain uncomplete. We have recently defined two transcription factors with distinct roles in sprouting of endothelial cells and their progenitors, HLX and FOXF1. Our data show that HLX is a VEGF-regulated homeobox transcription factor which controls a genetic program to upregulate UNC5B and other repulsive guidance cues and thus inhibits sprouting and neovascularization at normoxic conditions. Under hypoxia the HLX-mediated upregulation of UNC5B is largely reduced leading to strongly increased sprouting capabilities. HLX seems further to be involved in UNC5B expression in tip cells, which may be important to give direction to the growing sprout (1). The forkhead box transcription factor FOXF1 we found highly expressed by endothelial progenitors, its expression level decreasing with endothelial maturation. FOXF1 overexpression strongly induces sprouting of progenitors as well as mature endothelial cells by modulating the Notch/Dll pathway. The factor upregulates specifically Notch-2 and leads to increased exposure of Dll1 at the cell surface. In consequence, the expression of several receptors with important functions in neovascularization are modulated. This includes the upregulation of VEGFR-2 and ephrin B2, whereas EphB4 is downregulated. This suggests a crucial role of FOXF1 for the increased angiogenic sprouting capabilities of endothelial progenitors and suggests its involvement in arterial specification. This work was supported by FWF P21291 and EC Health-222995. (1) Testori et al., Blood 117, 2735 (2011)

Transcriptional profiling reveals a requirement for phosphodiesterase 10A during flow-induced vessel remodeling in the chick embryo

E C Hoggar*, M Placzek, T J Chico. [1]MRC Centre for Biomedical and Developmental Genetics, The University of Sheffield, Sheffield, United Kingdom, [2]NIHR Cardiovascular Biomedical Research Unit, Sheffield Teaching Hospitals, Sheffield, United Kingdom

Collateral vessel development occurs by remodelling of pre-existing endothelial communications between occluded and neighbouring vascular territories to restore blood flow following occlusion of a large conducting artery. The biological response of blood vessels to blood flow is central to collateral vessel development. We characterised the transcriptional response of the extra-embryonic vasculature of the chicken embryo during flow-induced remodelling in an attempt to identify potential drivers of this process. Right vitelline artery ligation induced remodelling of the contra-lateral vasculature, with collateral vessels supplying the ligated territory. Collaterals arose by enlargement of pre-existing vessels and the collateral network demonstrated active remodelling with an early peak in collateral number at 12h post ligation, followed by pruning and selection of a more efficient haemodynamic configuration of fewer, larger vessels over 48h. We characterised the global transcriptional changes at 4 and 12h post ligation and found that collateral vessels displayed a distinct gene expression profile. Among 164 differentially expressed protein coding mRNAs, we identified genes from the microarray which are unique to the developing collateral vessels and therefore suggested to be driven by shear stress. Phosphodiesterase 10A (PDE10A) is an enzyme involved in regulating the cellular concentration of cyclic nucleotides and able to hydrolyse both cAMP and cGMP. PDE10A was highly up-regulated at the site of flow-induced remodelling at 4h post ligation. Local application of the PDE10A inhibitor Papaverine Hydrochloride had no effect on normal vessel diameter (ctrl 466±34um, Papaverine [20uM] 600±90um) or development but significantly impaired collateral vessel formation at 24h post-ligation (ctrl 109±9 um, Papaverine [20uM] 49±5 um $P<0.0001$). Time course micrographs taken at 2h intervals following ligation in the absence or presence of Papaverine showed significantly reduced collateral development from 6h post-ligation, suggesting that Papaverine inhibited PDE10A at an early stage of collateral vessel growth. To demonstrate that PDE10A inhibition was specific to endothelial cells we isolated and dissected collateral vessels from the endodermic and ectodermic tissue of the midline area immediately posterior to the chick at 4 and 12h post-ligation. We explanted the endothelial tissue onto collagen beds and incubated with or without Papaverine for 15h. Explants were cultured with BrdU to measure proliferation in each group. Proliferation index revealed no difference in endothelial cell DNA replication in collateral vessels at 4h, treated with or without Papaverine (ctrl 34.5%±4.4, Papaverine [20uM]) 28.4%±3). However there was a significant decrease in the number of proliferating cells in collateral vessels dissected at 12h which had received Papaverine treatment compared to controls (ctrl 8.8%±0.1, Papaverine [20uM]) 2.8%±1.3 $P=0.04$). We therefore conclude that flow induced vascular remodelling in the chick induces dynamic changes in vessel architecture, associated with marked transcriptional changes distinct from normal vessel patterning. PDE10A is up-regulated during flow induced remodelling and pharmacologic inhibition significantly impairs this process. In vivo, the diameter and therefore the efficiency of collateral vessels are reduced. In vitro the effect of Papaverine also appears to be limited to vessels exposed to a change in flow, suggesting that PDE10A is involved in flow induced vessel remodelling in the chick embryo.

Hypoxia induced changes in vascular endothelial progenitor cell function: role for the extracellular metalloproteinase ADAMTS9?

Michelle B. Hookham*, Imran HA. Ali, Nathan Cantley, Heather Wilson, Alan W. Stitt and Derek P. Brazil. Centre for Vision and Vascular Science, School of Medicine, Dentistry, and Biomedical Sciences, Queen's University Belfast, Northern Ireland

Diabetic Retinopathy (DR) is the leading cause of visual impairment in working age populations worldwide. Current treatment for DR such as laser photo coagulation therapy does not address the early stages of this disease. Therefore, efficient treatments aimed at early intervention for DR represent a significant unmet clinical need. The aim of this work was to examine endothelial progenitor cells (EPCs) and their potential role as a cell based autologous therapy to revasularise the ischaemic tissue of the diabetic retina. Our group have isolated and characterised a distinctive human blood derived EPC with high proliferative capacity known as endothelial colony-forming cells (ECFCs). To mimic low O2 concentrations in the ischaemic retina, we have exposed ECFCs to 1 % O2 for varying times ranging from acute (less than 24 h), moderate (up to 72 h) and chronic (greater than 7 days). Exposure to chronic hypoxia resulted in reduced cell doubling compared to ECFCs maintained in 21 % O2. Moderate exposure to hypoxia delayed the ability of ECFCs to form tube-like structures in Matrigel, an in vitro measure of angiogenic activity. Hypoxia induced changes in ECFC shape, with increased F-actin staining evident in hypoxia-exposed cells and clearly visible punctuate focal adhesions visible compared to normoxic controls. Consistently, scratch wound assays indicated a higher number of cells in the wound, supporting an increased migratory phenotype in response to hypoxia. Microarray analysis of RNA extracted from ECFCs maintained in 1 % O2 for 8 h, 24 h and 48 h identified only 29 genes differentially regulated at all three time points. Of these, many were established hypoxic targets including genes involved in metabolism; ALDOC, ENO2 and SLC2A1 which are key components of the gluconeogenesis, glycolysis and glucose metabolic pathways. Upregulation of angiogenic factors such as adrenomedullin and ANGPTL4 was also detected, together with increased mitochondrial stress/autophagy genes BNIP3 and BNIP3L and the histone demethylase JMJD1A. Several novel genes not previously identified in hypoxia responses in ECFCs were identified, including altered expression of the extracellular metalloproteinase ADAMTS9 and its proprotein convertase Furin. A rapid and sustained decrease in ADAMTS9 mRNA in response to hypoxia was detected in ECFCs, with maximal reductions evident at 4 h. ADAMTS9 expression appears to be HIF1alpha dependent, as ECFCs treated with the prolyl hydroxylase inhibitor DMOG displayed high HIF1alpha levels and reduced ADAMTS9 mRNA in normoxia. The decrease in ADAMTS9 mRNA in response to hypoxia was further enhanced by the addition of the PI3K inhibitor LY294009. We are currently determining the significance of these gene expression patterns in ECFC angiogenesis. These data will advance our understanding of ECFC function in the ischaemic/hypoxic niche in diseased tissue in diabetic patients.

Neuropilin-1 expression is regulated by Gut microbial communities

Nives Hörmann[1,2]*, Jennifer Hahlbrock[1], Fredrik Bäckhed[3], Ulrich Walter[1], Christoph Reinhardt[1]. [1]Center for Thrombosis and Hemostasis (CTH), University Medical Center Mainz, Experimental Research, 55131 Mainz, Germany, [2]Graduate School for Immunotherapy, University Medical Center Mainz, 55131 Mainz, Germany, [3]Department of Molecular and Clinical Medicine & The Wallenberg Laboratory for Cardiovascular Research at Sahlgrenska Academy, University of Gothenburg, 423 45 Gothenburg, Sweden

Germfree (GF) mice show reduced villus vascularisation and thinner villus diameters in the distal part of the small intestine compared with conventionally raised (CONV-R) mice or conventional-derived (CONV-D) littermates. We found that tissue factor-dependent coagulation factor signaling is involved in these microbiota-induced changes in the small intestine mucosa. Collectively, these mechanisms contribute to altered tissue homeostasis in the small intestine and to a reduction in mucosal surface area observed upon colonization with a gut microbiota. Toll-like receptors (TLRs) sense microbial-associated molecular patterns (MAMPs) and TLR2 activation promotes angiogenesis. Here, we reveal mechanisms that are induced via TLR2 signaling by the gut microbiota and may promote tissue remodeling in the small intestine. Expression of TLR2 and its co-receptor TLR1 was increased in colonized mice, whereas expression levels of the co-receptor TLR6 remained unchanged. In addition, TLR4 mRNA levels were elevated upon colonization of ex-GF mice with a gut microbiota. This effect could be reversed by a 7 day treatment of CONV-R mice with the broad-spectrum antibiotics ampicillin and neomycin. An up-regulation of TLR2 was also found in the small intestinal epithelial cell line MODE-K in response to 2h and 4h stimulation with the TLR2/6-specific agonist macrophage-activating lipopeptide-2 (MALP-2) and the synthetic agonist Pam3CSK4. In contrast to stimulation with the TLR4-specific agonist Lipopolysaccharide (LPS) resulted in decreased TLR4 expression. Indeed, stimulation with TLR agonists resulted in massive downstream signaling as shown by increased phosphorylation of Erk1/2 and IκB. Neuropilin-1 (Nrp-1) is a known co-receptor and regulator of Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) signaling, but it also is a co-receptor for Plexin-dependent binding of class 3 semaphorins (Sema). We found that in the small intestine Nrp-1 is mainly expressed by enterocytes and its expression is decreased upon colonization with a gut microbiota. In the sterile infection MODE-K cell culture model we could demonstrate that this decrease in Nrp-1 expression is linked to TLR2 signaling. In addition, expression levels of Sema3F and its receptor Plexin A4 are also regulated by the gut microbiota. Collectively, our results obtained by the combined use of genetic and gnotobiotic mouse models indicate that the TLR2 / Nrp-1 signaling loop may be regulated by MAMPs.

Anti-inflammatory and anti-coagulant intervention strategies are not effective in reducing the effects of ionizing radiation on plaque size and phenotype in ApoE-/- mice

Saske Hoving[1]*, Sylvia Heeneman[2], Hans te Poele[1], Jeffrey Pol[2], Nicola Russell[1], Marion Gijbels[2], Fiona Stewart[1], Mat Daemen[3]. [1]Netherlands Cancer Institute, Amsterdam, The Netherlands, [2]Cardiovascular Research Institute Maastricht (CARIM), Maastricht, The Netherlands, [3]The Academic Medical Center, Amsterdam, The Netherlands

Earlier diagnosis and better treatment options have lead to improvements in cancer specific survival for most tumor types, but unfortunately this also results in an increased number of patients at risk for developing treatment related side effects. Several clinical trials have shown an increase in localized atherosclerosis after radiotherapy for Hodgkin's disease, breast and head and neck cancer and there is good evidence to identify radiation as an independent risk factor in vascular disease. In an earlier study we have shown that a single irradiation dose (14 Gy) to the carotid arteries of mice lacking functional ApoE (ApoE-/-) accelerated the development of macrophage-rich, inflammatory atherosclerotic lesions, prone to intra-plaque hemorrhage. The aim of the present study is to assess the potential of specific anti-inflammatory and anti-coagulant intervention strategies to circumvent radiation-induced effects on plaque development and phenotype. ApoE-/- mice were given single doses of 14 Gy or sham treatment (0 Gy) to the neck and the carotid arteries were harvested at 4-30 weeks after irradiation. Clopidogrel (20 mg/kg/day), atorvastatin (15 mg/kg/day), aspirin (ASA, 30 and 300 mg/kg/day) and nitric oxide releasing aspirin (NO-ASA, 60 mg/kg/day) were given in the chow at doses known to produce antiplatelet or anti-inflammatory effects, from 1 week before irradiation until termination of the experiment. Specific immunohistochemical stainings were performed to investigate expression levels of thrombotic and inflammatory markers of endothelial cell damage (e.g. thrombomodulin, ICAM-1, VCAM-1, eNOS and MCP-1) at early times after irradiation. This damage was correlated with the subsequent development of atherosclerotic lesions (plaque number, size and phenotype) at late times after irradiation. Clopidogrel and high dose ASA effectively blocked platelet aggregation, while the lower dose of ASA, NO-ASA and atorvastatin had no significant effect on platelet aggregation. Clopidogrel decreased MCP-1 expression and high dose ASA inhibited endothelial cell expression of VCAM-1 and thrombomodulin in the carotid arteries at 4 weeks after irradiation. Expression of ICAM-1, tissue factor and eNOS was unchanged in all treatment groups. Analysis of atherosclerotic lesions in this study confirmed that irradiation increased the number of lesions and predisposed to the formation of inflammatory, thrombotic plaque phenotype with reduced collagen content, compared with age-matched controls. NO-ASA significantly reduced the total number of lesions (44%) and the number of initial macrophage-rich lesions in age matched unirradiated mice (66%) and clopidogrel reduced the total plaque area (44%), but these effects were not maintained in the irradiated mice. High dose ASA did not reduce lesion number or size, but did lead to formation of collagen-rich advanced lesions in irradiated mice. In conclusion, the effects of radiation-induced atherosclerosis could not be circumvented by anti-inflammatory and anti-coagulant therapies, suggesting more complex underlying mechanistic pathways compared to age-related atherosclerosis. With increasing attention on quality of life aspects of cancer treatments, more knowledge is required on the mechanisms of development and management of late side effects. This knowledge should enable the development of effective intervention strategies to prevent or ameliorate the development of atherosclerotic changes in patients following radiation therapy.

19,20-DiHDPA, a product of the soluble epoxide hydrolase, promotes angiogenesis by direct inhibition of Notch signalling

Jiong Hu*, Rüdiger Popp, Khader Awwad, Timo Frömel, Ingrid Fleming. Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University, Frankfurt am Main, Germany

Cytochrome P450 (CYP) enzymes produce a number of bioactive epoxy-fatty acids from long-chain polyunsaturated fatty acids, e.g., from arachidonic acid and docasahexaenoic acid. The bioactivity of the epoxy-fatty acids is tightly regulated by the activity of the soluble epoxide hydrolase (sEH) which metabolizes them to the corresponding diols. Given that our previous work attributed arachidonic acid epoxides a role in angiogenesis, the aim of the present study was to investigate the role of sEH in postnatal retinal vascularization. Retinal vascularization was markedly delayed in sEH-/- mice at postnatal days (P) 2 and P5, and was associated with reduced tip cell numbers and filopoida extensions at the angiogenic front. This phenotype was associated with the induction of the Notch-dependent transcription factors Hes1 and Hey1, attenuated endothelial cell (EC) proliferation, and could be reproduced by pharmacological inhibition of sEH in wild-type (WT) mice, as well as by postnatal deletion of sEH in tamoxifen inducible sEH knockout mice. We found that the sEH was mainly expressed in Müller glia cells and that Müller cell specific sEH knockout mice displayed delayed retinal angiogenesis that was similar to that observed in the global sEH-/- mice. sEH expression was maintained in cultured murine Müller cells and conditioned medium from WT Müller cells stimulated EC proliferation. However, conditioned medium from sEH-/- Müller cells increased mRNA levels of Hes1 and Hey1 and decreased EC proliferation. The impaired Notch signaling in EC could be rescued either by the adenovirus mediated re-expression of sEH in sEH-/- Müller cells, or by the depletion of lipid components, rather than by blocking VEGF signaling. As our data indicated that a Müller cell-derived lipid was responsible for the effects observed we generated lipid profiles from WT and sEH-/- retinas (P5). We found a significant increase in epoxy-fatty acid levels i.e., 11,12- and 14,15-epoxyeicosatrienoic acid and 12,13-epoxyoctadecenoic acid, as well as a decrease in diol levels, especially the 19,20-dihydroxydocosapentaenoic acid (DiHDPA), in sEH-/- compared to WT littermates. Conditioned medium from sEH-/- Müller cells also showed a similar lipid profile. We observed that 19,20-DiHDPA significantly decreased the Dll4-induced cleavage of Notch intracellular domain in the absence and presence of the proteosome inhibitor MG-132. Moreover, 19,20-DiHDPA was able to inhibit gamma secretase-mediated processing of a constitutively active Notch derivative (Notchâˆ†E), indicating that 19,20-DiHDPA inhibits Notch signaling by direct inhibition of gamma-secretase activity. Finally, intravitreal injection of 19,20-DiHDPA resulted in a dramatic induction of primary network density, as well as a significant enhancement of tip cell and filopodia number in sEH-/- mice retina at P5. This is the first demonstration of an important role for Müller glia cells in the development of the retinal vasculature. Moreover, our data indicate that the sEH is required for retinal vascularization and that a sEH metabolite is essential for normal vascularization by directly affecting gamma-secretase mediated Notch signaling.

Endothelial cells act as a rheostat of liver regeneration through an autocrine Angiopoietin-2 loop

Junhao Hu[1]* , Kshitij Srivastava[1], Hellmut Augustin[1,2]. [1]Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), [2]Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim, Heidelberg University (CBTM), Germany

Liver regeneration requires precisely coordinated proliferation between different cellular compartments of the liver, most notably hepatocytes and liver sinusoidal endothelial cells (LSEC), to reconstitute liver structure and physiological function. Recent evidence has shown that LSEC, beyond their role as structural determinants of liver architecture, may play an active role during liver damage and liver regeneration by secreting angiocrine factors that promote hepatocyte proliferation. This was evidenced by landmark studies showing that the disruption of LSEC function by VEGFR2 deletion or VEGFR2 blockade severely impaired liver regeneration. In order to identify other positively or negatively acting endothelial cell-derived angiocrine factors, we performed gene expression analyses of partially hepatectomized and sham operated livers and assessed differential gene expression. LSEC-derived Angiopoietin-2 (Ang-2) expression was rapidly downregulated upon 2/3 partial hepatectomy. Correspondingly, partial hepatectomy experiments in Ang-2-deficient mice revealed that loss of Ang-2, which is exclusively expressed by LSEC, enhanced hepatocyte proliferation during the inductive phase of liver regeneration (day 0-4). The mechanistic analysis of this angiocrine crosstalk between LSEC and hepatocytes identified autocrine acting Ang-2 as regulator of LSEC TGFß1 expression, which is known to act as a negative regulator of hepatocyte proliferation. While LSEC-derived negative Ang-2 regulation served as an enhancer of hepatocyte proliferation, liver regeneration was surprisingly found to be slowed in Ang-2-deficient mice during the angiogenic phase of liver regeneration (day 4-8). Correspondingly, Ang-2 was in wildtype mice prominently upregulated during the angiogenic phase of liver regeneration suggesting that the balance of direct vascular vs. indirect parenchymal Ang-2 effects may be shifted towards direct direct angiogenesis regulating effects during the later stages of liver regeneration. Mechanistically, this interpretation was supported by the finding that Ang-2 during the angiogenic phase served as positive regulator of VEGFR2 expression and the established endothelial Wnt pathway regulator Wnt2. Collectively, the experiments identify Ang-2 as an endothelial rheostat of liver regeneration whose expression is dynamically regulated during the different stages of liver regeneration to differentially control endothelial cell function (direct effects) and hepatocyte proliferation (indirect effects through EC-derived angiocrine growth factors).

VEGF decreases leukocyte adhesion in vitro through interfering with NF-kappaB signaling pathways leading to endothelial activation

Hua Huang*, Elise Langenkamp, Sara Mangsbo, Angelica Loskog, Anna Dimberg. Dept of Immunology, Genetics and Pathology, Uppsala University, Rudbeck laboratory, 75185 Uppsala, Sweden

Tumor vessels are morphologically and functionally distinct from normal vessels due to constant stimulation by pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF). The role of VEGF signaling in inducing angiogenesis and vascular permeability is well established, but there are conflicting data regarding its potential function in endothelial activation and leukocyte recruitment. To understand the role of VEGF in modulating leukocyte recruitment, we have analyzed how VEGF treatment affects tumor necrosis factor (TNF-)alpha-induced endothelial activation of human dermal microvascular endothelial cells (HDMEC) in vitro. Using a microfluidic device, we found that VEGF decreased Jurkat adhesion to endothelial cells in flow conditions, and that the VEGFR-inhibitor Sunitinib (Sutent, Pfizer) restored adhesion. Microarray analysis and FACS analysis revealed a set of genes involved in leukocyte recruitment, including VCAM-1, CXCL10, CXCL11 and IL-1R1, that were induced by TNF-alpha and downregulated when TNF-alpha treatment was combined with VEGF. As expected, the TNF-alpha-induced gene expression that was inhibited by VEGF was restored by co-treatment with Sunitinib. VEGF interfered with TNF-alpha-induced I-kappaB degradation, suggesting that VEGF inhibits pro-inflammatory gene expression and endothelial activation through direct interference with the NF-kappaB pathway. These results suggest that Sunitinib treatment may increase T-lymphocyte recruitment in tumors through enhanced responsiveness of the endothelial cells to pro-inflammatory signaling. We are currently investigating if short-time Sunitinib treatment can be used to enhance adoptive T-cell therapy.

Paxillin phosphorylation mediates vascular endothelial cadherin internalisation during lymphocyte transmigration

Natalie Hudson[1]*, Roberta Martinelli[1,2], John Greenwood[1] and Patric Turowski[1]. [1]Department of Cell Biology, UCL- Institute of Ophthalmology, London, UK, [2]Present address: Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Endothelial cells are known to actively be involved in facilitating and aiding transendothelial migration (TEM) of leukocytes across the EC barrier into the underlying tissue. We have recently shown that phosphorylation of vascular endothelial (VE)-cadherin is instrumental in this process. Here we show that phosphorylation of the focal adhesion and actin associated protein paxillin mediates VE-cadherin internalisation during lymphocyte TEM. Lymphocyte transmigration across brain microvascular endothelial cells is critically dependent on adhesion to and signalling of intercellular adhesion molecule 1 (ICAM-1). Antibody-mediated activation of ICAM-1 in brain microvascular endothelial cells led to significant phosphorylation of paxillin via a signalling pathway involving Src, protein kinase C and the MAP kinase JNK, but not Erk or p38. Specific pharmaceutical or genetic neutralization experiments showed each component of this pathway to be important for lymphocytes TEM. Co-transfection experiments suggested that paxillin converged with VE-cadherin in regulating lymphocyte TEM. Indeed, rather than modulating focal adhesions, ICAM-1-activated paxillin associated with VE-cadherin and induced its internalisation in a JNK-dependent manner. This work suggests that paxillin and VE-cadherin co-operate to modulate the paracellular cleft during leukocyte TEM and that internalisation of endothelial junction components plays an important role during TEM. This works was funded by the Wellcome Trust and British Heart Foundation

BMP9 regulation of angiogenic sprouting through an Alk1-BMPRII/ActRII-ID1/ID3-dependent pathway: Implications for Hereditary Hemorrhagic Telangiectasia Type II

Jai-Hyun Kim, Matthew R. Peacock, Steven C. George and Christopher C.W. Hughes*. Department of Molecular Biology & Biochemistry. Department of Biomedical Engineering. Edwards Lifesciences Center for Advanced Cardiovascular Technology. University of California Irvine, Irvine CA 92697 USA

ALK1 (ACVRL1) is a member of the TGF-beta receptor family and is expressed predominantly by arterial endothelial cells (EC). Mutations in ACVRL1 are responsible for Hereditary Hemorrhagic Telangiectasia Type 2 (HHT2), a disease manifesting as fragile vessels, capillary overgrowth, and numerous arterio-venous malformations (AVMs). Arterial EC also express EphrinB2, which has multiple roles in vascular development and angiogenesis and is known to be reduced in ACVRL1 knockout mice. Using an in vitro angiogenesis model we find that the Alk1 ligand BMP9 induces EphrinB2 in EC, and this is entirely dependent on expression of Alk1 and at least one of the co-receptors BMPRII or ActRII. BMP9 induces both ID1 and ID3, and both are necessary for full induction of EphrinB2. Loss of Alk1 or EphrinB2 results in increased arterial-venous anastomosis, while loss of Alk1 but not EphrinB2 results in increased VEGFR2 expression and enhanced capillary sprouting. Conversely, BMP9 induces expression of jagged-1 and the VEGFR2 regulator Hey-1, and blocks EC sprouting, and this is dependent on Alk1. Finally, notch signaling overcomes the loss of Alk1 - restoring EphrinB2 expression in EC, and curbing excess sprouting. Thus, in an in vitro model of HHT2, Alk1 and notch act in concert to regulate expression of EphrinB2 and VEGFR2, and thereby, vascular sprouting and anastomosis.

AmotL2 enables the actomyosin contractility needed for cell shape changes during aortic lumen formation

Sara Hultin[1]*, Simona Vertuani[1], Mahdi Mojallal[1], Yuyuan Zheng[1], Staffan Nyström[1,3], Christin Bäth[1], Christian Gentili[1], Per Uhlen[3], Jean-Paul Borg[2], Arindam Majumdar[4], Lars Holmgren[1]. [1]Department of Oncology and Pathology, Cancer Center Karolinska, Karolinska Institutet, 17176, Stockholm, Sweden, [2]Plateforme de Spectrométrie de Masse et de Proteomique, Centre de Reserche en Cancérologie de Marseille U891, INSERM/Institut Paoli-Calmettes 27, Bd Leï Roure, BP 30059, 13273, Marseille, Cedex 9, France, [3]Department of Medical Biochemistry and Biophysics, Molecular Neurology, Karolinska Institutet, Scheeles väg 2, 17177, Stockholm, Sweden, [4]Department of Genetics and Pathology, Rudbecklaboratoret, Uppsala University, Dag Hammarskjöldsväg 20, 75185, Uppsala, Sweden

Abstract Tube formation is one of the major processes during embryonic development. For vascular tubes to be formed, several processes need to be coordinated; proliferation, migration, cell polarization, cell shape changes and junction assembly. By studying the aortic development in the zebrafish embryo, we have found AmotL2 (Angiomotin-Like-2) to be an important player during this event. Embryos, in which AmotL2 function has been knocked down with anti-sense morpholinos, cannot establish functional circulation, which is due to defect aortic lumen formation. Further, we show that AmotL2 interacts with Par3, a protein which has previously been shown to control apical-basal cell polarity and tight junctions in epithelial cells. The phenotypes of AmotL2 and Par3 knock-down overlap, which suggests that they act in the same signaling pathway. Interestingly, both cell-cell junctions and apical-basal polarity are unaffected in the AmotL2 knock-down embryos. Instead, we find Par3 to be important for correct localization of AmotL2 at the cell-cell contacts. In contrast to junctions and cell polarity, the actin cytoskeleton is severely defective in the AmotL2 knock-down embryos. In MS-1 cells the actin stressfibers, linking the cell to its neighbors, are almost totally absent. Interestingly, we find AmotL2 to interact with actin and the actin-binding protein Afadin. Embryos treated with the myosin inhibitor Blebbistatin shows a phenotype similar to the AmotL2 knock-down embryos. Myosin binds to actin and enables the contractility of the actin filaments. We suggest that AmotL2 and Afadin act together to regulate stress fiber formation in aortic endothelial cells. This, we hypothesize, is essential for the actomyosin generated contractility needed to perform synchronized cell shape changes required for the formation of a vascular tube.

Vinculin associates with endothelial VE-cadherin junctions to control their force-dependent remodelling

Stephan Huveneers[1]*, Joppe Oldenburg[1], Emma Spanjaard[1,2], Gerard van der Krogt[1], Ilya Grigoriev[3], Anna Akhmanova[3], Holger Rehmann[2] and Johan de Rooij[1]. [1]Hubrecht Institute for developmental biology and stem cell research and University Medical Centre Utrecht, Utrecht, The Netherlands, [2]Department of Molecular Cancer Research, Centre of Biomedical Genetics and Cancer Genomics Centre, University Medical Centre Utrecht, Utrecht, The Netherlands, [3]Cell Biology, Faculty of Science, Utrecht University, Utrecht, The Netherlands

To remodel endothelial cell-cell adhesion, inflammatory cytokine- and angiogenic growth factor-induced signals impinge on the VE-cadherin complex, the central component of endothelial adherens junctions. This study demonstrates that junction remodelling takes place at a molecularly and phenotypically distinct subset of VE-cadherin adhesions, defined here as Focal Adherens Junctions (FAJ). FAJs are attached to radial F-actin bundles and marked by the mechanosensory protein Vinculin. Endothelial hormones VEGF, TNFalpha, and most prominently thrombin induce a transient formation of FAJs from existing stable junctions. The actin cytoskeleton generates pulling forces specifically on FAJs and inhibition of Rho-Rock-actomyosin contractility prevents the formation of FAJs and junction remodelling. FAJs are normally formed in cells expressing a Vinculin-binding-deficient mutant of alpha-catenin showing that Vinculin recruitment is not required for adherens junction formation. Comparing Vinculin-devoid FAJs to wild type FAJs reveals that Vinculin protects VE-cadherin junctions from opening during their force-dependent remodelling. These findings implicate Vinculin-dependent cadherin mechanosensing in endothelial processes such as leukocyte extravasation and angiogenesis.

Angiopoietin-like protein 2 contributes to pathogenesis of diabetic retinopathy

Yasuhiro Ito*, Yuichi Oike Hidenobu Tanihara. Department of Ophthalmology and Visual science, Department of Molecular Genetics, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

Proliferative diabetic retinopathy (PDR) is the major cause of blindness in the working-age population. PDR is characterised by neovascularisation due to retinal ischaemia, and subsequent fibrovascular membrane formation, which can cause retinal detachment and blindness. Inflammation plays an important role in pathogenesis of diabetic retinopathy. Recent studies show that angiopoietin-like protein 2 (Angptl2) contributes to chronic inflammation in diabetes, tumor, rheumatoid arthritis and dermatomyositis, Angptl2 is expressed in endothelial cells, and increased by hypoxia and endoplasmic reticulum stress, serum level of angptl2 is increased in diabetes. Angptl2 acts on endothelial cells, resulting in promoting inflammation, angiogenesis and vascular leakiness. However, the function of Angptl2 in diabetic retinopathy is still unknown. In this study, we investigate the Angptl2 concentration in vitreous fluid and expression in fibrovascular membrane of PDR patients to evaluate the role of Angptl2 in pathogenesis of diabetic retinopathy. At first we collected vitreous samples at the time of vitreous surgery from 33 PDR and 29 nondiabetic patients. The concentration of Angptl2 in vitreous samples was measured by using the human Angptl2 enzyme-linked immunosorbent assay. The vitreous concentration of Angptl2 significantly increased in patients with PDR compared with controls ($P < .001$). Next, we examined Angptl2 protein expression in fibrovascular membrane from PDR patients by immunohistochemistry using both anti-Angptl2 and anti-CD31 or anti-Mac2 antibody. Double immunofluorescent staining showed that Aangptl2 was expressed in endothelial cells and macrophages in fibrovascular membrane. In vitro study, we found that Angptl2 expression was increased under 2% hypoxic condition in human retinal endothelial cells (HRECs), and promoted ICAM-1 expression on HREC through the activation of NFkB signaling. Taken together, Aangptl2 may play an important role in the pathology of diabetic retinopathy by causing the chronic inflammation in retinal endothelial cells. Therefore, Angptl2 may have a therapeutic potential for diabetic retinopathy.

The lymphatic system plays a vital role in maintaining tissue homeostasis by regulating tissue fluid and protein balance

Fumiko Itoh[1]*, Kei Ichikawa[1], Susumu Itoh[2], Guillermo Oliver[3], & Takuya Watanabe[1].
[1]Laboratory of Cardiovascular Medicine, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan, [2]Laboratory of Biochemistry and High Technology Research Center, Showa Pharmaceutical University,Tokyo , Japan, [3]Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA

The lymphatic system plays a vital role in maintaining tissue homeostasis by regulating tissue fluid and protein balance. During mammalian development, lymphatic endothelial cells (LECs) derived from a specific location of the arterial cardinal vein, bud off from the venous endothelium reacting to VEGF-C to form primary lymphatic sacs. These primitive sacs continue to sprout, proliferate, and migrate into the periphery and undergo maturation to generate the entire lymphatic vasculature network throughout the body. Many cytokines, which are important for blood vessel development, are also implicated in lymphangiogensis, Although TGF-β signaling is known to play key roles in angiogenesis, it remains veiled how TGF-β regulates lymphangiogenesis. To elucidate molecular mechanisms by which TGF-β pathway controls lymphatic vascular development, we conditionally inactivated TGF-β type II receptor (TβRII) in LECs using the Cre-ERT2 system driven by the Prox1 promoter. Prox1 is a homeobox transcription factor and highly expresses in LECs. After these Cre-ERT2 mice are bred with TβRIIfl/fl mice, we established TβRII5F/F; Prox1-CreER mice. The injection of Tamoxifen (TM) to these mice resulted in the deletion of these target genes in LECs specifically. When we administered TM at E10.5 and E11.5, TβRIIF/F; Prox1-CreER embryos showed edema at E13.5. Therefore we stained blood and lymphatic vessels with anti-PECAM-1 and anti-LYVE-1 antibodies, respectively. Interestingly, blood vessel seemed to be well-established, whereas lymphatic vessels merely existed though they were ragged and poorly organized. These data indicated that TGF-β signaling is indispensable for maintenance of lymphatic vessel integrities.

The role of flow-induced gene expression in vascular plasticity

Espen D. Jahnsen* and Elizabeth A.V. Jones Department of Biomedical Engineering, McGill University, Montreal, Canada

Within the embryo, the cardiovascular system is the first fully functional organ system to develop, with vascular endothelial cells forming a continuous layer on the luminal wall of blood vessels. The mechanical stress exerted by the flowing blood on the endothelium plays a significant role in regulating many physiological functions including numerous signaling pathways as well as inducing changes in the expression of numerous genes. Although there is expression of arterial and venous specific genes before the onset of flow in the embryo, there are no clear structural differences between the two types of vessels. With the onset of flow, morphological distinct arteries and veins develop. Several genes have been shown to be expressed exclusively in arteries and veins, including Notch1 and delta-like 1 (Dll1) in arteries and CoupTFII and EphB4 in veins. Several studies have suggested that although endothelial cells are genetically predetermined as arterial or venous, changes in hemodynamics can override these cues. In order to explore the role of flow-induced arterial and venous gene expression in vascular plasticity, we utilize a parallel plate flow chamber with a peristaltic and a syringe pump to mimic embryonic, oscillatory, pulsatile and steady laminar flow on human abdominal aortic endothelial cells (HAAEC). Genes of interest include kruppel-like factor 2 (KLF2), endothelial nitrous oxide synthase (eNOS), and platelet derived growth factor B (PDGFB). Additionally, we employ siRNA studies of Notch1 and Dll1 expression to assess their role in flow-induced vascular plasticity. Preliminary results suggest that the genes involved in arterial-venous differentiation are changing depending on the flow condition.

Follicular workload dynamically regulates thyroid gland vasculature through VEGF pathway

Jeon Yeob Jang[1]*, Kyuho Kim[1], Joon Seo Lim[1], Kibaek Choe[2], Seong Eun Lee[3], Pilhan Kim2, Minho Shong[3], Ook Joon Yoo[1] and Gou Young Koh[1]. [1]Graduate School of Medical Science and Engineering and, [2]Graduate School of Nanoscience and Technology, Korea Advanced Institute of Science and Technology, Daejeon, Korea, [3]Research Center for Endocrine and Metabolic Diseases, Chungnam National University School of Medicine, Daejeon, Korea

Thyroid gland is a highly vascularized organ required for active hormonal regulation. The thyroid gland vasculature has distinguishable characteristics such as endothelial fenestrations, and it also serves as a conduit between thyroid gland microenvironment and systemic circulation. However, it is poorly understood how thyroid gland blood vessels are remodeled under dynamic hormonal change and workload of thyroid gland. Here, we show that high follicular workload of thyroid gland as induced by thyroid-stimulating hormone (TSH) actively regulates thyroid gland vasculature through vascular endothelial growth factor (VEGF) during adult physiological state. We observed that daily administration of recombinant TSH increased the level of mature thyroid hormones such as T3 and T4 while inducing remodeling of follicular architecture. Immunohistochemical analysis revealed that under hormonal stimulation, thyroid gland vasculature is increased in its diameter and density. Moreover, intravital image after perfusion of FITC-dextran and rhodamine-leveled RBC showed that functional perfusion is also dynamically changed under hormonal stimulation. We screened and compared the physiological expression level of various angiogenic ligands in thyroid glands and other adjacent organs. Importantly, we observed robust and distinct VEGF/VEGFR2 expression in the thyroid gland of reporter mouse models, suggesting a paracrine role of VEGF in thyroid follicle. VEGF expression level was increased under high TSH level in both in vivo reporter mouse models and in vitro follicular cell culture system. In contrast, suppression of TSH resulted in the reduction of VEGF expression level, thus showing that VEGF expression is precisely regulated by TSH. We therefore suggest that follicular workload determined by TSH dynamically regulates thyroid gland vasculature through VEGF pathway.

The novel function of mannan binding lectin associated serine-protease-1 (MASP-1): Pro inflammatory activation of endothelial cells

Peter K. Jani[1]*, Marton Megyeri[2], Veronika Mako[1], Erika Kajdacsi[1], Peter Gal[2], Laszlo Cervenak[1]. [1]3rd Department of Internal Medicine, Semmelweis University, Budapest, [2]Institute of Enzymology, BRC, Hungarian Academy of Sciences, Budapest, Hungary

Introduction: Activation of the complement system can induce and enhance inflammatory reaction by means of activating endothelial cells. Endothelial cells are very important in the regulation of inflammation via production of pro-inflammatory cytokines and adhesion molecules. We previously described that MASP-1 activates Ca2+, p38-MAPK and NFkappaB pathways in endothelial cells by cleavage of PAR-4 receptor. Several other transcription factors and signal transduction molecules are involved in the process of endothelial inflammatory activation such as cAMP-responding element binding protein (CREB) or JNK, which have not yet been studied. Aim: Whether CREB and JNK signaling pathways are involved in MASP-1 induced endothelial cell activation and the pro-inflammatory pathways finally lead to cytokine production in endothelial cells and if so, effects of MASP-1 are comparable to known pro-inflammatory mediators such as TNFalpha or IL-1beta. Materials and methods: We used recombinant human MASP-1 catalytic fragment (CCP1-CCP2-SP) and cultured HUVEC primary cell line as a model for endothelial cells. HUVECs were seeded onto 96-well plates until confluency then treated with recombinant MASP-1 fragment or other stimulators for 24 hours. Signaling pathway inhibitors had been added to cells 30 minutes before treatment. IL-1alpha, IL-1ra, IL-6, IL-8, IL-10, TNFalpha and MCP-1 cytokines were measured from supernatants by commercial ELISA and xMAP. We analyzed data by GraphPad Prism 4.0 software, using one-way ANOVA and Dunnett's post test. Results: We found that MASP-1 can activate CREB phosphorylation and also JNK activation and it was comparable with the effect of TNFalpha or IL-1beta. MASP-1 treatment induced dose dependent IL-6 and IL-8 expression in endothelial cells and its effects were comparable with those of induced by IL-1beta, LPS, TNFalpha and thrombin and were higher than those of induced by bradykinin or histamine. Both IL-6 and IL-8 expression could be inhibited by p38 MAPK inhibitor, whereas blocking NFkappaB, ERK-1/2, JNK, PI-3Kinase signaling pathways had no effect. MASP-1 treatment did not induce MCP-1, TNFalpha IL 1alpha, IL 1ra and had no effect on the production of anti-inflammatory cytokine IL 10. Discussion: Although we found that CREB and JNK pathways were also activated, the p38-MAPK pathway seemed to be the most important in the cytokine production of endothelial cells induced by MASP-1 among the signaling pathways studied in our experiments. The unique cytokine pattern (increased IL-6 and IL-8, but not MCP-1 and other pro/anti-inflammatory cytokines) generated by MASP-1 may have regulatory role in early immune response to bacteria and fungi. Since mannan-binding-lectin or ficolins complexed with MASP-1 are activated soon after microbial infection and IL-8 is a potent chemotactic factor for neutrophil granulocytes, MASP-1 may be a specific, fast acting and effective activator of neutrophils the other most important anti-bacterial/fungal agent beside complement system. Our result implies a novel connection between complement system and neutrophil granulocytes.
(OTKA 100684)

HIF-regulated HO-1 and VEGF expression improves revascularization in ischemic limbs

Agnieszka Jazwa[1]*, Jolanta Jagodzinska[1], Mateusz Tomczyk[1], Chantal Pichon[2], Alicja Jozkowicz[1], Jozef Dulak[1]. [1]Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland, [2]Centre de Biophysique Moléculaire, CNRS, Orléans, France

Aims: Heme oxygenase-1 (HO-1) mitigates cellular injury by exerting antioxidant, anti-apoptotic, and anti-inflammatory effects. Vascular endothelial growth factor-A (VEGF-A) is a critical regulator of angiogenesis stimulating proliferation, migration, and proteolytic activity of endothelial cells. Their coordinated action may be desirable for post-ischemic tissue healing. Methods and results: Unilateral limb ischemia was induced in mice lacking HO-1 gene (HO-1-/-) and in wild-type controls (WT). Reparative neovascularization was reduced in HO-1-/- ischemic muscles resulting in impaired blood flow recovery, increased inflammation and muscle cell death compared with WT mice. Skeletal muscles injected with 10 μg of bicistronic plasmid encoding human HO-1 and human VEGF-A under the control of hypoxia response element (HRE) and minCMV promoter (pHRE-HO-1-VEGF-A) in the presence of 1×106 Vevo MicroMarker microbubbles, exposed to ultrasound at 1 MHz, 20% duty cycle for 60 seconds were efficiently transfected without toxicity. Moreover, this local simultaneous and transient activation of HO-1 and VEGF-A genes in hypoxic/ischemic gastrocnemius muscles of HO-1-/- mice was able to restore the reparative neovascularization and post-ischemic blood flow recovery to the level detected in WT controls. Additionally, overexpression of HO-1 in ischemic skeletal muscles influenced the expression of several agents (MyoD, Myogenin, miR-206, miR-146a, Pax3 and Pax7) involved in satellite cell differentiation and skeletal muscle regeneration.
Conclusion: Our results demonstrate that this gene delivery method is promising for clinical applications and that pHRE-HO-1-VEGF-A vector can be further developed as an effective tool in cardiovascular regenerative medicine.Supported by grants: POIG No. 01.01.02-00-109/09 (European Union structural funds) and Homing-Plus/2011-3/3 (Foundation for Polish Science).

Glucose and reactive glucose metabolite methylglyoxal induce pathophysiological blood vessel development in zebrafish

Kristina Jörgens[1,2]*, Jennifer Pohl[1,3], Thomas Fleming[4], Angelika Bierhaus[4], Peter P. Nawroth[4], Hans-Peter Hammes[3], and Jens Kroll[1,2]. [1]Vascular Biology and Tumorangiogenesis, Heidelberg University, [2]DKFZ-ZMBH Alliance, Heidelberg, [3]5th Medical Department, Medical Faculty Mannheim, Heidelberg University and [4]Department of Medicine I and Clinical Chemistry, Heidelberg University, Heidelberg, Germany

The zebrafish is a well established model organism to study vascular development. Diabetes mellitus is associated with a state of chronic hyperglycaemia and diabetic patients are known to develop various long-term micro- and macrovascular damages, which are mostly caused by the formation of reactive metabolites from glucose, called reactive dicarbonyls. One important reactive dicarbonyl is methylglyoxal (MGO), which is excessively formed as a result of increased glucose flux through glycolysis and is known to accumulate under diabetic conditions. MGO modifies proteins, lipids and nucleic acids and thereby leading to structural damages and dysfunctional molecules. In this study we have analyzed effects of high glucose and MGO concentrations on blood vessel formation in zebrafish and identified an abnormal vascular development in the trunk. Likewise, gene expression silencing of glyoxalase 1, a key enzyme in the main degradation pathway for MGO, induced a similar hyperbranching phenotype in zebrafish. Aminoguanidine, a known MGO scavenger, reduced hyperbranching of blood vessels following glucose treatment or glyoxalase 1 silencing. The enhanced angiogenic activity under high glucose and MGO is mediated by vascular endothelial growth factor (VEGF) receptor signalling via its downstream target phosphoinositide-3 kinase (PI3K), as use of the VEGF receptor inhibitor Vatalanib (PTK787) and the PI3K inhibitor LY294002 impaired hyperbranching of the trunk blood vessels. Together, the data support an important impact of metabolic pathways on vascular development in zebrafish.

Differential influence of extracellular adhesion protein (Eap) from Staphylococcus aureus on blood coagulation and fibrinolysis

Christiane Jünemann[1]*, Brian V. Geisbrecht[2], Mathias Herrmann[3] & Klaus T. Preissner[1].
[1]Department of Biochemistry, Medical School, Justus-Liebig-University, Giessen, Germany, [2]Division of Cell Biology and Biophysics, University of Missouri, Kansas City, USA, [3]Department of Med. Microbiology, Saarland University Hospital, Homburg/Saar, Germany

The basic adhesive protein Eap, which is secreted by S. aureus, was previously shown by our and other groups to exert potent anti-inflammatory and anti-angiogenic functions, thereby inhibiting wound healing and influencing tissue regeneration. Although several host proteins, including fibrinogen, vitronectin, or prothrombin were reported to directly bind to Eap, its role in blood coagulation and fibrinolysis is still obscure. In a plasma clot-lysis system, where the onset of coagulation, clot stability and the degree of clot lysis is measured, Eap was found to influence the clot stability by decelerating the coagulation process (due to an increase of the maximal clotting time) and by delaying the clot lysis in a concentration-dependent manner. The same result was found using activators of the intrinsic coagulation pathway such as kaolin (as determined by the kaolin clotting time or the activated partial thromboplastin time). In contrast, tissue factor-mediated induction of the extrinsic pathway of coagulation revealed an Eap-dependent decrease of the „prothrombin time“. These data are indicative for a differential effect of Eap on the extrinsic pathway (= initiation of thrombin formation) and intrinsic pathway (= amplification of thrombin formation) of coagulation as well as of an anti-fibrinolytic function of Eap in plasma. In an isolated system, the conversion of prothrombin to thrombin in the prothrombinase complex occurred in a retarded manner in the presence of Eap, while the rate and rigidity of fibrin formation was increased by Eap in a concentration-dependent fashion. The anti-fibrinolytic influence of Eap may be best explained by stabilizing plasminogen activator inhibitor-1, thereby delaying plasmin formation. Taken together, these data provide evidence for a modulatory role of Eap in blood coagulation and fibrinolysis, most likely at sites of vascular injury where sufficient quantities of the bacterial protein are available during wound infection. The observed differential effects of Eap may be related to the diverse binding interactions between the bacterial protein and prothrombin, fibrinogen and vitronectin. Further studies will aim to clarify the mechanistic aspects of the diverse role of Eap in blood coagulation and fibrinolysis, aiming to decipherer its contribution as putative causal factor for bad- or non-healing wounds during S. aureus infections.

Altered vasoactive peptide levels in hereditary angioedema patients

Erika Kajdacsi[1]*, Peter K. Jani[1], Dorottya Csuka[1], Jan Kunde[2], Zoltan Prohaszka[1], Henriette Farkas[1], Laszlo Cervenak[1]. [1]3rd Department of Internal Medicine, Semmelweis University, Budapest, Hungary, [2]Department of Global Medical Affairs, BRAHMS GmbH, Neuendorf str. 25, D-16761 Hennigsdorf, Germany

Introduction Hereditary angioedema (HAE) due to C1-inhibitor (C1-INH) deficiency is an autosomal dominant disorder. Increased endothelial permeability is evident during edematous attacks, but not during inter-attack periods. Edema and decreased blood pressure due to edema (hypovolemic shock) may be life-threatening consequences of these attacks in HAE. Since bradykinin is known to be the major pathogenetic factor of HAE, the regulation of vascular tone and vascular integrity controlled by other vasoactive peptides (such as endothelin-1 (ET-1), adrenomedullin (ADM), atrial natriuretic peptide (ANP) and arginine vasopressin (AVP)) may have substantial importance in HAE-C1-INH. Aims: Comparing the ET-1, ANP, ADM and AVP vasoactive peptides levels in HAE patients and healthy control subjects, in HAE patients during attack and attack free periods. Methods: In the first part of the study 103 patients with HAE-C1-INH and in 112 healthy control subjects were enrolled. Plasma ET-1, AMD, ANP and AVP levels were measured by BRAHMS KRYPTOR assay from EDTA-plasma of controls and inter-attack symptom-free HAE-C1-INH patients. In the second part of the study 19 HAE-C1-INH patients inter-attack symptom-free and during attack samples were also measured by BRAHMS KRYPTOR assay. Results: The level of ANP was significantly lower in HAE-C1-INH patients (median: 37.0 (28.0-46.8)) compared to healthy controls (median: 47.0 (37.0-63.0)), while the ET-1, ADM and AVP levels were not different. The level of ET-1 and the level of ADM increased during attack compared to the inter-attack symptom-free period. There was no alteration in the level of AVP and ANP in the same comparison. Conclusion: ANP is known to improve endothelial cell integrity and lower permeability. Therefore HAE patients, having low ANP levels, are even more predisposed to bradykinin induced attacks. The increased ADM and ET-1 levels during attacks in HAE-C1-INH patients can be biomarkers or a response to the impaired endothelial function. Henceforth we would like to clarify by in vitro endothelial cell experiments whether these alterations are involved in the pathomechanism of the HAE-C1-INH disease or, they are compensatory mechanism of the body response to the impaired vasoregulation.

(OTKA 100886, OTKA 100684)

Dissecting the role of Apelin-APJ signaling in gliomagenesis

Roland E. Kälin*, Anne Jarczewski, Raymond Monk, Stefanie Kraft, Josefine Radke, and Frank L. Heppner. Department of Neuropathology, Charité - Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

Glioma are the most frequent malignant primary tumors of the brain. They are highly invasive, infiltrate normal brain tissue and total surgical resection is difficult. Glioma survival and growth is determined by its interaction with brain parenchyma, which includes intense tumor angiogenesis. In previous work, we found that the novel peptide hormone apelin, which signals through its G-protein coupled receptor APJ, is conserved during vertebrate evolution and is closely associated with the initiation of angiogenic blood vessel growth in the frog Xenopus laevis as well as the mouse. The functional importance of apelin-APJ signaling in embryonic angiogenesis and its epistatic relationship with VEGF signaling was established in a series of loss- and gain-of-function experiments in Xenopus tadpoles. We further confirmed that apelin-APJ signaling is necessary and sufficient to promote angiogenic sprouting of intersomitic blood vessel in vivo. Apelin appears to act first in a paracrine fashion to induce intersomitic blood vessels outgrowth, while then it possibly serves for vessel guidance in an autocrine manner via the angiogenic tip cells. Finally, we detected apelin and APJ upregulation in human glioblastoma multiforme (GBM) specimens implying a role in tumor angiogenesis. To investigate apelin-APJ signaling in pathological vessel formation we recently established an orthotopic model of tumor formation in the mouse. Intracerebral xenotransplantation of the glioma cell line U87MG, expressing high levels of apelin in vitro, gave rise to massive and well-vascularized xenografts. To study the putative contribution of apelin to the formation and function of glioma vascular beds we knocked down apelin in U87MG cells by lentiviral shRNA, which resulted in a reduced tumor volume and attenuated the formation of the xenograft vasculature. Detailed analyses of glioma growth, vascularization and vascular permeability by immunohistology, stereomorphology and in vivo imaging suggests that apelin signaling is required for the formation of glioma vascular beds and, consequently, glioma growth. In ongoing experiments we are presently investigating gliomagenesis in mice overexpressing or lacking apelin to test for a potential complementary role of apelin originating from the glioma microenvironment. We are anticipating that our study will provide insights whether apelin-APJ signaling may serve as a future novel target for anti-angiogenic tumor therapy.

Repression of the miR-17-92a cluster by HDAC9 promotes angiogenesis of endothelial cells

D. Kaluza[1]*, S. Gesierich[2], C. Doebele[1], JN. Boeckel[1], A. Bonauer[1], J. Kroll[3], A. Zelent[4], L. Rössig[1], S. Dimmeler[1], C. Urbich[1]. [1]Institute for Cardiovascular Regeneration, Centre of Molecular Medicine, University of Frankfurt, Germany, [2]Joint Research Division Vascular Biology of the Medical Faculty Mannheim (CBTM), University of Heidelberg, Heidelberg, Germany, [3]Center for Biomedicine and Medical Technology Mannheim, Joint Research Division Vascular Biology Medical Faculty Mannheim, Germany, [4]Leukemia Research Fund Centre, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, United Kingdom

Histone deacetylases (HDACs) act as modulators of gene expression by deacetylation of histone proteins. Broad-spectrum HDAC inhibitors reduce postnatal neovascularization in response to hypoxia and in tumor angiogenesis. Therefore, broad-spectrum HDAC inhibitors are in clinical use as anti-cancer agents. Previous studies identified the class IIa histone deacetylase HDAC5 as a negative regulator of angiogenesis. Furthermore, HDAC5 and HDAC9 knockout mice develop cardiac hypertrophy upon hypertrophic stress, indicating that HDAC5 and HDAC9 exhibit a redundant function in the heart. However, the role of HDAC9 in endothelial cells (EC) is unknown. Knockdown of HDAC9 with siRNA oligonucleotides significantly decreases EC sprouting from spheroids and tube formation in a matrigel assay. To confirm the different roles of HDAC9 and HDAC5 in mammalian vessel formation in vivo, we performed a spheroid based matrigel plug assay in mice. Downregulation of HDAC9 significantly decreases human vessel formation in the plug (44±6%, $p<0.05$), the average size (62±6%, $p<0.05$) and perfusion of the vessels (19±6% of control vessels vs 1±1% of shHDAC9 vessels, $p<0.01$). In contrast silencing of HDAC5 significantly augments perfusion of human vessels in this model. To address the underlying mechanism of HDAC9 in EC, we used different mutated constructs and found that the pro-angiogenic effect requires the catalytic domain of HDAC9, nuclear localization and sumoylation of HDAC9. Recently, it was shown that broad spectrum HDAC inhibitors decrease the expression of the oncomiR cluster miR-17-92a in cancer cells thereby limiting cancer cell proliferation. In contrast, inhibition of HDAC activity in EC increases the expression of the miR-17-92a cluster. Since the miR-17-92a cluster exhibits crucial anti-angiogenic roles in EC, we focused on the regulation of this cluster by class II HDACs. Therefore, we silenced single class II HDAC isoenzymes by siRNA and assessed the expression of the miR-17-92a cluster. Silencing of HDAC9 significantly increases the expression of the primary transcript of the miR-17-92a cluster, while downregulation of the other class II members HDAC4, HDAC5, HDAC6 and HDAC7 was not significantly effective. Consistently, silencing of HDAC9 upregulates single microRNAs of the cluster. In EC HDAC9 is expressed as two splice variants: HDAC9 and MITR, which lacks the deacetylation domain. Silencing of MITR does not affect miR-17-92a expression, indicating that the deacetylation domain is required for miR-17-92a repression. Consistently, overexpression of HDAC9 in HEK cells diminishes luciferase activity driven by the miR-17-92a promoter, while MITR was not affective. Moreover, downregulation of HDAC9 decreases the mRNA expression of the predicted miR-17/-20 target VEGF-A and the protein expression of the described miR-17 target Jak1. In accordance, silencing of miR-17 and miR-92a partially rescues the sprouting defect of HUVECs after depletion of HDAC9. In summary, we found that HDAC9, in contrast to HDAC5, promotes angiogenesis in vitro and in vivo. Mechanistically, the pro-angiogenic effect of HDAC9 requires the catalytic domain, nuclear localization and sumoylation of HDAC9. In addition, knockdown of HDAC9 increases the expression of the anti-angiogenic miR-17-92a cluster and decreases the expression of the miR-17-92a targets VEGF-A and Jak1, providing for the first time a link between HDAC9 and microRNA expression.

Role of the endothelial-derived endogenous anti-inflammatory factor Del-1 in inflammation-mediated adrenal gland dysfunction.

Waldemar Kanczkowski[1]*, Antonios Chatzigeorgiou[1], David Sprott[1], Sylvia Grossklaus[1], Stefan Bornstein[2] and Triantafyllos Chavakis[1]. [1]Division of Vascular Inflammation, Diabetes and Kidney. Department of Medicine III. Technical University Dresden, [2]Division of Molecular Endocrinology, Diabetes and Kidney. Department of Medicine III. Technical University Dresden, Germany

Systemic inflammation, such as in the course of the systemic inflammatory response syndrome (SIRS) and sepsis, often results in adrenal dysfunction. The adrenal gland is highly vascularised; thus we hypothesized that inflammation-induced endothelial dysfunction may actively participate in adrenal insufficiency. To address this hypothesis, we utilized the properties of Del-1 (developmental endothelial locus-1), which is an endothelial-derived secreted molecule that acts in an anti-inflammatory manner by antagonizing integrin-dependent leukocyte adhesion to the endothelium. By quantitative real-time PCR (qPCR) analysis we identified the expression of Del-1 in the adrenal gland. Moreover, we took advantage of the fact that the Del-1-/- mice are LacZ knock-in transgenics, where the LacZ gene is controlled by the native Del-1 promoter, allowing for monitoring endogenous Del-1 expression and demonstrated expression of Del-1 in the adrenal gland by beta galactosidase staining. Furthermore, we found that in wildtype mice, adrenal gland expression of Del-1 was downregulated upon SIRS induction by systemic LPS administration. Consistent with the function of Del-1 as an endogenous anti-inflammatory factor, we observed that infiltration of innate immune cells (neutrophils, monocytes) in the adrenal glands of Del-1-deficient mice in the course of LPS-induced SIRS was increased as compared to wildtype mice. Immune cell recruitment to the adrenal glands was assessed by qPCR, immunohistochemistry and myeloidoperoxidase assay. In addition, Del-1-deficiency resulted in increased adrenal expression of inflammatory factors, such as, TNF alpha or IL-1 beta. The increase in inflammation and immune cell recruitment into the adrenal gland was not ICAM-1- or VCAM-1-dependent , since expression of these adhesion molecules did not differ in the adrenal gland ofDel-1-/- - and Wildtype-mice after systemic LPS administration. The increased adrenal gland inflammation due to Del-1-deficiency was also associated with a reduced adrenal corticosterone production which was significantly lower in Del-1-/- mice at 24h post LPS administration. Taken together, these data suggest that endothelial Del-1 acts as an important gatekeeper of inflammatory cell recruitment to the adrenal gland and adrenal gland inflammation thereby modulating adrenal gland (dys-)function in the course of SIRS.

Human endothelial colony forming cells and mesenchymal progenitor cells form functional blood vessels in rat myocardium after ischemia/reperfusion injury

Kyu-Tae Kang[1]*, Matthew Coggins[2], Chunyang Xiao[2], Anthony Rosenzweig[2], Joyce Bischoff[1]. [1]Vascular Biology Program and Department of Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA, [2]Cardiovascular Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Cell-based therapies intended to restore heart function after myocardial infarction have been tested in a variety of pre-clinical models and in human clinical trials. The cells tested have varied from bone marrow-derived cells to cardiac progenitor cells to differentiated embryonic stem cells, with the goal of regenerating the myocardium. However, few studies have focused on building new blood vessels in ischemic myocardium as a means to promote recovery of cardiac muscle. Previous work from a number of labs has shown that human endothelial colony forming cells (ECFC) combined with mesenchymal progenitor cells (MPC) can be used to "bio-engineer" functional human blood vessels in immune-deficient mice. Here, we investigated whether ECFC and MPC could form functional vascular networks within ischemic myocardium, and whether this would result in improved cardiac function. Myocardial ischemia/reperfusion (I/R) injury was induced in 12-week-old male immune-deficient rats by ligation of the left anterior descending coronary artery (LAD). After 40 minutes, the LAD ligature was released, and reperfusion was visually confirmed. ECFC and MPC (2X10^6 cells; 2:3 ratio) or PBS were then injected into the ischemic myocardium immediately after reperfusion. Initial infarct size at day 1 was no different between the two groups. ECFC retention was determined by injecting luciferase-labeled ECFC/MPC: luciferase enzyme activity assays showed that 2,000 ECFC were present at day 7, indicating a low but quantifiable level of cellular retention. Human ECFC-lined perfused vessels in the myocardium were directly visualized by tail vein injection of a mixture of fluorescently-tagged human- and murine-specific lectins. Perfused human vessels were seen in myocardial tissue injected with ECFC/MPC but not PBS, 7 days after I/R injury. A composite Left ventricular (LV) wall motion score demonstr! ated imp roved regional function in the cell-injected rats compared to the PBS-injected rats at Day 7 (1.2 ± 0.1 vs. 1.3 ± 0.1; p = 0.02; N = 17, 15). LV internal dimension in diastole was reduced after 4 months in the cell-injected rats compared to the PBS-injected rats (p = 0.04; N = 6, 5), indicating that ECFC/MPC injection can attenuate long-term adverse ventricular remodeling. Global LV function as indicated by fractional shortening was reduced at day 14 in the cell-injected rats, (43.7 ± 7.4 % vs. 46.7 ± 4.0 %; p = 0.03; N = 13, 12) but not different after 4 months between the two groups (41.3 ± 3.7 % vs. 43.7 ± 5.0 %; p = 0.15; N = 6, 5). These data suggest that delivery of ECFC/MPC directly into ischemic myocardium to enhance neovascularization could be a potential therapeutic approach to reduce I/R injury and restore cardiac function.

Deficiency of mitogen-activated proteinkinase activated proteinkinase 2 (MK2) prevents maladaptive vascular remodeling and promotes vascular regeneration after injury of the carotid artery

Piyushkumar R. Kapopara*, Johann von Felden, Kristina Sonnenschein, Johann Bauersachs, Bernhard Schieffer, Udo Bavendiek. Dept. Cardiology and Angiology, Hannover Medical School, Hannover, Germany

Background: Atherosclerosis as well as mechanical injury of the vascular wall (e.g. angioplasty) cause a maladaptive remodeling characterized by neointima formation and media hypertrophy causing narrowing of the affected vessel. This process is accompanied by an increased vascular expression of inflammatory mediators, an increased vascular recruitment of leukocytes as well as proliferation and migration of activated vascular smooth muscle cells (SMC). Moreover, mechanical injury of the vessel wall results in a loss of the vessel protecting endothelial cell layer. The MAP kinase-activated-protein-kinase 2 (MK2) regulates expression of inflammatory mediators, cell migration, and cell proliferation; processes known to be important for vascular remodeling after vascular injury. Therefore, we hypothesized an important role of MK2 in vascular remodeling and endothelial regeneration after injury. Methods & Results: The functional role of MK2 in arterial remodelling after vascular injury was investigated in hypercholesterolemic low-density-lipoprotein-receptor deficient mice (LDRKO) subjected to wire injury of the common carotid artery (CCA). LDLRKO and LDLRKO deficient for MK2 (LDLR/MK2KO) were fed a high cholesterol diet (1.25 % cholesterol) for 4 weeks before and after wire injury. Injured CCAs were harvested 28 days after wire injury and vascular remodelling was determined by histomorphometry. Neointima formation observed in LDLRKO was almost completely prevented in LDLR/MK2KO (n=8, $p<0.01$) and also the media hypertrophy was reduced in LDLR/MK2KO mice. Next, expression (western blot) of cell proliferation markers (p-Rb, cyclinD1) were analyzed in cultured primary murine SMC isolated from aortas of wild type (WT) and MK2-deficient mice (MK2KO). Induction of p-Rb and cyclinD1 by FCS or PDGF was markedly reduced in SMC from MK2KO compared to WT (n=3). To elucidate the underlying mechanism cell proliferation (BrdU-assay; expression of p-Rb, cyclinD1, Westernblot) was analyzed in cultured smooth muscle cells (SMC) isolated from aortas of wild type (WT) and MK2-deficient mice (MK2KO). MK2-deficiency significantly reduced cell proliferation (n=3, $p<0.05$) as well as expression of p-Rb and cyclinD1 induced by FCS or PDGF in SMC (n=3-6). Furthermore, MK2-deficiency decreased migration of SMC (wound scratch assay) induced by PDGF and FCS (n=6, $p<0.05$). Moreover, we investigated the functional relevance of MK2 for vascular regeneration in the model of re-endothelialization after electric injury of the CCA in WT and MK2KO mice (Evan´s blue staining of denuded area). MK2-deficiency enhanced re-endothelialization of the denuded area after injury of the CCA (n=6-9, $p<0.05$). Consistently, MK2-deficiency significantly increased endothelial cell proliferation in vivo (EdU-staining) across the denuded area of the CCA (n=3-4, $p<0.05$). Next, expression (western blot) of cell proliferation markers (p-Rb, cyclinD1) as well as cell proliferation (BrdU-/Alamar blue-assay) and migration (scratch-assay) were determined in cultured murine endothelial cells (EC) isolated from WT and MK2-deficient mice (MK2KO). MK2-deficiency markedly increased the induction of p-Rb and cyclinD1 in EC by FCS or endothelial growth medium (EGM) compared to WT (n=3). In addition, MK2-deficiency increased cell proliferation and migration of EC induced by FCS and EGM compared to WT (n=3-5, $p<0.05$). Conclusion: Deficiency of mitogen-activated protein kinase-activated protein kinase 2 (MK2) prevents maladaptive vascular remodelling and promotes vascular regeneration after vascular injury.

High-fat diet impairs lymphatic function

Katrin Sabine Blum[1], Sinem Karaman[1]*, Steven Proulx[1], Alexandra Ochsenbein[1], Paola Luciani[1], Jean-Christophe Leroux[1], Christian Wolfrum[2] and Michael Detmar[1]. [1]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, 8093 Zurich, Switzerland, [2]Institute of Food, Nutrition and Health, Swiss Federal Institute of Technology, ETH Zurich, 8603 Schwerzenbach, Switzerland

Lymphatic vascular defects and the lack of dermal lymphatic capillaries can result in adipose tissue accumulation as shown in mouse knockout models and patients with lymphedema. This suggests a link between lymphatic function and adipose tissue accumulation and proliferation. To elucidate changes of the lymphatic vasculature in obesity, we studied the morphology and function of the lymphatic capillaries and collectors in mice fed with high-fat diet (HFD) compared to chow fed controls. Histological analysis of HFD mice showed smaller dermal capillaries and a larger amount of subcutaneous adipose tissue in the tail. There was no evidence for edema in the skin, and the relative water content of the popliteal fat pad was not increased in HFD mice. All HFD mice, but not the control animals, had tortuous collecting lymphatic vessels in the knee region. Irregular pulsing and a weaker reaction to mechanostimulation of the foot were detected in lymphatic collectors of the HFD mouse hind-legs with in vivo near-infrared fluorescence imaging. Dermal backflow was detected in one mouse of the HFD group. Importantly, acute elevation of tissue pressure by s.c. injection of olive oil also resulted in decreased lymphatic vessel pulsing and reduced reaction to mechanostimulation. Together, these findings indicate that accumulation of adipose tissue in obesity impairs lymphatic function, at least in part by increasing tissue pressure.

Hyperglycemia and oxidative stress in cultured endothelial cells - comparison of primary endothelial cells with an immortalized endothelial cell line

Susanne Karbach[1,3*#], Thomas Jansen[1#], Sven Horke[2], Tjebo Heeren[1], Alexander Scholz[1], Meike Coldewey[1], Angelica Karpi[1], Michael Hausding[1], Swenja Schuhmacher[1], Matthias Oelze[1], Thomas Münzel[1], and Andreas Daiber[1]. [1]Medical Center of the Johannes Gutenberg University, Mainz, Germany, [2]2nd Medical Clinic, Department of Cardiology [3]Department of Pharmacology, Institute of Molecular Medicine [#]contributed equally

Objective: Diabetes mellitus is a major risk factor for the development of cardiovascular disease. Oxidative stress was postulated to play an important role in the progression of these cardiovascular complications. Accordingly, good vascular models for diabetic complications are of high clinical and pharmacological importance. In the present study we investigated the effects of hyperglycemia on the formation of reactive oxygen species (ROS) and nitric oxide (NO)-cGMP signaling in two different endothelial cell cultures. Methods: Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cords and were used besides an immortalized endothelial cell line EA.hy 926. Induction of hyperglycemia was performed by high concentrations of glucose supplied in the medium (30 or 35 mM). Oxidative stress was assessed by fluorescence and chemiluminescence based methods and NO-cGMP signaling by EIA kits as well as NO formation quantified by an HPLC assay. Mitochondrial aldehyde dehydrogenase activity (ALDH-2) was measured by HPLC based analysis. Results: HUVECs and EA.hy 926 cells showed increased oxidative stress and impaired NO-cGMP signaling in response to the induced hyperglycemia. The major difference between the two different cell types was the dramatic decrease in viability in HUVEC whereas EA.hy cells showed rather increased growth under hyperglycemic conditions. Starvation in the presence of low growth medium (0.5 % fetal calf serum) led to an additional substantial decrease in viability and increased superoxide formation in HUVECs. Conclusions: Both endothelial cell types, HUVEC and EA.hy 926, may be used as models for vascular hyperglycemic complications. However, high growth medium should be used to avoid starvation induced oxidative stress and cell death.

Peripheral nerve is critical for Vasa Vasorum maturation in injured vascular walls - effects of SubstanceP on pericytes-mediated vascular maturation-

Maki Kabara*, Jun-ichi Kawabe, Akira Asanome, Atsushi Yamauchi, Motoki Matsuki, Naofumi Takehara, Naoyuki Hasebe. Asahikawa Medical University, Department of cardiovascular Regeneration and Innovation, Department of Medicine, Asahikawa, Japan

Introduction: Immature Vasa Vasorum (VV), a microvasculature in the adventitial layer, induces the formation of unstable plaques. Normalization of VV would be attractive therapeutic approach for arteriosclerotic diseases. Peripheral nerve is important in vascular development; however, its effect on vascular maturation is not still clear. In this study, we investigated the role of peripheral nerve on vascular maturation of VV. Methods and Results: We've developed in vivo angiogenesis assay using collagen-coated tube (0.7 mm diameter) to observe the microvasculature around the injured femoral artery of C57BL6 mice. At 14 days after wire-mediated vascular injury, formation of VV was enhanced. Immunohistochemical analysis showed that peripheral nerve was already regenerated around the pre-matured VV microvessels. It is suggested the presence of new role of peri-vascular nerves in the angiogenesis except the vasoactive effects in pathophysiological condition. When biodegradable gel containing VEGF was settled around the injured femoral artery, total length of VV was significantly increased accompanied with enhanced vascular permeability and microbleeds. When regeneration of peripheral nerve was stimulated by nerve growth factor (NGF) in addition to VEGF released from gels, mean diameter of formed VV microvessels was increased and its permeability and microbleeds were attenuated. Although NGF increased the length of neovessels sprouting from aorta explants in nerve-free angiogenesis assay, vascular maturation which was estimated by the neovessel length covered with pericytes was not enhanced. To elucidate the mechanism of peripheral nerve-mediated vascular maturation, pericyte cells lines (PCs) was established from VV of temperature-sensitive SV40T-antigen transgenic mice. PCs were associated with endothelial tube to form matured vessels. Among neurotransmitters, substance P (SP) enhanced proliferation and migration of PCs and induced PCs-mediated vascular maturation. Conclusion: Regeneration of the peripheral nerve enhances vascular stability and maturation of VV and SP may contribute to VV maturation through the action on vascular pericytes.

Tumor-derived microvesicles induce proangiogenic phenotype in endothelial cells via endocytosis

Taisuke Kawamoto[1]*, Noritaka Ohga[1], Kosuke Akiyama[1], Naoya Hirata[1], Shuji Kitahara[2] Nako Maishi[1], Takahiro Osawa[1], Kazuyuki Yamamoto[1], Miyako Kondoh[1], Masanobu Shindoh[3], Yasuhiro Hida[4], Kyoko Hida[1]. [1]Department of Vascular Biology, Graduate School of Dental Medicine, University of Hokkaido, Sapporo 060-8586, Hokkaido, Japan, [2]Department of Anatomy and Developmental Biology, School of Medicine, Tokyo Women's Medical University, Tokyo, 162-8666, Japan, [3]Department of Oral Pathology and Biology, Graduate School of Dental Medicine, University of Hokkaido, Sapporo 060-8586, Hokkaido, Japan, [4]Department of Cardiovascular and Thoracic Surgery, Graduate School of Medicine, University of Hokkaido, Sapporo 060-8586, Hokkaido, Japan

Increasing evidence indicates that tumor endothelial cells (TEC) differ from normal endothelial cells (NEC). Our previous reports also showed that TEC were different from NEC. For example, TEC have chromosomal abnormality and proangiogenic properties such as high motility and proliferative activity. However, the mechanism by which TEC acquire a specific character remains unclear. To investigate this mechanism, we focused on tumor-derived microvesicles (TMV). Recent studies have shown that TMV contain numerous types of bioactive molecules and affect normal stromal cells in the tumor microenvironment. However, most of the functional mechanisms of TMV remain unclear. Here we showed that TMV isolated from tumor cells were taken up by NEC through endocytosis. In addition, we found that TMV promoted random motility and tube formation through the activation of the phosphoinositide 3-kinase/Akt pathway in NEC. Moreover, the effects induced by TMV were inhibited by the endocytosis inhibitor dynasore. Our results indicate that TMV could confer proangiogenic properties to NEC partly via endocytosis. We for the first time showed that endocytosis of TMV contributes to tumor angiogenesis. These findings offer new insights into cancer therapies and the crosstalk between tumor and endothelial cells mediated by TMV in the tumor microenvironment.

PHD3 silencing leads to increased tumor growth and enlarged tumor vessels

Antje Kettelhake[1]*, Maryam Rezaei[2], Aleksandar Kuzmanov[3], David Poitz[4], Ben Wielockx[1,5,6], Georg Breier[1,6]. [1]Department of Pathology, Technical University Dresden, Dresden, Schubertstraße 15, Germany, 01307, [2]Department of Anatomy and Vascular Biology, University of Muenster, Germany [3]Department of Biomedicine, University of Basel, Switzerland, [4]Department of Internal Medicine and Cardiology, Technical University Dresden, Germany, [5]Emmy Noether Research Group, [6]Center for Regenerative Therapies Dresden, Germany

The fast growth of solid tumors causes the development of hypoxic areas that are very often marked by HIF upregulation. HIF enables the tumor cells to survive under hypoxic conditions for example by increasing the production of angiogenic factors. One possibility to suppress the action of HIF in tumor tissue is the manipulation of the HIF-prolyl hydroxylases (PHD1-4). The function of PHD3 in the context of tumor progression and angiogenesis has not been investigated in detail so far. For that reason we stably silenced PHD3 in a murine osteosarcoma cell line (LM8) using a lentiviral transduction system. Surprisingly, we found that downregulation of PHD3 does neither affect the protein levels of HIF-1α and HIF-2α nor the expression levels of known HIF-target genes. qPCR analysis shows equally strong expression of the other PHD isoforms as well as factor inhibiting HIF-1 (FIH) in sh-PHD3 clones compared to controls. However, platelet-derived growth factor c (PDGF-C) is markedly upregulated and the angiopoietin 2 (Ang2) mRNA level is decreased in the sh-PHD3 clones. Subcutaneous injection of the manipulated tumor cells into C3H mice leads to the development of significantly larger tumors compared to control tumors. Interestingly, the density of tumor vessels is decreased but the vessels are enlarged as determined by staining for the endothelial cell marker PECAM. α-smooth muscle actin (αSMA) immunofluorescence staining reveals that more vessels in the PHD3 silenced tumors are covered with αSMA-positive cells. Downregulation of PDGF-C in shPHD3 tumor cells reduces tumor growth to control levels, increases the number of vessels per area and abates the vessel size. Our data demonstrate that in osteosarcoma PHD3 as well as PDGF-C play very important roles for tumor growth, tumor vessel shape and perivascular cell coverage. Both factors might be potential targets for anti cancer therapies.

Role of AP-1 transcription factor subunit Junb in microRNA regulation and its impact on lymphangiogenesis in zebrafish

Kristin Kiesow[1]*, Melanie Sator-Schmitt[1], Karin Strittmatter[1], Katrin Bennewitz[2], Peter Angel[1], Jens Kroll[2] and Marina Schorpp-Kistner[1]. [1]Division of Signal Transduction and Growth Control, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, k.kiesow@dkfz.de, [2]Department Vascular Biology and Tumorangiogenesis, Center for Biomedicine and Medical Technology Mannheim (CBTM), Ludolf-Krehl-Str. 13-17, 68167 Mannheim, Germany

AP-1 transcription factors are essential regulators of gene expression in response to a variety of extracellular stimuli. In particular, the AP-1 subunit Junb was recently identified as a critical regulator of angiogenesis in response to hypoxia in vivo. Complete as well as endothelial cell-specific ablation of Junb in mouse resulted in similar angiogenic defects with subsequent embryonic lethality. Junb was found to serve as a transactivator of several pro-angiogenic molecules including Vegf-a, the transcription factor Cbfb and its target Mmp13. In contrast, previous studies on the role of Junb in other physiological processes including haematopoietic cell differentiation and inflammation during cutaneous wound healing, have highlighted, that Junb can also exert repressor function. Hence, Junb must be considered as a context-dependent transcriptional regulator. The mechanisms underlying Junb-dependent gene repression have not been elucidated so far. A global microRNA expression profiling of murine wild-type and Junb-deficient cells of different origin provided a number of Junb-dependently expressed microRNAs. Morpholino-mediated knock-down of the corresponding microRNA homologues in tg(fli:EGFP) transgenic Zebrafish resulted in overall normal morphology and no delay in embryonic development. A more in-depth analysis of the developing vascular system revealed, that individual loss of either one of the two identified miRNAs or of Junb leads to intracranial hemorrhages and a very distinct defect in lymphangiogenesis. In 80-90% of morphant embryos, the parachordal lymphangioblasts (PL), precursors of the developing lymphatic vasculature, are completely absent. Via introduction of Junb mRNA we can partially rescue both phenotypes observed in the miRNA morphants. The findings provide additional evidence in line with previous reports in mouse that Junb is a critical regulator of vascular and angiogenic processes and suggest that a subset of miRNAs regulated by the stress-induced immediate early gene Junb is required for the fine-tuning of vascular development including lymphangiogenesis in the developing Zebrafish.

Intravital immunofluorescence in the mouse ear dermis

Witold W. Kilarski*, Esra Guc, Jeremy C. M. Teo S. Ryan Oliver, Amanda Lund and Melody A. Swartz. Institute of Bioengineering and Swiss Institute of Experimental, Cancer Research (ISREC), école Polytechnique Fédérale de Lausanne, Lausanne 1015, Switzerland

Intravital imaging of interstitial cells interacting with their microenvironment - including tumor cell invasion and leukocyte migration into blood or lymphatic vessels - is crucial for understanding mechanisms of immunological, metastatic and healing processes. While multiphoton and second harmonic generation imaging have brought tremendous insight into these processes, they are limited by model availabity and imaging expense. Here we describe an intravital immunofluorescence technique that allows live imaging of interactions between any cell types and extracellular compartments in living mouse ear skin. Surgically exposed skin was vastly unaffected by the procedure evident with minimal neutrophils extravasation and functional vessels. Immuno- and photo-toxicity of strong indirect fluorescent immunolabeling was minimized by blocking macrophage Fcγ receptors and the use of antioxidant ascorbate immersion. We demonstrate differential migration behaviors of melanoma cells, marrow-derived cells, blood-circulating leukocytes, and dermal dendritic cells, with the latter entering chemokine CCL21-positive pre-collecting lymphatics. We were able to simultaneously image multiple ear locations for up to 9 hours with identification of tissue structures or cells limited only by availability of cell surface receptor and extracellular markers. This intravital imaging platform can be used for variety of alternative applications, including study of skin parasites infection, mechanisms of angio- and lymphangiogenesis, tumor, inflammation and skin transplant immunity.

Novel regulatory mechanism for thrombin-dependent MMP-2 activity that is modulated by heparan sulfate

Bon-Hun Koo, Yeon Hyang Kim, and Doo-Sik Kim*. Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Korea

MMP-2 is synthesized as a zymogen that is activated by conformational change or proteolytic cleavage of the propeptide. Thrombin was demonstrated to be involved in MMP-2 activation via specific cleavages of the propeptide. It was also shown that thrombin is able to degrade MMP-2, but this degradation is greatly reduced under cell-associated conditions with a concomitant increase in its activation. However, the underlying molecular mechanisms remain to be elucidated. It is demonstrated in this work that heparan sulfate proteoglycan is essential for thrombin-mediated activation of pro-MMP-2. Binding of heparan sulfate to thrombin is primarily responsible for this activation process, presumably through conformational changes at the active site. It was interesting to note that interaction of MMP-2 with exosites 1 and 2 of thrombin is crucial for thrombin-mediated MMP-2 degradation, and inhibition of this interaction by heparan sulfate or hirudin fragment results in a decrease in MMP-2 degradation. We demonstrate interaction between thrombin exosite 1 and hemopexin-like domain of MMP-2, indicating a regulatory role of hemopexin-like domain in MMP-2 degradation. Taken together, our experimental data suggest a novel regulatory mechanism of thrombin-dependent MMP-2 enzymatic activity by heparan sulfate proteoglycans.

Hic-5 deficiency enhances mechanosensitive apoptosis and modulates vascular remodeling

Joori Kim-Kaneyama* and Akira Miyazaki. Department of Biochemistry, Showa University School of Medicine, Tokyo, Japan

Forces associated with blood flow are crucial not only for blood vessel development but also for regulation of vascular pathology. Although there have been many studies characterizing the responses to mechanical stimuli, molecular mechanisms linking biological responses to mechanical forces remain unclear. Hic-5 (hydrogen peroxide-inducible clone-5) is a focal adhesion adaptor protein proposed as a candidate for a mediator of mechanotransduction. In the present study, we generated Hic-5-deficient mice by targeted mutation. Mice lacking Hic-5 are viable and fertile, and show no obvious histological abnormalities including vasculature. However, after wire injury of the femoral artery in Hic-5 deficient mice, histological recovery of arterial media was delayed due to enhanced apoptosis of vascular wall cells, whereas neointima formation was enhanced. Stretch-induced apoptosis was enhanced in cultured vascular smooth muscle cells (vascular SMCs) from Hic-5 deficient mice. Mechanical stress also induced the alteration of intracellular distribution of vinculin from focal adhesions to the whole cytoplasm in SMCs. Immunoelectron microscopic study of vascular SMCs from a wire-injured artery demonstrated that vinculin was dispersed in the nucleus and the cytoplasm in Hic-5-deficient mice whereas vinculin was localized mainly in the sub-plasma membrane region in wild type mice. Our findings indicate that Hic-5 may serve as a key regulator in mechanosensitive vascular remodeling.

Do the regeneration of the normal vasculature in tumors suppress the tumor growth?

Shuji Kitahara[1]*, Yasuhiro Suzuki[2], Yoshikazu Kuwahara[3], Motoi Fukumoto[3], Asuka Yoshii[1], Manabu Fukumoto[3], Yasufumi Sato[2], Taichi Ezaki[1]. [1]Department of Anatomy and Developmental Biology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan, [2]Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, [3]Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan

[Aim] It has been known that tumor vessels are structurally and functionally different from normal vessels. Impaired blood supply hinders with the delivery of therapeutics to solid tumors. Hence, the strategy for the functional "normalization" of tumor vasculature would substitute the impairment to control the tumor proliferation. In this report, we sought to characterize whether transplanting normal endothelial cells to tumor-bearing mice would trigger vascular remodeling and reduce tumor volume. [Materials & Methods]SAS tumor cells (human squamous cell carcinoma: 2.5x106cells) were injected into the dosal subcutis of SCID mice. After 1 week, the tumor-baring mice were injected cysplatin (20mg/kg) intraperitoneally to regress tumors. And 2 weeks later, to induce the normal angiogenesis in the tumor, human microvascular endothelial cells (HUMVEC®: 5x105cells) were transplanted into necrosis areas of the tumor. To characterize the regeneration of blood vessels, all blood vessels were labeled with FITC-labeled tomato lectin and the multiple-immunostainings for various markers were performed for 3 dimensional imaging by the confocal laser-scanning microscopy. [Results & Discussion] Tumor vessels reconstituted by HUMVEC with angiopoietin in Matrigel® exhibited the normalization of tumor vasculature, resulting in a significant reduction (about 20% reduction) in the tumor volume. Microscopic observations revealed that the transplanted human endothelial cells formed anastomoses with the host mouse vasculature, and perfused vessels (as evidenced by injected FITC-Tomato lectin) were detectable after 7days. The normalization of tumor vasculatures may therefore contribute not only to attenuation of the innate resistance to chemotherapy/radiotherapy but also to potential improvement of the delivery of anticancer drugs to hypoxic tumors.

CD34 marks angiogenic tip cells in human vascular endothelial cell cultures

Martin J. Siemerink[1], Ingeborg Klaassen[1]*, Ilse M.C. Vogels[1], Arjan W. Griffioen[2], Cornelis J.F. Van Noorden[1], and Reinier O. Schlingemann[1,3]. [1]Ocular Angiogenesis Group, Department of Ophthalmology and Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands, [2]Angiogenesis Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands. [3]Department of Clinical and Molecular Ophthalmogenetics, The Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

The functional shift of quiescent endothelial cells into tip cells that migrate and stalk cells that proliferate is a key event during sprouting angiogenesis. Unfortunately, an in vitro model of the tip cell phenotype in vascular endothelial cell cultures is lacking. We previously showed that the sialomucin CD34 is expressed on tip cells in vivo, but also in a small subset of elongated endothelial cells that extend filopodia in endothelial cell cultures. As this is a hallmark of tip cells in vivo, we investigated if these CD34+ cells represent a model of tip cells in vitro. As predicted by our hypothesis, the CD34+ endothelial cells had low proliferation activity, and the CD34+ phenotype was upregulated by VEGF-A and downregulated by TNF-alpha and DLL4, three mechanisms known to regulate the tip cell phenotype in vivo. Real-time qPCR and microarray data analysis of the CD34+ cells identified increased expression of all known genes previously associated with tip cells in vivo. Genome-wide mRNA profiling analysis of CD34+ cells demonstrated enrichment for biological functions related to angiogenesis and migration, whereas CD34-negative cells were enriched for functions related to proliferation. Our findings suggest that cells with virtually all known properties of tip cells are present in vascular endothelial cell cultures, and that they can be isolated based on expression of CD34. In addition, we characterized the transcriptome of these cells and identified many novel genes with potential significance for angiogenesis. This novel strategy may open alternative avenues of research that may help to understand the molecular processes and functions in angiogenesis in general and of the specialized endothelial tip cell in particular.

Characterization of Macrophage Migration Inhibitory Factor (MIF) as a new B cell chemokine

Omar El Bounkari, Christina Klasen*, Jürgen Bernhagen. Institute of Biochemistry and Molecular Cell Biology, RWTH Aachen University, Aachen, Germany

Macrophage migration inhibitory factor (MIF) is an inflammatory cytokine with chemokine-like functions which plays a pivotal role in the pathogenesis of atherosclerosis and other inflammatory diseases. We showed that atherogenic monocyte and T lymphocyte recruitment by MIF is mediated through non-cognate interaction with the CXC chemokine receptors CXCR2 and CXCR4, respectively, which also interact with CD74, the surface form of MHC class invariant chain, functioning as a third MIF receptor. A role for B lymphocytes in atherogenesis has more recently been appreciated, but the underlying mechanisms and recruitment signals are poorly understood. B lymphocytes express substantial levels of CD74 as well as CXCR4. We thus set out to study the potential role of MIF in B cell migration. Applying Transwell-based in vitro chemotaxis assays, we found that primary B lymphocytes isolated from wild type (wt) C57/Bl6 mice migrated towards MIF in a dose-dependent manner with a maximum response observed at 200 ng/ml rMIF. The chemotactic index was comparable to that elicited by the typical B cell chemokine CXCL13. Interestingly, B cells from Cd74-deficient mice completely failed to migrate in response to MIF but not CXCL13, indicating that CD74 may play a specific role in MIF-driven B cell migration. This notion was confirmed by blockade experiments using neutralizing antibodies against CD74, which attenuated MIF-driven chemotaxis of wt B cells. Of note, MIF-mediated B cell chemotaxis also was abolished by blockade of CXCR4 with the pharmacological inhibitor AMD3100. This suggested that both MIF receptors contribute to MIF-mediated B lymphocyte chemotaxis, possibly by interdependent sequential signaling pathways and/or receptor complex formation. Additionally, intracellular MIF appears to have a fundamental basic effect on the migratory capacity of B lymphocytes, as Mif-/- B lymphocytes failed to respond to chemotactic stimulation by either MIF or CXCL13. Pending in vivo verification of these effects, our data suggest a novel function of MIF and its receptors in the recruitment of B lymphocytes which might play a role in atherogenesis and other inflammatory conditions.

Scar rupture and death upon myocardial infarction by NOX4-derived ROS

PWM. Kleikers[1]*, K. Wingler[1], K. Radermacher[1], N. Weissmann[2], HHHW. Schmidt[1].
[1]Maastricht University, CARIM, Department of Pharmacology, Vascular Drug Discovery Group (VDDG), Maastricht, Netherlands, [2]University Giessen Lung Centre, University Hospital, Giessen, Germany

Aim: Reactive oxygen species (ROS) are thought to be key players in ischemia-reperfusion-injury (IRI). However, clinical trials using antioxidants to scavenge ROS failed to show a benefit. Thus, inhibition of ROS-production may be a superior therapeutic strategy. We recently identified NOX4 as a major source of ROS in ischemic stroke in mice. Therefore, we hypothesized that NOX4 may also play a detrimental role in IRI of other organs such as heart and lung. Methods: We performed ischemia-reperfusion (I/R) of the heart in male and female NOX4 KO and matched WT mice by ligating the left descending coronary artery (LAD) for 45 min, followed by a 24 h or 4 weeks reperfusion and observation period. Basal and 24 h post-reperfusion ultrasounds were performed. At the end of the observation period, area at risk and infarct size and left ventricular haemodynamic function were determined. In another set of experiments, isolated perfused mouse lungs were subjected to 30 min of ischemia and 90 min of reperfusion. Post-ischemic vascular leakage and lung capillary filtration coefficient were measured as parameters of tissue damage. Results: In the hearts of NOX4 KO mice infarct size and area at risk were unchanged compared to WT mice. Functional parameters 24h post-reperfusion were impaired after IRI, but not different between KO and WT mice. Also, the changes in left ventricular haemodynamic function were not different between the genotypes. A longer follow-up of 4 weeks after I/R, and in addition, after myocardial infarction, did not show any difference between WT and NOX4 KO male mice in infarct size, collagen formation or cardiac function. However, in the MI group, WT mice showed a higher degree of acute mortality caused by cardiac rupture or heart failure. In the isolated perfused lung, I/R caused oedema formation with an increase in lung weight and increased capillary filtration coefficients, but again no differences between KO and WT mice. Conclusions: After I/R of the heart no significant differences in infarct size, area at risk or functionality were found between NOX4 KO and matched WT mice. Similarly, in lung IRI, no effect of NOX4 was observed. These results are contrasting with those found in ischemic stroke, where NOX4 KO mice showed a smaller infarct size and better neurological outcome. In addition, NOX4 might play a role in the acute mortality after MI since WT mice had a lower survival rate than NOX4KO mice Thus, the role of NOX4 in IRI is not straightforward, it might be organ- or even cell-specific. Also, the amount of ROS produced by NOX4 and the timing within the IRI play major roles. NOX4 may have a possible beneficial effect in angiogenesis and remodelling, while in the acute phase it rather seems detrimental. Therefore, we are currently investigating the role of NOX4 in the chronic ischemia model of the hindlimb and the possibility of cell-specific effects in ischemic stroke by using cell-specific NOX4 KO mice.

The transcription factor CREM is involved in the regulation of VSMC proliferation in response to vascular remodeling

Klugstedt, C.T*, Seidl, M.D, Steingräber, A.K, Schmitz,W, Müller,F.U. Institut für Pharmakologie und Toxikologie, Westfälische Wilhelms-Universität Muenster, Germany

Transcriptional response driven by the cAMP signaling pathway is mediated among others by the transcription factor CREB/CREM/ATF-1 family. These specific transcription factors require binding to cAMP-response elements (CRE) in promoter regions of respective target genes. Their supposed involvement in the regulation of proliferation and apoptosis of vascular smooth muscle cells (VSMC) makes them an important target in the research of vascular proliferative diseases. It has been shown that CREM deficient mice (global CREM knock-out, CREM-/-) exhibit an increased formation of a neointima three weeks after ligation of the carotid artery. In wild-type mice, Crem mRNA expression was significantly down-regulated by 48% (rel. expression, CREM-/- vs. WT, 0.52, SE 0.3-0.8; n=8; $p<0.05$) in ligated carotids compared to non-ligated as shown by real time PCR analysis. The finding of the increased neointima was accompanied by an increased proportion of proliferative cells as shown by Ki67-staining of vascular smooth muscle cells in the carotid media of CREM-/- mice (CREM-/- 0.8±0.3 %, WT 0.1±0.1 %; n=9-10; $p<0.05$). Media thickness was not altered between these samples. Proliferation in PDGF-BB treated (7.5 ng/ml for 24h) isolated CREM-/- VSMCs was significantly increased by 1.3 fold compared to WT controls while untreated cells showed no difference. To address the overall activation of target genes, VSMCs were transiently transfected with a CRE-activated luciferase reporter gene construct. A stimulation with PDGF for 9h led to a 3-fold increase in CRE-controlled promoter activity in CREM-/- VSMCs as compared to WT controls (CREM-/- 2.9±0.4, WT 1.0±0.2; n=5; $p<0.05$). To identify genes which are differentially regulated between the PDGF-stimulated VSMCs of CREM -/- and WT mice, a microarray analysis and verification by quantitative real time PCR analysis was performed. In conclusion, anti-proliferative effects of CREM are associated with a complete repression of CRE-mediated transcriptional activation by PDGF and suggest CREM as an important factor for gene regulation in the pathogenesis of vascular proliferative disorders. (Supported by the DFG)

Raman spectroscopy for stem cell detection and classification of viability states

Steffen Koch[1]*, Katja Schenke-Layland[2,3], Heike Walles[2,4], Karin Schütze[1]. [1]CellTool GmbH, Tutzing, Germany, [2]Fraunhofer IGB, Stuttgart, Germany, [3]University Tübingen, Tübingen, Germany, [4]University Würzburg, Würzburg, Germany

Introduction Raman spectroscopy is an optical spectroscopy method that allows label-free and non-contact characterization of cells and tissue under physiological conditions. Raman spectroscopy is based on focused laser light shone into cells to excite molecular vibrations. The shift in frequency of the emitted light is detected by a spectrograph and yields spectra with detailed chemical information about the sample. Like a „photonic fingerprint“ this information can be used to identify and characterize cell types, cellular states or the cellular response to drug treatment. In stem cell research cells have to be characterized unambiguously using different methods before use. Undifferentiated stem cells and differentiated cells often lack specific sites for identification and characterization. Raman spectroscopy is an innovative method to safely characterize cells and cell states on a single cell level without destruction or damage. Material & Methods In order to determine differences in the biochemical component patterns, Raman-spectra of cell groups were obtained and analyzed with multivariate statistics. The results were compared to standard techniques like fluorescence labelling and biochemical characterization of the cells. For cell discrimination and characterization cells were cultured under the same standard conditions and measured in suspension after detachment from the culture flask. For discrimination of viability state two cell types were characterized with Raman spectroscopy. The results were verified by fluorescent labelling and flow cytometry. Apoptosis and necrosis were induced by temperature changes to avoid chemical interactions that may influence Raman spectra. For apoptosis cells were cultured at room temperature for 0 - 196 h. Necrosis was induced heating the cells at 55°C for 90 minutes. Results Different cell types were analysed to prove the feasibility of Raman spectroscopy for quick and safe cell discrimination. The results of different cell types clearly showed that cell identification purely based on spectroscopy is possible. Spectral analysis is highly specific and allows discrimination of mesenchymal stem cells and fibroblasts. Apoptosis and necrosis were monitored by standard methods and compared to Raman spectroscopic results. Principal component analysis revealed changes in proteins and DNA in apoptotic cells. The Support vector machine analysis showed a high accuracy for the detection of viable and non-viable cells of 99.7 %; for the identification of viable, apoptotic and necrotic cells an accuracy of 96.3 % could be achieved. Discussion Raman spectroscopy is a new tool for cell analysis. The technology provides valuable information about various kinds of cells and tissue. The purely laser light based method is reliable and efficient for cell and tissue characterization especially when standard methods lack the ability for safe identification. Raman spectroscopy may become a powerful supportive technology in stem cell research and tissue engineering.

Involvement of HIF-2α, but not HIF-1α, in chronic hypoxia-induced inhibition of tube formation by human endothelial cells in 3D fibrin matrices

Pieter Koolwijk*, Monique C. Duyndam, Ester M. Weijers, Victor W.M. van Hinsbergh. Dept. Physiology, Institute for Cardiovascular Research (ICaR-VU), VU University Medical Center, Amsterdam, The Netherlands

It is generally accepted that angiogenesis is initiated/stimulated by acute hypoxia via the induction of growth factors e.g. VEGF. However, in chronically ischemic tissues commonly observed in congestive heart failure, diabetic legs and tissue-engineered scaffolds, endothelial cells (ECs) have significantly reduced angiogenic capacities. The majority of the hypoxia-induced processes are mediated by Hypoxia Inducible Factors, HIFs, a family of transcription factors that directly induce the expression of many genes in mammalian cells. Here we evaluated the involvement of HIF-1α and HIF-2α on the formation of tube-like structures by human ECs at chronic hypoxia (1% of oxygen). Using a hypoxia workstation, which allows us to compare cell characteristics of human ECs cultured at chronic hypoxia (> 1 week 1% oxygen without reoxygenation) with ECs cultured at 20% oxygen conditions, it was demonstrated that chronic hypoxia reduced tube formation by human microvascular ECs (hMVECs) in 3D fibrin matrices. This reduction in tube formation could be prevented by refreshment of the chronic hypoxia-cultured ECs using 20% saturated culture-medium, introducing a hypoxia-reoxygenation-hypoxia condition. The expression of the HIF-1α and HIF-2α proteins in hMVECs was examined at different time points after exposure to hypoxia. Hypoxia-induced HIF-1α protein expression was observed after 3 and 6 h and gradually reduced to baseline after 24 - 48 h. In contrast, HIF-2α protein expression was more stable; also detected after 3 h of hypoxia, but this expression was sustained up to 96 h. To study the involvement of the two HIF subtypes during the inhibition of tube formation down-regulation of HIF-1α and HIF-2α was accomplished using 3 different HIF-1α or HIF-2α-specific siRNAs from different companies. For each of the siRNAs, down-regulation of HIF-2α restored the hypoxia-reduced tube formation, whereas inhibition of the expression of HIF-1α didn't have any effect. These data indicate that at chronic hypoxia a HIF-2α-mediated pathway is involved in the down regulation of the angiogenic response and that HIF-2α interference can overcome the inhibition. Our data suggests that limitation of capillary sprouting – possible to achieve more stable vessels in the acute hypoxic situation – reduces the angiogenic response in chronic hypoxia.

Endothelial cell derived Wnt ligands control vessel branching in the postnatal retina

Claudia Korn[1]*, Junhao Hu[1], Kshitij Srivastava[1], Tabea Arnsberger[1,2], Michael Boutros[2,3], Hellmut G . Augustin[1,4], Iris Augustin[2]. [1]Div. of Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), [2]Div. of Signaling and Functional Genomics, German Cancer Research Center Heidelberg, [3]Dept. of Cell and Molecular Biology, and [4]Dept. of Vascular Biology and Tumor Angiogenesis, Center of Biomedicine and Medical Technology (CBTM), Medical Faculty Mannheim, Heidelberg University, Germany

Wnt signaling is involved in a variety of developmental processes and tissue homeostasis. Recent studies of the Wnt pathway point to a role of Wnt signaling in vascular morphogenesis and remodeling. Canonical/ß-catenin-dependent Wnt pathway is implicated in the development and differentiation of the retina and brain vasculature. Evidence for non-canonical/ß-catenin-independent Wnt pathways in regulating vessel formation is only beginning to emerge. It has been shown that non-canonical Wnt5a signaling induces proliferation, survival and migration of cultured endothelial cells and that loss of fzd4 function in mice impairs angiogenic response via non-canonical PCP signaling. Evi/Gpr177/Wntless is one of the few nonredundant components of the Wnt pathway. It acts as a specific regulator of pan-Wnt protein secretion resulting in global disruption of Wnt signaling upon genetic deletion of Evi. We therefore profiled the expression of Evi and Wnt family members in FACS sorted retina and lung endothelial cells. Wnt ligand as well as Evi expression was found to be increased in pups of wildtype mice compared to adult retina as well as lung endothelial cells. Moreover, non-canonical Wnt ligands were expressed more abundantly, indicating major input of non-canonical Wnt signaling. To investigate the role of endothelial cell-derived Wnt ligands in controlling angiogenesis, we generated conditional endothelial cell Evi null mice (EviECKO) by crossing VE-cadherinCre-ERT2 mice with Evifl/fl mice. Postnatal deletion of endothelial cell Evi expression revealed a reduction of vessel area in EviECKO mice. Furthermore, the vessel structure was less condensed as indicated by a reduced number of branches and branchpoints in EviECKO mice. Taken together, the experiments identified the Wnt-secretion factor Evi as an essential endothelial cell- derived regulator of vessel formation during early retinal development suggesting important functions of endothelial cell-specific and primarily non-canonical Wnt ligands in the control of vessel branching.

The Role of the extracellular matrix in leukocyte infiltration into the pancreas of Non-Obese Diabetic (NOD) mice

Éva Korpos[1]*, Nadir Kadri[2], Reinhild Kappelhoff[3], Christopher Overall[3,4], Ekkehard Weber[5], Dan Holmberg[6], Susanna Cardell[7] and Lydia Sorokin[1]. [1]Institute of Physiological Chemistry and Pathobiochemistry, Muenster University, Muenster, Germany, [2]Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden, [3]Departments of Oral Biological and Medical Sciences and, [4]Biochemistry and Molecular Biology, Centre for Blood Research, University of British Columbia, Vancouver, Canada, [5]Institute of Biochemistry, Martin-Luther University of Halle Wittenberg, Halle, Germany, [6]Center for Infection and Inflammation Research, Copenhagen University, Denmark, [7]Department of Microbiology and Immunobiology, University of Gothenburg, Gothenburg, Sweden

Type 1 Diabetes (T1D) results from the autoimmune destruction of insulin producing β-cells of the pancreatic islets. Several steps are crucial for induction of T1D; the first is extravasation of CD4+ T lymphocytes from blood vessels into the pancreatic tissue, the second is penetration of the peri-islet basement membrane (BM) surrounding the β-islets, and third the β-cell destruction which leads to appearance of disease symptoms. BMs act to separate tissue compartments and represent barriers to the movement of both soluble molecules and cells. Hence, cells penetrating such protein barriers must employ specialized mechanisms (1,2). The main question addressed here is what is the mechanism used by leukocytes to penetrate the peri-islet capsule of the pancreatic β-islets in order to reach their target, the insulin producing β-cells, in non-obese diabetic (NOD) mice during development of T1D and in recently diagnosed human T1D patients. The peri-islet capsule is composed of a BM and an underlying interstitial extracellular matrix layer. We demonstrate a global loss of the peri-islet BM and interstitial matrix components only at sites of leukocyte penetration of the peri-islet capsule. Stereological analyses reveal that the number of islets showing loss of the peri-islet BM versus islets with intact BMs correlates with incidence of insulitis, indicating that this is a critical disease-limiting step. Protease- and protease-inhibitor-specific microarray analyses (CLIP-CHIPTM) of laser dissected leukocyte infiltrated and non-infiltrated β-islets and confirmatory protein analyses have identified cathepsin S, W and C activity at sites of leukocyte penetration of the peri-islet BM in association with a macrophage subpopulation in NOD mice and human T1D samples. In conclusion, our data demonstrate that leukocyte penetration of the peri-islet BM is a critical disease-limiting step in T1D, the mechanism of which differs fundamentally from that involved in leukocyte extravasation from blood vessels (2), suggesting that the extracellular matrix milieu influences the mode employed by immune cells to infiltrate into tissues, and raising novel possibilities for tissue-specific immune modulatory therapies. 1) Korpos, E. et al, 2010. Cell Tissue Res. 339:47-57. 2) Wu, C. et al, 2009. Nat Med. 15:519-27.

eNOS traffic inducer NOSTRIN is an important regulator of vascular function

Igor Kovacevic[1]*, Tanja Hindemith[1], Mary Lee[2], Annemarieke Loot[2], Ingrid Fleming[2], Meike Hoffmeister[1], Stefanie Oess[1]. [1]Institute for Biochemistry II, Goethe University Frankfurt Medical School, Theodor Stern Kai 7 Hs 75, 60590 Frankfurt/Main, Germany, [2]Institute for Vascular Signalling, Goethe University Frankfurt Medical School, Theodor Stern Kai 7 Hs 75, 60590 Frankfurt/Main, Germany

NOSTRIN (eNOS traffic inducer) belongs to the F-BAR protein family. F-BAR proteins are multivalent adaptors that link plasma membrane and cytoskeleton and co-ordinate cellular processes such as membrane protrusion, migration and endocytosis. We have previously shown that NOSTRIN interacts with eNOS, dynamin, N-WASP and caveolin, and thereby participates in the regulation of eNOS subcellular localisation and activity. We have generated NOSTRIN knockout mice with complete and endothelial cell-specific deletion of the NOSTRIN gene. We compared the phenylephrine (PE)-induced vasoconstriction and acetylcholine (ACh)-induced vasodilation of isolated aortae and found that PE-induced vasoconstriction was unaltered, but ACh-induced vasodilation was significantly inhibited both in complete and endothelial cell-specific NOSTRIN knockout in comparison to control mice. Accordingly nitric oxide production from aortae isolated from both NOSTRIN knockout mouse lines was reduced. Finally the blood pressure in complete and endothelial-cell specific knockout mice was significantly increased in comparison to control mice. We conclude that NOSTRIN is an important regulator of eNOS activity and nitric oxide production in the vasculature of adult mice.

Vasohibin-2 expressed in human ovarian serous adenocarcinoma accelerates tumor growth by promoting angiogenesis

Takahiro Koyanagi[1,2]* Yoshifumi Takahashi[1,2], Yasuhiro Suzuki[1], Yasushi Saga[2], Naoki Kanomata[3], Takuya Moriya[3], Yasuhiko Tabata[4], Mitsuaki Suzuki[2], and Yasufumi Sato[1].
[1]Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan, [2]Department of Obstetrics and Gynecology, School of Medicine, Jichi Medical University, Tochigi, Japan, [3]Department of Pathology 2, Kawasaki Medical University, Kurashiki, JAPAN, [4]Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Vasohibin-1 (VASH1) is a VEGF-inducible endothelium-derived angiogenesis inhibitor, and vasohibin-2 (VASH2) is its homologue. Our previous analysis revealed that VASH1 is expressed in endothelial cells to terminate angiogenesis, whereas VASH2 is expressed in infiltrating mononuclear cells mobilized from bone marrow to promote angiogenesis in a mouse model of hypoxia-induced subcutaneous angiogenesis. To test the possible involvement of VASH2 in malignant tumor, we examined human ovarian cancer cells for the presence of VASH2. Immunohistochemical analysis revealed that VASH2 protein was preferentially detected in cancer cells of ovarian serous adenocarcinoma. We then used SKOV-3 and DISS, 2 representative human serous adenocarcinoma cell lines, and examined the role of VASH2 in the tumor. The knockdown of VASH2 by stable transfection of shRNA showed little effect on the proliferation of cancer cells in vitro, but notably inhibited tumor growth, peritoneal dissemination, and tumor angiogenesis in a murine xenograft model. In addition, intratumoral injection of VASH2 siRNA impregnated into cationized gelatin microspheres inhibited subcutaneous SKOV-3 tumor growth in vivo. Next, we stably transfected the human VASH2 gene into 2 types of murine tumor cell lines, EL-4 and MLTC-1, in which endogenous VASH2 was absent. When either EL-4 or MLTC-1 cells were inoculated into VASH2 (-/-) mice, the VASH2 transfectants formed bigger tumors when compared with the controls, and the tumor microvessel density was significantly increased. VASH2 stimulated the migration of endothelial cells and its increased expression in cancer cells related to the decrease of miR-200b. These results indicate that VASH2 expressed in ovarian serous adenocarcinoma cells promoted tumor growth and peritoneal dissemination by promoting angiogenesis.

The protease MT1-MMP drives a combinatorial proteolytic program in activated endothelial cells

Agnieszka Koziol[1]*, Pilar Gonzalo[1], Alba Mota[1], Ángela Pollán[1], Cristina Lorenzo[1], Nuria Colomé[2], David Montaner[3], Joaquin Dopazo[3], Joaquin Arribas[2], Francesc Canals[2], and Alicia G. Arroyo[1]. [1]Vascular Biology Department, Centro Nacional de Investigaciones Cardiovasculares (CNIC), 28029 Madrid, Spain, [2]Proteomics Laboratory and Medical Oncology Research Program, Vall d'Hebron Institute of Oncology, Vall d`Hebron University Hospital Research Institute, 08035 Barcelona, Spain, [3]Bioinformatics Department, Centro de Investigación Príncipe Felipe, 46012 Valencia, Spain

The mechanism by which proteolytic events translate into biological responses is not well understood. To understand the link of pericellular proteolysis to events relevant to capillary sprouting within the inflammatory context, we aimed at the identification of the collection of MT1-MMP substrates in endothelial tip cells induced by inflammatory stimuli. We applied quantitative proteomics to endothelial cells (ECs) derived from wildtype and MT1-MMP null mice to identify the substrate repertoire of this protease in TNFα-activated ECs. Bioinformatics analysis revealed a combinatorial MT1-MMP proteolytic program, in which combined rather than single substrate processing would determine biological decisions by activated ECs including chemotaxis, cell motility and adhesion, and vasculature development. MT1-MMP-deficient ECs inefficiently processed several of these substrates (TSP1, CYR61, NID1 and SEM3C) validating the model. This novel concept of MT1-MMP-driven combinatorial proteolysis in angiogenesis might be extendable to proteolytic actions in other cellular contexts.

The role of the GEF Trio in endothelial cell adaptation to laminar flow

Jeffrey Kroon[1]*, Jos van Rijssel[1], Floris P.J van Alphen[1], Dirk Geerts[2], Peter L. Hordijk[1] and Jaap D. van Buul[1]. [1]Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, [2]Department of Pediatric Oncology/Hematology, Erasmus University Medical Center, Rotterdam,The Netherlands

Endothelial cells that line the lumen of arteries are constantly exposed to pulsatile laminar flow. This results in high shear forces that play an important role in vascular remodeling and are atheroprotective. Turbulent flow on the other hand is associated with low shear stress and correlates with the onset of diseases, such as atherosclerosis. It has been shown by us and others that endothelial cells exposed to high laminar flow induce actin stress fiber formation, cell elongation and alignment in the direction of flow. However, it is not clear how the signals that are induced by the changes in flow are translated into endothelial cell alignment. One possible candidate that may play a critical role in remodeling the actin cytoskeleton upon flow changes is the guanine nucleotide exchange factor (GEF) Trio. Trio activates the small GTPases Rac1, RhoA and RhoG, key regulators of the actin cytoskeleton. Our current focus is to determine if the GEF Trio is able to re-organize the actin cytoskeleton upon induction of laminar flow and to elucidate possible other roles of Trio in endothelial cell functioning under shear stress conditions. Our lab uses IBIDI-microslides in combination with specialized rectangular flow chambers to study the adaptation of endothelial cells to laminar flow conditions. We show that Trio is upregulated by laminar flow and that this increases Trio localization at cell-cell junctions. Using shRNA to reduce Trio protein expression, we find that flow-induced endothelial cell alignment was abrogated in Trio-deficient endothelial cells. Furthermore, whereas induction of laminar flow increases the transendothelial electrical resistance, indicative for more stable cell-cell contacts, this response is lost in Trio-shRNA-transduced endothelial cells. Conversely, subsequent expression of a shRNA-resistant Trio construct rescues the loss in flow-induced resistance. These data show that re-arrangement of the actin cytoskeleton, cell alignment and increased endothelial barrier function induced by laminar flow, is mediated by the Rho-GEF Trio. This work thus identifies Trio as an important novel component in a mechanosensory signalling pathway, which regulates endothelial cell function in response to shear stress.

Myeloperoxidase interactions with thrombocytes

Kolarova H, Adam M, Gajdova S, Klinke A, Pekarova M, Baldus S, Kubala L*. Institute of Biophysics, Academy of Sciences of the Czech Republic Department of Animal Physiology, Faculty of Science, Masaryk University, Czech Republic International Clinical Research Center - Center of Biomolecular and Cell Engineering, St. Anne's University Hospital Brno, Brno, Czech Republic Department of Cardiology, University Heart Center Hamburg, University Hospital Eppendorf, Hamburg, Germany

Alternations in thrombocyte physiological functions contribute to pathogenesis of vascular inflammatory processes. Inflammation is connected with activation of blood polymorphonuclear neutrophils and release of myeloperoxidase (MPO), which has been suggested to play important role in acute and chronic vascular diseases. However, MPO effects on thrombocytes physiological function as a contributing mechanism to vascular inflammatory diseases are unexplored. Binding of MPO to both human and mouse thrombocytes was determined based on combination of immunohistochemistry, western blot, ELISA, determination of enzymatic activity, and flowcytometric, fluorimetric and spectrophotometric analysis. The activation status of thrombocytes was determined based on expression of surface receptors, shape changes, aggregation, release of granules, and interaction with polymorphonuclear leukocytes. The dose-dependent binding of MPO to thrombocytes was observed. The MPO presence on and in thrombocytes was in direct contrast with absence of MPO in megakaryocytes, the precursors of thrombocytes isolated from mouse bone marrow. MPO bound to thrombocytes retained its enzymatic activity, which was connected with the increased formation of reactive oxygen species and the decreased production of nitric oxide by thrombocytes. MPO increased the expression of surface thrombocyte receptors and increased the thrombocyte interaction with polymorphonuclear leukocytes and endothelial cells. MPO binds to thrombocytes and interferes with their physiological functions, which can further modulate physiological functions of blood vessels.

ADAMTS5 functions as an angiogenesis inhibitor and suppresses melanoma growth in mice

Saran Kumar*, Soheila Sharghi-Namini, Nithya Rao and Ruowen Ge. Department of Biological Sciences, National University of Singapore, Science Drive 4, Singapore, 117543

ADAMTS5 is a member of A Disintegrin and Metalloproteinase with ThromboSpondin Motifs (ADAMTS) family of matrix metalloproteinases with multiple proteoglycan substrates. Although well characterized as the major aggrecanase involved in cartilage degradation in osteoarthritis, how it influences cancer remains unclear. We have previously shown that the first thrombospondin type 1 repeat (TSR1, the central TSR) but not TSR2 (the C-terminal TSR) of ADAMTS5 is anti-angiogenic in vitro. Coupled with prior reports that ADAMTS5 expression is altered in several human cancers, we hypothesized that this proteoglycanase may play an important role in cancer and angiogenesis. Our work reports a previously un-realized function of ADAMTS5 as an anti-angiogenic/anti-tumorigenic protein independent of its function as a proteoglycanase. In this work, we demonstrate that full-length ADAMTS5 and its autocatalytic fragment inhibit angiogenesis in vitro. Further, stable overexpression of the full-length ADAMTS5 or its naturally existing autocatalytic fragments suppressed melanoma growth in mice. The reduced tumor growth is primarily a result of diminished tumor angiogenesis coupled with reduced tumor cell proliferation and increased tumor cell apoptosis. The catalytic activity of ADAMTS5 is dispensable for its anti-tumorigenic function since the full-length active site mutant E411A presented similar tumor suppression activity. Domain mapping and mechanistic studies revealed that ADAMTS5 inhibits tumor growth through its TSR1 by suppressing tumor angiogenesis, likely by reducing the production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), platelet-derived endothelial growth factor (PD-ECGF), insulin-like growth factor binding protein 3 (IGFBP-3) and plasminogen activator inhibitor-1(PAI-1) in the tumor milieu. Taken together, this study suggests that ADAMTS5 is a novel anti-angiogenic and a tumor suppressive metalloproteinase.

The recombinant lectin-like domain of thrombomodulin inhibits EGF-induced angiogenesis through interaction with Lewis Y antigen

Cheng-Hsiang Kuo[1-3]*, Po-Ku Chen[1-3], Bi-Ing Chang[1,3], Meng-Chen Sung[1], Chung-Sheng Shi[1], Jeng-Shin Lee[6], Chuan-Fa Chang[3,4], Guey-Yueh Shi[1,3], and Hua-Lin Wu[1,3,5].
[1]Department of Biochemistry and Molecular Biology, [2]Institute of Basic Medical Sciences, [3]Cardiovascular Research Center, and, [4]Department of Medical Laboratory Science and Biotechnology, College of Medicine, [5]Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan, [6]Harvard Gene Therapy Initiative, Harvard Medical School, Boston, MA, USA

Lewis Y antigen (LeY) is a cell surface tetrasaccharide that participates in angiogenesis and tumor-associated epidermal growth factor (EGF) receptor signaling. Recently, we demonstrated that LeY is a specific ligand of recombinant lectin-like domain of thrombomodulin (TM). However, the biological function of interaction between LeY and TM in endothelial cells has never been investigated. In this study, the roles of recombinant lectin-like domain of TM -TM domain 1 (rTMD1) - in antiangiogenesis were investigated. Surface plasmon resonance assay showed that rTMD1 interacted with soluble LeY. EGF receptor in human umbilical vein endothelial cells was LeY-modified. rTMD1 inhibited EGF receptor signaling, chemotaxis, and tube formation in vitro and EGF-mediated angiogenesis in vivo. We concluded that rTMD1 inhibits angiogenesis via interaction with LeY. Administration of rTMD1 or recombinant adeno-associated virus vector carrying TMD1 could be a promising antiangiogenesis strategy.

Novel co-regulators of nuclear receptor Nur77 in vascular disease

Kondababu Kurakula*, Claudia M van Tiel, Erik van der Wal, Carlie JM de Vries. Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Rationale Nur77 is a nuclear receptor that has been shown to promote endothelial cell survival, to modulate the inflammatory response of macrophages and to inhibit the formation of smooth muscle cell rich lesions, indicating a functional involvement of Nur77 in cardiovascular disease. Nur77 is referred to as an orphan receptor, because as yet no natural ligands that regulate its transcriptional activity have been identified. To gain insight into how Nur77 activity is regulated we performed a yeast two-hybrid screen to identify novel co-regulators. Methods & Results In this study, we identified Peptidyl-prolyl isomerase Pin1 and Four and a half LIM domains protein-2 (FHL2) as novel interacting proteins of Nur77 nuclear receptor by yeast two-hybrid screen and co-immunoprecipitation studies. We found that Pin1 enhances whereas FHL2 represses Nur77 transcriptional activity in a dose-dependent manner. Pin1 enhances the stability of Nur77 protein in an isomerase-dependent manner and FHL2 has no influence on Nur77 protein stability. ChIP experiments on the promoter of Enolase3, a Nur77 target gene revealed that FHL2 inhibits the association of Nur77 with DNA. FHL2 is highly expressed in human endothelial and smooth muscle cells, but not in monocytes or macrophages whereas Pin1 is expressed in all vascular cell types. To substantiate functional involvement of Pin1 and FHL2 in smooth muscle cell physiology, we demonstrated that Pin1 enhances and FHL2 decreases the anti-proliferative effect of Nur77. Conclusion Collectively, these studies demonstrate that Pin1 and FHL2 are co-regulators of Nur77 and play a pivotal role in vascular disease. Clinical Relevance Nur77 is protective in atherosclerosis and insight in regulation of its activity provides novel opportunities for intervention in vascular disease.

Characterization of blood vessels in the bone during development

Urs Langen*, Mara Pitulescu and Ralf H. Adams. Max-Planck-Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, and University of Muenster, Faculty of Medicine, Muenster, Germany

The skeletal system has an essential structural function and also harbors the hematopoietic system in mammalian organisms. Previous work established that the blood vessels in the bone, which are often referred to as the sinusoidal vasculature because of their peculiar morphology, are highly specialized and tightly linked to osteogenesis in development and regeneration. Likewise, important functional roles as components of the hematopoietic stem cell niche have been attributed to endothelial and perivascular mesenchymal cells. Despite these important functions, very little is known about the organization, angiogenic growth and molecular regulation of the bone sinusoidal vasculature in embryonic and postnatal life. The analysis of cellular and molecular interactions inside the skeletal system is complicated by the physical properties of the dense and hard bone tissue. Technical improvements addressing the processing, immunostaining and imaging of bone in combination with powerful cell type-specific and inducible mouse genetics now allow us to investigate the growth and regulation of sinusoidal vessels at high detail. Here, we report the characterization of sinusoidal vessel growth and its relationship to ossification from the first invasion of endothelial sprouts into embryonic mesenchymal condensations to the processes in adolescent animals.

Transcriptional profiling identifies CD93 as a novel tumor vessel marker in human glioblastoma

Elise Langenkamp*, Lei Zhang, Sofie Mellberg, Lothar Dieterich, Tommie Olofsson, Irina Alafuzoff, Fredrik Pontén, Anna Dimberg. Dept. of Immunology, Genetics and Pathology, Uppsala University, Dag Hammarskjöldsväg 20, 75185 Uppsala, Sweden

Glioblastoma, the most aggressive type of glioma (WHO Grade IV), is characterized by highly abnormal vessels. The aberrant tumor vasculature is malfunctioning and hyperpermeable and displays features of microvascular proliferations that are part of the diagnostic criteria for Grade IV glioma. In order to characterize these abnormal vessels, we have analyzed the molecular make-up of Grade IV glioma vasculature in comparison to Grade II and normal brain. To this end, glioma vessels were separated from the surrounding tumor tissue by laser-capture microdissection, and gene expression was analyzed using microarray technology. This revealed CD93 as one of the genes that was drastically upregulated in Grade IV vessels as compared to vessels in Grade II glioma and normal brain. CD93 is a transmembrane glycoprotein that has been reported to play a role in infiltration of inflammatory cells, yet its role in glioblastoma vasculature remains unknown. Analysis of a human brain tumor tissue microarray confirmed that the vast majority of Grade IV glioma vessels expressed CD93 protein at a high intensity, while in Grade II and normal brain tissue only the minority of vessels expressed CD93. Similar to human gliomas, the vasculature of the mouse GL261 glioma model displayed markedly enhanced CD93 expression compared to the surrounding normal brain tissue. CD93 was also detected on the majority of blood vessels in childhood brain tumors. CD93 expression was significantly upregulated by human dermal microvascular endothelial cells during VEGF-induced tube formation on a 3D collagen gel. Interestingly, siRNA-mediated knockdown of CD93 is associated with decreased formation of vessel-like structures in response to VEGF, suggesting an important role during tubular morphogenesis. We are currently investigating the functional role of CD93 in vascular development and inflammatory cell recruitment in glioblastoma.

Conceptualizing vascular remodelling via endothelial cell shape analysis in vivo

Eleonora Lapi*, Martin Jones and Holger Gerhardt. Vascular Biology Laboratory Cancer Research UK 44 Lincoln's Inn Fields London WC2A 3PX, United Kingdom

Angiogenesis is the physiological process of growing new blood vessels from pre-existing ones. It is fundamental in development, where it expands and remodels the primitive plexus formed via vasculogenesis into a functional network. Its dysfunction contributes to the pathogenesis of many diseases. Genetic loss of function models in mouse and zebrafish are powerful tools to study angiogenesis, identifying a growing number of genes involved in blood vessel formation. However, the detailed cell biology underlying many phenotypes often remains poorly understood as we lack methods and concepts for the analysis of endothelial cell shapes and their dynamic regulation in remodelling vascular networks. Here we present the first systematic approach towards endothelial cell shape analysis in vivo, unravelling the cell shape changes underlying vascular pattern formation. Using Cre recombinase-mediated activation of membrane-targeted GFP expression in sparse mouse retina endothelial cells we visualized single endothelial cells in confocal laser scanning microscopy. Analysing more than 1000 single cells, we used two converging approaches for shape classification in order to establish a complete catalogue of endothelial cell shapes in the developing plexus The first (visual grouping) was biology-led and biased by our preconceived knowledge of regions and stages of development, such as tip/stalk, artery, vein, primitive plexus, remodelling plexus etc. and led to the classification of cells in reference to the position and developmental phase of plexus development. The second was an unbiased computational approach in which image analysis following segmentation and shape-parameter extraction was able to define shape clusters irrespective of cell localization, developmental stage or other biological parameters. By analysing retinal samples of selected developmental stages via the visual grouping approach, we clustered cells in different shape classes. The cells belonging to the different classes were then analysed for their position and mapped back on a schematic representation to understand the percentage contribution and the spatial distribution of the various shapes on a remodelling vascular plexus. We identified cell shape classes that are typical of the plexus in remodelling (amoeba, crescent, star) and we observed that with time, during development, there is a change in cell shape and a shift towards spindle shaped cells, revealing a previously unappreciated degree of cell reshaping and cell-cell rearrangements during remodelling. With the computational analysis, by looking at the parameters of a single cell, we can see where in the „parameter space“ landscape it lies and identify likely transitions between cell shapes by the proximity of the different clusters. This can then be cross-correlated with the visual grouping and mapped back onto the spatial distribution, to deliver insight into the biological meaning of the cell shape and predict possible transitions between shapes.

Nrf-2-dependent and -independent mechanisms contribute to the cytoprotective actions of n-3 PUFAs carried in chylomicron remnants on aortic endothelial cells

SH Latham*, R Purcell, C Lawson, K Botham and CPD Wheeler-Jones. Department of Basic Sciences, Royal Veterinary College, London, United Kingdom

Chylomicron remnants (CR) are triacylglycerol-rich lipoproteins (TGRLs) present in the blood 3-9 hours after consumption of dietary fats. Whilst CRs are rapidly cleared by the liver in healthy individuals, CR circulation time is prolonged in individuals with diabetes, renal failure and familial hyperlipemia and there is emerging evidence linking CRs to endothelial dysfunction and vascular inflammation. Despite the fact that the majority of individuals consuming a typical Western diet are chronically post prandial, there is currently little understanding of the molecular basis of CR actions on vascular cell function. CR-like particles (CRLPs) are an established model for CRs and allow direct comparison of the cellular actions of lipoproteins with distinct compositions. We have previously shown that CRLPs modify human umbilical vein endothelial cell (HUVEC) signalling and gene expression in a manner that depends upon their fatty acid composition and oxidative state. Here, we have used CRLPs prepared using triglycerides (TGs) extracted from four natural dietary fats (fish, DHASCO (algal), corn and palm oils) and directly compared their effects on human aortic endothelial cells (HAECs) at equivalent TG concentrations. CRLPs containing TG high in n-3 polyunsaturated fatty acids (PUFA) derived from fish and DHASCO oils both strongly increased expression of the cyto-protective enzyme heme-oxygenase-1 (HO-1) in HAECs with comparable efficacy (4-6 hours). Exposure to CRLPs containing n-6- or saturated FA-rich TGs extracted from corn and palm oils, respectively, showed markedly less ability than n-3 PUFA-rich CRLPs to enhance HO-1 expression in both HAECs and HUVEC. Use of Nrf-2-specific siRNA in HAECs and HUVEC effectively depleted Nrf-2 and completely abolished the stimulatory effects of CRLPs on endothelial HO-1 expression. All four types of CRLPs triggered acute phosphorylation (5-30 min) of both ERK1/2 and Akt in HAECs whereas only n-3 PUFA-containing CRLPs enhanced phosphorylation of AMPK at 30 mins. Further studies, however, showed that pharmacological blockade of AMPK, MEK or p38MAPK did not significantly affect the ability of CRLPs to promote HO-1 induction. CRLPs prepared from all four oils showed varied ability to increase expression of superoxide dismutase 2 (MnSOD) and preliminary data suggest that the enhanced MnSOD expression in CRLP-stimulated HAECs is unaffected by Nrf-2 depletion but reduced by inhibition of AMPK activity. Together, our findings support the hypothesis that the exogenous remnant lipoprotein component of TGRLs in the post prandial phase directly regulates EC behaviour and provide evidence that CRs elicit cyto-protective actions on endothelial cells through Nrf-2-dependent and -independent mechanisms.

Slow release H2S donors protect human microvascular endothelial cells from oxidative stress-induced cell toxicity

S. Le Trionnaire*, J. Whatmore, M. E. Wood., A. Perry, M. Whiteman. Peninsula Medical School, University of Exeter, Exeter, United Kingdom

Significant microvascular endothelial dysfunction (MED) is observed in diabetic and hypertensive patients, although the underlying mechanisms are unclear. An increase in vascular oxidative stress and mitochondrial dysfunction have been suggested as possible causes for MED. Recently, we showed that lower levels of the gaseous mediator hydrogen sulfide (H2S) were observed in diabetic subjects compared to age and BMI-matched controls, and that H2S levels were strongly negatively correlated with impaired MED in vivo [1]. These observations suggest that reduced vascular synthesis of H2S contributes to MED in vascular diseases. We hypothesize that H2S is normally protective against oxidative stress induced endothelial cells damage. The aim of this study is to examine whether H2S can reduce oxidative-stress induced toxicity in human microvascular endothelial cells. Human microvascular endothelial cells (HMEC) were exposed to slow release H2S donors (SRHD) GYY4137 (100 µM), AP67 (500 µM), AP72 (500 µM) and the mitochondria-targeting SRHD AP39 and AP123 (0.1 µM), before or after the treatment with oxidative stress (OS) agents (SIN-1, H2O2 and 4-HNE). Cell viability was determined by alamarBlue®, intracellular levels of ROS were assessed using DHE and H2-DCFDA assay, and MitoSOX™ Red was used to measure the generation of superoxide in the mitochondria. The mitochondrial membrane potential (âˆ†Ψm) was established using the potentiometric dye TMRM. Also, to determine a mechanism of action for the observed cell protection of our drugs, we investigated whether individual SRHD induced effects on specific intracellular signalling pathways involved in cell survival and apoptosis. To this end, expression of Erk1/2, Akt, caspase 3 and caspase 9 was analyzed by Western Immunoblotting. Finally, we examined the effects of SRHD on apoptosis by measuring the caspase 3/7 activity: HMEC were exposed to an apoptosis-inducing agent staurosporine (SS-1µM), prior to treatment with SRHD, and caspase 3/7 activity was measured by a commercial kit. Incubation of HMEC with OS agents (10-1500 µM) significantly increased oxidative-stress induced cell toxicity and ROS production, and induced the loss of $\Delta\Psi m$ (each $p<0.01$ c.f. oxidant treatment alone). In sharp contrast, treatment of HMEC with SRHD significantly reduced oxidative stress-induced cell death and loss of $\Delta\Psi m$ ($p<0.02$), and reduced the cytoplasmic and mitochondrial generation of ROS ($p<0.04$-$p<0.0001$). The modulation of Akt, Erk1/2, caspase 3 and caspase 9 by SRHD paralleled protective effects of SRHD in that concentrations ranging from 0.1-500 µM. Finally, the water soluble SRHD AP67 and AP72 were able to significantly reverse the activation of caspase 3/7 ($p<0.007$-$p<0.0001$), whereas the mitochondria-targeting compounds AP39 and AP123 had no significant effect. Together these data suggest that SHRD can inhibit/reverse oxidative stress-mediated cell injury. The observed cell protection may be partially the result of SRHD interaction with survival and apoptotic signaling pathways. Strategies which increase vascular H2S bioavailability may represent a novel therapeutic strategy to limit micro/macrovascular endothelial dysfunction and maintain vascular health. [1]Whiteman et al., Diabetologia 2010;53:1722-6.

Angiopoietin-1 Induces vascular regeneration in ischemic retinopathies

Junyeop Lee[1,2]*, Kyung Eun Kim[1,2], Honsoul Kim[1,2], Toshio Suda[3], Naoki Mochizuki[4], Yoshikazu Nakaoka[5], Issei Komuro[6], Ook-Joon Yoo[2], Gou Young Koh[1,2]. [1]National Research Laboratory of Vascular Biology and Stem Cells, [2]Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Korea, [3]Department of Cell Differentiation, The Sakaguchi Laboratory, School of Medicine, Keio University, Japan, [4]Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Japan, [5]Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Japan

Ischemic retinopathies including retinopathy of prematurity and proliferative diabetic retinopathy involve vascular pathologies in the retina, such as avascular region formation, subsequent aberrant angiogenesis, and vascular leakage that eventually lead to blindness. Although intraocular delivery of VEGF-A blocking agents is a principal therapy for preventing and/or delaying the progression of these retinopathies, it cannot achieve fundamental or permanent recovery of the already disrupted vasculature of the ischemic retina. Here we demonstrated that intravitreal supplementation of angiopoietin-1 (Ang1) induced ordered retinal angiogenesis into central avascular retina, leading to reduced avascular regions, hypoxia and neovascular tuft formation by improving blood perfusion in the mouse oxygen-induced retinopathy (OIR) model. These newly-formed retinal vessels were leakage-resistant vasculatures that are covered well by pericytes. Thus, Ang1 improved the structural integrity of the regenerated blood vessels. Owing to these effect, Ang1 prevented vascular leakage and hypoxia-induced apoptosis of retinal neurons. Ang1-overexpressing mice showed that Ang1 promoted proliferation and sprouting of retinal endothelial cells and increased pericyte coverage. At the same time, these mice displayed increased density of astrocyte and fibronectin scaffolds which guide retinal angiogenesis during development. On the other hand, Ang1-depleted mice showed decreased proliferation and sprouting of retinal endothelial cells, as well as decreased density of astrocyte and fibronectin scaffolds. These findings led us to demonstrate that Ang1 reinforced fibronectin scaffolds in the sprouting endothelial cells and adjacent astrocytes, finally provided a neovascular track that guides directional angiogenesis into avascular retina in OIR model. Notably, this process was mediated through the integrin αvβ5 signaling pathway. Our results suggest Ang1 supplementation as a potential treatment strategy for the fundamental treatment of ischemic and permeable vascular retinopathies.

Perivascular adipose tissue-derived methyl palmitate in vascular tone regulation

Yuan-Chieh Lee, Hsi-Hsien Chang, Chih-Lung Chiang, Chin-Hung Liu, Mei-Fang Chen, Po-Yi Chen, Tony J.F. Lee*. Department of Life Sciences and Center for Vascular Medicine, College of Life Sciences, and Institute of Medical Sciences, College of Medicine, Tzu Chi University, Departments of Ophthalmology and Research, Buddhist Tzu Chi General Hospital, Hualien, Taiwan, and Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois, USA

The systemic blood vessels are surrounded by various amounts of perivascular adipose tissue (PVAT). Recent evidence indicates that PVAT-derived relaxing factor (PVATRF) significantly regulates vascular tone by opening the potassium channels on the smooth muscle cell. Furthermore, changes in PVAT mass and function have been suggested to contribute to increased vascular resistance in different models of experimental hypertension. The chemical identity of the PVATRF has been the focus of recent active research. We have demonstrated for the first time release of an endogenous potent vasodilator methyl palmitate or palmitic acid methyl ester (PAME) from the superior cervical ganglion the rat (1). We therefore examined if PAME is the PVATRF and if its release from the PVAT and/or its vasorelaxing activity diminished in established hypertension, using superfusion bioassay cascade technique, tissue bath myography, in vivo experimentation, and gas chromatography/mass spectrometry. The results indicated that the PVAT of WKY spontaneously and calcium dependently released PVATRF and PAME. Both induced aortic vasorelaxations, which were inhibited by 4-aminopyridine (2 mmol/L) and tetraethylammonium 5 and 10 mmol/L but were not affected by glibenclamide (3 μmol/L) or iberiotoxin (100 nmol/L). Aortic vasorelaxations induced by PVATRF- and PAME-containing Krebs solutions were not affected after heating at 70°C but were equally attenuated after hexane extractions. Culture mediums of differentiated adipocytes, but not those of fibroblasts, contained significant PAME and caused aortic vasorelaxation. On the other hand, the PVAT of the SHR released significantly less PVATRF and PAME with an increased release of angiotensin II (AII). In addition, PAME-induced relaxation of the SHR aortic smooth muscle diminished drastically, which was ameliorated significantly by losartan (2). Furthermore, incubation of isolated PVAT preparations of the SHR aorta in Krebs solution containing losartan (10 μM) for 30 min significantly enhanced PVAT-elicited vasorelaxation, with enhanced PAME release, suggesting inhibition of PAME release by AII acting on AT1 receptors. Also, chronic captopril treatment for 30 days significantly lowered the mean arterial blood pressure in adult SHR with enhanced PVATRF-induced vasorelaxation. These results indicate that PAME and PVATRF exhibit similar characteristics in biochemistry and pharmacology, suggesting that PAME is the PVATRF, causing vasorelaxation by opening voltage-dependent Kv channels on smooth muscle cells. The diminished PAME release and its vasorelaxing activity and increased release of AII in the PVAT of SHR aorta suggest a noble role of PVAT and PAME in pathogenesis of hypertension. The antihypertensive effect of losartan is partly attributed to its increasing PAME release and reversing AII-initiated diminishment of PAME-induced vasorelaxation (Supported by NSC, Tzu Chi Found & TCU). 1. HW Lin, CZ Liu, DS Cao, PY Chen, MF Chen, SZ Lin, M Mozayan, AF Chen, LS Premkumar, DS Torry, and TJF Lee (2008) Proc Natl Acad Sci, USA. 105:19526-31. 2. YC Lee, HH Chang, CL Chiang, CH Liu, JI Yeh, MF Chen, PY Chen, JS Kuo, TJF Lee (2011) Circulation 124(10):1160-71.

Sympathetic activation causes nitrergic vasodilation and increased blood flow in the brain stem

Hsi-Hsien Chang, Yuan-Chieh Lee, Mei-Fang Chen, Jon-Son Kuo, and Tony J. F. Lee*. Department of Life Sciences and Center for Vascular Medicine, College of Life Sciences, and Institute of Medical Sciences, College of Medicine, Tzu Chi University, Departments of Ophthalmology and Research, Buddhist Tzu Chi General Hospital, Hualien, Taiwan; and Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois, USA

The unique feature of close apposition between sympathetic and parasympathetic nerve terminals in the adventitia of large cerebral arteries of several species provides morphological evidence for axo-axonal interaction mechanism in regulating diameter and blood flow of these arteries. Via this interaction mechanism, sympathetic nerve activation causes parasympathetic nitrergic vasodilation of isolated basilar arteries (1). Specifically, norepinephrine released from depolarized sympathetic nerves binds the beta2-adrenoceptor located on the neighboring parasympathetic nitrergic nerves, causing release of nitric oxide (NO) and therefore relaxation of the smooth muscle cells. Results from in vivo experimentation indicate that electrical stimulation (ES) of sympathetic nerves originating in the superior cervical ganglion (SCG) and topical nicotine (10-30 μM) onto basilar arteries in anesthetized Wistar-Kyoto rats (WKY) significantly increase diameter of the basilar arteries and basilar arterial blood flow (BABF) measured by laser-Doppler flowmetry. Both increases due to electrical and chemical stimulations are inhibited by 7-nitroindazole (a neuronal nitric oxide synthase inhibitor) and ICI 118,551 (a beta2-adrenoceptor antagonist), but not by atenolol (a beta1-adrenoceptor antagonist). Topical norepinephrine onto basilar arteries also increases BABF, which is abolished by atenolol combined with 7-nitroindazole or ICI 118,551. These results further support the presence of functional axo-axonal interaction mechanism in vivo. Similar results are found in anesthetized normotensive, pre-hypertensive spontaneously hypertensive rat (SHR, 8-week old). However, in anesthetized adult SHR and renovascular hypertensive rats (RHR) with established hypertension, ES of sympathetic nerves or topical nicotine causes minimum or no increase of basilar arterial diameter and BABF. This is consistent with the reported morphological decrease of parasympathetic nerve terminals in basilar arteries of adult SHR and RHR comparing to age-matched WKY, thus interrupting the axo-axonal interaction mechanism. Pharmacological examination also rules out any possibilities of diminished presynaptic beta2-adrenoceptor activities and NO-cGMP coupling in neurovascular transmission in hypertensive rats. It is concluded that, unlike that found in most peripheral vascular beds, excitation of sympathetic nerves to basilar arteries in normotensive WKY causes parasympathetic nitrergic vasodilation with increased BABF. This axo-axonal interaction mechanism appears to operate in regulating cerebral blood flow in the human based on circumstantial evidence. This finding indicates, for the first time, an endowed functional neurogenic mechanism for increasing the BABF or brain stem blood flow in coping with increased local sympathetic activities in acutely stressful situations such as the „fight-or-flight response“. This defensive mechanism of increasing blood flow, however, diminishes in genetic and non-genetic hypertension due most likely to defected cerebral perivascular parasympathetic nitrergic nerves in this neurovascular disease (supported by NSC/TW). 1. Si ML and Lee TJF (2002) Circ Res 91(1):62-69

Carbon monoxide-exposed astrocytes induces angiogenesis by enhancing HIF-1alpha-mediated VEGF expression

Kyu-Sun Lee[1]*, Yoon Kyung Choi[1], Hansoo Lee[2], Dooil Jeoung[2], Kwon-Soo Ha[1], Young-Guen Kwon[3], Kyu-Won Kim[4], and Young-Myeong Kim[1]. [1]Vascular System Research Center and Department of Molecular and Cellular Biochemistry, School of Medicine, Kangwon National University, Chuncheon, Kangwon-do 200-701, Republic of Korea, [2]Division of Biological Sciences, College of Natural Sciences, Kangwon National University, Chuncheon, Kangwon-do 200-701, Republic of Korea, [3]Department of Biochemistry, College of Sciences, Yonsei University, Seoul 120-749, Republic of Korea, [4]NeuroVascular Coordination Research Center, College of Pharmacy and Research, Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Republic of Korea

We examined the molecular mechanism by which carbon monoxide (CO) regulates HIF-1-dependent expression of VEGF. We found that astrocytes stimulated with the CO donor (CORM-2) promoted angiogenesis by increasing HIF-1alpha-dependent VEGF expression. VEGF expression was inhibited by treatment with HIF-1alpha siRNA and a HO inhibitor, indicating that CO stimulates VEGF expression via upregulation of HIF-1alpha protein level. CORM-2 activated the translational regulatory proteins p70S6k and eIF-4E by activating Akt and ERK. These translational signal events and HIF-1alpha protein level were suppressed by inhibitors of PI3K, MEK, and mTOR, suggesting that the PI3K/Akt/mTOR and MEK/ERK pathways are involved in a translational increase in HIF-1alpha. In addition, CORM-2 increased stability of the HIF-1alpha protein by suppressing its ubiquitination, without altering HIF-1alpha degradation. CORM-2 increased HIF-1alpha/HSP90alpha interaction responsible for HIF-1alpha stabilization, and HSP90 inhibitor decreased this interaction, HIF-1alpha protein level, and VEGF expression. These results suggest that CO stimulates VEGF production by increasing HIF-1alpha protein level via its translational stimulation and protein stabilization.

Dissecting the mechanisms of integrin LFA-1 activation in neutrophils

Craig T. Lefort*,[1] Jan Rossaint,[2] Markus Moser,[3] Brian G. Petrich,[4] Alexander Zarbock,[2] Susan J. Monkley,[5] David R. Critchley,[5] Mark H. Ginsberg,[4] Reinhard Fässler,[3] and Klaus Ley.[1] [1]Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, [2]Department of Anesthesiology and Critical Care Medicine, University of Muenster, Muenster, Germany, [3]Department of Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried, Germany, [4]Department of Medicine, University of California San Diego, La Jolla, CA, USA [5]Department of Biochemistry, University of Leicester, Leicester, United Kingdom

β2 integrins, including LFA-1 (αLβ2), play critical roles in leukocyte recruitment during inflammation by binding to ICAM-1 expressed on the endothelium. The affinity of integrin for ligand is determined by its global conformation, regulated by the binding of proteins to the integrin cytoplasmic tails in a process called „inside-out“ activation. At least three different LFA-1 conformations with distinct affinities have been observed: low affinity with bent ectodomain, intermediate affinity with extended ectodomain, and high affinity with extended ectodomain and open headpiece. Intermediate affinity LFA-1 contributes to neutrophil rolling interactions with the endothelium, whereas high affinity LFA-1 mediates neutrophil arrest. In the current study, we have determined the roles for two integrin activating proteins, talin-1 and kindlin-3, in neutrophil rolling and arrest. Using established in vivo (intravital microscopy of post-capillary venules) and ex vivo (microfluidic flow chamber) models, we found that talin-1 is necessary for both LFA-1-dependent neutrophil rolling and arrest. In contrast, kindlin-3 was required only for neutrophil arrest and was dispensable for rolling mediated by intermediate affinity LFA-1. We further corroborate our results with imaging and in vitro studies using antibodies that directly report β2 integrin conformation. Our data indicate that talin-1 is sufficient to induce LFA-1 ectodomain extension, whereas kindlin-3 is critical for the transition of LFA-1 to a high affinity state. These studies represent the first demonstration that talins and kindlins serve distinct functions in integrin activation.

Hic-5 deficiency blocks the development of angiotensin II-induced abdominal aortic aneurysms in mice

Xiao-Feng Lei*, Joo-ri Kim-Kaneyama, Shigeko Arita, Takuro Miyazaki, Akira Miyazaki.
Department of Biochemistry, Showa University School of Medicine, Tokyo, Japan

Oxidative stress, generated by excessive reactive oxygen species (ROS), plays a key role in regulation of matrix metalloproteinases (MMPs) and contributes to the pathogenesis of abdominal aortic aneurysms (AAA). However, the precise mechanism of the cellular signaling by ROS in the pathogenesis of AAA has not been fully elucidated. Hydrogen peroxide-induced clone 5 (Hic-5), which is a focal adhesion protein expressed in smooth muscle cells (SMCs) of various tissues, is induced by hydrogen peroxide as well as by transforming growth factor-β. Using the angiotensin II (AngII)-induced AAA model in Apoe-/- mice, we show that Apoe-/- Hic-5-/- mice are completely protected from AngII-induced AAA formation and rupture in contrast to Apoe-/- Hic-5+/+ mice. Mechanistically, no difference was found in inflammatory cytokine expression and ROS generation between Apoe-/- Hic-5-/- mice and Apoe-/- Hic-5+/+ mice after AngII infusion. However, AngII-infused Apoe-/- Hic-5-/- mice lacked MT1-MMP induction and MMP2 activation in contrast to Apoe-/- Hic-5+/+ mice. In cultured vascular SMCs from Hic-5-/- mice, MT1-MMP induction and MMP2 activation by AngII were repressed compared with those from Hic-5+/+ mice. These in vivo and in vitro data indicate a crucial role of Hic-5 in activation of MMPs proposing a previously undescribed role for Hic-5 in AAA formation.

The role of endothelial JAM-C in inflammattory and vascular events associated with a murine model of ovarian cancer

David A Leinster[1]*, Bartomeu Colom[1], James R Whiteford[1], Triantafyllos Chavakis[3], Michel Aurrand-Lions[4], Beat A Imhof[5], Frances R Balkwill[2] & Sussan Nourshargh[1].
[1]William Harvey Research Institute and, [2]Barts Cancer Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, [3]Dresden University of Technology, 01307 Dresden, Germany, [4]Inserm, Centre de Recherche en Cancérologie de Marseille, Unité Mixle de Recherche (UMR) 1068, Marseille, France and 5Centre Médical Universitaire, CH-1211 Geneva, Switzerland

JAM-C is an adhesion molecule that plays a significant role in regulating endothelial cell (EC) functions, including leukocyte-EC interactions and angiogenesis and as such has been implicated to the pathogenesis of numerous inflammatory and vascular disorders. In the present study we have investigated the role of JAM-C in the development of a murine model of ovarian cancer. The model employed is one of syngeneic ovarian tumours that develop from the Mouse Ovarian Surface Epithelial Cell (MOSEC) line. The MOSEC line, developed by repeat passaging of ovarian surface epithelial cells, was generated as an alternative to implanting human tumours into immunocompromised animals1. Intraperitoneal (i.p) injection of the MOSECs forms tumours with a similar peritoneal spread to advanced human ovarian cancer. Within a 10-15 week time frame the tumour colonizes the peritoneal cavity with obvious deposits visible on the spleen, liver and ovary and stimulates the generation of ascitic fluid which is used as a surrogate marker for when the disease reaches an in vivo survival end point. As shown previously the MOSEC tumour deposits colonize the peritoneum recruiting tumour promoting T cells and macrophages followed by development of functional blood vessels2. The role of EC JAM-C on the progression of this model was investigated through the use of two mouse strains, a conditional EC specific JAM-C KO mouse (EC JAM-C KO) and transgenic mice that over-express JAM-C in their ECs. Wild type (WT) mice injected with MOSECs survived with a median of 88 days whilst EC JAM-C KO animals and transgenic mice showed an enhanced (median of 96 days, $p=0.04$) and reduced (median of 78.5 days, $p=0.03$) survival, respectively. To understand how JAM-C regulates tumour development, we looked at both the immune cell infiltrate and tumour vascularity. With respect to the EC JAM-C KO mice, no significant differences in their leukocyte infiltrate profile as compared to tumours of WT mice was noted. Furthermore, EC JAM-C KO mice showed no overall reduction in tumour vessel density but the number of α-SMA positive cells surrounding the tumour vessels was reduced suggesting a role for EC JAM-C in the development/maturation of functional tumour vessels. Analysis of vessel sprouts using the aortic ring angiogenesis assay also showed a similar trend towards reduced α-SMA positive cells surrounding newly formed vessels stemming from EC JAM-C KO aortic rings as compared with WT controls. The findings demonstrate a role for JAM-C in the development of tumours in a murine model of ovarian cancer and suggest that EC JAM-C supports tumour growth through recruitment of smooth muscle cells/pericytes to newly formed blood vessels. 1. Roby, K.F. et al. Development of a syngeneic mouse model for events related to ovarian cancer. Carcinogenesis 21, 585-591 (2000). 2. Leinster, D.A. et al. The peritoneal tumour microenvironment of high-grade serous ovarian cancer. J Pathol, doi: 10.1002/path.4002 (2012). This work was supported by funds from The Wellcome Trust

Maternal supraphysiological hypercholesterolemia leads to reduced endothelium-dependent vasodilation of human umbilical vein, reduced nitric oxide production and increased activity of arginase II in human umbilical vein endothelium

Leiva A*, Guzmán-Gutiérrez E, Abarzúa F, Westermeier F, Diez de Medina C, Pardo F, Sobrevia L. Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynecology, Medical Research Centre (CIM), School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago, Chile

Maternal supraphysiological hypercholesterolemia (MSPH) leads to aortic atherosclerosis in the fetus and children. Since nitric oxide (NO) synthesis is reduced in atherosclerosis, we hypothesize that MSPH will alters L-arginine transport (NO synthases (NOS) substrate) and NO synthesis in human umbilical vein endothelial cells (HUVEC) leading to altered vascular reactivity. Methods: Umbilical vein rings from women with maternal physiological hypercholesterolemia (MPH) or MSPH (cut-point >280 mg/dl for maternal blood cholesterol at term (n=71)) in pregnancy were mounted in a myograph and endothelium-dependent (calcitonin gene-related peptide (CGRP), 10-10-10-7 M) or independent (sodium nitroprusside (SNP), 10-5 M) vasodilatation was measured. L-Arginine transport (30-500 μM, 3 μCi/ml, 37ºC, 1 minute), L-[3H]citrulline formation (9 μCi/ml L-[3H]arginine, 60 minutes, 37ºC) in absence or presence of NG-nitro-L-arginine methyl ester (L-NAME, 100 μM), and urea formation from L-arginine (50 μM, 60 minutes, 37°C) was measured in HUVEC primary cultures (passage 3). eNOS, human cationic amino acid transporter 1 (hCAT-1) and arginase II protein abundance was evaluated by western blot. Results: MSPH associates with reduced CGRP-relaxation (IC50 ~3.2 nM) compared with MPH (IC50 ~0.15 nM) (Student's unpaired t test, $P<0.05$); however, SNP-vasodilatation was unaltered in MSPH. L-Arginine transport maximal velocity was higher in MSPH compared with MPH (Vmax ~12 and ~5 pmol/μg protein/minute, respectively), without altering the apparent Km (~188 and ~128 μM, respectively) or hCAT-1 protein expression. NOS activity was lower (~29%) in MSPH compared with MPH without alterations in eNOS expression. Arginase II activity and expression was increased in MSPH (~1.3 and ~2.4 fold, respectively) compared to MPH. Conclusion: MSPH associates with altered L-arginine bioavailability for NOS in HUVEC leading to reduce umbilical vein reactivity, a likely key phenomenon in MSPH-associated adult cardiovascular disease. CONICYT (ACT-73 PIA, AT-24100210), FONDECYT (1110977, 1120928, 11110059). FW and EG-G hold CONICYT-PhD fellowships.

L-Carnitine transport in human fetal endothelial cells and modulation of human umbilical vein reactivity

Leiva A[1]*, Guzmán-Gutiérrez E[1], Salomón C[1], Arroyo P[1], S Zambrano[2], MV Ruiz[2], A Blanca[2], Mate A[2], Vásquez C[2], Sobrevia L[1]. [1]Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynaecology, Medical Research Centre (CIM), School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago, Chile, [2]Department of Physiology, Faculty of Pharmacy, Universidad de Sevilla, Spain

Preeclampsia (PE) is a pregnancy syndrome coursing with increased maternal arterial pressure and placenta endothelial dysfunction. L-Carnitine acts as an antihypertensive, but its biological action on fetal vascular reactivity and membrane transport mechanisms in human fetal endothelium (HUVEC) is unknown. HUVEC express novel organic cation transporter 2 (OCTN2) mediating L-carnitine transport. We assayed L-carnitine transport in HUVEC and this molecule effect on human umbilical vein reactivity. Methods: Umbilical vein rings were mounted in a myograph and endothelium-dependent (intact vessels) or independent (endothelium denuded vessels) reactivity to L-carnitine, in absence or presence of NG-nitro-L-arginine methyl ester (100 µM) was assayed. L-Cartinine transport (1-40 µM, 5 µCi/ml, 37ºC, 20-300 s) was measured in HUVEC primary cultures (passage 2). Results: L-Carnitine transport showed a Na+-dependent (initial velocity (vi) = 0.244 fmol/µg protein/min) and a Na+-independent (vi = 0.064 fmol/µg proteína/min) component. Transport was saturable (maximal velocity (Vmax) = 0.316 fmol/µg protein/min, apparent Km = 10 µM). L-Carnitine caused an endothelium dependen dilation of vein rings with an EC50 ~49 µM, an effect that was partially blocked (~58%) by L-NAME.Conclusion: L-Carnitine also acts as a modulator of umbilical vein relaxation involving NO synthesis, most likely derived from HUVEC. Support: PN de I+D+I 2008-2011 (PS09/01395), Junta de Andalucía, Consejería de Salud (PI-0034), AECID (D/031187/10, A1/036123/11) (Spain). CONICYT (ACT-73 PIA, AT-24100210), FONDECYT (1110977, 11110059) (Chile). EG-G and CS hold CONICYT-PhD fellowships. PA holds a PUC-PhD (Chile) fellowship. SZ is a fellow of Junta de Andalucía (PI-0034), AB supported by PS09/01395.

The EF-hand calcium-binding protein S100A1 is critical for ischemic angiogenesis

Carolin Lerchenmueller[1,2]*, Walter J. Koch[3], Hugo A. Katus[1], Karsten Peppel[3], Patrick Most[1,2]. [1]Center for Molecular and Translational Cardiology, Department of Medicine III, Heidelberg University Hospital, Heidelberg, Germany, [2]Laboratory for Cardiac Stem Cell & Gene Therapy, Center for Translational Medicine, Jefferson Medical College, Philadelphia, USA 3) Center for Translational Medicine, Jefferson Medical College, Philadelphia, USA

Background - Impaired NO homeostasis in endothelial cells (ECs) lacking the EF-hand calcium (Ca2+) sensor protein S100A1 prompted investigation of its pathophysiological role in regenerative angiogenesis in humans and experimentally induced critical limb ischemia (CLI). Methods and Results - Patients with peripheral arterial disease (PAD) and chronic CLI of the lower extremity showed almost complete loss (decrease to 5%, P<0.05) of S100A1 mRNA expression in tissue distal of the obstructed blood flow (control n=3 vs. CLI n=5). Of note, experimentally induced CLI in S100A1 knock-out mice (SKO) due to unilateral femoral artery resection (FAR) caused a high rate of auto-amputation compared with wild type control mice post FAR (0/22 for WT vs. 10/18 for SKO, P<0.01). Assessment of post-FAR blood flow recovery and revascularization revealed insufficient tissue perfusion and defective capillary formation in ischemic SKO tissue compared to controls measured by Echo Doppler (SKO mice never achieved >25-30% perfusion of ischemic WT hind limbs 1 and 2 weeks post-FAR, P<0.01, n=8), microsphere uptake (80% reduction in blood flow in SKO ischemic limbs compared with controls, P<0.01, n=5), and histological and anatomical evaluation (capillary staining in skeletal muscle and corrosion casting of the hind limbs), respectively. Consistently, ECs from SKO mice fail to proliferate, migrate and form tube-like structures in vitro (as shown by 3[H]-Thymidine-incorporation assay, chemokinesis assay and matrigel tube formation assay) reflecting impaired capillary formation in in vivo angiogenesis assays in SKO mice (using a matrigel plug assay). Biochemical analyses in turn unveiled inhibitory eNOS phosphorylation (p-T495 phosphorylation increased by 3,5fold in SKO vs. WT ischemic skeletal muscle tissue, P<0.05, n=4, while p-S1177 was decreased), impaired NO bioavailability (3-fold vs. ischemic WT ischemic skeletal muscle tissue and SKO vs. WT ECs under hypoxic conditions, P<0.01, n=4), exaggerated VEGF production (5-fold vs. ischemic WT skeletal muscle tissue, P<0.05, n=4) and abnormal VEGF-dependent downstream signalling in SKO ischemic tissue (enhanced PI3K, PDK and AKT expression levels). Molecular assays then demonstrated that a Ca2+-dependent S100A1/eNOS interaction improves NO production of ischemic tissue-derived and recombinant eNOS by recombinant S100A1 in vitro. Accordingly, NO substitution by DETA-NO treatment rescued defective angiogenesis and salvaged limbs, respectively, in SKO-FAR mice (each phenotype n=6-7). Conclusions - Our study shows for the first time a downregulation of S100A1 expression in humans with CLI and characterizes S100A1 as an indispensable factor for postischemic regenerative angiogenesis. Loss of S100A1 seems to compromise key aspects of EC-dependent neovascularisation based on abnormal NO bioavailability and eNOS function. Its pathophysiological impact on CLI prompts further investigation of its therapeutic regenerative potency in PAD.

Shear-induced Aquaporin-1 expression regulates NO secretion in endothelial cells

Thomas Leyen[1]*, Ruud Fontijn[1], Josefien Baggen[1], Dirk Geerts[2], Etto Eringa[3], Anton Horrevoets[1]. [1]Department of Molecular Cell Biology and Immunology, VUmc, Amsterdam, [2]Department of Human Genetics, AMC, Amsterdam, 3Department of Physiology, VUmc, Amsterdam, The Netherlands

Introduction: Shear-induced expression of Krüppel-like factor 2 (KLF2) is an important mediator of endothelial response to laminar shear stress and results in an atheroprotected endothelial phenotype. KLF2 expression regulates many downstream target genes both directly and indirectly, influencing a large variety of cellular functions such as anti-inflammatory gene expression, changes in the actin-cytoskeleton and induction of the Nitric Oxide (NO)-producing enzyme endothelial Nitric Oxide Synthase (eNOS). The current study identifies Aquaporin-1 (AQP1) as a shear-induced gene in endothelial cells. Next to its function as a water-channel, AQP1 has also been identified as a NO channel. In this study we investigate the role of the shear-induced increase in eNOS and AQP1 expression on NO release and vasomotor changes. Methods & Results: We show that AQP1 is highly expressed in endothelial cells found at atheroprotected sites in the vasculature but is nearly absent at atheroprone sites. This coincides with the expression pattern that is known for KLF2. To test whether KLF2 regulates the expression of AQP1, we ectopically expressed KLF2 in endothelial cells grown under static conditions. KLF2 expression results in an increase of AQP1 mRNA and protein levels. Immunofluorescence analysis of AQP1 in endothelial cells ectopically expressing KLF2 shows that KLF2 expression results in a relocalization of AQP1 to cell-cell junctions. To test whether KLF2-induced AQP1 in endothelial cells functions as a NO channel, we tested the role of AQP1 in KLF2 induced NO secretion. By using a lentivirus overexpressing KLF2 in combination with a lentivirus expressing a shRNA against AQP1, we show that KLF2 induced NO release is partly mediated by AQP1. Conclusion: In this study we identify AQP1 as a shear-induced, KLF2-regulated NO channel in endothelial cells. Shear-induced increase of NO production combined with an increased NO secretion may point towards a shear-regulated mechanism for vasomotor changes. The role of KLF2-induction and subsequent AQP1 expression in regulating vasodilation are currently under investigation.

Targeting Olfactomedin-like 3, a BMP4 agonist, inhibits tumor growth by impairing angiogenesis and pericyte coverage

Marijana Miljkovic-Licina*, Philippe Hammel, Sarah Garrido-Urbani, Boris P.-L. Lee, Mehdi Meguenani, Chiraz Chabaane, Marie-Luce Bochaton-Piallat and Beat A. Imhof.
Department of Pathology and Immunology, CMU, University of Geneva, Geneva, Switzerland

Anti-angiogenic drugs have been used as anti-cancer agents to target tumor endothelial cells or pericytes. Due to limited efficacy of the current monotherapies, dual targeting of endothelial cells and pericytes provide more versatile anti-angiogenic strategies. Here, we identify Olfactomedin-like 3 (Olfml3) as a novel pro-angiogenic cue within the tumor microenvironment. It is produced by angiogenic endothelial cells and accompanying pericytes, and deposited in the peri-vascular compartment of the neovasculature. Olfml3 deficiency inhibits endothelial cell migration and sprouting in vitro. Blockade of Olfml3 by anti-Olfml3 antibodies reduces tumor vascularization, pericyte coverage and tumor growth in vivo. Olfml3 alone and through binding to bone morphogenetic protein 4 (BMP4) enhances the canonical SMAD1/5/8 signaling. Therefore, Olfml3 contributes to a pro-angiogenic microenvironment supporting remodeling and maturation of neovessels. Its blockade provides a novel strategy to control tumor growth by targeting a single molecule produced by endothelial cells and pericytes.

Expression of platelet CD40 promotes platelet-induced atherogenesis by enhancing leukocyte recruitment

Dirk Lievens[1]*, Tom Seijkens[2], Marijke Kuipers[3], Holger Winkels[1], Linda Beckers[2], Oliver Soehnlein[1], Remco Megens[1], Norbert Gerdes[1,2], Johan Heemskerk[3], Christian Weber[1], Esther Lutgens[1,2]. [1]Institute for Cardiovascular Prevention (IPEK), University Hospital of the LMU Munich, Munich, Germany, [2]Dept of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands, [3]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands

Beyond an eminent role in hemostasis and thrombosis, platelets are important mediators of inflammation and protagonists of atherogenesis. Here we investigated the inflammatory propensity of platelet-specific expression of the CD40 receptor, an integral membrane protein of the tumor necrosis factor receptor (TNF-R) family. Besides its presence on immune and other cell types the CD40 receptor is constitutively expressed on platelets where its function remains unknown. Platelets were isolated from Apoe-/- or Cd40-/-Apoe-/- mice, activated with thrombin, and injected (3x10E7 platelets, i.v.), into 17-week-old Apoe-/- mice every 5 days for 12 weeks. Compared to infusion of activated Apoe-/- (wild type) platelets, injection of activated Cd40-/-Apoe-/- platelets caused a 73% decrease in plaque size (Apoe-/- platelets 18.3x10E4 ± 2.7x10E4 µm2 vs Cd40-/-Apoe-/- platelets 6.7 x10E4 ± 2.1 x10E4 µm2, Vehicle 9.8 x10E4 ± 2.8 x10E4 µm2, $p<0.05$) in the aortic arch. Absence of CD40 on platelets reduced the absolute number of plaque macrophages (Mac-3) by 39% but did not affect the content of CD45+ cells and CD3+ T lymphocytes. Flow cytometric analysis revealed an elevated number of circulating Ly6G+ neutrophils (+32%) upon injection of activated platelets. However, this increase was absent when CD40-deficient platelets, were injected highlighting the inflammatory potential of platelets and a central role for CD40 in this process. In addition, we detected a 20% decrease in the formation of platelet-leukocyte aggregates in vitro while intravital microscopy in carotid arteries showed a 2-fold decrease of platelet adhesion to the vessel wall of mice, injected with activated Cd40-/- platelets, In sum, this study reveals that platelet CD40 promotes atherosclerosis by interaction with both neutrophils and endothelial cells, thereby amplifying leukocyte recruitment to sites of vascular injury.

SR-BI expressed by lymphatic vessels is required for removal of cholesterol from peripheral tissues

Hwee Ying Lim[1]*, Chung Hwee Thiam, Kim Pin Yeo, Chung Shii Hii, Kristine CY McGrath[2,3], Alison Heather, J. Steven Alexander[4], and Véronique Angeli[1]. [1]Dept. of Microbiology, Immunology Programme, Yoon Loo Lin School of Medicine, National University of Singapore, Singapore, [2]School of Medical and Molecular Biosciences, University of Technology, Sydney, Australia, [3]Heart Research Institute, Sydney, Australia, [4]Department of Molecular and Cellular Phyisology, LSU Health Sciences Center-Shreveport, USA

The lymphatic and blood vessels function to maintain tissue fluid homeostasis. With the exception of immune cell entry, the prevailing view is that the lymphatic vasculature plays a passive role in the uptake of fluid and macromolecules from the periphery. Of interest, high-density lipoprotein (HDL) macromolecules transport excess cholesterol from the peripheral tissues back to the bloodstream, a process referred to as reverse cholesterol transport (RCT). Although several lines of evidence support the role of lymphatics in RCT and less so via the venous capillaries, it has not been directly demonstrated. Here we sought to determine whether lymphatic vessels are essential in RCT and the mechanisms underlying cholesterol transport by lymphatics. Using fluorescent-labelling methods, we show that cholesterol and its major vehicle, HDL are normally transported by peripheral lymphatic vessels and, disruption of lymphatic drainage results in lipoprotein accumulation in tissues and reduced RCT. Lymphatic endothelial cells (LECs) express HDL transporters including adenosine tri-phosphate binding cassette receptor A1 (ABCA1) and scavenger receptor class B type I (SR-BI). Silencing RNA interference against SR-BI but not ABCA1 potently abrogated HDL uptake by LECs. Blocking SR-BI with neutralizing antibody prevented in vitro HDL uptake by LECs and reduced HDL transport by lymphatic vessels in vivo. Previously, using apolipoprotein-E deficient (apoE-/-) mice as a model of dyslipidemia, we reported that hypercholesterolemia is associated with impaired lymphatic drainage and increased lipid accumulation in peripheral tissues. We now demonstrate that restoring lymphatic drainage in these mice significantly improved lipid clearance. Collectively, this study challenges the current view that lymphatic endothelium is a passive exchange barrier for cholesterol clearance and provide further evidence for its interplay with lipid biology in health and disease.

Expression and function of acetyl-CoA carboxylase (ACC) isoforms in endothelial cells

S. Lindenmüller[1,2]*, D. Weigel[1], R. Heller[1]. [1]Universitätsklinikum Jena, Institut für Molekulare Zellbiologie, Hans-Knöll-Strasse 2, 07745 Jena, [2]Universitätsklinikum Jena, Center for Sepsis Control and Care, Erlanger Allee 101, 07747 Jena, Germany

Acetyl-CoA carboxylase (ACC) is a target enzyme of 5'AMP-activated protein kinase (AMPK), which is a major regulator of intracellular energy state and homeostasis. AMPK has recently been shown to exert angiogenic functions and ACC, which is inhibited by AMPK, has been suggested to be one of the responsible downstream targets. ACC exists in two isoforms, ACC1 and ACC2, which both generate malonyl-CoA but have distinct functions in fatty acid metabolism. Malonyl-CoA produced by ACC1 is a precursor for fatty acid synthesis, while ACC2-derived malonyl-CoA serves as an inhibitor of fatty acid transport into the mitochondria and therefore as an inhibitor of fatty acid oxidation. So far, nothing is known about the expression and function of ACC isoforms in endothelial cells. Thus, the goal of this study was to characterize ACC isoforms and to investigate their importance in regulating fatty acid metabolism in human umbilical vein endothelial cells (HUVEC). Furthermore, a possible role of ACC in angiogenesis induced by vascular endothelial growth factor (VEGF) was explored. The expression of ACC-isoforms was analysed by qRT-PCR. The ACC1 isoform was three-fold higher expressed than ACC2 and thus represents the major isoform in endothelial cells. Only ACC1 was detectable in Western blots. To investigate the functional importance of different ACC isoforms, they were downregulated by specific siRNA and fatty acid oxidation (14CO2 trapping method) and synthesis (14C-acetate incorporation) were measured. HUVEC were able to oxidize fatty acids from extra- and intracellular sources. Oxidation was prevented by an inhibitor of fatty acid transport into the mitochondria (etomoxir) and stimulated when glycolysis was blocked by 2-deoxyglucose. Downregulation of either ACC1 or ACC2 had only a minor stimulating effect on fatty acid oxidation, even when it was measured under conditions of glucose depletion. This corresponded to a small inhibitory effect of AMPK downregulation (siRNA against alpha1 and alpha2 catalytic subunits) on fatty acid oxidation. HUVEC were also able to incorporate 14C-acetate in their neutral lipid fraction. This was impaired by inhibitors of fatty acid synthase (C75) or 3-hydroxy-3-methylglutaryl-coenzyme A reductase (simvastatin) indicating that fatty acids as well as cholesterol were synthesized. Downregulation of ACC1 reduced 14C-acetate incorporation demonstrating that ACC1 is important for fatty acid synthesis in endothelial cells. Interestingly, VEGF led to a transient AMPK-dependent phosphorylation of ACC1 suggesting that VEGF may cause a temporary arrest of the energy-consuming fatty acid synthesis. However, downregulation of ACC1 had no effect on VEGF-induced angiogenesis as measured by spheroid assays. Thus, in long-term, an inhibition of fatty acid synthesis may be counterbalanced by an increased fatty acid uptake. Together, these data show that ACC isoforms play only a minor role in fatty acid oxidation supporting the concept that fatty acid oxidation does not essentially contribute to energy generation in HUVEC. ACC1, the major isoform in endothelial cells, is involved in fatty acid synthesis but its downregulation does not lead to an impairment of angiogenesis.

Inhibition of gelatinase B activity reduces cellular inflammation and restores function of transplanted pancreatic islets.

Neelam Lingwal[1]*, , Klaus T Preissner[2], Thomas Linn[1]. [1]Third Medical Department, 2Institute for Biochemistry, Faculty of Medicine, Justus-Liebig University, 35392 Giessen, Germany

Abstract Islet transplantation provides an approach to compensate for loss of insulin producing cells in patients with type 1 diabetes (1,2). However, the intraportal route of transplantation is associated with instant inflammatory reactions to the graft and subsequent islet destruction as well (3,4). While matrix metalloproteases (MMP) 2 and 9 are involved in both remodelling of extracellular matrix and leukocyte migration (5,6), their influence on outcome of islet transplantation has not been characterized. Analysis of MMP-2 and MMP-9 in Islets cells by zymography, gelatin dequenching assays, and western blot showed that in normal state islets were expressing less MMP but treatment with inflammatory cytokines increased MMP-2 and MMP-9 secretion from islet. However, islet transplantation to mice liver showed less MMP activity in transplanted islet and more with recipients liver. We observed comparable MMP-2 mRNA expressions in control and transplanted groups of mice, whereas MMP-9 mRNA and protein expression levels increased after islet transplantation. Immunostaining for CD11b (Mac-1) expressing leukocytes (macrophage, neutrophils) and Ly6G (neutrophils) revealed substantially reduced inflammatory cell migration into islet-transplanted liver in MMP-9-knockout (KO) recipients. Moreover, gelatinase inhibition resulted in a significant increase in the insulin content of transplanted pancreatic islets and reduced macrophage and neutrophil influx as compared to the control group. These results indicate that increase of MMP-9 expression and activity after islet transplantation is directly related to enhanced leukocyte migration and that early islet graft survival can be improved by inhibiting MMP-9 (gelatinase B) activity. REFERENCES 1. Sutherland DE, Matas AJ, Goetz FC, Najarian JS: Transplantation of dispersed pancreatic islet tissue in humans: autografts and allografts. Diabetes 29 Suppl 1:31-44, 1980 2. Brandhorst H, Brandhorst D, Hering BJ, Bretzel RG: Significant progress in porcine islet mass isolation utilizing liberase HI for enzymatic low-temperature pancreas digestion. Transplantation 68:355-361, 1999 3. Moberg L, Korsgren O, Nilsson B: Neutrophilic granulocytes are the predominant cell type infiltrating pancreatic islets in contact with ABO-compatible blood. Clin Exp Immunol 142:125-131, 2005 4. Yasunami Y, Kojo S, Kitamura H, Toyofuku A, Satoh M, Nakano M, Nabeyama K, Nakamura Y, Matsuoka N, Ikeda S, Tanaka M, Ono J, Nagata N, Ohara O, Taniguchi M: Valpha14 NK T cell-triggered IFN-gamma production by Gr-1+CD11b+ cells mediates early graft loss of syngeneic transplanted islets. J Exp Med 202:913-918, 2005 5. Gong Y, Hart E, Shchurin A, Hoover-Plow J: Inflammatory macrophage migration requires MMP-9 activation by plasminogen in mice. J Clin Invest 118:3012-3024, 2008 6. Khandoga A, Kessler JS, Hanschen M, Khandoga AG, Burggraf D, Reichel C, Hamann GF, Enders G, Krombach F: Matrix metalloproteinase-9 promotes neutrophil and T cell recruitment and migration in the postischemic liver. J Leukoc Biol 79:1295-1305, 2006

Overexpression of pro-angiogenic cytokins affects the proliferation, migration and tube formation abilities of human endothelial progenitor cells

Monika Litwin[1]*, Dagmara Baczynska [1,2], Agata Radwanska[1], Wojciech Witkiewicz[1], Tadeusz Dobosz[1,2]. [1]Regional Specialist Hospital in Wroclaw, Research and Development Center, Wroclaw, Poland, [2]Laboratory of Molecular Technique, Institute of Forensic Medicine, Medical University of Wroclaw, Wroclaw, Poland

Critical limb ischemia (CLI) represents the most severe stage of atherosclerotic lower extremity peripheral artery disease and is associated with high mortality rates. Gene therapy and therapeutic angiogenesis are considered as potential future treatments of CLI. Experimental preclinical studies of therapeutic angiogenesis using vascular endothelial growth factor (VEGF165)-mediated gene transfer in patients with CLI unsuitable for revascularization have shown promising results. In recent years special attention has been paid to find new pro-angiogenic factors which could be used in gene therapy of CLI. The aim of this study was to observe the effect of pro-angiogenic cytokins overexpression on proliferation, migration and tube formation abilities of human endothelial progenitor cells (EPC 55.1 and 55.2). Selected cell lines were isolated as described previously by Paprocka et al. (2011) and express stem cell/progenitor (CD133) and endothelial (CD202b, VEGFR2, CD146, CD105) cell markers. Vectors encoding cDNA for vascular endothelial growth factor (VEGF165), angiopoetin-1 (ANG-1), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), prostaglandin-I synthase (PGIS), hypoxia-inducible factor (HIFα) and bicistronic vectors encoding VEGF gene together with the above-mentioned genes were used for transfection of EPC cells. The overexpression of cytokins was confirmed by real-time RT-PCR and Western blotting analysis. We observed increased in proliferation rate of EPC cells overexpressing HGF, VEGF and ANG and in paticular those cells transfected with bicistronic vector encoding VEGF with PGIS, HGF and HIFα. These was accompanied by significant changes in cells migration measured on TranswellTM filters. Tube formation analysis was made on μ-slide angiogenesis plates coated with growth factor reduced Matrigel. Cell transfected with vectors encoding PGIS and HGF as well as those tranfected with bicistronic vectors VEGF/ANG, VEGF/HGF and VEGF/HIFαwere characterized by increased migration and tube formation abilities. Our experimental data indicate the potential for the use of vectors encoding selected pro-angiogenic cytokins in cell-gene therapy of critical ischemic disease of lower limbs. This publication is part of project „Wrovasc - Integrated Cardiovascular Centre“, co-financed by the European Regional Development Fund, within Innovative Economy Operational Program, 2007-2013 realized in Provincial Specialized Hospital, Research and Development Center in Wroclaw. M. Paprocka, A. Krawczenko, D. DuÅ›, A. Kantor, A. Carreau, C. Grillon, C. Kieda. CD133 positive progenitur endothelial cell lines from human cord blond. Cytometry Part A 79A: 594-602 (2011).

Impact of the EphrinB/EphB system on pro-inflammatory monocyte-endothelial cell interaction

Hui Liu*, Markus Hecker, Thomas Korff. Institute of Physiology and Pathophysiology, University of Heidelberg, Germany

The expression of ephrinB ligands and EphB receptors is a prerequisite for an orchestrated development of the vasculature. However, only little is known about the function of this ligand/receptor system in the adult vasculature. Our results show that ephrinB2 as well as ephrinB1 are localized on the luminal surface of endothelial cells and become up-regulated during inflammation, enabling their interaction with circulating leukocytes. Whereas forward signaling downstream of the EphB receptors promotes the migratory and pro-inflammatory activity of monocytes, reverse signaling induces granulocyte-macrophage colony-stimulating factor(GM-CSF) release and a c-Jun N-terminal kinase (JNK)-dependent increase in expression of adhesion molecules such as VCAM-1 or E-selectin in the endothelial cells. Therefore, monocyte attachment to EphB2-stimulated endothelial cells is enhanced. Moreover, ephrinB1 and ephrinB2, which both are partially localized in the intercellular junctions of the endothelial cells, are crucial for the transmigration of monocytes through the endothelial cell monolayer, and their expression is affected by pro-inflammatory stimuli such as tumor necrosis factor-alpha (TNF-α) . Thus, ephrinB1/2 expression on endothelial cells promotes the adhesion and transmigration of EphB2-expressing monocytes. In order to analyse these ephrinB/EphB-dependent interactions between monocytes and endothelial cells in more detail, we generated EphB2-overexpressing mouse myeloma cells, which in fact are capable of up-regulating adhesion molecule expression in and inducing the release of von Willebrand factor from the cultured endothelial cells. We conclude that reverse signaling processes promote the pro-inflammatory differentiation of endothelial cells whereas forward signaling facilitates monocyte diapedesis and subsequent differentiation into macrophages in the vessel wall.

CCN3, a potential angiogenic factor involved in endothelial dysfunction observed in the pregnancy disease preeclampsia

Haiyi Liu*, Liska Horsch, Stefan Radke, Bernd Giebel, Elke Winterhager, Alexandra Gellhaus. Institut für Molekularbiologie, Universität Essen, Germany

The placental microvasculature is essential for efficient transfer of gases, nutrients and waste between the mother and fetus. The pregnancy disease preeclampsia (PE) leading to a reduced fetal oxygen and nutrition support is characterized by hypoxia and endothelial dysfunction with reduced capillarization of the terminal villi. The matricellular proteins CCN1 and CCN3 are mainly expressed in placental endothelial cells and both protein levels are decreased in early-onset severe PE. The aim of this study is to clarify the role of CCN1 and CCN3 for proper endothelial function in the healthy placenta versus preeclamptic conditions. Therefore primary cultures of the human placental vessel endothelial cells (HPLVEC) from a whole placenta at term was established by developing a perfusion system and compared to primary cultures of umbilical cord endothelial cells (HUVEC). The HUVEC cells were positive for vWF and CD31 while negative for CD34/CD29/CD44 analysed by FACS which confirmed the properties of endothelial cells. The primary placental vessel endothelial cells were confirmed to be endothelial cells by CD31, CD144 positive markers and negative for CD29, CD34, CD44, CD45, CD90, and the purity was more than 95%. Next, expression of CCN1, CCN3 and of the marker genes Cx43, sFlt-1 - both are highly increased in preeclampsia - was analysed on mRNA and protein level and their different response to hypoxia (1% O2) was tested. Preliminary results revealed that in comparison with the other three target genes (CCN1/Cx43/sFlt-1), CCN3 changed mostly upon hypoxia conditions. In contrast to HUVEC which showed an upregulation of CCN3 after 24h with a strong downregulation up to 96h of hypoxic treatment, HPLVEC revealed a short upregulation of CCN3 in the first 4h followed by a strong downregulation up to 96 hours hypoxia. In summary, these studies showed a hypoxic effect on CCN3 in HPLVEC and HUVEC which could contribute to the observed endothelial dysfunction in preeclampsia.

Expression profile analysis of microRNAs 221 and 222 in human umbilical vein endothelial cells: Anti-angiogenic effects of hydroxyurea

Flavia Cristine Mascia Lopes*, Regiane Ferreira, Dulcinéia Martins Albuquerque, Fernando Ferreira Costa, Nicola Conran. 1INCT de Sangue, Hematology and Hemotherapy Center, School of Medical Sciences, University of Campinas - UNICAMP, Campinas, Sao Paulo, Brazil

Hydroxyurea (HU) is a drug that has been used successfully for therapy in Sickle Cell Disease (SCD). SCD results from mutations in the β chain of hemoglobin and it is associated with a complex pathophysiological process that involves endothelial activation, decreased nitric oxide (NO) bioavailability, inflammation and a newly recognized pro-angiogenic state. Recently, microRNAs (miRNAs) have emerged as key regulators of several cellular processes, including angiogenesis. MiRNAs are a class of small non-coding RNAs that regulate gene expression at post-transcriptional level. Furthermore, MiRNAs studied in endothelial cells have been implicated in responses to angiogenic stimuli, growth factor stimulation and hypoxia, suggesting that they may be an integral component of angiogenic signal transduction pathways. Our previous data suggest that some of the therapeutic effects of HU may function through novel vascular mechanisms since HU was capable of regulating angiogenesis by attenuating migration, proliferation and capillary-like structure formation in human umbilical vein endothelial cells (HUVECs). Although vascular endothelial cells are considered significant targets of HU in the context of SCD and HU is thought to benefit SCD individuals through several mechanisms; the mechanisms underlying the regulation of angiogenesis by HU remain unclear. The aim of this study was to analyze the expression profile of miRNAs 221 and 222 which have been previously described as involved in the regulation of endothelial cell functions and angiogenesis. HUVECs were treated with 100 μM HU for 24 hours. After the incubation period, the cells were collected for the miRNA extraction, transcription to cDNA and quantitative PCR to analyze miRNA expression. Results demonstrate that the expression profile of miRNA 221 was considerably increased in the presence of HU (100 μM) compared to control cultures ($p=0.0190$). Inversely, miRNA 222 was down regulated in the HUVEC cultures treated with HU under the same conditions ($p=0.0159$). According to these studies, our data suggest that the anti-angiogenic effects of HU may be due to changes in miRNA 221 and 222 expression. These miRNAs play different roles in several cells and in a variety of metabolic pathways. In addition, they also regulate Endothelial Nitric Oxide Synthase (eNOS) in the endothelium and the regulatory mechanisms of NO are essential for angiogenesis. Since miRNAs seem to be tightly linked to transcription factors in complex regulatory networks, we are currently conducting new studies to evaluate the transcription factors that could be potential targets of miRNA 221 and 222. In conclusion, the recent discovery of the involvement of miRNAs in the control of angiogenesis renders them very attractive for the development of new approaches for restoring the angiogenic balance.

Investigating the role of podoplanin and CLEC-2 in the integrity of the blood-cerebrospinal fluid barrier

Kate L. Lowe[1]*, Brenda A. Finney[1], Ana M. Gonzalez[2], Edina Schweighoffer[3], Victor L, J. Tybulewicz[3] and Steve P. Watson[1]. The University of Birmingham, [1]Centre for Cardiovascular Sciences, [2]Neuropharmacology and Neurobiology Section, Institute of Biomedical Research, College of Medical and Dental Sciences, Edgbaston, Birmingham, [3]MRC National Institute for Medical Research, London, United Kingdom

INTRODUCTION: The C-type lectin-like receptor (CLEC-2) is a transmembrane protein expressed at high levels on platelets and megakaryocytes. CLEC-2 interacts with podoplanin on lymphatic endothelial cells (LECs) to mediate the separation of blood and lymphatic vessels. Mice deficient in podoplanin, CLEC-2 or Syk develop a blood mixing phenotype, believed to result from a migration defect in LECs. Furthermore, CLEC-2 and Syk deficient mice develop hemorrhaging in the brain at mid-gestation. The absence of lymphatic vessels in the brain proposes an additional role for podoplanin which is expressed on choroid plexus epithelial cells which form the blood-cerebrospinal fluid (blood-CSF) barrier. Here we begin to investigate whether circulating platelets interact with choroid plexus epithelial cells to influence the formation of the blood-CSF barrier. METHODS: Brains from wild-type mice, mice with a constitutive (CLEC-2-/-) or platelet/megakaryocyte specific (CLEC-2fl/flPF4-Cre) loss of CLEC-2 and mice with a constitutive loss of Syk (Syk-/-) were fixed in 4% formadeldehyde in PBS and embedded in paraffin. Tissue sections were either stained with haematoxylin and eosin (H&E), or immunostained for podoplanin using peroxidase and brightfield microscopy. Immunofluorescence staining for both podoplanin and the choroid plexus marker transthyretin (TTR) were visualised by confocal microscopy. Additionally, choroid plexus epithelial cells were seeded on transfilters and trans-epithelial electrical resistance (TEER) was measured over time. RESULTS & CONCLUSIONS: CLEC-2-/-, CLEC-2fl/flPF4-Cre and Syk-/- mice all displayed a haemorrhaging phenotype as early as E12.5. In CLEC-2-/- mice haemorrhagic areas were localised to both the ventricles and parenchyma, while in Syk-/- mice they were restricted to the parenchyma. Currently, the localisation of haemorrhages in CLEC-2fl/flPF4-Cre mice has not been identified. Expression of podoplanin was confirmed to co-localise with TTR on the surface of choroid plexus epithelial cells in tissue sections of all mice investigated. To investigate whether platelets interact with podoplanin at the blood-CSF barrier, a choroid plexus cell line has been employed and initially characterised to form monolayers with increasing TEER up to confluency, supporting the formation of junctions. These results show both CLEC-2 and Syk are required for the integrity of the brain vasculature. Studies on the choroid plexus cell line will aim to provide the molecular basis for this phenotype. This work was supported by the Wellcome Trust, British Heart Foundation and Medical Research Council.

T cell extravasation across the Blood-Brain Barrier endothelium: Extensive T cell crawling and transcellular versus paracellular T cell diapedesis

Ruth Lyck[1]*, Christoph Wyss[1], Michael Abadier[1], Oliver Steiner[1], Giuseppe Cagna[2], Ruth Linnepe[2], Dietmar Vestweber[2], Urban Deutsch[1], Britta Engelhardt[1]. [1]Theodor Kocher Institute, University of Bern, Freiestrasse 1, CH-3012 Bern, Switzerland, [2]Max-Planck Institute for Molecular Biomedicine, Röntgenstrasse 20, D-48149 Muenster, Germany

The blood-brain barrier (BBB) is formed at the level of central nervous system (CNS) microvessels by highly specialized endothelial cells. Through an elaborate network of continuous and complex tight junctions and a low pinocytotic activity the BBB protects the CNS from harmful substances in the blood stream. Likewise, immune cell entry into the CNS is strictly limited by the BBB. Though, during neurological disorders such as multiple sclerosis (MS) or its animal model experimental autoimmune encephalomyelitis (EAE) autoreactive T cells traverse the BBB and enter the CNS parenchyma where they destroy the myelin sheath of the neurons. To study the process of T ell extravasation across the BBB, we have established an in vitro imaging model that allows visualizing the dynamic interaction of T cells with primary mouse brain microvascular endothelial cells (pMBMECs) under physiological flow in vitro. Using this model we have demonstrated an essential role for endothelial ICAM-1 in mediating T cell arrest, T cell polarization and T cell crawling preferentially against the direction of flow on the luminal surface of pMBMECs. While T cells harbor all essential cues to mechanosense the direction of flow, the extended migration distances of T cells required to find a site permissive for diapedesis are directed by unique characteristic of pMBMECs. Little is known about the last step of T cell extravasation, the diapedesis, across the BBB. We hypothesize that the extreme tightness of BBB endothelium translates into transcellular over paracellular diapedesis of T cells. To study the route of T cell diapedesis we isolated pMBMECs from transgenic mice expressing VE-cadherin-GFP under control of the endogenous genetic VE-cadherin locus. Because of their continuous junctional GFP staining the route of T cell diapedesis can be identified in live cell imaging experiments. After having characterized VE-cadherin-GFP expressing pMBMECs for their junctional integrity and cell adhesion molecule profile, we currently analyze which pathway T cells take for their diapedesis across pMBMECs. In particular, we aim to clarify the active participation of pMBMECs for defining the pathway of T cell diapedesis. To this end, we analyze the route of T cell diapedesis after different cytokine stimulations of pMBMECs and upon deficiency of endothelial ICAM-1 and ICAM-2.

Contribution of tumor endothelial cells to tumor metastasis

Nako Maishi[1]*, Yusuke Ohba[2] Kazuyuki Yamamoto[1], Noritaka Ohga[1], Junichi Hamada[3], Kosuke Akiyama[1], Taisuke Kawamoto[1], Takahiro Osawa[1], Miyako Kondoh[1], Hitomi Ohmura[1], Masanobu Shindoh[4], Yasuhiro Hida[5] and Kyoko Hida[1]. [1]Dept. Vascular Biology, Hokkaido Univ.Grad.Sch.Dent.Med., [2]Dept. Molecular and Cellular Pathology, Hokkaido Univ.Grad.Sch.Med., [3]Division of Cancer Related Genes, institute for Genetic Medicine, Hokkaido Univ., [4]Dept. Oral Pathol. biol., Hokkaido Univ.Grad.Sch.Dent.Med., [5]Dept. Cardiovascular and Thoracic Surgery, Division of Cancer Medicine

Stromal cells in tumor microenvironment interact with tumor cells, and frequently acquire abnormalities. Blood vessels provide an escape route for tumor cells to leave the tumor and enter into the circulation. Interaction between tumor endothelial cells (TECs) and tumor cells plays a key role in the early stage of hematogenous metastasis. We have reported TECs are different from normal endothelial cells (NECs) in many aspects, such as gene expression profiles and chromosomal abnormalities. To analyze the interaction between TECs and tumor cells, we isolated two types of TECs from human tumor xenografts in nude mice; HM-TEC isolated from highly metastatic tumor (HM tumor) and LM-TEC from low metastatic tumor (LM tumor). We also isolated NEC from dermis of normal nude mice. HM-TEC showed higher proliferative and invasive activity than LM-TEC, suggesting that TEC differs depending on their original malignancy status of the tumor. We hypothesized that TECs may also contribute to tumor metastasis, especially in the beginning step. To address this hypothesis, LM tumor cells were co-xenografted with each type of EC into nude mice and analyzed metastasis. The numbers of circulating tumor cells (CTCs) were analyzed by flow cytometry. The largest number of CTCs was detected in tumors co-implanted with HM-TEC among other groups. In addition, the incidence of lung metastasis was increased when tumor was co-injected with HM-TEC. These results suggested that HM-TEC instigate LM tumor to metastasize with induction of intravasation. We investigated the roles of TECs in tumor intravasation; 1) migration of tumor cells towards TECs, 2) adhesion to endothelial layer, 3) crossing the endothelium, in vitro. Tumor cells migrated towards HM-TEC more than LM-TEC or NEC. Tumor cells adhered to HM-TEC the most. They migrated through the HM-TEC monolayer most among all ECs. These results suggested that HM-TEC may help intravasation of tumor cells. To address the mechanism how HM-TEC attract LM tumor cells, we compared gene expression profiles between the three types of ECs, and scouted for molecules upregulated in HM-TEC. We focused on a secreted protein, biglycan, which we had reported as a novel TEC marker. In order to investigate the effects of biglycan upregulated in HM-TEC on LM tumor cells, biglycan expression was knockdowned in HM-TEC using siRNA. Tumor cell migration decreased towards to biglycan knockdown HM-TEC, suggesting that TEC - derived biglycan induces tumor cell chemotaxis towards TEC. Considering the differences between HM-TEC and LM-TEC, we speculated HM-TEC acquired their specific characteristics caused by tumor derived factors. Then we assumed that NEC could acquire a part of characteristics of HM-TEC by exposure to HM tumor derived factors. The effects of tumor derived factors on NECs were investigated using HM tumor - conditioned medium (CM). mRNA expression levels of biglycan was upregulated by tumor CM. Migration and adhesion of LM tumor increased when NEC was treated by tumor CM. These results demonstrated that NECs acquire „HM-TEC like“ phenotype under the influence of HM tumor derived factors. Our findings suggest that TECs „educated“ in tumor microenvironment, may actively induce tumor metastasis.

Inflammatory signaling synergizes with TGFb2 in the induction of endothelial to mesenchymal transition

Monika Maleszewska*, Jan-Renier Moonen, Guido Krenning, Martin C. Harmsen. Cardiovascular Regenerative Medicine Research Group (CAVAREM), Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, EA11 NL-9713 GZ Groningen, The Netherlands

Key words: EndMT, inflammation, IL-1β, TGFβ2, NFκB Endothelial to mesenchymal transition (EndMT) is characterized by loss of endothelial properties and acquisition of mesenchymal-like phenotype. EndMT contributes to various inflammation-related pathologies, in particular to organ fibrosis (blood vessels, heart, kidney, lungs, intestine), e.g. as the result of ischemia-reperfusion injury. The process of EndMT can be investigated by monitoring of changes in expression of endothelial and mesenchymal markers. We used primary HUVEC in an in vitro model to investigate the role of inflammatory co-stimulation (IL-1β) in TGFβ2-dependent EndMT. IL-1β and TGFβ2 co-stimulation causes EndMT in HUVEC. This is observed as transition to spindle-shaped cells, along with the time-dependent upregulation of mesenchymal markers SM22αand calponin on gene and protein level, and the downregulation of endothelial markers eNOS and vWF. Stimulation of HUVEC with either IL-1β or TGFβ2, or both, revealed that IL-1β and TGFβ2 synergistically upregulate the early-induced EndMT markers SM22αand calponin. TGFβ2 was the only TGFβ isoform that was continuously and synergistically upregulated during EndMT. On the other hand, IL-1β expression was suppressed in presence of TGFβ2, compared to the response observed upon IL-1β treatment alone. Furthermore, IL-1β stimulation was dispensable once EndMT was induced. Therefore, after the synergistical co-induction of EndMT TGFβ2 is likely to play the driving role in this process. The activity of the inflammatory transcription factor NFκB was necessary for the induction of EndMT, as its inhibition prevented the induction of the process, but it also seemed to be required for further progression of EndMT. Our study shows that inflammatory signaling strongly enhances TGFβ2-dependent EndMT. The synergistic effects of combined IL-1β and TGFβ2 stimulation suggest that these mediators might also underlie endothelial dysfunction in diseased tissue. The interplay between pro-inflammatory and pro-fibrotic signaling can therefore be crucial for the contribution of EndMT to the organ fibrosis.

Microvessel thrombosis as an instrument of innate immunity

Manukyan D.[1,6]*, von Bruehl M.L.[2], Pfeiler S.[1], Grahl L.[1], Bidzhekov K.[1], Khandagale A.B.[1], Konrad I.[2], Reinhardt C.[1,6], Kennerknecht E.[2], Reges K.[1], Braun S.[2], Goosmann C.[3], Brinkmann V.[3], Spannagl M.[4], Preissner K.T.[5], Massberg S.[2], Engelmann B.[1]. [1]Ludwig-Maximilians-Universität, Institut für Klinische Chemie, Munich, Germany, [2]Deutsches Herzzentrum, Munich, Germany, [3]Max-Planck-Institut für Infektionsbiologie, Berlin, Germany, [4]Justus-Liebig-Universität, Institut für Biochemie, Giessen, Germany, [5]Hämostaseologie, Ludwig-Maximilians-Universität, Munich, Germany, [6]present address: Johannes Gutenberg-Universität, Centrum für Thrombose und Hämostase (CTH) Mainz, Germany

Background: Systemic infection is associated with activation of blood coagulation in microvessels of different organs. We studied the effect of this activation on bacterial virulence and analyzed the trigger mechanisms involved. Methods and Results: Wild type (WT) mice were infected intravenously with E. coli. After 6 h, fibrin formation (FF) was observed in liver and spleen microvessels (immunohistochemistry). 8-10% of microvessels were occluded by fibrin. FF was inhibited by anti-tissue factor (TF) antibody. FXIIa inhibitor PCK also reduced FF. GFP-E. coli. were mostly located inside microvessels. In contrast in mice deficient for the antimicrobial effectors neutrophil elastase and cathepsin G (NE/CG-/-), FF and vessel occlusion were reduced and bacteria were localized in the perivascular tissue. In WT mice, inhibition of FF with hirudin and anti-histone antibody led to extravasation of E. coli. In NE/CG-/- mice, hirudin further enhanced bacterial extravasation while bacteria were retained inside vessel after increasing FF with rVIIa. We prepared tissue factor pathway inhibitor (TFPI) mutant T87F/L89A resistant to cleavage by NE and CG (site-directed mutagenesis). The mutant inhibited microvessel FF and vessel occlusion with higher efficiency than nTFPI. Concomitantly it increased extravasation of bacteria and enhanced bacterial survival, which was elucidated by determination of colony forming units (CFU) using homogenized organs. Conclusions: Our findings suggest for the first time a physiological role for microvessel thrombosis as it restricts the spreading and inhibits the survival of bacteria and thus directly supports innate immunity. Physiological microvessel thrombosis is initiated and maintained by TF, neutrophil serine proteases, extracellular nucleosomes and factor XII. Microvessel and large vessel thrombosis thus share several common activator mechanisms.

Azathioprine, a novel treatment for aneurysms with an old drug

Goran Marinkovic*, Stijntje Hibender, Carlie JM de Vries and Vivian de Waard. Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, The Netherlands

Objective: An aortic aneurysm is a dilatation of the arterial wall caused by destruction of its integrity, which results in often lethal vessel ruptures. Currently, only surgical treatment is available for patients with growing aortic aneurysms, therefore there is a clinical need to identify drugs to attenuate aneurysm growth. Chronic inflammation is fundamental in aneurysm pathology with excessive leukocyte infiltration. We studied the role of the immunosuppressive drug Azathioprine (AZA) in aneurysm formation. Methods and Results: To investigate AZA in aneurysm formation, we used the Angiotensin-II (AngII) aneurysm mouse model. AngII in ApoE-/- mice on a Western Type diet resulted in 11/14 (79%) mice with aneurysms, whereas only 3/13 (23%) of the AZA-treated mice developed aneurysms. In the aorta altered signalling downstream of the AngII receptor type 1 was observed upon AZA treatment; phosphorylation of Jak2 and JNK was decreased and of p38 and p44/42 was increased. In addition, reduced levels of matrix metalloproteinases (MMP)2 and MMP9 and decreased leukocyte influx was observed in AZA-treated mice. To study aneurysm progression, AZA treatment was started 10 days after AngII infusion; all mice developed aneurysms, however the aneurysm type was more severe in the control group, demonstrating the inhibitory effect of AZA on aneurysm progression. To delineate the mechanism of AZA protection, we performed experiments with macrophages and endothelial cells in the presence of 6-mercaptopurine (6MP), the active metabolite of AZA. Macrophages produce less IL-12 and IFNgamma and more IL-10 after 6MP incubation and endothelial cells express less CCL5 (Rantes), IL-12 and VCAM1. Adhesion of monocytes to activated endothelial cells is reduced in the presence of 6MP. These data underscore the anti-inflammatory effect of AZA on macrophages and endothelial cells, which may explain the inhibition of aneurysm formation. Conclusion: We report a protective effect of AZA in aortic aneurysm initiation and, most importantly, inhibition of progression of the disease. AZA modulates the inflammatory response of macrophages and endothelial cells, inhibits monocyte adhesion to endothelial cells, and modulates AngII signalling in the aortic vessel wall.

Regulation of microvasculature by estrogen receptors using mouse models

Margaret Markiewicz*, Anna Ludwicka-Bradley, Gary Gilkeson. Division of Rheumatology and Immunology, Medical University of SC. Charleston, SC, USA

Objective: The goal of this study was to investigate the role of ERalpha and/or ERbeta on microvasculature in the skin using unique genetic models: knockout ERalpha (ERa-/-) and ERbeta (ERb-/-) female mice. Methods: We first visualized and analyzed microvasculature in the skin of ERa-/-, ERb-/- and control mice by injecting FITC-dextran into the heart of mice. Next, the microvessels were visualized by immunostaining with pericyte specific antibodies such as a-smooth muscle actin and desmin. In vivo permeability assay was performed to establish whether vessel stabilization is impaired in the knockout mice. Furthermore, we isolated mouse microvascular endothelial cell from WT,ERa-/-,ERb-/- mice followed by determination of genes important in stabilization of neovessels. Results: In both ERa and ERb knockout mice the vascular network was disorganized and the diameter of blood vessels was irregular as compared to the wild type animals. The expression levels ofa-SMA and desmin were significantly decreased in the skin vessels of ERa-/- and to a lesser degree in ERb-/- mice as compared to WT mice. The extraction of Evan's blue was significantly increased in the skin of ERb-/- mice as compared to WT animals. Interestingly, the extraction of Evans blue was also increased in the lung, heart and brain of these mice suggesting that vascular leakage take place in these mice. Since the vascular changes were observed in dermal microvessels of ERa and ERb knockout mice we determined the genes important in stabilization of neovessels in cultured endothelial cells isolated from ERb-/- and control mice. To examine mRNA levels of PDGFB, VE-cadherin and N-cadherin qPCR analysis was performed. Expression level of the following genes was downregulated: PDGFB (~15 folds), VE-cadherin (~ 28 folds),N-cadherin (~ 1.6 folds). This data may suggest that vessel stabilization is impaired in the skin of ERb-/- mice. The same analyses for ERa-/- mice are in process. Furthermore, the proteoglycans Lumican and Decorin have been shown to influence the process of angiogenesis in in vitro and in vivo studies. In our additional analyses of the whole skin punches obtained from ERb-/- mice we observed that mRNA levels of Decorin were significantly diminished (~7.7 folds), while Lumican levels decreased ~2.4 folds. On the other hand, we observed significantly increased levels of Lum (~9.9 folds) and slightly elevated levels of Dcn (~1.9 folds) in ERa-/- mice. These results suggest a stimulatory role of ERb in the expression of Dcn and Lum which may affect endothelial cell migration and tube formation in these mice. The results obtained from the skin of ERa-/- mice suggest an inhibitory role of ERa in the expression of these proteoglycans. Conclusions: Our data indicate that ERa and ERb play an important role in the process of neovascularization. Specifically, our results suggest that both receptors regulate the genes implicated in the vessel stabilization (resolution phase). However, estrogen receptors may differentially regulate selected matrix components such as Lumican and Decorin which might affect activation phase of angiogenesis.

Neuron-specific prolyl-4-hydroxylase domain 2 knockout reduces brain injury after transient cerebral ischemia

Reiner Kunze[1], Wei Zhou[2], Roland Veltkamp[2], Ben Wielockx[3], Georg Breier[3], Hugo H. Marti[1]*. [1]Institute of Physiology and Pathophysiology, University of Heidelberg, Germany, [2]Department of Neurology, University of Heidelberg, Germany, [3]Institute of Pathology and DFG Research Center for Regenerative Therapies, University of Technology Dresden, Germany

Upon ischemic stroke decline of cellular oxygen level initiates a complex endogenous response that prevents acute neuronal cell death and accounts for long-lasting cerebral regeneration. A multitude of factors involved in this response, including erythropoietin (Epo) and vascular endothelial growth factor (VEGF) are transcriptionally regulated by hypoxia-inducible factors (HIFs). During normoxia, a family of prolyl-4-hydroxylase domain (PHD) proteins hydroxylates HIF-αsubunits, resulting in their degradation. Recent studies have shown that pharmacological PHD inhibition can reduce infarct size after stroke. However, currently used inhibitors also influence the enzymatic activity of proteins others than PHDs and, moreover, non-selectively inhibit all PHD family members. Hence, we generated transgenic mice bearing a neuron-specific knockout of Phd2, the most abundant PHD family member in brain tissue, and studied the outcome from acute ischemic stroke in mice. Neuron-specific ablation of Phd2 significantly increased the protein stability of HIF-1αin forebrain and resulted in significantly enhanced expression of the neuroprotective HIF target genes Epo and VEGF, as well as glucose transporter and enzymes related to anaerobic glucose metabolism under hypoxic and ischemic conditions in vivo. Mice with Phd2-deficient neurons subjected to transient cerebral ischemia exhibited a reduction in infarct size by more than 50 %. In addition, cell death of highly vulnerable hippocampal CA1 neurons located in peri-infarct region was dramatically reduced in these mice. Further histological analysis revealed that vessel density in forebrain subregions, except for caudate-putamen, was not significantly different in nPhd2 knockout animals as compared to wild-type, strongly indicating that angiogenesis is not making a significant contribution to the reduction in infarct size. In conclusion, our findings denote that the endogenous adaptive response upon hypoxic/ischemic insults in the brain is at least partly dependent on the activity of HIFs, and identify PHD2 as the key regulator for the protective hypoxia response. The results suggest that specific inhibition of PHD2 may provide a useful therapeutic strategy to protect brain tissue from ischemic injury.

Strongly reduced establishment of blood outgrowth endothelial cell colonies in subjects with subclinical cardiovascular disease

Javier Martin-Ramirez[1]*, Menno Hofman[1], Maayke GM Kok[2], Esther Creemers[3], Joost Meijers[2], Jan Voorberg[1] and Sara-Joan Pinto[2,4]. [1]Department of Plasma Proteins, Landsteiner Laboratory of AMC and Sanquin Blood Supply Foundation, Amsterdam, The Netherlands, [2]Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands, [3]The Heart Failure Research Center, Academic Medical Center, Amsterdam, The Netherlands, [4]Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, Amsterdam, The Netherlands

Introduction: Atherosclerotic coronary artery disease (CAD) represents a major cause of death in developed countries. The clinical manifestation of CAD consists of acute or chronic heart-muscle damage caused by a decrease in supply of oxygen-rich blood. This sequel of events is due to the formation of atherosclerotic plaques resulting in the tightening of the lumen in the affected coronary vessels. Several cellular components, including endothelial cells from the arterial wall are involved in this process. Here, we established cultures of blood outgrowth endothelial cells (BOECs) from peripheral blood of patients with premature cardiovascular disease (CVD) and their first degree 'healthy' relatives. Methods: At the outpatient clinic of the Academic Medical Centre, Amsterdam, the Netherlands, patients with premature CVD (men <51 yr, women < 56yr) and their 'healthy' first degree relatives were seen. From 28 patients and 30 'healthy' first degree relatives blood was drawn for cultures of endothelial cells. Furthermore, all 'healthy' first degree relatives underwent multi-detector computed tomography (CT), to establish coronary calcification (CAC), as a marker of subclinical CVD. On average from 14 to 21 days after isolation and establishment of cultures, endothelial cell colonies were observed. These cultures were further propagated to establish their proliferative capacity and cryopreserved. Results: We included 28 patients and 30 'healthy' first degree relatives. Of these relatives, 20 (66.67%) present a normal CAC score and 10 (33.33%) display a CAC score > 80th percentile. We were able to establish BOEC colonies in 23 (82%) of the patients, in 16 (80%) of the relatives without subclinical CVD and in 5 (50%) of the relatives with subclinical CVD ($p=0.1085$). When concerning the ability of the isolated colonies to proliferate, we were able to expand colonies in 17 (60%) of the patients, in 13 (65%) of the relatives without subclinical CVD and in 2 (20%) of the relatives with subclinical CVD ($p<0.05$). Surprisingly, the success rate of the patients was practically the same as for the 'healthy' first degree relatives with normal CAC score, which might be due to statin therapy. Conclusion: These observations demonstrate that the number of circulating BOEC precursors in relatives with subclinical CVD is reduced when compared to relatives without subclinical CVD and patients. Furthermore, the proliferative capacity of the isolated endothelial cells was the same between relatives without subclinical CVD and patients but significantly different when compared to relatives with subclinical CVD. Our findings suggest that establishment of BOEC from individuals with subclinical atherosclerosis is impaired. The mechanism underlying the reduced frequency of BOEC precursors in the circulation of these subjects is currently under investigation.

Drug-induced macrophage autophagy in atherosclerosis: For better or for worse?

W. Martinet*, I. De Meyer, S. Verheye, D.M. Schrijvers, H. Bult, G. R.Y. De Meyer. Laboratory of Physiopharmacology, University of Antwerp, Belgium

Autophagy is a reparative, life-sustaining process by which cytoplasmic components are sequestered in double membrane vesicles and degraded upon fusion with lysosomal compartments. A growing body of evidence suggests that autophagy is present in advanced atherosclerotic plaques, even though its role in atherosclerosis remains poorly understood. Most likely, autophagy safeguards plaque cells against cellular distress, in particular oxidative injury, by degrading damaged intracellular material. In this way, autophagy is anti-apoptotic and contributes to cellular recovery in an adverse environment. Because basal autophagy can be intensified by specific drugs such as mammalian target of rapamycin (mTOR) inhibitors or Toll-like receptor 7 (TLR7) ligands, we investigated whether these drugs could promote a stable plaque phenotype. Stent-based delivery of the mTOR inhibitor everolimus in atherosclerotic plaques from cholesterol-fed rabbits triggered selective macrophage autophagy and led to a marked reduction in macrophage content via autophagic death without affecting smooth muscle cells (SMCs). This finding suggests that everolimus may be a promising compound for stabilization of vulnerable atherosclerotic plaques. However, local in vivo administration of TLR7 ligand imiquimod to rabbit plaques via osmotic minipumps induced macrophage autophagy, but without induction of cell death. Moreover, it triggered cytokine production, upregulation of vascular adhesion molecule-1, infiltration of T-lymphocytes, accumulation of macrophages and plaque enlargement. Treatment with dexamethasone suppressed these pro-inflammatory effects. In vitro treatment of macrophages with imiquimod caused activation of NF-kB and release of pro-inflammatory cytokines and chemokines independent of autophagy. To investigate whether these adverse effects were also associated with mTOR inhibition, macrophages in culture were either treated with everolimus or starved to inhibit mTOR. Everolimus led to inhibition of protein translation, activation of p38 MAPK and the release of pro-inflammatory cytokines (e.g. IL-6, TNFa) and chemokines (e.g. MCP1, Rantes) prior to induction of autophagic death. These effects were also observed with rapamycin, but not after starvation. Everolimus-induced cytokine release was unaffected by deleting the essential autophagy gene Atg7 in macrophages, suggesting autophagy-independent cytokine synthesis, but was inhibited when macrophages were co-treated with p38 MAPK inhibitor SB202190 or the glucocorticoid clobetasol. Combined stent-based delivery of clobetasol and everolimus in rabbit plaques downregulated TNFa expression as compared to everolimus-treated plaques, yet did not affect the ability of everolimus to induce macrophage clearance. The contradictory in vivo effects of everolimus and imiquimod might be misleading because the level of autophagy induced by both compounds in the plaque could be entirely different. Stents eluting everolimus may yield high local concentrations of the eluted drug which could stimulate autophagic macrophage death. In contrast, imiquimod delivered to the vessel wall via osmotic minipumps, may be rapidly diluted in the surrounding tissue. In vitro experiments demonstrated that high concentrations of imiquimod are needed to induce autophagic death in macrophages, whereas low concentrations already activate NF-kB and induce cytokine synthesis. We may therefore conclude that the stent-based release of everolimus promotes a stable plaque phenotype via selective induction of macrophage autophagic death. Moderate autophagy is induced in macrophages of imiquimod-treated plaques, which does not lead to cell death, but to prolonged NF-kB-dependent cytokine release and plaque inflammation.

The Eph/ephrin and ADAM work in concert to regulate pulmonary endothelial barrier against tumor cells

Yoshiro Maru*. Department of Pharmacology Tokyo Women's Medical University 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666 Japan

The Eph is the largest family of receptor tyrosine kinase and unique in that their ligands ephrin are also membrane-bound. The recent findings indicate their tight connection with vascular biology. For example, ephrinB2 is required for VEGFR2 to exert biological effects and knockout mice of EphA2 showed a decreased number of pericytes and enhanced microbe-induced lung permeability for leukocytes. We now show that EphA1, the prototype of the Eph family and originally identified by us (Science, 1987), is co-expressed with its sole ligand ephrinA1 in lung endothelial cells but not pericytes. Lung endothelial cells make cell-cell contacts by EphA1 binding to ephrinA1 in a tyrosine kinase-independent manner and participate in the establishment of a pulmonary endothelial barrier. EphA1 knockout mice revealed increased lung permeability as judged by Evans blue assay. We have previously shown that an artificially-produced soluble form of ephrinA1-Fc activated EphA1 tyrosine kinase, which in turn signals through integrin-linked kinase (ILK) and focal adhesion kinase (FAK) to cause defect in endothelial cell spreading (J.Cell Sci., 2009). EphrinA1-Fc also augments lung permeability in vivo, which efficiently allows tumor cell extravasation into the lungs. Furthermore, we have found a naturally-occurring soluble form of ephrinA1 in the serum of tumor-bearing mice. Yeast two-hybrid screening with extracellular domain of EphA1 as a bait identified ADAM12, which cleaves the membrane-bound form of ephrinA1 to the soluble form. Moreover, ephrinA1 shedding by ADAM12 was found to be up-regulated by TGFbeta. Given our previous findings that TGFbeta is partially required for primary tumor-induced expression of endogenous ligands for TLR4 in the pre-metastatic lungs (Nat.Cell Biol., 2006; Nat.Cell Biol., 2008) and that those ligands promote expression of ephrinA1 in endothelial cells (Adv Exp Med Biol., 2011), we suppose that the TGFbeta-ADAM-Eph/ephrin axis plays an important role in lung permeability and metastasis.

EGF-like domain 7 in the Placenta: Expression and role

Micol Massimiani[1]*, Lucia Vecchione[2], Francesca Amati[2], Heidi Stuhlmann[3] and Luisa Campagnolo[1]. [1]Department of Public Health and Cell Biology, University of Rome Tor Vergata, [2]Department of Biopathology, University of Rome Tor Vergata, [3]Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY, USA

In the present study we investigated the expression profile of Egfl7 (Epidermal growth factor-like domain 7) in the human placenta. Egfl7 has been identified in a genetic screen for endothelial restricted genes and encodes a secreted protein that is involved in angiogenesis [Fitch et al. 2004; Schmidt et al. 2007]. Non-endothelial expression of Egfl7 has been reported in primordial germ cells [Campagnolo et al. 2005], neural stem cells [Schmidt et al. 2009] and cancer cells [Huang et al. 2010; Diaz et al. 2008; Wu et al. 2009; Delfortrie et al. 2011]. In the present report we identify trophoblast cells as a novel source of Egfl7. In particular, we show by immunofluorescence analysis that EGFL7 is not exclusively expressed by endothelial cells of the fetal blood vessels but also by trophoblast cells of human placentas from first and third trimester of gestation. Trophoblast expression of Egfl7 was confirmed by co-localization studies with cytokeratins. In addition, we compared the expression profiles of Egfl7 between samples of physiologically developed placentas and placentas affected by pre-eclampsia (PE). The analysis was carried out on ten samples from healthy controls and ten from pre-eclampsia affected patients, all obtained by cesarean section during the third trimester of gestation. PE is a placentopathy that affects around 5-8% of all pregnancies worldwide and is characterized by inadequate cytotrophoblastic invasion of the spiral uterine arteries and the insufficient remodeling of the vascular structures that causes placental ischemia. Both immunofluorescence and real-time PCR analysis showed that Egfl7 is significantly reduced in PE placentas, while the expression of CD31 was unchanged. We also investigated the expression of Vegf, since conflicting data have previously been published for PE placentas [Sgambati et al. 2004; Chung et al. 2004; Park et al. 2010]. Similarly to Egfl7, an overall decrease of Vegf expression was detected in all pre-eclamptic samples compared to the healthy controls. In conclusion, this study represents the first evidence of trophoblast as a novel source of Egfl7; its reduced expression in PE placentas suggests a role for Egfl7 in placental development and in defective cytotrophoblastic invasion characterizing pre-eclampsia. On this premise, we investigated the role of Egfl7 in trophoblast cells using gain- and loss-of-function approaches in human choriocarcinoma cell line Jeg-3 and in primary cytotrophoblast cells. Lentiviral infections of both cell types were performed to obtain long-term Egfl7 overexpression or downregulation. Preliminary results suggest that Egfl7 positively regulates proliferation and migration of Jeg-3 and primary cytotrophoblast cells.

RSK4 regulates endothelial differentiation through suppression of cAMP/PKA-mediated Flk1 induction

Taichi Matsunaga[1,2]* Kohei Yamamizu[1], Kazuko Koshiba-Takeuchi[3,4], Mizuyo Kojima[3], Jun K.Takeuchi[3,4,5], Jun K. Yamashita[1,2]. [1]Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, [2]Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan, [3]Division of Cardiovascular Regeneration, Research Center for Epigenetic Disease, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 4Biological Sciences, Graduate School of Science, the University of Tokyo, 5 JST PRESTO

Endothelial cell (EC) differentiation is strictly regulated to generate functional blood vessel. To elucidate the mechanisms at the cellular and molecular level, we previously demonstrated that Flk1+ cells derived from ES cells serve as vascular progenitors and can constructively reproduce the early differentiation of both ECs and Mural cells (MCs; vascular smooth muscle cells and pericytes). We recently reported that cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling enhanced EC differentiation from Flk1+ vascular progenitors via induction of VEGF receptors expression. We also revealed that expression of Ets variant2 (Etv2), a key molecule for hemato-endothelial fate, is triggered by PKA signaling. To further dissect these sequential processes of vascular formation, we investigated a novel molecule target and its function during EC differentiation. We performed global gene expression analysis at various differentiation stages ES cells to vascular cells; undifferentiated ES cells, Flk1+ vascular progenitors, ECs and MCs. Then, we found that four isoforms of a serine/threonine kinase, p90 ribosomal protein S6 kinase (RSK), have a distinct expression pattern during EC and MC differentiation. RSK1 was predominately expressed in undifferentiated ES cells. RSK2 and RSK3 were highly expressed in ECs. RSK4 was expressed in Flk1+ vascular progenitors and diminished during EC differentiation, whereas it remained expressed in MCs. Although RSKs regulate diverse cellular processes such as proliferation and motility, the biological functions of RSKs in EC differentiation processes are still poorly understood. To investigate roles of RSKs in EC differentiation, we examined the effect of a broad-spectrum RSKs inhibitor, SL0101, in our ES cell system. SL0101 treatment enhanced VEGF receptor expression and EC differentiation specifically in the presence of the simultaneous cAMP signal activation. SL0101-elicited EC differentiation was abolished under condition of PKA catalytic subunit alpha (PKAc) knock down, suggesting that the regulation by RSK during EC differentiation is dependent on PKA pathway. To identify specific RSK isoform that is responsible to the regulation of EC differentiation, we investigated EC differentiation using shRNA against each RSK isoform. Whereas transfection of RSK1 to 3 shRNA did not affect the ratio of EC appearance, specific RSK4 knockdown in Flk1+ vascular progenitors reproduced the effects of SL0101 on EC differentiation via increasing Flk1 expression. Immunoprecipitation-western blot experiments indicated that RSK4 has a potential to bind with PKAc. RSK4 overexpression significantly decreased PKA activity, suggesting a direct negative effect of RSK4 on PKA activity. Furthermore, we performed an ex vivo whole embryo culture assay. Embryos cultured in the presence of SL0101 displayed an aberrant surface of yolk sac which was formed by drastically increased area of CD31+ vasculature in yolk sac. Taken together, our findings are first evidences that RSK4 is a novel negative regulator for EC differentiation through inhibition of PKA activity. RSK4 expression regulates endothelial cell fate through suppression of PKA-mediated Flk1 induction. Fine tuning of PKA and Flk1 signaling by RSK4 is critical to normal vascular development in mouse embryogenesis. Elucidation of the new-mode cell fate determination by RSK4 would provide novel insights in developmental biology, stem cell biology, and regenerative medicine.

Immortalized endothelial cell lines

Roland Schucht[1], Franziska Schuller[2], Jeannette Zauers[1], Tobias May[1]*. [1]InSCREENeX GmbH; Inhoffenstr. 7, 38124 Braunschweig, Germany, [2]Helmholtz Centre for Infection Research, Dept. Gene Regulation and Differentiation, Inhoffenstr. 7, 38124 Braunschweig, Germany

Endothelial cells are specialized cells that line blood vessels. They control blood pressure, form a barrier between vessel and surrounding tissue, are involved inflammation processes and form novel blood vessels. Dysfunction of the endothelium is observed in severe diseases like diabetes, hypertension, cancer and coronary artery disease. For the elucidation of novel targets or novel treatments a novel endothelial cell system is highly desirable. To be most effective and to identify relevant targets/drug candidates such a cell system should closely reflect the in vivo properties of endothelial cells. Therefore we have established novel endothelial cell lines with in vivo like properties. For this purpose, we identified genes that fully retain the functions of endothelial cells and induce their immortalization. Primary endothelial cells from the umbilical cord and from the skin were transduced with these immortalizing genes which led to the establishment of endothelial cell lines with a robust proliferation phenotype. The resulting cell lines were cultivated for more than nine months which corresponds to more than 120 cumulative population doublings. An in-depth characterization of these cell lines was performed side-by-side with primary cells and demonstrated that the established cell lines retained the expression of endothelial specific marker proteins as well as endothelial specific functions. Importantly, this phenotype was stable throughout the whole cultivation period. We regard these novel endothelial cell lines as physiological relevant in vitro test systems which can greatly support the drug discovery process.

Modeling angiogenesis in vitro: Comparing two human co-culture approaches

Belinda O'Clair[1,3], Ashley Wolfe[1,3], Tim Dale[2], Kevin Allard[1], Del Trezise[2], Vince Groppi[1], and Dyke P. McEwen[1]*. [1]Essen BioScience, Inc. Ann Arbor, MI, USA; [2]Essen BioSceince, Ltd. Welwyn Garden City, United Kingdom; [3]Authors contributed equally to this work

Angiogenesis is a multi-step, complex process regulated by growth factors, enzymes, and extracellular matrix molecules. In vivo, the angiogenic process involves multiple cell types acting in concert to cause endothelial cell proliferation, migration, differentiation, and, ultimately, micro-vascular arrays. Under pathologic conditions, changes in the micro-environment stimulate new vessel production. Anti-angiogenic therapies have shown promise at slowing disease, such as solid tumors, yet have proven transitory due to either inherent or acquired resistance. Advancement of in vitro angiogenesis models to study drug resistance and more complex pharmacologic paradigms, i.e. combination regimens, are paramount to developing the next generation therapeutics. Here, we investigate two different in vitro models of angiogenesis. The first model uses co-cultures of human umbilical vein endothelial cells (HUVEC) with normal human dermal fibroblasts (NHDF) and the other uses co-cultures of endothelial colony forming cells (ECFC) with adipose-derived stromal cells (ADSC). Both models form networks that phenotypically mimic in vivo microvasculature. When HUVEC are seeded with NHDF, the HUVEC recapitulate the major phases of the angiogenic process, initially proliferating and migrating into endothelial clusters followed by differentiation and anastomosis into complex networks over the 10-14 day assay. Co-cultures of ECFC with ADSC develop angiogenic networks over a much shorter, 4-day time course. Cell proliferation is not a significant factor in the ECFC/ADSC model. However, the stromal cells in this model do differentiate into pericytes as demonstrated by the expression of smooth muscle actin and PDGFR-β. As is the case in in vivo networks, pericytes have a supportive and trophic role in maintaining and protecting established microvascular networks. Data will be presented that highlight the vascular nature of these models, the ability to investigate multiple angiogenic pathways, and demonstrate the use of combination regimens to overcome resistance and restore anti-VEGF antibody sensitivity. Taken together, this study characterizes two translatable human models to study mechanism of action of pro- and anti-angiogenic factors and therapeutic agents in vitro.

Inflammatory mediators differentially regulate macro- versus micro-vascular endothelial cell survival

A C McSloy*, C E Farrar, E Garonna and CPD Wheeler-Jones NA. The Royal Veterinary College, London, United Kingdom

Endothelial cell (EC) functions during pathological and physiological angiogenesis are regulated by diverse mitogens and inflammatory mediators (e.g. growth factors, eicosanoids, adipokines and proteases) and ultimately depend upon the balance between proliferation and apoptosis. We have recently reported that leptin, an adipose-tissue derived cytokine, and thrombin, a multi-functional serine protease, both exert pro-angiogenic actions on human umbilical vein endothelial cells (HUVEC) through distinct pathways but whether these growth promoting activities are accompanied by modulation of apoptotic signaling is not defined. In the present study we have investigated the potential anti-apoptotic actions of leptin and thrombin on both macro-vascular (HUVEC) and micro-vascular ECs (human lung microvascular ECs; HLMEC). Leptin attenuated serum deprivation-induced HUVEC and HLMEC apoptosis in a concentration-dependent manner as assessed by quantification of calcein fluorescence in Calcein-AM loaded cells and by measurement of caspase-3/-7 activity. In parallel experiments leptin and thrombin promoted time- and concentration-dependent increases in phosphorylation of both ERK1/2 and Akt in HUVEC and HLMEC which were sustained for up to 30 minutes. Analysis of gene expression by PCR array (Human apoptosis RT2 Profiler™) showed that exposure to thrombin (2 hours) selectively up-regulated mRNA expression for BCl-2, BCl-XL and AIP1. Thrombin and leptin also significantly increased expression of the anti-apoptotic proteins, survivin and BCl-2 and reduced PARP cleavage in serum-starved HUVEC, further supporting roles in limiting apoptosis. Pharmacological blockade of MEK1/2 (PD184352) or Akt (Akti1/2) abrogated the ability of both leptin and thrombin to protect HUVEC from apoptosis, as assessed by measurement of caspase-3/-7 activity and induction of BCl-2 protein expression, supporting a role for these agonists in promoting EC survival by engagement of the MEK-ERK and Akt signaling pathways. In marked contrast to its protective effects in HUVEC, exposure of HLMEC to thrombin (0.01-3 U/ml) decreased cell viability (calcein fluorescence) to a level which was greater than that observed with serum starvation alone and, as measured by caspase-3/-7 activity, significantly increased apoptosis above that promoted by serum deprivation. In keeping with its pro-apoptotic actions on microvascular ECs thrombin also concentration-dependently suppressed basal tubulogenesis by HLMEC on Matrigel. Neutralization of thrombin with hirudin prevented the thrombin-stimulated increase in HLMEC caspase-3/-7 activity and reversed its attenuation of MLEC differentiation. Additional studies showed that thrombin-stimulated HLMECs exhibited weak and transient changes in ERK1/2 and Akt phosphorylation. Collectively, these data show that pro-angiogenic inflammatory mediators enhance cytoprotection by limiting EC apoptosis through regulation of anti-apoptotic protein expression and provide evidence for differential actions of thrombin on conduit vessel ECs versus microvascular ECs. These findings have relevance for understanding mechanisms of EC survival and angiogenesis in tissue microvasculature.

The angiogenic potential of Myeloid Angiogenic Cells (MACs) is regulated by cell density and associated alterations in the secretome

Reinhold J. Medina*, Christina L. O'Neill, Michele O'Doherty, Sarah EJ. Wilson, Jasenka Guduric-Fuchs, Alan W. Stitt. Centre for Vision and Vascular Science, School of Medicine, Dentistry, and Biomedical Science, Queen's University Belfast, United Kingdom, BT12 6BA.

Endothelial progenitor cells (EPCs) have the potential to re-vascularise ischaemic tissues. We recently demonstrated that a pro-angiogenic EPC subpopulation, termed MACs, show molecular and functional characteristics of alternative activated M2 macrophages. It has now been shown that MACs significantly promote vascular repair in the ischaemic retina by a paracrine effect. IL8 was identified as a critical component of MACs secretome, as IL8 antibody blocked MACs pro-angiogenic effects. IL8 is capable of inducing phosphorylation of VEGFR2 and ERKs independent of VEGF. A parallel, in vitro co-culture approach using MACs and retinal microvascular endothelial cells (RMECs) was used as an in vitro angiogenesis model to study the effects of different MAC densities. As expected, a ratio of 4/1 for RMECs/MACs respectively, significantly increased RMECs tube formation ($p<0.01$). However, higher density of MACs showed no pro-angiogenic effect. An angiogenesis protein array was used to assess conditioned media components. Interestingly, the MACs high density group secretome showed a specific upregulation of anti-angiogenic PTX3, PAI1, CXCL16; significant lower IL8 levels and an imbalance in the ratio MMP9/TIMP1. In conclusion, our data indicate that MACs induce angiogenesis by acting as M2 macrophages. However, the MAC phenotype is highly plastic and in certain conditions, such as high MAC densities, the cells alter their secretome profile by increasing anti-angiogenic cytokine release and therefore reduce their vasoreparative potential.

Regulation of cells shape in angiogenesis by the formin FMNL3

Hetheridge C, Scott AN, Swain RK, Copeland JW, Higgs HN, Bicknell R, Mellor H*.
University of Bristol, United Kingdom

The process of angiogenesis requires endothelial cells to undergo profound changes in shape and polarity. This must involve remodelling of the endothelial cell cytoskeleton; however, we know little of this process or of the proteins that control it. We used a co-culture assay of angiogenesis to examine the cytoskeleton of endothelial cells actively undergoing angiogenic morphogenesis. We find that elongation of endothelial cells during angiogenesis is accompanied by stabilisation of microtubules and their alignment into parallel arrays directed at the growing tip. In other systems, similar microtubule alignments are mediated by the formin family of cytoskeletal regulators. We screened a library of human formins and indentified the novel formin FMNL3/FRL2 as a critical regulator of endothelial cell elongation during angiogenesis. We show that activated FMNL3 triggers microtubule alignment and that FMNL3 is required for microtubule alignment during angiogenic morphogenesis. FMNL3 is highly-expressed in the endothelial cells of Zebrafish during development and embryos depleted for FMNL3 show profound defects in developmental angiogenesis that are rescued by expression of the human gene. We conclude that FMNL3 is a novel regulator of endothelial microtubules during angiogenesis and is required for the conversion of quiescent endothelial cells into their elongated angiogenic morphology.

The endothelial expressed chemokine CXCL16 (SR-PSOX) induced the adhesion and rolling of platelets: Comparision to fractalkine (CX3CL1) mediated adhesion

Sascha Meyer dos Santos[1]*, Nadejda Monsefi[2], Ute Klinkhardt[1], Sebastian Harder[1].
[1]Clinical Pharmacology, [2]Cardiovascular and Thoracic Surgery, Goethe University Hospital Frankfurt, Frankfurt, Germany

CXCL16 is an inflammatory chemokine that is expressed in membrane-anchored form on the endothelium in all stages of atherosclerosis and can mediate the adhesion of blood monocytes. To date a possible adhesive capacity of CXCL16 toward platelets is completely unexplored. The present study wishes to elucidate the mechanisms and relevance of endothelial CXCL16 in the accumulation of platelets at atherosclerotic plaques or predisposition sites. A flow-based adhesion assay was used to study the adhesion of platelets to immobilised CXCL16 in µ-slides under physiologic flow conditions. Platelets adhered negligible to immobilised CXCL16 at 150 s-1 wall shear rate. However, platelet adhesion to vWf (von Willebrand factor) (19,30 ± 4,053 adherent platelets at 600 s-1) was increased in the presence of CXCL16 (31,45 ± 6,028). Likewise, the number of rolling platelets was increased by the presence of CXCL16 in the adhesion matrix (116,1 ± 3,5 % of vWf mediated rolling at 600 s-1 and 116,4 ± 3,7 % at 1800 s-1). Control ELISA experiments confirmed that the presence of CXCL16 did not induce changes in vWf coating density (103.6 ± 0,9 % of vWf alone) or conformational changes in vWf (GPIb binding to vWf in the presence of CXCL16 was 98,3 ± 2,3 % of binding to vWf alone). Therefore we conclude that our observed effects are specifically attributable to CXCL16. Coating density in our µ-slides was 130 molecules CXCL16 per µm2 and therefore comparable to fractalkine (418 molecules per µm2), the only other chemokine beyond CXCL16 that can be expressed in membrane-anchored form and mediate platelet adhesion. Further experiments are underway to understand the molecular mechanisms by which CXCL16 mediate the adhesion and rolling of platelets on the endothelium and to shed light on the in vivo relevance of this new mechanism by which platelets accumulate on atherosclerotic plaques and compare the mechanistics to fractalkine mediated platelet adhesion. We hope the transmembrane chemokines CXCL16 and fractalkine might be important and valuable pharmacological targets in the futures to control atherosclerotic disease.

Proangiogenic gene expression level in Critical Limb Ischemia (CLI)

Michalowska Dagmara[1]*, Baczynska Dagmara[1], Skora Jan[2], Barc Piotr[2], Dobosz Tadeusz[1]. [1]Molecular Techniques Unit, Department of Forensic Medicine, Wroclaw Medical University, Poland, [2]Department and Clinic of Vascular, General and Transplantation Surgery, Wroclaw Medical University, Poland

Critical limb ischemia (CLI) is the most advanced form of peripheral arterial disease (PAD) and is associated with severe morbidity and mortality risks. The most common clinical symptoms of CLI are significant chronic ischemic at-rest pain, non-healing ulcers and gangrene of the affected foot. CLI is mostly caused by atherosclerotic lesions in arteries and vasculitis as well as thromboangiitis obliterans. The major problem in CLI disease is the failure of angiogenesis process and insufficient growth of new vessels. Therefore further understanding of the molecular basis of CLI is needed. In our project we evaluated the gene expression level of proangiogenic cytokines and their regulation. The aim of this study was to compare the profile of proangiogenic gene expression in popliteal arteries of patients with critical limb ischemia and in healthy donors. We examined the level of mRNA for 13 genes previously described as proangiogenic factors: vascular endothelial growth factor (VEGF), angiopoietin-1 (ANG-1), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), prostaglandin-I synthase (PGIS), hypoxia-inducible factor 1 alpha (HIF1α), hypoxia-inducible factor 2 alpha (HIF2 α), slit homolog 3 Drosophila (SLIT3), secreted frizzled-related protein 2 (SFRP2), vascular endothelial growth factor receptor (FLT1), fibroblast growth factor receptor 2 (FGFR2), hepatocyte growth factor receptor (MET) and tyrosine kinase (TEK). Popliteal arteries from Hunter's canal were collected during limb amputation from 40 patients suffering from CLI and from 14 healthy donors during multi-organ transplantation. Total RNA was extracted and mRNA level was analyzed by real-time RT PCR using TaqMan Gene Expression Assay. We have no observed any differences between mRNA level of examined cytokines in patients suffering from CLI and in healthy donors. However the mRNA level for secreted frizzled-related protein 2 (SFRP2) and vascular endothelial growth factor receptor (FLT-1) was increased 5,19-fold and 2,84-fold respectively in CLI. Our study will provide a new insight into molecular events responsible for development of critical lower limb ischemia and will improve therapeutic angiogenesis strategies. This study has been funded by Grant of the Ministry of Science and Higher Education, Poland, nr N N402300636. „Polymorphism and methylation status of gDNA in patients with critical lower limb ischemia. Identification of molecules responsible for angiogenesis process".

VEGF-mediated activation of calcineurin-NFAT-DSCR-1 axis in lung endothelium is critical for the establishment of lung metastases

Takashi Minami[1]*, Jun-ichi Suehiro[1], Tsuyoshi Osawa[1], William C. Aird[2], and Sandra Ryeom[3]. [1]The Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, 153-8904 Japan, [2]Department of Medicine, Center for Vascular Biology Research, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, MA 02215, [3]Department of Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104 USA

The pre-metastatic niche is thought to be a future site for metastatic tumor growth, primed and awaiting the influx of tumor cells for seeding and growth. The presence of VEGF receptor (VEGFR) 1-positive bone marrow-derived progenitor cells as well as myeloid cells has been shown to be critical for the establishment of metastatic lesions. While there have been a number of cell types recruited to the pre-metastatic niche, the role of the vasculature at these distant sites has not been extensively examined. We have previously shown that the calcineurin-NFAT pathway is a critical intracellular mediator of VEGF signaling in endothelial cells. VEGF activation of calcineurin-NFAT leads to the expression of pro-angiogenic and pro-inflammatory targets and also induces the short form of Down Syndrome Critical Region-1 (DSCR-1s), an endogenous inhibitor of calcineurin. This transactivation of DSCR-1s forms a negative feedback loop modulating NFAT-dependent angiogenesis. In this study, we show organ specific activation of calcineurin-NFAT-DSCR-1s signaling in the lung vasculature but not in liver or kidney leading to preferential metastasis to the lung. Using genetically engineered mouse models with DSCR-1 loss and DSCR-1 overexpression, we first inoculated our Dscr-1-null mice by systemic, subcutaneous and orthotopic implantation of melanoma, Lewis lung carcinoma and Renal cell carcinoma cells, respectively. Our studies reveal the formation of a pre-metastatic niche specifically in the lung. These sites were characterized by the presence of VEGFR2-positive endothelial cells and preceded increased lung metastases with no correlation to primary tumor size. To test whether endothelial overexpression of DSCR-1s was sufficient to suppress lung metastases, we examined our DSCR-1s transgenic mouse driven by the Tie2 promoter. Both the growth of transplanted tumors and lung metastases were dramatically inhibited in this model when DSCR-1s is constitutively overexpressed in the endothelium, due to the inhibition of NFAT activation. Further, our studies reveal significant upregulation of Angiopoietin (Ang)-2 in Dscr-1-null, which we show here to be an NFAT-dependent target. In contrast, Ang-1 and Tie2 were severely declined after the tumor metastasis in lung from Dscr-1-null mice, leading the reduction of vessel-integrity. Treatment of Dscr-1-null mice with a soluble Ang-2 receptor, soluble Tie2 (sTie2), decreased the incidence of lung metastases and suppressed the migration of tumor-associated myeloid cells to the lung. Collectively, these data suggest that inhibiting calcineurin-NFAT activation in the lung vasculature may block lung metastases and that sequestration of the NFAT target Ang-2 may be sufficient for this effect. Thus our studies provide new insights into the formation of the VEGF-VEGFR2 pre-metastatic niche in the lung and offers DSCR-1s and sTie2 as new therapeutic targets for lung metastases.

Distinct profiles of angiogenic mediators produced by mesenchymal stem cells of patients with chronic heart failure and co-morbidities

I. Minullina*, R. Dmitrieva, I. Meln, P. Butylin, S. Anisimov, A. Zaritskey. Almazov Federal Heart, Blood and Endocrinology Centre, Saint-Petersburg, Russia

Stimulation of angiogenesis represents one of the mechanisms contributing to regenerative action of mobilized or resident stem cells. There is accumulating evidence that mesenchymal stem cells (MSC) mediate angiogenesis and arteriogenesis via the release of paracrine factors. The goal of this study was to investigate paracrine-mediated properties of MSC in patients with heart failure (HF) and comorbid diabetes mellitus (DM) and/or obesity (O) compared to healthy donors (HD). Bone marrow (BM) and subcutaneous adipose tissue (Ad)-derived MSC cultures were evaluated at passage 3 for production of key factors involved in neovascularisation. The concentration of Angiopoietin-2 (Ang2), HGF, VEGF, TGFb1, MCP-1, SDF-1, IL-8, IL-1b, PDGF-BB, TNFa in conditioned medium (CM) of MSC cultured for 48 h was quantiï¬ed via ELISA or Bioplex. VEGF and TGFβ1 secretion did not differ in BM-MSC derived from HF patients compared to HD. The amount of immunoreactive Ang2, HGF, MCP-1 and IL-8 was higher in BM-MSC in all HF groups, though the difference was not always significant (251.9±34.4 vs 123.0±43.7 pg/ml, p=0.09 for Ang2; 3100±363.8 vs 1313±433.3 pg/ml, p=0.02 for HGF; 597.0±32.5 vs 428.5±63.4 pg/ml, p=0.02 for MCP-1; 460.0±127.2 vs 56.2±10.5 pg/ml, p=0.12 for IL-8). VEGF, IL-8, and MCP-1 secretion was down-regulated in Ad-MSC of patients with isolated HF compared to HD (1353.7±161.2 vs 2385.8±262.4 pg/ml for VEGF, p=0.003; 157.9±37.7 vs 471.5±71.4 pg/ml for IL-8, p=0.0006; 230.6±41.1 vs 340.4±24.7 pg/ml for MCP-1, p=0.08). The level of TGFβ1 released by Ad-MSC was unchanged in all HF groups compared to HD. Ad-MSC of HF subjects with comorbid obesity produced higher amounts of immunoreactive Ang-2 compared to non-obese patients (38.3±4.9 vs 19.6±6.5 pg/ml, p=0.05). There was a tendency to decreased SDF-1 secretion in HF+O+DM group compared to HD in both MSC populations (463.5±161.9 vs 660.5±169.7 pg/ml for Ad-MSC, p=0.4; 1566.6±549.3 vs 2071.4±389.4 pg/ml for BM-MSC, p=0.4). IL-1b, PDGF-BB, and TNFa could not be detected in the CM from either of the MSC populations. Our data show that two populations of MSC of HF patients with comorbid conditions display differences in secretion of paracrine factors implicated in neovascularisation. There is a common tendency to increased BM-MSC secretion of proangiogenic mediators in all HF groups compared to HD, and a tendency to decreased Ad-MSC release of angiogenesis-modulating factors in patients with isolated HF. However, TGFβ1 secretion is unchanged in both MSC populations derived from HF patients. These findings suggest that proangiogenic effects mediated by MSC mobilization may be altered in patients with HF suffering from DM and obesity. Knowledge of paracrine mediator secretion profiles of MSC derived from patients with HF can provide insight into ways of modifying these cells ex vivo to enhance their therapeutic potential.

Retinoic acid induces blood-brain barrier development

Mark R. Mizee[1]*, Desiree Wooldrik[1], Kim A.M. Lakeman[1], Bert van het Hof[1], Joost A.R. Drexhage[1], Dirk Geerts[2], Marianna Bugiani[3], Eleonora Aronica[4], Reina E. Mebius[1], Alexandre Prat[5], Helga E. de Vries[1], and Arie Reijerkerk[1]. [1]Blood-Brain Barrier Research Group, Department of Molecular Cell Biology and Immunology, VU university medical center, Amsterdam, The Netherlands, [2]Department of Pediatric Oncology/Hematology, Sophia Children's Hospital, Erasmus University Medical Center, Rotterdam, The Netherlands, [3]Department of Pathology, VU university medical center, Amsterdam, The Netherlands, [4]Department of Neuropathology, Academic Medical Center, University of Amsterdam, The Netherlands, [5]Neuroimmunology Research Laboratory, CHUM Notre-Dame Hospital, Faculty of Medicine, Université de Montréal, Montréal, Quebec, Canada

The blood-brain barrier (BBB) is crucial in the maintenance of a controlled environment within the brain to safeguard optimal neuronal function. The endothelial cells (ECs) of the BBB possess specific properties which restrict the entry of cells and metabolites into the central nervous system (CNS). The specialized BBB endothelial phenotype is induced during neurovascular development by surrounding cells of the CNS. However, the molecular differentiation of the BBB endothelium remains poorly understood. Retinoic acid (RA) plays a crucial role in the brain during embryogenesis. Since radial glial cells supply the brain with RA during the developmental cascade and associate closely with the developing vasculature, we hypothesize that RA is important for the induction of BBB properties in brain ECs. Analysis of human post-mortem fetal brain tissue shows that the enzyme mainly responsible for RA-synthesis, retinaldehyde dehydrogenase, is expressed by radial glial cells. Moreover, pharmacologic inhibition of RAR activation during the differentiation of the murine BBB resulted in leakage of serum proteins into the developing brain and reduced the expression levels of important BBB determinants. In addition, the most important receptor for RA-driven signaling in the CNS, RA-receptor beta (RARbeta), is markedly expressed by the developing brain vasculature and not detectable in post-natal stages. Our findings have been further corroborated by in vitro experiments showing RA and RARbeta dependent induction of different aspects of the brain endothelial cell barrier. Together, our results point to an important role for RA in the induction of the BBB during human and mouse development.

In vivo Tie2 downregulation induced by lipopolysaccharide is caused by haemodynamic effects and is controlled by NF-κB activation

Neng F. Kurniati[1], Matijs van Meurs[1,2], Rianne M Jongman[1,3], Franziska vom Hagen[4], Guido Krenning[1], Jan-Renier A.J. Moonen[1], Martin C. Harmsen[1], Michel M.R.F. Struys[3], Hans-Peter Hammes[4], Jan G. Zijlstra[2], Peter Heeringa[1], Grietje Molema[1]*. [1]Department of Pathology and Medical Biology, Medical Biology Section, [2]Department of Critical Care, [3]Department of Anesthesiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, and [4]5th Medical Clinic, University-Clinic Mannheim, Medical Faculty of the University of Heidelberg, Heidelberg, Germany

Rationale: Tie2 is predominantly expressed by endothelial cells and involved in vascular integrity control. Changes in Tie2 expression during endotoxemia (van Meurs et al, AJP Renal 2009) may contribute to loss of microvascular integrity and increased inflammatory activation. Understanding the kinetics and molecular basis of these changes may assist in the development of therapeutic intervention to counteract the detrimental microvascular dysfunction in endotoxemia and shock. Objective: To investigate the molecular mechanisms underlying the changes in Tie2 expression upon lipopolysaccharide (LPS) challenge in vivo in mice. Methods and Results: Eight hours after LPS challenge, Tie2 mRNA loss was observed in all major organs, while loss of Tie2 protein was predominantly observed in lungs and kidneys, a similar loss could be induced by secondary cytokine TNF-α. Ang2 protein administration did not affect Tie2 protein expression nor was Tie2 protein rescued in LPS-challenged Ang2-deficient mice, excluding a major role for Ang2 in this process. In vitro, endothelial loss of Tie2 was observed upon lowering of shear stress, not upon LPS and TNF-α stimulation, suggesting that inflammation related hemodynamic changes play a major role in loss of Tie2 in vivo. In vitro, this loss was partially counteracted by pre-incubation with NF-κB inhibitor BAY11-7082, an effect that was further substantiated in vivo by pre-treatment of mice with the NF-κB inhibitor prior to the inflammatory challenge. Conclusions: Loss of Tie2 mRNA and protein in the microvasculature in vivo upon LPS challenge is an indirect effect caused by a change in endothelial shear stress. No role for Ang2 was observed. The loss of Tie2 mRNA, not Tie2 protein, is controlled by NF-κB signaling that is likely induced by the diminished shear stress.

Endothelial-to-mesenchymal transition underlies atherosclerosis

Jan-Renier A.J. Moonen[1]*, Ee Soo Lee[1], Marc Schmidt[2], Monika Maleszewska[1], Theo G. van Kooten[3], Clark J.A.M. Zeebregts[4], Guido Krenning[1], Martin C. Harmsen[1]. [1]Cardiovascular Regenerative Medicine Research Group (CAVAREM), Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands, [2]Zentrum für Dermatologie, Venerologie und Allergologie, Universitätsklinikum Gießen, Justus-Liebig-Universität Gießen, Gießen, Germany, [3]Department of Biomedical Engineering, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, [4]Department of Surgery, Division of Vascular Surgery, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Atherosclerosis is characterized by formation of neointimal lesions comprised of smooth muscle-like cells. It has long been thought that these cells arise through migration and proliferation of smooth muscle cells from the media. In line with the 'response-to-injury' hypothesis this process is thought to be initiated by endothelial dysfunction. However, to date, a role for the endothelium as a primary source of smooth muscle-like cells has been largely disregarded. Here we show that endothelial-to-mesenchymal transition (EndMT) underlies neointima formation. In both physiological and experimentally induced models of atherosclerosis, as well as in clinical samples, we found neointimal cells of endothelial origin. The presence of neointimal lesions is known to depend on local differences in fluid shear stress. By flow modulation in vivo and in vitro we found a key role for high laminar shear stress in inhibition of EndMT through activation of MEK5/ERK5 signalling. Our results demonstrate that the endothelium can act as a source of neointimal cells through EndMT and how shear stress modulates this process. These findings provide new insights in the basic understanding of atherosclerosis and challenges the long standing dogma of the primary role of media-derived smooth muscle cells in this disease. This provides new perspectives in treatment of atherosclerosis, for example through modulation of MEK5/ERK5 signalling.

Apoptosis is critical for vascular inflammation and aneurysm progression in a murine abdominal aortic aneurysm model

Stephanie Morgan*, Dai Yamanouchi, Calvin Harberg, Bo Liu. Department of Surgery at University of Wisconsin-Madison in Madison, WI, USA

Objective: The presence of apoptotic markers is a prominent histological feature of abdominal aortic aneurysm (AAA). Previously, our group has shown that inhibition of apoptosis in the angiotensin II model of AAA in mice diminishes pro-inflammatory signaling and thus aneurysm development. To further explore the hypothesis that apoptosis of aortic smooth muscle cells (SMCs) contributes to pathogenesis of aneurysm progression through promoting vascular inflammation, we sought to enhance SMC apoptosis by modifying the mouse CaCl2 model. Methods and Results: Treatment of cultured aortic SMCs with a sequential application of CaCl2 and PBS to produce CaPO4 induced significant apoptosis as identified by flow cytometry (61% increase as compared to PBS control). Apoptosis induction was attenuated by blocking CaPO4 crystal formation with sodium pyrophosphate (53% decrease as compared to CaPO4 treatment). In vivo, CaPO4 crystal formation was similarly produced by CaCl2 and PBS applied to the adventitial surface of abdominal aorta. Compared to CaCl2, CaPO4 induced a faster growing aneurysm. At day 7 post injury when the CaCl2 treated-vessels showed no significant aneurysmal dilation, the CaPO4-treated vessels exhibited a 1.99 ± 0.14-fold expansion in diameter with massive apoptosis of SMCs and inflammatory infiltration as well as severe degradation of elastin fibers. Since both treatments caused a comparable degree of elastin damage initially, we postulated that the accelerated aneurysm progression seen in the CaPO4-treated model is due to the greater magnitude of apoptosis induced by the CaPO4 crystal. To test this hypothesis, we administered daily injection of the pan-caspase inhibitor QVD-OPh to the peritoneal cavity of mice for 7 days starting 6 hours before aneurysm induction. Animals were sacrificed at 7 and 42 days after surgery. At 7 days, QVD-OPh-treated mice exhibited minimum aortic expansion with profound decrease in SMC apoptosis and inflammatory infiltration. More remarkably, the protective effect of caspase inhibitor persisted to 42 days, 3 weeks after the drug treatment halted: QVD-OPh-treated mice displayed reduced formation of aneurysm (1.61 ± 0.11 vs. 2.39 ± 0.31 as compared to DMSO-treated control). Conclusion: Our data show the CaPO4 model to be an accelerated murine AAA model, with enhanced apoptosis as a mechanism. Furthermore, these results demonstrate the importance of apoptosis in vascular inflammation, likely through stimulation of cytokine production.

NRP1 negatively regulates VEGF-induced angiogenesis and tumor growth in trans

Sina Koch, Eric Morin*, Laurens van Meeteren, Chiara Testini, Sebastien Le Jan, Niklas Friberg, Lena Claesson-Welsh. Uppsala University, Dept Immunology, Genetics and Pathology, The Rudbeck Laboratory, Dag Hammarskjöldsv. 20, 751 85 Uppsala, Sweden

Vascular endothelial growth factor A (VEGFA) plays a central role in vascular development and angiogenesis. VEGFA induces complex formation between VEGFR2 and its membrane bound coreceptor Neuropilin-1 (NRP1; see Koch Biochem J. 2011 Jul 15;437(2):169-83). We have previously shown that NRP1 is essential for the 3-dimensional organization of endothelial cells into vessels in mouse embryoid bodies, zebrafish and subcutaneous matrigel plugs in mice (Kawamura et al., Blood. 2008 Nov 1;112(9):3638-49). NRP1 is expressed on endothelial cells but also on a range of other cell types such as tumor cells. We therefore asked, whether VEGFA could be presented to VEGFR2 in trans by NRP1 expressed on adjacent cells. Here, we have identified the distinct consequences of VEGFA-induced VEGFR2-NRP1 complexes in trans (i.e. individual cells express either VEGFR2 or NRP1) and in cis (VEGFR2 and NRP1 expressed on the same cell) in vitro and in vivo. In situ proximity ligation assays showed that trans-complexes formed with slower kinetics and were arrested/trapped in the plasma membrane, while cis-complexes formed more rapidly and were internalized. These spatial and temporal differences in complex formation had consequences for downstream signaling, manifested as prolonged PLCγ and ERK2 activation. Moreover, cis-complexes, but not trans-complexes, mediated activation of ERK1. We suggest that NRP1 in trans retains VEGFR2 at the cell surface, where it can activate PLCγ and ERK2, while ERK1 requires VEGFR2 internalization to be activated. Furthermore, we investigated the in vivo consequences of trans-complex formation using an endothelial cell-specific inducible NRP1 knockout mouse. Subcutaneous tumor growth (T241 fibrosarcoma) was studied under conditions where NRP1 was expressed or not in tumor endothelial cells and/or tumor cells. In sharp contrast to cis, NRP1 expression in tumor cells (trans) inhibited initiation of tumor formation. To further investigate tumor angiogenesis independent of tumor initiation, we measured vessel density in tumors growing in subcutaneous matrigel plugs. In the matrigel plugs, vessel density was significantly decreased in trans compared to cis. We suggest, that spatiotemporal alterations in trans complex formation and subsequent signal transduction contribute to decreased vessel ingrowth resulting in reduced tumor formation. Interestingly, T241 tumor cells expressing NRP1 lacking the C-terminal tail (NRP1ΔC) also formed trans-complexes with VEGFR2 (transΔC), but did not inhibit tumor initiation, as compared to cis tumors. The underlying mechanism involves a faster release of VEGFA from NRP1ΔC as compared with full length NRP1. We propose that NRP1 in trans is a negative regulator of VEGFA-induced VEGFR2 signaling, and that this regulation requires the C-terminal domain of NRP1. This research was supported by a fellowship to LAvM by the Dutch Cancer Society and by a grant to LCW from the Swedish Cancer Society, the Swedish Science Council and the Wallenberg Foundation.

Microglia modulate vascularization in glioblastoma multiforme

Annett Müller[1]*, Susan Brandenburg[1], Christin Schmidt[1], Kati Turkowski[1], Frank L. Heppner[2], Peter Vajkoczy[1]. [1]Charité Universitätsmedizin Berlin, Department of Neurosurgery, Institute of Experimental Neurosurgery, [2]Charité Universitätsmedizin Berlin, Department of Neuropathology, Institute of Neuropathology, Berlin, Germany

Microglia are the brain's resident immune cells and are important for maintaining the integrity of the central nervous system (CNS). Although their function during many CNS pathologies is well characterized, their role in tumour progression is still under discussion. Among brain tumours, glioblastoma multiforme is the most malignant one and treatment is often not successful. In our research, we are interested in identifying the specific part of microglia in tumour angiogenesis and tumour growth. Therefore, we implanted syngeneic GL261 cells intracranially into CD11b-HSVTK and wildtype mice and evaluated microglia depletion, vessel density, maturation of vessels and infiltration of immune cells by immunofluorescence stainings. Furthermore, we established tube formation assays of endothelial cells together with primary microglia analysing their interaction in vitro. CD11b-HSVTK transgenic mice express the thymidin kinase of herpes simplex virus in macrophages, microglia and granulocytes. Applying the antiviral drug Ganciclovir via an osmotic pump into the ventricle, microglia were ablated. In our model we established approximately 70% depletion of Iba-1+ cells in the mouse brain. Surprisingly, in the tumour area the microglia number decreased only about 20-40%. Although the depletion was low the vessel density was reduced by 50%. Nevertheless the vessel covered area remained constant and maturation of the blood vessels was unchanged. However, the tumour size was slightly reduced. Furthermore, other immune cells from the periphery were not replacing the killed microglia in the transgenic mice. Performing tube formation assays of endothelial cells in co-culture with primary microglia we observed that tube size as well as number of tubes and intersections were raised over time. Our studies suggest that microglia can positively affect tumour vascularization and thereby facilitating tumour progression. Consequently, blocking the pro-angiogenic functions of microglia may be a new approach in tumour therapy.

AGGF1 and the role of PI3K/Akt signaling in endothelial cells and vascular smooth muscle cells - a new angiogenic factor on old paths

S. Müller*, S. Eschricht, K.-U. Jarr, H.A. Katus, E. Chorianopoulos. Department of Cardiology, University Hospital Heidelberg, Heidelberg, Germany

Insufficient or aberrant angiogenesis is responsible for various diseases. AGGF1 (Angiogenic Factor with G-patch and FHA domain 1) was identified as susceptibility gene after mutation analysis of patients suffering from Klippel-Trenaunay syndrome (KTS), a rare congenital disease harbouring malformations of both blood and lymph vessels as well as abnormal growth of soft and bone tissue. In this disease, gain of function mutation of AGGF1 was shown to lead to excessive angiogenesis. In order to further characterise the yet unknown biological and functional traits of AGGF1, we studied its molecular background and performed functional analyses. Initially, we obtained an overview over the localisation of the molecule by analysing its distribution in different murine tissues via immunofluorescence. Thereby, we detected an intensive accumulation of AGGF1 in small arterial, but interestingly to a lesser extent in venous vessels. Thereafter we investigated the molecular signature of AGGF1 in cell lines contributing to angiogenesis (endothelial and vascular smooth muscle cells). After transfection and stimulation of the cells with AGGF1 we identified the involvement of PI3K/Akt pathway in AGGF1 signaling by Western blotting. Intriguingly, specific inhibition of PI3 kinase activity in the above mentioned cell lines proved the Akt pathway to be the main target of AGGF1. By performing cell viability and proliferation assays (MTS assay and BrdU labeling) with transfected cells we further demonstrated the proangiogenic function of the molecule in vitro. Furthermore, we unraveled a link of AGGF1 to another important proangiogenic factor, that also operates via the Akt pathway, - Platelet derived growth factor B (PDGF-B). Transfection of murine vascular smooth muscle cells with AGGF1 caused a downregulation of PDGF receptor beta on mRNA level, whereas the receptor`s ligand PDGF-B itself wasn't differentially expressed between transfected and non-transfected samples, thus implicating that AGGF-1 does not act via PDGF-B itself. On the contrary, when stimulating or transfecting the cells with PDGF-B, we did not observe any changes in the expression levels of AGGF1. In conclusion, we not only created an appropriate in vitro model of AGGF1, but also established the basis for future in vivo experiments. By identifying the Akt pathway as the main molecular signature of AGGF1, proving its angiogenic potential and pinpointing relevant interaction partners, we introduce novel potential therapeutic targets for vascular diseases.

A novel mode of VE-cadherin endocytosis controls VE-cadherin recycling

Takashi Sakurai, Masahiro Murakami*. Cardiovascular Research Center and Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, USA

While active remodeling of endothelial cell-cell junctions is important for the maintenance of vascular homeostasis, it also controls basal and acute vascular permeability by disrupting and reassembling cell junctions. VE-cadherin which mediates cell-cell adhesion by forming homophilic binding at the extracellular domain is a central player regulating these processes. Endocytic turnover of VE-cadherin plays a critical role in junction dynamics; however, its precise regulation is not well characterized. To understand the mechanism of VE-cadherin endocytosis, we designed various VE-cadherin constructs fused with fluorescent proteins and analyzed their trafficking in live cells using spinning disk confocal microscopy. By mixing two populations of cells expressing VE-cadherin-GFP and VE-cadherin-RFP, respectively, we found that internalized vesicles contain VE-cadherin derived from neighboring cells. This suggests that VE-cadherin is internalized together with the counterpart VE-cadherin molecule without disrupting hemophilic interactions. The internalized vesicles containing VE-cadherin from the neighboring cell colocalize with EEA1 and Rab5, and then traffic with Rab11 with a small subset being detected in Rab7-positive endosomes. These VE-cadherin-positive vesicles appear to contain other transmembrane proteins such as connexin 43. This mode of internalization does not occur in cells not expressing VE-cadherin at adherens junctions, indicating it is dependent on the formation of VE-cadherin-based junctions between two neighboring cells. Together, our data indicate that VE-cadherin is internalized from cell-cell contacts without dismantling the homophilic interaction with the VE-cadherin counterpart expressed on the opposing cell. The complex undergoes transportation via the conventional endocytic pathway and traffics back to the membrane, implying the role in VE-cadherin recycling and cell-cell junction remodeling.

Prostaglandin D2-DP signal promotes endothelial barrier function via cAMP/PKA/Rac1 pathway

Koji Kobayashi[1], Yoshiki Tsubosaka[1], Masatoshi Hori[1], Shuh Narumiya[2], Hiroshi Ozaki[1], Takahisa Murata[1]*. [1]Department of Veterinary Pharmacology, The University of Tokyo . [2]Department of Pharmacology, Faculty of Medicine, Kyoto University, Japan

its mechanism is unclear. We here investigated the signal pathway underlying PGD2-mediated barrier promotion in endothelial cells (HUVECs). PGD2 or its receptor (DP) agonist, BW245C increased transendothelial electrical resistance (TER) and decreased dextran permeability in ECs, both indicating the decrease in endothelial permeability. Pretreatment with a DP antagonist (BWA868C) abrogated the both PGD2- or BW245C-induced TER increase. Immuno-staining showed that BW245C caused cytoskeletal rearrangement of ECs including the formation of cortical actin rim and the assembly of adherens junction. These phenomena were accompanied with Rac activation in ECs. Consistently, pretreatment with a Rac inhibitor (NSC-23766) abolished the BW245C-induced TER elevation and cytoskeletal rearrangement. We next investigated the signal pathway underlying DP receptor-mediated endothelial barrier enhancement. BW245C increased intracellular cAMP level. cAMP elevation is known to activate protein kinase A (PKA) and/or exchange protein directly activated by cAMP 1 (Epac1) to represent its physiological function. Pretreatment with cell permeable PKA inhibitory peptide attenuated BW245C-induced TER increase, while gene depletion of Epac1 did not influence the effect of BW245C. We further confirmed that pretreatment with PKI significantly inhibited BW245C-induced Rac1 activation. In vivo, application of croton oil and histamine caused vascular leakage indexed using dye extravasation in DP naïve (WT) mice. DP deficient mice exhibit greater permeability compared with WT mice. Pretreatment with BW245C attenuated the dye leakage in WT mice but not in DP deficient mice. Additional treatment with PKI significantly reduced DP-mediated barrier enhancement. These results showed that PGD2-DP signal stimulates cAMP/PKA-dependent, Epac1-independent Rac1 activation that results in endothelial barrier enhancement.

Alternative splicing in the vascular response to pathological shear stress

Patrick Murphy*, Richard Hynes. MIT - Koch Institute for Integrative Cancer Research

Frictional forces exerted by blood flow on the vascular endothelium play an essential role in a range of vascular pathologies, most notably atherosclerosis and aneurysm. In both of these diseases, a switch from normal laminar flow to low and reversing flow is believed to drive progression. Although some of the immediate shear-responsive signaling pathways have been identified, the downstream signaling which coordinates the vascular response remains unclear. We hypothesize that alternative splicing is an important mechanism in the vascular response to low and reversing flow, and have focused on fibronectin (FN), a critically important and alternatively spliced extracellular matrix protein, as an archetype of this mechanism. Alternatively spliced isoforms of FN, which include EIIIA and EIIIB domains, are highly expressed in atherosclerosis, aneurysm and pathological vascular remodeling. Normally absent in adult vasculature, these isoforms are abundant in developing vasculature across species, including chicken, mice, and humans. Impaired EIIIA inclusion has been linked to an elevated risk for aneurysm growth in a genetically susceptible patient group. While genetic mutations that prevent the formation of the isoforms containing EIIIA and EIIIB inhibit developmental vascular remodeling in mice, the role and regulation of alternative FN splicing in aneurysm progression remains unclear. Here, we present data that low and reversing flow promotes the inclusion of EIIIA and EIIIB domains through alternative splicing of fibronectin in the endothelium in vivo. This occurs as early as 48hrs after the switch from laminar flow to low and reversing flow. Surprisingly, this occurs without a detectable increase in total FN production at this early time point. Furthermore, in a genetically engineered mouse model expressing constitutive forms of FN but unable to include the EIIIA and EIIIB domains, exposure of the carotid artery to low and reversing flow through surgical intervention results in defects in vascular remodeling resembling aneurysm and vessel dissection. Our results suggest that alternative splicing of FN is an early and important mediator of the vascular response to low and oscillatory flow conditions. Ongoing work is aimed at determining the mechanism underlying the aneurysm phenotype and the regulation of alternative splicing in the vascular response to low and oscillatory flow.

Identification and characterization of a resident vascular stem/progenitor cell population in preexisting endothelial cells

Hisamichi Naito*, Hiroyasu Kidoya, Susumu Sakimoto, Taku Wakabayashi, Kazuhiro Takara, Nobuyuki Takakura. Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Japan

The existence of vessel-resident endothelial stem cells remains a matter of debate. Here, we identify a CD31+CD45-endothelial progenitor/stem-like population located at the inner surface of preexisting blood vessels using the Hoechst method in which stem cell populations are identified as side populations (Naito et al, EMBO 2012). This population is dormant in the steady state but possesses colony-forming ability, produces large numbers of endothelial cells (ECs) and when transplanted into ischemic lesions, restores blood flow completely and reconstitutes de-novo long-term surviving blood vessels. Moreover, although surface markers of this population are very similar to conventional ECs and they are committed to endothelial linage confirmed by VE cadherin-GFP mouse, the gene expression profile is completely different. Our results presented in this study support the existence of a CD31+CD45− side population in mouse vascular endothelia that carry features of endothelial stem cells/progenitors and this heterogeneity of ECs may lead to the identification of new targets for vascular regeneration therapy.

Modification of a novel angiogenic peptide, AG30, for the development of novel therapeutic agents

Hironori Nakagami[1]*, Yasufumi Kaneda[2], Ryuichi Morishita[3]. [1]Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development, [2]Division of Gene Therapy Science, Osaka University Graduate School of Medicine [3]Department of Clinical Gene Therapy, Osaka University Graduate School of Medicine 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan

We previously identified a novel angiogenic peptide, AG30, with antibacterial effects that could serve as a foundation molecule for the design of wound healing drugs. Toward clinical application, in this study we have developed a modified version of the AG30 peptide characterized by improved antibacterial and angiogenic action, thus establishing a lead compound for a feasibility study. Since AG30 has an α-helix structure with a number of hydrophobic and cationic amino acids, we designed a modified AG30 peptide by replacing several of the amino acids. The replacement of cationic amino acids (yielding a new molecule, AG30/5C), but not hydrophobic amino acids, increased both the angiogenic and the antimicrobial properties of the peptide. AG30/5C was also effective against methicillin-resistant Staphylococcus aureus (MRSA) and antibiotic-resistant Pseudomonas aeruginosa. In a diabetic mouse wound-healing model, the topical application of AG30/5C accelerated wound healing with increased angiogenesis and attenuated MRSA infection. To facilitate the eventual clinical investigation/application of these compounds, we developed a large-scale procedure for the synthesis of AG30/5C that employed the conventional solution method and met Good Manufacturing Practice guidelines. In the evaluation of stability of this peptide in saline solution, RP-HPLC analysis revealed that AG30/5C was fairly stable for 12 months. Therefore, we propose the use of AG30/5C as a wound healing drug with antibacterial and angiogenic actions.

Endothelial Gab1 deletion accelerates angiotensin II-dependent vascular inflammation and atherosclerosis in apolipoprotein E knockout mice

Yoshikazu Nakaoka*, Kaori, Higuchi, Wataru Shioyama, Yoh Arita, Takahiro Hashimoto, Taku Yasui, Kuniyasu Ikeoka, Keiko Yamauchi-Takihara, Issei Komuro. Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Japan

Docking protein Grb2-associated binder 1 (Gab1) has critical roles in signal transduction of various growth factors, cytokines and numerous other molecules. Gab1 undergoes tyrosine-phosphorylation and associates with SH2-domain containing signaling molecules such as protein tyrosine phosphatase SHP2 and phosphatidylinositol-3 kinase p85 subunit upon stimulation with extracellular stimuli. We have recently reported that Gab1 is essential for postnatal angiogenesis through the analysis of endothelium-specific Gab1 knockout (Gab1ECKO) mice (Shioyama, Nakaoka et al. Circ.Res. 108, 664-675, 2011). However, the role of Gab1 in atherosclerosis remains unknown. In the preset study, we aim to elucidate the role of endothelial Gab1 in vascular inflammation and atherosclerosis. We intercrossed Gab1ECKO mice with apolipoprotein E (ApoE) knockout (ApoEKO) mice. Six-month-old male ApoEKO/Gab1ECKO and littermate control (ApoEKO) mice were treated with angiotensin II (AngII) via an osmotic infusion minipump. After AngII treatment, ApoEKO/Gab1ECKO mice showed significantly enhanced atherosclerosis and aneurysm formation compared with control mice. The pro-inflammatory cytokines including interleukin-6, interleukin-1-beta, and tumor necrosis factor-alpha in the aorta were significantly upregulated in ApoEKO/Gab1ECKO mice compared with control mice. Furthermore, the expression levels of Krüppel-like factor (KLF) 2 (KLF2) and KLF4, key transcription factors for endothelial homeostasis and vascular protection, were significantly reduced in the aortic endothelium of ApoEKO/Gab1ECKO mice compared with those of control mice. Consistently, both vascular cell adhesion molecule-1 expression and macrophage infiltration on the aortic walls were enhanced in ApoEKO/Gab1ECKO mice compared with control mice. Collectively, endothelial Gab1 deletion accelerates AngII-dependent vascular inflammation and atherosclerosis on ApoE-null background presumably in association with downregulation of KLF2 and KLF4.

Ephrin-B2 regulates PDGFRβ function in vascular smooth muscle cells

Akiko Nakayama*, Masanori Nakayama and Ralf Adams. Max-Planck-Institute for Molecular Biomedicine, Muenster, Germany

Morphological and functional changes in the vascular smooth muscle (vSMC) layer are hallmarks in the development of vascular diseases such as atherosclerosis and aortic aneurysms. Previous work has shown that platelet-derived growth factor (PDGF-B) is a key regulator of smooth muscle cell proliferation, migration and phenotypic modulation. Ephrin-B2 is a transmembrane protein and ligand that can activate Eph family receptor tyrosine kinases on adjacent cells, which is termed termed 'forward' signalling. In addition, binding to Eph molecules can also trigger receptor-like ('reverse') signaling downstream of the ligand. The latter regulates the internalization and signaling activity of vascular endothelial growth factor receptors in endothelial cells, but it is currently unclear whether ephrin-B2 regulates other tyrosine kinase receptors in the vascular system. Here we show that the vSMC-specific inactivation of the gene encoding the ephrin-B2 (Efnb2) with a Cre-loxP approach was compatible with the survival of the resulting mutant mice. However, vessel wall integrity was compromised in adult mutants, which was particularly obvious in the aortic arch region. To investigate the molecular basis for these defects, we investigated PDGFRβ function in cultured ephrin-B2-deficient and control vSMCs. Changes affecting the localization, internalization and signaling downstream of PDGFRβ were investigated. Our analysis indicates that ephrin-B2 has a pivotal role in PDGF-B/PDGFRβ signal transduction and thereby in the regulation of smooth cell behavior.

Physiological roles of collagen-binding integrins on endothelial cells in angiogenic sprouting and vessel integrity

S. Niland[1]*, T. Bracht[1], C. Westerhausen[2], D. Komljenovic[3], T. Bäuerle[3], S.W. Schneider[2], J.A. Eble[1]. [1]Goethe University Frankfurt, Center for Molecular Medicine, Dept. of Vascular Matrix Biology, [2]Mannheim University Hospital, Dept. of Experimental Dermatology, [3]German Cancer Research Center Heidelberg, Division Medical Physics in Radiology, Heidelberg, Germany

Angiogenesis is the formation of blood vessels from preexisting ones by sprouting of endothelial cells. The collagen-binding integrins α1β1 and α2β1 seem to play an important role in the entire angiogenic cascade. In order to characterize the role of integrin α2β1 at both molecular and cellular level, the integrin-dependent anchorage of ECs on collagen as the basis of mechanical force exertion and EC migration and infiltration is analyzed. Furthermore, the effect of collagen-binding integrins on vessel stability and integrity is assessed. To define and disclose the role of integrin α2β1 on endothelial cells in angiogenesis, an integrin α2β1 specific mini-collagen has been generated. Integrin α2β1 recognizes the peptide sequence GFOGER (O = 4 hydroxy-proline) presented as a trimer within a collagenous triple-helical framework. We produced a recombinant non-hydroxylated mini-collagen, termed FC3, which harbors this recognition site. FC3 consists of a foldon-stabilized host triple helix with 10 GPP-repeats, into which the integrin binding motif was inserted. Force spectroscopy was used to determine the binding of integrin α2β1 to its triple-helical recognition motif (GFPGER)3 at the molecular level. The strong binding of integrin α2β1 to its ligand underlines its importance as cellular mechanotransducer. Since recombinant mini-collagen FC3 is unhydroxylated, it predominantly binds to integrin α2β1 it is fully competent to agonistically elicit integrin α2β1-induced cell reactions. Hence, when a substratum is biofunctionalized with mini-collagen FC3, it acts as an agonist for α2β1 integrin-mediated cell functions, similar to collagen type I, in terms of adhesion and spreading, as it induces lamellipodia formation and recruitment of integrin α2β1 into focal adhesions. Integrins α2β1, and to a lesser extent α1β1, are crucial for sprouting angiogenesis. Inhibition of either integrin by blocking antibodies or the snake venom-derived antagonist rhodocetin significantly reduces the outgrowth of sprouts in the mouse aortic ring assay. In a mouse tumor model, the effect of intravenously administered rhodocetin was studied by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). Intravenously administered rhodocetin selectively accumulates in the tumor tissue, where it leads to a decrease in blood perfusion, simultaneously increasing the vessel permeability significantly.

AMP-activated protein kinase inhibits IGF-I Signaling and cell proliferation in vascular smooth muscle cells via suppressing activation of extracellular signal-regulated kinases 1 and 2

Junyu Ning*, Cong Jin. Beijing Center for Disease Control and Prevention, P. R. China

As a metabolic sensor and effector, the serine/threonine protein kinase AMP-activated protein kinase (AMPK) regulates the adaptation of cells to signals arising from nutrients, hormones and growth factors. AMPK has been reported to inhibit some IGF-I actions but the mechanism through which AMPK functions has not been well described. We previous reported that AMPK inhibits IGF-I-stimulated phosphorylation of Akt/mTOR/p70S6K and protein synthesis via phosphorylating IRS-1 at serine 794 in porcine vascular smooth muscle cells. In this study we utilized mutagenesis and RNAi to identify the AMPK inducing inhibition of IGF-I signaling through suppressing activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in porcine vascular smooth muscle cells. The AMPK activator metformin increased phosphorylation of AMPK Thr172 and inhibited IGF-I-stimulated phosphorylation of ERK1/2. Constitutively active AMPK suppressed IGF-I-stimulated phosphorylation of ERK1/2 and cell proliferation whereas AMPK knocking-down induced greater response to IGF-I stimulation. The results suggest that AMPK plays an inhibitory role in modulating IGF-I-stimulated cell proliferation and that AMPK inhibits IGF-I signaling via multiple entries in vascular smooth muscle cells.

Analysis of cell-based mechanisms involved in angiogenic morphogenesis by biological and computational approaches

Kei Sugihara[1], Koichi Nishiyama[1]*, Takashi Miura[2], Satoshi Arima[1], Yuji Hakozaki[1], Yuichiro Arima[1], Ki-Sung Kim[1], Yasunobu Uchijima[1], Yukiko Kurihara[1], and Hiroki Kurihara[1]. [1]Dept. of Physiol. Chem. and Metab., Grad. Sch. of Med., Univ. of Tokyo MD Scientist Training Program, Fac. of Med., Univ. of Tokyo, [2]Dept. of Anat. and Dev. Biol., Grad. Sch. of Med., Kyoto Univ., Japan

Angiogenic morphogenesis is a complex process accomplished by repetition of modules such as sprouting, elongation, and bifurcation, which configures branching vascular networks. In the process, vascular endothelial cells (ECs) and mural cells move in a coordinated fashion. However, the details of how vascular structures are formed as a collective consequence of a multi-cellular assembly process remain largely unknown. Using a newly developed time-lapse imaging and computer-assisted analysis system, we have recently characterized collective EC movements in ex vivo VEGF-induced branch elongation: individual ECs migrated forwards and backwards at different speeds, changing their relative positions („cell-mixing“), even at the tip („overtaking of the tip cell“). Based on an EC-tracking analysis of murine retina, the „cell-mixing“ phenomenon also very likely to take place during in vivo angiogenesis. Furthermore, quantitative experiments characterized the VEGF-induced angiogenic EC behaviors: VEGF increased mean EC speed, improved its directionality, and enhanced tip cell behaviors, resulting in branch elongation. Next to further clarify how EC behaviors are integrated into angiogenic morphogenesis, we formulated a one-dimensional mathematical model for branch elongation. Based on the characterized EC dynamics, we firstly assumed that cell motility is defined by speed (still or motile) and direction (back or forth), which changes stochastically (Model 1), and angiogenic EC behaviors were simulated using the parameters extracted mainly from biological experiments. In a preliminary study, Model 1 apparently reproduced the patterns of VEGF-stimulated angiogenic cell behaviors but not completely the tip cell dynamics in biological experiments. To fill in the gap, an additional assumption was incorporated: (1) a cell stops if it stays at the tip longer than given period and (2) the cell starts moving again when overtaken by another cell (Model 2). Qualitative and quantitative analysis indicated that both the models largely reproduced VEGF-stimulated angiogenic behaviors in biological experiments in terms of the patterns and the analyzed indices including elongating distance of the branch, mean speed, and directionality. However, tip cell behaviors such as number of tip cell overtaking were mimicked more accurately in Model 2 than in Model 1, suggesting that the incorporated rule for tip cells may reflect a biological phenomenon contributing to proper branch elongation. In conclusion, we have developed a novel mathematical model to simulate collective EC behaviors during branch elongation, which could in turn yield new insight for understanding the elongating aspects of angiogenesis. Combination of biological and computational approaches might enable us to explore cell-based mechanisms behind angiogenic morphogenesis.

Activation of Tie2 is regulated by dimerisation and conformation

Alengo Nyamay-Antu[1]*, Christoph Ballestrem[1], Richard Kammerer[2]. [1]Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, [2]Paul Scherrer Institut, Villingen, Switzerland

The receptor tyrosine kinase Tie2 and its ligands, the angiopoietins, have been shown to act as a key regulatory system for vascular quiescence and angiogenesis. Although the angiopoietin/Tie2 signalling pathways have been well characterized, the molecular mechanism by which the ligands regulate Tie2 activity remains unclear. To address this question, we aim to identify whether the activation mechanism of Tie2 is induced by its dimerisation alone, or whether subsequent relative rotation of the kinase domain is required. In the present study, we have employed a coiled-coil based protein engineering approach to monitor the effect of constraining the relative orientation of the transmembrane and intracellular domains on the kinase activity of Tie2. By replacing the extracellular domain of Tie2 with a dimeric parallel coiled-coil motif, we have generated seven ligand-independent chimeric homodimers of the kinase domain (Put3cc-Tie2 I-VII), which have distinct orientations of their kinase domains. The chimeras Put3cc-Tie2I-VII, ectopically expressed in mouse fibroblasts, localised to the cell surface and were highly phosphorylated in comparison to full-length Tie2. Furthermore, amongst the chimeras, we observed significant differences in kinase activity, suggesting that dimerisation and the relative rotation of the kinase domains both play a role in the activation mechanism of Tie2. To further understand the mechanism by which dimerisation of the kinase domain is regulated, we have generated mutants of the kinase domain in order to assess the impairment of dimerisation by monitoring their kinase activity. Our findings propose a mechanism in which the auto-phosphorylation of Tie2 is determined by dimerisation and conformation.

Outgrowth endothelial cell ex vivo expansion is associated with a pro-inflammatory phenotype and impaired vasoreparative function

T.M. O Doherty*, R.J. Medina, C.L. O Neill, S. Wilson, J. Guduric-Fuchs, A.W. Stitt. Centre for Vision and Vascular Science. Queens University Belfast, United Kingdom

Endothelial Progenitor Cells (EPCs) represent a novel therapeutic approach for the revascularisation of ischaemic tissues. Generating sufficient numbers of cells is a major factor hindering the implementation of EPC cell therapy; consequently ex vivo expansion methodologies are employed to amplify cell numbers. This study characterises EPC growth dynamics in culture conditions, subsequent senescence, and the impact this has on the vasoreparative function. Due to the lack of a standardized cell definition, the term EPC describes a diverse range of different cell types. We have focused on one EPC subtype, Outgrowth Endothelial Cells (OECs); widely accepted as a bona fide progenitor fully committed to the endothelial lineage. OECs exhibited significant expansion capability ex vivo, however with serial passages a progressive decline in proliferative capacity was observed, eventually reaching a Hayflick limit of approximately 30 and 60 population doublings for peripheral and umbilical cord blood-derived OECs, respectively. A permanent cell cycle arrest in G1 was verified by growth curves and FACS analysis. A significant decreased of BrdU uptake ($p<0.01$) was also demonstrated at higher passages. Morphologically, these OECs displayed a significant increase of cytoplasmic volume linked to the expansion of mitochondrial and lysosomal mass, along with increased β-galactosidase activity ($p<0.01$) and significant accumulation of γ-H2AX foci ($p<0.01$). In addition, a significant decrease in telomerase activity coupled with telomere shortening indicated a process of replicative senescence. Whole-genome transcriptome array comparison of early passage (EP) versus senescent OECs identified 828 significantly over-expressed and 705 under-expressed transcripts. Bioinformatics analysis highlighted an upregulated inflammatory component in senescent OECs. qRT-PCR validation of microarray results confirmed a significant increase in gene expression levels of IL1A, IL1B, IL6, and IL8. Protein arrays confirmed that senescent OECs, when compared to EP-OECs, exhibited higher levels of IL8, IL1B, and CCL2, and their secretome was highly enriched for IL6, IL8, CCL2, and CXCL1. As NFκB is an important regulator of inflammation, we blocked its transcriptional activation to assess the effects on OEC growth. Interestingly, when OECs were cultured in the presence of sulfasalazine (NFκB inhibitor), there was a significant increase in OEC replicative capacity. In vitro functional assays indicated that when compared to EP-OECs, senescent OECs had significantly impaired capacity to migrate and form tubes compared to EP controls ($p<0.05$). A model of murine ischaemic retinopathy was used to evaluate the regenerative potential of senescent OECs in vivo. Senescent OECs demonstrated impaired capacity to integrate and re-vascularise ischaemic retina compared to EP-OECs. In conclusion, EPCs have limited replicative potential and after long term culture become senescent. In vitro and in vivo, these senescent cells display impaired vasoreparative function. This phenotype is associated with the increased expression of pro-inflammatory cytokines under transcriptional control by NFκB. Therefore the limitations associated with restricted cell expansion in vitro and a senescence phenotype are important factors to consider in the development of EPCs for cytotherapy.

Infection of human endothelial progenitor cells with Bartonella henselae induces vessel-like growth in vitro

Fiona O'Rourke[1]*, Tanja Mändle[2], Carmen Urbich[3], Stefanie Dimmeler[3], Kirsten Lauber[4], Volkhard A.J. Kempf[1]. [1]Universität Frankfurt, Medizinische Mikrobiologie und Krankenhaushygiene, Frankfurt am Main, [2]Universität Tübingen, Medizinische Mikrobiologie und Krankenhaushygiene, Tübingen, [3]Universität Frankfurt, Institut für Kardiovaskuläre Regeneration, Frankfurt am Main, [4]Universität München, Molekulare Onkologie, München, Germany

Endothelial progenitor cells (EPCs) are a heterogeneous mixture of adult stem cells that play an essential role in revascularization after vascular damage. Their discovery over a decade ago led to various pre-clinical and clinical trials investigating the use of these cells in regenerative medicine for ischemic injury. In our work we investigated an unconventional method of improving the angiogenic potential of EPCs through bacterial infection. Bartonella spp. are facultative intracellular pathogens and the only known bacteria to induce angiogenesis in humans. Here we describe the course of a bacterial infection of EPCs with the vasculotropic bacterium B. henselae. Our data demonstrate that EPCs are highly susceptible to B. henselae infection. Upon infection EPCs show a strong activation of hypoxia inducible factor-1 (HIF-1), the key transcription factor in angiogenesis. This is followed by the signature HIF-1-dependent pro-angiogenic cell response including production of cytokines such as vascular endothelial growth factor (VEGF) and adrenomedullin (ADM). Furthermore, B. henselae prevents apoptosis of EPCs and induces cell migration along a stromal cell-derived factor (SDF)-1 gradient, both essential functional components of the angiogenic response. Finally, when culture plates are coated with a basement membrane which simulates the extra-cellular matrix (MatrigelTM), infected EPCs assemble into complex vessel-like structures in vitro. Cumulatively, our data demonstrate that infection with B. henselae can improve the angiogenic capacity of EPCs and induce vessel-like growth in vitro. At present we are working to phenotypically and genetically characterize the transformation of EPCs from circulating progenitor cells to vessel-like structures and identify genes and pathways involved in this bacterial induced process.

An important role of semaphorin 3A/neuropilin-1 signaling in lymphatic vessel maturation

Giorgia Jurisic[1], Hélène Maby-El Hajjami[2], Sinem Karaman[1], Alexandra M. Ochsenbein[1]*, Annamari Alitalo[1], Shoib S. Siddiqui[1], Carlos Ochoa Pereira1, Tatiana V. Petrova[1,2] and Michael Detmar[1]. [1]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, 8093 Zurich, Switzerland, [2]Division of Experimental Oncology CePO, CHUV and University of Lausanne, 1066 Epalinges, Switzerland

Lymphangiogenesis plays important roles during embryonic development and in a number of diseases including chronic inflammatory diseases and advanced cancer. However, only a few regulators mediating lymphangiogenic processes are known. In a transcriptional profiling study of ex vivo isolated endothelial cells from blood versus lymphatic vessels of the mouse colon, we found that Sema3A was specifically expressed by lymphatic vessels but not by blood vessels. Immunostains confirmed these findings and also revealed that the Sema3A receptor neuropilin was expressed by mural cells and by cells of the lymphatic valves. To investigate the functional role of Sema3A and its receptor neuropilin-1 in lymphatic vessel development, we administered antibodies blocking the binding of Sema3A or of VEFGA to NRP-1 to pregnant mice. Analysis at P5.5 revealed that Sema3A/NRP-1 signaling blockade resulted in irregularly shaped & bulging collecting mesenteric lymphatic vessels and in an altered patterning of developing tail lymphatic vessels, whereas no such phenotypes were seen after VEGFA/NRP-1 signaling blockade or in isotype control treated pups. Importantly, the Sema3A/NRP-1 signaling blockade led to increased smooth muscle coverage on mesenteric lymphatic valves, suggesting that Sema3A signaling repels pericytes from the lymphatic valve region. Our in vitro data support this hypothesis by demonstrating that pericytes express NRP-1, that recombinant Sema3A inhibits their migration in a dose-dependent manner, and that the Sema3A/NRP-1 signaling blocking antibody reverses this inhibitory effect in a chemo-haptotaxis transwell experiment. Preliminary data obtained in tamoxifen-induced endothelial cell-specific Sema3A ko mice are in line with the Sema3A signaling blocking antibody studies by showing aberrant tail lymphatic patterning in P7 pups. Taken together, these data indicate an important role of Sema3A/NRP-1 signaling in lymphatic vessel maturation.

Stimulation of blood mononuclear cells with bacterial virulence factors triggers the release of pro-coagulant and pro-inflammatory microparticles

Sonja Oehmcke*, Matthias Mörrgelin, Johan Malmström, Adam Linder, Michelle Chew, Henrik Thorlacius and Heiko Herwald. University Rostock IMIKRO, Schillingallee 70, Rostock, Germany, Division of Infection Medicine, Department of Clinical Sciences, Lund University, Lund, Sweden

The pro-coagulant activity of plasma microparticles (MPs) has been related to the exposure of tissue factor and phosphatidylserine (PS) on their outer vesicle membrane. Here we report that blood mononuclear cells, stimulated with bacterial virulence factors, produce not only pro-coagulant, but also pro-inflammatory MPs. Our results show that contact system activation on MPs contributes to these two effects. PS plays an important role in these processes, as its up-regulation on MPs trigger contact system activation, followed by the release of bradykinin, a potent vascular mediator. Furthermore our data clearly show, that the activation of the contact system stabilizes a tissue factor induced clot. Monocyte-derived MPs were identified in plasma samples from septic patients and further analysis of MPs from these patients revealed that their pro-coagulant activity is dependent on the tissue factor- and contact system-driven pathway.

The F-BAR protein NOSTRIN participates in FGF signal transduction and vascular development

Igor Kovacevic[1], Jiong Hu[2], Ann Siehoff-Icking[1], Nils Opitz[1], Aliesha Griffin[3], Andrew C. Perkins[3], Alan L. Munn[4], Werner Müller-Esterl[1], Rüdiger Popp[2], Ingrid Fleming[2], Benno Jungblut[5], Meike Hoffmeister[1], Stefanie Oess[1]*. [1]Institute for Biochemistry II, Goethe University Frankfurt Medical School, Theodor Stern Kai 7 Hs 75, 60590 Frankfurt/Main, Germany, [2]Institute for Vascular Signalling, Goethe University Frankfurt Medical School, Theodor Stern Kai 7 Hs 75, 60590 Frankfurt/Main, Germany, [3]Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia, [4]School of Medical Science and Molecular Basis of Disease Programme, Griffith Health Institute, Griffith University (Gold Coast), Southport, Queensland 4222, Australia, [5]Max Planck Institute for Heart and Lung Research, Parkstraße 1, 61231 Bad Nauheim, Germany

F-BAR proteins are multivalent adaptors that link plasma membrane and cytoskeleton and co-ordinate cellular processes such as membrane protrusion and migration. Yet, little is known about the function of F-BAR proteins in vivo. Here we report, that the F-BAR protein NOSTRIN is necessary for proper vascular development in zebrafish and postnatal retinal angiogenesis in mice. The loss of NOSTRIN impacts on the migration of endothelial tip cells and leads to a reduction of tip cell filopodia number and length. NOSTRIN forms a complex with the GTPase Rac1 and its exchange factor Sos1 and overexpression of NOSTRIN in cells induces Rac1 activation. Furthermore, NOSTRIN is required for FGF-2 dependent activation of Rac1 in primary endothelial cells, and the angiogenic response to FGF-2 in the in vivo matrigel plug assay is greatly diminished in NOSTRIN knockout mice. We propose a novel regulatory circuit, in which NOSTRIN assembles a signalling complex containing FGFR1, Rac1 and Sos1 thereby facilitating the activation of Rac1 in endothelial cells during developmental angiogenesis.

Role of VEGF- and Notch-signaling for liver vascularization in zebrafish

Nils Ohnesorge*, Arndt F. Siekmann. Max Planck Institute for Molecular Biomedicine Muenster, Germany

The liver is a highly vascularized organ with a distinctive pattern of specialized blood vessels. Although blood supply is vital for proper liver development and function, only little is known what signals coordinate angiogenesis in the developing liver and how the blood vessels form a functional network. The zebrafish is a model organism that provides an easily accessible system to study these early developmental processes. In this study we have analyzed the interactions of hepatocytes and endothelial cells in the zebrafish liver. In order to do so, we utilized transgenic zebrafish in which different fluorescent proteins were specifically expressed in these cells. Our studies revealed that the blood vessels of the liver plexus (LIV) first form by sprouting angiogenesis from the common cardinal vein and the subintestinal vessels and connect around 50 hours post fertilization at the migrating front of the hepatocytes. Over the next three days the first liver lobe forms. Blood vessels of the LIV follow the migrating and proliferating hepatocytes ventrally by elongation of existing vessels and cross-connecting larger gaps by sprouting angiogenesis. The formation of two more liver lobes continues throughout larval stages. To further elucidate the signaling mechanisms behind these processes we analyzed zebrafish mutants in dll4, flt4 and kdrl to investigate the role of the Notch- and VEGF-signaling pathways. We will present an initial characterization of the mutant phenotypes during liver vascularization.

Novel functional relationship between VEGF and skeletal muscle metabolism. Evidence for crosstalk between angiogenesis and metabolism?

Sara Olenich[1], Gerald N. Audet[1], Viola Szeszel-Federowicz[2], I. Mark Olfert[1,3,4]*. West Virgina University School of Medicine [1]Division of Exercise Physiology, [2]Dept. of Otolaryngology, [3]Center for Cardiovascular and Respiratory Sciences, [4]Mary Babb Randolph Cancer Center, USA

Blood vessels form an important interface between the environment and organs they perfuse. It is not surprising that metabolic alterations have been observed in conditions (such as cancer) known to stimulate angiogenesis. However the potential crosstalk between angiogenic and metabolic pathways is poorly understood. Vascular endothelial growth factor (VEGF) is a multifunctional protein critical in vascular growth/development and essential for angiogenesis, particularly in response to exercise. Using microarray analysis, we have examined skeletal muscle (gastrocnemius) gene responses in sedentary (Sed) and 8-week treadmill trained (Tra; 1 hr, 5 d/wk) muscle-specific VEGF deficient mice (KO-Sed and KO-Tra) compared to littermate control mice (WT-Sed and WT-Tra) to gain insight into the potential non-angiogenic mechanisms involving VEGF. When comparing sedentary groups (KO-Sed vs WT-Sed) and trained groups (KO-Tra vs WT-Tra) there were 349 and 126 differentially expressed genes, respectively. Ingenuity pathway analysis revealed potential novel functions of VEGF in association with lipid and carbohydrate metabolism between KO-Sed and WT-Sed. Between KO-Tra and WT-Tra there were associations with gene networks involved in cell signaling, protein synthesis, endocrine system disorders and metabolic disease. Our data also confirm the vital association of VEGF in skeletal muscle structure and function, but more importantly identify potential novel relationships between VEGF and metabolism, endocrine and metabolic disorder not previously recognized. Future studies examining the role of VEGF as a metabolic regulator may open new avenues linking angiogenesis and metabolic alteration in chronic cardiovascular and neoplastic disease.

Platelets mediate the accelerated angiogenic switch in mice lacking histidine-rich glycoprotein

Maria Ringvall[1], Åsa Thulin[1], Jessica Cedervall[1], Zhang Lei[1], Wilhelm Jahnen-Dechent[2], Agneta Siegbahn[3] and Anna-Karin Olsson[1]*. [1]Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden, [2]Department of Biomedical Engineering, RWTH Aachen University, Germany, [3]Department of Medical Sciences, Uppsala University, Sweden

Histidine-rich glycoprotein (HRG; alternatively, HRGP/HPRG) is a heparin-binding plasma protein that can suppress tumor angiogenesis and growth in vitro and in vivo. Mice lacking the HRG-deficient mice are viable and fertile, but have an enhanced coagulation resulting in decreased bleeding times (1). To address whether HRG deficiency affects tumor angiogenesis and growth, we have crossed HRG knockout mice with the RIP1-Tag2 mouse, a well established orthotopic model of multistage carcinogenesis. HRG-/- RIP1-Tag2 mice display a significantly elevated angiogenic switch, as well as two-three times larger tumor volume compared to their RIP1-Tag2 HRG+/+ littermates, supporting a role for HRG as an endogenous regulator of tumor growth (2,3). In the present study we show that platelet activation is increased in mice lacking HRG. To address whether this elevated platelet activation contributes to the increased pathological angiogenesis in HRG-deficient mice, they were rendered thrombocytopenic before the onset of the angiogenic switch by injection of the anti-platelet antibody GP1bα. Interestingly, this treatment suppressed the increase in angiogenic neoplasias seen in HRG knockout mice. However, if GP1bαtreatment was initiated at a later stage, after the onset of the angiogenic switch, no suppression of tumor growth was detected in HRG-deficient mice. Our data show that increased platelet activation mediates the accelerated angiogenic switch in HRG-deficient mice. Moreover, we conclude that platelets play a crucial role in the early stages of tumor development but are of less significance for tumor growth once angiogenesis has been initiated. References: 1. Tsuchida-Straeten N, Ensslen S, Schafer C, Woltje M, Denecke B, Moser M, Graber S, Wakabayashi S, Koide T and Jahnen-Dechent W. „Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (HRG)“. J Thromb Haemost. 2005 May;3(5):865-72. 2. Thulin A, Ringvall M, Dimberg A, Karehed K, Vaisanen T, Vaisanen MR, Hamad O, Wang J, Bjerkvig R, Nilsson B, Pihlajaniemi T, Akerud H, Pietras K, Jahnen-Dechent W, Siegbahn A and Olsson AK. Activated platelets provide a functional microenvironment for the antiangiogenic fragment of histidine-rich glycoprotein. Mol Cancer Res. 2009 Nov;7(11):1792-802. 3. Ringvall M, Thulin A, Zhang L, Cedervall J, Tsuchida-Straeten N, Jahnen-Dechent W, Siegbahn A and Olsson AK. Enhanced platelet activation mediates the accelerated angiogenic switch in mice lacking histidine-rich glycoprotein. PLoS One. 2011;6(1):e14526.

High glucose induces cellular dysfunction and premature senescence in Endothelial Progenitor Cells (EPCs)

Christina L. O`Neill*, Reinhold J. Medina, Michelle T. O' Doherty, Sarah E. J. Wilson, Alan W. Stitt. Centre for Vision and Vascular Science, School of Medicine, Dentistry and Biomedical Science, Queens University Belfast, United Kingdom

EPCs have been shown to promote angiogenesis and revascularise ischaemic tissue. Recent clinical trials have demonstrated that these cells play an important role in vascular repair and therefore represent an ideal candidate for therapeutic revascularisation. However, in many vascular diseases such as diabetes, the resident vasculature is functionally impaired. Endothelial dysfunction is a common problem in diabetes resulting in diminished vascular regeneration and progression of vascular complications. Furthermore, it has recently been shown that EPCs may also be impaired in diabetes. It has also been demonstrated that the number and function of circulating EPCs are reduced in patients with cardiovascular risk factors such as hyperglycaemia. The purpose of this study was to examine the effect of the diabetic milieu on EPCs, by mimicking diabetic conditions in vitro. We chose to examine the effect of high glucose conditions on a particular EPC subset called Outgrowth endothelial cells (OECs), as this subset has been thoroughly characterised by our group as a bona fide endothelial progenitor cell population. Long-term exposure (3-6 weeks) to high D-Glucose (DG) (25mM) resulted in a significantly decreased proliferation rate of OECs when compared to untreated control (5mM DG) or L-glucose (LG) (osmotic control) treated cells ($p<0.001$). High DG also negatively affected OEC function, as shown by a significant reduction in OEC migratory capacity using an in vitro scratch wound assay ($p<0.001$) and a significant decrease in OEC tubulogenic capacity using an in vitro 3D matrigel model ($p<0.001$). Gene expression analysis highlighted increased expression of pro-inflammatory genes CCL2 and ICAM. Interestingly, long-term exposure to high DG resulted in premature senescence of OECs, which was characterised by a significant increase in senescence-associated β-galactosidase activity ($p<0.001$); and a significant increase in 53BP1 foci ($p<0.05$). Our results demonstrate the deleterious effect of high D-glucose on OECs and indicate that this effect may be due to the induction of premature senescence mechanisms. This finding is extremely important as it indicates that OECs isolated from diabetic patients may be dysfunctional and therefore, will require full functional evaluation, and consecutive modulation/repair if found to be dysfunctional, before using them as an autologous cell therapy.

Chemically defined conditions for derivation and expansion of endothelial cells from human Pluripotent Stem Cells (hPSCs).

Valeria Orlova[1,2]*, Christian Freund[2], Dorien Ward-van Oostwaard[2], Konstantinos Gkatzis[2], Richard Davies[2], Peter ten Dijke[1] and Christine Mummery[2]. [1]Department of Molecular Cell Biology and, [2]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands

Human pluripotent stem cells (hPSCs) remain a valuable supply of patient specific cells for tissue graft engineering and modeling of human cardiovascular diseases. More important, hPSCs could serve as an unlimited source of cells with restricted life span and expansion capacities, such as primary human endothelial cells (ECs) in particularly. Thus, ECs derived from hPSCs could be potentially useful to study molecular mechanisms of cardiovascular diseases, as well as for personalized therapy and high scale drug screenings. Despite multiple reports published on derivation of endothelial cells from hPSCs, the need in efficient serum-free protocol remains indispensible. Furthermore, chemically defined conditions are needed for standardized functional assays, and more accurate and reproducible studies on growth factor and matrix specific mediated responses in endothelial cells. We have developed a protocol for derivation and expansion of endothelial cells in fully chemically defined conditions. The protocol facilitates efficient generation of endothelial cells from human ES, as well as iPS cell lines from control subjects and patients with genetic vascular disorder - Hereditary Hemorrhagic Telangiectasia (HHT). hPSCs derived ECs expressed endothelial-specific markers (CD31, VE-Cadherin, VEGFR2, vWF) and could be expanded up to five passages, without loss of endothelial cell morphology, or a significant decrease in proliferation rate. Morphologically, hPSCs derived endothelial cells were similar to human microvascular endothelial cells, with formation of tight cell-cell junctions and cortical actin being co-localized with VE-Cadherin in quiescent monolayer. Pro-inflammatory stimuli caused opening of cell-cell junctions and re-organization of cortical actin cytoskeleton with the formation of actin stress fibers demonstrating that ECs retained intrinsic mechanisms to regulate endothelial cell barrier permeability. Similar to HUVECs hPSCs derived ECs formed 2D sprouting network on matrigel. Interestingly, hPSCs derived ECs exhibited mixed arterial/venous identity, similar to embryonic endothelial cells. Studies towards generation of ECs from hPSCs with more prominent arterial phenotype and in vivo potential are ongoing at the present moment.

Cross-talk of RANKL and Renin-Angiotensin II system in vascular calcification

Mariana Kiomy Osako[1,2,3]*, Hironori Nakagami[2], Hiromi Rakugi[1], Ryuichi Morishita[3].
[1]Department of Geriatric Medicine, [2]Division of Vascular Medicine and Epigenetics [3]Department of Clinical Gene Therapy

RANKL (Receptor Activator of NF-ï□«B Ligand), its receptor RANK, and decoy receptor osteoprotegerin (OPG) are key molecules in bone metabolism, which we have recently reported to be expressed in vascular cells, and to regulate calcification of vascular smooth muscle cells (VSMC). In this study we show that renin-angiotensin system (RAS) modulates RANKL signaling in vasculature and conversely the activation of RANKL signaling increase the expression of components of RAS in VSMC. We found that Angiotensin II (AngII) dramatically increased calcium deposition followed by the osteogenic differentiation of VSMC, and expression of osteogenesis-related genes such as cbfa1 and msx-2 detected by western blotting. Interestingly, the addition of anti-RANKL neutralizing antibody significantly decreased the ability of AngII to induce calcification. We have previously shown that female ApoE-/- mice under estrogen deficiency by ovariectomy (OVX) and high fat diet (HF) presented high RANK-RANKL expression in vasculature and increased calcification incidence in a 3 months model. The infusion of a subpressor dose of AngII (100 ng/kg/min) in this animal model significantly increased the expression of RANKL system and calcification in vasculature, as detected by Real Time PCR and Von Kossa staining respectively. Ang II infusion in other animal model, OPG-/- mice, which present unopposed RANKL activation and vascular calcification at young age, induced a dramatic increased in vascular calcification after 1 month compared to wild type (WT) mice and independently of sex and estrogen level. Treatment of OVX/HF ApoE deficient mice with angiotensin II receptor blocker (Olmesartan 3 mg/kg/day) decreased the calcification incidence and osteogenic-markers expression in vasculature. Similarly OPG-/-/AT1R-/- mice showed inhibition of calcification compared to OPG-/- mice. The lack of AT1R inhibited the calcification also in vitro: primary culture of aortic VSMC from WT, OPG-/- , and OPG-/-/AT1R-/- confirmed that OPG-/-/AT1R-/- showed low expression levels of msx2 and cbfa1 under osteogenic differentiation medium compared to OPG-/- VSMC. Interestingly, OPG-/- VSMC showed high basal ACE and AT1R expression, and pERK level compared to WT VSMC. Inhibition of ERK phosphorylation by PD98059 decreased the expression and activity of ACE. Conversely, AngII stimulation increased the expression of RANK and RANKL in VSMC. This study demonstrates that RAS activation leads to RANK-RANKL system activation, and conversely, unopposed RANKL stimulation activates tissue RAS, and highlights a broader contribution of RANKL system to vascular pathophysiology.

Paradoxically impaired development of atherosclerosis in hyperlipidemic down's syndrome candidate region-1-/- and apolipoprotein E-/- double-knockout mice

Tsuyoshi Osawa[1,2]*, Kazuhiro Toyama[3], Suguru Nakagawa[3], Mai Miura[1], Jun-ichi Suehiro[1], Toshiya Tanaka[2], Hiroyuki Aburatani[3], Tatsuhiko Kodama[2], and Takashi Minami[1]. [1]Laboratory for Vascular Biology, [2]Laboratory for Systems Biology and Medicine, and, [3]Genome Science Division, The University of Tokyo, Japan

The epidemiological incidence of cancer and atherosclerosis is significantly reduced in Down's syndrome individuals. Down's syndrome candidate region-1 (DSCR1, also known as RCAN1) suppresses vascular endothelial growth factor (VEGF)-mediated angiogenesis via calcineurin-NFAT pathway, resulting in suppression of tumor growth. However, a role of DSCR1 in development of atherosclerosis is still unknown. Here we show that DSCR1 null mutant (DSCR1-/-) mice impaired the development of atherosclerosis in hyperlipidemia model in combination with ApoE null mutant (ApoE-/-) mice. The DSCR1-/- and ApoE-/- double-knockout (DSCR-/-ApoE-/-) are partially embryonic lethal. Moreover, Survived adult DSCR-/-ApoE-/- mice significantly increased plasma cholesterol and frequently developed corneal opacity and lipoma. After administration of a high-fat/high-cholesterol diet, plasma cholesterol level was significantly increased in DSCR-/-ApoE-/- mice compared with that of wild type, DSCR1-/-, or ApoE-/- mice. Moreover, lipid profiles such as percentage of CM, VLDL, and LDL were similarly altered in ApoE-/- and DSCR1-/-ApoE-/- mice compared to the wild type and DSCR1-/- mice. However, DSCR1-/-ApoE-/- mice showed significant reduction of body weight, atherosclerotic lesion size, lipid accumulation in the skin, and infiltration of F4/80+ macrophages and CD11b+ monocytes into the atherosclerotic lesion compared with those of ApoE-/- mice. By using the DSCR1 promoter coupled to the lacZ reporter (DSCR-lacZ) mice, the lacZ activities was rarely detected in postnatally. However, interestingly combined the DSCR1-lacZ and ApoE-/- mice, the DSCR1 promoter mediated lacZ activities were clearly observed with hepatocyte and smooth muscle cells in atherosclerosis. Number of total lymphocytes was decreased, but CD11b+/Gri1- monocyte cells were increased in ApoE-/- mice, which were compensated in DSCR1-/-ApoE-/- mice at the level of wild type mice. Collectively, we demonstrated a connection between the calcineurin inhibitor DSCR1 and metabolic diseases including hyperlipidemia, atherosclerosis, and corneal opacity. The molecular mechanism of these phenomenon are need to be further elucidated.

Optimized method for culturing outgrowth endothelial progenitor cells from human umbilical cord blood and adult peripheral blood

Noriko Oshima-Sudo[1,3,4]*, Yuko Hoshino[1], Motohiro Komaki[2], Ken-ichi Nakahama[1], Toshiro Kubota[3], Mayumi Abe[2], Ikuo Morita[1,4]. [1]Department of Cellular Physiological Chemistry, Graduate School, [2]Department of Nanomedicine (DNP), Graduate School, [3]Department of Comprehensive Reproductive Medicine, Graduate School, [4]Global COE, Tokyo Medical and Dental University, Japan

Objective: Outgrowth endothelial progenitor cells (OECs) are expected to be a valuable source of blood vessels for regenerative medicine. Especially, OECs obtained from umbilical cord blood have a higher proliferative potential and are useful for vascular biology research and clinical applications for ischemic diseases. Here, we established a protocol for isolation of OECs from umbilical cord blood, and furthermore we attempted to obtain OECs from adult peripheral blood. Methods: To obtain OECs, we isolated MNCs from human umbilical cord blood and determined the OEC colony formation rate (OEC-CFR) under various conditions on the basis of the following 4 points: 1, cell density; 2, pre-selection of CD45(-) cells; 3, culture medium; and 4, influence of cryopreservation. Next, we obtained from mononuclear cells from human peripheral blood of healthy adult volunteers. We cultured MNCs from adult peripheral blood using optimized method to obtain OECs from umbilical cord blood. We examined senescence marker of OECs from umbilical cord blood and adult peripheral blood using senescence associated beta-gal (SA-beta-gal) staining. Results: The OEC-CFR from CD45(-) cells was 0.250 colony/5 x 107 cells and was dependent on the initial cell density, while the OEC-CFR from total mononuclear cells was 0.347 colony/5 x 107 cells. This result suggested that pre-selection of CD45(-) cells was not necessary to obtain OECs. Supplementation of the culture medium with microvascular endothelial growth medium (EGM-2-MV) caused an increase in the OEC-CFR. Furthermore, we obtained at least 1 colony from each sample of total mononuclear cells after cryopreservation, although the OEC-CFR was lower than that from fresh cells. When we use MNCs from adult peripheral blood, OEC-CFR were lower than that from cord blood. The percentage of SA-beta-gal positive cells is higher in OECs from adult peripheral blood than that from umbilical cord blood. Conclusion: This method could be useful for the clinical application of OECs from umbilical cord blood. We conclude that if we would use this optimized method, it would be difficult to obtain OECs from adult peripheral blood.

The role of FOXQ1 in monocytes transmigration

Ilja Ovsiy[1]*, Ioannis Manousaridis[1], Vladimir Riabov[1], Aida Avdic[1], Aliyah Sanders[1], Sergij Goerdt[1], Elisabeth Kremmer[2], Guido Krenning[3], Marco Harmsen[3], Alexei Gratchev[1], and Julia Kzhyshkowska[1]. [1]Department of Dermatology, Venerology and Allergology, Medical Faculty Mannheim, Ruprecht-Karls-University of Heidelberg, Mannheim, Germany, [2]Helmholtz Center Munich, Institute of Molecular Immunology, Munich, Germany, [3]Department of Pathology and Laboratory Medicine, Medical Biology Section, University of Groningen Medical Center, Groningen, The Netherlands

Interaction between monocytes and endothelial cells plays a pivotal role during inflammation and cardiovascular diseases. Under pathological conditions activated endothelium mediates extravasation of monocytes into the tissues, where they migrate to the site of inflammation and differentiate into macrophages. But monocytes are exposed in blood stream to the broad range of pathological soluble factors as cytokines, pathogen products or modified lipoproteins. Activation of monocytes in the circulation is a result of co-operative action of systemic and locally produced factors. However, the exact molecular mechanism of the specific activation of monocytes in blood stream is still poorly understood. Recent studies showed that the levels of cytokine IL-4 are increased in the circulation during Th2-associcated inflammation level. Using Affymetrix chip assay we found that monocyte respond to IL-4 by strong overexpression of novel transcription factor Forkhead box Q1 (FOXQ1). Using Real-time PCR analysis we demonstrated that FOXQ1 is also overexpressed in monocytes of patients with acute atopic dermatitis. FOXQ1 is a member of forkhead transcription factors family that share specific DNA-binding motif known as the ‘winged helix’ domain. In human FOXQ1 is expressed on a high level in the stomach, trachea, bladder, salivary gland and has an overexpression in colorectal adenocarcinoma and lung carcinoma cell lines. FOXQ1 is actively involved in epithelial mesenchymal transition and downregulates transcription of E-cadherin. However there is no evidence about the function of FOXQ1 in monocytes or macrophages. In the presented study we investigated the expression regulation of FOXQ1 in human monocyte-derived macrophages and analyzed effect of FOXQ1 on monocyte transmigration. Analysis of FOXQ1 expression in human monocyte-derived macrophages by qRT-PCR confirmed that Th2 derived cytokine IL-4 induces FOXQ1. We showed that TGF-β1 in combination with dexamethasone amplifies the effect of IL-4. In order to identify FOXQ1-induced genes and analyse function of FOXQ1 we generated cell-based model system. Murine macrophage-like RAW264.7 cells were stably transfected with pEF6/V5-HisB-mFOXQ1 or empty vector pEF6/V5-HisB. Comparison of RAW-FOXQ1 and RAW-vector single cell-derived clones revealed, that overexpression of recombinant FOXQ1 does not affect basic macrophage functions endocytosis and phagocytosis. Supernatants of FOXQ1 expressing clones increased chemotaxis of RAW264.7 cells suggesting that FOXQ1 stimulates monocyte chemotaxis in an autocrine manner. Affymetrix chip assay revealed that FOXQ1 target genes can be involved in motility and cytoskeletal dynamics. We have established in vitro transmigration assay and demonstrated that RAW-FOXQ1 clones migrate toward nutritional gradient 9.5 times more efficient compared with RAW-vector cells. FOXQ1-dependent migration toward MCP-1 gradient was even more pronounced. The stimulatory effect of FOXQ1 on monocyte migration correlated with its ability to suppress expression of receptor Plexin C1 known to inhibit migration of monocytes and dendritic cells. In parallel with FOXQ1, Plexin C1 was downregulated in human monocytes uponr IL-4 stimulation and in monocytes of patients with acute atopic dermatitis.Our data indicate that FOXQ1 is upregulated by IL-4 and TGF-β1 and stimulates monocyte migration in response to inflammatory stimuli by suppression of Plexin C1. We hypothesise that FOXQ1 supports increased monocytes extravasation through the activated endothelium during chronic inflammation.

Aspartame induces angiogenesis in chick chorioallontoic membrane

Ozgurtas T*, Aydin F, Fermanli O, Tekin S, Yesildal F. Department of Biochemistry, Gulhane Military Medical Academy, 06018, Ankara, TURKIYE School of Medicine, Gulhane Military Medical Academy, 06018, Ankara, TURKIYE

Angiogenesis is the process of generating new blood vessels from preexisting vessels and is considered essential in many pathological conditions. Aspartame is the most widely used artificial sweetener and is added to a wide variety of foods, beverages, drugs, and hygiene products. The purpose of the present study was to evaluate the effect of aspartame in chick chorioallantoic membrane angiogenesis model in vivo. In this well characterized model, aspartame induces angiogenesis in a concentration-dependent manner. Compared with the normal group, aspartame group has significant increased vessels proliferation. These results provide evidence that aspartame induces angiogenesis and may be important for angiogenesis-dependent human diseases.

Cancer signaling in human colorectal cancer

Narendra Padhan[1]*, Peter Åkerud[2], Helgi Birgisson[2] and Lena Claesson-Welsh[1]. [1]Uppsala University, Rudbeck Laboratory, Dept. Immunology, Genetics and Pathology, Sweden, [2]Uppsala University, Uppsala University Hospital, Dept. Surgical Sciences, 751 85 Uppsala, Sweden

Cancer includes more than 200 diseases with the common characteristic of uncontrolled cellular growth. Apart from the many tumor-type specific aspects, which remain poorly understood, the challenge to efficiently treat cancer is complicated by the complexity of the disease process in each individual patient. A growing cancer tissue develops hypoxia due to rapid growth, in parallel with insufficient and functional vascularization. Hypoxia in turn promotes increased production of growth factors and growth factor receptors (e.g. EGF, VEGF, PDGF and their receptors) in the tumor. Resistance to cancer therapy involves exaggerated signaling in pathways regulating cell proliferation and cell motility. Here, we used nanofluidic isoelectric focusing technique to study signaling via extracellular regulated kinase 1 and 2 (ERK1/2), the cytoplasmic tyrosine kinase Src, PLCγ and S6 Kinase. To identify activated forms of ERK1/2, Src, PLCγ and S6 Kinase, we analyzed endothelial cells treated with vascular endothelial growth factor (VEGF) for different time periods. To determine signal transduction in human cancer, biopsies from benign mucosa, stage II and IV colorectal cancer (CRC) were analyzed. In total about 35 CRC biopsies were examined. Interestingly, ERK1/2 expression gradually decreased with advanced disease. Moreover, accumulation of phosphorylated Erk1 significantly decreased in cancer stage II and IV biopsies. Conventional western blotting on these samples did not allow detection of phosphorylated ERK1/2 in most samples. Analysis of Src and S6 kinase also showed decreased expression with advanced disease. In contrast, expression of PLCγ was significantly increased at cancer stage II, while it returned to the same levels as in the benign tissue, at cancer stage IV. Our results suggest desensitization of several signal transduction pathways in advanced colorectal cancer. Future plans include to isolate different cell populations from human colorectal cancer to identify which cell types in the tumor microenvironment are subject to desensitization; the ultimate goal of our studies is to improve diagnosis and optimize therapy of cancer.

Study of the anti-inflammatory effects of a novel endothelial cell-matrix formulation conditioned media

Laura Paneghetti*, Yin-Shan Ng. UCL Institute of Ophthalmology, London, United Kingdom

Endothelial injury occurs after percutaneous angioplasty and in approximately 30-50% of the treated vessels it causes intimal hyperplasia (IH), a disease characterised by local inflammation and activation, migration and proliferation of vascular smooth muscle cells (VSMC) and myofibroblasts in the intima. This process leads to critical narrowing or restenosis of the treated vessel within 6-12 months. Restenosis is also responsible for transplanted organs, bypass grafts and arteriovenous fistulas failures. Currently there is still no effective treatment to prevent IH and the resulting stenosis of the affected vessels. Many recent publications have shown how endothelial cells (EC) grown on collagen-based 3D matrixes (EC/matrix formulation) were highly effective in inhibiting IH in various animal models. The anti-IH effect provided by the EC/matrix formulation appeared to be, at least in part, the result of the secretion of EC-based products which suppressed local inflammation and VSMC/myofibroblasts activation. Based on this observation we started producing the EC/matrix-conditioned media (ECPCM). Our goals are to use ECPCM as a therapeutic tool and also to identify the anti-inflammatory factor(s) in it. Although cell-based therapy has a great potential, it also presents several therapeutic, technical and also ethical challenges. For this reasons the possibility to use a cell-conditioned media or better still the purified factor(s) as a therapy for IH is of great advantage. By establishing an in-vitro functional assay for the ECPCM we were able to demonstrate that ECPCM contains a broad-spectrum and strong anti-inflammatory activity against several pro-inflammatory and pro-thrombotic cytokines on ECs. Moreover, some progress was made in the purification and identification of the active factor(s) in ECPCM responsible for the anti-inflammatory activity. We have also started to investigate the molecular mechanism of action of the ECPCM in suppressing the pro-inflammatory activities of platelet factor 4 (PF4) and interleukin-6 (IL-6) on ECs. Lastly, we are currently working on the development of a mouse model to test ECPCM beneficial effects in-vivo.

Cross-talk and interdependence of hydrogen sulfide and nitric oxide in angiogenesis

Andreas Papapetropoulos[1]*, Ciro Coletta[2], Panagiotis Panopoulos[1], and Csaba Szabo[2].
[1]Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece, [2]Department of Anesthesiology, University of Texas Medical Branch, Galveston TX, USA

Hydrogen sulfide (H2S) has recently emerged as a novel gaseous transmitter, with regulatory roles in the cardiovascular system. We have previously shown that H2S, like nitric oxide (NO), promotes angiogenesis. It was originally believed that H2S and NO exert their effects in vascular cells via discrete signalling pathways, ATP-sensitive K+ channels and cGMP, respectively. Exposure of endothelial cells to H2S increased intracellular cGMP in a NO-dependent manner and activated protein kinase G (PKG) and its downstream effector, VASP. Inhibition of eNOS or PKG abolished the H2S-stimulated endothelial proliferation, migration and sprout formation, demonstrating a requirement for NO in H2S signaling, and the convergence of the vascular actions of H2S and NO at the cGMP/PKG pathway. In addition, silencing of the H2S-producing enzyme cystathionine-γ-lyase (CSE) abolished NO-stimulated cGMP accumulation and angiogenesis, indicating the requirement of H2S in the vascular actions of NO. Using recombinant phosphodiesterase 5 we could demonstrate that H2S exerted a potent inhibitory effect on enzyme activity in vitro. The in vivo relevance of the current findings is highlighted by the fact that H2S-induced wound healing and angiogenesis in vivo was suppressed by pharmacological inhibition or genetic ablation of eNOS. In conclusion, NO and H2S are mutually required for the physiological control of new blood vessel formation. This research has been co-financed by the EU (ESF) and Greek national funds through the Operational Program „Education and Lifelong Learning“ of the NSRF - Research Funding Program: Thalis. Investing in knowledge society through the European Social Fund

DKK1,2 role in tumor angiogenesis

Hongryeol Park[1]*, Jeong-Ki Min[2], Hye-yoon Jung[1], Ji-Young Yoo[3], Seung-sik Rho[1], Chae-Ok Yun[3], Young-Myoung Kim[4], Young-Guen Kwon[1]. [1]Department of Biochemistry, College of Sciences, Yonsei University, Seoul 120-752, Korea, [2]Therapeutic Antibody Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea, [3]Brain Korea 21 Project for Medical Science, Institute for Cancer Research, Yonsei Cancer Center, Yonsei University College of Medicine, Seoul 120-752, Korea, [4]Vascular System Research Center, Kangwon National University, Kangwon-Do 200-701, Korea

WNT canonical and non-canonical signaling pathways contributes greatly during angiogenesis. WNT canonical pathway activates beta-catenin which up-regulates VEGF, DLL4 expression and enhances VE-CAD junction. While WNT non canonical pathway induces endothelial cell proliferation and cell survival. Previously, our lab estabiliched that DKK2, one of the known WNT antagonists, enhances angiogenesis in vitro and in vivo. DKK2 enhanceds angiogenic ability through WNT independent LRP6/APC/Asef2/CDC42 cascade. However, DKK1 inhibits DKK2,VEGF-induced angiogenesis. We further analyzed whether DKK1 and DKK2 induce consistent results in tumor angiogenesis. We injected B16F10 tumor cells to flank of C57BL/6 mice and adenovirus expressing DKK1,2. Compared to control, mice treated with DKK2 adenovirus showed accelerated tumor growth, and those treated with DKK1 adenovirus showed delayed tumor growth. Through tumor section staining, adenoviral DKK2-treated and adenoviral DKK1-treated tumors showed increase, decreased vessel density compared to control mice. The same results abserved in tumor xenograft models of DKK1,2 transgenic mice. In the other hand, we previously discovered that DKK2 induces vessels with enhanced vessel maturity than VEGF-induced vessels. We co-stained the tumor vessels with CD31 for endothelial cells and NG2 for pericyte cells or SMA for smooth muscle cells. The staining results showed that adenoviral-DKK2-treated-tumor and DKK2 over-expression mice xenograft tumor vessels were well-coated with pericytes or smooth muscle cells than control vessels. Interestingly, SMA positive endothelial cells were detected in adenoviral-DKK1-treated-tumor and DKK2 over-expression mice xenograft vessels.

Nephronectin regulates axial vein morphogenesis in zebrafish

Chinmoy Patra[1]*, Filomena Ricciardi[1], Iva Nikolic[2], Mirko HH Schmidt[2], Benno Jungblut[1], Felix B. Engel[1]. [1]Max Planck Institute for Heart and Lung Reserch, Parkstrasse 1, 61231-Bad Nauheim, Germany, [2]Institut für Mikroskopische Anatomie und Neurobiologie Forschungsschwerpunkt Translationale Neurowissenschaften Universitätsmedizin der Johannes Gutenberg-Universität, Langenbeckstraße 1 D-55131, Mainz, Germany

Angiogenesis is the development of new vessels from pre-existing vessels. This is a critical morphological event both in organ development as well as in diseases. Like in other vertebrates, in zebrafish vessels form a complex network in order to fulfill tissue oxygen demands. Development of complex vascular networks is dependent on the directional migration of groups of endothelial cells, which is called angiogenic sprouting. Here we have demonstrated that in zebrafish the extracellular matrix protein, nephronectin, is transiently expressed in the caudal vein plexus forming region at the time of caudal vein sprouting at around 30 hours post fertilization (hpf). Morpholino-mediated nephronectin depletion resulted in the malformation of the caudal vein plexus and the ventral vein and in the frequent loss of inter-segmental veins. Time-lapse analysis from 28 hpf to 40 hpf indicated a decreased in the frequency of caudal vein sprout formation in nephronectin morphants. In addition, existing sprouting appeared multi-directional indicating a navigation problem. Biochemical analysis demonstrated that nephronectin is able to bind to the integrin αV/β3 heterodimer. Importantly, integrin αV and nephronectin expression overlapped in the region of the caudal vein plexus. Moreover, morpholino-mediated integrin αV knockdown in zebrafish phenocopied nephronectin depletion. Taken together, our data indicate that nephronectin regulates directional sprouting of the axial vein in zebrafish, which might be via integrin αV.

Anti-inflammatory vascular effects of resveratrol are mediated by the RNA binding protein KSRP

Julia Art[1], Verena Besche[2], Matthias Bros[2], Huige Li[1], Norbert Handler[3], Florian Bauer[3], Thomas Erker[3], Felix Behnke[5], Bettina Mönch[6], Ulrich Förstermann[1], Verena M. Dirsch[4], Oliver Werz[6], Hartmut Kleinert[1], Andrea Pautz[1]*. [1]Department of Pharmacology, Johannes Gutenberg-University Medical Center, Mainz, Germany, [2]Department of Dermatology, Johannes Gutenberg-University Medical Center, Mainz, Germany, [3]Department of Pharmaceutical/Medicinal Chemistry,University of Vienna, Vienna, Austria, [4]Department of Pharmacognosy, University of Vienna, Vienna, Austria, [5]Pharmaceutical Institute, University Tuebingen, Tuebingen, Germany, [6]Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University, Jena, Germany

Atherosclerosis, the most common cause of cardiovascular diseases, is a chronic inflammatory disease. A number of different risk factors, such as hypercholesterolemia, smoking or high blood pressure, contribute to the onset of atherosclerosis. It has been demonstrated that the polyphenolic compound resveratrol, found in fresh grapes and wine, has several cardiac protective effects mediated by reduction of vascular inflammation, inhibition of low-density lipoprotein oxidation, inhibition of platelet aggregation and cell proliferation, as well as vasorelaxation. Moreover in different animal models resveratrol treatment reduced the size and density of atherosclerotic lesions. Despite intensive research, only a few direct resveratrol target proteins have been identified but their role in resveratrol-mediated cardio protection is still elusive. In target fishing experiments using immobilized resveratrol and extracts of human peripheral blood mononuclear cells we identified the „KH-type splicing regulatory protein“ KSRP as a direct and high affinity binding target of resveratrol. KSRP is an RNA-binding protein involved in the regulation of pro-inflammatory gene expression and miRNA biogenesis. KSRP initiates the decay of mRNAs encoding for pro-inflammatory mediators by binding to AU-rich elements in the 3'-untranslated region of those mRNAs. Thereby it recruits the exosome, a multi protein complex of 3'-5' exoribonucleases, which degrades the mRNA. In human DLD-1 cells we could demonstrate that resveratrol treatment reduced the mRNA expression of IL-8, TNF-αand iNOS, which are all known to be implicated in atherosclerotic processes and which are targets of KSRP-mediated mRNA degradation. We provided evidence that these resveratrol effects rely on an increase of KSRP dependent destabilization of those mRNAs rather than on modulation of their promoter activity. Moreover, we detected in resveratrol treated DLD-1 cells an increased expression and activity of hsa-miR155, a miRNA whose biogenesis is promoted by KSRP. It has been assumed that many of the cardio protective effects of resveratrol are mediated by the histone/protein deacetylase sirtuin 1 (SIRT-1). In our experiments inhibition of SIRT-1 activity had no effect on resveratrol-mediated mRNA destabilization. Therefore we can exclude an involvement of SIRT-1. Rather, our data indicate that resveratrol promotes the decay of pro-inflammatory mRNAs by enhancing the activity of KSRP. So we demonstrated that resveratrol prevented a p38 MAPK dependent inhibitory phosphorylation at threonine residues of the protein, without blocking p38 MAPK activation or activity. Experiments in apolipoprotein E deficient mice confirmed our cell culture data. In mice orally treated with resveratrol we detected also reduced phosphorylation of KSRP and consecutively decreased expression of KSRP-regulated pro-inflammatory mRNAs such as TNF-αor IL-12 in different tissues. This implicates that resveratrol directly binds to KSRP in-vivo and thereby induces KSRP activity. Therefore KSRP may be an important denominator of the anti-inflammatory effects of resveratrol in the vasculature.

The role of Jagged1 in physiological angiogenesis

Pedrosa, Rita[1,2]*; Trindade, Alexandre[1,2]; Carvalho, Catarina[1,2]; Fernandes, Ana Carina[1,2]; Adams, Ralf[3]; Duarte, António[1,2]. [1]Centro de Investigação Interdisciplinar em Sanidade Animal, Faculty of Veterinary Medicine, Technical University of Lisbon, [2]Instituto Gulbenkian Ciência, [3]Max-Planck Institute for Molecular Biomedicine, Muenster, Germany

Over the past few years, it has become clear that the Notch signaling pathway plays a key role in coordinating multiple aspects of endothelial behavior during vessel patterning and thus in shaping the remodeling of the vascular network. While understanding of Dll4 function in angiogenesis is already advanced, the role of Jagged-1 (Jag1) in adult neoangiogenesis remains elusive. Recently published studies, suggest antagonistic roles for these two Notch ligands in the development of the mouse retinal vasculature, with Jag1 loss-of-function phenotype being opposite to the described Dll4 loss-of-function mutants (Benedito, R. et al, Cell 137, 1124-1135, 2009). Here we show the role of Jag1 in physiological neoangiogenesis, and how it affects wound healing. For this experiment we used endothelial-specific Jag1 conditional gain- and loss-of-function mutants. Our results suggest a role of this ligand in promoting physiological angiogenesis. In the Jag1 loss-of-function there was a delay in the healing rate of dermal wounds, while in the Jag1 gain-of-function wound healing was accelerated. At the end-point the Jag1 loss-of-function mutants had a two-day delay relative to the healing of control wounds, while the Jag1 gain-of-function mutants displayed a two-day advance in the closure of the dermic wounds, when compared with the respective controls. In the first case, this phenotype can be explained by a decrease in vascular density and the presence of immature vessels, that are less functional and more leaky. In the Jag1 overexpression mutants the opposite phenotype was observed. We also performed genetic analyses (RT-PCR) of several genes involved in the Notch pathway and other signaling pathways involved to better understand the mechanism of Jag1/Notch signaling. We observed that there is a direct relationship between the Jag1/Notch pathway and the VEGF-A/VEGF-R2. In the Jag1 loss-of-functions mutants there was down-regulation of the VEGF signaling pathway, whereas in Jag1 over-expression mice the opposite was observed. This interaction between these two signaling pathways is opposite of what was observed for the Dll4/Notch signaling. We also observed that the other Notch ligand Dll1, expression levels, relates inversely with the level of Jag1 expression, and that some effectors, like Hes2 and Nrarp respond in the same way as Jag1 expression. Our results suggest that contrary to what is known for Dll4, Jag1 has a pro-angiogenic role. These results point to the possibility of using enhancers of the Jag1/Notch signaling in therapeutical strategies for promoting physiological angiogenesis or using inhibitors on therapeutical strategies against tumour angiogenesis.

Wnt1 is anti-lymphangiogenic in a melanoma mouse model

Heide Niederleithner & Peter Petzelbauer. Skin & Endothelium Research Division (SERD), department of Dermatology, Medical University of Vienna, Austria

Wnt signals contribute to melanoma progression by boosting their proliferation and survival. Initially, we expected that activated Wnt signaling also improves their proficiency to recruit blood and lymph vessels. To assess this, we added cell-culture supernatants of Wnt1+ and Wnt1- melanoma to endothelial spheroids. Whereas supernatants of Wnt1- melanoma cells induced lymphatic sprouts, those of Wnt1+ cells were unable to do so and this was restored by VEGF-C. Subsequent testing of several human melanoma lines revealed that Wnt1 suppressed their VEGF-C expression. This Wnt1 effect did not depend on GSK3ß, ß-catenin or AP-1, but was blocked by cyclosporine A. To analyze Wnt1 effects in melanoma in vivo, we selected Wnt1- melanoma cell lines, over-expressed Wnt1 and injected them subepidermal into SCID mice. We found reduced VEGF-C expression, reduced lymphangiogenesis and delayed metastasis to sentinel nodes in Wnt1+ as compared with Wnt1- melanoma ($p<0.05$). Concomitant over-expression of VEGF-C or feeding of animals with cyclosporine A restored lymphangiogenesis and metastasis in Wnt1+ melanoma. In conclusion, Wnt1 is anti-lymphangiogenic by suppressing melanoma-derived VEGF-C expression.

Control of arterial remodeling by nuclear expulsion of myocardin in vascular smooth muscle cells

Larissa Pfisterer*, Anja Feldner, Markus Hecker, Thomas Korff. Insitute of Physiology and Pathophysiology, Division Cardiovascular Physiology, University of Heidelberg, Germany

Myocardin forms a ternary complex with the serum response factor (SRF) and as such it binds with a high affinity to the DNA-recognition site (CArG- motif) of genes controlling the contractile capacity of vascular smooth muscle cells (SMC). Therefore myocardin is known as a major regulator of SMC phenotype, especially preventing a shift from the contractile to the synthetic phenotype. Arterial remodeling processes typically require this shift to the synthetic phenotype to adapt the SMC mass and the architecture of the arterial vessel wall to a prolonged increase in blood pressure. Herein, we focused on the role of myocardin in vascular remodeling in the adult. At first, we investigated the protein abundance of myocardin in remodeling arteries in mice evoked either by hypertension (DOCA-salt treatment) or arteriogenesis (hindlimb ischemia model) and noted a down-regulation of myocardin in the SMCs of the media. As both pathological conditions are associated with an increase in wall stress (WS), we hypothesized that this biomechanical force controls myocardin activity. Consequently we studied the impact of a supraphysiological rise in WS on myocardin levels in the medial SMCs of isolated pressure-perfused mouse arteries and confirmed the decline. Moreover, in human arterial cultured SMCs exposed to cyclic stretch for 24 hours -thus mimicking one critical component of WS- myocardin abundance was significantly reduced both on the mRNA- and protein- level. Interestingly, this in vitro approach revealed a stretch-induced transport of myocardin from the nucleus to the cytoplasm where it was subsequently degraded by the proteasome. This stretch-induced export of myocardin from the nucleus was stimulated by the activation of ERK1/2 through MEK1/2 and resulted in a serine phosphorylation of myocardin in the nucleus. In conclusion, these findings suggest that increased wall stress elicits the export of myocardin from the nucleus and its proteasomal degradation in the cytoplasm of arterial SMCs. This chain of events may critically determine the switch from the contractile to the synthetic phenotype of SMCs in the course of pressure- induced arterial remodeling processes.

Lysyl oxidase-like protein-2 mediated assembly of type IV collagen is required for sprouting angiogenesis

C. Pichol-Thievend[1,2]*, M. Bignon[1,2], M. Malbouyres[3], J. Hardouin[4], N. Brechot[1,2], A. Barret[1,2], E. Guillon[3], F. Ruggiero[3], C. Monnot[1,2], S. Germain[1,2], L. Muller[1,2]. [1]Centre Interdisplinaire de recherches en Biologie (CIRB) - INSERM U1050 - 11 place Marcelin Berthelot 75005- Paris, [2]College de France - 11 place Marcelin Berthelot 75005- Paris, [3]Institut de Génomique Fonctionnelle, ENS-Lyon, UMR CNRS 5242, Université de Lyon, Lyon Cedex, France

Sprouting angiogenesis is associated with extensive hypoxia-driven extracellular matrix (ECM) remodeling. The molecular mechanisms involved in building the vascular microenvironment and its impact on capillary formation remain elusive. In reponse to hypoxia, we identified lysyl oxidase like protein 2 (LOXL2) which accumulates in the endothelial ECM. We showed that LOXL2 is expressed in endothelial cells during developmental angiogenesis, both during vascularization of the rat retina and in growing intersomitic vessels of zebrafish embryos. LOXL2 was also detected in neovessels in a mouse model of hindlimb post-ischemic revascularization. The functional role of LOXL2 in sprouting angiogenesis was investigated by loss of expression in the zebrafish embryo. Knocking-down LOXL2 proper organization of endothelial cells and formation of capillaries, resulting in non functional intersegmental vessels (ISV). Surprisingly, pharmacological inhibition of lysyl oxidase activity did not affect ISV formation. Further investigation in a 3D culture model using loss and gain of function experiments confirmed that LOXL2 expression was required for capillary formation. We showed LOXL2 depletion affected endothelial cell migration and proliferation whereas pharmacological inhibition only slightly affected lumen formation, suggesting that mechanisms independent of LOXL2 enzymatic activity were responsible for defective capillary morphogenesis. As i) LOXL2 belongs to the lysyl oxidase family of secreted proteins involved in collagens and elastin crosslinking and ii) is indeed colocalised with type IV collagen in the endothelial basal lamina we hypothesized that LOXL2 could regulates organization of the vascular basement membrane. Whereas knocking-down LOXL2 expression led to inhibition of type IV collagen assembly, inhibition of LOXL2 enzymatic activity only affected type IV collagen crosslinking. In conclusion, we show that LOXL2 regulates neovessel formation through assembly of the vascular basal lamina and type IV collagen organization and provide further novel evidence that LOXL2 regulates sprouting angiogenesis independently of its lysyl oxidase activity.

Imaging of adhesion molecules in a murine model of vascular dementia

Brigit den Adel[1], Ernst Suidgeest[2], Carmen Burtea[3], Robert, Muller[3], Louise van der Weerd[1,2], Robert E Poelmann[1]*. [1]Anatomy and Embryology, Leiden University Medical Center, Leiden, Netherlands, [2]Radiology, Leiden University Medical Center, Leiden, Netherlands, [3]General, Organic and Biomedical Chemistry, Université de Mons, Mons, Belgium

Alzheimer's disease (AD) and vascular dementia (VD) are the most common forms of dementia in older individuals living in Western societies. Although AD is considered a neurodegenerative disorder, in the last decade a consistent amount of evidence has suggested that vascular factors might be involved also in the pathogenesis of AD. Interestingly, it has been demonstrated that the vast majority of brains affected by AD display micro-vessels degeneration and periventricular white matter lesions. Of consequence, it is reasonable to hypothesize that an impairment in the normal endothelial function (so-called „endothelial dysfunction“) might contribute to the pathogenesis of AD. In the presence of noxious molecules in the blood, such as reactive oxygen metabolites or inflammatory cytokines, endothelial cells express adhesion molecules that are responsible for the adhesion and activation of leukocytes before their transmigration into sub-endothelial space. This is considered one of the first steps in the development of atherosclerosis. In fact, endothelial dysfunction has been related to cerebrovascular disease. Increased serum levels of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) have been linked to vascular dementia in patients, yet data about the role of these adhesion molecules in the onset and pathogenesis of dementia is lacking. We studied the spatiotemporal distribution of both VCAM-1 and E-selectin in aged ApoE-/- mice, a model for vascular dementia using both VCAM-1 and E-selectin targeted Ultra Small Particles of Iron Oxide. 12-14 months old male ApoE-/- mice were randomized to 3 different diet groups: chow, Western diet or Western diet supplemented with atorvastatin. After 6 weeks of diet mice (n=5 per group) were injected via the tail vein with E-selectin USPIOs, VCAM-1 USPIOs or untargeted USPIOs. 2 hours after injection animals were sacrificed and brains taken out. Brains were stored in 4%PFA and 24 hours later MRI was performed on a 7T horizontal PharmaScan (Bruker biospin, Ettlingen, Germany). A T1 weighted 3D FLASH sequence was used with the following parameters: TR 21ms, TE 14ms, FOV 1.6 cm3, MTX 200*200*128. Focal hypointensities were analyzed using ImageJ and USPIO accumulation was correlated to histology. Mice fed a high fat diet and to a slightly lesser degree the chow fed had enhanced accumulation of VCAM-1 USPIOs in the thalamus, hippocampus and prefrontal cortex. This effect was abrogated by treatment with atorvastatin. E-selectin accumulation was observed in the brain stem, thalamus and to a lesser extend the hypothalamus. Treatment with atorvastatin restored the accumulation levels in the thalamus to control values. Different spatial accumulation was observed between the 3 treatment groups and the different contrast agents that was confirmed by histology. Accumulation of iron particles was observed at the vascular interface in these brains. It is known that VCAM-1 expression is increased at the level of the blood brain barrier, by an increased flow of circulating free β amyloid in AD and some reports suggest a similar role for E-selectin. Our results potentially allow the use of VCAM-1 and E-selectin imaging to follow temporal brain changes and diagnose vessel wall inflammation early in the pathogenesis of AD.

Visualization of the initial steps of lymphatic vessel development and the role of CCBE1 in this process using ultramicroscopy

R. Hägerling[1], C. Pollmann[1]*, M. Andreas[1], L. Kremer[1], Uwe Schröer[3], Volker Andresen[3], S. Schulte-Merker[2] and F. Kiefer[1]. [1]Cell Signaling Laboratory, Department of Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, Röntgenstraße 20, D-48149 Muenster, Germany, [2]Developmental Biology, Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, [3]LaVision BioTec GmbH, Astastraße 14, D-33617 Bielefeld, Germany

The lymphatic system, the second vascular systems of vertebrates, plays an essential role in fluid homeostasis, the initiation of immune reactions and fat absorption, but also contributes to pathological conditions. After the initial formation of blood vessels during embryonic development, a subpopulation of endothelial cells in the cardinal vein in response to an as yet unknown signal express the transcription factor prospero-related homeobox 1 (PROX1), subsequently followed by vascular endothelial growth factor receptor 3 (VEGFR-3) and other lymphatic-specific markers. Lymphatic endothelial progenitor cells then leave the cardinal vein (CV) and follow a gradient of VEGF-C to form the first lymphatic structures and the thoracic duct. The primary superficial lymphatic plexus finally is formed by radial sprouting of lymphatic endothelial cells (LECs). Several competing models for the process of initial lymphangiogenesis exist. To resolve the mechanisms of the initial steps of lymph vessel formation, we generated high resolution 3-dimensional renderings of mouse developmental stages between E9.5 and E12.5. We analyzed immunofluorescence stained and PROX1-driven mOrange2-genetically-labeled mouse embryos, using the novel light sheet imaging modality ultramicroscopy. Our analysis revealed that initially LECs migrate away from the CV as streams of non-luminized, loosely connected cells, forming a mesh network of LECs. The LECs stream coalesces into a first peripheral longitudinal lymphatic vessel (PLLV), located lateral to the somites and subsequently forms a large luminized structure, we refer to as the primary thoracic duct (pTD) near the cardinal vein. Collectively, these first lymphatics structures have been referred to as lymph sacs. An area of highest level PROX1 expression demarks the site of closest juxtaposition between the CV and pTD and likely gives rise to the first venous-lymphatic valve. LECs sprouting from the PLLV form superficial lymphatics. In CCBE-1-deficient embryos, Prox-1-positive LECs arose normally in the CV and between E10 and E10.5 the initial migration of LECs away from the CV was unperturbed. Unexpectedly, the PROX1 expression domain in the CV was broadened in CCBE1-deficient embryos, which displayed increased VEGFR-3 expression and sprouting of venous endothelium. Concomitantly in CCBE1 KO embryos, we observed a distinct loss of PROX1-positive cardiomyocytes. Our study suggests that ultramicroscopy is an extremely powerful tool for the analysis of developmental processes and allowed us to develop a novel model for the initial steps of blood lymphatic separation and initial lymphangiogenesis. Our future goals include investigation of the mechanisms controlling LEC migration and identification the molecules regulating the special control of this process.

Both lipophilic and hydrophilic statins displayed strong in vitro anti-cytomegalovirus activity with lipophilic statins holding a higher in vivo potential

Ponroy N*, Taveira A, Mueller NJ, Millard AL. Division of Infectious Diseases and Hospital Epidemiology, University Hospital, Zurich; Switzerland

Human cytomegalovirus (HCMV) sero-positivity is associated with higher risk of cardiovascular disease. Statins, in addition to their cholesterol lowering function, exhibit antiviral activity against several viruses. Fluvastatin restrains the in vitro replication of HCMV in endothelial cells (EC), a potential in vivo reservoir for this virus. So far, nothing is known about the efficiency of other statins against HCMV. Here, we compared the in vitro anti-CMV activity of three lipophilic statins (atorva-, fluva- and simvastatin) and one hydrophilic statin (pravastatin) and evaluated if they potentiate the activity of ganciclovir „the gold standard anti-CMV drug“. Human EC and fibroblasts were treated for 24-hours with statins at 3 doses: a sub-inhibitory dose (SD) and two doses (IC20, IC50) inhibiting 20% and 50% of cell proliferation, respectively. Cells were subsequently infected with HCMV at an MOI of 1. HCMV titers were determined after 6 days post infection (dpi) using the TCID50 assay and expression of viral antigens was assessed by western blotting from 1 to 6 dpi. All statins dose-dependently inhibited the production of HCMV virions. Interestingly, at IC50 doses, fluvastatin reduced HCMV titers by 0.8 log in EC but did not affect HCMV replication in fibroblasts. Simvastatin decreased HCMV titers by 2 log in EC and 1.5 log in fibroblasts, while atorvastatin was the most efficient lipophilic statin reducing titers by 2.5 log in both EC and fibroblasts. The hydrophilic pravastatin exhibited the strongest anti-CMV activity with 3 and 4 log reductions of HCMV titers in EC and fibroblasts, respectively. Whereas statins did not affect HCMV entry, major changes in the pattern of viral antigen expression were observed. As opposed to fluvastatin, that only slightly restrained the expression of all viral antigens, atorva-, simva-, and pravastatin dramatically reduced immediate early antigens and completely abrogated early and late antigens. The specificity of statins' anti-CMV activity was assessed by co-treatment with mevalonate or cholesterol. Mevalonate almost completely abolished the anti-CMV activity of all statins whereas cholesterol had no effects. Finally, ganciclovir alone decreased HCMV titer by 1 log. Co-treatment with all statin SD reduced HCMV titers by 1.5 log, showing an additive effect of the drugs. Moreover, at IC20 and IC50 doses, a synergistic effect, illustrated by 2.5 log decreased titers, was observed with atorvastatin and fluvastatin. In conclusion, we demonstrated that all statins exhibited a potent anti-CMV activity in vitro. Atorva-, fluva- and simvastatin were efficient at doses ranging their plasma peak concentrations (Cmax), whereas pravastatin, was only effective at concentrations >1000-fold higher than Cmax. Lipophilic statins are thus more likely to have an effective anti-CMV activity in patient.

2-acetylphenothiazine inhibits superoxide ion generation in human platelets and impairs collagen-dependent thrombus formation

D.S. Vara[1], M. Campanella[2,3], G. Pula[1]*. [1]Department of Pharmacy and Pharmacology, University of Bath, [2]Department of Veterinary Basic Sciences, Royal Veterinary College, University of London, [3]Consortium for Mitochondrial Research (CfMR), University College London, United Kingdom

Background and purpose: The physiological functions of NADPH oxidases (NOXs) include host defence, post-translational processing of proteins, cellular signalling, regulation of gene expression and cell differentiation. NOX enzymes also contribute to a wide range of pathological processes, in particular cardiovascular diseases and neurodegeneration. Previous studies have suggested that NOXs regulate platelet activation. In this study, we investigated the effect of the novel NOX inhibitor 2-acetylphenotiazine (2-APT) on human platelet intracellular signalling and functional responses. Experimental approach: Superoxide ion and overall ROS generation were assessed with dihydroethidium (DHE) and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CM-H2-DCFDA) in single cell fluorescence imaging experiments, respectively. Whole blood thrombus formation, washed platelet aggregation, integrin αIIbβ3 activation, and Syk phosphorylation were analysed to understand the functional consequences of NOX inhibition by 2-APT. Key results: We demonstrated that in addition to NOX2 human platelets also express NOX1. In accordance, platelet treatment with the NOX1-selective inhibitor 2-APT significantly inhibited superoxide ion generation in response to collagen and fibrinogen adhesion (IC50 = 306 nM and 227 nM, respectively). The functional relevance of NOX inhibition by 2-APT was highlighted by the abolishment of collagen-dependent whole blood thrombus formation and washed platelet aggregation by 2-APT. As suggested by Syk phosphorylation and protein kinase C activation experiments, platelet treatment with 2-APT impairs the early signalling events downstream of GPVI, a key collagen receptor in human platelets. Conventional NOX inhibitors apocynin and diphenylene iodonium (DPI) also inhibited platelet aggregation and thrombus formation in response to collagen in a GPVI-dependent manner, although at much higher concentrations compared to 2-APT. Conclusions: Our results suggest that 2-APT is a potent inhibitor of superoxide generation in human platelets and that the activity of NOXs is critical for the intracellular signalling of the collagen receptor GPVI, collagen-dependent platelet activation and consequent thrombus formation.

Domains I and IV of annexin A2 inhibit the formation of a vascular-like network in a co-culture system

Aase Raddum*, Lasse Evensen, Hanne Hollås, James B. Lorens, Anni Vedeler
Department of Biomedicine, University of Bergen, Norway

It has been suggested that Annexin A2 (AnxA2) has a role in angiogenesis by acting as a co-receptor for plasminogen and its activator, tPA, hence being responsible for local plasmin generation (1). The production of plasmin and other proteases of the plasminogen activator system, in addition to the matrix metallo-proteinases (MMP) are required for the breakdown of the extracellular matrix (ECM) that is a prerequisite to give room for the emerging, new blood vessel (2). The role of AnxA2 in angiogenesis has been studied in a co-culture system consisting of Green Fluorescent Protein (GFP)-expressing human umbilical vein endothelial cells (HUVEC) and vascular smooth muscle cells (vSMC) mimicking most of the features of VEGF-dependent neo-vascularisation (3). The heterotetrameric AnxA2-p11 complex inhibited the formation of a vascular-like network in this system by ~30% while the equivalent concentration of p11 or recombinant AnxA2 had no significant effect. However, the same molar concentrations of a mutated, soluble and partly folded domain IV (DIV) (4) and domain I (DI) of AnxA2 inhibit this network formation by ~50%. It should be noted that DI was expressed with part of the N-terminus of the full-length protein for stability reasons. For comparison, PTK787, an inhibitor of the vascular endothelial growth factor receptor 2 also inhibits network formation by ~50%. While PTK787 is only able to inhibit network formation, DI and DIV are also able to disintegrate a preformed mature vasculature. DI was initially included as a negative control as we postulated that this domain would have no effect in the co-culture system since tPA binds to Cys-8 in the N-terminus (5). However, there may be additional interaction sites (6). Surprisingly, DI, like DIV, inhibits network formation in a dose-dependent manner. Therefore, another negative control, lysozyme with no effect on network formation in the co-culture system, was used as this protein has similar physical-/chemical properties as the domains, in terms of size and pI. Furthermore, AnxA2 monoclonal antibodies directed against aa 123-328 or aa 1-50 inhibited network formation by about 15% and 30%, respectively. The most potent inhibition (50%) was displayed by the monoclonal antibodies directed specifically against Tyr phosphorylated AnxA2, suggesting a pivotal role for endogenous Tyr phosphorylated AnxA2 present on the HUVEC surface. Consequently, it is likely that exogenously added DI and DIV may sequester soluble factors in the cell medium that interact with the endogenous extracellular AnxA2 and that are required for functional vascular network formation in angiogenesis. REFERENCES (1) Flood, EC. et al. 2011, Vascul. Pharmacol., doi 10.1016/j.vph.2011.03.003 (2) Adams, RH. et al., 2007, Nat Rev Mol Cell Biol 6, 464-478 (3) Evensen, L. et al., 2009, PLoS ONE 4, e5798 (4) Aukrust, I. et al., 2006, J Mol Biol 363, 469-481 (5) Hajjar, KA. et al. 1998, J Biol Chem 16, 9987-9993 (6) Roda, O. et al. 2003, J Biol Chem 8, 5702-5709

The co-operative role of tenascin-C and fibronectin in angiogenenic endothelial cells

Agata Radwanska[1,2]*, Dominique Grall[1,2], Benoit Langlois[3], Gertraud Orend[3], Ellen Van Obberghen-Schilling[1,2]. [1]University of Nice-Sophia Antipolis, Nice, France, [2]Institute of Biology Valrose, UMR CNRS 7277 INSERM 1091, Parc Valrose 06108 Nice cedex 2, France [3]University of Strasbourg, Inserm U682, 3 Avenue Moliere, 67200 Strasbourg, France

Angiogenesis, the sprouting of new vasculature from a pre-existing vascular network, is an essential process during development, maintenance of tissues and metastatic spread of cancer. Considerable evidence indicates that besides cytokines, the extracellular matrix components, fibronectin (FN) and tenascin-C (TNC) play an important role in this process. TNC and FN share a similar expression pattern in vivo, TNC can physically interact with FN and it exerts a modulatory role in cell adhesion-dependent processes. We have previously shown that cellular FN variants (containing EDB and EDA domains) play an important role in matrix assembly, cell motility and junctional integrity in endothelial cells (Cseh et al., J. Cell Sci. 2010). Although adhesive and counter-adhesive effects are respectively attributed to FN and TNC, the precise mechanisms by which these two matrix components functionally interact in endothelial cells remain to be further elucidated. Using different endothelial cell models (BAEC, HUVEC, HMEC) in 2D and 3D culture configurations we set out to characterize the coordinate role of TNC and cellular FN in matrix assembly, adhesion signaling and capillary morphogenesis. Whereas secretion and basal assembly of cellular FN variants was observed in all endothelial cells examined, we were unable to detect TNC expression (by Western analysis or RT-PCR) or assembly (by immunofluoresence) under standard 2D culture conditions. When cells were seeded on human recombinant TNC-coated surfaces, the fibrillar organization of the FN network was altered and the distribution of Integrin-linked Kinase in fibrillar matrix-forming adhesions was perturbed. Altered matrix assembly by endothelial cells was accompanied by F-actin remodeling, disruption of adherence junctions and increased cell motility. In our 3D system, endothelial cells grown on Cytodex beads were embedded in fibrin gels overlayed with telomerase-immortalized fibroblasts (TIFs) that secrete and incorporate considerably large amounts of TNC into their FN-rich extracellular matrix. Under these 3D conditions, HUVECS migrate into the fibrin matrix and form multicellular sprouts within 4-8 days. Addition of recombinant TNC to the fibrin gels enhanced sprouting of HUVECs. In contrast, sprout formation was impaired when the cells were cultured in the presence of TIFs deficient for TNC expression (TNC-targeted shRNA expression). Although it remains to be determined whether TNC is expressed by endothelial cells in vivo, our results demonstrate that TNC has an impact on FN fibrillogenesis and capillary morphogenesis in endothelial cells in vitro. The molecular mechanisms that underlie TNC-induced effects on FN matrix assembly and adhesion signaling in endothelial cells will be discussed.

Dual inhibition of Ang-2 and VEGF via a novel human bispecific bivalent IgG1 CrossMAb shows potent anti-angiogenic, anti-tumoral, and anti-metastatic efficacy and leads to a reduced side effect profil

Romi Rämsch*, Yvonne Kienast, Werner Scheuer, Erica Lorenzon, Frank Herting, Marielle Odin, Wolfgang Schäfer, Jörg Regula, Kay Stubenrauch, Christian Klein, Markus Thomas. The authors are employees of Roche Diagnostics GmbH, Penzberg, Germany

VEGF-A blockade has been extensively clinically validated as a treatment for human cancers. Angiopoietin-2 (Ang-2) expression has been shown to function as a key regulator of blood vessel remodeling, tumor angiogenesis, and metastasis. In tumors, Ang-2 is up-regulated and associated with poor prognosis. Recent data demonstrated that Ang-2 inhibitors, both as single agents or in combination with chemo- or anti-VEGF therapy, mediate anti-tumor effects. Additionally, it has been shown that the Ang-2/-1/Tie and the VEGF/VEGFR systems act in complementary ways suggesting that dual targeting may be more effective than targeting each pathway alone. Based on bevacizumab and the Ang-2 selective antibody LC06 we have generated a novel human bispecific bivalent IgG1 CrossMab antibody blocking VEGF-A and Ang-2 function simultaneously. Here we show in multiple subcutaneous and orthotopic in vivo models including models (semi-) resistant to anti-VEGF treatment that the systemic application of the Ang-2-VEGF CrossMAb effectively reduces angiogenesis, tumor growth and metastasis. Furthermore, we demonstrate that a highly selective anti-Ang-2 approach has safety related advantages over an unselective treatment with an antibody targeting Ang-1 and Ang-2 simultaneously. Whereas anti-Ang-1/Ang-2 long-term treatment resulted in regression of healthy vessels in the mouse trachea, an anti-Ang-2 selective treatment did not affect the physiological vessels in the trachea of the mice at all. These results imply a clear differentiation between selective Ang-2 and unselective Ang-1/Ang-2 inhibition. Although anti-tumoral efficacy is retained selective Ang-2 inhibition did not lead to a further impairment of healthy vessels compared to anti-VEGF-A treatment only. Finally, we demonstrate a clear disadvantage of Ang-2 monotherapy compared to Ang-2-VEGF dual inhibition due to strong up-regulation of VEGF resulting not only in revascularization and tumor growth, but also in toxicity as observed in peliosis-like liver pathology. These pathological effects were completely inhibited by dual inhibition of Ang-2 and VEGF-A. Taken together, our data indicate that Ang-2 and VEGF-A exhibit angiogenic synergy in a mutually compensatory fashion and that their inhibition via the novel Ang-2-VEGF CrossMab mediates potent anti-tumoral, anti-metastatic and anti-angiogenic efficacy. Additionally the CrossMAb is expected to exhibtit a better side effect profile compared to the respective monotherapies and thereby represents a promising therapeutic agent for the therapy of cancer. These data support the investigation of the Ang-2-VEGF CrossMAb in PhI clinical trials scheduled for 2012.

Atherogenic effects of thrombin via regulation of sphingosine kinase-1 (SPHK-1) expression in vascular smooth muscle cells

Anja Flößer, Andreas Böhm, Andrea Fischer, Heyo K. Kroemer, Bernhard H. Rauch*. Universitätsmedizin Greifswald, Institut für Pharmakologie, Abteilung Allgemeine Pharmakologie, Felix-Hausdorff-Str. 3, 17487 Greifswald, Germany

Sphingosine-1-phosphate (S1P) is a cellular signaling lipid generated by sphingosine kinase-1 (SPHK-1). The present study investigates the regulation of SPHK-1 in thrombin-stimulated human vascular smooth muscle cells (SMC) and in an atherosclerosis model in mice treated with the direct oral thrombin inhibitor dabigatran. Expression of SPHK-1 was determined by Taqman® real-time PCR, Western blotting and immunohistochemistry in human saphenous vein SMC and in ApoE-deficient mice. Mitogenesis was determined via DNA synthesis, proliferation by cell counting. Binding of the mRNA stabilising factor human antigen R (HuR) was measured by immunoprecipitation (pulldown PCR). ApoE mice were treated with dabigatran etixilate (DE)-supplemented chow (10 mg DE/g chow) or matching placebo for 6 months. Plaque coverage was determined by oil red O staining. Thrombin induced a time- and concentration-dependent (1-100 nM) increase in SPHK-1 mRNA and protein expression in human vascular SMC, determined by real-time PCR and Western blotting, n=6-7. Inhibition of SPHK-1 attenuated thrombin-induced SMC proliferation but not SMC migration (n=5). The regulatory action of thrombin on SPHK-1 was mimicked by a synthetic PAR-1 ligand and reduced by siRNA against the mRNA stabilising protein HuR. Thrombin was also shown to promote HuR binding to SPHK-1 mRNA, associated with increased nucleo-cytosolic shuttling of HuR and SPHK-1 mRNA stabilisation in the presence of actinomycin D. In ApoE-deficient mice, long-term treatment with the direct thrombin inhibitor dabigatran significantly reduced aortic SPHK-1 expression by 50% (n=5) and plaque size by 35% compared to control animals (n=10). In conclusion, thrombin induces SPHK-1 expression and S1P synthesis in vascular SMC via the mRNA stabilising protein HuR. This leads to increased SMC proliferation. Inhibition of thrombin by dabigatran treatment in vivo attenuates progression of plaques possibly by reducing SPHK-1 expression.

Inhibition of IGF-1 receptor signaling increases serum cholesterol and plaque burden in ApoE deficient mice

Anton Razuvaev*, Jeanna Joneberg, Olle Larsson, Magnus Axelson, Ulf Hedin, Joy Roy.
Karolinska Institutet, Stockholm, Sweden

Insulin like growth factor-1 has been shown to have a prominent effect on smooth muscle cell proliferation, migration and apoptosis. We and others have previously shown that inhibiton of IGF-1 related signaling can attenuate intimal hyperplasia by attenuating smooth muscle cell proliferation and promoting apoptosis after vascular injury. We hypothesized with support from preliminary work in our laboratory that IGF-1 inhibition will decrease atherosclerosis burden, but may negatively affect plaque stability by increasing SMC apoptosis. Picrpodophyllin is a specific inhibitopr of the IGF-1 receptor phosphorylation and is currently under development as an anti-cancer drug. The aim of this study was to study the short and long term effects of IGF-1 inhibition using PPP in ApoE deficient mice Methods and Results: 12 wk old Apo E mice (n=64) were treated with an oral preparation of PPP or vehicle (controls) for a period of 10 and 18 weeks. Blood samples were collected before sacrifice and aortas, brachiocephalic trunk and liver samples harvested. Semi quantitative analysis of the aortic and brachiocephalic trunks showed an increase in plaque burden and was significant after 18 weeks. There were no differences in weight, serum creatinine, albumin or glucose between the two groups. However, we noted 18% lower levels of IGF-1, doubled levels of growth hormone, 30% increase in serum cholesterol levels in the PPP treated group. RT-PCR analysis showed a 38% decrease in LDL receptor expression in the liver in the PPP group. Conclusion: PPP treatment increased plaque burden. The proposed mechanism is via an increase in serum cholesterol, however further work has to be performed to confirm the pathways involved.

microRNAs control brain endothelial cell barrier function and immune quiescence, implications for MS.

Arie Reijerkerk*, MA Lopez-Ramirez, Bert van het Hof, Kim A.M. Lakeman, Joost A.R. Drexhage, Gijs Kooij, Martijn E. Blommaart, Joost B. Vos, Dirk Geerts, Reuven Agami, Alex Prat, Anton J. van Zonneveld, Anton. J. Horrevoets, Ignacio A. Romero, and Helga E de Vries. Blood-Brain Barrier Research Group, Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands. Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands. Department of Pediatric Oncology/Hematology, Sophia Children's Hospital, Erasmus University Medical Center, Rotterdam, The Netherlands Division of Gene Regulation, Netherlands Cancer Institute, Amsterdam, The Netherlands. Neuroimmunology Research Laboratory, Center of Excellence in Neuromics, CHUM-Notre-Dame Hospital, Faculty of Medicine, Université de Montréal, Montréal, Quebec, Canada. Department of Nephrology and Einthoven Laboratory for Experimental Vascular Research, Leiden University Medical Center, Leiden, The Netherlands

In normal physiology the blood-brain barrier (BBB) tightly regulates brain homeostasis. Perturbations of BBB function, including the loss of brain endothelial cell barrier integrity and immune activation are hallmarks of multiple sclerosis (MS). Therefore, understanding of the BBB in health and disease may lead to novel approaches for MS treatment. Using a combined genetic and bioinformatics approach, we uncovered a novel mechanism which regulates different aspects of the BBB, including barrier formation and immune quiescence, i.e. through microRNAs. microRNAs are recently discovered endogenous, small, noncoding RNAs which regulate the production of about 30% of human proteins and have been shown to be involved in cell biology and pathology. Using a genomics approach, we have identified a microRNA (miR-125a-5p) which targets the activity of the transcription factor myc-associated zinc finger protein and plays a major role in the formation of a tight brain endothelial cell barrier and the paracellular trafficking of immune cells. Interestingly, lower levels of miR-125a-5p were associated with the inflamed BBB in vitro and in brain endothelial cells obtained form MS patients by laser capture. Most importantly, our recent analyses in brain capillaries which were isolated from MS patients have revealed that a large panel of BBB stabilizing microRNAs is significantly reduced in MS lesions. We conclude that therapeutic application of microRNAs (such as miR-125a-5p) potentially could re-establish normal functioning of the BBB to prevent inflammation in multiple sclerosis.

Endothelial Wnt/β-catenin signaling reduces vascularization, Blood-Brain Barrier breakdown and tumor growth in a mouse glioma model

Marco Reis[1]*, Cathrin J. Czupalla[1], Nicole Ziegler[1], Kavi Devraj[1], Jenny Zinke[1], Sascha Seidel[1#], Rosario Heck[2], Sonja Thom[1], Jadranka Macas[1], Ernesto Bockamp[2], Stefanie Dimmeler[3], Karl H. Plate[1], Stefan Liebner[1]. [1]Institute of Neurology (Edinger Institute), Johann Wolfgang Goethe-University Frankfurt Medical School, [2]Medical Center of the Johannes Gutenberg-University Mainz, III. Department of Internal Medicine, Division of Experimental and Translational Oncology, [3]Institute for Cardiovascular Regeneration, Johann Wolfgang Goethe-University Frankfurt Medical School. [#]Current address: Institute of Neuropathology, Justus Liebig-University Medical School, Giessen, Germany

Endothelial Wnt/β-catenin signaling is necessary for developmental angiogenesis of the central nervous system and differentiation of the blood-brain barrier (BBB), but it seems to be inoperable in the adult. In particular, its relevance for angiogenesis and vessel differentiation in tumors is unknown. We aimed to elucidate the role of endothelial Wnt/β-catenin signaling during brain tumor vascularization, in particular the effect on angiogenesis and BBB characteristics. To this end we generated mouse GL261 glioma cell lines expressing either Wnt1 or the soluble Wnt signaling inhibitor Dickkopf-1 (Dkk1) in a doxycycline-dependent manner. We show that in subcutaneous and intracranial transplanted glioma, endothelial Wnt/β-catenin signaling resulted in significantly diminished tumor growth, due to reduced vascular density and a quiescent vessel phenotype with increased mural cells attachment. Accordingly, Wnt1 glioma vessels showed increased BBB characteristics indicated by reduced IgG permeability and distinct junctional staining of the tight junction marker claudin-3. Furthermore, a tumor experiment performed with endothelial β-catenin gain-of-function mice phenocopied the quiescent and normalized vascular phenotype of Wnt1 expressing tumors. Conversely, Dkk1 promoted glioma vascularization and an angiogenic endothelial phenotype thereby increasing tumor growth. Wnt1 activated the Dll4/Notch pathway in tumor endothelia, blocking an angiogenic and favoring a quiescent phenotype by inhibiting tip cell markers such as neuropilin-1 and inducing stalk cell genes as jagged-1 and VEGFR1. Furthermore, we observed an increased expression of the platelet-derived growth factor subunit B (pdgfb) upon endothelial Wnt/β-catenin signaling, which might support the recruitment of mural cells to the tumor vasculature, thus supporting vascular quiescence. In conclusion, sustained and reinforced Wnt/β-catenin signaling leads to inhibition of angiogenesis with stabilized and less permeable vessels, which might proof to be a valuable therapeutic target for edema and anti-angiogenic cancer therapy.

FDG-6-phosphate in experimental atherosclerotic lesions: Translatable marker of plaque inflammation that reflects glucose metabolic trapping but does not require PET imaging

Richard Conway, Eyassu Chernet, David De Rosa, Robert Benschop, Anne Need, Emily Collins, Michael Kalbfleisch, James Bean, Mark Rekhter*. Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA

Rationale. 18F-Fluorodeoxyglucose (FDG)-positron emission tomography (PET) imaging of atherosclerosis is based on the hypothesis that plaque cells metabolize glucose more actively than surrounding tissues. Despite clinical success, it is challenging to use FDG-PET in mouse models due to limited sensitivity and resolution. Objective. We aimed to (a) develop a technique that would also quantify glucose metabolic trapping in atherosclerotic plaques but would not require PET imaging, and (b) validate this technique by demonstrating vascular response to a pharmaceutical with known anti-atherogenic properties. Methods and Results. We have developed a novel quantitative method that is based on mass spectrometry of FDG-6-phosphate, a metabolite of FDG, in the mouse arteries. Nonradioactive FDG was injected 30 minutes before euthanasia. Arteries were dissected, and lipids were extracted with chloroform-methanol. Delipidated arteries were re-extracted with 50% acetonitrile-50% methanol-0.1% formic acid. A daughter ion of FDG-6-phosphate was quantified in the extracts using liquid chromatography and mass spectrometry (LC/MS/MS). This approach facilitated analysis of traditional (cholesterol) and novel (FDG-6-phosphate) markers in the same tissue. FDG-6-phosphate was accumulated in atherosclerotic lesions associated with carotid ligation of the Western diet fed ApoE-/- mice (5.9 times increase compare to unligated carotids, $p<0.001$). Treatment with LXR agonist T0901317 significantly (2.1 times, $p<0.01$) reduced FDG-6-phosphate accumulation 2 weeks after surgery. Anti-atherosclerotic effects were independently confirmed by reduction in lesion size, macrophage number, cholesterol ester accumulation, and macrophage proteolytic activity. FDG-6-phosphate also readily accumulated in the macrophage cell line (THP-1 cells) in vitro when FDG was added to a culture media. FDG-6-phosphate accumulation was proportional to the cell number ($r=0.98$). Thus, non-radioactive FDG can be a valuable tool for both in vivo and in vitro studies. Conclusions. Mass spectrometry of FDG-6-phosphate in experimental atherosclerosis provides potential translational link to the clinical studies utilizing FDG-PET imaging.

The effect of acute and prolonged blockade of store operated channels on the response of the neonatal pulmonary circulation to hypoxia

Parrau D[1,3], Ebensperger G[1], Santos D[1], Herrera EA[1,2], Montt C[1], Chubretovich M[1], Diaz M[1], Moraga FA[4], Ulloa C[1], Hernandez I[1], Llanos AJ[1,2], Reyes VR[1]*. [1]Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Pantiago, Chile, [2]International Center of Andean Studies (INCAS), Universidad de Chile, Putre, Chile, [3]Facultad de Medicina, Universidad Diego Portales, Santiago, Chile, 4Facultad de Medicina, Universidad Catolica del Norte, Coquimbo, Chile

Pulmonary arteries respond to acute hypoxia with a rapid and reversible vasoconstriction, known as hypoxic pulmonary vasoconstriction (HPV), increasing transiently the pulmonary arterial pressure (PAP) and resistance (PVR). Chronic hypoxia results in persistent pulmonary hypertension (PPH), a disease characterized by a sustained elevation in PAP and PVR and cardiopulmonary remodelling. Newborns exposed to chronic hypoxia in utero show a high incidence of PPH (Herrera et al, 2007). Since intracellular calcium increase is a key event in pulmonary vascular contraction and remodelling (Firth et al, 2007), we investigated the role of store operated channels (SOC), a calcium-permeable cationic channel class in both HPV and PPH, using low altitude newborn lamb with normal PAP (LANB) and high (HANB) altitude newborn lambs with pulmonary hypertension. In vivo, a single dose of 2-aminoethyldiphenylborinate (2-APB), a compound known to inhibit SOC, attenuates HPV in both LANB and HANB. This response is markedly stronger in HANB, where also basal PAP and PVR are reduced. Systemic arterial pressure and cardiac output are not modified by 2-APB during acute administration. Ex vivo studies showed that 2-APB induces relaxation in isolated pulmonary arteries, with a greater sensitivity in HANB than LANB. Pulmonary gene expression of TRPC4 and Stim1, two putative SOC-components, that participates in pore formation and in the calcium-sensing of internal stores respectively, and are higher in HANB than LANB. Furthermore, we studied the effect of treatment with 2-APB for a longer period in the pulmonary circulation. In vivo administration of 2-APB for 10 days resulted only in a partial reduction of basal PAP in HANB. Isolated pulmonary arteries from HANB treated with 2-APB showed a significant reduction of contractile response to thromboxane mimetic U46619 and thinning of the pulmonary artery wall, but also displayed an increase of the sensitivity and response to the phospodiesterase V inhibitor sildenafil, as well as to the Rho-kinase inhibitor fasudil. Taken together these results suggest that SOC are involved in acute and chronic responses of pulmonary circulation to hypoxia, during the perinatal period. Further studies should reveal the mechanisms involved and will elucidate the potential therapeutic use of SOC blockade to treat PPH. References -Herrera et al. Am J Physiol 292: R2234, 2007 -Firth et al. Biochem Biophys Acta 1772: 895, 2007. Supported by FONDECYT 1080663, 1090355 and 1110595.

Reciprocal regulation of platelet derived growth factor-BB and bone morphogenetic protein-2 by Factor VII activating protease in vascular smooth muscle cells

Rödel, E.[1]*, Plöger, F.[2], Schwarz, E.[3], Kanse, S.M.[4]. [1]Institute of Biochemistry, Justus-Liebig University, Giessen, Germany, [2]Biopharm GmbH, Heidelberg, Germany, [3]Institute of Biotechnology, Martin-Luther University, Halle, Germany, [4]Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

Background: Human genetic studies show that functional defects in FSAP are a risk factor for atherosclerosis and vascular calcification. This serine protease has high homology to plasminogen and was shown to be a potent inhibitor of platelet derived growth factor-BB (PDGF-BB) with respect to the proliferation and migration of vascular smooth muscle cells (VSMC). Bone morphogenetic protein-2 (BMP-2) belongs to the transforming growth factor beta (TGF-beta) superfamily that promotes cell differentiation and matrix deposition and has an inhibitory effect on VSMC proliferation. BMP-2 contributes to vascular calcification and atherosclerosis and is in turn regulated by various extracellular inhibitors such as Noggin and Bmper. We have uncovered a reciprocal regulation of PDGF-BB and BMP-2 by FSAP in VSMC. Methods: Influence of FSAP and other haemostasis factors on the regulation of BMP-2 and its latent pro-form was tested by Western blotting, various cellular assays, realtime PCR analysis and amino-terminal protein sequencing. Results: Mature BMP-2 was cleaved by FSAP and this increased its biological activity. This was demonstrated by decreased proliferation and cellular differentiation of C2C12 myoblasts into osteoblasts in a dose and time dependent manner. Cleavage and activation was further demonstrated to lesser extend by Plasmin, but Thrombin, FVIIa, FXa and APC were not effective. Same effects were found by treatment of latent pro-BMP-2 with FSAP and Plasmin. Amino-terminal sequencing of cleaved pro-BMP-2 fragments by FSAP showed generation of the active mature BMP-2 whereas cleavage of mature BMP-2 generated a N-terminally truncated form, characterized by enhanced biological activity. These cleavage sites differed compared to Plasmin treatment of BMP-2 and latent pro-BMP-2. The VSMC expression of calcification-relevant genes was enhanced. BMP-2 cleavage and activation was abolished by enzymatically inhibited FSAP or the BMP-2 inhibitor Noggin. Conclusion: FSAP can inhibit the effects of PDGF-BB on VSMC proliferation and migration (Sedding et al., J. Exp. Med. 2006). Whereas BMP-2, a factor that inhibits VSMC proliferation and induces calcification, is enhanced by FSAP. Thus, FSAP drives the inhibition of VSMC proliferation and enhances differentiation and calcification. This could be relevant for atherosclerosis since a polymorphism in the FSAP gene which leads to a loss of enzymatic activity correlates with atherosclerosis and vascular calcification.

Long Pentraxin-3 inhibits FGF-dependent angiogenesis and growth of steroid hormone-regulated tumors

Roberto Ronca[1]*, Patrizia Alessi[1], Emanuela Di Salle[1], Daria Leali[1], Daniela Coltrini[2], Michela Corsini[1], Giorgia Nardo[3], Stefano Indraccolo[3], Cecilia Garlanda[4] and Marco Presta[1]. [1]Unit of General Pathology and Immunology and, [2]Unit of Histology, Department of Biomedical Sciences and Biotechnology, School of Medicine, University of Brescia, Brescia, [3]Istituto Oncologico Veneto-IRCCS, Padua, Italy, [4]Istituto Clinico Humanitas-IRCCS, Rozzano, Italy

Steroid hormone-regulated tumors, including breast and prostate cancers, represent a class of epithelial lesions whose growth is finely tuned by steroid hormones. However, as these tumors progress, they may become independent from steroid hormones for growth, limiting the effectiveness of hormonal ablation therapies. Fibroblast growth factors (FGFs) are potent angiogenic factors that exert non-redundant autocrine/paracrine functions in various tumors types. Experimental and clinical evidences indicate that the FGF/FGF receptor (FGFR) axis can drive the progression of steroid hormone-dependent cancers to a hormone-independent state, thus representing a possible alternative target for the treatment of hormonal cancers. Previous observations had shown that the soluble pattern recognition receptor long pentraxin-3 (PTX3) is a natural selective antagonist for a restricted number of FGF family members, inhibiting FGF2 but not FGF1 and FGF4 activity. Here, we extended these findings and assessed the capacity of PTX3 to antagonize also FGF8b and to inhibit the vascularization and growth of steroid hormone-regulated breast and prostate cancers. Surface plasmon resonance analysis demonstrates that PTX3 binds FGF8b with high affinity. As a consequence, PTX3 prevents the binding of FGF8b to its receptors, inhibits FGF8b-driven ERK1/2 activation, cell proliferation and chemotaxis in endothelial cells, and suppresses FGF8b-induced neovascularization in vivo. Also, PTX3 inhibits testosterone and FGF8b-driven proliferation in androgen-regulated Shionogi 115 (S115) mouse breast and LNCaP human prostate tumor cells and the FGF8b/FGF2-driven proliferation of TRAMP-C2 murine prostate cancer cells. Furthermore, when transfected into S115 or TRAMP-C2 cells, PTX3 impairs FGF/testosterone induced cell proliferation and angiogenic activity in the chick embryo chorioallantoic membrane (CAM) assay. Accordingly, hPTX3_S115 and hPTX3_TRAMP-C2 cells show a dramatic decrease of their tumorigenic capacity in vivo. These results identify PTX3 as a novel FGF2/FGF8b antagonist endowed with antiangiogenic and antineoplastic activity with possible implications for the therapy of breast and prostate steroid hormone-regulated tumors.

Motor coordination in a new mouse model of atherosclerotic plaque rupture

Roth L.[1]*, Van Dam D.[2], Van der Donckt C.[1], Schrijvers D.M.[1], Vanhoutte G.[3], Verhoye M.[3], Martinet W.[1], Van der Linden A.[2], Bult H.[1] and De Meyer G.R.Y.[1]. [1]Laboratory of Physiopharmacology, [2]Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge and [3]Bio-Imaging Lab, University of Antwerp, Belgium

Apolipoprotein E deficient (apoE-/-) mice with a heterozygous mutation in the fibrillin-1 gene (fbn1C1039G+/-, Marfan phenotype) show an increase in arterial stiffness due to fragmentation of the elastin fibres. We recently showed that this results in exacerbated atherosclerosis and spontaneous plaque ruptures, leading to atheroembolic stroke (as assessed on MRI), accompanied by neurological symptoms (e.g. head tilt) and sudden death. The present study focused on motor coordination of the apoE-/- fbn1C1039G+/- mice, which might provide useful information on the neurological aspects of this mouse model. Female apoE-/- (control) and apoE-/- fbn1C1039G+/- mice (6 weeks old) were fed a Western type diet (WD) for up to 20 weeks (n=20 mice in each group). A group of female apoE-/- fbn1C1039G+/- mice on normal chow diet (ND, n=20) was also included to study potential effects of the Marfan phenotype on motor performance. Coordination was assessed every two weeks starting at 10 weeks of WD or ND by the following tests: gait analysis, stationary beam, wire suspension and accelerating rotarod. From 12 weeks onward, the gait analysis test revealed a significant increase in track width (distance between left and right paw) of the apoE-/- fbn1C1039G+/- (WD) mice as compared with the apoE-/- (WD) and apoE-/- fbn1C1039G+/- (ND) mice (2.81±0.05 mm vs. 2.64±0.03 mm and 2.68±0.05 mm) and this effect remained consistent throughout the experiment (at 20 weeks: 3.07±0.14 mm vs. 2.62±0.05 mm and 2.62±0.07 mm). This finding indicates that the apoE-/- fbn1C1039G+/- (WD) mice tried to correct for a loss in balance and coordination by widening the distance between the left and right paw. Moreover, the increase in track width was observed at a time point before head tilt occurred, indicating that gait analysis might detect neurological symptoms at an early stage. The stationary beam test showed a trend towards a decrease in motor coordination of the apoE-/- fbn1C1039G+/- (WD) mice as compared to the controls. Currently, experiments are ongoing to confirm these results. The wire suspension test (grip strength) and the rotarod (motor coordination and balance) did not reveal significant differences. In conclusion, gait analysis showed the early development of differences in motor coordination between apoE-/- fbn1C1039G+/- (WD), apoE-/- (WD) and apoE-/- fbn1C1039G+/- (ND) mice and can therefore be used to evaluate neurological symptoms at an early stage (before head tilt occurred) in the apoE-/- fbn1C1039G+/- mouse model of plaque rupture. This test might also be of value to assess the effect of potential plaque stabilizing drugs to prevent atherothrombotic vascular disease.

Identification of novel tumor endothelial markers in human muscle invasive bladder carcinoma by laser capture microdissection and transcriptional profiling

Filip Roudnicky[1]*, Cedric Poyet[2], Fabrizia Negrini[1], Peter Wild[3], Vivianne Otto[1], Tullio Sulser[2], Michael Detmar[1]. [1]Institute of Pharmaceutical Sciences, ETH Zürich, Switzerland, [2]Department of Urology, University Hospital Zürich, Zürich, Switzerland, [3]Department of Pathology, University Hospital Zürich, Zürich, Switzerland

The importance of angiogenesis in tumor growth and metastasis by supplying nutrients and oxygen is well established. However, the role of endothelial cells as active participants in the tumor environment is poorly understood. To identify factors contributed by the tumor-associated endothelium, we performed immuno-laser capture microdissection (LCM) of blood vascular endothelial cells in conjunction with transcriptional profiling from surgically harvested non-neoplastic and neoplastic tissue of six patients with confirmed muscle-invasive bladder cancer (MIBC). We found that most of the previously known markers of angiogenic endothelium showed increased expression in bladder cancer-associated endothelium, and we also identified upregulation of several novel tumor angiogenesis markers. Endocan (endothelial-specific molecule-1) was one of the genes that was highly elevated in tumor-associated blood vascular endothelium. We confirmed this upregulation of endocan both by quantitative real time PCR and immunohistochemical staining. Notably, blood vascular endothelial cells were found to express higher levels of endocan in patients with MIBC (N=13) compared to patients with noninvasive bladder cancer (N=36) as shown by immunohistochemical staining. In addition, levels of endocan were significantly elevated in the plasma of patients with MIBC (N=30) compared to healthy individuals (N=30). We also found that endocan is upregulated in vitro and in a transgenic in vivo mouse model by VEGF-A. Furthermore, we detected increased VEGF-A concentrations in the plasma of patients with MIBC (N=40) compared to healthy individuals (N=30). Endocan silencing in cultured human primary endothelial cells abolished cell migration and tube formation induced by VEGF-A. These data indicate that endocan is an important mediator of VEGF-A-induced angiogenesis and a potential biomarker for MIBC.

Rabbit iliac artery model to evaluate novel drug-eluting stents

Matthijs S. Ruiter[1]*, Robbert J. de Winter[2], Carlie J.M. de Vries[1]. [1]Department of Medical Biochemistry, [2]Department of Cardiology, Academic Medical Center, University of Amsterdam, The Netherlands

Background Drug-eluting stents (DES) are widely used to maintain perfusion to occluded coronary arteries and prevent in-stent restenosis. Although the current generation of DES prevent excessive smooth muscle cell growth, these devices increase the risk for thrombotic events, with potentially lethal consequences. We aim to develop novel DES that not only prevent restenosis, but also promote re-endothelialization of the stented vessel wall to prevent thrombosis. Effectiveness of prototypes is evaluated in rabbit iliac arteries. Methods and results In male New Zealand White rabbits (2.5-3.0 kg), stent protoypes were inserted at 3mm diameter in both the left and right iliac artery after balloon denudation of these vessels. Stent patency was confirmed by angiography. After 28 days, stent patency was again angiographically confirmed and animals were euthanized after which stented arteries were perfusion fixed and harvested. Stented arteries were dehydrated and embedded in MMA/BMA (1:1). Sections were cut from the middle of the stent using a microtome and sections were stained to study vessel morphology. Successful histochemical stainings include Hematoxilin/Eosin, Lawson elastin and Masson trichrome. Intimal lesion formation was quantified by morphometric analysis. Immunohistochemical staining for smooth muscle alpha-actin (1A4) and macrophages (RAM-11) was performed, showing localization of small groups of macrophages around the stent struts. Conclusion The procedure to evaluate stents of human dimension in rabbit iliac arteries with 28-day follow-up is now fully operational in our institute. We have minimal animal drop-out and a very high success rate. The lesions are composed predominantly of smooth muscle cells with some macrophage infiltrates around the stent struts. Current experiments will reveal the effectiveness of new stent prototypes to prevent intimal lesion formation after 28 days and re-endothelialization 7 days and 14 days after stent placement.

VEGF modulates NMDA receptors activity in cerebellar granule cells through Src-family kinases before synapse formation

Claire Meissirel[1,2,17], Carmen Ruiz de Almodovar[3,4,17]*, Ellen Knevels[3,4,17], Cathy Coulon[3,4], Naura Chounlamountri[1,2], Inmaculada Segura[3,4], Pierre de Rossi[1,2] Stefan Vinckier[3,4], Kristof Anthonis[3,4], Bérangère Deléglise[1,2] Maria de Mol[3,4], Carine Ali[5,6,7], Karel Dassonville[1,2], Ellen Loyens[8], Jérôme Honnorat[1,2], Yvette Michotte[8], Véronique Rogemond[1,2], Ilse Smolders[8], Thomas Voets[9], Denis Vivien[5,6,7], Pieter Vanden Berghe[10], Ludo Van Den Bosch[4,11], Wim Robberecht[4,11,12], Alain Chédotal[13], Salvatore Oliviero[14], Mieke Dewerchin[3,4], Dietmar Schmucker[4, 15], Nicole Thomasset[1,2,17], Paul Salin[1,2,16,17], and Peter Carmeliet[3,4,17]. [1]Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 1028, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5292, Neurooncology and Neuroinflammation, Lyon Neuroscience Research Center, F-69000 Lyon, France, [2]University Lyon 1, F-69000 Lyon, France, [3]Laboratory for Angiogenesis and Neurovascular Link, Leuven, Belgium, [4]Vesalius Research Center, VIB, K.U. Leuven, B-3000 Leuven, Belgium, [5]INSERM, Unité 919, Serine Proteases and Pathophysiology of the Neurovascular Unit, Caen Cedex, France, [6]Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6232, Center for Imaging Neurosciences and Applications to Pathologies, Cyceron, F-14074 Caen Cedex, France, [7]University of Caen Basse-Normandie, F-14074 Caen Cedex, France, [8]Department of Pharmaceutical Chemistry, Drug Analysis, and Drug Information, Vrije Universiteit Brussel, 1090 Brussels, Belgium, [9]Laboratory of Ion Channel Research, K. U. Leuven, B-3000 Leuven, Belgium, [10]Center for Gastroenterological Research, K. U. Leuven, B-3000 Leuven, Belgium, [11]Laboratory of Neurobiology, K. U. Leuven, B-3000 Leuven, Belgium, [12]Department of Neurology, University Hospital Leuven, B-3000 Leuven, Belgium, [13]INSERM, Unité Mixte de Recherche S968, Department of Development, Institute of Vision, F-75012 Paris, France, [14]Department of Molecular Biology, Università di Siena, 53100 Siena, Italy, [15]Laboratory of Neuronal Wiring, K. U. Leuven, B-3000 Leuven, Belgium, [16]INSERM Unité 1028, Centre National de la Reserche Scientifique Unité Mixte de Reserche 5292, Physiopathology of the Sleep Neuronal Networks, Lyon Neuroscience Research Center, F-69000 Lyon, France, [17]Authors contributed equally to this work

NMDA type glutamate receptors (NMDARs) are best known for their role in synaptogenesis and synaptic plasticity. Much less is known about their developmental role before neurons form synapses. We report here that VEGF, which promotes migration of granule cells (GCs) during postnatal cerebellar development, enhances NMDAR-mediated currents and Ca(2+) influx in immature GCs before synapse formation. The VEGF receptor Flk1 forms a complex with the NMDAR subunits NR1 and NR2B. In response to VEGF, the number of Flk1/NR2B coclusters on the cell surface increases. Stimulation of Flk1 by VEGF activates Src-family kinases, which increases tyrosine phosphorylation of NR2B. Inhibition of Src-family kinases abolishes the VEGF-dependent NR2B phosphorylation and amplification of NMDAR-mediated currents and Ca(2+) influx in GCs. These findings identify VEGF as a modulator of NMDARs before synapse formation and highlight a link between an activity-independent neurovascular guidance cue (VEGF) and an activity-regulated neurotransmitter receptor (NMDAR).

A novel mouse model of liver tumor progression to study angiogenesis in hepatocellular carcinoma

Anja Runge[2]*, Carolin Mogler[2,3], André Neumann[2], Dorde Komljenovic[4], Jan-Philip Bergeest[6], Bernd Arnold[5], Peter Schirmacher[3], Karl Rohr[6], Tobias Bäuerle4, Sergij Goerdt[7], Hellmut G Augustin[1,2]. [1]Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim (CBTM), Heidelberg University and, [2]Vascular Oncology and Metastasis, German Cancer Research Center, Heidelberg (DKFZ-ZMBH Alliance), [3]Institute of Pathology, University Hospital, Heidelberg, [4]Dept. Medical Physics in Radiology, DKFZ [5]Dept. Molecular Immunology, DKFZ, [6]Dept. Bioinformatics and Functional Genomics, Biomedical Computer Vision Group, BIOQUANT, [7]Dept. Dermatology, Venereology, and Allergology, University Medical Center Mannheim, Germany

Intratumoral hepatocellular carcinoma (HCC) angiogenesis is complicated by the presence of two vascular systems in the liver. The arterial as well as the sinusoidal vascular system are both believed to contribute to HCC angiogenesis. HCC has recently been shown to be the only type of tumor (beyond renal cell carcinoma) in which anti-angiogenic monotherapy exerts some clinical efficacy. Yet, the molecular mechanisms of HCC angiogenesis towards tumor progression are poorly understood. We have developed a novel conditional transgenic liver tumor model, which allows the initiation of liver tumor progression by intravenous adenoviral-Cre injection versatily enabling the temporally controlled initiation of liver tumorigenesis. Upon early nodule formation, liver sinusoidal endothelial cells (LSEC) undergo intratumoral capillarization accompanied by changes in peri-endothelial cell coverage. Interestingly, VEGFR2 expression was reduced in liver tumor nodules compared to the constitutive VEGFR2 expression in normal liver. In contrast, the expression of the angiogenic factor, Angiopoietin-2 (Ang-2), was dramatically upregulated during liver tumorigenesis suggesting an important role of Ang/Tie signaling in liver tumor progression. Treatment of tumor mice with the clinically HCC approved multi-kinase inhibitor Sorafenib decreased tumor development with decreased vessel area and tumor size, and established the tumor model as a useful tool for the validation of anti-angiogenic drugs.

Intracavernous delivery of blocking antibody to nerve injury-induced protein 1 (Ninjurin 1) restores erectile function through enhanced endothelial regeneration in the streptozotocin-induced diabetic mouse

Ji-Kan Ryu*, Guo Nan Yin, Hai-Rong Jin, Min Ji Choi, Kang-Moon Song, Mi-Hye Kwon, Woo Jean Kim, and Jun-Kyu Suh. National Research Center for Sexual Medicine and Department of Urology, Inha University School of Medicine, Incheon 400-711, Republic of Korea

Patients with diabetic erectile dysfunction (ED) often have severe endothelial dysfunction and peripheral nerve damage, which results in poor response to oral phosphodiesterase-5 inhibitors. Nerve injury-induced protein 1 (Ninjurin 1) has been known to be up-regulated after peripheral nerve injury and to be involved in vascular regression during embryonic period. In the present study, we examined differential expression of Ninjurin 1 in the penis of diabetic mice and also determined whether and how Ninjurin 1 blocking antibody restores erectile function in diabetic animals. Eight-week-old C57BL/6J mice were used and diabetes was induced by intraperitoneal injection of streptozotocin (50 mg/kg/d for 5 days). At 8 weeks after induction of diabetes, the animals were divided into 5 groups: controls, diabetic mice, and diabetic mice treated with a single intracavernous injection of IgG (20 μl) or Ninjurin 1 blocking antibody (1.0 μg or 2.5 μg/20 μl, respectively). Both immunohistochemical staining and western blot analysis revealed that cavernous Ninjurin 1 expression was significantly higher in diabetic mice than in controls. Local delivery of Ninjurin 1 blocking antibody significantly increased cavernous endothelial content through enhanced endothelial cell proliferation, and induced phosphorylation of Akt and eNOS compared with that in the untreated or IgG-treated diabetic group. Endothelial protective effects, such as decreased expression of p47phox and inducible NOS, and decrease in the generation of reactive oxygen species including superoxide anion and peroxinitrite, and decrease in the number of apoptotic cells in the cavernous endothelium, was noted in the diabetic mice treated with Ninjurin 1 blocking antibody. The angiopoietin-1 (Ang1) expression was down-regulated and Ang2 expression was up-regulated in the diabetic penis as compared with control penis, which was reversed by treatment with Ninjurin 1 blocking antibody. High-dose of Ninjurin-1 blocking antibody (2.5 μg/20 μl) induced profound restoration of erectile function in the diabetic mice (up to 90% of the control values), whereas low-dose of Ninjurin-1 blocking antibody (1 μg/20 μl) elicited partial improvement. The Ninjurin 1 blocking antibody rescues erectile function through restoration of cavernous endothelial integrity both functionally and structurally probably by activating Ang1-Tie2 pathway. The results suggest that inhibition of Ninjurin 1 will be a novel therapeutic strategy for the treatment of vasculogenic ED. Supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs (Jun-Kyu Suh, A110076), Republic of Korea.

Radiation-induced vascular late reactions in the rat cervical spinal cord: a longitudinal magnetic resonance imaging (MRI) study

M. Saager[1,2]*, C. Gillmann[1,2], T. Welzel[2], M. Jugold[1], P. Peschke[1], P. Huber[1,2], J. P. Debus[2], C. P. Karger[1]. [1]German cancer research center (DKFZ), Heidelberg, Germany, [2]Department of Radiation Oncology, University of Heidelberg, Germany

Purpose: In oncology, the treatment field for head and neck cancer is often directly adjacent to the cervical spinal cord (CSC), which then may receive high doses of radiation. Since the CSC is a late reacting tissue, radiation induced myelopathy may appear months or years after the treatment. Until now, there is an ongoing discussion whether the target of irradiation are the oligodendrocytes or/and the vascular endothelial cells which finally leads to myelopathy. Our in vivo studies with the CSC of rats showed that the time between irradiation and the onset of paresis as consequence of myelopathy is significantly shorter for heavy ions than for photons. One possible explanation might be a differential mode of cell inactivation of the vascular endothelial cells after photon and heavy ion irradiation. In order to examine the role of vascular late reactions in radiation-induced paresis, we established a longitudinal study supported by contrast enhanced MRI. Ultimate goal is to show a blood-spinal cord barrier (BSCB) dysfunction and to determine whether the BSCB dysfunction occurs before or at the same time when the movement restrictions begin. Methods and Materials: The CSC of female Sprague Dawley rats was irradiated with photons (61 Gy) or heavy ions (12C, 23 Gy) in six fractions on consecutive days. The irradiation field included the cervical segments C1-C5. After irradiation rats were monitored monthly with a 1.5T MRI scanner (Siemens Symphony) in combination with a custom-made radio-frequency coil. Measurements were performed using a sagittal and axial T2-weighted spin-echo sequence. Before and after i.v. injection of contrast agent (Magnevist®, 0.2 mmol/kg) sagittal T1-weighted images were taken. Besides MRI examination, rats were also checked weekly for weight and paresis. So far, the photon study has been completed. The carbon ion study is in progress. Results: Results of the completed photon study showed a contrast agent uptake in the irradiated field after 190 days in the T1-weighted images. An oedema formation was also observed in the T2-weighted images. At this time the animals exhibited no signs of locomotion disorders. Our observations also reveal that the time frame between contrast agent uptake and the first sign of movement restriction is very short. After 213 days (± 20 days) all animals developed paresis grade II. The T1-weighted MR images showed a prominent contrast agent uptake in the irradiated field. In addition to the oedema in the axial T2-weighted images, a dilatation of the canalis centralis was observed in the sagittal T2-weighted images. Conclusion: So far, the study has shown that irradiation of the CSC leads to a BSCB dysfunction which occurs before the onset of paresis. Furthermore, it was observed that the time frame between enhancement and onset of movement restriction is in the order of a few days. Besides this, an oedema appeared along the irradiated field. Histological examinations are in progress to correlate and verify these results and to determine the nature of irradiation induced structural changes.

Transcriptional control of genes within the Notch signalling pathway during vessel growth

Sacilotto N[1]*, Liu K[2], Pinheiro P[3], Gerhardt H[4], Bou-Gharios G[2], Patient R[3] and De Val S[1].
[1]Ludwig Institute for Cancer Research, Oxford University, [2]Kennedy Institute of Rheumatology, Oxford University, [3]Weatherall Institute of Molecular Medicine, Oxford University, [4]London Research Institute, Cancer Research United Kingdom

The Notch signalling pathway plays a significant role in vessel differentiation and angiogenic growth, and is consequently an attractive candidate for anti-angiogenesis therapy. The aim of my work is to understand the regulatory networks that control transcription of genes within the Notch pathway during vessel growth through the systematic identification, validation and characterization of regulatory elements. The characterization of enhancers directing specific expression patterns within the sprouting angiogenic front and during arterio-venous differentiation, will significantly enhance our current knowledge of the transcriptional cascades during embryonic, physiological and pathological vessel growth. We utilize multiple in silico criteria to identify putative endothelial cell-specific enhancers, which are validated through the use of moderate-throughput transient Tol2 transposon-based transgenic zebrafish, and confirmed through the generation of transgenic mice. This system has been used to locate multiple novel enhancers regulating genes in the Dll4-Notch pathway. These are capable of directing expression of linked reporter genes in patterns mimicking those of the endogenous genes throughout embryonic development in both fish and mouse models. Analysis of these enhancers using in vivo and in vitro techniques is providing novel information about the transcriptional cascades upstream of Dll4 and Notch in both arterial and angiogenic remodelling.

Neutrophil extracellular traps directly induce epithelial and endothelial cell death: A predominant role of histones

Mona Saffarzadeh[1]*, Christiane Juenemann[1], Markus A. Queisser[1,2], Guenter Lochnit[1], Guillermo Barreto[3], Sebastian P. Galuska[1], Juergen Lohmeyer[4] and Klaus T. Preissner[1].
[1]School of Medicine, Institute of Biochemistry, Justus-Liebig-University, Giessen, Germany, [2]Pulmonary & Critical Care Medicine, Northwestern University, Chicago, Illinois, USA, [3]Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany, [4]Department of Internal Medicine II, Justus-Liebig-University, Giessen, Germany

Neutrophils play an important role in innate immunity by defending the host organism against invading microorganisms. Antimicrobial activity of neutrophils is mediated by release of antimicrobial peptides, phagocytosis as well as formation of neutrophil extracellular traps (NET). Bacteria, fungi and inflammatory stimuli such as interleukin-8, lipopolysaccharide (LPS) or phorbol myristate acetate (PMA) provoke „NETosis“ of neutrophils. NET is composed of DNA in association with histones, as the most abundant proteins in NET, as well as granular proteins such as elastase and myeloperoxidase and several cytoplasmic proteins. These structures can bind and kill bacteria and fungi, whereby NET-associated proteins such as elastase and histones exhibit bactericidal and leishmanicidal activity. Elevated amounts of NET were also observed in several pathophysiological conditions in vivo and exaggerated NET formation was correlated with damaging effects and impaired tissue function. However, the direct effect of NET and their components on host cells have not been investigated. This study focused on the influence of NET on host cell functions, particularly on human alveolar epithelial cells as the major cells responsible for gas exchange in the lung. To inspect the direct effect of NET on host cells, human neutrophils were isolated from healthy donors, and neutrophils were stimulated with PMA to produce NET. Incubation of NET with human lung epithelial cells (A549), mouse lung epithelial cells (MLE-12), human pulmonary artery endothelial cells (HPAEC), primary-isolated human umbilical vein endothelial cells (HUVEC) and murine alveolar type II (AT-II) induced cytotoxic effects in a dose-dependent manner. Complete or partial digestion of DNA in NET did not change NET-mediated cytotoxicity, indicating that DNA component in NET (either undigested or fragmented) is not responsible for its cytotoxic effect. To investigate the role of major NET-associated proteins in cytotoxicity, NET was preincubated with antibodies against histones, with polyanionic compounds, activated protein C (APC) and with elastase or myeloperoxidase inhibitor. While pretreatment of NET with antibodies against different histones decreased NET-mediated cytotoxicity to a varying degree, incubation of both isolated histones and NET with polysialic acid significantly reduced cytotoxicity. Although APC did decrease the histone-induced cytotoxicity in a purified system, it did not change NET-induced cytotoxicity, indicating that histone-dependent cytotoxicity of NET is protected against APC degradation. Preincubation of NET with elastase inhibitor did not reduce NET-mediated cytotoxicity although elastase activity significantly increased after DNA digestion. However, preincubation of NET with myeloperoxidase inhibitor moderately decreased NET-mediated cytotoxicity, indicating that histones and myeloperoxidase are responsible for NET-mediated cytotoxicity. Moreover, in LPS-induced acute lung injury mouse model, NET formation was documented in lung tissue as well as in the bronchoalveolar lavage fluid, and tissue destruction appeared to occur in vicinity to NET. These data reveal the important role of protein components in NET, particularly histones, which may lead to host cell cytotoxicity and tissue destruction. Antibodies against histones and polyanionic compounds such as polysialic acid are recommended to dampen cytotoxicity induced by exaggerated NET formation in pathological conditions.

Effects of IL-1β and IL-1 receptor antagonist in hepatocellular cancer

O. Salnikova*, K. Breuhahn, J. Schmidt, E. Ryschich. Dept. of General Surgery, University of Heidelberg, Heidelberg, Germany, Dept. of Pathology, University of Heidelberg, Heidelberg, Germany

Background/Aim: Tumor cells produce pro- and anti-inflammatory cytokines which determine local microenvironment and affect the phenotype of tumor-associated endothelial cells and the cellular immune response. The aim of the present study was to examine the effects of IL-1β and IL-1 RN (IL1 receptor antagonist) on hepatic endothelial cells and T-cell function. Methods and results: The transgenic HCC mouse model (AlbTag) was used. To characterize the pro- and anti-inflammatory intratumoral microenvironment in vivo, the production of forty cytokines was analysed using protein antibody array and ELISA. We found that normal liver tissue produces numerous cytokines whereas the cytokine production in HCC was completely disbalanced. Among various anti- and pro-inflammatory cytokines, a remarkable increase of IL-1RN was found both in tumor tissue and in the blood of HCC-bearing mice. The expression of IL-1RN was also up-regulated in 52% of human HCCs. Using fluorescence-labelled antibodies, we examined endothelial cell markers and adhesion molecules expressed on hepatic sinusoidal endothelial cells (HSEC) and tumor endothelial cells (TEC) after isolation and cultivation in vitro. To assess the in vivo expression of the same markers, HCC and normal liver tissues were analysed by immunohistochemistry. In vivo hepatic sinusoidal endothelial cells expressed the liver-specific markers LYVE-1, Stab2 and accumulated acLDL. Tumor endothelial cells were CD31+, LYVE-1-, Stab2- and did not show endocytosis of acLDL. Interestingly, hepatic and tumor endothelial cells showed an almost identical phenotype in vitro. They expressed LYVE-1, Stab2, ICAM-1, VCAM-1, CD31 and accumulated acLDL. The effects of IL-1RN on expression of adhesion molecules and on leukocyte adhesion to endothelial cells was studied in HSEC. IL-1β stimulated HSEC, that resulted in a 1.3 and 1.5-fold elevation of ICAM-1 and VCAM-1 expession, respectively. Furthermore, pre-treatment of HSEC with IL-1β significantly increased the number of adherent CD4+ and CD8+ T cells. This effect of IL-1β was effectively abrogated by IL-1RN, but the addition of anti-IL-1RN blocking mAb reconstituted the expression of adhesion molecules and T cell adhesion induced by IL-1β. Conclusion: HCC is characterized by disbalanced cytokine production which may affect the function of endothelial cells and leukocyte recruitment. Highly elevated level of IL-1RN may represent one of the most important factors which determines the anti-inflammatory microenvironment in HCC.

Neuroligins 1 and 2, neuronal proteins with differential synaptic roles, modulate separate function in angiogenesis.

Anna Valeria Samarelli*, Tatiana Nunes Silveira, Alessia Bottos, Federico Bussolino[#], Marco Arese[#]. Dept of Oncological Sciences, IRCC, University of Torino , Italy [#]equal contribution

A large number of cues that govern the growth and remodeling of the vascular system also play roles in neurons. We have recently shown that two central modulators of the synaptic function, the transmembrane proteins Neurexin (Nrxn) and Neuroligin (Nlgn), are expressed in the vascular system and modulate angiogenesis, zebrafish embryonic vascular development and vascular tone. 1 2 3 We are now concentrating on the role of Nlgns in Endothelail Cells (EC) biology. Among five human isoforms, Nlgn1 and 2 are the most expressed in EC. Neurobiologist generally accept that Nlgn1 and Nlgn2 exert their main roles at excitatory and inhibitory synapses, respectively. Synaptic activity per se, however, appears distant from the vascular functions and this differentiates Nlgn from the proteins that mediate axon patterning (e.g. Semaphorins, Netrins, Ephrins), whose role can easily be linked to many cellular events of angiogenesis (especially migration and adhesion, but also proliferation and cell survival). Hence, we are analyzing the role of Nlgn1 and Nlgn2 in „classical angiogenic phenotypes“ (adhesion migration and proliferation) along with more „general“ biological functions such as the release of autocrine-paracrine factors from cells. Data show differential roles of Nlgns 1 and 2 in EC: Nlgn1 is preferentially involved in EC adhesion on ECM proteins while NLGN2 is involved in the release of an angiogenic factor, angiopoietin 2, from the same cells. In conclusion our major aims are to well characterize the different roles of Neuroligin 1 and 2 in endothelial cells such as in CNS and to investigate the different molecular pathways on which they are based. 1 Bottos, A., Rissone, A., Bussolino, F., and Arese, M., Cell Mol Life Sci. 2 Bottos, A. et al., Proc Natl Acad Sci U S A (2009). 3 Rissone et al , submitted.

The cooperative effect of apelin with 17-beta estradiol on lymphatic integrity

Mika Sawane[1,2]*, Hiroyasu Kidoya[2], Masaya Takagi[1], Fumitaka Muramatsu[2], Nobuyuki Takakura[2] and Kentaro Kajiya[1]. [1]Shiseido Innovative Science Research and Development Center, Yokohama, Japan, [2]Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

We have previously shown that apelin/ APJ signaling promotes lymphatic integrity, resulting in attenuation of inflammation. The interplay between apelin and sex hormones is totally unknown although apelin gene maps to the X chromosome. Estrogen, which is one of the female sex hormones, plays a crucial role in the regulation of vascular responses and angiogenesis. Clinical studies have revealed that postmenopausal women are at a higher risk of stroke and cardiovascular diseases. Typical symptoms of postmenopausal women is hot flushes, which are caused by estrogen deficiency induced the disruption of the thermoregulatory systems including vascular defects. In addition, recent report demonstrated that lymphatic flow decreased in postmenopausal women. However, the direct role of estrogen in lymphatic vessels has not been clear. Here we first describe that the estrogen is a novel lymphangiogenesis factor and acts cooperatively to stabilize lymphatic integrity with apelin. First, we have confirmed that estrogen receptor-α(ERα) and estrogen receptor-β (ERβ) were expressed by human lymphatic endothelial cells (LECs) as well as human umbilical vein endothelial cells (HUVECs). In addition, we investigated the in vitro effect of 17-beta estradiol (E2) on proliferation and formation of lymphatic vessels. The number of LECs increased after the treatment with E2 dose-dependently. Moreover, E2 induced migration and cord formation of LECs. Interestingly, permeability assay revealed that E2 blocked hyperpermeability of LECs synergically with apelin. Taken together, these results indicate that estrogen is a lymphangiogenesis factor and that E2 has the cooperative effect with apelin on lymphatic integrity. Apelin might have a potential to improve menopausal symptoms by estrogen deficiency induced vascular dysfunction.

RANKL enhances macrophages paracrine pro-calcific activity: Dependence on IL-6 and TNFα

Andrea Callegari, Kate Deuell, Matthew Coons, Cecilia M. Giachelli, Michael E. Rosenfeld, Marta Scatena* Andrea Callegari, Kate Deuell, Matthew Coons, Cecilia M Giachelli, Marta Scatena. Department of Bioengineering, University of Washington, Seattle, WA USA Michael E Rosenfeld Department of Pathology, University of Washington, Seattle WA USA

Vascular calcification is highly correlated with morbidity and mortality, and it is often associated with inflammation. In vitro, macrophages have been shown to enhance vascular smooth muscle cells (SMCs) calcification partly in TNFα-dependent manner. RANKL inhibition in vivo has been shown to reduce vascular calcification in mouse atherosclerosis models. Besides regulating osteoclast formation, RANKL has been suggested to modulate macrophage and dendritic cell cytokines production. We found that treatment of bone marrow derived macrophages (BMDMs) with RANKL resulted in increased production of IL-6 and TNFα. To address whether RANKL may regulate macrophage-enhanced SMC calcification we used a macrophage/SMC co-culture system and examined the effects of RANKL on SMC matrix calcification. As expected, BMDMs placed in co-culture with SMCs, increased SMCs phosphate-induced calcification compared to SMC single culture. When RANKL was added to the macrophage/SMC co-cultures there was a further enhancement of SMC calcification. However, treatment with RANKL did not stimulate single culture SMC calcification. We, thus, reasoned that IL-6 and TNFα, may mediate RANKL-induced macrophage enhanced SMC calcification in a paracrine fashion. Addition of neutralizing IL-6 and TNFαantibodies together with RANKL treatment to macrophage/SMC co-cultures significantly reduced the RANKL induction of SMC calcification. Further, TNFαand IL-6 treated SMC single cultures calcified to greater extent than vehicle treated cells, suggesting that these factors may regulate calcification genes. qPCR analysis determined that TNFαand more strongly TNFαin combination with IL-6 induced upregulation of Runx2, Msx2, Pit1, Alkaline Phosphatase, osteopontin, and downregulation of matrix gla protein in SMCs. RANKL treatment of SMCs did not modulate these genes. RANKL activation of pro-inflammatory and pro-calcific pathways in macrophages may contribute to vascular calcification and inflammation.

Control of leukocyte transmigration: Inside-out regulation of endothelial ICAM-1 function through actin-binding proteins

Antje Schaefer*, Eloise C. Anthony, Mark Hoogenboezem, Jaap D. van Buul and Peter L. Hordijk. Department of Molecular Cell Biology, Sanquin Research and Academic Medical Center, University of Amsterdam, The Netherlands

Extravasation of leukocytes describes the multi-step process of leukocyte migration across the vascular endothelium into the underlying tissue. The tightly regulation of this process is crucial for the efficient operation of the immune system. Uncontrolled and excessive leukocyte transmigration is thus a relevant aspect of chronic inflammatory diseases such as atherosclerosis or rheumatic arthritis. One of the first steps of leukocyte extravasation is the adhesion of the leukocytes which is mediated through adhesion molecules such as the endothelial transmebrane protein ICAM-1 which is a ligand for leukocyte beta2 integrins. Leukocyte binding induces the clustering of ICAM-1 which in turn leads to the recruitment of actin-binding proteins to the adhesion sites, the activation of Rho-like GTPases and the re-organization of the actin cytoskeleton within the endothelium to ensure the transmigration of leukocytes. The clustering of ICAM-1 and thus leukocyte transmigration are not only regulated through these outside-in signaling events but also in an inside-out fashion. Very little is known about the inside-out signaling pathways within the endothelium, in particular about the control of ICAM-1 clustering and its biological function through actin-binding proteins and thus the actin cytoskeleton. Here, we focus on the interaction of the actin-binding adapter proteins Filamin B (280 kDa), alpha-actinin 4 (100 kDa) and cortactin (65 kDa) with the short cytoplasmic ICAM-1 tail (28 aa) and the consequences for the clustering of ICAM-1 and the transmigration of leukocytes. Transmigration assays under static and physiological flow conditions demonstrate that siRNA-mediated depletion of these adapters in human primary endothelial cells results in distinct adapter-specific defects in adhesion, spreading and transmigration of neutrophils. We found that clustered ICAM-1 is able to form different complexes with these adapter proteins in human primary endothelial cells. The interaction of ICAM-1 with Filamin B does not require alpha-actinin 4 and cortactin and vice versa while alpha-actinin 4 and cortactin form another independent complex with ICAM-1. Moreover, the binding of these adapters is regulated through the dynamics of the actin cytoskeleton in an adapter-specific manner. Monitoring the ICAM-1 induced recruitment of Filamin B, alpha-actinin 4 and cortactin in live-cell imaging experiments as well as FRAP studies indicate distinct functional roles of these adapter proteins in the inside-out regulation of the ICAM-1 clustering and thus leukocyte transmigration.

Inflammation and the development of late vascular damage after radiotherapy

Scharpfenecker M[1]*, Floot B[1], Russell NS[2], Stewart FA[1]. [1]Department of Biological Stress Response and, [2]Department of Radiotherapy, the Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands

Microvascular damage in normal tissues is a serious late complication of cancer patients after radiotherapy. Vessel injury develops from months to years after irradiation and manifests as telangiectasia, which are characterized as dilated and thin-walled blood vessels. Problems arise when telangiectasia rupture, leading to excessive bleedings which may impair organ function and require surgical intervention. The vascular phenotype implies that an imbalance between vascular repair and homeostasis pathways causes blood vessel dilation; however, the mechanism is still unclear. We have identified the transforming growth factor-beta (TGF-β) co-receptor endoglin as being critically involved in the development of late normal tissue damage: mice with reduced endoglin levels (Eng+/-) display less vascular injury and fibrosis after kidney irradiation compared to Eng+/+ littermates. Endoglin is mainly expressed on endothelial cells, but also on monocytes/macrophages. Therefore, the observed differences in the repair capability after irradiation may be either due to changed endothelial cell function or due to an altered inflammatory response in Eng+/- mice. We show that in the mouse kidney, irradiation induced an inflammatory infiltrate, which mainly consisted of macrophages. Quantitative image analysis demonstrated that macrophage numbers were only slightly decreased in irradiated Eng+/- mice compared to their wild type littermates. This was accompanied by reduced mRNA levels of the macrophage chemoattractant CCL2 (MCP-1) and its receptor CCR2. In addition, we investigated whether differential endoglin levels affected macrophage function in the irradiated tissue. Analysis of macrophage-derived pro-inflammatory cytokines in whole kidney lysates revealed that irradiation induced an upregulation of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) mRNA in Eng+/+ mice, which was significantly reduced in Eng+/- mice. Double stainings for the macrophage marker F4/80 and interleukins demonstrated that IL-1β and IL-6 were indeed produced by macrophages in the irradiated kidney. Moreover, isolation of bone marrow cells (containing macrophage precursors) showed aberrant/impaired cytokine expression in response to an inflammatory stimulus (LPS) in Eng+/- mice compared to Eng+/+ mice. Furthermore, studies with skin biopsies derived from the normal tissue of breast cancer patients treated with radiotherapy showed that vascular changes after irradiation were accompanied by macrophage infiltration and by changes in endoglin expression levels. We are currently investigating how endoglin modulates signalling pathways and the production of cytokines in macrophages, and how macrophage depletion affects kidney function after irradiation. In summary, our studies suggest that endoglin mediates the inflammatory response in irradiated tissues by regulating the expression of IL-1β and IL-6 in macrophages. As these cytokines not only have pro-inflammatory, but also pro-angiogenic and pro-fibrotic properties, this strongly indicates that in this way they contribute to the development of late normal tissue damage after irradiation.

Angiopoietin-2 differentially regulates angiogenesis through Tie2 and integrin signaling

Felcht M[1,2], Schering A[1], Seidel P[1], Srivastava K[1], Hu J[1], Bartol A[1,3], Kienast Y[4], Vettel C[5], Loos EK[1], Kutschera S[1,3], Bartels S[1], Appak S[1,3], Besemfelder E[1], Terhardt D[1], Chavakis E[6], Wieland T[5], Klein C[4], Thomas M[4], Uemura A[7], Goerdt S[2], Augustin HG[1,3]. [1]Division of Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Heidelberg, Germany, [2]Department of Dermatology, Venerology, and Allergology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [3]Department of Vascular Biology and Tumor Angiogenesis (CBTM), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [4]Roche Diagnostics GmbH, Penzberg, Germany, [5]Institute of Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [6]Department of Internal Medicine, Cardiology and Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, Goethe University, Frankfurt am Main, Germany, [7]Division of Vascular Biology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan

The Tie2 ligand Angiopoietin-2 (Ang-2) exerts context-dependent effects on endothelial cells (EC). It acts as a negative regulator of Ang-1/Tie2 signaling during angiogenesis and vessel maturation thereby controlling the responsiveness of EC to exogenous cytokines. Ang-2 may under certain conditions also act as pro-angiogenic molecule. The molecular mechanisms of differential Ang-2 functions are poorly understood which poses a critical limitation in the rational exploitation of Ang-2 as a target of anti-angiogenic therapy. We show here that the activated endothelium during angiogenesis harbors a subpopulation of Tie2-negative EC (Tie2low). As such, Tie2 and angiogenic EC integrins are differentially expressed by sprouting tip cells and remodeling stalk cells. Ang-2 binds to αvβ3, αvβ5 and α5β1 integrins in Tie2low EC, subsequently inducing in a Tie2-independent manner FAK phosphorylation at Tyr397, Rac1 activation, migration and sprouting angiogenesis. Correspondingly, Ang-2 blockade in vivo interferes with integrin signaling and inhibits FAK[Tyr397] phosphorylation and sprouting angiogenesis of Tie2low EC. The data establish a contextual model of pro-angiogenic and vessel-destabilizing functions of Ang-2 which is controlled by differential Tie2 vs. integrin expression, binding and activation.

VEGF-C application pattern improves the regeneration of autotransplanted lymph node fragments and the reconnection with the lymphatic system

Schindewolffs L[1]*, Buettner M[2], Hadamitzky C[3], Breves G[4], Pabst R[1]. [1]Institute of Immunmorphology, Hannover Medical School, Hannover, Germany [2]Institute of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany, [3]Clinic of Plastic, Hand and Reconstructive Surgery, Hannover Medical School, Germany, [4]Institute of Physiology, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

An effective transport of body fluids is only guaranteed by a functional lymph system. Within the context of axillary dissection during the conservative breast cancer treatment this balance can be disturbed, resulting in a secondary lymphedema in ~30% of the patients. The therapy of secondary lymphedema is, so far, only symptomatical. There is no adequate surgical therapy available at this point but we could establish a technique of transplanting fragments of autologous lymph nodes to prevent lymphedema in rats. VEGF-C revealed a beneficial effect on the regeneration of the transplants. The purpose of this study was to improve the application pattern of VEGF-C to achieve a higher regeneration rate by using this animal model for lymphedema. We focused on the time point, location and injected dose. Eighty-nine adult, healthy female Lewis rats (~200g) underwent surgery, in which all right popliteal and inguinal lymph nodes had been removed. Three inguinal lymph nodes of each rat were fragmented and transplanted back into the subcutaneous fat tissue of their right groin. Group A (n=10) received 6.67µg VEGF-C per rat intradermally into the right abdominal wall close to the transplantation area on day 1,2,3 post OP. Group B (n=19) received 6.67µg VEGF-C per rat intradermally into the medial side of the right thigh on day 1,2,3 post OP. Group C (n=20) received the same dose of VEGF-C, which was injected in the same location as group B, but on day 14,15,16 post OP. Group D (n=20) received 13.34µg VEGF-C per rat on the same time points and location as group B. Group E (n=20) was left without further intervention after surgery (control). After four weeks the lymphatic drainage and the connection of lymphatic vessels and the transplants was tested. Therefore, Patent Blue was injected intradermally. The distribution of the dye was examined and the transplants were set up for immunohistochemistry to detect T- and B-lymphocytes, HEVs and lymphatic endothelial cells. The transplants were graded as „regenerated“ or „not regenerated“ due to the cell distribution detected by fluorescent immunohistochemistry and the connection of the transplants to the remnant lymph vessels. The evaluation showed that the location does not make any difference, whether in terms of the connection or the regeneration of the transplants. However, it also illustrates that an early application, such as in group B, has a positive influence on the regeneration of the lymph node fragments (89%) compared to group C with a later time point (85%). Regarding the reconnection, group B also showed improved results (group B: 53%, group C: 35%). A higher dose, such as given in group D, results in a rate of 95% regenerated lymph node fragments whereas the control group (E) ends up with only 70%. The reconnection of the lymph node fragments also benefits from a high VEGF-C dose: with an outcome of 80% in group D compared to 15% in the control group, the reconnection rate is significantly enhanced. Acknowledgement: This work was supported by the Deutsche Forschungsgemeinschaft: PA 240/10-1.

EGFL7 ligates alphavbeta3 integrin to enhance vessel formation

Iva Nikolic[1], Jeannette Meister[1], Helene Braun[2], Khader Awwad[3], Emmanouil Chavakis[4,5], Chinmoy Patra[2], Patrick N. Harter[1], Michel Mittelbronn[1], Karl H. Plate[1], Felix B. Engel[2], Stefanie Dimmeler[4], Johannes A. Eble[6], Benno Jungblut[2], Ingrid Fleming[3], Mirko HH Schmidt[1,7]*. [1]Institute of Neurology (Edinger Institute), Johann Wolfgang Goethe University School of Medicine, Heinrich-Hoffmann-Str. 7, D-60528 Frankfurt am Main, Germany, [2]Max-Planck-Institute for Heart and Lung Research, Parkstr. 1, D-61231 Bad Nauheim, Germany, [3]Institute for Vascular Signalling, Center for Molecular Medicine, Johann Wolfgang Goethe University School of Medicine, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany, [4]Institute for Cardiovascular Regeneration, Center for Molecular Medicine and, [5]Department of Cardiology, Johann Wolfgang Goethe University School of Medicine, Frankfurt am Main, Germany, [6]Department of Vascular Matrix Biology, Center for Molecular Medicine, Johann Wolfgang Goethe University School of Medicine, Frankfurt am Main, Germany, [7]Institut für Mikroskopische Anatomie und Neurobiologie, Johannes Gutenberg-University, Mainz, Germany

Angiogenesis, defined as blood vessel formation from a preexisting vasculature, is governed by multiple signal cascades including integrin receptors, in particular integrin alphaVbeta3. Here we identify the endothelial cell-secreted factor epidermal growth factor-like protein 7 (EGFL7) as a novel specific ligand of integrin alphaVbeta3 thus providing mechanistic insight into its proangiogenic actions in vivo and in vitro. Specifically, EGFL7 attaches to the extracellular matrix and by its interaction with integrin alphaVbeta3 increases the motility of ECs, which allows endothelial cells to move on a sticky underground during vessel remodeling. We provide evidence that the deregulation of EGFL7 in zebrafish embryos leads to a severe integrin-dependent malformation of the caudal venous plexus, pointing towards the significance of EGFL7 in vessel development. Consequently, we are able to show for the first time that the expression levels of EGFL7 in human specimens of diseases rely largely on the remodeling state of the existing vasculature but not on the origin of the disease. Our work now draws the first comprehensive picture on the molecular mechanism EGFL7 engages to govern physiological and pathological angiogenesis.

Dynamics of Junctional Adhesion Molecule (JAM) A during atherosclerotic lesion formation

M.M.N. Schmitt[1]*, R.T. Megens[2], O. Soehnlein[2], M.A. van Zandvoort[3], T.M. Hackeng[3], C. Weber[2], and R.R. Koenen[2]. Institute for Molecular Cardiovascular Research (IMCAR), University Hospital Aachen, RWTH Aachen University, Germany, [2]Institute for Cardiovascular Prevention (IPEK), LMU, Munich, Germany and [3]Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands

Leukocyte transendothelial migration (TEM) is a crucial event in atherosclerotic lesion formation and is controlled by a multi modal cascade comprising adhesion molecules, integrins and cytokines. Junctional Adhesion Molecule- (JAM-) A, as a transmembrane protein mainly located in endothelial cell (EC) junctions, on leukocytes and CD34+ cells, displays a wide variety of functions in cell polarity, barrier function, leukocyte adhesion as well as stem cell adhesion and differentiation. Aim of this study was to investigate the role endothelial JAM-A (eJAM-A) plays in hyperlipidemia mediated induction of leukocyte TEM and thus its effect on the early stage atherogenesis. Preliminary data indicate that genetic reduction of eJAM-A in apolipoprotein E-deficient female mice fed on high fat diet for 12 weeks significantly reduces plaque size in the aortic root and the descending aorta. In line with those results, we could show a significant reduction of macrophage content in plaque of aortic root and brachiocephalic artery in female mice after genetic reduction of eJAM-A and high fat diet. Mechanistically data were obtained by using Two Photon Laser Scanning Microscopy of atherosclerotic carotid arteries ex vivo, which display a significant redistribution of eJAM-A out of the endothelial junctions to the luminal site of the endothelial cells (EC). Those data were validated by an in vitro experiment using cultured human aortic endothelial cells (HAoEC) under hyperlipidemic conditions and fluorescent microbeads coupled to JAM-A antibodies. Microbeads, because of an approximate size of 1 µm too big for entering endothelial junctions, adhered more often to the luminal site of HAoECs under hyperlipidemic conditions (2.84±0.27 beads per EC) compared to untreated HAoECs (1.55±0.16 beads per EC). Finally, a significant increase of monocyte transmigration through HAoECs under hyperlipidemic conditions compared to untreated HAoECs could be demonstrated (21.6±2.01% vs. 6.25±1.65% transmigrated cells of adherent cells, respectively). By blocking eJAM-A, transmigration levels went back to basal level (4.0±1.67% transmigrated cells of adherent cells). All data obtained in this study indicate that hyperlipidemia induced eJAM-A redistribution from the junctions to the luminal site facilitates leukocyte TEM by displaying a higher accessibility of JAM-A for passing leukocytes and thus has a big influence on atherogenesis. In future, eJAM-A could be used as a suitable biomarker for non-invasive imaging of atherosclerotic plaque and could be beneficial as a worthwhile target in immunotherapy of patients suffering from bad predictions for atherosclerosis.

Angiopoietin-2 promotes myeloid cell infiltration in a beta-2 integrin-dependent manner

Alexander Scholz[1]*, Victoria Lang[2], Reinhard Henschler[2], Marcus Czabanka[3], Peter Vajkoczy[3], Emmanouil Chavakis[4], Janina Drynski[1], Patrick N. Harter[1], Michel Mittelbronn[1], Daniel J. Dumont[5], Karl H. Plate[1], and Yvonne Reiss[1]. [1]Edinger Institute/Institute of Neurology, Frankfurt University Medical School, Frankfurt, Germany, [2]Institute of Transfusion Medicine and Immune Hematology, German Red Cross Blood Donor Service Baden-Württemberg-Hessen GmbH, [3]Department of Neurosurgery, Charité-Universitätsmedizin, Berlin, Germany, [4]Institute of Cardiovascular Regeneration, Frankfurt University Medical School, Frankfurt, Germany [5]Sunnybrook Research Institute, Toronto, Canada

In human inflammatory diseases, we identified endothelial Angiopoietin-2 (Ang-2) expression to be strongly associated with inflammations mediated by myeloid cells but not lymphocytes. To identify the underlying mechanism, we made use of a transgenic mouse model with inducible endothelial cell-specific expression of Ang-2. In this model, in the absence of inflammatory stimuli, long term expression of Ang-2 led to a time-dependent accumulation of myeloid cells in numerous organs, suggesting that Ang-2 is sufficient to recruit myeloid cells. In models of acute inflammation, such as delayed-type hypersensitivity and peritonitis, Ang-2 transgenic animals showed an increased responsiveness. Intravital fluorescence video microscopy revealed augmented cell adhesion as an underlying event. Consequently, we demonstrated that Ang-2 is able to induce strong monocyte adhesion under shear in vitro which could be blocked by antibodies to beta-2 integrin. Taken together our results describe Ang-2 as a novel, endothelial-derived regulator of myeloid cell infiltration that modulates beta-2 integrin-mediated adhesion in a paracrine manner.

Balancing life and death: The role of autophagy in atherosclerosis

Schrijvers D.M., Grootaert M., Bult H., De Meyer G.R.Y., Martinet W. Laboratory of Physiopharmacology, University of Antwerp, Belgium

A growing body of evidence gathered by our group and others suggests that autophagy occurs in atherosclerotic plaques. Autophagy represents a reparative, life-sustaining process for degradation of long-lived or damaged organelles and proteins via lysosomes, but when continuously stimulated it results in cell death. Despite the increasing knowledge on autophagy, its role in atherosclerosis is unclear. Smooth muscle cells (SMC) are important to provide stability for atherosclerotic plaques because they are the major source of collagen. Hence, loss of SMC is detrimental for plaque stability. We therefore investigated the effect of autophagy inhibition in SMC on cellular function, atherogenesis and atherosclerotic plaque stability. Mechanistic studies indicated that autophagy is crucial for adequate SMC function. Genetic targeting of ATG7, essential in autophagy, in SMC resulted in a pronounced decrease in proliferation and migration capacities of these cells. Interestingly, ATG7-null SMC initially seemed unaffected with regard to sensitivity for cell death stimuli. However, this was attributed to a heavily upregulated protective back-up mechanism via glutathione S-transferase (GST). Inhibition of this pathway using ethacrinic acid rendered ATG7-null SMC more sensitive to cell death, as compared to ATG7 expressing SMC. In contrast to SMC, ATG7-null macrophages do not upregulate GST and hence are more susceptible to cell death than ATG7 expressing macrophages. To determine how these events impact atherosclerotic plaque stability, we crossbred apolipoprotein E knockout mice with cell specific ATG7-deficient mice. Effects on atherogenesis and plaque stability are currently being investigated. Understanding the role of autophagy in atherosclerosis is fundamental and may lead to novel therapeutical strategies to prevent atherothrombotic vascular disease.

Pericytes regulate vascular stability after kidney injury through TIMP3 and ADAMTS1

Claudia Schrimpf[1,2,3]*, Cuiyan Xin[2], Gabriella Campanholle[2], Sean E. Gill[2], William Stallcup[5], Shuei-Liong Lin[4], George E. Davis[5], Benjamin D. Humphreys[3] and Jeremy S. Duffield[2]. [1]Medizinische Hochschule Hannover, [2]Center for Lung Biology, & Institute for Stem Cell & Regenerative Medicine, University of Washington, Seattle, WA, [3]Laboratory of Inflammation Research, Renal Division, Brigham & Women's Hospital and Harvard Medical School, Boston, MA, Division of Nephrology, [4]National Taiwan University Hospital, Taipei, Taiwan, [5]Cancer Center, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

Backround: Recently a central role for kidney pericytes as progenitors of myofibroblasts after injury have been described, but regulatory mechanisms by which pericytes differentiate into myofibroblasts remain unknown and effects on the microasculature of the kidney are unexplored. Methods: Using microarray analysis, InSitu Hybridization and Immunofluoresent stainings we identified novel regulated genes in kidney pericytes during detachment and differentiation in response to injury in vivo. Using a 3D vascular regression assay we proof the importance of Timp3 and Adamts-1 on vascular stability and microvascular integrity and confirm our findings by studies in Timp3 deficient mice. Results: Under physiological conditions kidney pericytes strongly express the tissue inhibitor of matrix metalloproteinase 3 (Timp3), while Adamts-1 a disintegrin and metalloproteinase with thrombospondin motive 1 is barely present. In response to injury, the expression of Timp3 gets downregulated, while Adamts-1 levels rise. In a functional 3D regression assay primary kidney pericytes stabilize the microvasculature similar to brain pericytes and pericyte derived Timp3 and Adamts1 are regulating factors involved in the underlying mechanism and mice deficient in Timp3 have a spontaneous microvascular phenotype and impaired angiogenesis in response to kidney injury. Conclusion: These studies dissect pericyte dependent mechanisms involved in microvascular stability of the kidney and elucidate endothelial to pericyte crosstalk.

Platelet dense granule secretion mediates platelet-dependent enhancement of tumor cell transmigration and formation of metastases

Dagmar Schumacher*, Boris Strilic, Nina Wettschureck, Stefan Offermanns. Max-Planck-Institute for Heart and Lung Research, Department of Pharmacology, Ludwigstr. 43, 61231 Bad Nauheim, Germany, Medical Faculty, J.W. Goethe University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany

Tumor cell metastasis to distant organs is the primary cause of mortality in cancer patients. Tumor cells leave the primary tumor, intravasate, survive in the circulation and extravasate through the endothelial cell layer to grow in the target organ. It has long been known that blood platelets play an important role in tumor cell survival and dissemination, but the mechanism by which platelets promote metastasis remained unclear. Given that platelets are found closely associated with tumor cells shortly after vascular arrest, we explored whether platelets can facilitate the transmigration of tumor cells through the endothelium and thereby promote extravasation of tumor cells into the organ parenchyma. The ability of various mouse and human tumor cells like Lewis-Lung carcinoma cells (LLC1), B16F10 melanoma cells or human neuroblastoma cells (SH-SY5Y) to transmigrate through an endothelial cell layer was strongly enhanced by seeding tumor cells together with mouse or human platelets onto the endothelial cell layer. This indicates that platelets facilitate tumor cell transmigration in vitro. We found that platelet granule secretion is involved in this process as supernatant from platelets incubated with tumor cells but not from resting platelets was sufficient to enhance tumor cell transmigration. Additionally, no platelet-mediated increase of tumor cell transmigration was observed in dense granule secretion-defective platelets of Munc13-4 deficient mice. Thus, dense granule secretion is required for platelet-dependent tumor cell extravasation in vitro. While the growth and weight of primary tumors after subcutaneous injection of LLC1 and B16 cells was indistinguishable between wild-type mice and animals lacking Munc13-4, the number of metastases in the lung was strongly reduced in Munc13-4-deficient animals. The strong decrease in formation of metastases in Munc13-4 deficient mice was also observed after i.v. injection of LLC1 and B16F10 cells. Thus, platelet dense granule secretion plays a critical role in tumor cell metastasis by enhancing tumor cell transmigration through the endothelial cell layer.

Integrin alpha6 controls endothelial podosome rosettes formation in tumor angiogenesis

Giorgio Seano[1,2]*, Laura di Blasio[1,2], Roberto Sessa[1,2], Paolo Armando Gagliardi[1,2], Federico Bussolino[1,2], Guido Serini[1,2], and Luca Primo[2,3]. [1]Department of Oncological Sciences, University of Torino, Turin, Italy, [2]Institute for Cancer Research and Treatment, Candiolo, Turin, Italy, [3]Departments of Clinical and Biological Sciences, University of Torino, Turin, Italy

The mechanisms, by which angiogenic endothelial cells (EC) break the physical barrier of vascular basement membrane (vBM) and consequently sprout in a new vessel, are not clear. It is known that cultured EC show specialized plasma-membrane microdomains, called podosome, and in EC they are organized in rosettes; the latter combine adhesive and proteolytic activities to spatially restricted sites of matrix degradation. We demonstrate that angiogenic EC and tumor vessels show a significant increase of podosome rosettes in comparison to quiescent EC. The increment of the endothelial podosome rosettes corresponds to an increased ability to degrade extracellular matrices. Integrins are the principal adhesion regulator of podosome-type structures. We show that integrin alpha6, whose expression is up-regulated in angiogenic EC, is recruited in podosome-rosettes both in cultured EC and in tumor vessels. Moreover, down-regulation and functional blocking of integrin alpha6 impairs podosome formation and maturation. Similarly, high level of laminin - the major component of vBM and the ligand of integrin alpha6 - blocks podosome formation. We demonstrate that podosome rosettes do not form in EC when integrin alpha6 is not available for trafficking into nascent podosomes due to the binding to laminin in focal adhesions. Only over-expression of integrin alpha6 - simulation of angiogenic factors stimulation effects - is able to overcome the anti-podosome effects of laminin. To understand the physiological relevance of the involvement of integrin alpha6 in endothelial podosome rosettes, we treat a genetic tumor mice model with anti-alpha6 integrin neutralizing antibody. The functional blocking of integrin alpha6 impairs podosome rosettes formation in vivo, by impairing tumor angiogenesis. Taken together, these results suggest that laminin in vBM stabilize mature vessels by recruiting integrin alpha6 in focal adhesions. The endothelial increase of integrin alpha6 expression, induced by angiogenic growth factors stimulation, overcomes the anti-angiogenic effect of vBM, by increasing integrin alpha6 availability to form podosome rosettes, necessary to degradate vBM and form new vessels. Moreover, these studies provide evidence that endothelial podosome rosettes play a crucial role in angiogenic in vivo models and that their inhibition impairs tumor angiogenesis.

Glutathione peroxidase-1-deficiency enhances age-dependent vascular dysfunction

Sebastian Steven[1]*, Swenja Schuhmacher[1], Matthias Oelze[1], Markus Bachschmid[2], Christopher Doppler[1], Steffen Daub[1], Alexandra Schuff[1], Alexander Scholz[1], M. Torzewski[3], Eberhard Schulz[1], Karl J. Lackner[3], Thomas Münzel[1], and Andreas Daiber[1].
[1]2nd Medical Clinic and [3]Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany, [2]The Boston University Medical Center, Boston, USA

Goal: In previous studies we were able to show, that genetic deficiency of the mitochondrial antioxidant proteins manganese superoxide dismutase (SOD2) and aldehyde dehydrogenase-2 (ALDH-2) contribute to age-related vascular dysfunction and mitochondrial oxidative stress. In the present study we investigate, whether the genetic deficiency of glutathione peroxidase (GPx-1) enhances ageing-dependent vascular dysfunction and the formation of reactive oxygen and nitrogen species (RONS). Methods: Mice of 3 different age groups were used: 2, 6 and 12 months. Vascular function was assessed via isometric tension studies using isolated aortae. Formation of RONS was assessed by optical, HPLC- and immunoblotting-based techniques. Dysfunction/uncoupling of endothelial NO synthase (eNOS) was assessed by the phosphorylation and S-glutathionylation pattern of immunoprecipitated eNOS. Results: Vascular function was significantly impaired in aged GPx-1-/- -mice, whereas six months old mice showed no altered vascular phenotype. However, GPx-1-/- -mice of various age groups had significantly impaired vascular function, compared to their wild type littermates. RONS-formation in mitochondria, membraneous fraction, heart-tissue and serum was enhanced with increased age, but (except 3-nitrotyrosine staining) not significantly different compared to wild type littermates. The dramatic endothelial dysfunction in old GPx-1-/- mice may be due to protein kinase C and protein tyrosine kinase-dependent phosphorylation as well as S-glutathionylation and inactivation of eNOS. GPx-1 deficiency also increased the adhesion of leukocytes to cultured endothelial cells. Conclusion: GPx-1-/- -mice were expected to display a higher level of RONS in their tissues caused by the impaired breakdown of hydrogen peroxide or lipid peroxides. However, we could only detect minor differences between RONS-formation in GPx-1-/--mice and their wild type littermates, which could not completely explain the differences observed for vascular function with increasing age. Further investigations will focus on the expression of other antioxidant proteins, which could act as a compensatory mechanism in GPx-1-deficiency. Also the role of GPx-1 for vascular inflammation will be addressed in future experiments.

Analysis of CD99L2 in vivo function in neutrophil transendothelial migration by means of CD99L2 conditional knock-out mice

Ruth Seelige[1]*, Christiane Natsch[2], Sigrid März[1], Ding Jing[1], Stefan Butz[1], Gabriele Bixel[1], and Dietmar Vestweber[1]. [1]Max Planck Institute for molecular biomedicine, Muenster [2]Department of chemistry and pharmacy, Westfaelische Wilhelms University, Muenster, Germany

Leukocyte extravasation depends on a tightly regulated cascade of receptors and adhesion molecules on leukocytes as well as at endothelial cell contacts. One of the most recently identified proteins participating in this process is CD99L2. CD99L2 is a small, highly O-glycosylated transmembrane protein with no resemblance to any known protein family. It is distantly related to CD99, a well known glycoprotein involved in leukocyte diapedesis. Although the two molecules only share 32% sequence identitiy, they are highly related in function. CD99L2 is expressed on neutrophils, lymphocytes and endothelium, where it locates to the intercellular junctions. Interestingly, its expression on neutrophils is only found in the periphery in contrast to the bone marrow, which makes it one of very few antigens induced with neutrophil maturation. With the help of polyclonal antibodies that we generated against CD99L2, we could recently show that it is involved in the transmigration of neutrophils through endothelial cells in vitro as well as into inflamed peritoneum and cremaster tissue. Despite of its high expression on lymphocytes, CD99L2 was dispensable for lymphocyte extravasation in vivo and in vitro. Because CD99L2 is expressed both on neutrophils and the endothelium, we sought to further investigate the mechanism by which it exerts its effect on transmigration. By using antibodies, we could already demonstrate in vitro that CD99L2- mediated transmigration was only inhibited when the endothelial cells, but not the neutrophils, were preincubated with antibodies or F(ab)2 fragments, respectively. In agreement with this, intravital microscopy of cremaster venules revealed CD99L2 to be involved in the actual diapedesis step because antibodies arrested leukocytes at the level of the basement membrane while adhesion and rolling was not affected. To further elucidate the role of CD99L2 in leukocyte diapedesis and to evaluate its function independent of antibody effects, we generated CD99L2 conditional knock-out mice. Here, we show that knocking out the CD99L2 gene indeed results in the reduction of leukocyte extravasation. By using intravital microscopy in the inflamed cremaster muscle, we were able to clarify that CD99L2 deficiency does not affect adhesion or rolling of leukocytes, but only the actual diapedesis step. Importantly, neutrophil extravasation was only decreased when CD99L2 was knocked out in endothelial cells, but not when it was missing on leukocytes. Thus, for the first time, we were able to demonstrate in vivo that CD99L2 is exclusively required for leukocyte extravasation on endothelial cells. To further study CD99L2 function in vivo, we isolated primary lung endothelial cells from CD99L2 deficient mice. Neutrophil transmigration through these cells is indeed diminished. In the future, we plan to transfect these cells with mutated forms of CD99L2, e.g. a truncated construct and constructs with mutated glycosylation sites, to investigate if and how this might affect leukocyte transmigration.

The transcription factor CREB promotes atherosclerotic plaque progression

M.D. Seidl[1]*, A.K. Steingräber[1], C. Klugstedt[1], K. Tiemann[2], S. Offermanns[3], W. Schmitz[1], F.U. Müller[1]. [1]Westfälische Wilhelms-Universität, Institut für Pharmakologie und Toxikologie, Domagkstr 12, 48149 Muenster, Germany, [2]Isar Kliniken, Sonnenstraße 24-26, 80331 München 3MPI Bad Nauheim Pharmakologie, Ludwigstr. 43, 61231 Bad Nauheim, Germany

The transcription factor CREB (cAMP responsive-element binding protein) is supposed to regulate differentiation, proliferation and apoptosis of vascular smooth muscle cells (VSMCs) by contributing to the transcriptional control of functionally relevant target genes. In particular, CREB was linked to vascular injury and plaque progression. Here we studied the vascular role of CREB in mice with a smooth muscle cell specific, tamoxifen induced inactivation of CREB (CREB-KO) in comparison to non-induced control animals of the same genotype (Ctr). In aortic sections a detailed histochemical analysis revealed an increased proportion of apoptotic nuclei in CREB-KO vs. Ctr mice (TUNEL-positive cell; CREB-KO, 1.09±0.21‰*; Ctr, 0.47±0.09 ‰; n=6; *P<0.05 vs. Ctr), while the number of proliferating Ki67-positive cells was unaltered. In the aorta we found no differences in the mRNA levels of B-cell lymphoma 2 (Bcl2) and platelet derived growth factor receptor, alpha polypeptide (Pdgfra), which are prominent CREB target genes in the vasculature involved in the regulation of apoptosis and proliferation. To address the possible functional role of CREB in the response to vascular injury the development of a neointima in a model of carotid ligation was investigated. There were no detectable differences between the genotypes. To study the role of CREB in atherogenesis we determined the development of atherosclerotic plaques in CREB-KO and Ctr mice on an ApoE-/- background after 30 weeks of atherogenic diet. In this model CREB inactivation led to a significant increase by 38% of the plaque area in the aorta (Oil-red-O staining, n=7-11) and an increase of 57% of mean plaque area in the aortic arch and the carotids shown by the use of ultrasound analysis (n=8-14). In conclusion, CREB is of functional relevance in VSMCs showing anti-apoptotic and anti-atherogenic properties in mouse aortae. (Supported by the IZKF Muenster).

Nerve Growth Factor overexpression in Clara cells suppresses metastasis and tumor growth in a mouse model of experimental lung metastasis

Katrin Seidler[1]*, Akylbek Sydykov[2], Sabine Müller-Brüsselbach[3], Rolf Müller[3], Norbert Weißmann[2] , Harald Renz[1], Andreas Nockher[1]. [1]Institute of Laboratory Medicine and Pathobiochemistry, Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany [2]Universities of Giessen and Marburg Lung Center (UGMLC), Excellence Cluster Cardiopulmonary System (ECCPS), Giessen, Germany 3Institute of Molecular Biology and Tumor Research (IMT), Philipps-University of Marburg, Marburg, Germany

Introduction: Metastasis of tumor cells to distant organs remains the major cause of mortality in cancer patients. Regarding lung cancer research, the pathological mechanisms underlying tumor dissemination are still barely understood. Metastasis is a multistep process terminating in extravasation of cancer cells and growth at the secondary tumor site. Generally, the lung is often affected by metastasis since it is one of the first organs reached by systemic venous drainage. In view of new findings, one possible mediator of metastasis and tumor growth is the neurotrophin, nerve growth factor (NGF). NGF was originally identified as an essential factor for the development and maintenance of the nervous system. Recent studies show that neurotrophins also possess a variety of other biological functions in several organs and cell types. In lung tumors, the secretion of neurotrophins promotes tumor cell proliferation in an autocrine manner and a lack of NGF signaling leads to apoptosis of carcinoma cells. On the other hand, NGF inhibits proliferation of small cell lung carcinoma cells (SCLC) in vitro and abrogates their tumorigenic properties. Previously, our group has shown that NGF is an autocrine growth factor for airway epithelial cells and leads to the augmentation of airway inflammation in a mouse model of experimental asthma. Objective: To investigate the impact of NGF on lung metastasis and tumor growth. Methods: In this study, a mouse model of experimental lung metastasis was used. Surface metastasis and tumor growth in wild type mice (WT) and transgenic mice overexpressing NGF in Clara cells in the airway epithelium (NGF-Tg) were examined. Tumor volume was quantified histologically by use of a computer assisted stereological toolbox system (CAST) Local inflammation was assessed by histology and measurement of cytokines and chemokines in bronchoalveolar lavage fluid using a cytometric bead array system and ELISA. Since tumor cells have to cross the vascular barrier to form metastases, measurement of vascular permeability in the lungs was performed in an ex vivo model of ischemia and reperfusion. Results: In an in vivo model of experimental lung metastasis, we found tumor growth significantly reduced in NGF-Tg mice. This result was verified by the use of two cancer cell lines with different metastatic potential (LLC cells and B16/F10 melanoma cells). These findings were not accompanied by any differences in cellular or humoral anti-tumor immune responses neither in lung tissue nor in bronchoalveolar lavage of NGF-Tg and WT mice. Interestingly, the measurement of vascular permeability in the lungs in an ex vivo model of ischemia and reperfusion revealed the protection of NGF-Tg animals from reperfusion damage and edema. Conclusion: Our recent findings indicate that NGF overexpression alters blood vessel structure leading to reduced vascular permeability and specific retention of cancer cells in the vasculature.

Novel mechanism regulating endothelial permeability via T-cadherin dependent VE-cadherin phosphorylation and clathrin-mediated endocytosis

Semina E.[1]*, Rubina K.[2], Sysoeva V.[2], Tkachuk V[2]. [1]Russian Cardiology Research Center, Moscow, Russia, 121552, [2]Faculty of Basic Medicine, Lomonosov Moscow State University, Moscow, Russia, 119192

Pathological processes such as inflammation, atherosclerosis, wound healing and angiogenesis cause a dissociation and rearrangement of endothelial cell junctions and results in enhanced endothelial cell permeability. Adherent junctions formed by classical cadherins provide stable and semipermeable barrier of endothelial monolayer in blood vessels. VE-cadherin is a major classical cadherin located at adherent junctions and responsible for control of vascular permeability. Atypical cadherin T-cadherin belongs to a cadherin superfamily of adhesive molecules. Like other cadherins, T-cadherin consists of extracellular part, including five Ca2+-binding domains, but lacks transmembrane and cytoplasmic domains and is anchored to the plasma membrane via glycosylphosphatidylinositol. T-cadherin expression is altered in endothelial and smooth muscle cells in pathological conditions, which correlates with changes in endothelial permeability. To study the role of T-cadherin in the regulation of endothelial barrier function, we created lentivirus constructs for effective overexpression and suppression of T-cadherin in HUVEC. We demonstrate that changes in T-cadherin expression in endothelial cells modulate permeability: T-cadherin overexpression increases permeability of HUVEC monolayer to FITC-dextran in vitro, in contrast downregulation of T-cadherin decreases permeability of HUVEC monolayer to FITC-dextran. In HUVEC, high level of T-cadherin expression results in increased EC monolayer permeability due to induced gap formation in the area of VE-cadherin cell-cell contacts and accumulation of VE-cadherin in cytoplasm. The mechanism underlying the increased permeability of endothelial monolayer upon T-cadherin overexpression is the phosphorylation of intracellular domain of VE-cadherin on Y731 (which is known as a beta-cathenin binding site), but not of Y658 (p120ctn binding site). Confocal images of HUVEC doubles stained with anti-VE-cadherin and anti-clathrin antibodies show co-localization of VE-cadherin and clathrin in cell cytoplasm. Double immunofluorescent staining of VE-cadherin and Lysotracker Red® verified increased VE-cadherin content in lysosomes. These results suggest VE-cadherin internalization via clathrin-mediated endocytosis and degradation in lysosomes. Overexpression of T-cadherin in HUVEC results in RhoA, Rac1 and Cdc42 activation as well as their downstream signaling adaptors ROCK2, PAK1 and LIMK1, and also in actin stress fibers formation and microtubule depolymerization. We hypothesize that T-cadherin regulates endothelial barrier function via VE-cadherin phosphorylation and its clathrin-dependent internalization and degradation in lysosomes through activation of RhoA, Rac1 and Cdc42.

Cerebral cavernous malformations proteins in cardiac morphogenesis

Marc Renz, Cécile Otten, Franziska Rudolph, Johan Duchene, and Salim Abdelilah-Seyfried*. Max Delbrück Center for Molecular Medicine Berlin, Robert-Rössle Str. 10, 13125 Berlin, Germany

Familial cerebral cavernous malformations (CCM) are inherited vascular abnormalities with a poorly understood etiology that are caused by mutations in CCM1/KRIT1, CCM2, or CCM3/PDCD10. While the CCM complex has been mainly implicated in endothelial vessel formation and maintenance of vascular integrity, little is known about the putative cardiac function of these proteins. In zebrafish and mouse, loss of CCM components causes massive cardiac dilation phenotypes that have not been further characterized. We used zebrafish ccm2 mutant hearts to identify the underlying molecular mechanism involved in cardiac morphogenesis defects this group of diseases. Our study revealed that the ccm mutant cardiac phenotypes involve increased angiogenesis signaling and that Krüppel-like factor 2 (Klf2) activity is a central component in this pathway. In zebrafish, expression of shear-stress responsive Klf2 is not only positively regulated by blood flow, but also repressed by Ccm proteins within endocardium and that disturbance of this mechanism underlies the molecular and cardiac morphogenetic alterations in ccm mutants. Our analyses provide new and unexpected insights into early cardiac morphogenesis. This work indicates the role of endocardial signaling for early myocardial morphogenesis. Taken together, our work may also have implications not only for CCMs but also for other vascular diseases that involve altered angiogenesis signaling.

Visualization and tracing of tumor-associated pericytes

Ann LB. Seynhaeve[1,2]*, Hanna M. Eilken[1], Susanne Adams[1], Timo LM ten Hagen[2], and Ralf H. Adams[1]. [1]Max Planck Institute for Molecular Biomedicine, Department Tissue Morphogenesis and University of Muenster, Faculty of Medicine, Muenster, Germany [2]Erasmus MC, Laboratory for Experimental Surgical Oncology, Department of Surgery, Rotterdam, The Netherlands

The vasculature consists of endothelial cells forming vascular tubes and supporting mural cells, including smooth muscle cells and pericytes. In the adult the vasculature remains predominantly quiescent and activation is mainly found under pathological conditions as tumor growth and progression. Next to tumor cells, tumor-associated endothelial cells are the most targeted cell type in cancer therapy. However, in recent years attention is also drawn to perivascular cells as a potential therapeutic target. Although their significance in healthy and diseased angiogenesis is under intense observation many questions remain unclear. One limitation is the lack of a proper model to observe the dynamic angiogenic processes. As a new tool to study the biological behavior and genetic profile of pericytes we have generated a new mouse line carrying inducible Cre recombinase under the control of PDGFRb, a specific perivascular cell gene. This model allows time-controlled recombination of loxP flanked sequences for loss- and gain-of-function experiments and genetic profiling. As vessel growth is extremely dynamic and even distinct according to tumor types these mice are creating a unique tool to investigate pericyte recruitment and behavior intravitally. Furthermore, lineage tracing experiments, addressing the issue of a pericyte precursor cell, will unravel the origin and fate of these cells. This investigation will provide more information about their significance in tumor progression and also increase their therapeutic impact. For lineage tracing, the PDGFRb-CreERT2 is bred with Rosa26-YFP in which the offspring switch on the fluorescent marker YFP in PDGFRb-expressing cells after tamoxifen administration at desired time points. Two syngeic tumor types are used; the B16BL6 melanoma and the Lewis Lung carcinoma (LLC). Confocal analysis of tumor sections showed that most of the YFP positive perivascular cells are allied with the endothelial lining and are also stained positive for NG2 and SMA. However, a small subfraction of YFP cells are not associated with vessels and do not express NG2 and SMA. These cells can primarily be found in the skin tissue surrounding the tumor crafting the possibility of a tumor pericyte progenitor cell. These first results indicates that this mouse line is a good tool for lineage tracing and implies the potential of a pericyte precursor that plays a major role in tumor angiogenesis.

Reduced miR-223 expression enhances mouse pulmonary hypertension and right ventricle failure response to chronic hypoxia

Lei Shi[1]*, Bhola Kumar Dahal[2,3], Baktybek Kojonazarov[2,3], Robert Voswinckel[2,3], Beate Fisslthaler[1], Werner Seeger[2,3], Ralph T. Schermuly[2,3], Ingrid Fleming[1]. [1]Institute for Vascular Signalling, Centre for Molecular Medicine, Johann Wolfgang Goethe University, Theodor Stern Kai 7, 60590 Frankfurt, Germany, [2]University of Giessen Lung Center, Department of Internal Medicine II/V,Justus-Liebig-University Giessen, Germany, [3]Max-Planck-Institute for Heart and Lung Research, Department of Lung Development and Remodeling, Bad Nauheim, Germany

The factors contributing to the development of chronic hypoxia-induced pulmonary hypertension and the subsequent right heart failure are not well understood. As microRNAs (miRs) are important regulators of differentiation, proliferation and cell signaling pathways, this study aimed to determine which miRs are regulated by hypoxia in the mouse lung and their functional significance in hypoxia-induced pulmonary hypertension and right ventricular dysfunction. miR-223 was the most significantly decreased miR in the hypoxic mouse lung. The insulin like growth factor 1 receptor (IGF1R) is a direct target of miR-223 (3'UTR reporter gene assay) and IGF1R protein levels were increased in lungs and right ventricles from hypoxic mice via a mechanism involving HIF1α/2αand C/EBPα. In vitro, the overexpression of pre-miR-223, decreased IGF1R expression and attenuated IGF1-induced Akt phosphorylation, cell migration and proliferation in pulmonary artery smooth muscle cells. In lungs from patients with primary pulmonary hypertension, miR-223 levels were also decreased while IGF1R expression was elevated. Decreasing endogenous miR-223 in mice using specific antagomiRs increased hypoxia-induced pulmonary artery pressure, pulmonary artery remodeling, right ventricular hypertrophy and dysfunction. A similar phenomenon was also observed in miR-223-deficient mice. Administration of an IGF1R inhibitor attenuated the hypoxia induced pulmonary remodeling and markedly improved right ventricular function. miR-223 was also reduced in the hypoxic right (but not the left) ventricle and increasing the right ventricular work load by pulmonary artery banding in the absence of hypoxia was also able to reduce right ventricular miR-223 after 3 weeks. In this model, antagomiR application further impaired right ventricular function and the IGF1R inhibitor again improved heart function. These data indicate that hypoxia reduces the expression of miR-223 in the lung and right ventricle and that this contributes to the development of pulmonary hypertension and right ventricular dysfunction by the expression of the IGF1R. Inhibition of the IGF1R may be a novel approach to prevent the advancement of pulmonary hypertension and the development of cor pulmonale.

Role of miR-223 in the regulation of angiogenesis

Lei Shi*, Beate Fisslthaler, Nina Zippel, Ingrid Fleming. Institute for Vascular Signalling, Centre for Molecular Medicine, Johann Wolfgang Goethe University, 60590 Frankfurt, Germany

The role of microRNAs (miRs) in the regulation of vascular function is a topic of great scientific interest. However, as the vast majority of the screens for „endothelial cell miRs“ have been performed using cultured cells, the miRs that regulate endothelial cell differentiation/dedifferentiation have been largely ignored. The aim of this study was to identify miRs altered in freshly isolated versus cultured (first passage) human umbilical vein endothelial cells (HUVEC) and to determine their importance for in the regulation of endothelial cell phenotype and function. miRNA profiling revealed that miR-223 is highly expressed in freshly isolated endothelial cells but dramatically decreased in cultured HUVEC and mouse lung endothelial cells. There was no effect of pre-miR-223 overexpression on the proliferation (BrdU incorporation assay) of cultured endothelial cells but pre-miR-223 did result in a change in phenotype in that the cells lost their typical cobblestone morphology and became elongated, i.e. more similar to the endothelial cells observed in situ. Pre-miR-223 also inhibited migration in a scratch wound assay and migration towards VEGF or bFGF in a transwell assay. In parallel studies we also observed that miR-223 overexpression inhibited endothelial cell tube formation on Matrigel and prevented endothelial cell sprouting in a modified spheroid assay. These in vitro findings could be translated to an in vitro model as an increase in the neovascularization of VEGF- and bFGF-impregnated Matrigel plugs was detected in miR-223 knockout mice. Recovery of blood flow after hind limb ischemia was also significantly faster in miR-223 knockout mice that their wild-type littermates. Using a proteomic approach we found that 41 proteins were increased more than 30% and 37 proteins decreased more than 30% (versus control) in the presence of miR-223. Among the down-regulated proteins were beta1 integrin, septin 2, RhoB and the endothelial nitric oxide synthase (eNOS). beta1 integrin, septin-2 and RhoB were identified as direct targets of miR-223 and in particular the knockdown of septin-2 (a GTP binding protein involved in cytokinesis, and cell polarity) and beta1 integrin were able to reproduce the elongated morphology seen in pre-miR-223-treated endothelial cells as well as to inhibit endothelial cell tube formation. Taken together, these results suggest that miR-223 is an „anti-angiogenic“ miR that preserves the quiescent phenotype of endothelial cells in situ - at least partially by repressing septin-2 and beta1 integrin expression.

A possible role of the podoplanin positive cells as an extravascular pathway during wound healing in the mouse tongue

Kazuhiko Shimizu* and Taichi Ezaki. Department of Anatomy and Developmental Biology, School of Medicine, Tokyo Women's Medical University, Tokyo, Japan

[Aim] Regeneration of the microcirculation including lymphatic vessels is very important for the remodeling of tissue organization. In spite of several reports on the mechanism of lymphatic vessel regeneration in wound healing, there have been very few reports about the cell migration pathway before the microcirculation regenerate. In our previous study, we found that the podoplanin positive cells expressing a chemokine, CCL21 played a role of an extravascular pathyay as a cell migration pathway in the urine spleen (Shimizu et al., 2009). In the present study, we sought to characterize the podoplanin positive cells appearing during wound healing in the mouse tongue to study their roles in the tissue repair. [Materials and Methods] We made a 1mm-deep laceration with a sharp sterile razor on the tongue in 5 to 8 weeks-old C57BL/6 mice under anesthesia. For labeling of blood vessels, FITC-conjugated tomato lectin was injected in intravenously. The mice were perfusion-fixed with 4% paraformaldehyde in PBS for 5 min at a pressure of 120 mmHg at various times after injury. The tongue were excised, cut into small pieces and further immersed in 30% sucrose in PBS at 4 ºC for over night. The tongues were then snap-frozen in lequid nitrogen and their cryosections of 10 to 14μm tickness were made for various morphological analysis. All samples stained by the immunofluorescent method were examined with a Leica TCS-SL confocal laser-scanning microscope (Leica, Wetzlar, Germany). Extraction of total RNA from the wounded tongues was performed using ISOGEN kit (Nippon Gene Co. LTD, Tokyo, Japan). PCR analysis were performed with CCL21 and podoplanin. The detection of signals for CCL21 in situ hybridization was done using an alkaline phosphatase conjugated anti-DIG antibody and visualized by NBT/BCIP solution (Roshe Diagnostics, Mannheim, Germany). [Results and Discussion] By day 5 after injury, the epithelium completely healed and the granulation was formed in the submucus where many active fibroblast-like cells were populated and formed fine meshworks without any tubular formation of lymphatic vessels. In addition, these fibroblast-like cells strongly expressed podoplanin, but not LYVE-1. In situ hybridization studies showed that the podoplanin positive cells expressed mRNA for CCL21 chemokine. The RT-PCR also revealed that CCL21 and podoplanin mRNAs increased as early as on day 1 after injury. In our previous study, podoplanin positive reticular cells construct fine meshworkes and co-expressed CCL21 in the mouse spleen (Shimizu et al., 2009). These reticular cells have a role of an extravascular pathway to lymphatic vessels in T cell migration in the normal mosue spleen. Since these podoplanin positive cells in wound areas of the tongue coexpressed a chemokine CCL21, similarly to the splenic podoplanin positive cells, the podoplanin positive cells may also have roles of an extravascular pathway in cell migration mechanisums during wound healing in the mouse toungue. And this cell migration via the extravascular pathway during the tissue regeneration may have indirect roles in the local angiogenesis and/or lymphangiogenesis caused by various migrating cells in the mouse tongue.

Vascular endothelial cadherin cleavage: A perturbation of adherens junction gatekeeper occurs during rheumatoid arthritis

Adama Sidibé[1,2,3]*, Tiphaine Mannic[1,2,3], Mélanie Arboleas[1,2,3], Mariela Subileau[1,2,3], Danielle Gulino-Debrac[1,2,3], Laurence Bouillet[1,2,3,4], Mary Jan[5,6] Thibault Vandhuick[5,6], Xavier Le Loët[5,6], Olivier Vittecocq[5,6], Isabelle Vilgrain[1,2,3]. [1]INSERM, Unité 1036, Biology of Cancer and Infection, Grenoble, F-38054, France, [2]UJF-Grenoble 1, Biology of Cancer and Infection, Grenoble, F-38041, France, [3]CEA, DSV/iRTSV, Biology of Cancer and Infection, Grenoble, F-38054, France, [4]Centre Hospitalier Universitaire de Grenoble, Division of Internal Medicine, Grenoble, F-38043, France, [5]Centre Hospitalier Universitaire de Rouen, ROUEN, F-76031, France, [6]INSERM Unité 905, Pathophysiology and biotherapies of dysimmune and inflammatory diseases F-76031 Rouen, France.

Background: Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that principally attacks synovial joints. However, accelerated atherosclerosis and increased cardiovascular morbidity and mortality are major clinical consequences of endothelial dysfunction in rheumatoid arthritis patients. The observed inflammation of blood vessels is essentially due to the release of both soluble and cell-bound mediators including proinflammatory cytokines. Tumor necrosis factor alpha (TNFa) is a key factor which is highly expressed in RA and has been considered to be one of the most important cytokine in the pathogenesis of the disease. Although TNFa provokes the biosynthesis and release of multiple endogenous mediators that can directly/indirectly contribute to the EC response, it directly influences endothelial barrier function. TNFa is known to alter VE-cadherin function, the major cell-cell adhesion molecule at endothelial adherens junctions, by inducing VE-cadherin tyrosine phosphorylation and increasing microvascular permeability. But the effect of TNFa on VE-cadherin extracellular domain cleavage by proteases has not been extensively explored. Objective: We designed the present study to determine whether TNFa could induce structural modifications of VE-cadherin that could account for decreased adhesive properties of the protein and increased permeability. We examined whether TNFa could induce VE-cadherin cleavage and whether tyrosine kinases and metalloproteinases were involved in this process. Importantly, we addressed for the first time the question of the clinical relevance of soluble VE-cadherin (VE-90) in RA patients. Methods: Human umbilical vein endothelial cells were used in primary culture and treated with recombinant TNFa to study VE-cadherin cleavage. Cell lysates and conditionned media were analyzed respectively for VE-cadherin cytoplasmic and extracellular domain (VE-90) generation by Western blotting. VE-90 was analyzed at basal line in sera from sixty-three patients having arthritis, evolving for less than 6 months (VErA cohort) Results: In this study, we found TNFa to be a powerful inducer of VE-cadherin extracellular domain cleavage at concentrations that correlate with pathophysiologic conditions in a time-dependent manner. That was prevented by tyrosine kinase inhibitors (genistein and PP2) or by knocking down Src demonstrating the involvement of tyrosine kinases (particularly Src) in this process. In contrast, tyrosine phosphatase blockade enhanced VE-cadherin cleavage, confirming the requirement of tyrosine phosphorylation processes. This was noticeable in extracellular domain shedding and subsequent cytoplasmic tail generation. MMPs are involved in this cleavage as the use of metalloproteinase activator (APMA) and inhibitor (GM6001) has demonstrated. Examining different metalloproteinase secretion profiles indicated the particular contribution of MMP-2 in VE-cadherin cleavage. Of importance, VE-90 was present in 63 RA patient sera and positively correlated with disease activity score DAS at baseline and after one year follow-up. Interestingly, this relationship seems independent of CRP levels. Conclusion: These findings provide the first evidence of VE-cadherin proteolysis upon TNFa and suggest that VE-90 could constitute a new marker of disease activity followup, particularly in the subset of RA patients with no CRP level increase. This work has been published in: Sidibé et al. (2012) Arthritis Rheum 64, 1, 77-87 Acknowledgment: Adama Sidibé and Tiphaine Mannic received grants from the Courtin Arthritis Foundation

Association between the expression of plasminogen activator inhibitor 1 and protease urokinase-type plasminogen activator – as a discriminator between normal and cancerous prostate cells

Siegert, G.[1]*, Hagelgans, A.[1], Arneth, B.[1], Tiebel, O.[1], Füssel, S.[2], Menschikowski, M.[1].
[1]Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus at the Technische Universität Dresden, Fetscherstraße 74, 01307 Dresden, [2]Urology, University Hospital Carl Gustav Carus at the Technische Universität Dresden, Fetscherstraße 74, 01307 Dresden, Germany

Aims: Proteases of the fibrinolytic system, especially urokinase-type plasminogen activator (uPA), play a crucial role in the pathogenesis of vascular diseases as well as in cancer progression influencing cell proliferation, extracellular matrix degradation, invasion, and metastasis. In the present study we examined the expression of uPA and plasminogen activator inhibitor 1 (PAI-1) together with vitronectin as matrix component in normal (PrEC) and three prostate cancer cell lines with different metastatic potentials. Methods: ELISA and quantitative RT-PCR techniques were used to measure protein and mRNA levels of uPA, PAI-1, and vitronectin. The influence of epigenetic regulation was investigated using methylation-specific high resolution melting (MS-HRM) analyses of bisulfite-modified genomic DNA. Results: Lowest protein and mRNA levels of uPA showed the LNCaP cell line with low metastatic potential. The uPA levels were lower in comparison to those found in normal PrEC. High uPA protein and mRNA levels were measured in the androgen-independent cell lines DU-145 and PC-3 which exhibit moderate and high metastatic potentials. MS-HRM analyses revealed a reverse correlation between uPA gene expression and methylation degrees of CpG sites in the proximal part of the gene. PAI-1 expression was distinctly down-regulated in cancer cells both at protein and mRNA levels and the lowest PAI-1 expressions were found in LNCaP and DU-145 cells. Treatment of prostate cancer cells such as DU-145 cells with DNA-demethylating agent, 5-aza-2´-deoxycytidine, resulted in about 3-fold increased PAI-1 mRNA levels indicating an involvement of epigenetic mechanisms in the regulation of PAI-1 expression. This conclusion is consistent with data obtained by MS-HRM analyses showing the highest methylation degree of the PAI-1 gene in DU-145 cells. The ratio between PAI-1 and uPA gene expression was elevated in normal prostate cells (~ 10), whereas this ratio was approximately 1.0 in PC-3 and < 1.0 in LNCaP and DU-145 cells. Vitronectin, an abundant plasma and matrix glycoprotein, which stabilizes PAI-1 in its active conformation, was not detectable at protein levels in the analysed cell lines. At mRNA levels, vitronectin expression was only observed in DU-145 cells. Conclusion: The study shows that the serine protease uPA and its inhibitor PAI-1 are differentially expressed in normal and prostate cancer cell lines. Epigenetic mechanisms are involved in the regulation of uPA and PAI-1 expressions. The relation between PAI-1 and uPA expressions was the best distinguishing biomarker between normal and cancer cells, but not a discriminator between cancer cells with different metastatic potential.

Properties of PAI-1 released after platelet activation

Siegert, G.*, Arneth, B., Gehrisch, S., Tiebel, O. Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus at the Technische Universität Dresden, Fetscherstraße 74, 01307 Dresden, Germany

Introduction: Plasminogen activator inhibitor-1 (PAI-1) in blood circulation is stored mainly in platelet alpha-granules and released by platelet activation. Only a small proportion of this released PAI-1 is functional active. Vitronectin, a major protein for cell adhesion and motility is responsible for the stabilisation of PAI-1 in its active conformation. The aim of the study was to investigate whether the supplementation with vitronectin influences the proportion of the active, t-PA binding part of PAI-1, released from activated platelets. Methods: Citrate blood was collected from healthy volunteers without medications. Platelet rich (PRP) and platelet poor plasma (PPP) samples were obtained by standard procedure. PRP was incubated with vitronectin (35 mg/l and 135 mg/l) and the platelet aggregation induced by collagen (10 µg/l). PAI-1 antigen and active PAI-1 antigen were analyzed by tests from Technoclone GmbH Vienna Austria. In a second experiment the t-PA binding capacity of PAI-1 released by platelet aggregation in t-PA supplemented PRP was measured by formation of t-PA-PAI-1-complexes.
The binding of PAI-1 and t-PA on the platelet surface without and with TRAP stimulation in dependence of a supplementation with vitronectin was examined by flow cytometry. Results: Collagen induced platelet aggregation was associated with a 10-fold increase of the total PAI-1 antigen in plasma. In contrast only a small increase of the active t-PA binding PAI antigen was measured. The supplementation of plasma with vitronectin could not change this result significantly. Concentration of t-PA-PAI complexes increased very moderate by platelet aggregation in t-PA supplemented PRP. Applying flow cytometry we could show the expression of PAI-1 as well as t-PA on the surface of TRAP activated platelets. Both were not altered by vitronectin supplementation. Conclusion: One part of platelet born PAI-1 is bound on the platelet surface. This PAI is able to bind t-PA and obviously responsible for thrombus lysis resistance. Only approx. 3% of the unbound part of active PAI-1 is able for binding to t-PA and form tPA-PAI-1-complexes. This result is independent from a vitronectin supplementation indicating that the majority of released active PAI is already bound on the platelet surface via mediation by intrinsic vitronectin. The released latent PAI is not able for vitronectin binding and therefore without influence on the vitronectin mediated cell migration.

The role of hepatic stellate cells during inflammation and metastasis

Nicole Simonavicius[1]*, Annika Armulik[2], Guillem Genove[2], Scott Friedman[3] and Mathias Heikenwälder[1]. [1]Institute of Virology, Helmholtz Zentrum Munich and Technical University Munich, Schneckenburgerstr. 8, 81675 Munich, Germany, [2]Division of Vascular Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Scheeles väg 2, SE-171 77, Stockholm, Sweden, [3]Division of Liver Diseases, Mount Sinai School of Medicine, 1425 Madison Ave., New York, NY 10029-6574, USA

Recent advances in cancer research highlighted the importance of an inflammatory tissue microenvironment in influencing carcinogenesis. However, the exact molecular and cellular mechanisms that respond to and maintain the inflammatory environment, leading to cancer and metastasis, remain elusive. Hepatocellular carcinoma (HCC), the third-leading cause of cancer-related death worldwide, is thought to be underpinned by inflammation. Hepatic stellate cells (HSCs), the pericyte equivalent in the liver, are located in the Space of Disse and are closely associated with endothelial cells building the blood vessels. HSC represent the main liver stromal constituent with an established role in the development of fibrosis; however the roles of HSCs in an inflamed microenvironment that drive liver fibrosis, carcinogenesis and metastasis are incompletely understood. Pro-inflammatory cytokines of the tumour necrosis superfamily (TNF) are upregulated during chronic liver inflammation and damage. Therefore, the modulation of HSC behaviour by these inflammatory stimuli mimicking a pro-carcinogenic environment was examined both in vitro and in vivo. The liver vasculature of mice treated with inflammatory mediators displayed abnormal morphologies and phenotypic changes were observed in HSCs by immunohistochemistry. Furthermore, the gene expression profiling of pericyte-like 10T1/2 fibroblasts and human LX-2 HSCs treated with pro-inflammatory cytokines revealed changes in a panel of genes important for pericyte-endothelial cell interactions (e.g. MMPs). To examine if the changes in the HSCs triggered upon inflammatory stimulation affect tumour cell extravasation, a novel in vitro trans-endothelial/pericyte migration assay was developed. Interestingly stimulation of pericyte-like 10T1/2 fibroblasts with inflammatory mediators led to an increase of the endothelial cell permeability barrier in vitro monitored by the amount of FITC-dextran that passed through the endothelium. MMPs were strongly upregulated in mouse pericyte-like 10T1/2 cells and human LX-2 HSCs treated with pro-inflammatory cytokines, indicating that MMPs might play a role in changing the permeability of the vasculature during inflammation. To gain better understanding of the contribution of HSCs to changes that occur to the endothelium upon inflammatory stimuli in vivo, the frequency of experimental liver metastases will be examined in mice that have been treated with pro-inflammatory cytokines. Together, this will provide new insights into the biology of HSCs in inflammation-driven fibrosis, carcinogenesis and metastasis.

SIRT 1 induction and resveratrol treatment revert senescence in endothelial progenitor cells from low birth weight neonates

Stéphanie Simoncini[1,2‡]*,Paula Frizera Vassallo[1,2‡], Isabelle Ligi[1,2,3], Richard Bachelier[1,2], Stéphane Robert[1,2], Edwige Tellier[1,2], Julia Morère[4], Florence Sabatier[1,2,5], Maxime Marcelli[6], Alain Pascal[7] , Umberto Simeoni[1,2,3]. [1]Inserm, UMR 1076 Endothélium, Pathologies vasculaires et Cibles thérapeutiques, Marseille, [2]Université Aix-Marseille, [3]Assistance Publique-Hôpitaux de Marseille, Département de Néonatologie, Hôpital de la Conception, [4]Inserm, UMR 910, Génétique Médicale et Génomique Fonctionnell, [5]Assistance Publique-Hôpitaux de Marseille, Laboratoire d'Hématologie, Hôpital de la Conception, Marseille, [6]Assistance Publique-Hôpitaux de Marseille, Département d'Obstétrique, Hôpital de la Conception, Marseille, [7]Inserm, Unité Mixte de Recherche 608, Physiopathologie de l'Endothélium Marseille, Marseille, France

Low birth weight (LBW) is known to critically increase cardiovascular diseases risk factor at adulthood. Numerous epidemiologic studies described clinical evidence of a developmentally programmed endothelial dysfunction associated to LBW. However, the impact of LBW on the vascular endothelium is not clearly established.Because senescence of endothelial cells has been proposed as a determinant of vascular dysfunction,we hypothesized that developmentally-associated premature aging could representa mechanistic link between LBW andincrease cardiovascular diseases risk factor at adulthood In this study, weinvestigated whether LBW alters the angiogenic properties of cord blood endothelial colony forming cells in 25 preterm neonates (LBW-ECFC) by comparison to term neonates (CT-ECFC).In LBW-ECFC, we observed a significant decrease in the number of colonies formed by ECFC anda delayedtime of appearance of their clonal progeny.LBW dramatically reduced LBW-ECFCcapacity to form sprout and capillary-like structures,to migrate and to proliferate in vitro. This angiogenic defect of LBW-ECFC was confirmed in vivo by their inability to form robust capillary networks in matrigel plugs implanted in nu:nu mice.Moreover, gene profile analysis of LBW-ECFC demonstrated an increased expression of anti-angiogenic genes. This imbalance toward an angiostatic state provides a mechanistic link between LBW and impaired angiogenic properties of ECFC. Such a defect of ECFC angiogenicproperties together with enlarged and flattened cell morphologylet us to hypothesize that premature senescence could be a determinant of LBW-ECFC dysfunction. In agreement with this hypothesis, LBW-ECFC displayed a significant increase of the senescence associated ß-galactosidase(SA-ß-Gal) activity, a sustained growth arrest and an alteration of cell cycle markers,which were inversely correlated with birth weight. Consistent with a stress-induced rather than with a replicative senescence, telomeres are not significantly shortened but a significant increase of p16 levels was observed in LBW-ECFC.Moreover, we analyzed the expression of SIRT1, a nicotinamide adenine dinucleotide (NAD(+))-dependent histone/protein deacetylase, that plays a crucial role in various physiological processes, such as aging. Interestingly, SIRT1mRNA and protein levels are significantly decreased in LBW-ECFC compared to CT-ECFC and negatively correlated with endothelial senescence.We also demonstrated here that this decreased expression was partly due to epigenetic silencing of the SIRT1 promoter enriched in the histone H3K9me3 repressive mark.We then investigated the pathways that linkSIRT1 variation and defects of LBW-ECFC. Overexpression of SIRT1 or chemical-induction after resveratrol treatment of LBW-ECFC cause a significant reduction of the senescent phenotype accompanied by changes in cell morphology, increased cell proliferation and decrease in SA-ß-Gal staining. Finally, SIRT1 induction afterresveratrol treatment in LBW-ECFC rescues theirangiogenic capacityin vitro. This is the first report providing evidence of a deleterious role of LBW on ECFC angiogenic properties by demonstrating a mechanistic link withpremature senescence linked to SIRT1 silencing. Deciphering the mechanism triggering premature senescence of LBWECFC will improve our understanding of events impacting on cardiovascular complications at adulthood. Modulating SIRT1 expression may hold promises in the prevention and the targeted therapy of cardiovascular disease in adults born prematurely with a low birth weight.

The role of endothelial G-proteins of the G12/13 family in tumor angiogenesis

Kishor Kumar Sivaraj*, Stefan Offermanns and Nina Wettschureck. Dept. of Pharmacology, Max-Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

Tumor angiogenesis is a hallmark of cancer. Tumors induce blood vessel growth by secreting growth factors that facilitate endothelial cell proliferation, survival, and motility, most prominent among them vascular endothelial growth factor (VEGF). In addition to VEGF, several agonists at G-protein-coupled receptors (GPCRs) have been suggested to play a role in angiogenesis, for example thrombin or lysophospholipids, but the relevance and mechanistic details of G-protein-mediated signaling in tumor angiogenesis are not well understood. GPCRs mediate their intracellular effects through four families of heterotrimeric G-Proteins, Gαs, Gαi/o, Gαq/11 and Gα12/13. Studies in constitutive and conditional knockout mice suggested a role of the G-protein α-subunit Gα13 in developmental angiogenesis1,2, but the relevance in the adult organism is unclear. To study the role of endothelial Gα13 signaling in tumor angiogenesis we used a genetic mouse model that allows tamoxifen-inducible, endothelial cell (EC)-specific inactivation of the G-protein α-subunits Gα12 and Gα13 (EC-Gα12/13-KO). We subcutaneously implanted Lewis lung carcinoma (LLC1) and Melanoma (B16/F10) cells into EC-Gα12/13-KOs and found that tumor growth and tumor weight were significantly reduced compared with littermate controls. Interestingly, tumor angiogenesis was significantly reduced in EC-Gα12/13-KOs, and VEGF-induced angiogenic responses were abrogated both in in vivo and in vitro angiogenesis assays. We currently investigating the role of Gα12/13 in VEGF dependent effects in endothelial cells. REFERENCES 1.Offermanns S. et al., 1997. Science. 2. Ruppel M. K. et al., 2005, PNAS.

Reversal of hypoxia in murine atherosclerotic plaques prevents apoptosis and necrotic core expansion

Thomas L. Theelen[1,2], Matthijs van Gink[1,2], Jeroen Otten[1,2], Mike Jeurissen[1,2], Anique Janssen[1,2], Chris M. Van der Loos[4], Linda Beckers[1,2], Erwin Wijnands[3], Marion J. Gijbel[1,2], Mat J.A.P. Daemen[4] , Judith C. Sluimer[1,2]*. [1]Cardiovascular Research Institute Maastricht (CARIM), [2]Department of Pathology, Maastricht University Medical Center, Maastricht, [3]Department of Molecular Genetics, Maastricht University Medical Center, Maastricht, [4]Department of Pathology, Amsterdam Medical Centre (AMC), Amsterdam, The Netherlands

Recently we were the first to demonstrate hypoxia in advanced murine and human plaques. Although hypoxia stimulates processes detrimental for atherogenesis, i.e. inflammation and apoptosis, its actual role in the development of atherosclerosis is unknown. In the present study we investigated whether plaques are reoxygenated by in vivo carbogen (95% O2, 5% CO2) breathing, and whether carbogen-induced reoxygenation affects atherogenesis. Low-density-lipoprotein-receptor (LDL-r)-/- mice (11-wk-old males, n=10/group) were fed a 0.25% cholesterol diet for 0, 4, 8, 12 and 16 weeks. Mice were injected with the hypoxia-specific marker pimonidazole (100mg/kg ip) one hour before sacrifice to detect hypoxic cells ex vivo using immunohistochemistry. Surprisingly, after 4 weeks of diet plaque hypoxia could already be demonstrated in fatty streaks in the aortic root, while being absent in media of diseased and non-diseased vessel wall. Multispectral analysis (Nuance VIS-FL detector and software 3.0) of double immunohistochemistry showed that 69±10.3% of MAC3+ plaque macrophages were hypoxic, whereas alpha smooth muscle actin+ smooth muscle cells and CD3+ T-cells were mostly normoxic. LDLR-/- mice with advanced plaques were subjected to a single 90min exposure of carbogen or compressed air (21% O2) at 5L/min. Mice were injected with pimonidazole half-way the exposure and sacrificed directly thereafter. Single carbogen treatment of advanced plaques led to a dramatic 80% reduction of plaque hypoxia in the aortic arch (p=0.029) compared to control. Therefore, the effects of chronic carbogen exposure on atherogenesis were studied in LDL-r-/- mice (20-wk-old males, n=15/group) were fed 0.25% cholesterol diet for four weeks. During the following four weeks of diet, mice were exposed daily to carbogen or air for 90 min. Twenty-four hours after the last exposure, mice were sacrificed and pimonidazole injected to study plaque hypoxia and atherogenesis in the aortic arch and root. Even 24 hours after the last exposure, chronic carbogen exposure resulted in a 42% decrease of plaque hypoxia in the aortic root (p=0.028) compared to control. Chronic carbogen exposure did not alter plaque size or macrophage content in aortic root or arch, but reduced necrotic core size by 37% in advanced plaques of the aortic root compared to control (p=0.0003). In parallel, carbogen treatment halved plaque apoptosis (TUNEL+cells/total cell) (-50%, p=0.03) compared to compressed air, likely due to improved efferocytosis of apoptotic cells (+36%, p=0.03). These plaque-stabilizing effects were independent of serum cholesterol levels and haematological parameters (Hb, Ht, erythrocyte-, leukocyte-, platelet counts). In addition, extensive FACS analysis of inflammatory cell-/subtypes in blood, spleen, lymphe nodes, and bone marrow did not show differences in systemic inflammation parameters. Thus, we conclude that local plaque reoxygenation prevented necrotic core expansion. Carbogen exposure successfully enhanced plaque oxygenation without systemic side-effects. Although carbogen did not affect plaque initiation (aortic arch), plaque reoxygenation of advanced plaques (aortic root) drastically decreased plaque core size and apoptosis, most likely by enhancing efferocytosis. Reversal of plaque hypoxia thus prevents necrotic core expansion and may provide a new therapeutic avenue for plaque stabilization.

Human umbilical vein endothelium from gestational diabetes and normal pregnancy are differentially responsive to insulin without altering insulin receptor β subunit phosphorylation

Westermeier F, Salomón C, Arroyo P, Guzmán-Gutiérrez E, Pardo F, Leiva A, Sobrevia L*. Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynecology, Medical Research Centre (CIM), School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago, Chile

Human umbilical vein endothelium (HUVEC) from pregnancies with gestational diabetes (GD) exhibit reduced adenosine transport via the human equilibrative nucleoside transporter 1 (hENT1) compared with cells from normal pregnancies. Since insulin modulates hENT1 expression and activity, we aim to identify whether insulin receptor β-subunit (β-IR), Akt (metabolic signaling), p44/42mapk (mitogenic signaling) and hENT1 activity are differentially modulated by insulin in HUVEC from normal or GD pregnancies. Methods: Primary cultured HUVEC (passage 3) from full-term normal and diet-treated GD pregnancies (n=44) were used. Insulin (1 nM, 8 hours) was assayed on hENT1 adenosine transport (4 μCi/ml, 20 seconds, 22ºC). Total and phosphorylated β-IR, Akt and p42/44mapk (western blot) was determined in absence or presence of PD-98059 (p42/44mapk inhibitor) or wortmanin (PI3K inhibitor). Results: hENT1-mediated adenosine transport was reduced (~75%) (Student's unpaired t test, ANOVA, $P<0.05$) in cells from GD compared with normal pregnancies, effect that was blocked by insulin. Insulin effect on transport was blocked by PD-980259, but unaltered by wortmanin. Insulin increased phosphorylated/total Akt (wortmanin-inhibited) protein ratio in normal (~4 fold) and GD (~3 fold) pregnancies, but blocked GD-increased p42/44mapk phosphorylation (PD-98059–inhibited) to values in normal pregnancies in absence of insulin. Insulin caused a similar increase of β-IR phosphorylation in normal and GD (SC50 ~0.28 nM) pregnancies. Conclusion: HUVEC from GD pregnancies exhibit a differential response to insulin associated with a preferential mitogenic, rather than a metabolic phenotype. This phenomenon could play crucial roles in the regulation of the feto-placental vascular tone by insulin in GD. CONICYT (ACT-73 PIA, AT-24100210), FONDECYT (1110977, 1120928, 11110059), Faculty of Medicine, PUC (PMD 03/10). FW, CS and EG-G hold CONICYT-PhD fellowships. CS and PA hold Faculty of Medicine, Pontificia Universidad Católica de Chile-PhD fellowship.

Insulin induces human equilibrative nucleoside transport 2 involving SREBP-1c in human umbilical vein endothelial cells

Pardo FN, Westermeier F, Salomón C, Guzmán-Gutierrez E, Leiva A, Sobrevia L*. Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynecology, Medical Research Centre (CIM), School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, P.O. Box 114-D, Santiago, Chile

Human umbilical vein endothelial cells (HUVEC) maintain normal extracellular levels of adenosine due to an efficient uptake of this nucleoside via human equilibrative nucleoside transporters 1 (hENT1, ~80%) and 2 (hENT2, ~20%). Insulin increases hENT2 expression and activity in HUVEC; however, there are no reports addressing whether this hormone alters SLC29A2 (for hENT2) gene promoter activity. Since sterol responsive element binding protein 1c (SREBP-1c) activity is increased by insulin in human hepatocytes, and the promoter region of SLC29A2 contains consensus sequences for this transcription factor (in silico), we hypothesize that insulin will modulate SLC29A2 expression in HUVEC, and that SREBP-1c may play a role in this phenomenon. Methods. HUVEC were isolated from normal pregnancies (n=6) and cultured up to passage 3 under standard conditions (5% CO2, 37ºC) in 20% sera-containing M199 medium. Insulin effect on SLC29A2 promoter activity was assayed in pGL3-vector reporter constructs (pGL3-hENT2-1998, pGL3-hENT2-1491, pGL3-hENT2-1086, pGL3-hENT2-864 and pGL3-hENT2-602 for -1998, -1491, -1086, -864 and -602 bp from ATG, respectively). HUVEC were electroporated (320 V, 975 µF, 9-11 ms), exposed to insulin (1 nM, 8 hours), and promoter reporter activity measured by luciferase assay. SREBP-1c protein abundance was determined in whole cell extract, and cytoplasm and nucleus fractions by western blot, and mRNA determined by SQ-PCR. Results: Insulin caused a similar increase (~1.2 fold, $P<0.05$, ANOVA) of pGL3-hENT2-1998 and pGL3-hENT2-1491 transcriptional activity; however, shorter constructs activity was unaltered by this hormone. Insulin increased the SREBP-1c protein abundance and translocations to the nucleus, and the mRNA level. The in silico analysis revealed at least one SREBP consensus sequence between -1133 and -1119 bp from ATG. Conclusion: The results suggest that insulin increases SLC29A2 expression requiring responsive elements within -1491 and -1086 bp from ATG of the promoter region. In addition, SREBP-1c could play a role in this phenomenon. CONICYT (ACT-73 PIA, AT-24100210), FONDECYT (1110977, 1120928, 11110059), Faculty of Medicine, PUC (PMD 03/10). CS, EG-G and FW hold CONICYT-PhD fellowships. CS holds Faculty of Medicine, Pontificia Universidad Católica de Chile-PhD fellowship.

Regulation of AMPK by cAMP signaling pathways

K. Spengler[1]*, A. Woods[2], D. Carling[2], R. Heller[1]. [1]Klinikum der Friedrich-Schiller-Universität Jena, Institut für Molekulare Zellbiologie, Hans-Knöll-Strasse 2, 07745 Jena, Germany [2]Cellular Stress Group, MRC Clinical Sciences Centre, Imperial College, Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom

5'AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that regulates intracellular energy state and homeostasis. AMPK activation is essentially dependent on phosphorylation of threonine 172 (T172) which is mediated by calcium/calmodulin-dependent protein kinase kinase beta (CaMKKbeta) or by the tumor suppressor kinase LKB1. Other phosphorylation sites including serine 485 (S485) with yet unknown functions have also been identified. Previous studies have shown that vascular endothelial growth factor (VEGF) stimulates AMPK via a CaMKKbeta-dependent pathway and that the AMPKï¡1 isoform is required for VEGF-induced angiogenesis. In muscle, liver and adipose tissue many of the metabolic pathways regulated by AMPK are affected by cAMP signaling systems and synergistic and antagonistic interactions between the two systems have been described. The present study was aimed at exploring a possible crosstalk between cAMP-dependent pathways and AMPK activation in endothelial cells in response to VEGF. Experiments were performed in human umbilical vein endothelial cells (HUVEC). AMPK activity was analysed in cell lysates by monitoring T172 phosphorylation and in AMPKalpha1 immunoprecipitates by measuring the phosphorylation of a specific substrate. VEGF led to AMPK activation, which was associated with phosphorylation of T172. In addition, a delayed phosphorylation of S485 was detectable. In contrast, intracellular cAMP elevation induced by a combined treatment with forskolin (adenylate cyclase activator) and isobutyl-methylxanthin (IBMX, phosphodiesterase inhibitor) triggers S485 phosphorylation without affecting T172. Downregulation of PKA with specific siRNA revealed that cAMP-induced phosphorylation of S485 was mediated by PKA. Interestingly, preincubation with forskolin/IBMX reduced the subsequent activation of AMPK in response to VEGF, which was demonstrated by both reduced T172 phosphorylation and impaired enzyme activity. The cAMP-mediated effect was in part due to an interference with AMPK phosphorylation by CaMKKbeta. Staining of CaMKKbeta immunoprecipitates with an antibody against phosphorylated PKA substrates revealed that PKA phosphorylates CaMKKbeta. This was accompanied by an inhibited CaMKKbeta activity as demonstrated by a decreased phosphorylation of CaMKIV, a well-known CaMKKbeta substrate. In addition, S485 phosphorylation by PKA may also exert an inhibitory effect on AMPK. Accordingly, cAMP elevation leading to S485 phosphorylation inhibited AMPK in response to a CaMKKbeta-independent stimulus, aminoimidazole carboxamide ribonucleotide (AICAR). Together, these data describe an important inhibitory effect of the cAMP/PKA pathway on AMPK activation in response to VEGF, which may play a role in the control of VEGF-induced permeability.

Adjuvant anti-Ang-2 therapy inhibits postsurgical metastasis

Kshitij Srivastava[1], Junhao Hu[1], Alexander Schering[1], Manfred Jugold[2], Markus Thomas[3], Hellmut Augustin[1,4]. [1]Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), [2]Medical Physics in Radiology, Small Animal Imaging Center (SAIC), German Cancer Research Center Heidelberg, [3]Discovery Oncology, Roche Diagnostics GmbH, Penzberg, [4]Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim, Heidelberg University (CBTM), Germany

The Angiopoietin/Tie system is a vascular receptor tyrosine kinase system playing a pivotal role in controlling the angiogenic cascade and vessel homeostasis. Genetic inactivation of Angiopoietin-2 (Ang-2) results in a non-responsive, refractory vessel phenotype. Based on this distinct Ang-2 knockout phenotype, we studied the effect of antibody-mediated Ang-2 inhibition on physiological and pathological angiogenesis. Systemic delivery of neutralizing Ang-2 antibody was effective in potently interfering with postnatal retinal angiogenesis during the early phase of sprouting angiogenesis (postnatal days 1-6) as well as during later vessel remodeling (postnatal days 9-20). Based on these findings, we examined the potency of antibody-based Ang-2 inhibition as a strategy for postsurgical adjuvant tumor therapy. Subcutaneous Lewis lung carcinomas (LLC) and orthotopic 4T1 breast cancers were surgically removed, after which the mice were treated with Ang-2 neutralizing antibody or control antibody. Ang-2 antibody treated mice had no to very few lung metastases in both models. In contrast, more than 80% of control mice developed overt lung metastases. Frequent bone metastasis and associated bone damage was observed in control antibody treated group of the breast cancer model. Ang-2 antibody treatment decreased the incidence of bone metastases and preserved the structure of long bones as well. The Ang-2 antibody also conferred a significant overall survival advantage in comparison to control antibody treatment in a separate postsurgical adjuvant therapy trial. We next compared Ang-2 adjuvant therapy to standard postsurgical chemotherapy regimen alone or to the combination of anti-Ang-2 with metronomic chemotherapy. When assessing parameters like therapeutic efficacy, body weight loss, myelosupression, ovarian toxicity and intestinal villi damage, the combination of anti-Ang-2 with metronomic chemotherapy was found to provide not only the best survival benefits, but also turned out to be as the safest approach in toxicological studies. Mechanistically, Ang-2 neutralization led to the downregulation of key proinflammatory molecules (ICAM-1, VCAM-1). This corresponded to reduced recruitment of prometastatic tumor associated macrophages to sites of pre-seeded lung metastases. Taken together, the data provide strong evidence for an attractive therapeutic window of anti-angiogenic drugs for postsurgical adjuvant treatment and identify the combination of anti-Ang-2 with metronomic chemotherapy as a particularly promising combination therapy.

Neutrophil extracellular traps contribute to DVT formation by providing a prothrombotic and procoagulant surface

Konstantin Stark[1]*, Marie-Luise von Brühl[1], Alexander Steinhart[1], Sue Chandraratne[1], Anca Tirniceriu[1], Michael Lorenz[1], Annekathrin Eckart[1], Selgai Haidari[1], Susanne Pfeiler[2], Davit Manukyan[2], Jerry Ware[3], Volker Brinkmann[4], Klaus Preissner[5], Bernd Engelmann[2] and Steffen Massberg[1]. [1]Deutsches Herzzentrum and I. Medizinische Klinik, TUM, Munich, Germany, [2]Institut für Klinische Chemie, Ludwig-Maximilians-Universität, Munich, Germany, [3]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, USA, [4]Max-Planck-Institut für Infektionsbiologie, Berlin, Germany, [5]Department of Biochemistry, University of Giessen, Germany

Objective: Neutrophils have been primarily implicated in host defence, but it is increasingly recognised, that they also contribute to coagulation. One mechanism how they could do that is exposure of Neutrophil extracellular traps (NET). These extracellular DNA structures have been found in deep venous thrombosis (DVT), but how they participate in thrombus formation in vivo is unclear. In this study we wanted to assess the dynamics of NET formation in vivo and their impact on DVT development in a murine flow reduction model of the inferior vena cava (IVC). Methods: Thrombosis was induced in C57Bl6 mice by placing a narrowing ligature around the IVC, resulting in a reduction of blood flow velocity (n=16). NET formation in vivo was visualized by intravital 2-photon microscopy. Thrombogenesis and NET formation were quantified in GPIb-/-, DNase and heparin treated animals (n=7 each). The ability of NETs to bind and activate factor XII was assessed in vitro. Results: Neutrophils were recruited very early after initiation of flow reduction in the IVC, supported by platelets. NET formation in the IVC, triggered by platelets, could be detected in vivo as early as 3h after flow reduction. We found that NETs were binding platelets, tissue factor, and fibrinogen, demonstrating a concentration of procoagulatory and prothrombotic factors on their surface. This is highlighted by the fact that coincubation of activated platelets and neutrophils resulted in significant FXII activation. Inhibition of NETs by an antibody directed against the H2A-H2B-DNA complex significantly attenuated FXII activation. The functional impact of NETs for DVT formation is indicated by the finding that disruption of NETs by DNase (n=5) treatment resulted not only in a reduced number of NETs, but also in a markedly reduced thrombus weight compared to wt control. Surprisingly, injection of heparin resulted in a diminished number of NETs inside the IVC, which could add to its antithrombotic effect (n=3). Conclusion: Here we show that neutrophils contribute to DVT by NET formation, which is triggered by adherent platelets. This provides a platform for platelet adhesion and concentration of procoagulatory factors on their surface, linking inflammation and thrombosis at the cellular level. Thus, disruption of NETs could be an interesting new therapeutic approach for prophylaxis and treatment of DVT.

Von Willebrand Factor regulates angiogenesis in Von Willebrand disease patient's blood outgrowth endothelial cells

Richard D Starke[1]*, Koralia E Paschalaki[1,2], Sarah Mazi[1], Tuncay Sahutoglu[1], Jacqueline Cutler[3], Elspeth Payne[1], Carolyn Millar[4], Mike A Laffan[4] and Anna M Randi[1]. [1]Cardiovascular Sciences, NHLI, Imperial College, London, UK, [2]Airway Disease Department, NHLI, Imperial College, London, UK, [3]Molecular Haemostasis Laboratory, Department of Haemostasis and Thrombosis, GSTS Pathology, London, UK, [4]Department of Haematology, Imperial College, London, UK. EP's present address: UCL Cancer Centre, POGB 314, London UK

Endothelial Von Willebrand factor (VWF) is a large plasma glycoprotein stored in specialised organelles called Weibel-Palade bodies (WPB). VWF directs the formation of WPB and regulates the storage of other components. VWF can be released from EC constitutively or upon agonist stimulation and is best known for its role in mediating platelet adhesion to sites of EC damage and stabilising coagulation factor VIII. A qualitative or quantitative deficiency of VWF causes Von Willebrand disease (VWD), the most common congenital bleeding disorder. We have demonstrated that VWF can also regulate angiogenesis. Inhibition of VWF expression by siRNA in human umbilical vein EC (HUVEC) resulted in loss of WPB and increased release of the growth factor Angiopoietin (Ang)-2. Moreover, VWF siRNA caused a decrease in integrin αVβ3 expression, a known angiogenic regulator. VWF-deficient HUVEC showed increased MAPK signalling, with raised ERK1/2 phosphorylation. In vivo, we have previously shown increased angiogenesis and vascular density in the Matrigel plug model and in the ear of VWF deficient mice. These findings were expanded using the retinal angiogenesis model in newborn mice, which showed increase vascular density, branching and EC sprouting in the VWF deficient mouse compared to controls. Angiogenesis is essential for normal development and for physiological processes in the adult. Excessive angiogenesis is linked to angiodyplasia, vascular malformations in the gastrointestinal tract that can cause severe bleeding in VWD patients. To investigate the role of VWF in regulating angiogenesis in patients with VWD, we used blood outgrowth EC (BOEC) isolated from patients with VWD and normal controls. Here we focus on one patient with severe type I VWD with associated angiodysplastic lesions. The patient exhibited reduced plasma VWF antigen (0.11 IU/mL, N.R. 0.5-2), ristocetin cofactor activity (<0.08 IU/mL, N.R. 0.5-2), collagen binding activity (0.03 U/mL, N.R. 0.5-2) and loss of high molecular weight VWF multimers. The patient did not respond to treatment with the vasopressin analogue DDAVP, which induces VWF release from WPB. Sequencing of the VWF gene revealed that the patient is a compound heterozygote for mutations that cause truncation of the mature protein. VWF mRNA levels were very low in the patient's BOEC compared to controls. Immunofluorescence and VWF ELISA confirmed the almost total absence of VWF and WPBs. Both constitutive and phorbol 12-myristate 13-acetate (PMA)-stimulated release of VWF from the cells were also significantly reduced, as expected, given the lack of intracellular VWF and the lack of clinical response to DDAVP. We hypothesised that the absence of VWF and WPB might lead to the constitutive release of other WPB components, amongst these the pro-angiogenic growth factor Ang-2. Interestingly, BOEC from the VWD patient showed increase constitutive release of Ang-2, similar to that observed in VWF-deficient HUVEC and an increase in in vitro angiogenesis assays, namely capillary network formation on Matrigel and proliferation. In conclusion, our data demonstrate that VWF regulates angiogenesis through intracellular and extracellular pathways, and that EC from VWD patients present a pro-angiogenic phenotype. These findings may help explain the pathogenesis of angiodysplasia in patients with VWD.

Fatty acid amide hydrolase deficiency is associated with a vulnerable plaque phenotype in atherosclerotic mice

Sébastien Lenglet[1], Aurélien Thomas[2], Fabrizio Montecucco[1], Fabienne Burger[1], Graziano Pelli[1], Katia Galan[1], Benjamin Cravatt[3], Christian Staub[2], Sabine Steffens[1]*.
[1]Division of Cardiology, University Hospital, 1211 Geneva, Switzerland, [2]Unit of Toxicology, University Center of Legal Medicine; 1211 Geneva, Switzerland, [3]The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, La Jolla, CA, USA

Elevated endocannabinoid levels are linked with the development of atherosclerotic vascular disease. However, it remains unclear whether endocannabinoid levels might represent a risk factor or diagnostic biomarker for acute atherosclerotic vascular events. Here, we studied the involvement of fatty acid amide hydrolase (FAAH) deficiency, the major enzyme responsible for endocannabinoid anandamide degradation, in atherosclerotic plaque vulnerability. We generated apolipoprotein E-deficient (ApoE-/-) FAAH-/- mice by interbreeding ApoE-/- with FAAH-/- mice and measured serum levels of anandamide and related FAAH metabolites palmitoyl- and oleoylethanolamide (PEA, OEA). We assessed atherosclerosis in ApoE-/- and ApoE-/-FAAH-/- mice after 5, 10 and 15 weeks on high cholesterol diet (HCD; 1.25% cholesterol) and analyzed weight, serum cholesterol and atherosclerotic plaque composition. Levels of FAAH metabolites anandamide, PEA and OEA were 1.4 to 2-fold higher in FAAH-/-ApoE-/-mice. FAAH deficiency attenuated atherosclerotic plaque size increase (by ~50% in thoraco-abdominal aortas after 15 weeks HCD; n=7-10; P=0.007), but plaques had significantly lower content of smooth muscle cells (reduced by 36% at 10 weeks HCD in aortic sinuses; n=10-15; P=0.01) and increased matrix metalloproteinase MMP-9 expression (by 73%; P=0.049). There was no difference in macrophage content, but a 65% increase in neutrophil infiltrates (P=0.0007) in aortic sinus plaques from ApoE-/-FAAH-/- mice compared to ApoE-/- controls. This was accompanied by 1.9-fold increased chemokine CXCL1 mRNA levels (P =0.004) in mouse aortas. CXCL1 expression in plaques was confirmed by immunostaining. MMP-9 mainly colocalized with neutrophils rather than macrophages and positively correlated with their intraplaque infiltration (r= 0.6529; P=0.006). Interestingly, we also found 2.4-fold increased mRNA levels of the Th17 signature cytokine IL-17 in spleens of ApoE-/-FAAH-/- mice compared with ApoE-/- controls (P=0.0078), suggesting a switch of T lymphocyte polarization towards Th17. In conclusion, increased endocannabinoid anandamide and related FAAH metabolite levels are associated with smaller atherosclerotic plaques with more vulnerable phenotype. Whether systemic or local increases in CXCL1 and/or IL-17 production may contribute to plaque neutrophil infiltration is subject of ongoing studies.

Role of platelet interleukin-17 receptor A in platelet activation, aggregation and adhesion over human endothelial cells in vitro

Kateryna Sopova[1], Katerina Gatsiou[2], Carolin Amrhein[1], Christian Erbel[2], Alexandros Tselepis[2], Voahanginirina Randriamboavonjy[4], Konstantinos Stellos[1]*. [1]Vascular Inflammation Group, Institute of Cardiovascular Regeneration, Centre of Molecular Medicine, Goethe University Frankfurt am Main, [2]Department of Chemistry, University of Ioannina, [3]Department of Cardiology, University of Heidelberg, [4]Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University, Frankfurt, Germany

Background: Interleukin 17 (IL-17) is a pro-inflammatory cytokine, which plays a vital role in inflammation. The aim of the present study was to evaluate the expression of IL-17 receptor A (IL-17RA) on platelets and its possible role in platelet activation, aggregation and adhesion in vitro. Methods: Evaluation of the expression of IL-17RA on platelets was determined by flow cytometry and western blot analysis. The possible role of IL-17 alone or in combination with other platelet agonists in platelet activation (glycoprotein-GPIIb/IIIa activation and P-selectin expression) has been studied in platelet rich plasma (PRP) and isolated platelets with the help of flow cytometry. The possible role of IL-17 in platelet aggregation was determined with the help of light transmission aggregometry. The role of IL-17A in platelet adhesion over cultured resting and TNF-alpha/INF-gamma- activated endothelial cells (HUVECs) was investigated under high shear stress in vitro. Results: IL-17RA is expressed on platelets, as shown by flow cytometry and western blot. Its expression is elevated upon TRAP-6-induced platelet activation. IL-17 significantly enhanced the PAR-1-mediated GPIIb/IIIa activation studied in both PRP and isolated platelets, but did not have any effect on P-selectin expression ($P<0.05$). In order to evaluate the possible effect of IL-17 alone or in combination with ADP, TRAP or collagen on platelet aggregation we performed a series of LTA experiments. IL-17 neither increased nor decreased platelet aggregation after pretreatment with IL-17 alone or in combination with different concentrations of ADP or TRAP-6 or collagen. Next, in order to evaluate the possible effect of IL-17 on platelet adhesion, we performed a series of flow chamber experiments of washed platelets, pretreated with IL-17 or ADP or control vehicle, over resting and activated endothelial cells under high shear stress (2000-s). Pretreatment of platelets with IL-17 significantly increased their adhesive properties over both resting and activated endothelial cells ($P<0.05$ for both). The initial step of platelet adhesion (rolling of platelets over HUVECs) was also significantly increased after pretreatment with IL-17. Conclusion: Expression of IL-17RA on the surface of platelets may be involved in IL-17-induced vascular inflammation.

In vitro biological activity of different transgenic anti-VEGF molecules as potential treatment options for retinal neovascular disorders

Stieger K*, Wimmer T, Lorenz B. Department of Ophthalmology, Justus-Liebig-University Giessen, Giessen, Germany

Purpose: Ocular neovascularisation due to uncontrolled growth of new vessels into the retina following overexpression of vascular endothelial growth factor (VEGF) is the main cause of visual impairment in retinal neovascular diseases such as age-related macular degeneration (AMD) or diabetic retinopathy (DR). The intraocular expression of anti-VEGF molecules potentially represents a therapeutic strategy to block neovascularization in these pathologies. The aim of this study was to characterize the optimal composition of the expression cassette for the production of these molecules in standard cell lines in vitro. Methods: Both antibody chains of Ranibizumab, the light and part of the heavy chain were designed containing secretory leader sequences or restriction sites for subsequent subcloning. The fragments were either expressed separately using an IRES containing expression cassette (Ra01), or were cloned together into one reading frame containing either a glycine or glycine-proline anchor in between (Ra02-Ra06). Plasmids were transfected into HEK293 and Hela cell lines and the expression of the molecules verified by Western blot analysis. A Ranibizumab specific ELISA was developed in order to measure the concentration of the anti-VEGF molecules. The biological activity was tested using HUVEC (human umbilical vein endothelial cell) tube formation assays and HUVEC migration assays. Results: All Ra compositions were detected in the supernatant of transfected Hela and HEK293 cells. Generation of long cellular tubes in the HUVEC tube formation assay, which is predominantly due to VEGF activity, was reduced to 50% with Ra01 and similar levels were achieved with Ra02-Ra06. VEGF activity in the HUVEC migration assay was reduced by about 50% for all Ra compositions. Similar VEGF inhibition results were obtained using commercially available recombinant Ranibizumab (Lucentis®) at equal concentrations. Conclusion: Transgenic Ranibizumab, either expressed separately or as one molecule have similar inhibitory effects on VEGF activity as commercially available Ranibizumab in vitro. These results lay the foundation for the development of an alternative treatment strategy for patients with AMD or DR, in which Ranibizumab is produced at low doses directly in retinal cells.

The transcription factor MEF2C downmodulates the sprouting capacity of endothelial cells

Sturtzel Caterina*, Testori Julia, Lipnik Karoline, Schweighofer Bernhard, Bilban Martin and Hofer Erhard. Medical University Vienna, Department of Vascular Biology and Thrombosis Research,Vienna, Austria

The MADS-box transcription factor myocyte enhancing factor 2C (MEF2C) has been recognized as an important switch in the control of differentiation of many cell types. We have detected MEF2C to be specifically induced by the pro-angiogenic growth factors VEGF-A and bFGF in endothelial cells. Upregulation by VEGF-A was mediated via VEGFR-2. To elucidate a potential role of MEF2C in angiogenesis we performed gain and loss-of-function studies in the in vitro spheroid sprouting assay. Adenoviral overexpression of MEF2C in HUVEC revealed a strong inhibitive effect on sprouting, whereas a dominant-negative MEF2 variant stimulated sprouting in this setting. Furthermore, in a wound healing assay MEF2C overexpression inhibited migration, but proliferation was unaffected. To define potential transcriptional targets of MEF2C involved in these effects gene profiling of HUVEC overexpressing MEF2C was conducted. This identified alpha-2 macroglobulin (A2M) as the most prominently induced gene. When we tested shRNA-mediated silencing of A2M expression, the inhibitive effect of MEF2C on sprouting was reduced. In line with an increased production of A2M supernatants of MEF2C overexpressing cells inhibited sprouting and this soluble inhibitory activity was diminished following shRNA-mediated knock-down of A2M. These findings support that A2M, which has been previously described as an effective inhibitor of serine proteases as well as a scavenger molecule for growth factors like VEGF or bFGF, is indeed involved in mediating the inhibitory effects of MEF2C on sprouting. Additional data show that the upregulation of A2M observed at normoxic conditions is largely reduced by hypoxia. We therefore propose that MEF2C via up-regulation of A2M functions in a negative feed-back loop to suppress sprouting under normoxic conditions. (This work was supported by a DOC-fFORTE-fellowship of the ÖAW and FWF grant P21291-B11).

Genome-wide approaches reveal functional VEGF-NFATc-mediated RND1 induction is necessary for endothelial barrier potential

Jun-ichi Suehiro*, Yasuharu Kanki, and Takashi Minami. Laboratory for Vascular Biology, RCAST, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo, Japan

Endothelial cell activation and dysfunction underlie many vascular diseases, including atherosclerosis, thrombosis, and pathological angiogenesis. Vascular endothelial cell growth factor (VEGF) is a key regulator for endothelial cell survival, migration, and proliferation, which leads the VEGF receptor-2 regulated several activation-signaling cascades in endothelial cells. Among them, we focused on the calcineurin-NFATc pathway. It has been well considered that NFATc activation and the nuclear localization play an important role in bone formation, and heart development. However, the NFATc regulated endothelial function, especially, the specific targets for NFATc in endothelial cells did not extensively elucidated. Here, we show the newly mapping of genome-wide NFATc binding events in VEGF-stimulated primary cultured endothelial cells, by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). We succeeded in identifying total 4,119 NFATc binding regions in response to VEGF stimulation, with high confidence score, P value less than 10e-50. Compared with referenced human genome, 50 % of the NFATc binding regions were located at the promoter proximal transcriptional start site or 5' untranslated region, and 17 % of which located at the gene body. NFATc occupied sites were also co-enriched the transcription factor binding regions of CREB or C/EBP factors. Combined the NFATc ChIP-seq profile and the epigenetic histone marks revealed that predominant NFATc-occipied peaks were overlapped with promoter marking; trimethylated (me3) lysine 4 in histone 3 (H3K4me3), or enhancer marking; H3K4me1 and Acetylated H4, but not overlapped with silencer marking; H3K27me3. DNA microarrays with NFATc modulation indicated the almost NFATc binding targets were correlated with induced patterns, consistent with the epigenetic histone microenvironment in endothelial cells. Moreover by using the ChIP-seq profiles, we newly identified NFATc regulated downstream gene, Rho family GTPase 1(RND1). VEGF stimulated RND1 induction was attenuated in the presence of NFAT inhibitor, Cyclosporine A. In contrast, RND1 was markedly induced by the NFATc overexpression. siRNA-mediated knockdown of RND1 in endothelial cells impaired VEGF-mediated migration, capillary-like tube formation, and barrier function. Rho GTPase pull down assays and the immunohistochemical stainings against VE-cadherin or actin, indicated that NFATc mediated RND1 stimulation negatively regulated VEGF-RhoA signaling, followed by the disruption of actin stress fiber formation. Collectively, these findings suggest that calcineurin-NFATc activations are one of the predominant pathways in VEGF stimulated endothelial cells. NFATc-mediated RND1 plays critical roles for migration, tube formation and barrier formation in endothelial cells.

Erectile dysfunction occurs prior to systemic vascular disease due to incompetent penile endothelial cell-cell junctions

Ji-Kan Ryu, Hai-Rong Jin, Guo Nan Yin, Mi-Hye Kwon, Kang-Moon Song, Min Ji Choi, Woo Jean Kim, and Jun-Kyu Suh*. National Research Center for Sexual Medicine and Department of Urology, Inha University School of Medicine, Incheon 400-711, Republic of Korea

Erectile dysfunction (ED) is often regarded as a silent marker for serious systemic vascular diseases. Exact mechanism by which ED occurs prior to systemic vascular diseases remains to be elucidated. We, herein, performed a comprehensive study to answer this question. For histological evaluation, cavernous tissues from patients with ED were obtained by percutaneous needle biopsy. Either hypercholesterolemia or diabetes was induced by feeding a high-cholesterol diet or by intraperitoneal injection of streptozotocin, respectively, in 8-week-old C57BL/6J mice. The cavernous expression of endothelial cell-cell (EC-EC) junction proteins was evaluated by immunohistochemistry and by western blot analysis. We determined vascular endothelial permeability in the penis, heart, hindlimb, brain, and testis after injection of a variety of vascular space markers (350 Da to 2000 kDa) into the jugular vein. The cavernous expression of EC-EC junction proteins, including VE-cadherin, claudin-5, ZO-1, and PECAM-1, was significantly lower in hypercholesterolemic or diabetic mice than in controls and similar expression pattern was also observed in human cavernous tissue from normal or pathologic condition. These EC-EC junction proteins were more sparsely distributed in the endothelium of cavernous sinusoids than in the endothelium of coronary or femoral arteries. We observed a significant leakage of fluorescent tracer across the cavernous endothelium, whereas minimal leakage in the heart and the hindlimb or no leakage in the brain and the testis was noted in normal mice. Moreover, endothelium of cavernous sinusoids was much more permeable in hypercholesterolemic and diabetic condition than in normal condition, whereas endothelial layer from heart and hindlimb did not show a notable difference in permeability between the conditions. Intracavernous injection of recombinant angiopoietin-1 protein decreased cavernous endothelial permeability by restoring EC-EC junction proteins and induced recovery of erectile function in diabetic mice. This is the first report demonstrating that endothelium of cavernous sinusoid in normal and pathological conditions is incompetent in both structurally and functionally compared with the vasculature from other part of body, which give us an important clue to understanding why ED is highly prevalent and often precedes the occurrence of other systemic vascular diseases. Supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs (Jun-Kyu Suh, A110076), Republic of Korea.

Immunomodulatory functions of lymphangiogenic vessels

Melody A. Swartz[1]*, Amanda W. Lund[1], Sachiko Hirosue[1], Francesca Capotosti[1], Fernanda Duraes[2], Stéphanie Hugues[2]. [1]Institute of Bioengineering, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne Switzerland, [2]Department of Pathology and Immunology, University of Geneva Medical School, Centre Médical Universitaire (CMU), Geneva Switzerland

Tumor expression of the lymphangiogenic factor VEGF-C is correlated with metastasis and poor prognosis, and although VEGF-C enhances transport to the draining lymph node (dLN) and antigen exposure to the adaptive immune system, its role in tumor immunity remains unexplored. First, we demonstrate that VEGF-C-driven lymphangiogenesis promotes immunological tolerance in murine melanoma. In B16 F10 melanomas expressing a non-endogenous antigen (OVA), VEGF-C protected tumors against pre-existing anti-tumor immunity and promoted local deletion of OVA-specific CD8+ T cells. Naïve OVA-specific CD8+ T cells, transferred into tumor-bearing mice, became dysfunctional and apoptotic. Next, we show that lymphatic endothelial cells (LECs) in dLNs cross-presented OVA, and naïve LECs could scavenge and cross-present OVA in vitro. Cross-presenting LECs expressed the T cell inhibitory ligand PDL-1, and drove the proliferation and apoptosis of OVA-specific CD8+ T cells ex vivo. Finally, we show how LECs interact with both immature and mature dendritic cells to suppress their activation of T cells. Our findings introduce a tumor-promoting role for lymphatics in the tumor and dLN, and suggest that lymphangiogenic vessels may be a novel target for immunomodulation.

Angiogenic inhibitors induce mature/drug-resistant blood vessels in the fibrous cap (the vasculature outside of tumor parenchyma)

Takara Kazuhiro*, Naito Hisamichi, Wakabayashi Taku, Kidoya Hiroyasu, Takakura Nobuyuki. Department of signal transduction, RIMD, Osaka University, Japan

It was previously reported that most blood vessels in the tumor microenvironment are immature because mural cell (MC) coverage to endothelial cells (ECs) is broadly lacking. Hyperpermeability of the tumor vasculature finally induces interstitial hypertension that relieves against penetration of anti-cancer drugs into the depths of the tumor. Therefore new concept has emerged that normalization of tumor blood vessels by anti-angiogenesis therapy results in restoration of normal permeability and improves drug delivery. However recent reports suggest that cancer cell invasion is induced from the edge of the tumor into peripheral areas after angiogenesis inhibitor treatment. Therefore, it is important to analyze the status of blood vessels in the fibrous cap at the tumor rim after anti-angiogenesis therapy. In the present study, we found that mature blood vessels in which ECs are covered with MCs are present in the fibrous cap. Immature blood vessels were destroyed after treatment with angiogenesis inhibitors, but maturing blood vessels remained visible. Furthermore, we evaluated whether the increased numbers of blood vessels covered by MCs in the fibrous cap resulted in any functional changes. Although the fibrous cap is not composed of tumor tissue, the permeability of the blood vessels in it was significantly increased. This result indicates that the vasculature in the fibrous cap is influenced by VEGF secreted from the tumor cells and is functionally abnormal. Therefore, administration of angiogenesis inhibitor not only normalizes blood vessels in the tumor parenchyma, but also in the fibrous cap. Maturing blood vessels showed a less dilated character after treatment. It is widely accepted that well-matured blood vessels are sheathed with extracellular matrix (ECM) and that cancer cells migrate along tracks made of ECM collagen fibers. Therefore, our date suggest that destroying the maturing blood vessels outside of tumor parenchyma is important to prevent cancer cell invasion. We would like to show the trajectory of cancer cell invasion from the edge of tumor into peripheral area to consider and provide a new paradigm to inhibit invasion of cancer cells.

Impaired macrophage resolution at inflammatory sites in mice lacking leukocyte ADAM17

Jingjing Tang*, Carole L. Wilson, Ivan G. Gomez, and Elaine W. Raines. University of Washington, Department of Pathology, Seattle, WA. USA

Resolution of inflammation is essential for maintenance of tissue homeostasis. Tissue level resolution is spontaneously initiated to promote reduction of inflammatory cell numbers, but its failure can lead to excessive and chronic inflammation. Thus, there is a need to better define molecular mechanisms regulating the resolution process. One mechanism that could promote resolution is specific proteolysis of macrophage cell surface proteins, which can immediately alter macrophage responsiveness and adhesion. Using spontaneous resolution of inflammation in the peritoneal cavity, we have tested the role of a major transmembrane protease, ADAM17, which has been shown to proteolytically shed many macrophage inflammatory proteins. Hematopoietic chimeras lacking leukocyte ADAM17 have normal levels of all circulating cells, but their number of resident peritoneal macrophages are two-fold elevated relative to hematopoietic chimeras wildtype (WT) for ADAM17. Even in mice reconstituted with a combination of WT and ADAM17 null leukocytes, the number of ADAM17 null resident peritoneal macrophages is increased two-fold indicating a cell-intrinsic mechanism. However, ADAM17 null resident macrophages leave the peritoneal cavity with normal kinetics following stimulation with the sterile irritant thioglycollate, and recruited ADAM17 null inflammatory macrophages subsequently stimulated with lipopolysacharide also rapidly disappear from the peritoneum. Thus, stimulus-induced loss of ADAM17 null macrophages is comparable to WT macrophages. In contrast, ADAM17 null macrophages persist in the peritoneal cavity relative to WT in the spontaneous resolution of inflammation following peritoneal injection of thioglycollate, a property that is also cell-intrinsic. By 9 days after thioglycollate, a time point where macrophage number has returned to that of resident peritoneal cells, ADAM17-null macrophages remain two-fold elevated relative to WT macrophages. The ADAM17-null macrophages also show higher surface levels by flow cytometry of three adhesion molecules: L-selectin, CD11b and CD18. On-going experiments are evaluating the potential roles of these candidate ADAM17 substrates in the impaired resolution of macrophages lacking ADAM17. In summary, our data suggest that ADAM17-mediated proteolysis does not impact stimulus-induced macrophage exiting, but does impair spontaneous macrophage resolution, potentially mediated through elevated surface levels of candidate substrates. This work was supported by grants from the National Institutes of Health HL018645 and HL067267 to EWR.

Platelet flow-induced protrusion (FLIPR) formation is a shear and calpain induced process leading to platelet disintegration and the formation of procoagulant microparticles

C Tersteeg[1,2]*, HF Heijnen[1], G Pasterkamp[2], CE Floris[1], RT Urbanus[1], C Maas[1], I. Hoefer[2], EM Mak-Nienhuis[1,2], V De Angelis[1], PhG de Groot[1], M Roest[1]. [1]Department of Clinical Chemistry and Hematology, UMC Utrecht, the Netherlands, [2]Department of Experimental Cardiology, UMC Utrecht, The Netherlands

Activated platelets are a major source of pro-coagulant and pro-inflammatory microparticles. Despite their importance in circulation, the formation of microparticles after platelet activation was only visualized under static conditions up till now. We studied the formation of microparticles under physiological conditions of flow in whole blood or platelet rich plasma from healthy donors. Perfusion chambers with stenosis-like obstructions were used to disturb the laminar flow profiles. Long membrane strands of up to 250 micrometer were observed downstream of the stenosis, and these strands originate from spread platelets (further named „flow-induced protrusions“ (FLIPR)). FLIPR formation was observed predominantly from disintegrating platelets that were spread on physiological surfaces including fibrinogen, fibronectin, vWF, collagen peptides and collagen. After 30 min of flow FLIPRs were formed from 3.1 +/- 1.1% of the platelets spread in areas with non-linear flow profiles. Similarly, aggregates formed on collagen at high shear show FLIPR formation directly downstream the aggregates, within 15 minutes of perfusion. Platelets that formed FLIPRs expressed anionic phospholipids, determined by Annexin-V and prothrombin binding. FLIPRs further disintegrate into procoagulant microparticles. Isolation of these disintegrated FLIPRs showed that they were able to accelerate thrombin generation in a thrombin generation assay. Destabilizing microtubules with nocodazole increased the FLIPR formation to 30.5 +/- 4.2 %, while inhibiting actin polymerization using cytochalasin D showed no effect. FLIPR formation is depending on the presence of extracellular calcium and was completely blocked with the calpain inhibitor calpeptin. Our study introduces a novel mechanism of platelet microparticle formation from protruding platelets in a calpain and shear dependent process. Platelet disruption will lead to FLIPR formation and the formation of procoagulant microparticles. This research was supported by the Center for Translational Molecular Medicine and the Netherlands Heart Foundation (CIRCULATING CELLS).

Redundant functions of phospholipases D1 and D2 in platelet granule release

Ina Thielmann[1]*, David Stegner[1], Peter Kraft[2], Ina Hagedorn[1], Christoph Kleinschnitz[2], Guido Stoll[2] & Bernhard Nieswandt[1]. [1]University of Wuerzburg, Chair of Vascular Medicine, University Hospital and Rudolf Virchow Centre, DFG Research Centre for Experimental Biomedicine and the [2]Department of Neurology, University of Wuerzburg, Wuerzburg, Germany

Background – Platelet activation and thrombus formation are crucial for primary hemostasis but can also result in pathological thrombus formation. Agonist-induced platelet activation involves different signaling pathways leading to the activation of phospholipases (PL). While the role of PLCs is well established, less is known about the relevance of PLDs. Phospholipase D (PLD) is a phosphodiesterase which hydrolyzes phosphatidylcholine to phosphatidic acid and choline. Both PLD isoforms PLD1 and PLD2 are expressed in platelets. Recently, we demonstrated a role for PLD1 in GPIb-dependent aggregate formation, while the role of PLD2 remained elusive. Methods & Results –To investigate the functional relevance of PLD2 and to address a possible functional redundancy of the two PLD isoforms in platelet signaling and thrombus formation, Pld2-/- and Pld1-/-/Pld2-/- mice were generated and analyzed in parallel to Pld1-/- mice. Adhesion, activation and aggregation of Pld-deficient platelets were analyzed by using in vitro and in vivo assays. The absence of Pld2 resulted in reduced Pld activity in platelets but it had no detectable effect on the function of the cells in vitro and in vivo. The combined deficiency of both Pld isoforms lead to a defect in ï¡-granule release which resulted in protection in a model of ferric chloride induced arteriolar thrombosis, which was not observed in mice lacking only one Pld isoform. Still, the Pld1-/-/Pld2-/- mice displayed normal tail bleeding times. Thus, interfering with Pld activity might be a promising and safe strategy for antithrombotic therapy. Conclusion - These results reveal redundant roles of Pld1 and Pld2 in -granule secretion and experimental occlusive thrombus formation.

Fibrosis and matrix remodelling in foamy mouse macrophages

Anita C. Thomas[1]*, Wouter J. Eijgelaar[2], Mat J.A.P. Daemen[2] and Andrew C. Newby[1].
[1]Bristol Heart Institute, University of Bristol, Bristol, United Kingdom and, [2]CARIM, Maastricht, The Netherlands

Foam cell macrophage (FCM) formation is an early event in atherosclerosis that precedes fibrous cap development and subsequent cap rupture and thrombus formation; FCM have been implicated in all events. To understand this further we compared FCM and non-foamy macrophages (NFM) produced in fat-fed ApoE null (FCM) or C57Bl6 mice fed a normal chow diet (NFM). Sponges were implanted into fat-fed ApoE null or C57Bl6 mice fed a normal diet to produce FCM or NFM, respectively (n=4 each), and cells harvested from the sponges 4 weeks later. Cells were purified on their buoyant density and/or differential adherence. RNA samples of high quality were compared using Illumina bead chips. Differential expression was classified as significant ($P<0.05$ after Bonferoni correction for multiple testing) or suggestive ($P<0.05$ unadjusted). Gene expression was examined using Ingenuity Pathway analysis, GO annotation and clustering (DAVID Bioinformatics Resources). Data was confirmed using q-PCR (n=5-7) and immunohistochemistry. The functions found to be enriched/upregulated in FCM included cell death, development, regulation, differentiation and proliferation; metabolism, biosynthesis, endocytosis, transport and localisation; immune response, cell signalling; connective tissue development, function and disorders; and haematological system development, function and disorders. The primary canonical pathway upregulated in the FCM was liver X receptor/retinoic acid receptor (LXR/RXR) activation. Hepatic fibrosis/hepatic stellate cell activation and LPS/IL-1 mediated inhibition of RXR function pathways were also highly upregulated. Compared with NFM, FCM had more mRNA for many matrix proteases (including cathepsins and matrix metalloproteinases (MMPs)), without a corresponding difference in their inhibitors (cystatins and TIMPs). There was also an increase in mRNA for many of the proteins of the extracellular matrix (including collagens 1, 4, 6, 8, and glycosaminoglycans (biglycan, decorin). The FCM also had significantly more mRNA for the LXR-related genes (including LXR's obligate partner RXR, and target genes). Other genes associated with fibrosis that were upregulated in the FCM were connective tissue growth factor (CTGF), bone marrow protein-1 and Fos/FosB/Jun/JunB. Using immunohistochemistry, we established that many cells within the sponges contained CTGF; some had nuclear LXRï¡ or cFOS immunostaining. A few cells also had LXRï¡ or cFOS present in their cytoplasm. Our finding of changes in fibrosis and matrix degradation and deposition is in agreement with our previous studies and other literature. The interaction of these pathways, particularly in lipid-loaded cells, may account for the ambiguous behaviour of FCM in matrix synthesis (plaque growth) and degradation (plaque rupture), and deserves further investigation.

DNA-dependent protein kinase (DNA-PK) modulates proliferation of vascular smooth muscle cells through the orphan nuclear receptor NOR1

Veronika Thonn[1]*, Senad Medunjanin[1], Frank Burgbacher[2], Ruth H. Strasser[2], Dennis Bruemmer[3], Alexander Schmeisser[1], Ruediger C. Braun-Dullaeus. [1]Internal Medicine, Department of Cardiology, Pneumology and Angiology, Magdeburg University, Magdeburg, Germany [2]Internal Medicine, Department of Cardiology, Dresden University of Technology, Dresden, Germany, [3]Division of Endocrinology and Molecular Medicine, University of Kentucky College of Medicine, Lexington, Kentucky USA

Background: The NR4A subfamily member NOR1 plays a crucial role in vascular smooth muscle cell (VSMC) proliferation and neointima formation. Here, we hypothesize that DNA-dependent protein kinase (DNA-PK) modulates the signaling of NOR1 and hence participates in cell cycle progression. Results: Human aortic VSMC were maintained in culture and treated with the DNA-PK-specific inhibitor NU7026. FCS-induced cell cycle progression of VSMC was reduced by 70 % through NU7026 treatment ($P < 0.05$) as measured by BrdU incorporation experiments. Additionally, NU7026 treatment resulted in the inhibition of the FCS-stimulated upregulation of NOR1, of the cell cycle promoting protein PCNA (proliferating cell nuclear antigen) as well as the inhibition of cyclin D1 and the hyperphosphorylation of the retinoblastoma protein (RB). The same results were obtained when DNA-PK had been downregulated by siRNA. In addition, downregulation of DNA-PK by siRNA induced a reduction of the luciferase signal when reporter constructs of the NOR-1 binding site (NBRE) had been overexpressed in VSMC. Through immuno-fluorescence and co-immuno-precipitation studies we were able to demonstrate that DNA-PK forms a complex with NOR1 in the lysates of VSMC. To investigate the phosphorylation of NOR1 by DNA-PK, mutanional analysis and kinase assays were performed. We showed that NOR1 is a substrate of DNA-PK and is phosphorylated at the N-terminus. Both, the DNA-PK subunits and NOR1 are expressed in human atherosclerotic plaque specimens, predominantly in the neointimal VSMC as demonstrated by immuno-histochemical experiments. Conclusion: VSMC proliferation is modulated through the direct phosphorylation of NOR1 by DNA-PK. To understand cellular responses to vascular injury the identification of new key molecules involved in VSMC function will be crucial and may provide future targets for therapeutic interventions.

The Role of IKKalpha in atherosclerosis

P. Tilstam*, H. Noels, M. de Winther, C. Weber. IMCAR, Aachen, Germany

The transcription factor NF-kappaB deserves particular attention in cardiovascular research, as it is known to play a central role in inflammatory reactions associated with atherogenesis. Two main pathways activate NF-kappaB: while inflammatory signals induce „canonical“ NF-kappaB activation through the IKKβ/IKKgamma kinase complex, „alternative“ NF-kappaB activation, triggered by e.g. RANKL and CD40L, depends on the IKKalpha kinase. In addition, IKKalpha terminates canonical NF-kappaB activation in macrophages and thereby contributes to the resolution of inflammation. Despite this function of IKKalpha as a regulator of both canonical and alternative NF-kappaB activity, the contribution of IKKalpha to atherogenesis remains unexplored. Therefore, the aim of this project is to identify the role of IKKalpha activation in hematopoietic versus resident vascular cells in atherosclerosis. In order to study the function of IKKalpha in hematopoietic cells, ApoE-/- mice were transplanted with ApoE-/- bone marrow carrying a mutated, inactivation-resistant IKKalpha gene (IKKalpha AA/AA ApoE-/-), or with ApoE-/- bone marrow as control. The mice received a high-fat diet for either 8 or 13 weeks. The atherosclerotic progression was analyzed in the aortic root and the aorta. The hematopoietic profile was studied by flow cytometric analysis of leukocytes isolated from bone marrow, blood, spleen and lymph nodes. After 8 and 13 weeks of high-fat diet the lesion analysis of aortic root and aorta did not show any significant differences between IKKalpha AA/AA ApoE-/- bone marrow-transplanted mice and controls. However, the hematopoietic profile showed reduced levels of B-cells in bone marrow and blood of IKKalpha AA/AA ApoE-/- bone marrow-transplanted mice, corresponding with a known role of IKKalpha in B-cell development. Interestingly, relative numbers of regulatory T-cells (Tregs) in the total T-cell population were decreased. This indicates an important role for IKKalpha in Treg generation, and requires further attention. In future experiments, functional in vitro studies on primary IKKalpha AA/AA ApoE-/- macrophages will be performed. Furthermore, we will study atherogenesis in IKKalpha AA/AA ApoE-/- mice to explore the importance of IKKalpha activation in resident vascular cells in the context of atherosclerosis.

The guanine-nucleotide exchange factor Trio regulates endothelial adherens junction formation through the activation of the small GTPase Rac1

Ilse Timmerman[1]*, Jos van Rijssel[1], Dirk Geerts[2], Peter L. Hordijk[1] and Jaap D. van Buul[1].
[1]Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands, [2]Department of Pediatric Oncology/Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands

Adherens junctions serve to maintain a restrictive endothelial barrier, but are dynamically regulated during inflammation and angiogenesis. The assembly of adherens junctions can be affected by activities of Rho GTPases. Conversely, cadherins can signal in an outside-in fashion to Rho GTPases, in particular Rac1, thereby relaying information from adhesion to the organization of the actin cytoskeleton. It is still unclear which GEF functions in endothelial cells to locally activate Rac1 during formation of VE-cadherin-mediated cell-cell adhesion. We show that the Rho-GEF Trio localizes at endothelial cell-cell junctions, where it interacts with the VE-cadherin complex. Trio silencing by shRNA results in more punctate junctions, accompanied by a decrease in the resistance of the endothelial monolayer. This decrease in endothelial monolayer resistance upon Trio depletion is rescued by expression of the N-terminal GEF-D1 domain of Trio, but not by the C-terminal GEF-D2 domain, indicating that Trio-induced Rac1 or RhoG activation is involved. Expression of the N-terminal Trio domain results in a morphological conversion from punctate to more linear junctions, similar to the effect seen by expression of a constitutively active mutant of Rac1 but not RhoG. In addition, Trio localizes primarily at newly forming contacts, suggesting that Trio, like Rac1, is involved in the initiation of novel cell-cell contacts. Finally, using beads coated with the ectodomain of VE-cadherin to mimic nascent junction formation, we demonstrated that VE-cadherin-dependent cell-cell adhesion activates Rac1, but not RhoG. Interestingly, Trio silencing blocked the increase in Rac1 GTPase activity observed after VE-cadherin-dependent contact formation. Collectively, we show that Trio is important for VE-cadherin-dependent Rac1 activation upon cell-cell contact formation. Our data suggest that Trio-mediated signals that are induced upon novel cell-cell contact formation promote VE-cadherin-dependent endothelial cell-cell adhesion and hence junction stabilization.

Role of aPKC in endothelial cells and vascular integrity

Filip Claes, Wouter Vandevelde, Sabine Harenkamp, Christin Lössl, Jose Arencibia, Erhard Seifried, Reinhard Henschler, Ricardo Biondi, Lieve Moons, Marc Tjwa*. Lab of Vascular Hematology / Angiogenesis, Institute for Transfusion Medicine, Goethe University Frankfurt, Germany

Atypical Protein Kinase C (aPKC) is a subfamily of the PKC family, which regulates signal transduction and is implicated as promotor of various diseases including cancer and chronic inflammation but its role in endothelial cells and angiogenesis remains unknown. We found that cultured endothelial cells expressed the aPKC member PKCiota but not PKCzeta. Upon VEGF stimulation, cultured endothelial cells showed increased phosphorylation of aPKC, suggesting cell-intrinsic angiogenic properties of aPKC. In 3D sprouting assays, inhibition of aPKC by the clinically available compound aurothiomalate (ATM) dose-dependently impaired angiogenic sprouting and, importantly, reduced VEGF-stimulated sprouting to levels of sprouting, found in non-VEGF-stimulated conditions. When PKCiota was genetically knocked-down in zebrafish by morpholinos, the main axial vessels formed normally, but dose-dependently induced vessel collapse and blood flow defects. These data suggest that aPKC plays an important role in vessel integrity by regulating VEGF signalling. As miR-126 promotes vessel integrity by regulating VEGF signalling, we studied a potential interaction between aPKC and miR-126. Inhibition of aPKC by ATM dose-dependently reduced the expression of miR-126 in cultured endothelial cells. To test their genetic interaction further, we injected zebrafish with miR-126 morpholinos at suboptimal dose, and treated these mutants with a suboptimal dose of ATM. While miR-126 knock-down or ATM at suboptimal only caused vessel collapse in a minority of fish, the combination of both induced a highly penetrant phenotype of vessel collapse and blood flow defects. These genetic interactions were further confirmed by the use of PKCiota morpholinos, other commercially aPKC inhibitors and newly developed aPKC inhibitors, with greater selectivity and efficacy for aPKC inhibition. Together, these data suggest a novel cell-intrinsic role for aPKC in endothelial cells, which involves microRNA-mediated regulation of VEGF signalling, and which is required for maintaining vessel integrity. These findings might have implications for the use of VEGF inhibitors in cancer and beyond.

The 8Cys subgroup of tetraspanins regulate ADAM10 expression in platelets, endothelial cells and erythrocytes

Elizabeth J. Haining[1], Jing Yang[1,2], Kabir Khan[1], Richard Collier[1], Schickwann Tsai, Steve P. Watson[3], Jon Frampton[4], Paloma Garcia[4], Michael G. Tomlinson[1]*. 1School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Birmingham, UK, 2Division of Hematology, Department of Medicine, University of Utah, Salt Lake City, USA, 3School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK, 4School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

A Disintegrin and Metalloproteinase 10 (ADAM10) is a ubiquitous transmembrane metalloprotease that cleaves the extracellular regions from over 40 different transmembrane target proteins. ADAM10 is essential for embryonic and vascular development through its cleavage and activation of Notch, which is critical for cell fate decisions during development. ADAM10 also impacts arterial thrombosis, atherosclerosis and angiogenesis. This is achieved through its cleavage of targets such as the platelet collagen receptor GPVI, vascular endothelial growth factor receptor 2 (VEGFR2), the junctional adhesion molecule VE-cadherin, and transmembrane chemokines CX3CL1 and CXCL16. Despite its importance in health and disease, ADAM10 regulation remains poorly understood. ADAM10 is compartmentalised into membrane microdomains formed by tetraspanins, which are a superfamily of 33 transmembrane proteins in humans that regulate clustering and trafficking of certain other transmembrane 'partner' proteins. This is achieved by specific tetraspanin-partner interactions. However, it is not clear which tetraspanins specifically interact with ADAM10. The aims of this study were to identify which tetraspanins interact with ADAM10 and how they regulate this metalloprotease. Co-immunoprecipitation identified specific ADAM10 interactions with Tspan14, Tspan15 and Tspan33/Penumbra. These are members of the largely unstudied 8Cys subgroup of tetraspanins, all six of which promoted ADAM10 maturation. Different cell types expressed distinct repertoires of 8Cys tetraspanins. Megakaryocytes and human umbilical vein endothelial cells (HUVECs) expressed relatively high levels of Tspan14, while erythrocytes expressed high levels of Tspan33/Penumbra. Consistent with these expression data, ADAM10 surface expression was substantially reduced on Tspan14-knockdown HUVECs and Tspan33/Penumbra-deficient mouse erythrocytes, but normal on platelets from these mice. These results define 8Cys tetraspanins as essential regulators of ADAM10 maturation and trafficking to the cell surface. This finding has therapeutic implications, since focusing on specific 8Cys-ADAM10 complexes may allow cell-type and/or substrate-specific ADAM10 targeting.

VEGF164 over-expression in skeletal muscle induces vascular enlargement and intussusceptive angiogenesis through reciprocal activation of Notch-1 in contiguous endothelial cells

Marianna Trani[1]*, Roberto Gianni-Barrera[1], Silvia Reginato[1], Ruslan Hlushchuk[2], Michael Heberer1, Valentin Djonov[2] and Andrea Banfi[1]. [1]Cell and Gene Therapy, Departments of Biomedicine and of Surgery, Basel University Hospital, Switzerland, [2]Institute of Anatomy, University of Bern, Switzerland

VEGF is the fundamental regulator of angiogenesis both in development and in adult tissues. We have previously found that over-expression of murine VEGF164 can induce either normal or aberrant angiogenesis depending strictly on its dose in the microenvironment around each producing cell in vivo. The best understood mode of angiogenesis is sprouting. The proper formation of sprouts relies on the generation of an alternate pattern of Dll4 expression and Notch1 activation in contiguous endothelial cells, leading to a balanced formation of tip and stalk cells. Here we sought to determine how the switch between normal and aberrant angiogenesis takes place and, in particular, how the Dll4/Notch1 pathway is regulated during the initial stages of normal and aberrant angiogenesis induced by different VEGF doses. Primary myoblasts were retrovirally transduced to express mouse VEGF164 and individual clones were isolated. Clones expressing low (60 ng/106 cells/day) or high VEGF levels (120 ng/106 cells/day), which induce normal and aberrant angiogenesis, respectively, were implanted in skeletal muscle of SCID mice. Four days after implantation, both VEGF doses caused a similar initial response, namely homogeneous enlargement of pre-existing micro-vessels without any evidence of filopodia-bearing tip cells. By 7 days, enlarged vessels remodeled into either normal pericyte-covered capillaries (with low VEGF) or aberrant angioma-like structures (with high VEGF). Since no evidence of sprouting could be found, we asked whether vascular growth was taking place by intussusception. Scanning electron microscopy analysis of vascular corrosion casts revealed that, by 4 days, the enlarged vessels induced by both VEGF doses clearly displayed the formation of intravascular pillars, the tell-tale sign of intussusception, or splitting angiogenesis. Remarkably, we could never detect an alternate pattern of Dll4 expression and Notch1 activation in the growing endothelial structures. With both VEGF doses, Dll4 was expressed on long stretches of several contiguous endothelial cells, in which Notch1 was contemporaneously activated. After 7 days, neither the normal nor the aberrant vascular structures that were generated showed any Dll4 expression or Notch1 activation. Pharmacological inhibition of the Notch pathway by DAPT disrupted the initial vascular enlargements and led to disorganized aggregates of endothelial cells. To better understand the role of Dll4/Notch1 axis in intussusceptive angiogenesis, we took advantage of a recently developed computational model (Bentley, K. 2008). Three different possible scenarios have been found to predict either patches of synchronicity or oscillation of Dll4 expression and Notch1 activation in consequence of different VEGF doses. In vivo experiments to test the model predictions are currently ongoing. In summary, these data indicate that: 1) both normal and aberrant angiogenesis induced by over-expression of different VEGF doses in non-ischemic skeletal muscle arise from a similar initial stage of vessel enlargement, followed by intussusceptive remodeling, rather than sprouting; 2) the initial vascular enlargement depends on the simultaneous activation of Notch1 on contiguous endothelial cells, leading to an all-stalk phenotype, rather than an alternate pattern of tip and stalk cells; 3) the remodeling to either normal or aberrant vascular structures by intussusception is not regulated by differential Notch1 activation.

Inhibition of Notch signaling by Dll4-Fc promotes reperfusion of acutely ischemic tissues

Trindade, Alexandre[1,2]*; Liu, Ren[3]; Sun, Zhanfeng[4]; kumar, Ram[5]; Weaver, Fred[4]; Krasnoperov, Valery[6]; Kranthi, Naga[6]; Gill, Parkash S.[3]; Duarte, António[1,2]. [1]Centro de Investigação Interdisciplinar em Sanidade Animal, Faculty of Veterinary Medicine, Technical University of Lisbon, Portugal, [2]Instituto Gulbenkian Ciência, Oeiras, Portugal, [3]Department of Pathology, University of Southern California, Los Angeles, California, United States of America, [4]Department of Vascular Surgery, 2nd Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China, [5]Department of Surgery, University of Southern California, Los Angeles, California, USA, [6]Vasgene Therapeutics, Los Angeles, California, USA

Notch pathway regulates vessel development and maturation. Dll4, a high-affinity ligand for Notch, is expressed predominantly in the arterial endothelium and is induced by hypoxia among other factors. Inhibition of Dll4 has paradoxical effects of reducing the maturation and perfusion in newly forming vessels while increasing the density of vessels. We hypothesized that partial and/or intermittent inhibition of Dll4 may lead to increased vascular response and still allow vascular maturation to occur. Thus tissue perfusion can be restored rapidly, allowing quicker recovery from ischemia or tissue injury. Our studies in two different models (hindlimb ischemia and skin flap) show that inhibition of Dll4 at low dose allows faster recovery from vascular and tissue injury. This opens a new possibility for Dll4 blockade's therapeutic application in promoting recovery from vascular injury and restoring blood supply to ischemic tissues.

Monocyte ADAM17 regulates transendothelial migration by promoting diapedesis

Yoshiaki Tsubota[1]*, Jeremy M. Frey[1], Phillip W.L. Tai[2], Robert E. Welikson[2], and Elaine W. Raines[1]. University of Washington, Departments of [1]Pathology and [2]Biochemistry, Seattle, WA, USA

Inflammation is a key process in disease pathogenesis, and leukocyte recruitment and transendothelial migration into inflamed tissues control both initiation and progression of acute and chronic inflammatory diseases, including atherosclerosis. Despite expanded definition of the leukocyte adhesion cascade and mechanisms underlying individual steps, very little is known about regulatory mechanisms controlling sequential shifts from one step to the next. We have tested the hypothesis that metalloproteinases provide mechanisms to rapidly transition monocytes between different steps. Our study demonstrates that metalloproteinase activity promotes human monocyte diapedesis on unactivated and inflamed human endothelium under static conditions and with physiological flow. Using time-lapse video microscopy and a broad spectrum inhibitor that blocks metalloproteinases expressed on both monocytes and endothelial cells, neither adhesion of monocytes nor their locomotion over the endothelial surface is altered. However, it takes monocytes approximately twice as long to complete the diapedesis step with metalloproteinase blockade. Thus, metalloproteinase activity is rate limiting, and is required for optimal diapedesis of monocytes across endothelial monolayers. To define the enzymes involved in this regulatory mechanism, we focused on two major metalloproteinases expressed on both monocytes and endothelial cells, ADAM10 and ADAM17, which have been reported to cleave and shed ectodomains of adhesion molecules required for transendothelial migration. Each of these ADAMs was eliminated from endothelial cells or primary monocytes using siRNAs. No significant change in diapedesis is observed with endothelial-targeted knockdown of ADAM10 or ADAM17 despite significant increases in barrier function with knockdown of ADAM10. In contrast, depletion of ADAM17, but not ADAM10, from primary monocytes delays the diapedesis step to a similar extent as that observed with metalloproteinase inhibition. Together, our data establishes that monocyte ADAM17, but not endothelial and monocyte ADAM10 or endothelial ADAM17, is a significant contributor to the metalloproteinase-mediated promotion of the diapedesis step. Thus, metalloproteinase activity, specifically monocyte ADAM17, facilitates the rapid and coordinated completion of transendothelial migration.

Deciphering the role of GPR97 - an orphan adhesion G protein coupled receptor highly expressed in the lymphatic endothelium

Valtcheva N.[1]*, Jurisic G.[1,2], Primorac A.[1] and Detmar M.[1]. [1]Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli Strasse 10, 8093 Zurich, Switzerland, [2]Developmental & Molecular Pathways, Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland

Our study aimed at the identification and molecular characterisation of lymphatic-specific G protein coupled receptors (GPCRs) to assess new targets for pharmacological manipulation of the lymphatic vascular system. We used a Taqman® Low Density Array to determine the GPCR expression profiles of ex vivo isolated intestinal mouse lymphatic and blood vascular endothelial cells in a 384-well format. We found striking differences between lymphatic and blood vascular endothelial cells regarding the expression patterns of the novel and not well-characterised subclass of adhesion GPCRs which are proposed to play a role in sensing flow and/or extracellular matrix composition. For further analysis we focused on GPR97 - an orphan adhesion GPCR of unknown signalling and function that was specifically expressed in mouse lymphatic endothelium. Primary human lymphatic endothelial cells were studied in common functional assays including adhesion, wound healing and transwell migration assay after transient siRNA knock down of GPR97. Our results indicate that GPR97 modulates lymphatic endothelial cell migration and adhesion via cytoskeletal rearrangements. The biological role of GPR97 in the lymphatic vasculature in vivo is currently being investigated in a GPR97 knock out mouse model.

Transglutaminase determines atherosclerotic plaque composition at locations exposed to oscillatory shear stress

Hanke L. Matlung[1], Annette E. Neele[1], Harald C. Groen[2], Kim van Gaalen[2], Bilge Guvenc[1], Angela van Weert[1], Jolanda J. Wentzel[2], Jaap D. van Buul[3], Ed vanBavel[1]* and Erik N.T.P Bakker[1]. [1]Dept of Biomedical Engineering, Academic Medical Center, Amsterdam. [2]Dept of Biomedical Engineering, Erasmus Medical Center, Rotterdam, [3]Sanquin, Amsterdam, The Netherlands

Aims: Atherosclerosis preferentially develops at sites of disturbed blood flow. We tested the hypothesis that transglutaminase activity plays a role in plaque development at these locations. Methods and results: Cultured endothelial cells were exposed to either oscillatory flow or steady flow for 24 h. Transglutaminase activity was significantly higher in cells exposed to oscillatory flow. Under oscillatory flow, inhibition of transglutaminase decreased the expression of MCP-1, but not of ICAM-1, VCAM-1 or E-selectin. In vivo, oscillatory flow was induced by placement of a tapered perivascular cast around the carotid artery of type 2 transglutaminase (TG2) knockout mice and WT counterparts. After 2 days, the number of monocytes adhering to the endothelium was significantly lower in TG2 knockout mice. ApoE knockout mice that were equipped with the flow-modifying cast showed lesions proximal to the cast (low shear stress), and distal to the cast (oscillatory shear stress). Inhibition of transglutaminase activity in this model did not affect the lesion proximal to the cast. However, the lesion that developed distal to the cast showed a marked reduction in macrophage and fat content. In addition, lesion size was increased in this area, which was attributed to an increase in smooth muscle content. Conclusions: Oscillatory shear stress increases endothelial transglutaminase activity. In turn, transglutaminase activity affects the expression of MCP-1 in vitro and monocyte recruitment in vivo. In a mouse model of atherosclerosis, transglutaminase activity has a major effect on plaque composition under conditions of oscillatory shear stress. supported by the Netherlands Heart Foundation and the FP7 Marie Curie network SMART

Proteomic screen identifies IGFBP7 as a novel component of endothelial cell-specific Weibel-Palade bodies

Dorothee van Breevoort[1]*, Ellen L. van Agtmaal[1], Bieuwke S. Dragt[1], Jacqueline Klein[1], Gebbinck[1], Ilze Dienava-Verdoold[1], Anton J.G. Horrevoets[2], Karine M. Valentijn[3], Jeroen C. Eikenboom[4], Mar Fernandez-Borja, Alexander B. Meijer[1,5] and Jan Voorberg[1,5]. [1]Department of Plasma Proteins, Sanquin-AMC Landsteiner Laboratory, Amsterdam, The Netherlands, [2]Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam, The Netherlands, [3]Department of Molecular Cell Biology, Section Electron Microscopy, Leiden University Medical Center, Leiden, The Netherlands, [4]Department of Thrombosis and Hemostasis, Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands. Department of Molecular Cell Biology, Sanquin-AMC Landsteiner Laboratory, Amsterdam, The Netherlands, [5]Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Vascular endothelial cells contain unique storage organelles, designated Weibel-Palade bodies (WPBs) that deliver inflammatory and hemostatic mediators to the vascular lumen in response to agonists like thrombin and vasopressin. The main component of these cigar-shaped WPBs is von Willebrand factor (VWF), a multimeric glycoprotein crucial for platelet plug formation. In addition to VWF, several other components are known to be stored in WPBs which are involved in vascular homeostasis, like osteoprotegerin, monocyte chemoattractant protein-1 and angiopoetin-2 (Ang-2). To gain more insight in the physiological role of WPBs and to identify novel WPB cargo proteins we used an unbiased proteomics approach. We performed mass spectrometry analysis of WPB-enriched endothelial subcellular fractions. This analysis revealed the presence of several known WPB components such as VWF, Ang-2 and P-selectin. Thirty-five novel candidate WPB residents were identified which included insulin-like growth factor binding protein-7 (IGFBP7), which has been proposed to regulate angiogenesis. Immunocytochemistry revealed that IGFBP7 is a bona fide WPB component. Co-transfection studies showed that IGFBP7 trafficked to pseudo-WPB in HEK293 cells. Using a series of deletion variants of VWF we showed that targeting of IGFBP7 to pseudo-WPBs was dependent on the carboxy-terminal D4-C1-C2-C3-CK domains of VWF. IGFBP7 remained attached to ultralarge VWF strings released upon exocytosis of WPBs under flow. The presence of IGFBP7 in WPBs highlights the role of this subcellular compartment in regulation of angiogenesis and maintaining vascular homeostatis.

Endothelial outgrowth cells derived from human blood samples; isolation and in vitro performance

Nynke M.S. van den Akker[1,2,3]*, Fabienne Jeukens[1,2], Mick Gagliardi[1,2], Mark J. Post[2], Daniel G.M. Molin[2], and Johannes Waltenberger[1,3]. [1]Departments of Cardiology and [2]Physiology, CARIM, Maastricht University, Maastricht, the Netherlands and, [3]Department of Cardiology and Angiology, Universitätsklinikum Muenster, Westfälische Wilhelmsuniversität Muenster, Germany

The use of synthetic vascular grafts for replacement of small diameter vessels is complicated by high thrombogenic and immunogenic risks. Endothelial coverage is considered an essential design parameter for tissue engineered vascular grafts. Different subpopulations of cells derived from the mononuclear fraction of peripheral blood have been proposed as a cellular source for autologous endothelial cells. Both early and late outgrowth endothelial progenitor cells from peripheral blood have been identified in the past decade, the latter also referred to as endothelial outgrowth cells (EOCs). Here, mononuclear cells were isolated from venous (healthy individuals) or arterial (coronary artery disease-patients undergoing percutaneous coronary intervention) blood using gradient centrifugation. We used the Monocyte Isolation Kit II (Miltenyi) to isolate non-monocytes. Isolated cells were seeded on fibronectin-coated flasks and cultured in endothelial cell-defined culture medium. mRNA and protein expression analysis for endothelial (CD31, CD34, VEGFR2, VE-cadherin), mesenchymal (alpha-SMA) or inflammatory (ICAM, VCAM) markers was performed on early passage cells (below P4) to determine endothelial phenotype. Endothelial function was assessed by means of determination of NO and PGI2-production, platelet and monocyte adhesion and Ac-LDL uptake-assay. We observed outgrowth of cells only once when using venous blood (n=15), which could not be analysed due to insufficient cell number. In contrast, in the arterial samples (n=99) we found outgrowth in 52% of cases, with around three colonies per sample. These cells were able to grow to full confluence in a monolayer after subsequent passaging. Although interindividual differences in general morphology and proliferation rates were seen, we altogether observed an endothelial phenotype on both mRNA and protein level, suggesting these cells to be EOCs. Moreover, functional assays confirmed endothelial properties of these EOCs. EOCs show profound endothelial characteristics already within the first passages with characteristics of differentiated rather than of progenitor cells, suggesting that these cells are outgrowing circulating endothelial cells. In conclusion, we state that EOCs can be isolated from arterial but only very limited from venous blood. Although these cells hold highly qualified endothelial potentials, additional research is required to identify the origin of these cells (bone marrow, intima), differences between cells derived from arterial and venous blood, the role of physiological status of the patient (healthy vs coronary artery disease) and usefulness of this cell source for clinical vascular tissue engineering.

Disruption of CD200L-CD200R axis results in increased local inflammation after triggering arteriogenesis in CD200L-deficient mice

P. van den Borne[1]*, A. Hoogendoorn[1], T.P. Rygiel[2], L. Meyaard[2], I.E. Hoefer[1], G. Pasterkamp[1]. [1]Department of Experimental Cardiology, University Medical Center, Utrecht, The Netherlands, [2]Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Background The growth of collateral arteries (arteriogenesis) is known as an inflammatory process that is triggered by local obstruction of larger arteries due to, for instance, atherosclerosis. It is a natural process in which pre-existing anastomoses develop into functional collateral arteries to restore blood flow in ischemic tissues and depends on the influx of inflammatory cells, mainly monocytes. The CD200 ligand-CD200 receptor (CD200L-CD200R) interaction is known as an inhibitory axis, which is critical to bridle massive inflammatory responses in the case of infections or inflammation. CD200L is a membrane glycoprotein that is present on a wide range of cells, including neurons, endothelium and immune cells. The expression of its receptor, CD200R, is solely expressed on cells from the myeloid lineage, but also on T cells and B cells, and NK cells. In both mice and human, CD200L only interacts with CD200R, which suggests a specific role for the CD200L-CD200R interaction in controlling myeloid cell functions. In this study, we want to investigate the role of the inhibitory CD200L-CD200R axis in the process of arteriogenesis. Methods Using a murine hind limb ischaemia model, 15 wildtype (WT) and 15 CD200L-/- (KO) mice underwent an unilateral femoral artery ligation and perfusion recovery was measured over 7 days using Laser-Doppler analysis. After termination at 7 days, blood and bone marrow was collected for RNA and protein analysis. Histology was performed on the adductor muscle of both hind limbs for determination of influx of inflammatory cells (MAC-3 for macrophages; CD-3 for T cells). Cell infiltration was measured in the perivascular space of at least 3 different collateral arteries, averaged per mouse and expressed as number of positive cells per vessel. Results At 3 and 7 days after operation, perfusion recovery was increased in KO mice compared to WT, however this was not significant (Day 3; p=0.07, Day 7; p=0.76; 15 WT vs 15 KO). At 7 days, macrophage infiltration in the tissue was higher in KO mice compared to WT, but this was also not significantly different between WT and KO (p=0.21; 10 WT vs 10 KO). However, T cell infiltration was significantly higher in KO mice compared to WT at day 7 (p=0.04; 10 WT vs 10 KO). Conclusions In this study, we did not see significant effects on perfusion recovery in CD200L-/- mice compared to WT mice. However, the preliminary data on histology do show an effect of tissue inflammation after disrupting the CD200L-CD200R axis in a CD200L-/- mouse model for hind limb ischaemia. Future research will reveal why the local increased tissue inflammation does not associate with the effects on perfusion recovery.

Medial elastin fragmentation leads to intraplaque neovascularization, acute plaque rupture, atheroembolic stroke and sudden death in atherosclerotic mice

Carole Van der Donckt[1]*, Dorien M. Schrijvers[1], Greetje Vanhoutte[2], Annemie Van der Linden[2], Marleen Verhoye[2], Wim Martinet[1], Hidde Bult[1], Arnold G. Herman[1] and Guido R.Y. De Meyer[1]. [1]Laboratory of Physiopharmacology, [2]Bio-Imaging Lab, University of Antwerp, Belgium

Atherosclerotic plaque rupture remains the leading cause of acute cardio- and cerebrovascular events. Current animal models flaw because they merely generate an unstable plaque phenotype without spontaneous rupture and clinical sequels. We have previously shown that elastin fragmentation of the vessel wall - as seen in humans during aging - leads to increased vascular stiffness, vessel dilation and exacerbated atherogenesis in fat fed apolipoprotein E deficient mice (apoE-/-) that have a mutation (C1039G+/-) in the fibrillin-1 (fbn1) gene. Female apoE-/- and apoE-/- fbn1C1039G+/- mice were fed a Western type diet for 35 weeks (n=20 mice in each group). In contrast to standard apoE-/- mice, atherosclerotic plaques of apoE-/- fbn1C1039G+/- mice were 2-fold larger, highly unstable (as shown by a large necrotic core and a decreased amount of smooth muscle cells and collagen fibers) and showed prominent neovascularization and hemorrhage, important features of vulnerable human plaques. Neovessels were commonly observed in the smaller vessels such as the brachiocephalic and left common carotid artery. Microvessel density was related to the degree of medial elastin fragmentation and stenosis of the vessel, indicating that both permeability of the media and size of the plaque (hypoxia) contribute to intraplaque neovascularization. Moreover, these neovessels appeared to be leaky, as seen by the extravasation of red blood cells into the plaque. Acute plaque rupture was observed in 70% of apoE-/- fbn1C1039G+/- mice, but never in apoE-/- mice. Furthermore, apoE-/- fbn1C1039G+/- mice showed neurological symptoms such as head tilt, disorientation and motor disturbances, and died suddenly (15 out of 20 mice, of which 50% after 20 weeks). In all apoE-/- fbn1C1039G+/- mice, magnetic resonance imaging (MRI) showed brain lesions that contained cholesterol clefts, macrophages and smooth muscle cells, indicating embolization of plaque debris after rupture. ApoE-/- fbn1C1039G+/- mice represent the first mouse model developing spontaneous plaque rupture with relevant clinical endpoints as seen in humans, including most of the features of vulnerable human plaques. The presence of abundant intraplaque neovascularization and the occurrence of acute plaque rupture in apoE-/- fbn1C1039G+/- mice point out a pivotal role for neovascularization in intraplaque hemorrhage, lipid core expansion, and plaque rupture. This mouse model is a unique tool to explore and validate novel plaque stabilizing therapies.

Early diagnostic markers for NSTEMI preceeding troponine.

CTM van der Pouw Kraan[1]* F.J.P. Bernink[2], JM Baggen[1] L. Timmers[3], A.M. Beek[2], M. Diamant[4], W.J.Y Chen[4], A.C. v Rossum[2], N. v Royen[2], A.J.G. Horrevoets[1] Y. Appelman[2].
[1]Molecular Cell Biology & Immunology, VU Medical Center, Amsterdam, [2]Cardiology, VU Medical Center, Amsterdam, [3]Cardiology, University Medical Center Utrecht, [4]Endocrinology and Diabetes Center, VU Medical Center, Amsterdam, The Netherlands

Background. The majority of patients that present at the CCU with suspicion of Acute Coronary Syndrome, do not show ST elevations on ECG and need additional testing of serum cardiac troponinT over time (9-12h), to detect or rule out cardiac disease. The discovery of novel biomarkers that enable diagnostic testing is of importance to detect/rule out MI at an earlier stage, reducing unnecessary hospitalization. Methods and results. We evaluated the mRNA expression levels of TLR2 and TLR4 by realtime PCR in whole blood samples obtained from 35 patients with acute myocardial infarction with ST elevation, enrolled in the EXAMI trial, at presentation at the CCU and after 4 months (considered baseline). We confirmed that TLRs are upregulated during MI. We identified for the first time a correlation of both TLR2- and TLR4 mRNA levels with infarct size (R=0.59, p=0.0003 and R=0.46, p=0.007). Furthermore we showed that both TLRs are upregulated earlier than troponinT: 25 of 26 patients who still had negative troponinT tests, did already show significantly elevated TLR4 mRNA levels, while 9 of these 26 troponinT-negative patients showed elevated TLR2 levels. Conclusions. TLR2 and TLR4 expression levels correlate with infarct size and are expressed earlier than troponinT, indicating that TLR2 and 4 are early and sensitive markers for MI, which will be evaluated as a diagnostic tool in our future research to rule out MI at an early stage.

Frequent night dialysis is associated with reduced platelet responsiveness

T.C. van Holten[1]*, B.C. van Jaarsveld[2], M. van Wijk[3], F. van Reekum[3], Ph.G. de Groot[1], M. Roest[1]. [1]Department for Clinical Chemistry and Haematology, University Medical Centre Utrecht, [2]Nephrology, VU University Medical Centre, Amsterdam, [3]Department for Internal Medicine and Dermatology, University Medical Centre Utrecht, The Netherlands

End-stage renal disease (ESRD) is associated with severe calcifications of the vasculature, and high cardiovascular disease mortality. Intense renal replacement therapy improves the vasculature patency. We hypothesize that intensity of renal replacement therapy is associated with platelet responsiveness. Different treatment strategies of ESRD patients were compared: 1) nocturnal dialysis (5-7 times per week), 2) conventional centre dialysis, and 3) peritoneal dialysis. These were compared with healthy controls. Platelet responsiveness was measured with increasing concentrations of adenosine diphophosphate (ADP), GPVI specific collagen related peptide (CRP-XL), and PAR-1 activating peptide (TRAP) in presence and absence iloprost. Platelet activation was determined via P-selectin expression using fluorescence-activated cell sorting. The area under the curve, expressed in arbitrary units, was taken as the responsiveness of platelets. Statistical tests were performed using Wilcoxon's test. Peritoneal dialysis was associated with an increased ADP response (mean: 4787, SEM: 357) as compared to night dialysis (mean: 2882, SEM: 357; $P<0.05$) and conventional dialysis (3391, SEM 325; $P=0.08$). The response to CRP-XL and TRAP was comparable across the different renal replacement therapies. All renal replacement therapies were associated with decreased platelet responsiveness for TRAP, and CRP-XL ($P<0.01$), but not for ADP when compared with a healthy control group. We conclude that more intense night dialysis is associated with a reduced platelet ADP responsiveness compared with the less intense peritoneal dialysis. In addition, ESRD patients have a reduced platelet responsiveness compared with controls for TRAP and CRP-XL. This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project CIRCULATING CELLS (grant 01C-102), and supported by the Netherlands Heart Foundation.

Regulation of CAMP dependent weibel-palade body exocytosis from endothelial cells

Kathinka WEM van Hooren*, Ellen A van Agtmaal, Mar Fernandez-Borja, Jan Voorberg and Ruben Bierings. Department of Plasma Proteins, Sanquin Research, Amsterdam, the Netherlands

Vascular endothelial cells provide a dynamic interface between circulating blood and underlying tissues that is critically involved in maintaining vascular integrity and homeostasis. Rapid recruitment of bio-active components from intracellular storage pools has been shown to contribute to the critical role of endothelial cells in maintaining vascular homeostasis. A significant number of haemostatic components and inflammatory mediators originate from endothelial cell-specific, cigar-shaped organelles called Weibel-Palade bodies (WPBs). WPBs function as storage vesicles for von Willebrand factor (VWF), a multimeric adhesive glycoprotein crucial for platelet plug formation, the leukocyte receptor P-selectin and a number of bioactive compounds that include the chemoattractants IL-8 and eotaxin-3. WPBs release their content following stimulation with agonists increasing intracellular Ca2+, like thrombin, or agonists increasing intracellular levels of cAMP, such as epinephrine. The physiological importance of this cAMP mediated pathway is illustrated by the rise in VWF levels in patients with von Willebrand's disease and mild hemophilia A following administration of the vasopressin analogue desmopressin (DDAVP) or epinephrine. We have previously shown that this cAMP mediated WPB release is partly dependent on protein kinase A and involves the activation of the small GTPase RalA. Here, we have investigated a possible role for another, PKA-independent cAMP-mediated signaling pathway in the regulation of WPB exocytosis, namely the guanine nucleotide exchange factor Epac1 and its substrate, the small GTPase Rap1. Epinephrine stimulation of endothelial cells leads to Rap1 activation in a PKA-independent fashion. siRNA-mediated knockdown of Epac1 abolished epinephrine induced activation of Rap1 and resulted in decreased epinephrine-induced WPB exocytosis. Downregulation of Rap1 expression and prevention of Rap1-activation through over-expression of Rap1GAP effectively reduced epinephrine- but not thrombin induced WPB exocytosis. Taken together, these data uncover a new Epac-Rap1 dependent pathway by which endothelial cells can regulate WPB exocytosis in response to agonists that signal through cAMP.

Differences between basal and agonist-induced nitric oxide production in the isolated mouse aorta

J. van Langen*, P. Fransen, C.E. Van Hove and H. Bult. Laboratory of Pharmacology, University of Antwerp, Wilrijk 2610, Belgium

Aim. Endothelial nitric oxide (NO) synthase (eNOS) is a key protector against atherosclerosis. This study addresses differences between basal and agonist-induced release of NO in the isolated mouse aorta. Methods. Segments (2mm) of the thoracic aorta of C57/Bl6 mice were mounted in an organ bath to measure isometric forces. Basal NO was assessed by its ability to suppress phenylephrine (PE, 10-6 M) or prostaglandin F2α(PGF2α10-5 M)-induced contractions at 1h intervals. Relaxations induced by acetylcholine (ACh) or the alpha-2 adrenoceptor agonist UK 14,304 in rings constricted with PE or PGF2αwere used as index of agonist-induced NO release and were determined at 1h intervals. Immunoblotting was used to investigate phosphorylation status of Akt. Results. Exactly 1h after dissection PE or PGF2α-induced contractions were very small, but they gradually increased every hour. In the presence of the pan-NOS inhibitor NΩ-nitro-L-arginine methyl ester (L-NAME), or after endothelium-removal, contractions were much larger and did not increase. In contrast, PE contractions were unaffected by selective inhibitors of neuronal NOS (nNOS) or inducible NOS (iNOS). In the presence of superoxide dismutase, PE contractions were lower, but they increased similarly to the untreated segments, whereas catalase had little effect. ACh and UK 14,304 induced relaxations of pre-constricted segments, which remained unaltered throughout the experiment. ACh relaxations were abolished by L-NAME and endothelium-removal, but were unaffected by superoxide dismutase. Catalase did not affect ACh responses at 1h, but strongly inhibited ACh relaxations at 3h. The phosphoinositide kinase-3 (PI3K) inhibitor wortmannin selectively increased PE contractions, without affecting ACh relaxations. Western blotting showed that Akt phosphorylation significantly declined in time and was abolished by wortmannin treatment. Conclusions. These results indicate that basal NO production is initially very high, but quickly disappears in the organ bath. The selective NOS inhibitors showed that basal NO is exclusively produced by eNOS in the endothelium, and not formed by nNOS or iNOS. In contrast to basal NO release, there were no apparent changes in ACh or UK 14,304-stimulated eNOS activity, implying different regulatory mechanisms. The selective inhibition of basal eNOS activity by wortmannin implies an exclusive role for the PI3K/Akt pathway. The observation that catalase had no effect on ACh responses at 1h, but strongly inhibited ACh relaxations at 3h points to a time-dependent uncoupling of eNOS, leading to a shift from NO to O2- production and subsequent formation of H2O2, which is known to act as a potent vasodilator.

ROCK2 regulates thrombin receptor-mediated vascular permeability: Role of baseline junctional forces

Cora M.L. Beckers[1], Nebojsa Knezevic[2], Mohammad Tauseef[2], Ramaswamy Krishnan[3], Kavitha Rajendran[3], Jurjan Aman[1], Jan van Bezu[1], Victor W.M. van Hinsbergh[1], Dolly Mehta[2], Geerten P. Van Nieuw Amerongen[1]*. [1]Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, van der Boechorststraat 7, 1081BT Amsterdam, The Netherlands, [2]Department of Pharmacology, Center for Lung and Vascular Biology, University of Illinois, College of Medicine, Chicago, IL 60612, [3]Molecular and Integrative Physiological Sciences, Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA

Rationale Several studies suggest distinct roles for the highly homologous isoforms of Rho kinase -ROCK1 and ROCK2- in regulating vascular function, but their involvement in regulating vascular permeability remained unclear. Objective To study the individual contributions of ROCK1 and ROCK2 to vascular hyperpermeability responses. Methods In the intact lungs of untreated and mice transduced with ROCK1/2 siRNAs, vascular permeability was induced by TFLLRN, an agonist of the thrombin receptor PAR1 and was assessed by Evans blue dye extravasation and lung wet-dry weight ratios. Endothelial hyperpermeability was induced in vitro by thrombin and was assessed by the passage of a tracer or by Electrical Cell-substrate Impedance Sensing (ECIS). Endothelial contractile forces were mapped by traction force microscopy. Measurements and Main Results Silencing of ROCK2, but not ROCK1 attenuated PAR1-mediated hyperpermeability of the pulmonary microvasculature and of endothelial monolayers. Simultaneous downregulation of both Rho kinase isoforms further reduced endothelial hyperpermeability. Surprisingly, ROCK2 inhibition did not affect the thrombin-induced contractile force enhancements mediated by the F-actin cytoskeleton, but lowered baseline tension on the intercellular junctions, thereby protecting the endothelium from barrier disruption. Conclusions These data point to ROCK2, rather than ROCK1 as the critical Rho kinase for regulation of endothelial barrier function. They identify regulation of basal isometric cellular tone (the so-called prestress) as a novel mechanism by which Rho kinase regulates endothelial hyperpermeability responses. Thus, ROCK2 activity is pivotal to a critical junctional tension for opening of the endothelial barrier in response to inflammatory mediators. NHS2011T072

Association of Smooth Muscle Cell Pathologies with Functional p27kip1 promoter-SNP

Claudia M. van Tiel[1]*, Peter I. Bonta[1], J. Wouter Jukema[2], Robbert J. de Winter[3], Alexander W. Clowes[4], Michael S. Conte[5], Carlie J. de Vries[1]. [1]Department of Medical Biochemistry and [3]Department of Cardiology, Academic Medical Center, Amsterdam, The Netherlands, [2]Department of Cardiology, Leiden University Medical Center, The Netherlands, [4]Department of Surgery, University of Washington, Seattle, USA, [5]Division of Vascular and Endovascular Surgery, UCSF, San Francisco, USA

Rationale The cyclin-dependent kinase inhibitor p27kip1 is an inhibitor of smooth muscle cell and leukocyte proliferation in vascular disease. We hypothesized that common genetic variations in p27kip1 may serve as a useful tool in risk stratification for smooth muscle cell proliferation-specific pathologies such as in-stent restenosis, bypass graft failure and transplantation arteriosclerosis. Methods & Results The p27kip1-838C>A single nucleotide polymorphism (SNP) (rs36228499) was determined in the GEISHA-cohort of 715 patients undergoing coronary angioplasty and stent placement. We discovered that the p27kip1-838C>A SNP is associated with clinical in-stent restenosis; the -838AA genotype decreases the risk of target vessel revascularization (hazard ratio, 0.28; 95% confidence interval, 0.10 to 0.77). This finding was replicated in the second GENDER-cohort study of 2309 patients (HR 0.61; 95% confidence interval, 0.40 to 0.93). We subsequently studied the functional importance of the -838C>A polymorphism in luciferase-reporter assays. Basal p27kip1 transcriptional activity of the -838A allele containing promoter is 20-fold increased compared to the -838C allele. In silico analysis of the p27kip1 promoter sequence revealed that the flanking regions of this SNP are highly conserved throughout different species and that a change at position -838 from C to A resulted in additional consensus binding sites for several transcription factors. The p27kip1-838C>A SNP was also determined in a small cohort of 204 patients undergoing peripheral artery bypass surgery in the leg. Also in this pathology a -838AA genotype decreases the risk of developing stenosis in the graft (hazard ratio, 0.40; 95% confidence interval 0.17-0.93). Conclusion Patients with the p27kip1 -838AA genotype have a decreased risk to develop smooth muscle cell proliferation-specific pathologies corresponding with enhanced promoter activity of the -838A allele of this cell-cycle inhibitor. Clinical Relevance Our data provide novel opportunities for risk stratification of individual patients to support the intervention cardiologist to triage patients with low risk of restenosis for treatment with a bare-metal stents rather than with a drug-eluting-stent. In addition, this SNP gives the vascular surgeon crucial information which patients are likely to develop stenosis in peripheral venous grafts.

Platelet-derived deoxyribose-1-phosphate promotes endothelial cell motility in vivo and angiogenesis in vivo

Dina S. Vara[1]*, Tim Woodman[1], Warwick B Dunn[2], Elena Garonna[3], Caroline PD Wheeler-Jones[3], Giordano Pula[1]. [1]Department of Pharmacy and Pharmacology, University of Bath, [2]Manchester Centre for Integrative Systems Biology, University of Manchester, [3]Royal Veterinary College, University of London, United Kingdom

Objectives: To understand the physiological role of deoxyribose-1-phosphate (dRP) release by human and mouse platelets. Results: Micromolar concentrations of the pro-angiogenic metabolite deoxyribose-1-phosphate (dRP) were detected by mass spectrometry (MS) and nuclear magnetic resonance (NMR) in the supernatants of collagen- and thrombin-stimulated platelets. The expression of the dRP-generating enzymes thymidine phosphorylase (TP), uridine phosphorylase (UP), and purine nucleoside phosphorylase (PNP), was investigated by immunoblot, revealing expression of all three nucleoside phosphorylases in human platelets, but only UP and TP in mouse platelets. The specific inhibition of UP by 5-phenylthiocyclouridine (PTAU) in human platelets and the genetic silencing of UP and TP in mouse platelets (TP-/-/UP-/-) significantly reduced the ability of protein-free platelet supernatants to enhance human umbilical vein endothelial cell (HUVEC) motility in monolayer scratch repair and transmigration assays. In either case, the addition of dRP restored the ability of platelet supernatants to stimulate HUVEC migration. The stimulation of endothelial cell migration by platelet-derived dRP correlated with the upregulation of integrin β3, which was induced in a reactive oxygen species–dependent manner and resulted significantly reduced for platelet supernatants with depleted dRP levels (i.e. PTAU-treated human platelets and mouse TP-/-/UP-/- platelets). As tested with selective inhibitory antibodies, the activity of integrin αvβ3 was necessary for the stimulation of HUVEC motility, thus confirming the involvement of integrin β3 in the response of endothelial cells to protein-free platelet supernatants. Finally, chick chorioallantoic membrane (CAM) vascularisation assays performed with protein-free supernatants suggested that the release of dRP by platelets is also responsible for the stimulation of angiogenesis in vivo. Conclusions: The pro-angiogenic metabolite dRP mediates the redox-dependent upregulation of integrin β3 and the cell migratory responses observed in endothelial cells treated with protein-free platelet supernatants. The paracrine regulation of endothelial cell motility by platelet-derived dRP is likely to play an important role in the stimulation of postnatal angiogenesis and tissue repair.

Shear stress regulates endothelial microparticle release

Anne-Clémence Vion[1,2]* , Bhama Ramkhelawon[1,2], Xavier Loyer[1,2], Gilles Chironi[1,2,3], Cecile Devue[1,2], Gervaise Loirand[4], Alain Tedgui[1,2], Stéphanie Lehoux[1,5]*, Chantal M. Boulanger[1,2]*. [1]INSERM, U970, Paris Cardiovascular Research Center PARCC, Paris, France, [2]Université Paris Descartes, Sorbonne Paris Cité, UMR-S970, Paris, France, [3]Centre de Médecine Préventive Cardiovasculaire, AP-HP, Hopital Européen Georges Pompidou, Paris, France, [4]INSERM UMR 1087, Institut du Thorax, Nantes, France, [5]Lady Davies Institute, Mac Gill University, Montreal, Canada

Endothelial activation and apoptosis release membrane-shed microparticles (EMP) that emerge as important biological effectors. As laminar shear stress (SS) is a major physiological regulator of endothelial survival, we tested the hypothesis that SS regulates EMP release. Plasma CD144+EMP levels were inversely correlated with basal shear rate in asymptomatic healthy subjects. In vitro, EMP levels quantified by flow cytometry in medium of endothelial cells subjected to low or high SS (2 and 20 dyne/cm2) augmented with time in low- compared to high-SS conditions. This effect was sensitive to ERK1/2 and ROCK inhibitors, but unaffected by caspases inhibitors. Low SS-stimulated EMP release was associated with increased endothelial ROCK and ERK1/2 activities and cytoskeletal reorganization. Overexpression of constitutively active RhoA stimulated EMP release under high SS. We also examined the effect of nitric oxide (NO) in mediating SS effects. L-NAME increased high SS-induced EMP levels by 3-fold, whereas the NO donor SNAP decreased it. L-NAME and SNAP did not affect ROCK and ERK1/2 activities. Then, we investigated NO effect on membrane remodelling as microparticle release is abolished in ABCA1-deficient cells. ABCA1 expression, which was greater under low SS than under high SS, was augmented by L-NAME under high SS and decreased by SNAP under low SS conditions. Altogether, these results demonstrate that sustained atheroprone low SS stimulates EMP release through activation of ROCK and ERK1/2 pathways, whereas atheroprotective high SS limits EMP release in a NO-dependent regulation of ABCA1 expression. These findings therefore identify endothelial SS as a physiological regulator of microparticle release.

Endothelial signaling pathways controlling the dissociation of the receptor phosphatase VE-PTP from VE-cadherin

Matthias Vockel*, Astrid Nottebaum, Andre Broermann and Dietmar Vestweber. Max-Planck-Institute for Molecular Biomedicine, Muenster, Germany

During inflammation, leukocytes extravasating from the blood into the surrounding tissue have to migrate through the endothelial barrier of the blood vessel wall. While endothelial cells form a stable physical barrier, they simultaneously have to allow regulated opening of junctions to facilitate transmigration of leukocytes. The integrity of endothelial cell junctions is mainly controlled by the adhesion molecule VE-cadherin. We have recently shown that the endothelial-specific receptor protein tyrosine phosphatase VE-PTP associates with VE-cadherin and that this interaction is required for contact integrity. Furthermore, leukocyte adhesion to endothelial cells or VEGF stimulation triggers the rapid dissociation of VE-PTP from VE-cadherin. This established VE-PTP as an endothelial junction protein that is involved in the regulated weakening of endothelial cell contacts during leukocyte transmigration. The dissociation of VE-PTP from VE-cadherin also occurs in vivo, as either LPS-induced inflammation in the mouse lung or systemic administration of VEGF stimulates the dissociation of the complex. These results suggested that the VE-cadherin/VE-PTP dissociation is a prerequisite for efficient leukocyte extravasation in vivo. To test this hypothesis, we generated knock-in mice expressing modified fusion proteins of VE-cadherin and VE-PTP containing additional protein domains that allow stabilization of their association by a chemical heterodimerizer. We found that intravenous application of the heterodimerizer strongly inhibited neutrophil extravasation in the inflamed cremaster and the LPS-inflamed lung. This clearly shows that this dissociation is indeed necessary for the opening of endothelial cell contacts during leukocyte extravasation in vivo. However, the signaling steps leading to VE-cadherin/VE-PTP dissociation in endothelial cells remained elusive. Also, the endothelial leukocyte receptor activating this signaling pathway was still unknown. We could identify this adhesion receptor triggering the leukocyte-induced dissociation and verify its requirement by using blocking antibodies to inhibit the dissociation. Furthermore, we identified downstream signaling pathways using pharmacological inhibitors of endothelial signaling mediators. Our results show that leukocytes are engaging specific intracellular pathways following adhesion to endothelial cells, leading to the dissociation of VE-cadherin and VE-PTP to faciliate an efficient transmigration.

Angio-associated migratory cell protein contributes to progenitor cell adhesion and migration and to neoatherosclerosis development in the rat in-stent restenosis model

Mauricio Borinski*, Christian Flege, Nicole Krott, Annika Grahl, Nikolaus Marx, Felix Vogt. University Hospital Aachen, Department of Cardiology, Pneumology, Angiology and Intensive Care, Aachen, Germany

Angio-associated migratory cell protein (AAMP) is a recently identified small protein with a molecular weight of 52 kDa. It is predominantly expressed in cells with a migratory phenotype, including endothelial cells, activated T-cells, and glia cells, as well as in malignant tissue such as human melanoma cells. AAMP shares sequence homology with immunoglobulin superfamily members, including cellular adhesion molecules such as leukocyte function antigen-2; immunoglobulin superfamily proteins mediate adhesion and migration of various malignant circulating cells. However, the role of AAMP for the development of atherosclerosis and of neointimal hyperplasia after vascular injury is poorly understood. Here, we aimed at the characterization of AAMP expression patterns and AAMP-dependent adhesive and migratory activity of various progenitor cell subpopulations and of the monocytic cell line THP-1; in addition, we sought to characterize the in vivo - role of AAMP during leukocyte adherence and the AAMP expression in the vessel wall after injury. An affinity-purified polyclonal antibody against recombinant AAMP was generated in rabbits; for histopathological evaluation, modified Movat's pentachrome stainings were performed on paraffin-embedded sections. Also, methodology included quantification of AAMP expression intensity by pixel analysis of AAMP staining intensity, static adhesion assays, transwell migration chamber assays, fluorescence microscopy or quantitative Western blotting. As in vivo model, the rat model of in-stent restenosis after direct trans-abdominal stent implantation of conventional coronary stents (2.5 x 8 mm) into the abdominal aorta has been employed. Lysates of endothelial and smooth muscle cell progenitor cells and of peripheral blood mononuclear cells and of THP-1 cells evidenced time-dependent expression patterns of AAMP. Adhesive capacity of cells upon TNF-alpha acitivated human umbilical vein endothelial cells was dose-dependently inhibited by anti-AAMP antibody and cellular migration was concentration-dependently reduced by anti-AAMP antibody. Post stent implantation, AAMP was over-expressed in neointimal tissue. Taken together, this is the first desription of AAMP expression in circulating vascular progenitor cells and in vivo in the rat in-stent restenosis model. Also, a regulatory role during adhesion and migration of different cells was evidenced, suggesting a contribution of AAMP to inflammatory dysregulation during atherosclerosis or neoatherosclerosis development. This identifies AAMP as a potential target to limit neoatherosclerotic lesion formation.

Dual regulation of CD84 by ADAM10 and calpain in platelets

Sebastian Hofmann[1] Timo Vögtle[1]* Markus Bender[1] Stefan Rose-John[2] Bernhard Nieswandt[1]. [1]University of Würzburg, Chair of Vascular Medicine, University Hospital and Rudolf Virchow Center for Experimental Biomedicine, Würzburg, Germany, [2]Institute of Biochemistry, Christian-Albrechts-University of Kiel, Kiel, Germany

Platelet receptor ectodomain shedding is a mechanism to modulate signaling and to downregulate platelet reactivity. Proteins of the a disintegrin and metalloproteinase (ADAM) family are involved in this process, with ADAM17 mediating the cleavage of GPIb, whereas shedding of GPV or GPVI can occur through either ADAM10 or ADAM17, depending on the shedding-inducing stimulus. CD84 is type I transmembrane glycoprotein of the signaling lymphocyte activation molecule (SLAM) family that is highly expressed in platelets and different immune cell populations, including T and B cells, monocytes/macrophages and mast cells. It belongs to the Ig superfamily of cell surface receptors, potentially binding to the cytoplasmic adaptors via their immunoreceptor tyrosine switch motif (ITSM). Because of its ability to form homodimers, CD84 has been suggested to mediate contact-dependent signaling and to contribute to thrombus stability, but its role in platelet physiology is not well explored. Here we show that CD84 is cleaved from the surface of human and murine platelets in response to shedding inducing agents as well as platelet receptor agonists. Studies in transgenic mice identified ADAM10 as the principal sheddase responsible for CD84 cleavage, whereas ADAM17 was not required. Moreover, western blot analysis revealed calpain-mediated cleavage of the intracellular CD84 C-terminus, occurring simultaneously with, but independently of ectodomain cleavage. Finally, analysis of plasma and serum samples from transgenic mice demonstrated that CD84 is constitutively shed from the platelet surface by ADAM10 in vivo and that ADAM10 is the only proteinase that sheds CD84 during blood clotting. These results reveal a tight regulation of platelet CD84 by simultaneous extra- and intracellular cleavage that may influence platelet-platelet as well as platelet-immune cell interactions.

Quantification of intravascular microparticles during Shiga toxin induced hemolytic uremic syndrome

Shuwang Ge, Barbara Hertel, Sang Hi Emden, Jan Beneke, Hermann Haller, and Sibylle von Vietinghoff*. Department of Medicine, Hannover Medical School, Hannover, Germany

E. coli shiga toxin can induce a hemolytic uremic syndrome (STEC-HUS) that is characterized by hemolysis, intravascular micro- and macrothrombi, renal failure and various degrees of central nervous system involvement. The pathophysiology of STEC-HUS is poorly understood. Data from animal models on the role of leukocytes and platelets are controversial. Circulating microparticles were elevated in small groups of pediatric patients. Here we investigated circulating microparticles in a group of 30 adult STEC-HUS treated at a tertiary care centre during the STEC-HUS outbreak in Germany in 2011. Microparticles from fresh patient plasma were analyzed by multi-color flow cytometry and immunofluorescence. Microparticle concentration, provenience and thrombocyte microparticle seeding to leukocytes were assessed. In patients with acute STEC-HUS, plasma microparticle concentrations expressing both platelet and leukocyte markers were significantly elevated compared to healthy controls. Higher plasma microparticle concentrations were detected in patients with renal failure receiving hemodialysis for renal replacement therapy. Platelet microparticles were detected on a high proportion of monocytes and granulocytes, but not lymphocytes in the circulation. Severely affected patients were treated by plasma exchange and/or inhibition of complement. Plasma exchange reduced platelet marker expression on leukocytes. Inhibition of complement did not alter platelet microparticle binding to leukocytes. It moderately and transiently decreased the number of circulating microparticles. In STEC-HUS, elevated numbers of circulating microparticles were detected. Microparticles may amplify systemic disease by distribution of leukocyte and platelet constituents and/or shiga toxin to leukocytes and endothelium.

CXCL1-CXCR2 signaling promotes arteriogenesis during ischemic hind limb conditions in the rat

M.H.M. Vries*, A. Wagenaar, D.G.M. Molin, M.J. Post. MUMC+, CARIM, Department of Physiology, Maastricht, The Netherlands

Arteriogenesis is a tightly regulated process in which pre-existing collateral vessels grow in diameter due to vascular wall remodeling after occlusion of a more proximal artery. Herein, monocyte recruitment is essential and may be governed by an elaborate interplay between endothelial cells and monocytes. The importance of CXC chemokines in this interplay has become increasingly evident in recent years and in this study we investigated the role of one particular ligand and receptor axis, namely CXCL1 and its cognate receptor, CXCR2. In a rodent ischemic hind limb model, rat femoral arteries were ligated and placebo, CXCL1 agonist (50 ng/h) or CXCR2 blocker (1 ng/h) was administered via an osmopump. After 1, 3 and 12 hours and 1, 3, 7 and 14 days, perfusion recovery was measured with Laser-Doppler. Subsequently, mRNA was isolated from explanted collateral vessels and processed for Affymetrix chip analysis. Furthermore, hind limb tissue sections were stained for macrophage marker CD68. CXCL1 agonist treatment significantly increased perfusion recovery at 3 days post-ligation (39+/-8% CXCL1 agonist vs. 22+/-6% placebo) and at 7 days post-ligation (51+/-4% CXCL1 agonist vs. 34+/-7% placebo; n=15, $p<0.05$). CXCR2 antagonist treatment significantly decreased perfusion recovery at both 7 and 10 days post-ligation (52+/-9% CXCR2 antagonist vs. 77+/-4% placebo and 68+/-2% CXCR2 antagonist vs. 87+/-7% placebo, respectively; n=8, p=0.01). In non-treated conditions, CXCL1 mRNA expression in collaterals was dramatically upregulated already 1 hour after ligation (ratio ligated/sham 2log 2.8) and from thereon decreased to normal levels at 3 days post-ligation. CD68 mRNA was upregulated from 12 hours after ligation and was prolonged for 3 days (peak ratio ligated/sham 2log 2.3). This increase in macrophage signal was supported by distinct CD68 macrophage staining for the collateral vessels. We conclude that CXCL1-CXCR2 signaling plays an important role in monocyte recruitment and arteriogenesis. Studies are currently being carried out to unravel the mechanisms via which CXCL1-CXCR2 signaling affects arteriogenesis.

Lipid binding Protein C Inhibitor (PCI) modulates the activity of the phosphoinositide-specific phosphatase SHIP2 in vitro

Wahlmüller F.*, Sokolikova B., Rieger D. and Geiger M. Center for Physiology and Pharmacology, Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Austria

Protein C inhibitor (PCI) is a multispecific protease inhibitor and a member of the serpin (serine protease inhibitor)-superfamily. It circulates at low levels (90 nM) in blood plasma and is involved in the regulation of blood coagulation and fibrinolysis (Geiger 2007, Suzuki 2008, Meijers 2011). Furthermore PCI binds to nonprotease ligands like heparin, retinoic acid and DNA aptamers.Many of these interactions lead to a stimulation of protease inhibition. The interaction of PCI with phospholipids was first analyzed in 2007 (Malleier et. al). Phosphatidylserine and phosphatidylethanolamine (PE) bound to PCI, and the inhibition of activated protein C (APC) was stimulated by those lipids in a heparin-like manner. In addition Baumgärtner et al. (2007) showed that PCI acts as a lipid-transferase for PE, which supports the internalization of PCI by cells. Here we examined the interaction of PCI with phosphoinositides (PIs) and the potential regulatory function of this interaction. Protein overlay assays, native PAGE, and ELISA were used to study the binding of PCI to PIs. To prove a functional effect we examined APC-inhibition by PCI in the presence of PIs as well as phosphate release of PIs by lipid-specific phosphatases in the presence of PCI. All mono- and bisphosphorylated PIs as well as phosphatidylinositol-3,4,5-trisphosphate (PtdIns3,4,5P3) bound to PCI as judged from binding assays. Dot blot analysis showed strongest interaction with the three monophosphorylated PIs ($p < 0.01$) and moderate stimulation of APC-inhibition. PCI incubated with unsaturated phosphatidylinositol-4,5-bisphosphate showed different electrophoretic mobility of PCI antigen on native gels. Furthermore overexpression of PCI in HEK293 cells lead to the phosphorylation of AKT (protein kinase B). This might be explained by the potential influence of PCI on intracellular PI-levels as well as the effect of PCI on the modulation of PI-specific enzymes mediating the interconversion of PIs. Indeed PCI stimulated the activity of the PI-specific phosphatase SHIP2 in vitro indicating that PCI might affect insulin signaling by blocking PtdIns3,4,5P3 mediated pathways. In future experiments we will study the influence of PCI on intracellular PI-levels, the generation of IP3 and diacylglycerol and its possible role in calcium signaling and protein kinase activation. We conclude that PCI is an additional intracellular interaction partner of PIs and affects intracellular lipid signaling.

Vascular endothelial side population cells in the mouse liver blood vessels contribute to angiogenesis in vivo

Taku Wakabayashi*, Hisamichi Naito, Hiroyasu Kidoya, Kazuhiro Takara, Susumu Sakimoto, Nobuyuki Takakura. Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Japan

We have previously identified vascular endothelial side population cells (EC-SPs) using Hoechst33342 efflux methods in the hind limb muscle which is distinct from bone marrow-derived EPCs (Naito et al. EMBO J 2012). EC-SPs represent 1% of the total CD31+CD45-ECs in the hind limb muscle, have high CD133 expression, and produce large number of ECs when transplanted into ischemic lesions, suggesting progenitor/stem cell properties. Here we assessed the characteristics of EC-SPs in the mouse liver. Liver EC-SPs represented 1-3% of the total liver CD31+CD45-ECs and showed high colony forming capacity in vitro compared with endothelial main population cells (EC-MPs). To investigate whether EC-SPs contribute to angiogenesis in vivo, EC-SPs from CAG-EGFP mice were transplanted into the adult mouse with extensive liver EC damage created by intraperitoneal monocrotaline (MCT) injection. After 1 month, transplanted EC-SPs gave rise to vascular networks which were integrated in the liver sinusoids. The integrated EGFP(+) vascular networks contained both EC-SPs and EC-MPs based on side population analysis. When EC-MPs were transplanted into the mouse with extensive liver EC damage, only a few vascular networks were observed and those networks contained only EC-MPs but not EC-SPs. This study revealed that EC-SPs exist not only in the limb muscle but also in the liver, and possess high colony-forming ability and vascular regenerating capacity. Furthermore, based on in vivo transplantation model, a novel hierarchy of vascular endothelial progenitor/stem cell may exist in the peripheral blood vessels.

Activation of signaling and transcriptional networks during TGF-β-induced endothelial-mesenchymal transition (EndMT)

Tetsuro Watabe*, Hajime Mihira, Takashi Kokudo, Hiroshi I. Suzuki, Yuichi Akatsu, Yasuhiro Yoshimatsu, and Kohei Miyazono. Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Japan

Epithelial-mesenchymal transition (EMT) plays important roles in various physiological and pathological processes, and is regulated by signaling pathways mediated by cytokines including transforming growth factor (TGF)- β. Endothelial cells also undergo differentiation into mesenchymal cells during various physiological and pathological processes including heart valve formation and cancer progression, respectively. However, the molecular mechanisms that regulate such endothelial-mesenchymal transition (EndMT) remain to be elucidated. Here we show that TGF-β plays important roles during mesenchymal differentiation of mouse embryonic stem cell-derived endothelial cells (MESECs) and mouse pancreatic microvascular endothelial cells (MS-1). TGF-β2 induced the differentiation of MESECs into mesenchymal cells with decrease in expression of an endothelial marker, claudin-5, and increase in that of mural markers, smooth muscle α-actin (α-SMA), while a TGF-β type I receptor kinase inhibitor inhibited it. Among transcription factors involved in EMT, Snail was induced by TGF-β2 in MESECs. Tetracycline-regulated expression of Snail induced the differentiation of MESECs into mural cells, while knockdown of Snail expression abrogated TGF-β2-induced mural differentiation of MESECs. These results indicate that Snail mediates the actions of endogenous TGF-β signals that induce EndMT. By addition of TGF-β2, MS-1 cells underwent mesenchymal transition characterized by re-organization of actin stress fiber and increased expression of various mesenchymal markers such as α-SMA. In contrast to MESEC, TGF-β2-induced EndMT of MS-1 cells is not dependent on Snail but on the activation of Rho signals. Whereas activation of Rho signals via TGF-β-induced non-Smad signals has been implicated in EMT, we found that Arhgef5, a guanine nucleotide exchange factor, is induced by Smad signals and contributes to the TGF-β2-induced α-SMA expression in MS-1 cells. We also found that TGF-β2 induces the expression of myocardin-related transcription factor-A (MRTF-A) in a Smad-dependent fashion and its nuclear accumulation in MS-1 cells and that MRTF-A is required and sufficient for TGF-β2-induced α-SMA expression. These results indicate that activation of Smad signals by TGF-β2 have dual effects on the activation of Rho signals and MRTF-A leading to the mesenchymal transition of MS-1 endothelial cells. Taken together, these findings suggest that TGF-β2 activates multiple transcriptional and signaling networks during mesenchymal transition of various types of endothelial cells.

Hey/Hes bHLH factors show great redundancy in target genes

David Weber[1]*, Julia Heisig[1], Eva Englberger[1], Chia-Lin Wei[2]. 1University Wuerzburg, Biocenter, Department of Developmental Biochemistry, 2Genome Institute of Singapore, Singapore

The Notch signaling pathway plays an important role during vertebrate vascular and cardiac development. Hey and Hes basic helix-loop-helix (bHLH) factors are essential transcriptional repressors that convey Notch signaling effects in many different cell types. Analysis of knock-out mice has defined critical roles for Hey1, Hey2 and HeyL in cardiac development, with Hey2 and Hey1/L knock-out mice showing a valve-septum-defect. Combined loss of Hey1/2 or Hes1/Hey2 leads to defects in vascular development. To identify target of Hey/Hes repressors we have generated human HEK293 and murine ES cells that expressed tagged Hey/Hes proteins in a highly regulated manner and we analyzed target genes by microarray and chromatin-IP to genome-wide map all Hey bound loci. Hey genes were found to modulate target gene expression to a rather limited extent, but with functional interchangeability between Hey factors. Chromatin immunoprecipitation revealed a much greater number of potential binding sites that largely overlap between Hey/Hes factors. However, for HeyL we identify many additional binding sites, which are not bound by the other Hey/Hes genes. There is also a great overlap in binding sites between ES cells and HEK293 cells. Binding sites are clustered in the proximal promoter region especially of transcriptional regulators or developmental control genes. Multiple lines of evidence suggest that Hey proteins primarily act as direct transcriptional repressors, while gene activation seems to be due to secondary or indirect effects. Mutagenesis of putative DNA binding residues supports the notion of direct DNA binding. While class B E-box sequences (CACGYG) clearly represent preferred target sequences, there must be additional and more loosely defined modes of DNA binding since many of the target promoters that are efficiently bound by Hey proteins do not contain an E-box motif. These data establish the Hey and HES bHLH factors as highly redundant transcriptional repressors, which explains the combinatorial action observed in different tissues with overlapping expression.

Proteolytic processing of Semaphorin 3G determines its vascular function

Anja Weick[1]*, Lise Roth[1], Simone Kutschera[1], Maria Riedel[1], Christer Betsholtz[2], Hellmut G. Augustin[1]. [1]Joint Research Division Vascular Biology, Medical Faculty Mannheim (CBTM), Heidelberg University, and German Cancer Research Center (DKFZ ZMBH Alliance), Heidelberg, Germany, [2]Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden

The neuronal guidance class 3 semaphorins have been identified as critical determinants of vascular guidance, assembly, and network formation in physiological and tumor-induced angiogenesis. Class 3 semaphorins consist of seven soluble family members, Sema3A through 3G that are proteolytically processed by proprotein convertases. Sema3G has largely been ignored, most likely due to its limited expression in neuronal cells. We have characterized Sema3G in the vascular system and determined that it is predominantly expressed by developing arterial endothelial cells in vascular rich organs such as the heart, the kidney and the lung. Uniquely, the unprocessed and processed forms of Sema3G have opposing functions. While cleaved Sema3G acts pro-angiogenic and enhances sprouting of blood endothelial cells, unprocessed Sema3G inhibits growth factor induced tube formation, proliferation and migration of lymphatic endothelial cells (LEC). Nevertheless, Sema3G-deficient mice are viable and fertile without overt anatomical defects suggesting that both unprocessed and processed Sema3G can be compensated by other class 3 semaphorins. Corresponding to the observed cellular anti-lymphangiogenic effect of unprocessed Sema3G, pathological challenge of mice in tumor experiments revealed a pronounced effect of Sema3G as negative regulator of intratumoral lymphangiogenesis (enhanced lymphangiogenesis in Sema3G silenced pancreatic MiaPaCa tumors). In conclusion, these data identify Sema3G as a novel regulator of vascular function that acts pro-angiogenic and anti-lymphangiogenic depending on its presentation as processed or unprocessed molecule, respectively.

Naturally occurring fibrin variants modify scaffold vascularization in tissue engineering.

Ester M. Weijers*, Geerten P. van Nieuw Amerongen, Victor W.M. van Hinsbergh and Pieter Koolwijk. Dept. Physiology (ICaR-VU), VU University Medical Center, Amsterdam, The Netherlands

Fibrin provides a temporary matrix structure for invading cells during angiogenesis in wound healing and tumor growth, which makes fibrin an interesting scaffold-protein for tissue engineering. Previously we showed that two naturally occurring fibrinogen variants support tube formation and angiogenesis to different extents. High molecular weight (HMW) fibrin facilitates an increased and accelerated tube formation, conversely low molecular weight (LMW) fibrin showed a reduced tube formation, both in vitro and in vivo. On the other hand, HMW- and LMW-fibrin do not influence adipose tissue-derived mesenchymal stem cell differentiation towards chondrogenic, osteogenic and adipogenic lineages. These results indicate that naturally occurring fibrin variants may provide a proper approach to modify scaffold vascularization, but knowledge is desired on the functionality of the newly formed vessels. This study aims to investigate the formation, integrity, morphology and activation of endothelial monolayers on HMW-fibrinogen, LMW-fibrinogen and gelatin coatings. On HMW-fibrinogen human umbilical vein endothelial cells (HUVEC) have similar adhesion rates as HUVEC on LMW-fibrinogen, but spread faster. The confluent monolayers on HMW-fibrinogen have a better monolayer integrity, as shown by their higher electrical resistances (124.7 ± 6.1%) than HUVEC on LMW-fibrinogen. Confluent endothelial monolayers on HMW-fibrinogen display a condensed peripheral F-actin band and fewer F-actin stress fibers, when compared to their counterparts on LMW-fibrinogen. On the contrary, the endothelial monolayer integrity on HMW- and LMW-fibrinogen showed a similar decrease in electrical resistance after monolayer activation with thrombin. In conclusion, endothelial cells on HMW- and LMW-fibrinogen show distinct monolayer characteristics. The endothelial monolayer on purified HMW-fibrinogen shows a better integrity with a more quiescent phenotype than on LMW-fibrinogen. This is accompanied by differences in the gene expression profile, but it is uncertain whether this is the cause or consequence of the altered monolayer characteristics and how they relate to the increased vascularization in HMW-fibrin. Together, these data provide a new perspective for modifying angiogenesis for therapeutic and tissue engineering applications.

Ovine carotid artery-derived cells as an optimized supportive cell layer in 2-D capillary network assays

Weinandy S[1]*, Babczyk P[1]*, Schiklenk C[1], Unger R[2], Kirkpatrick CJ[3], Flanagan T[4],Sachweh JS[1], Zenke M[5], Klee D[2], Jockenhoevel S[1]. [1]Department of Tissue Engineering & Textile Implants, AME-Helmholtz Institute for Biomedical Engineering, RWTH Aachen, Germany, [2]Institute of Technical and Macromolecular Chemistry (ITMC), RWTH Aachen, Germany, [3]Institute of Pathology, Johannes-Gutenberg-University of Mainz, [4]School of Medicine & Medical Science, Health Sciences Centre, University College Dublin, Dublin, Ireland, [5]Department of Cell Biology, Institute for Biomedical Engineering, RWTH Aachen, Germany

Background: The endothelial cell co-culture assay is a differentiation assay which simulates the formation of capillary-like tubules with the help of a supportive cell layer. For this supportive layer, cells of different origin, such as pulmonary artery smooth muscle cells and fibroblasts from human breast tissue have been successfully employed. Unfortunately, human tissue-derived cell sources are limited. In this study, we investigated alternative supportive cells from more accessible human or animal tissues, including umbilical cord and ovine carotid artery. The use of a more readily accessible cell source would simplify the endothelial cell co-culture assay. Methods and Results: Human umbilical artery smooth muscle cells (HUASMCs) and ovine carotid artery-derived cells were seeded in 96-well plates, followed by addition of human umbilical vein endothelial cells (HUVECs). Nine days after co-culture, the cells were fixed, immunostained and analysed with an in vitro angiogenesis assay (Angioquant®). Capillary-like structures were detected on the ovine carotid artery-derived supportive cell layers. The initial cell number as well as pro- and anti-angiogenic factors (VEGF, PDGF-BB and Bevacizumab) had an influence on the number of tubular-like structures. Furthermore, HUVECs from different donors show distinct levels of VEGF receptor-2, which correlated with the amount of tubular-like structures. In the case of HUASMC supportive cell layers, HUVECs detached almost completely from the surface. Conclusions: Cells of different origin have a varying applicability regarding the endothelial cell co-culture assay: under the conditions described in this study, ovine carotid artery-derived cells seem to be more suitable than HUASMCs for an endothelial co-culture assay. Furthermore, the ovine carotid artery-derived cells are easier to obtain and are in more abundant supply than dermal or breast tissue cells which are currently used. This simplifies the endothelial co-culture assay in respect to testing large amounts of pro- and anti-angiogenic factors.

T box expressed in T cells (T-bet) is a mediator of angiotensin II induced vascular dysfunction, oxidative stress and inflammation

Schmidgen, Maria Isabel[1]*, Kossmann, Sabine[1,2], Hausding, Michael[1], Schwenk, Melanie[2,3], Knorr, Maike[1,2], Karbach, Susanne Helena[1,4], Yogev, Nir[4], Brandt, Moritz[1,2], Waisman, Ari4, Oelze, Matthias[1], Kröller-Schön, Swenja[1], Becker, Christian[3], Daiber, Andreas[1], Münzel, Thomas[1,2], Wenzel, Philip[1,2]*. [1]2nd Medical Clinic Klinik, Universitätsmedizin Mainz, Mainz, Germany, [2]Center for Thrombosis and Hemostasis (CTH), Universitätsmedizin Mainz, Mainz, Germany, [3]Department for Dermatology, Universitätsmedizin Mainz, Mainz, Germany, [4]Institute for Molecular Medicine, Universitätsmedizin Mainz, Mainz, Germany

Background: Recent studies have shown a protection from atherosclerosis by genetical knockout of the Th1 key transcription factor T box expressed in T cells (T-bet) in apoE-/- mice. It remains to be established, whether T-bet is also involved in mediating angiotensin II (ATII) induced vascular dysfunction and oxidative stress. Methods and results: 1 week of ATII infusion (1mg/kg/d, administered by osmotic minipumps) caused a similar blood pressure increase in T-bet deficient mice (Tbet-/-) as compared to wild type littermates (WT). However, Tbet-/- showed ameliorated endothelial and smooth muscle dysfunction (assessed by isometric tension studies in isolated aortic rings) as compared to WT, which showed an increased expression of T-bet protein (as assessed by western blot) in aortic tissue. Likewise, dihydroethidine staining of aortic cryosections and lucigenin enhanced chemiluminescence revealed a reduced ROS-signal in all layers of the vascular wall, and whole blood respiratory burst measured by L0-12 was blunted in ATII-infused Tbet-/-, as well as protein tyrosin nitration. Increased levels of of T helper type 2 (Th2) cytokines released by isolated splenic CD4+ cells measured by ELISA were paralleled by blunted interferon gamma production in ATII infused Tbet-/- as compared to WT. Circulating myelomonocytic cells in response to ATII were decreased in Tbet-/-, as was the presence of these cells in the vacular wall. In addition, FoxP3 protein expression and Il-10 formation was strongly induced in spleens of Tbet-/-, underlining the antiinflammatory phenotype of these mice in this experimental model of vascular dysfunction. Conclusions: T-bet deficiency partially protects from ATII induced vascular dysfunction and oxidative stress by switching a proinflammatory Th1 to a Th2 and Treg T cell phenotype. We conclude, that T-bet mediates, at least in part, ATII induced vascular damage and might represent a novel target to treat vascular dysfunction and inflammation in arterial hypertension.

Role of VE-cadherin tyrosine phosphorylation for the control of endothelial cell contacts

Florian Wessel*, Astrid Nottebaum, Alexander Zarbock, Ruth Linnepe, and Dietmar Vestweber. Max-Planck-Institute of Molecular Biomedicine, Muenster, Germany

The regulation of endothelial cell contacts is important for the control of the barrier function of the endothelium and Vascular Endothelial Cadherin (VE-cadherin) is crucial for the stability and regulation of endothelial junctions. We believe that VE-cadherin and signaling processes that affect VE-cadherin are key for the regulation of leukocyte extravasation and the control of induced vascular permeability. Our group has established the receptor-type Vascular Endothelial Protein Tyrosine Phosphatase (VE-PTP) as a major regulator of VE-cadherin function and endothelial cell contact stability. Since VE-PTP associates with VE-cadherin and leukocytes as well as VEGF trigger the dissociation of VE-PTP from VE-cadherin, it is likely that phosphorylation of tyrosine residues of components of the VE-cadherin-catenin complex is involved in the opening of endothelial junctions. In vitro studies revealed that two VE-cadherin tyrosine-to-phenylalanine replacement mutants (Y658F and Y731F) inhibit the transmigration of monocytes through primary human endothelial cells (Allingham et al. J. Immunol. 2007, 179: 4053). However, a study by Turowski et al. (J. Cell Sci. 2008, 121:29) reproduced only one of these mutations (Y731F) to inhibit transmigration whereas the Y658F mutation seemed to have no inhibitory effect. Both mutations were also reported to affect the regulation of endothelial permeability in vitro. A third tyrosine (Y685) had been reported to be a major site for VEGF induced VE-cadherin phosphorylation, but seemed to be irrelevant for leukocyte transmigration. To investigate whether and if so, which tyrosine residues within the cytoplasmic tail of VE-cadherin are indeed relevant for the regulation of endothelial junctions in vivo, we generated various knock in mice where wt VE-cadherin was replaced by the respective Y/F point mutated version of VE-cadherin (Y658F, Y685F, or Y731F). All three mouse lines are viable and fertile. Interestingly, different tyrosine residues were relevant for the regulation of vascular permeability and for leukocyte extravasation. Based on the generation of antibodies specific for the different tyrosine phosphorylation sites in VE-cadherin we also found that phosphorylation of these tyrosine residues is differentially regulated in vivo and in vitro. We conclude that tyrosine phosphorylation of VE-cadherin is indeed an essential step in the regulation of leukocyte extravasation and vascular permeability in vivo, but the regulation and function in the control of endothelial junctions in vivo is more complex than would be expected from previously published in vitro results.

Protease-activated receptors 2 and 4 differentially utilise cyclo-oxygenase-2 activity and AMP kinase to regulate the pro-angiogenic functions of human endothelial cells

E Garonna[1], C Farrar[1]*, A McSloy[1], N Kirby[2], E Paleolog[1] and CPD Wheeler-Jones[1].
[1]Department of Basic Sciences, Royal Veterinary College, Royal College Street, London, U.K., [2]William Harvey Research Institute Barts and The London, Queen Mary's School of Medicine and Dentistry, London, United Kingdom

Protease-activated receptors (PAR1-4) are a novel family of G protein-coupled receptors implicated in inflammatory signalling in a range of vascular cell types and have emerging, but ill-defined roles as regulators of the angiogenic functions of endothelial cells (EC). In the present study we have investigated the molecular control of PAR-mediated angiogenesis. PAR-2-(2-furoyl(2f)-LIGRLO) and PAR-4 (AYPGKF)-selective agonist peptides (PAR-2-AP; PAR-4-AP) promoted HUVEC tubulogenesis on Matrigel, increased sprouting from EC spheroids in vitro and stimulated neo-angiogenesis in vivo (chick CAM assay). Activation of PAR-2, but not PAR-4, increased COX-2 expression and enhanced synthesis and release of 6-keto-PGF1alpha and PGE2. Blockade of COX-2 (but not COX-1) activity reduced PAR-2-mediated angiogenesis in vivo and siRNA knockdown of COX-2 or treatment with COX-2-selective NSAIDs (NS398; rofecoxib), abrogated PAR-2-AP-stimulated EC differentiation which was rescued by exogenous iloprost or PGE2. PAR-2-AP increased Akt phosphorylation and pharmacological inhibitors of PI3-kinase (LY294002) and Akt1 and 2 (Akti1/2) attenuated PAR-2-mediated COX-2 induction and PAR-2-driven angiogenesis. PAR-2-AP retained its ability to promote phosphorylation of GSK3beta and to enhance COX-2 expression in cells depleted of Akt1 expression using siRNA. Preincubation with inhibitors of PI3-K gamma (AS605240) or mTOR (rapamycin; torin 2) had no significant effect on PAR-2- or VEGF-stimulated COX-2 expression. Further experiments showed that PAR-2-AP and PAR-4-AP stimulated rapid phosphorylation of AMPK and acetyl-CoA carboxylase; PAR-4-driven phosphorylation of AMPK and PAR-4-mediated tubulogenesis were decreased in the presence of a PAR-4 antagonist peptide and blockade of AMPK attenuated PAR-4 and PAR-2-stimulated EC differentiation without inhibiting the effects of PAR-2-AP on COX-2 expression. These results define a role for COX-2-derived prostanoids in the autocrine regulation of EC functions mediated by PAR-2 activation and additionally identify AMPK as a key intermediary coupling both PAR-4 and PAR-2 to EC angiogenic functions in a COX-2-independent manner.

Syndecan extracellular core proteins have anti-angiogenic properties

Giulia De Rossi, Sussan Nourshargh and James R. Whiteford*. William Harvey Research Institute, Barts and The London School of Medicine & Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, United Kingdom

Syndecans are multifunctional heparan sulphate proteoglycans with roles in cell adhesion, migration and growth factor interactions. ECM molecules, inflammatory mediators and growth factors interact with syndecan HS chains and this is central to syndecan functionality. In addition, syndecans also have adhesion regulatory domains contained within their extracellular core proteins. These domains have been shown to regulate cell adhesion and migration. We have previously characterised the adhesion regulatory properties of the ectodomains of syndecan-2 and -4 on fibroblasts (1). We have also shown that the ectodomain of syndecan-2 stimulates cell adhesion through the protein tyrosine phosphatase receptor CD148 signalling to β1 integrin via a Src and PI3 kinase dependant pathway (2). The aim of the present work was to investigate the potential effects of the extracellular core proteins of all four syndecan family members on endothelial cell function particularly with regard to angiogenesis. We examined the effect of exogenously added bacterially expressed syndecan ectodomain fusion proteins on EC migration using invasion and 'scratch wound assays'. In addition we incorporated these proteins into Collagen I matrices to see their effect on vessel outgrowth from rat aortic ring explants. We report that all four syndecan extracellular core proteins exert an anti-angiogenic effect. Vessel outgrowth from aortic rings is significantly reduced in the presence of syndecan-1,-2,-3 and -4 ectodomain fusion proteins. All four proteins significantly inhibit EC migration in both 2D and 3D culture systems. We also show that the core proteins do not affect VEGF signalling in ECs but studies with mutant syndecan-2 and -4 ectodomain constructs suggest that the inhibition of angiogenesis and EC migration occurs through receptor crosstalk pathways leading to changes in integrin behaviour. These observations indicate an important role for shed syndecan core proteins in regulating angiogenesis. 1. Whiteford JR, Behrends V, Kirby H, Kusche-Gullberg M, Muramatsu T & Couchman JR. (2007). Syndecans promote integrin-mediated adhesion of mesenchymal cells in two distinct pathways. Exp Cell Res. 313, 3902-13. 2. Whiteford, J.R., Xian, X., Chaussade, C., Vanhaesebroeck, B., Nourshargh, S. & Couchman, J.R. (2011). Syndecan-2 is a novel ligand for the protein tyrosine phosphatase receptor CD148. Mol Biol Cell. 22, 3609-24. This work was supported by funds from Arthritis Research UK and the William Harvey Research Foundation (to JRW) and The Wellcome Trust (to SN).

Micro RNA-146a and its role in endothelial cells during vascular remodeling processes

Rebecca Widmer-Teske*, Wiebke Bielenberg, Jan-Marcus Daniel, Christian Troidl, Holger Nef, Stephan Achenbach, Christian Hamm, Daniel Sedding. Dept. of Cardiology/Angiology Giessen University, Giessen, Germany

Background: MicroRNAs (miRNAs) are a new class of small noncoding RNA molecules, comprising key regulators for major cellular events including proliferation, differentiation and apoptosis. Targeting miRNAs that influence functions in cells of the human vasculature like endothelial cells may offer an interesting approach for the prevention or treatment of vascular proliferative diseases. Methods/Results: Using microarray based expression analysis, we screened for regulated miRNAs during neointima formation. Restenosis was induced in C57BL6/N by dilation of the femoral artery, and miRNA was isolated 10 and 21 days after injury. About 59% of all known miRNAs was found to be aberrantly regulated after 10 days what was even enhanced to 88% after 21 days. Noticeably, miR-146a appeared to be one of the most regulated miRNAs during restenosis. Analysis on isolated cells of the human vasculature like monocytes/macrophages, smooth muscle cells and endothelial cells showed a strong expression of miR-146a, especially in endothelial cells. In vitro, the upregulation of miR-146a could be attributed to the inflammatory stimulus Il-1β. To further assess the functional role of miR-146a after its overexpression, endothelial cells were transfected with the precursor form of miR-146a that led to an attenuated migr by NFκB. In complementing in vivo experiments, inhibition of miR-146a following dilation of the femoral artery was performed. The data of Evan's Blue- and vWF staining showed significantly enhanced reendothelialization after 10 and 21 days. Conclusion: Determining the expression profile of differentially regulated miRNAs in restenosis development, we identified miR-146a likely involved in the disease development and ation, sprout formation and vessel network formation. On the other hand, using 2'-O methylated RNA targeting miR-146a as inhibitor, sprout formation, vascular network formation and cell migration were significantly enhanced. In the following, computational miRNA target prediction, the „TargetScan database", was used to find potential target genes for miR-146a. Quantitative Real-Time-RT-PCR tests were performed after overexpression of miR-146a. The transcripts for TRAF6 and IRAK1, two key adapter molecules in TLR- and IL-1 receptor signaling cascades, were significantly downregulated and hence represent molecular targets for miR-146a. Further in vitro analysis showed that miR-146a induction seems to be mediated progression and could further assess the impact of miR-146a. Thus, these observations add substantially to our understanding of the impact miRNAs have on vascular proliferative diseases.

S1P carrier-dependent regulation of endothelial barrier: HDL-S1P prolongs endothelial barrier enhancement as compared to albumin-S1P via effects on levels, trafficking and signaling of S1P1

Brent A. Wilkerson*, G. Daniel Grass, Shane B. Wing, W. Scott Argraves and Kelley M. Argraves Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425, USA

Sphingosine-1-phosphate (S1P) is a blood borne lysosphingolipid that acts to promote endothelial cell (EC) barrier function. In plasma, S1P is associated with both high density lipoproteins (HDL) and albumin, but it is not known whether the carriers impart different effects on S1P signaling. Here we establish that HDL-S1P sustains EC barrier longer than albumin-S1P. We showed that the sustained barrier effects of HDL-S1P are dependent on signaling by the S1P receptor, S1P1, and involve persistent activation of Akt and eNOS. Total S1P1 protein levels were found to be higher in response to HDL-S1P treatment as compared to albumin-S1P, and this effect was not associated with increased S1P1 mRNA or dependent on de novo protein synthesis. Several pieces of evidence indicate that long term EC barrier enhancement activity of HDL-S1P is due to specific effects on S1P1 trafficking. First, the rate of S1P1 degradation, which is proteasome mediated, was slower in HDL-S1P treated cells as compared to cells treated with albumin-S1P. Second, the long-term barrier promoting effects of HDL-S1P were abrogated by treatment with the recycling blocker, monensin. Finally, cell surface levels of S1P1 and levels of S1P1 in caveolin-enriched microdomains were higher after treatment with HDL-S1P compared to albumin-S1P. Together, the findings reveal S1P carrier-specific effects on S1P1 and point to HDL as the physiological mediator of sustained S1P-S1P1-dependent EC barrier function.

Wnt2 stimulates smooth muscle cell migration

Helen Williams* and Sarah J George. University of Bristol, United Kingdom

Increased levels of vascular smooth muscle cell (VSMC) migration lead to neointimal thickening. This process causes restenosis following angioplasty and stent implantation and vein graft failure after coronary artery bypass grafting. By investigating factors involved in promoting VSMC migration we hope to improve therapies to reduce VSMC migration and therefore improve the longevity of these treatments. The Wnts are a family of proteins best known for their role in embryological development and cancer and are known to be involved in migration of various cell types. The aim of this study was to see which of the Wnts are involved in VSMC migration. We used both an in vitro (scratch wound assay) and in vivo (left carotid ligation) wounding assay to study migration of VSMCs following wounding. We also analysed sections of human coronary arteries with and without neointimal thickening. Using a Wnt pathway array we showed that Wnt-2 mRNA was increased by 2.4±0.35 fold (P=0.048, n=3 arrays, each from cells of 2 or 3 mice) in mouse VSMCs stimulated to migrate by PDGF and wounding. We confirmed this increase using QPCR (2.8±0.88 fold increase in Wnt-2 mRNA, P=0.02, n=6). This increase in mRNA led to a concomitant rise in Wnt-2 protein of 163±9.24% (P=0.02, n=3) as shown by Western blotting. In mouse vessels wounded using carotid ligation we also observed an increase in Wnt-2 protein expression using immunohistochemistry. Addition of recombinant Wnt-2 protein produced a 106±38.9% increase in cell migration, while knocking down Wnt-2 by SiRNA led to a 41.7±11.3% reduction in cell migration ($P<0.05$ ANOVA, n=3). Addition of the Wnt-2 protein also increased levels of WISP-1 (Wnt inducible soluble protein 1) mRNA by 1.67±0.14 fold n=4, P=0.016. It was also observed that knock down of Wnt-2 led to a significant reduction in WISP-1 mRNA (64.8±6.13%, n=3, P=0.008). Addition or knock down of WISP-1 stimulated or inhibited migration in a similar way to Wnt-2, but the effects of WISP-1 and Wnt-2 were not additive, indicating that the effect of Wnt-2 on migration of cells may be via WISP-1. We then investigated whether Wnt-2 and WISP-1 were colocalised in human carotid arteries with neointimal thickening. Not only did Wnt-2 and WISP-1 appear to be present in similar areas of the media and intima in these lesions, but the correlation between levels of Wnt-2 and WISP-1in the media of vessels was significant ($r=0.647$, $r2=0.419$, n=13 $P<0.02$). This study shows that Wnt-2 promotes VSMC migration via upregulation of WISP-1 and may be responsible for enhanced VSMC migration and neointimal thickening in vivo. Design of novel treatments to inhibit Wnt-2 and/or WISP-1 may be useful for the reduction of restenosis.

Role of ALCAM in lymphatic endothelial cell biology

Maria Iolyeva, Ann-Helen Willrodt*, Stephanie Weingartner, Cornelia Halin Winter. ETH Zurich, Switzerland

Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166) is an adhesion molecule of the immunoglobulin superfamily, which is expressed on neurons, leukocytes, blood vascular endothelial cells and certain tumor cells. ALCAM engages into homophilic interactions, but also heterophilic interactions with CD6, L1CAM or Galectin-8 have been reported. Performing gene expression analyses of ex vivo sorted and in vitro cultured lymphatic endothelial cells (LECs) we have recently detected expression of ALCAM in this cell type. ALCAM expression was confirmed at the protein level on in vitro cultured LECs by FACS analysis and by Western blot. In addition, immunofluorescence performed on mouse and human lymph nodes and on human skin detected ALCAM expression on lymphatic vessels in vivo. To address the role of ALCAM expression in LECs, we performed functional in vitro assays using an ALCAM blocking antibody. These studies revealed that ALCAM was involved in distinct cellular processes in LECs, such as LEC-LEC adhesion, migration and tube formation, indicating a role for ALCAM in lymphatic vessel biology. In agreement with previous reports, ALCAM was also detected on murine and human dendritic cells (DCs). ALCAM proved to be important for the interaction of DCs with lymphatic endothelium: Blockade of ALCAM significantly reduced the adhesion of DCs to LEC monolayers. Furthermore, the transmigration of DCs generated from the bone marrow of ALCAM-/- mice through murine LEC monolayers was significantly reduced compared to transmigration of wild-type DCs. Besides mediating leukocyte migration, lymphatic vessels also play a major role in tumor cell metastasis. Upregulation of ALCAM in melanoma cells and other cancers reportedly correlates with metastastatic spread. We therefore investigated whether ALCAM could support tumor cell - LEC interactions. The presence of an ALCAM blocking antibody significantly reduced the adhesion of ALCAM-expressing melanoma cells to LEC monolayers. These data for the first time document the expression of ALCAM on LECs and provide first insights into the functional relevance of ALCAM expression in lymphatic vessels.

Endogenous soluble VEGFR-2 in embryonic development and in neuroblastoma progression

Jörg Wilting[1]*, Jürgen Becker[1], Christina Zelent[1], Johanna Fröhlich[1], Herbert A. Weich[2], Jayakrishna Ambati[3]. [1]Center of Anatomy, Department of Anatomy and Cell Biology, University Medicine Goettingen, Goettingen, Germany, [2]Div. Molecular Biotechnology, Helmholtz Centre for Infection Research, Braunschweig, Germany, [3]Department of Ophthalmology and Visual Sciences, University of Kentucky, Lexington, Kentucky, USA

Several years ago, endogenous pro-lymphangiogenic factors have been detected. These are Vascular Endothelial Growth Factor-C (VEGF-C) and VEGF-D. They bind and activate VEGF receptor (R)-3, a transmembrane receptors of lymphatic endothelial cells (LECs). The proteolytically processed form of VEGF-C also possesses affinity, though weaker, to VEGFR-2, which also is a transmembrane receptors of LECs and BECs. An endogenous anti-lymphangiogenic variant of the VEGF family has been detected very recently. Here we describe an endogenous secreted (soluble) splice variant of VEGFR-2, called esVEGFR-2, which is produced by alternative splicing in various cell types, e.g. in the corneal epithelium, epidermis, leukocytes and adrenal medulla. The knock-out of esVEGFR-2 induces i) the growth of lymphatics into the cornea of early postnatal mice and ii) hyperplasia of dermal lymphatics. EsVEGFR-2 binds VEGF-C but not VEGF-A, inhibits VEGF-C-induced activation of VEGFR-3 and reduces proliferation of human lymphangioma-derived LECs, indicating its potential use as a therapeutic for lymphatic malformations. Down-regulation of esVEGFR-2 in progressed neuroblastoma (NB), an embryonic tumour derived from sympathetic ganglia and the adrenal medulla, and characterized by lymphogenic spread of tumor cells, indicates a function for esVEGFR-2 in tumour lymphangiogenesis. This is also supported by the up-regulation of esVEGFR-2 in differentiating neuroblasts in vitro and high expression in differentiating, low grade NB in vivo. We present the first endogenous anti-lymphangiogenic VEGF family member, and discuss its functions in embryonic development and in disease.

A role for PLVAP in the regulation of endothelial permeability and angiogenesis

Joanna Wisniewska-Kruk[1]*, Ilse M.C. Vogels[1], Cornelis J.F. Van Noorden[1], Reinier O. Schlingemann[1,2], Ingeborg Klaassen[1]. [1]Ocular Angiogenesis Group, Department of Ophthalmology and Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands, [2]Department of Clinical and Molecular Ophthalmogenetics, The Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Endothelial cells of different vascular beds acquire specialized characteristics that are fine-tuned to specific organs. The outstanding example is the brain and the eye microvasculature, where highly differentiated endothelial cells maintain the blood-brain barrier (BBB) and the blood-retinal barrier (BRB) due to well developed cell-to-cell junctions, restricted caveolar transport, a series of specific transporters and cross-talk with astrocytes and pericytes. Loss of blood-retinal barrier (BRB) properties is an important feature in the pathology of diabetic retinopathy (DR). Up to date BRB dysfunction was mainly attributed to leaky junctions, however recent evidence highlights the importance of transcellular permeability as well. To reveal the impact of caveolar transport on the loss of BRB, we focused on plasmalemma vesicle associated protein (PLVAP, PV-1). PLVAP is an endothelium-specific integral membrane glycoprotein that is associated with both stomatal diaphragms of caveolae, transendothelial channels, as well as diaphragms of fenestrae. Whereas expression of PLVAP is completely absent from barrier endothelia of brain and eye, in pathological conditions, such as brain tumors, diabetic macular edema, and experimental VEGF-induced retinopathy, its expression co-localizes with barrier loss. PLVAP was inhibited by shRNA in primary Bovine Retinal Endothelial Cells (BRECs) that were cultured on transwell filters and stimulated with rhVEGF. Efficiency of silencing was confirmed by qPCR and ELISA analysis. Endothelial permeability was measured using fluorescently labeled dextrans of different sizes. The changes in endothelial junctions were visualized by immunocytochemistry and examined with confocal microscopy. The oxygen-induced retinopathy (OIR) model was used to study the changes in BRB permeability and neovascularization in mouse eyes injected with siRNA against PLVAP. FD70 was injected intracardial to visualize BRB leakage. In addition the effect of PLVAP inhibition on angiogenic sprouting was studied in the mouse aortic ring assay. Inhibition of PLVAP in vitro significantly reduced the permeability of 70 kDa dextran, which travels through the caveolar pathway, but not of small molecules (767Da), which are reported to travel through the paracellular pathway. Furthermore no significant changes in morphology of endothelial junctions were observed. In the OIR model, in which retinas of mice were injected with siRNA against PLVAP, decreased leakage of FD70 was found as well. Moreover, retinas in which PLVAP was silenced, were characterized by reduced vasculature, decreased number of neovascular tufts and increased avascular areas, suggestion a role of PLVAP in angiogenesis. In the aortic ring assay, silencing of PLVAP blocked angiogenic sprouting. Our results suggest an important role for PLVAP in as well angiogenesis as transcellular permeability. The inhibition of PLVAP seems to block caveolar transport, since permeability of high molecular dextrans was significantly reduced and that of small molecules was not. We will further investigate the role of PLVAP in angiogenesis and vascular permeability and whether it will be a successful therapeutic target in the treatment of diabetic retinopathy.

CD40L deficiency ameliorates diet-induced adipose tissue inflammation - but does not protect from insulin resistance and hepatic steatosis in mice

Dennis Wolf[1]*, Felix Jehle[1,2], Ingo Hilgendorf[1], Peter Stachon[1], Florian Willecke[1], Natalie Hoppe[1], Bianca Dufner[1], Alexandra Ortiz-Rodriguez[1], Nora Bukosza[1], Christoph Bode[1], Karlheinz Peter[2], Andreas Zirlik[1]. [1]Atherogenesis Research Group, Department of Cardiology, University of Freiburg, Freiburg, Germany, [2]Baker IDI Heart and Diabetes Institute, Melbourne, Australia, [3]Department of Laboratory Medicine, Medical University of Vienna and Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria

Background: Adipose tissue inflammation fuels the metabolic syndrome. We recently reported that CD40L - an established marker and mediator of cardiovascular disease - induces inflammatory cytokine production in adipose cells in vitro. Here, we tested the hypothesis that CD40L deficiency modulates adipose tissue inflammation in vivo. Methods and Results: WT or CD40L-/- mice consumed a high fat diet (HFD) for 20 weeks (n≥15 per group). Inflammatory cell recruitment was impaired in mice lacking CD40L as shown by a decrease of adipose tissue macrophages, B-cells, and an increase in protective T-regulatory cells. Mechanistically, CD40L-deficient mice expressed significantly lower levels of the pro-inflammatory chemokine MCP-1 both, locally in adipose tissue and systemically in plasma. Moreover, levels of pro-inflammatory IgG-antibodies against oxidized lipids were reduced in CD40L-/- mice. Accordingly, CD40L deficiency partially protected from weight gain and fat deposition in the early stages of diet-induced obesity (DIO). Also, circulating low-density lipoproteins and insulin levels were lower in CD40L-/- mice. However, CD40L-/- mice consuming HFD were not protected from the onset of insulin resistance and hepatic steatosis, suggesting that CD40L selectively limits the inflammatory features of diet-induced obesity rather than its metabolic phenotype. Interestingly, CD40L-/- mice consuming a low fat diet (LFD) showed both, a favorable inflammatory and metabolic phenotype characterized by diminished weight gain, improved insulin tolerance, and attenuated plasma adipokine levels. Conclusion: We present the novel finding that CD40L deficiency limits adipose tissue inflammation in vivo. These findings identify CD40L as a potential mediator at the interface of cardiovascular and the metabolic disease.

Insulin resistance at the endothelial cell level is a pathogenic link between psoriasis and its cardiovascular co-morbidities

Kathrin Woth*, Katja Hardt, Sandra Diehl, Claudia Buerger, Wolf-Henning Boehncke. J.W. Goethe Universityhospital, Frankfurt am Main, Germany

Psoriasis, a chronic inflammatory disease, appears mainly on the skin, but is associated with severe co-morbidities such as diabetes or coronary atherosclerosis. The common denominator of these co-morbidities is insulin resistance. Pathomechanisms in endothelial cells leading to the manifestation of the cutaneous phenotype on one hand and to the co-morbidities on the other hand are not sufficiently investigated. Due to the similarities between a psoriatic and an atherosclerotic plaque, we hypothesize that the underlying mechanism and pathways that are known to play a role in the development of atherosclerosis also contribute to the pathogenesis of psoriasis and its co-morbidities By measuring insulin-dependent PKB phosphorylation in primary endothelial cells (HDBEC [human dermal blood endothelial cells]), we could show that a mix of „psoriatic“ cytokines (interleukin (IL)-1beta, IL-17, IL-22, IL-23 and TNFα) induces insulin resistance. Moreover we could elucidate which signaling components are involved in mediating insulin resistance by using chemical inhibitors. The expression of adhesion molecules such as E-Selectin and ICAM-1 is repressed by insulin and altered under conditions of insulin resistance. Therefore we suggest that insulin is not only cardio protective, but as well anti-inflammatory and that under conditions of systemic inflammation as in psoriasis the disturbed insulin response contributes to the pathogenesis of psoriasis and its co-morbidities. Thus therapeutic approaches interfering with the altered insulin response in psoriasis might by very effective by targeting both the dermal as well as the cardiovascular dimension of psoriasis.

High density lipoproteins inhibit vascular endothelial inflammation by increasing 3β-hydroxysteroid-Δ24 reductase expression and inducing heme oxygenase-1

Ben J. Wu*, Kang Chen, Kwok L. Ong, Philip J. Barter and Kerry-Anne Rye. Lipid Research Group, Heart Research Institute, Sydney, Australia

Discoidal reconstituted high density lipoproteins containing apolipoprotein A-I (apoA-I) and phosphatidylcholine, (A-I)rHDL, inhibit inflammation in vitro by increasing 3β-hydroxysteroid-Δ24 reductase (DHCR24) expression. Heme oxygenase-1 (HO-1) is a protein that protects against atherosclerosis. This study asks if HDL increase DHCR24 expression by inducing HO-1. In vivo: A single iv infusion of lipid-free apoA-I (8 mg/kg) administered to normocholesterolemic NZW rabbits 24 h prior to insertion of a non-occlusive carotid collar decreased collar-induced endothelial expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and intima/media neutrophil infiltration. It also increased DHCR24 and HO-1 mRNA levels in the collared arteries. Local knockdown of DHCR24 and HO-1, and treatment with the HO-1 inhibitor, tin protoporphyrin-IX, SnPP, prevented apoA-I from reducing vascular inflammation. In vitro: Human coronary artery endothelial cells (HCAECs) were pre-incubated for with PBS or (A-I)rHDL then stimulated with tumor necrosis factor (TNF)-ï¡. Pre-incubation with (A-I)rHDL inhibited TNF-ï¡-induced VCAM-1 and ICAM-1 expression, and increased DHCR24 and HO-1 mRNA levels 5.7-fold and 10.5-fold, respectively. These anti-inflammatory effects were abolished in HCAECs transfected with DHCR24- and HO-1-siRNA, and with SnPP treatment. Induction of HO-1 by (A-I)rHDL was reduced in HCAECs transfected with DHCR24-siRNA. The (A-I)rHDL-mediated increase in DHCR24 expression was unaffected in HCAECs transfected with HO-1-siRNA or treated with SnPP. Incubation with the PI3K/Akt-specific inhibitor LY294002 inhibited the (A-I)rHDL-mediated increase in HO-1, but not DHCR24 expression. The ability of (A-I)rHDL to increase PI3K/Akt signalling was inhibited in HCAECs transfected with DHCR24 siRNA. HDL inhibit inflammation by increasing DHCR24 expression and inducing HO-1.

PAR-2 inhibition reverses experimental pulmonary hypertension

G. Kwapiszewska[1,2], P. Markart[1], B.K. Dahal[1], B. Kojonazarov[1], L.M. Marsh[2], R.T. Schermuly[1], C. Taube[3], A. Meinhardt[4], H.A. Ghofrani[1], M. Steinhoff[6], W. Seeger[1], K.T. Preissner[5], A. Olschewski[2], N. Weissmann[1], M. Wygrecka[5]*. [1]Department of Internal Medicine, [4]Anatomy and [5]Biochemistry, University of Giessen Lung Centre, Giessen, Germany, [2]Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria, [3]III Medical Clinic, Johannes Gutenberg-University Mainz, Mainz, Germany, [6]Department of Dermatology and Surgery, University of California San Francisco, UCSF, San Francisco, CA, USA

Rationale: A hallmark of the vascular remodeling process underlying pulmonary hypertension (PH) is the aberrant proliferation and migration of pulmonary arterial smooth muscle cells (PASMC). Accumulating evidence suggests that mast cell mediators play a role in the pathogenesis of PH. Objective: In the present study we investigated the importance of protease-activated receptor (PAR)-2 and its ligand mast cell tryptase in the development of PH. Methods and Results: Our results revealed strong increase in PAR-2 and tryptase expression in the lungs of idiopathic pulmonary arterial hypertension (IPAH) patients, hypoxia-exposed mice, and monocrotaline (MCT)-treated rats. Elevated tryptase levels were also detected in plasma samples from IPAH patients. Hypoxia and PDGF-BB upregulated PAR-2 expression in PASMC. This effect was reversed by HIF (hypoxia inducible factor)-1αdepletion, PDGF-BB neutralizing antibody or the PDGF-BB receptor antagonist Imatinib. Attenuation of PAR-2 expression was also observed in smooth muscle cells of pulmonary vessels of mice exposed to hypoxia and rats challenged with MCT in response to Imatinib treatment. Tryptase induced PASMC proliferation and migration as well as enhanced synthesis of fibronectin and matrix metalloproteinase-2 in a PAR-2- and ERK1/2-dependent manner suggesting that PAR-2-dependent signaling contributes to vascular remodeling by various mechanisms. Furthermore, PAR-2-/- mice were protected against hypoxia-induced PH and PAR-2 antagonist application reversed established PH in the hypoxia mouse model. Conclusions: Our study identified a novel role of PAR-2 in vascular remodeling in the lung. Interference with this pathway may offer novel therapeutic options for the treatment of PH.

CXCR4a is required for blood vessels formation during fin regeneration in zebrafish

Cong Xu* and Arndt Siekmann. Lab for Cardiovascular Patterning, Max-Planck-Institute for Molecular Biomedicine, 48149 Muenster, Germany

Postnatal neo-angiogenesis is required during both physiological and pathological conditions, such as the female reproductive cycle, wound healing and tumorigenesis. However, the mechanisms underlying this process still remain largely unknown. We use zebrafish fin as a model to study neo-angiogenesis during fin regeneration, which is a remarkable capability of teleosts after fin amputation. As an entry point, we compared neo-angiogenesis to developmental angiogenesis using Tg(fli1:EGFP; flt1enh:tdTomato) transgenic fish in which the EGFP is expressed in all vessels while the tdTomato expression is higher in arteries. Furthermore, we are aiming at understanding the molecular pathways involved in neo-angiogenesis. Previous studies have documented that CXCR4a chemokine signaling is essential for angiogenesis in the embryo. Analysis of zebrafish cxcr4a-/- mutants showed specific defects in the patterning of vessels in both angiogenesis and neo-angiogenesis settings. Surprisingly, homozygous mutant of cxcl12b which is the ligand of cxc4a in zebrafish did not phenocopy the defects of cxcr4a-/- adult fish. Our work shows that zebrafish is an excellent model for studying neo-angiogenesis. Our results so far indicate that CXCR4a signaling pathways are indispensible for develomental angiogenesis as well as neo-angiogenesis in zebrafish.

Tie1 and Tie2 regulate blood vessel formation in a mutually dependent manner

Daishi Yamakawa*, Hiroyasu Kidoya, Susumu Sakimoto, Weizhen Jia and Nobuyuki Takakura. Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Japan

Endothelial specific receptor tyrosine kinase Tie2 has distinct roles by cell condition. Under cell-cell contact, Tie2 induces PI3K-Akt activation strongly for vascular stabilization. On the other hand, Tie2 induces ERK activation strongly under cell-matrix contact for angiogenesis. However, it is unknown how activation of these signaling molecules is altered by Tie2. We focused on Tie1, orphan receptor that belongs to Tie family. Previous reports have shown that Tie1 is trans-activated by Tie2 phosphorylation and Tie2 signal is enhanced in Tie1-silenced endothelial cell (EC). Therefore, we investigated Tie1 function as a negative regulator of Tie2 activation. To analyze Tie2-Tie1 interaction, we used Bimolecular Fluorescence Complementation assay and isolated the dimerization domain of Tie2. Insertion of Tie2 dimerization domain into Tie1 enhanced dimerization and phosphorylation of Tie1. Interestingly, Tie1 phosphorylation activated p38 and suppressed Tie2-dependent ERK phoshorylation. In the mouse tissue, mural cell uncovered pro-angiogenic EC had little Tie1 expression but mural cell covered mature EC had high expression of Tie1 protein. Therefore, we considered that Tie1 protein expression was regulated by angiogenic stimulation. In fact, PMA (PKC activator) stimulation, which induce endothelial tube formation, promote Tie1 ectodomain shedding in short-term through ERK-dependent MMP activation. ã€€Consequently, we found that Tie1 regulates Tie2-dependent angiogenic signal through p38 for maturation of blood vessels. We propose that shift from ERK to Akt in stable EC is mediated through Tie1-dependent p38 pathway.

Biglycan is a specific marker and an autocrine angiogenic factor of tumor endothelial cells

Kazuyuki Yamamoto[1,2]*, Noritaka Ohga[1], Yasuhiro Hida[3], Nako Maishi[1], Taisuke Kawamoto[1], Kazuko Kitayama[1], Kosuke Akiyama[1], Takahiro Osawa[1], Miyako Kondoh[1], Kichizo Kaga[3], Nobuo Shinohara[4], Masanobu Shindoh[5], Satoshi Hirano[2], Kyoko Hida[1].
[1]Department of Vascular Biology, Graduate School of Dental Medicine, [2]Department of, Gastroenterological Surgery II, Graduate School of Medicine, [3]Department of, Cardiovascular and Thoracic Surgery, Graduate School of Medicine, [4]Department of Renal and Genitourinary Surgery, Graduate School of Medicine, [5]Department of Oral Pathology and Biology, Graduate School of Dental Medicine, University of Hokkaido, Sapporo 060-8586, Hokkaido, Japan

Tumor angiogenesis is necessary for solid tumor progression and metastasis. Tumor blood vessels are morphologically different from their normal counterparts. We isolated tumor endothelial cells (TECs), demonstrated their abnormalities, compared gene expression profiles of TECs and normal endothelial cells (NECs) by microarray analysis and identified several genes upregulated in TECs. We focused on the gene encoding biglycan, a small leucine-rich repeat proteoglycan. Biglycan is strongly expressed in inflammatory and fibrotic tissue. A recent study has shown that the proteoglycans contribute to tumor progression. However, no report is available on biglycan expression or function in TECs. We investigated function of biglycan in TECs, which were isolated from tumors. Real-time PCR, western blotting and immunocytochemistry revealed higher biglycan expression levels in TECs than in NECs. Furthermore, we confirmed that biglycan was secreted from TECs. Biglycan knockdown inhibited cell migration and tube formation in TECs. TLR2 and TLR4 are the biglycan receptors. TLR2 and TLR4 blocking antibodies suppressed biglycan mediated cell migration and tube formation. Furthermore, immunostaining revealed strong biglycan expression in vivo in several human tumor vessels, as in mouse TECs. Biglycan was detected in the sera of cancer patients but was hardly detected in those of healthy volunteers. Biglycan expression was induced in NECs by tumor conditioned medium (CM). Tumor CM contained a significantly high level of TGF-b2. Biglycan was upregulated in NECs by TGF-b2. Thus, TGF-b2 secreted from tumor might be one of the factors causing biglycan upregulation in ECs. In conclusion, biglycan is an autocrine angiogenic factor stimulating of tumor endothelial cell migration and tube formation. These findings suggested that biglycan is a novel TEC marker and a target for anti-angiogenic therapy.

Inflammatory endothelial microparticles (EMPs) contribute to cellular interaction as a bioactive carrier

Seiji Yamamoto[1]*, Erika Azuma[1,2], Tsutomu Yanagibashi[3], Masashi Ikutani[3], Yoshinori Nagai[3], Kiyoshi Takatsu[3,4], Kenji Miyazaki[1], Mitsuko Dohmoto[5], Masashi Muramatsu[6,7], Naoyuki Matsuda[8], Shumpei Niida[6], Yuichi Hattori[1]. [1]Department of Molecular and Medical Pharmacology, University of Toyama, Toyama, Japan, [2]Astellas Pharma Tech Co., Ltd. Toyama Technology Center, Toyama, Japan, [3]Department of Immunobiology and Pharmacological Genetics, University of Toyama, Toyama, Japan, [4]Toyama Prefectural Institute for Pharmaceutical Research, Toyama, Japan, [5]Kanazawa Institute of Technology, Genome Biotechnology Laboratory, Ishikawa, Japan, [6]Laboratory of Genomics and Proteomics, National Center for Geriatrics and Gerontology, Aichi, Japan, [7]Department of Cancer Genetics, Roswell Park Cancer Institute, NY, USA, [8]Department of Emergency and Critical Care Medicine, Nagoya University, Nagoya, Japan

Recently, several lines of experimental evidence have shown that endothelial microparticles (EMPs) were significantly increased in the circulating blood due to inflammatory diseases such as metabolic syndrome, preeclampsia and sepsis. However, production mechanism of the EMPs from inflamed endothelial cell membrane, and the nature of the EMPs are largely unknown. To address these issues, we developed in vitro quantitative EMPs production system using an endothelial cell line. When endothelial cells were subjected cytokine combination including TNF-α, IL-1β and IFN-γ with lipopolysaccharide (CytoCombo+LPS), inflammatory genes such as Il6 and Icam1 expressed and peaked in 3hrs. In this procedure, production of EMPs was successfully measured by flow cytometry, and EMPs significantly increased in 3hrs. Interestingly, miRNA array showed that CytoCombo+LPS altered drastically miRNA composition of the EMPs compared to that of unstimulated controls. Further analysis by scanning electron microscopy showed that EMPs shed from endothelial cell surface was observed immediately after stimulation by CytoCombo+LPS. We demonstrated that EMPs are produced by endothelial cells using inflammatory stimulants, such as CytoCombo+LPS, and are measured by flow cytometry quantitatively. MiRNA contained in EMPs may be altered by various constituents of the inflammatory stimuli. Our data suggest that EMPs act as the bioactive carrier in pathophysiological status, and miRNA contained in EMPs may potentially be the nature of the cellular interaction in inflammatory conditions.

Apurinic/apyrimidinic endonuclease (Ape1) in Endotherial Progenitor Cells (EPCs) has an important role in healing injured vascular wall through adhesion on the sites with much oxidative stress

Atsushi Yamauchi*, Jun-Ichi Kawabe, Maki Kabara, Akira Asanome, Motoki Matsuki, Naofumi Takehara, Naoyuki Hasebe. Asahikawa Rehabilitation Hospital, 2the department of cardiology Asahikawa Medical University

Introduction: Bone marrow derived endothelial progenitor cells (EPCs) are recruited at injured vascular walls and positively contribute to re-endothelialization, vascular repair. However, its effects are limited by cardiovascular risk factors such as aging. Ape1 is a multifunctional protein possessing both DNA-repair and controlling cellular response to oxidative stress. Ape1 reduce several important oxidized and inactivated transcription factor. We hypothesized that the expression of anti-oxidative protein, Ape1 in EPCs could function against unfavorable situation of inflammation-induced oxidative stress in vascular remodeling process. Methods and Results: EPCs were isolated from bone marrow (BM) of young (12-week old) or aged (1.5-year old) C57BL6 male mice. Among several cellular functions of EPCs, adhesion property was very sensitive to oxidative stress. Adhesion of EPCs to fibronectin was apparently inhibited by either H2O2 or TNFalpha in a dose dependent manner. In aged EPCs, adhesion ability was reduced to 46.0%of that in young EPCs, and further reduced to 24.7% in the presence of TNFalpha. EPCs were Ape1 expressing cells at high level among BM cells. The level of Ape1 mRNA in aged EPCs was significantly reduced by 10 fold compared to young EPCs. When Ape1 was further overexpressed in EPCs using recombinant adenovirus harboring Ape1 gene, H2O2-induced inhibition of adhesion ability was decreased compared to control, LacZ-expressing EPCs (75 ±12 % vs 49 ±8 %). In contrast, specific knock down of endogenous Ape1 by si-RNA further increased H2O2-induced inhibition of EPCs adhesion compared to control EPCs (32 ± 4 % vs 53 ± 9 %). In addition, E3330 which is a specific inhibitor of redox function in Ape1 did not inhibit the cell viability but decreased the ability of adhesion. Mouse femoral artery was injured by wire, and vascular neointimal thickness was estimated at 4 weeks after vascular injury. Neointimal thickness was significantly reduced by infusion of LacZ-expressing EPCs compared to the saline-infusion control (I/M ratio 1.6±0.2 vs 2.5±0.2), and was further reduced by infusion of Ape1-expressing EPCs compared to that of LacZ-EPCs (0.81±0.02 vs 1.6±0.2). Conclusion: Ape1 in EPCs is important to functions against oxidative stress and have their in vivo vascular repair effectively. Dysfunction of aged EPCs might be related to attenuated expression of Ape1 gene, and transplantation of Ape1-overexpressing EPCs may serve as a novel and useful therapeutic strategy.

Deficiency of plasma SCUBE1, a novel platelet adhesive protein, impairs thrombus stabilization and protects mice against thrombosis

Meng-Ying Wu, Yuh-Charn Lin, Wei-Ju Liao, Ruey-Bing Yang*. [1]Graduate Institute of Life Sciences, National Defense Medical Center; [2]Institute of Biomedical Sciences, Academia Sinica; [3]Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan

SCUBE1 (signal peptide-CUB-EGF domain-containing protein 1) is a novel secreted, membrane-associated protein expressed in platelet and endothelial cells. Our previous studies demonstrated that surface-expressed SCUBE1 acts as a novel adhesive molecule and plasma SCUBE1 is a potential biomarker of platelet activation in acute thrombotic diseases (J. Am. Coll. Cardiol, 51:2173, 2008). However, the precise pathophysiological role of plasma SCUBE1 in platelet biology remains largely unknown. In this study, we generated a new mutant mouse model with a targeted deletion of the Scube1 gene (named D allele) deficient in secretion of SCUBE1 protein into the circulation. At baseline, wild-type (WT) mice actively produced plasma SCUBE1 protein at concentration of 146 ng/ml, whereas production of the secreted form of SCUBE1 was eliminated (i.e., plasma SCUBE1 concentration was virtually undetectable) in the Scube1 D/D mice. The mutant mice have normal coagulation parameters, platelet counts and platelet expression of major signaling receptors. Whereas loss of secreted SCUBE1 does not prevent initial platelet aggregation, this deficiency impairs subsequent stabilization of platelet aggregates. Furthermore, isolated WT platelets failed to irreversibly aggregate in response to low concentration of platelet agonist in SCUBE1-deficient plasma. Conversely, addition of WT plasma or purified recombinant SCUBE1 protein improved aggregation of SCUBE1-deficient platelets. Importantly, the Scube1 D/D mice are significantly protected from ferric-chloride-induced arterial thrombosis but have only a mild prolonged tail-cut bleeding time. Our results demonstrated for the first time that plasma SCUBE1 functions as an important mediator of ischemic cardiovascular events by stabilizing thrombus formation, and suggested that plasma SCUBE1 may serve as a novel anti-thrombotic target.

Determination of platelet stimulation by ADP, Thrombin and Aspirin by evaluating the role of phosphatidylserine as an Annexin V binding molecule

Saman Yazdani[1]* Mohammad Reza Aghanoori[2] Yasaman Yazdani[3]. [1]Laboratory Medicine, Lund University, Sweden [2]Human Genetics, SUMS (Shiraz University of Medical Science), [3]SUMS (Shiraz University of Medical Science)

Background:Phosphatidylserine (PS) is a phospholipid and essential to the function of cells; it normally locates to the cytosolic side of the plasma membrane except in apoptotic cells. On the surface of apoptotic cells, a proportion of PS is exposed depending on the type and the nature of the stimuli to be recognized by phagocytes. The normal distribution of PS is altered during platelet stimulation and cellular apoptosis. PS modulates the activity of several enzymes involved in cellular signaling accelerating apoptosis. Class B scavengers, the thrombospondin receptor CD 36, CD 14, CD 68, β2 glycoprotein I, gas-6 and annexins have been proposed to mediate phosphatidyl-L-serine recognition. Annexin V can bind and polymerize PS through protein-protein interactions on membrane patches expressing PS. Type II Phosphatidylserine Decarboxylase (PSD), encoded by the PISD gene (22q12.2), converts PS into Phosphatidylethanolamine (PE). Methods: Fresh human platelets from platelet-rich plasma were activated by ADP (10, 20, 50,100 μM) and thrombin (0.01, 0.03, 0.10, 0.30, 1.00 units/ml) and inhibited by aspirin (1, 2, 5, 10 mM) in different concentrations by HEPES buffer respectively. By FACS, we detected a population of platelets exposing PS bound to fluorescein-labeled annexin V. By RT-PCR we evaluated the expression of the PISD gene in activated platelets, by measuring the relative amount of mRNA of PISD compared to the expression of the Actin housekeeping gene. Results: Successful activation and inhibition were confirmed by FACS which in average showed: 55.09%, 59.99%, 65.98%, 67.7% of population of ADP-activated platelets in different concentrations and 64.64%, 70.2%, 78.61%, 83.11%, 87.88% of population of thrombin-activated platelets in different concentrations, were exposing PS bound to fluorescein-labeled annexin V. On the other hand in average 43%, 40.01%, 39.6%, 33.77% of aspirin-inhibited platelets in different concentrations were exposing PS bound to fluorescein-labeled annexin V. By RT-PCR, relative amount of mRNA of PISD gene was measured indicating at least 32 and 41.1-43.5 fold increased expression of PISD gene following ADP and thrombin activation respectively. Discussion: The exposure of PS bound to fluorescein-labeled annexin V was augmented following ADP and thrombin activation and diminished following by Aspirin inhibition, in outer leaflet of platelets compared to quiescent platelets. Due to externalization of PS in stimulated platelets, PISD gene activity would increase to encode PSD to convert PS, as a mechanism to regulate PS level. Although no single gene producing PS was identified, however over expression of PISD gene can indirectly (instead of measuring the amount of PE on the surface of platelets prior and post stimulation) denote on over production of PS respecting phospholipid biosynthetic process. Evaluating the expression of PISD gene following by inhibition by aspirin, assumed to decrease, is suggested too. Although the relative expression of the PISD gene was evaluated by RT-PCR, however measuring ‘‘PSD β subunit“ (35.934 kD) enzyme, encoded by PISD gene, to observe activity by Western Blot or ELISA is highly recommended. Repeating FACS by using Milk fat globule-EGF factor 8 proteins (Mfge8) instead of annexin V, because of its higher affinity binding to PS, is also recommended.

Lymphadenectomy promotes tumor growth and cancer cell dissemination

Yeo Kim Pin*, Karen Khoo, Tan Karwai, Anne-Laure Puaux, Jean-Pierre Abastado, and Véronique Angeli. National University of Singapore, Singapore

Cancer cells disseminate through the hematogenous or the lymphatic route to colonize its tumor-draining lymph nodes (TDLNs) or region beyond. TDLNs can act as filters preventing cancer cell dissemination or it can serve as cancer cell reservoirs for further dissemination and metastasis though the primary tumor has been removed. In spite of limited convincing clinical benefits from randomized prospective trials, resection of TDLNs is a standard practice in several types of cancers including breast cancer and cutaneous melanoma. Hence, the role of TDLNs is complex and the effect of LN resection on cancer progression needs further evaluation. Using a spontaneous model of uveal melanoma, RET/AAD mouse, we found that mandibular and superficial parotid LNs drain the eyes. Growth of the primary eye tumor was accompanied by increased lymphangiogenesis in the mandibular lymph nodes while only peritumoral lymphatic vessels were observed in the tumor-bearing eyes. In addition, colonization of cancer cells in the draining lymph nodes is associated with the primary tumor size. We went on to remove both mandibular and superficial parotid LNs. Unexpectedly, early resection of the TDLNs increased tumor growth and cancer cell dissemination to the lungs and skin. Close examination of the primary tumor revealed increased primary tumor nodules as well as intratumoral blood vessel density. Here, using the spontaneous melanoma mouse model that closely mimic human cancer cases, we provide evidence that resection of TDLNs promotes cancer cell dissemination, tumor growth and metastasis.

Deleterious effect of IFN-β on pro-angiogenic cells is mediated by calpain1

Cansu Yildirim[1]*, Julie Favre[1], Ruud D. Fontijn[1], Reinier A. Boon[2], Michiel H. van Wijhe[3], Pieter Koolwijk[3], S. van Vliet[1], Tineke C.T.M. van der Pouw-Kraan[1] and Anton J.G. Horrevoets[1]. [1]Department of Molecular Cell Biology and Immunology, Institute for Cardiovascular Research (ICaR-VU), VU University Medical Center, Amsterdam, The Netherlands, [2]Institute for Cardiovascular Regeneration, Centre for Molecular Medicine, Goethe University, Frankfurt, Germany, [3]Department of Physiology, Institute for Cardiovascular Research (ICaR-VU), VU University Medical Center, Amsterdam, The Netherlands

Background Collateral artery formation termed arteriogenesis is an important natural process in the heart to rescue heart tissue from critical ischemia after a coronary artery obstruction. Circulating pro-angiogenic (CACs) are monocyte-derived cells with certain endothelial characteristics, which significantly contribute to collateral artery formation. During collateral artery formation, the CACs get educated by different growth factors and become angiogenic. Hence, CACs enhance collateral formation by secreting several angiogenic growth factors to the growing collateral vessels. Recently, we have shown that interferon-beta (IFN-ß) inhibits collateral artery formation in a murine hind limb model of arteriogenesis. We hypothesized that IFN-ß inhibits arteriogenesis by affecting CACs. Calpain (CAPN) is a cysteine protease which has been shown to modulate cell detachment, migration and induce apoptosis in many cell types. We also hypothesized that IFN-ß may induce the expression of CAPN leading to CAC detachment and/or apoptosis. In the present study, we explored the effect of IFN-ß and possible involvement of calpain on CACs during their differentiation and after differentiation, by analyzing the CAC number and function e.g. adhesion, and apoptosis. Methods CACs were obtained from peripheral blood mononuclear cells (PBMCs) by culturing them in endothelial specific growth media on fibronectin. Three days after seeding, the non-adherent cells were removed, and the adherent cells were characterized at day 4-7 as CACs by the uptake of acetylated LDL and binding of Ulex-lectin. To elucidate the effect of IFN-ß and calpain on CACs during their differentiation, PBMCs were directly stimulated with IFN-ß for three days with or without the chemical calpain 1 (CAPN1) inhibitor. For the effect of IFN-ß and calpain on CACs after the differentiation, CACs at day 3 were stimulated with IFN-ß for three days with or without CAPN1 inhibitor. After three days of stimulation, we determined the CAC numbers. Finally, the function of these cells was analyzed by performing FACS staining for apoptosis, and cell adhesion assays with analysis of integrin expression. Results Our results show that IFN-β slightly reduces CAC number during differentiation by ±20 %, whereas after their differentiation the effect of IFN-β is ±40%. IFN-β affected the CAC numbers by impairing the adhesion to fibronectin, without affecting apoptosis. Adhesion was dependent on α5β1 (VLA-5) integrin expression, however IFN-β did not decrease the expression levels of α5β1 integrins. Notably, IFN-β induced the expression of Calpain 1. The IFN-β induced reduction of differentiated CAC numbers was reversed by Calpain 1 inhibition. Conclusion Here we show that pharmacological inhibition of CAPN1 in differentiated CACs might increase the CAC number in the collateral vessels by stimulating their adhesion. In conclusion, inhibition of calpain activity in CACs from patients with bad collaterals may serve as a novel approach to stimulate coronary collateral artery growth.

Roles of BMP-9 signals in the lymphatic vessel formation

Yasuhiro Yoshimatsu*, Yuka Suzuki, Yuichi Akatsu, Yasuyuki Morishita, Tetsuro Watabe and Kohei Miyazono. Department of Molecular Pathology, Graduate School of Medicine, and the Global Center of Excellence Program for Integrative Life Science Based on the Study of Biosignaling Mechanisms, University of Tokyo, Bunkyo-ku, Tokyo, Japan and PRESTO, Japan Science Technology Agency, Kawaguchi, Saitama, Japan

Members of bone morphogenetic protein (BMP) family have been implicated in the formation of blood vessels. While members of BMP-2/4 and osteogenic protein-1 groups signal via activin receptor-like kinases (ALK)-2, -3, -6, BMP-9/10 signal via ALK-1. Of note, since circulating BMP-9 proteins in peripheral blood has been reported to be abundant and ALK-1 is one of the responsible genes for a vascular disorder, hereditary hemorrhagic telangiectasia, the physiological significance of BMP-9/ALK-1 signals is intriguing. We have previously reported that BMP-9/ALK-1 signals enhance the proliferation of blood vascular endothelial cells. However, the roles of BMP-9/ALK-1 signals in lymphatic vessel formation remain largely unknown. Here we examined the effects of BMP-9/ALK-1 signals on lymphangiogenesis both in vitro and in vivo. BMP-9 significantly inhibited the proliferation of human dermal lymphatic endothelial cells (HDLECs) in vitro. BMP-9 increased the subpopulation of HDLECs which underwent apoptosis, in accordance with decreased expression of anti-apoptotic BCLXL and mitogenic C-MYC. Importantly, we found that BMP-9 decreased the expression of Prox1, a transcription factor critical for the differentiation and maintenance of lymphatic endothelial cells, concomitantly with decreased expression of lymphatic markers such as VEGFR3 and with increased expression of blood vascular markers such as VEGFR2. Furthermore, in order to study the in vivo functions of BMP-9, we used BxPC3 human pancreatic cancer xenograft model. We observed the decreased formation of lymphatic vessels in tumors derived from BxPC3 cells lentivirally transduced with BMP-9 as compared with that from control BxPC3 cells. Taken together, these results suggest that BMP-9 inhibits lymphangiogenesis both in vitro and in vivo.

Laminin isoform expression and function in perivascular basement membranes

L Yousif[1]*, E Bakker[2], E Van Bavel[2], L Sorokin[1]. [1]Institute of Physiological Chemistry and Pathobiochemistry, University of Muenster, Germany, [2]Academic Medical Center, Amsterdam, The Netherlands

Vascular remodelling, the structural adaptation of the vessel wall, occurs in response to changes in mechanical forces and inflammatory processes in resistance arteries. Vascular components, including smooth muscle cells (SMCs), pericytes, and endothelial cells and their respective basement membranes (BM), all play crucial roles in vessel integrity and remodelling. The biochemical composition of the endothelial BM, underlying the endothelial monolayer and encasing pericytes, differs considerably from the smooth muscle BM, ensheathing each myocyte [1]. Of the many BM constituents, the heterotrimeric laminins (composed of an α, β and γ chain) are considered to be the biologically active component, controlling processes such as cell proliferation, differentiation and migration, and have also been implicated in transduction of mechanical signals from the vessel lumen to the vessel wall [2]. We here describe the laminin isoform expression in perivascular BM and, through the use of conditional KO mice, address the role of one of the major vascular laminins, laminin α5, in vessel function. Electron and immunofluorescence microscopy suggest that loss of laminin α5 from perivascular BM in a SMC and pericyte double KO (DKO) mouse affects SMC morphology and organization, suggesting an altered vessel function. To address this latter point more specifically, current studies focus on the physiological response of excised resistance arteries from WT and DKO vessels to shear, pressure and stretch stimuli. In addition, a mesenteric artery ligation model is employed that permits long-term redirection of blood flow, resulting in vessels of high, low or normal blood flow in the same animal. The high blood flow condition therefore mimics the effects of hypertensive conditions. Mesenteric artery ligation has been performed on WT and DKO mice and high, low and normal flow vessels are analyzed for cellular and extracellular matrix components as well as physiological response. [1] Hallmann, R. et al. (2005). Physiol. Rev. 85, 979-1000 [2] Gloe, T. and Pohl, U. (2002). News Physiol Sci. 17, 166-169

Nogo-B stabilizes atherosclerotic lesion through attenuation of ER-dependent free cholesterol induced macrophage apoptosis

Xinbo Zhang[1,2], Yuanyuan Zhang[1,2], Carlos Fernandez-Hernando[1] and Jun Yu[1,2]*.
[1]Department of Internal Medicine, Section of Cardiovascular Medicine and [2]Vascular Biology & Therapeutics Program, Yale University School of Medicine, New Haven, CT 06519, USA

Atherosclerotic plaque rupture is the most common cause of cardiac events, which cause most death in the world. Endoplasmic reticulum (ER) stress occurs in macrophage-rich areas of advanced atherosclerotic lesions and contributes to macrophage apoptosis and subsequent plaque necrosis and rupture. The reticulon-4 (Rtn-4) family of protein is primarily localized to the ER and consists of 3 splice variants of a common gene called Rtn-4A, Rtn-4B, and Rtn-4C. Rtn-4B, aka Nogo-B, is expressed in endothelial cells, vascular smooth muscle cells (VSMC) and monocytes/macrophages and, is the only isoform detectable in the vessel wall at protein level. Previously, we have shown that Nogo-B regulates macrophage homing and VSMC proliferation in response to ischemia and arterial injury in vivo. In human, the expression of Nogo-B is negatively correlates with the severity of atherosclerosis. Thus, we hypothesize that the local reduction of Nogo-B might contribute to plaque formation and/or instability. In current study, we used ApoE deficient mouse model to investigate the role of Nogo-B in atherogenesis. We have been able to show that Nogo and ApoE double knock-out (Nogo-/-ApoE-/-) mice developed larger and more advanced atherosclerotic lesion compared to ApoE-/- mice after Western diet feeding. The plaque to medium ratio and lipid content measured by Oil-Red-O staining was also increased in the brachiocephalic arteries and the aortic root of Nogo-/-ApoE-/- mice. While the macrophage and VSMC content in plaque was not changed, the loss of Nogo-B led to a marked increase in apoptosis and necrotic core area in the plaque. Meanwhile, Sirus Red and Trichrome staining displayed a significantly decreased collagen content in late atherosclerotic lesions of Nogo-/-ApoE-/- compared to ApoE-/- mice. In vitro, peritoneal macrophages from Nogo-/- mice were much more prone to apoptosis in response to free cholesterol (FC) loading compared to those from wild-type mice. Similar findings were observed following incubation with ac-LDL and ox-LDL in macrophage. Furthermore, Lentiviral reconstitution of Nogo-B decreased FC induced Nogo-/- macrophage apoptosis to the level comparable to wild-type. Mechanistically, loss of Nogo-B enhanced the accessibility of ER stress induced apoptotic pathways. Taking together, our results revealed a novel role of Nogo-B in decreasing ER-stress induced macrophage apoptosis and stabilizing atherosclerotic lesion in vivo, which may shed light in developing new strategies in treating late stage atherosclerosis.
This work is supported in part by an award from the American Heart Association to J.Y.

Role of protein arginine methyltransferase (PRMT) 1 in the pathogenesis of idiopathic pulmonary fibrosis (IPF)

Zakrzewicz D*, Zakrzewicz A, Miroslava Didiasova, Preissner KT and Wygrecka M. Department of Biochemistry, University of Giessen Lung Center, Giessen Laboratory of Experimental Surgery, Department of General and Thoracic Surgery, Justus-Liebig-University Giessen, Germany

Objectve: IPF has a poor prognosis and limited responsiveness to available treatments. It is characterised by epithelial cell injury, fibroblast activation, proliferation, migration and excessive extracellular matrix deposition. Protein arginine methylation is a posttranslational protein modification, which is performed by the class of enzymes called PRMTs. PRMT1, by methylation of specific protein targets, alters protein-protein interaction, signal transduction and histone function thereby influencing various cellular events. Increased PRMT expression has been reported in many renal and pulmonary disorders, however, the impact of PRMT1 activity and dysregulated intracellular protein methylation on the pathogenesis of IPF has not been investigated so far. Results: Expression of PRMT1 was strongly elevated in the lung homogenates from IPF patients compared to healthy donors, as revealed by Western Blot analysis. Immunohistochemical staining of IPF and donor lungs localized PRMT1 to epithelial type II cells, pulmonary arterial smooth muscle cells, as well as to IPF myo/fibroblasts in fibrotic foci. Fibroblasts isolated from IPF demonstrated enhanced PRMT1 expression compared to donor fibroblasts. Interestingly, in vitro overexpression of PRMT1 in fibroblasts resulted in significant increase of fibroblast migration and proliferation. This effect was reversed by pharmacological inhibitor of PRMT activity AMI-1 and treatment with PRMT1 siRNA. Conclusions: Altered fibroblast expression of PRMT1 may influence intracellular methylation, which in turn, controls pathogenic cellular events observed in lung fibrosis.

Proteomic profile of endothelial morphogenesis unveils tumor angiogenic markers and metabolic remodeling

Sara Zanivan[1,2]*, Federica Maione[3,4], Marco Y. Hein[1], Katrien De Bock[5], Maria Georgiadou[5], Pawel Ostasiewicz[1,6], Christian Frezza[2], Sandra Cauwenberghs[5], Liang Zheng[2], Juan Ramon Hernandez-Fernaud[2], Enrico Giraudo[3,4] ,Eyal Gottlieb[2], Peter Carmeliet[5], and Matthias Mann[1]. [1]Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany, [2]Beatson Institute for Cancer Research (Cancer Research UK), Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK, [3]Laboratory of Transgenic Mouse Models, Institute for Cancer Research and Treatment (IRCC), 10060 Candiolo, Italy, [4]Department of Oncological Sciences, University of Torino, School of Medicine, 10060 Candiolo, Italy, [5]Laboratory of Angiogenesis & Neurovascular link, Vesalius Research Center, K.U.Leuven, B-3000 Leuven, Belgium, [6]Department of Pathology, Wroclaw Medical University, 50-368, Wroclaw, Poland

Vessel growth is a critical process during development and tumor progression. We investigated this mechanism by SILAC-based mass spectrometry and profiled proteomic changes of human endothelial cells forming tubules on matrigel. To dissect functional changes specifically associated with morphogenesis but not general cell adhesion we quantified also proteomic changes in endothelial cells plated on different extracellular matrices, where cells spread, but do not form tubular structures. We identified and quantified a large proportion of the proteome which permitted a detailed investigation of the proteins and processes implicated in endothelial morphogenesis. We identified CLEC14A and MMRN2 as endothelial basement membrane proteins functionally involved in endothelial morphogenesis. We verified Clec14a and Mmrn2 as tumor angiogenic markers in different mouse models of carcinogenesis. Additionally, our approach revealed distinct adhesion mechanism in endothelial cells forming tubules and spreading on other matrices. Membrane adhesion proteins are upregulated in morphogenesis and spreading. In contrast, adhesion cytosolic proteins are downregulated in matrigel but not when cells spread on other matrices. Finally, we unveiled that endothelial cells forming tubules on matrigel change their metabolism and we provided evidence that this metabolic remodeling is functional in endothelial morphogenesis. Our results provide a step forward in the characterization of endothelial cells morphogenesis and we conclude that our strategy of integrating in vitro model of endothelial morphogenesis and spreading with unbiased MS approach is uniquely suited for discovering novel markers and mechanisms that accompany angiogenesis.

Importance of VEGFR-2 and VEGFR-3 signaling in postnatal vascular development

Georgia Zarkada[1]*, Krista Heinolainen[1], Tuomas Tammela[1], Erwin Wagner[2], Kari Alitalo[1].
[1]Molecular/Cancer Biology Program, Biomedicum Helsinki, University of Helsinki, Finland, [2]Genes, Development and Disease Group, Spanish National Cancer Research Centre, Madrid, Spain

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is involved in many physiological and pathological processes including embryonic cardiovascular development as well as tumor growth and metastasis. The key molecules regulating angiogenesis are vascular endothelial growth factors (VEGFs) and their cognate receptors (VEGFRs). VEGFR-3 is primarily involved in lymphangiogenesis and lymphatic metastasis. However VEGFR-3 is also expressed in endothelial tip cells, is essential for embryonic blood vessel development, and contributes to solid tumor growth and wound healing. We have previously demonstrated that VEGFR-2 activation results in VEGFR-3 upregulation in vivo (1), and that VEGFR-2 blocking antibodies are able to rescue the hypervascular phenotype occurring after VEGFR-3 genetic deletion in blood vessels (2). Here, using a mouse model of spatially and temporally controlled gene deletion, we show that the VEGFR-3 deficient mice display increased VEGFR-2 protein levels and excessive VE-Cadherin internalization in vivo, consistent with increased VEGFR-2 signaling. Deletion of both receptors in postnatal mice leads to severely compromised angiogenesis, plus changes in lymphatic vessels. Our results point to novel and distinct regulatory functions for VEGFR-2 and VEGFR-3 in angiogenesis. 1. T. Tammela et al., Nature 454, 656 (Jul 31, 2008). 2. T. Tammela, G Zarkada et al., Nat Cell Biol 13, 1202 (Sep 11, 2011).

Chemical oxygen and glucose deprivation rapidly impairs blood-brain barrier integrity in an in vitro model of the neurovascular unit

Christoph M. Zehendner[1,3]*, Laura Librizzi[2,3], Marco de Curtis[2], Christoph R. W. Kuhlmann[1], Heiko J. Luhmann[1]. [1]Institute of Physiology and Pathophysiology, University Medical Center of the Johannes Gutenberg-University of Mainz, Mainz, Germany, [2]Unit of Experimental Neurophysiology and Epileptology, Fondazione Istituto Neurologico Carlo Besta, Milano, Italy, [3]equal contribution

The aim of our study was to develop an in vitro model of the blood brain barrier (BBB) which includes neurons, astrocytes and brain endothelial cells (BEC) to mimic the neurovascular unit (NVU) and to evaluate the acute effects of chemical oxygen and glucose deprivation (OGD) on BBB parameters. We established an improved coculture consisting of organotypical slice cultures (Stoppini et al., 1991; Duport et al., 1998) and the immortalized BEC line bend3, which can be analyzed with confocal live cell imaging, immunohistochemistry (IHC) and electrical impedance sensing in real time under various conditions (Zehendner et al., 2009). OGD was induced by exposing the coculture to sodium cyanide and replacing glucose with 2-deoxy-D-glucose. We used IHC to analyze cellular integrity of cell types and BBB parameters (e.g. tight junction proteins ZO-1, Cl-5) as well as trans-endothelial electrical resistance (TEER) measurements. IHC revealed a proper morphology of neurons, astrocytes, BEC and cortical microvessels. Furthermore, we detected elevated levels of caspase-3 in BEC 30 minutes after induction of OGD. This was accompanied by a strong impairment of TEER and disruptions of tight junction proteins ZO-1 and Cl-5, which was partly reduced by preincubating the cells with 50 µmol/l of the irreversible caspase-3 inhibitor Z-DEVD-fmk 2 hours ahead of OGD (Zehendner et al., 2011). We established a novel NVU model with proper BBB integrity parameters and demonstrate that BBB integrity in this model is rapidly altered after 30 minutes of OGD without reoxygenation. This challenge rapidly resulted in BBB breakdown, tight junction disruption and led to activation of caspase-3. The inhibition of caspase-3 by Z-DEVD-fmk was partly protective on BBB integrity. Our results suggest a role for caspase-3 in rapid BBB damage (Zehendner et al., 2011). Our results may be of relevance for analyzing and understanding pathological processes within the NVU. References: - Duport, S., Robert, F., Muller, D., Grau, G., Parisi, L., and Stoppini, L. (1998). An in vitro blood-brain barrier model: cocultures between endothelial cells and organotypic brain slice cultures. Proc. Natl. Acad. Sci. U.S.A. 95, 1840-1845. - Stoppini, L., Buchs, P.A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods 37, 173-182. - Zehendner, C.M., Librizzi, L., de Curtis, M., Kuhlmann, C.R.W., and Luhmann, H.J. (2011). Caspase-3 contributes to ZO-1 and Cl-5 tight-junction disruption in rapid anoxic neurovascular unit damage. PLoS ONE 6, e16760. - Zehendner, C.M., Luhmann, H.J., and Kuhlmann, C.R.W. (2009). Studying the neurovascular unit: an improved blood-brain barrier model. J. Cereb. Blood Flow Metab. 29, 1879-1884.

Inhibition of the SDF-1/CXCR4 axis alleviates chronic and acute skin inflammation

Silvana Zgraggen*, Reto Huggenberger, Alexandra M. Ochsenbein, Steven Proulx, David Aebischer and Michael Detmar. Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, 8093 Zurich, Switzerland

Recent studies indicate that the SDF-1/CXCR4 axis may be involved in the pathogenesis of several inflammatory diseases. However, it is not known whether this chemokine axis also plays a role in inflammatory skin diseases. This study was aimed at evaluating the relevance of the SDF-1/CXCR4 interaction in the initiation or maintenance of chronic and acute cutaneous inflammation. We found that CXCR4 and SDF-1 were significantly up-regulated during inflammation in the established keratin 14 (K14) VEGF-A transgenic (tg) mouse model of chronic cutaneous inflammation and that CXCR4 is expressed on different inflammatory cells in the inflamed ear skin, including T cells, dendritic cells and macrophages. To investigate the functional importance of the SDF-1/CXCR4 axis in vivo, we inhibited CXCR4 signaling by AMD3100, a specific CXCR4 antagonist, and by a neutralizing anti-SDF-1 antibody in the K14-VEGF-A tg mice. Both treatments significantly reduced inflammatory edema formation and inflammatory cell infiltration in the inflamed ear skin. Moreover, we found that in the inflamed ear skin, CXCR4 was only expressed on blood vessels but not on lymphatic vessels and that inflammatory angiogenesis was significantly reduced by inhibiting CXCR4 signaling. Furthermore, incubation with AMD3100 or anti-SDF-1 antibody inhibited SDF-1 induced chemotaxis of CD11b+ splenocytes in vitro. Finally, we investigated the role of the SDF-1/CXCR4 axis in acute skin inflammation using FVB wild-type mice. Shortly after the induction of an oxazolone-induced skin inflammation, AMD3100 treatment was started. Inhibition of the CXCR4 signaling significantly limited acute skin inflammation with a reduction in dermal edema formation. Taken together, these results demonstrate that inhibition of the CXCR4/SDF-1 axis might serve as a novel strategy to treat patients with inflammatory skin disorders such as psoriasis.

Transcription regulation of coronary endothelial cell differentiation

Zhao Z[1]*, Sacilotto N[1], Lammerts van Bueren K[2], Black BL[2], Bou-Gharios G[3], and De Val S[1]. [1]Ludwig Institute for Cancer Research, University of Oxford, Oxford, UK, [2]University of California, San Francisco, San Francisco, U.S.A., [3]Kennedy Institute of Rheumatology, University of Oxford, Oxford, United Kingdom

Cardiovascular diseases have been a major threat to public health for the Western society in the recent decades. A profound understanding of the origin and specification of coronary vasculature is a critical step towards developing molecular interventions of revascularization in a failing heart. The coronary endothelium forms the inner layer of coronary vessels, and its position in the differentiation hierarchy of endothelium is under debate. Here we describe an enhancer of Neuropilin1 (Nrp1) that can drive specific expression to the coronary endothelium during coronary vascular formation. The Nrp1-LacZ positive endothelial cells in the developing coronary initially expand from around the sinus venosus at E11.5 and eventually contribute to capillary, vein and arterial compartment of the coronary vessel but not the endocardium. This expression pattern is consistent with that reported for the Apelin-nlacZ line(Red-Horse, 2010, Nature) and make the Nrp ehancer the first identified mammalian coronary-specific regulating element. Among the identified cis elements in this enhancer is an Octamer element and various POU-domain factors can bind in vitro. Our continued study of the molecular mechanism controlling the Nrp1 enhancer will lead to a better understanding of transcription regulation of coronary endothelial cell specification and may find the master transcription factor that implements coronary commitment.

ROCK2 deficiency in bone marrow-derived cells increases cholesterol efflux and decreases atherosclerosis

Qian Zhou[1,2]*, Goo Taeg Oh[1], Pat Ongusaha[1], Kunzhong Zhang[1], Karol Kaminski[1], Hannah Schmitt[2], Takuhito Shoji[1], Martin Moser[2], Christoph Bode[2], James K. Liao[1].
[1]Vascular Research Unit, Brigham & Women's Hospital and Harvard Medical School, Boston, MA, USA [2]Department of Cardiology, University Hospital Freiburg, Freiburg, Germany

Background: The Rho-associated coiled-coil containing kinases (ROCKs) are serine-threonine protein kinases that regulate the actin cytoskeleton. Increasing data also demonstrate a non-actin cytoskeletal effect of ROCK. There are two isoforms of ROCK, both having distinct downstream targets. Recent studies suggest that ROCKs are important mediators of cardiovascular diseases. While ROCK1 deficiency has been shown to ameliorate atherosclerosis in mice, the role of ROCK2 in atherosclerosis still remains unknown. Methods: ROCK expression and activity in bone marrow (BM)-derived cells (BMDCs) were analyzed by Western blot. Cell migration was determined using a modified Boyden chamber. Cholesterol uptake and efflux were evaluated in BMDCs. Expression of cholesterol transporters and PPARγ signaling was determined by RT-PCR, Western blot or Dual Luciferase assay. For in-vivo study, BM from wild-type (WT) and ROCK2+/- mice were transplanted into irradiated recipient LDLr-/- mice, followed by a 16-week diet containing 15.8% wt/wt fat and 1.25% cholesterol. The degree of atherosclerosis in the en face aorta and plaque composition was evaluated. Statistical analysis was performed using Student's t-test or Mann-Whitney U-test. A p value <0.05 was considered significant. Results: ROCK2 expression and activity in BMDCs was increased by OxLDL. Migration towards MCP-1 was reduced by 1.46 ± 0.72 fold in ROCK2+/- BMDCs. Foam cell formation were reduced in ROCK2+/- BMDCs. Accordingly, scavenger receptors were upregulated and cholesterol efflux was enhanced in ROCK2+/- BMDCs. This was prevented by a PPARγ inhibitor, indicating that ROCK2 mediated cholesterol efflux was dependent on PPARγ signaling. Indeed, PPARγ and LXRαexpression were upregulated in ROCK2+/- BMDCs and aortic lysates. Furthermore, transient transfection of the PPAR and LXR response elements were strongly activated by ROCK inhibition, while activation of ROCK reversed the effect. The pathophysiological effect of ROCK on cholesterol efflux was investigated in an in-vivo atherosclerosis model. ROCK2+/- BMT mice showed substantially less atherosclerosis in the aorta (9.8% vs. 15.64%, p<0.01) and subaortic sinus (330.1 x103 μm2 vs. 520.2 x103 μm2, p<0.01) in comparison to WT BMT mice. This was associated with less lipid, MOMA-2 and collagen expression within the plaque area and a down-regulation of pro-inflammatory factors. Conclusions: Our findings indicate that ROCK2 deficiency in BMDCs enhances reverse cholesterol transport through the activation of the PPARγ-LXRαpathway leading to decreased atherosclerosis. These findings suggest that inhibition of ROCK2 signaling in BMDCs may have therapeutic benefits in promoting cholesterol efflux and preventing atherosclerosis.

Angiopoietin-2 is a key regulator in septic hypercirculation

V. Pfetsch[1], J. Horstkotte[1], T. Ziegler[1*], C. Schwab[2], Ina Rohwedde[3], Melanie Schröder[1], R. Hinkel[1], Q. Di[1], S. Dietzel[4], U. Deutsch[2], C. Kupatt[1]. [1]Medizinische Klinik und Poliklinik I, Klinikum der Universität München, Campus Großhadern, München, [2]Theodor-Kocher-Institut, Bern, Schweiz, [3]Max-Planck-Institut für Biochemie, Martinsried, [4]Walter-Brendl-Zentrum für experimentelle Medizin, München, Germany

Background: Angiopoietins play a central role in the quiescence of the endothelium. Pathological stimuli, such as inflammation, lead to a destabilization of the endothelial cell layer via Angiopoietin-2 and its receptor Tie-2. In clinical studies, elevated Angiopoietin-2 levels in sera of patients suffering severe sepsis have been described (Kümpers 2008). Recently, it has been demonstrated, that treatment with an Ang-1 adenovirus in septic mice resulted in improved cardiac function as well as enhanced survival (Witzenbichler 2005). Here, we assessed the protective effect of Ang-2 antibodies (Ang2-ab) in a murine model of lipopolysaccharide (LPS)-induced sepsis. Methods: To induce Sepsis, LPS [20mg/kg] was injected intraperitoneally into C75BL/6 mice. 24 hours before sepsis induction, groups were pre-treated with Ang2-ab or an unspecific antibody (control-ab) as a control. After sepsis induction a sepsis-severity-score was assessed after 6 and 12 hours, followed by invasive and non-invasive hemodynamic measurements or observation of further survival. The sepsis-severity-score includes five different parameters (behaviour, pain, ascites, dispnea, weight loss), according to the severity points between 0 and 20 are given. Blood samples and organs were harvested for histological and molecular analysis. Results and Conclusion: Whereas in control-ab treated mice, hemodynamic function was severely depressed 12 hoursafter LPS injection, as seen in decreased left ventricular developed bloodpressure (74 ± 9 mmHg) and severely reduced systemic blood pressure (45 ± 3 mmHg), in Ang2-ab treated mice left ventricular developed blood pressure fell only to 92 ± 6 mmHg. Similarly, the drop in systemic blood pressure was less pronounced (77 ± 10 mmHg). This resistance to LPS-induced hemodynamic changes was reflected by reduced sepsis-severity-score results after 6 (5 ± 1 points vs.7 ± 1 points) and 12 hours (10 ± 1 points vs.14 ± 1 points) and at least by trend improved survival rate of the Ang2-ab treated mice. Histological analysis revealed that the dropout in pericytes seen in septic mice is abolished by Ang2-ab treatment. These findings highlight the pivotal role of Ang-2 in the onset and progression of sepsis and identify Ang-2 as a potential target for treatment of Sepsis.