Involvement of Chemokine Receptor 4/Stromal Cell–Derived Factor 1 System during Osteosarcoma Tumor Progression

Eliana Perissinotto,[1] Giuliana Cavalloni,[1] Francesco Leone,[1] Valentina Fonsato,[1] Stefania Mitola,[2] Giovanni Grignani,[1] Nadia Surrenti,[1] Dario Sangiolo,[1] Federico Bussolino,[2] Wanda Piacibello,[1] and Massimo Aglietta[1]

Department of Oncological Sciences and Laboratories of [1]Clinical Oncology and [2]Molecular Angiogenesis, University of Turin Medical School, IRCC Institute for Cancer Research and Treatment, Turin, Italy

ABSTRACT

Despite intensive chemotherapy and surgery treatment, lung and bone metastasis develop in about 30% of patients with osteosarcoma. Mechanisms for this preferential metastatic behavior are largely unknown. We investigated the role of the chemokine receptor 4 (CXCR4)/stromal cell–derived factor 1 (SDF-1) system to drive the homing of osteosarcoma cells. We analyzed the expression of the CXCR4 and SDF-1 proteins on several osteosarcoma cell lines and the effects of SDF-1 on migration, adhesion, and proliferation of these cancer cells. *In vitro* assays showed that the migration of osteosarcoma cells expressing CXCR4 receptor follows an SDF-1 gradient and that their adhesion to endothelial and bone marrow stromal cells is promoted by SDF-1 treatment. Moreover, the production of matrix metalloproteinase-9 is increased after SDF-1 exposure. We finally proved in a mouse model our hypothesis of the CXCR4/SDF-1 axis involvement in the metastatic process of osteosarcoma cells. Development of lung metastasis after injection of osteosarcoma cells was prevented by the administration of a CXCR4 inhibitor, the T134 peptide. These data show a possible explanation for the preferential osteosarcoma metastatic development into the lung, where SDF-1 concentration is high, and suggest that molecular strategies aimed at inhibiting the CXCR4/SDF-1 pathway, such as small-molecule inhibitors or anti-CXCR4 antibodies, might prevent the dissemination of osteosarcoma cells.

INTRODUCTION

Osteosarcoma is the most common pediatric primary bone tumor characterized by a high local aggressiveness with a tendency to metastasize to the lung and to the bone. Without adjuvant chemotherapy, more than half of the patients treated by surgery alone develop metastases within 6 months and more than 80% develop recurrent disease within 2 years of diagnosis (1). About 95% of the patients who died of metastatic disease has lung involvement as indicated by autopsy (2). At the same time, about 15% to 30% of the patients develop skeleton bone metastases (3). Although integrated multimodal therapies gained a great improvement on overall survival, 30% to 40% of the patients will eventually develop pulmonary and skeletal metastases (4). The reasons behind this peculiar metastatic tropism are largely unknown. Nevertheless, their comprehension could represent the starting point for innovative therapeutic strategies.

Metastasis development is a complex mechanism in which many factors, such as properties of tumor cells as well as the microenvironment in which they localize, can potentially influence tumor dissemination (5). Recent studies indicate that the metastatic process shares many similarities with leukocyte trafficking (6).

Chemokines are a superfamily of cytokine-like proteins that are grouped into CXC and CC chemokines that selectively attract and activate different cell types. These secreted proteins induce, through their interaction with G-protein-coupled receptor, cytoskeleton rearrangement, firm adhesion to endothelial cells and directional migration (7, 8).

The CXC chemokine stromal cell–derived factor 1 (SDF-1 or CXCL12; ref. 7) is expressed on the surface of vascular endothelial cells and is secreted by stromal cells from a variety of tissues such as bone marrow, lung, and liver (9). Its chemotactic effect is mediated by interaction with the chemokine receptor 4 (CXCR4 or CD184; refs. 10–12). It has been shown that endothelial cells expressing the CXCR4 receptor are strongly chemoattracted by SDF-1 (13). Hematopoietic stem cells are selective in their migratory response to SDF-1, which regulates their specific homing in marrow microenvironment (14–16).

Involvement of the CXCR4/SDF-1 axis has been shown during tumor progression of breast, prostate, and pancreatic cancer as well as in rhabdomyosarcoma and lymphoma (17–20). Owing to the importance of the CXCR4/SDF-1 axis in homing and in promoting cell migration in different kinds of tumor, it is possible that also osteosarcoma uses this system to produce metastases.

Recent studies show that CXCR4 inhibition can affect tumor cell dissemination in non-Hodgkin lymphoma, melanoma, and breast cancer (21, 22). The polyphemusin II peptide T22 and its derived T134 have shown *in vitro* the ability to prevent HIV

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Requests for reprints: Giuliana Cavalloni, Department of Oncological Sciences, Institute for Cancer Research and Treatment, Strada Provinciale 142, 10060 Candiolo, Turih, Italy. Phone: 39-011-9933523; Fax: 39-011-9933524; E-mail: giuliana.cavalloni@ircc.it.

type 1 (HIV-1) infection by blocking HIV-1 binding to its coreceptor CXCR4. T134 peptide exhibits higher activity and significantly less cytotoxicity in comparison with T22; it competes with SDF-1 at much lower concentration and shows a higher affinity than SDF-1 for the binding to CXCR4 (23–27).

Here we analyzed CXCR4 expression on some osteosarcoma cell lines and evaluated *in vivo* the potency of T134 peptide to modulate SDF-1-mediated chemotaxis and migration of osteosarcoma cells. In this study, we showed the ability of T134 to block CXCR4/SDF-1 interaction and to prevent osteosarcoma cell dissemination in the metastatic progression.

MATERIALS AND METHODS

Cell Cultures

Human osteosarcoma cell lines SJSA, U2-OS, and HOS were maintained in DMEM (Invitrogen SRL, Milan, Italy) supplemented with 10% FCS (EuroClone, Wetherby, West York, United Kingdom); MG-63 cell line was maintained in MEM (Invitrogen SRL) with 10% FCS. All cell lines derive from primitive bone osteosarcoma; they are immortalized, but not further transformed. Because in preliminary experiments we observed a decrease of CXCR4 expression on confluent cells, all the experiments were done during the exponential growth phase in which CXCR4 expression is high and reproducible.

Bone marrow stromal cells from normal donors were obtained, after informed consent was given, by culturing 10^7 bone marrow mononuclear cells as previously described (28).

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord, after informed consent was given, by collagenase treatment and cultured in dishes precoated with 0.04% gelatin according to Jaffe et al. (29). Briefly, the cells were maintained in M199 medium supplemented with 100 units/mL penicillin/streptomycin, 2 mmol/L L-glutamine (all from Biochrom, Berlin, Germany), 20% FCS, 40 μg/mL endothelial cell growth factor (Boehringer Mannheim, Mannheim, Germany), and 50 μg/mL heparin (Sigma-Aldrich, Deisenhofen, Germany). Cells were subcultured after trypsinization and used throughout passages 2 to 4. Bone marrow stromal cells and HUVEC proved to be a constant and reproducible source of SDF-1.

Flow Cytometric Analysis

Cells were washed in 1× PBS containing 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 0.01% sodium azide. The cells were labeled for 45 minutes at 4°C with mouse monoclonal antibody (mAb) R-phycoerythrin-conjugated anti-human CXCR4 (clone 12G5, R&D Systems, Minneapolis, MN). For intracellular CXCR4 staining we blocked cell surface expression by incubating with 10 μg/mL of nonconjugated anti-human CXCR4 mAb for 1 hour at 4°C. The cells were than washed, fixed, and permeabilized to stain the intracellular CXCR4 with anti-human CXCR4-PE mAb. After an additional wash, the receptor expression on 10,000 cells was determined using a FACS Vantage (Becton Dickinson, San Jose, CA). Cell acquisition and analysis were done using CellQuest software program (Becton Dickinson).

Within the range of cell concentrations used [(1 to 5) × 10^5] the cell density did not influence the result.

Western Blot Analysis

Five to 10 million cells were washed with 1× PBS and lysed with lysis buffer (50 mmol/L Tris-HCl pH 7.5, 250 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 0.5% Nonidet-P40, 1 mmol/L DTT) and a mixture of protease inhibitors (Sigma-Aldrich) for 15 minutes at 4°C and centrifuged at 14,000 rpm for 15 minutes. The protein concentration of cell lysates was measured using the Bio-Rad DC protein assay kit (Hercules, CA) and 20 μg of proteins were resolved on 10% SDS-PAGE and electrotransferred to 0.45-μm polyvinylidene difluoride (Amersham Pharmacia Biotech, Piscataway, NJ) at 180 mA for 90 minutes at 4°C. Nonspecific sites were blocked by incubating for 1 hour with 5% nonfat dry milk (Bio-Rad Laboratories) in 1× TBST 20 mmol/L Tris-HCl pH 7.5, 500 mmol/L NaCl, 0.1% Tween 20. The membrane was first incubated overnight with 1 μg/mL of mouse mAb anti-CXCR4 (clone 12G5, BD PharMingen, San Diego, CA) and then with 1 μg/mL horseradish peroxidase–conjugated secondary anti-mouse antibody (Amersham Pharmacia Biotech) in 1× TBST with 1% nonfat dry milk. After each incubation step, the membrane was washed for 30 minutes with 1× TBST, revealed with a chemiluminescence reagent (ECL, Amersham Pharmacia Biotech) and exposed to autoradiography film.

Determination of SDF-1 Production by Reverse Transcription–PCR

Total RNA was isolated from 5 × 10^6 to 10 × 10^6 cells using Trizol reagent (Invitrogen SRL) according to the manufacturer's instructions; 2.5 μg of total RNA were reverse transcribed for 45 minutes at 42°C with 100 pmol oligo(dT) primers (Roche Diagnostics, Milan, Italy) and 200 units of cloned Moloney murine leukemia virus reverse transcriptase in 1× reverse transcription buffer supplemented with 0.01 mol/L DTT, 1 mmol/L of each deoxynucleotide triphosphate and 200 units of RNase inhibitor (all from Invitrogen SRL) in a final volume of 25 μL. The resulting cDNA was subjected to a 50-μL PCR reaction containing 1× PCR buffer (Invitrogen SRL) supplemented with 1.5 mmol/L MgCl2, 200 μmol/L deoxynucleotide triphosphates, and 20 pmol of each primer. The primers for human SDF-1 were 5′-GGGGGAATTCCATGAACGCCAAGGTCGTGGTC-3′ (forward) and 5′-GGGGTCTAGAGGGCATGGATGAATA-TAAGCTGC-3′ (reverse). Thermal cycle conditions were 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute for two cycles followed by 41 cycles of 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute. All PCR products were analyzed on a 2% agarose gel with ethidium bromide staining.

SDF-1 ELISA

To determine SDF-1 secretion in conditioned medium, primary human osteosarcoma cell lines were plated in medium containing 10% FCS. The medium was changed on days 3 and 5. After the cells had reached confluence, the supernatants were collected following the manufacturer's instructions. The media were then analyzed by antibody sandwich ELISA (R&D Systems) with a detection range of 156 to 10,000 pg/mL SDF-1.

Modulation of CXCR4 Expression on Osteosarcoma Cells by Exposure to Recombinant Human SDF-1

Modulation of CXCR4 was done on SJSA cells; 5×10^5 cells were incubated in a 24-well plate with serum-free medium for 1 hour and were then cultured with or without 100 ng/mL of recombinant human SDF-1 (rhSDF-1, R&D Systems) diluted in bovine serum albumin, and incubated at 37°C for 1 and 3 hours. They were then washed in 1× PBS and stained to analyze CXCR4 expression by FACS analysis as described above.

Zymographic Analysis

Gelatinolytic activities of matrix metalloproteinases (MMPs) were assessed under nonreducing conditions using a modified SDS-PAGE. The supernatants of osteosarcoma cell lines were collected after 48 hours of standard culture condition. Aliquots of supernatants for each cell line were mixed with Laemmli buffer and applied onto an 8% polyacrylamide gel copolymerized with 1 mg/mL gelatin (Sigma-Aldrich). Electrophoresis was done under constant voltage (150 V) for 2 hours. The gel was washed 3 × 20 minutes each with 2.5% Triton X-100 (Sigma-Aldrich) to remove SDS and to allow the electrophoresed enzymes to renature before incubation in zymography buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, 10 mmol/L $CaCl_2$, 0.06% Brij 35 solution 30% pH 7.5) for 24 hours at 37°C. The gel was then stained with 0.5% Coomassie brilliant blue G-250 (Sigma-Aldrich) in 1:3:6 acetic acid/methanol/water for 15 minutes and destained with 1:4.5:4.5 acetic acid/methanol/water. Prestained standard high-range protein marker (Amersham Pharmacia Biotech) was used to determine the molecular weight of the gelatinase. The clear bands represented gelatinase activity. To determine whether zones of lysis detected by zymography were produced by MMPs, one gel was incubated with zymography buffer in the presence of 5 mmol/L EDTA. To evaluate the SDF-1 effect on MMPs modulation, we cultured the MG-63 and SJSA cell lines for 24 hours with and without rhSDF-1 (100 ng/mL) and did the zymographic assay on the supernatants as described above.

Migration Assay

The invasion potential was evaluated using Transwell chambers (1 cm^2 per well, Costar, Bodenheim Germany). The upper and lower culture were separated by an 8-μm pore-size polyvinylpyrrolidone-free polycarbonate filters (Nucleopore, Corning Costar Corp., Cambridge, MA). Briefly, the lower compartment of the chamber was loaded with aliquots of serum-free medium plus rhSDF-1 (100 ng/mL). Before the invasion assay, filters were coated with 100 μg/well Matrigel (Becton Dickinson) diluted in culture medium. Tumor cells were seeded onto the reconstituted basement for 48 hours (5×10^4 cells per well) at 37°C in humidified atmosphere with 5% CO_2. Cells that passed the synthetic basement were fixed with ethanol and stained with Giemsa solution (Diff-Quik kit, Baxter Diagnostics, Milan, Italy). Each experiment was done in triplicate. Cells migrated to the underside of the filter were counted in five fields (magnification ×100) in each well by light microscopy. To evaluate random migration, in one set of experiments rhSDF-1 was added to both upper and lower compartments.

Adhesion Assay

To evaluate the SDF-1 contribution on adhesion, bone marrow stromal and HUVEC cells (5×10^4 per well) were plated for 24 hours in a 24-well plate at a density compatible with monolayer confluence and were treated with 100 ng/mL rhSDF-1 for 1 hour at 37°C to produce the active form of SDF-1. SJSA cells (1×10^6 cells/mL), labeled before assay with the fluorescent dye calcein-AM (Calbiochem, Darmstadt, Germany) for 5 minutes at 37°C, were detached and subsequently added to bone marrow stromal or HUVEC cell monolayer for 30 and 60 minutes at 37°C in humidified atmosphere. Unattached cells were removed, and the remaining cells were washed with PBS and harvested to quantify the adherent cell fluorescence in a 96-well fluorescent plate reader (HTS 7000 Bio Assay Reader, Perkin-Elmer). To show CXCR4-dependent adhesion we pretreated SJSA cells with 10 ng/mL neutralizing anti-CXCR4 antibody for 1 hour at 4°C before the adhesion to SDF-1-unstimulated bone marrow stromal and HUVEC cells.

Blockade of CXCR4 Receptor

For CXCR4 neutralization in *in vitro* studies, the interaction between CXCR4 and SDF-1 was blocked by treating SJSA cells for 1 hour at 4°C with a nonconjugated anti-human CXCR4 mAb (clone 12G5, 10 μg/mL, R&D Systems) before migration and adhesion assays.

For *in vivo* neutralization, CXCR4 receptor was blocked by using T134 (150 μg/kg), a small analogue of T22 peptide (14 amino acid residues, Sigma-Genosys, The Woodlands, Texas), for 1 hour at 4°C before *in vivo* assays.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done according to the manufacturer's recommendation (Sigma-Aldrich). Briefly, 1×10^4 SJSA cells were seeded in 96-well plate in 150 μL DMEM containing 10% of FCS with or without rhSDF-1 stimulation (100 ng/mL). After 24 and 48 hours, MTT solution was added to each well and plates were incubated for 3 to 4 hours; subsequently, DMSO (Sigma-Aldrich) was added to the wells for 5 minutes. The plates were then read at 595 nm using an automate plate reader (Perkin-Elmer).

In vivo Metastasis Studies

Female BALB/c mice (6 to 8 weeks old) were supplied by The Jackson Laboratory (Bar Harbor, ME), maintained at the animal facilities of CIOS (Turin, Italy), and handled according to institutional regulations under sterile conditions in cage micro-isolators.

To investigate the involvement of the CXCR4/SDF-1 system during osteosarcoma metastatic process *in vivo*, one group of mice was given an injection of 10^6 SJSA cells alone into the tail vein and another group of mice was given an injection of SJSA cells and T134 (150 μg/kg) together. The animals were then given i.v. injections of T134 peptide thrice per week at the same concentration. No toxicity for the anti-human CXCR4 peptide was observed during preliminary *in vivo* experiments.

All mice were sacrificed 5 weeks after injection and lungs were examined macroscopically and microscopically for the

presence of metastases. For histology and immunohistochemistry evaluation, lungs were collected and fixed in 10% formalin and embedded in paraffin. Sections 4 μm thick were stained with H&E for conventional histology.

Statistical Analysis

Each chemoinvasion and adhesion experiment was done in triplicate. The results were analyzed using Student's *t* test. All *P* values are two tailed with a significance of $P < 0.05$. For *in vivo* experiments, pulmonary metastasis development was compared in different groups of treatment by using Student's *t* test.

RESULTS

CXCR4 and SDF-1 Expression on Osteosarcoma Cell Lines

CXCR4 expression was evaluated by FACS and Western blot analysis on four osteosarcoma cell lines SJSA, MG-63, HOS, and U2-OS.

Flow cytometric analysis showed that cell surface receptor expression was detectable on all tested cell lines, at different levels and in each cell line with high variability of expression, as shown in Fig. 1*A*. We observed that the CXCR4 level expression was influenced by the culture condition of the cells. In confluent cells the CXCR4 staining decreased compared with growing cells. Because SJSA cells expressed the highest level of CXCR4, we have chosen this cell line for our study.

The presence of CXCR4 receptor was confirmed by Western blot analysis (Fig. 1*B*).

The expression of the SDF-1 mRNA on the four osteosarcoma cell lines was evaluated by reverse transcription–PCR. SDF-1 transcript was detected in all cell lines tested with different levels of expression. High level of SDF-1 transcript was found in MG-63 and U2-OS (Fig. 2*A*).

To verify whether the presence of SDF-1 transcript corresponds to SDF-1 protein secretion, osteosarcoma cell line supernatants were analyzed by rhSDF-1 ELISA kit after the cells had reached confluence. We found that only MG-63 cell line secreted high levels of SDF-1 (60 ng/mL; Fig. 2*B*).

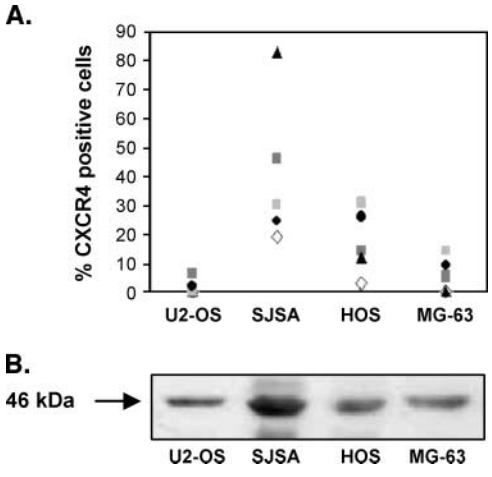

Fig. 1 CXCR4 expression on osteosarcoma cell lines. *A,* FACS analysis of CXCR4 protein expression. Each symbol represents an independent experiment. *B,* Western blot analysis.

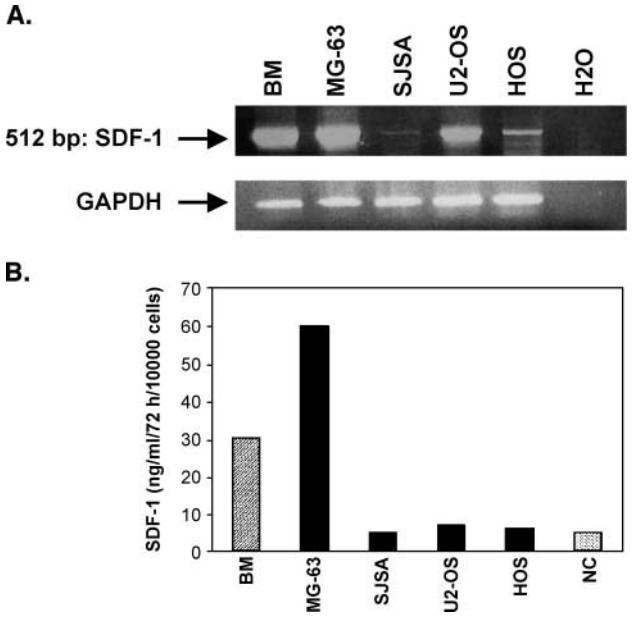

Fig. 2 SDF-1 expression on osteosarcoma cell lines. *A,* expression of SDF-1 mRNA by reverse transcription–PCR. Bone marrow (*BM*) stromal cells were used as positive control. H_2O, negative control of PCR. *B,* SDF-1 protein levels measured by ELISA in conditioned medium from osteosarcoma cell lines. *NC,* negative control (level of SDF-1 in DMEM with 10% FCS, without cells).

SDF-1 Induces Chemoinvasion and Adhesion in Osteosarcoma Cells

SDF-1 stimulation induces a down-regulation of CXCR4 on SJSA cells; in a representative experiment shown in Fig. 3*A*, the expression of CXCR4 significantly decreased from 86% (untreated cells) to 16% and 8% after 1 and 3 hours of SDF-1 treatment, respectively. In separated experiments we analyzed modulation of CXCR4 also in confluent SJSA cells (about 20% of $CXCR4^+$ cells) and we obtained a significant down-regulation (to 6%; data not shown). Down-regulation of the receptor is the result of CXCR4 internalization as shown by the increase of intracellular CXCR4 expression (Fig. 3*B*).

Tumor secretion of MMPs increases the metastatic potential of malignant cells (30, 31). We analyzed the extracellular matrix–degrading activity of osteosarcoma cells, evaluating MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) expression on supernatants of osteosarcoma cell lines. We observed pro-MMP-9 secretion in all of the supernatants tested; moreover, we detected a band of 83 kDa (Fig. 4*A*), corresponding to the active form of MMP-9 (32). We did not find an MMP-2 band.

The highest level of pro-MMP-9 was found in MG-63 supernatant. This result could correlate to the SDF-1 endogenous production by MG-63, according to previous studies showing that pro-MMP-9 production increases after SDF-1 or growth factor stimulation (33, 34).

To evaluate whether SDF-1 acts on MMPs modulation, with consequent increase of the metastatic potential, MG-63 and SJSA cell lines were stimulated for 24 hours with rhSDF-1 and then checked for the change of gelatinolytic activity. We found an increase on pro-MMP-9 secretion on MG-63 cell line supernatant

but no modulation in SJSA cell line. Moreover, after rhSDF-1 treatment we found an increased expression of the lower band (83 kDa) in both cell lines (Fig. 4*B*). By contrast, SDF-1 treatment did not induce MMP-2 secretion on supernatants (data not shown).

To verify the involvement of CXCR4/SDF-1 complex during osteosarcoma progression, we did a Matrigel migration assay with osteosarcoma cells toward medium with or without rhSDF-1 (100 ng/mL). As shown in Fig. 5*B*, we observed a massive migration of SJSA cells in response to rhSDF-1. On the contrary, MG-63 cell migration in response to rhSDF-1 was lower as compared to SJSA cell locomotion (Fig. 5*D*).

CXCR4 down-regulation by treating SJSA cells with rhSDF-1 for 1 hour before the migration assay reduced the number of the migrating cells (Fig. 5*E*). The inhibition of CXCR4/SDF-1 axis by treating the cells with neutralizing mAb against CXCR4 significantly impaired osteosarcoma cell migration toward SDF-1 (Fig. 5*F*). These results suggest that the phenomenon is dependent on CXCR4 levels.

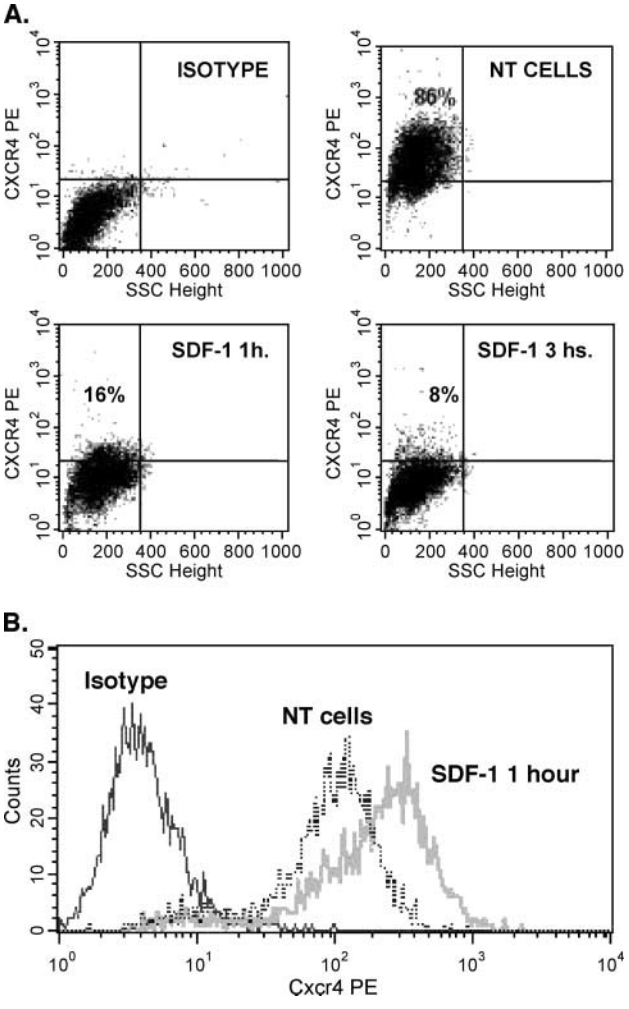

Fig. 3 Representative FACS analysis of CXCR4 modulation on SJSA cells after rhSDF-1 treatment. *A,* the SJSA cell line was cultured with rhSDF-1 (100 ng/mL) for 1 and 3 hours and stained with anti-CXCR4-PE for extracellular CXCR4 expression analysis. The percentage in the *top left quadrant* indicates CXCR4 positive cells. *B,* for intracellular staining, CXCR4 cell surface expression was blocked by incubating the cells with nonconjugated anti-human CXCR4 mAb and, after fixation and permeabilization, the cells were stained with anti-human CXCR4-PE mAb. *NT,* cells maintained in culture with DMEM + 10% FCS without SDF-1 for 3 hours.

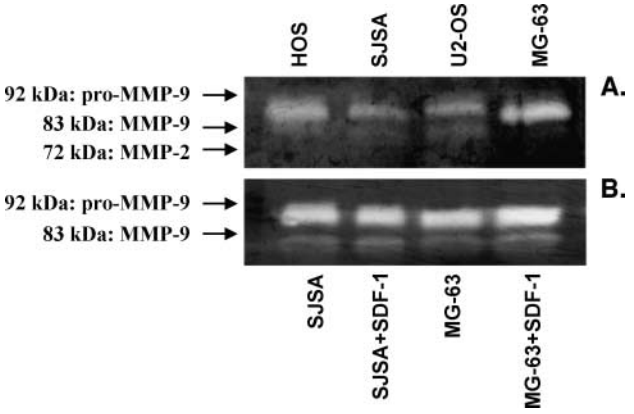

Fig. 4 Zymography assay on osteosarcoma cells supernatants. *A,* MMPs secretion on supernatant osteosarcoma cell lines. *B,* pro-MMP-9 and MMP-9 modulation by SDF-1 on SJSA and MG-63 cells. Cells were treated for 1 hour with rhSDF-1 (100 ng/mL) (SJSA + SDF-1 and MG-63 + SDF-1) and the supernatants subjected to zymographic assay.

Moreover, we analyzed the role of SDF-1 in osteosarcoma cell adhesion. The rhSDF-1 pretreatment of the monolayer of endothelial and bone marrow stromal cells was done to enhance the biological activities of endogenous SDF-1 as described by Amara et al. (35). Figure 6*A* shows that the adhesion of SJSA cells to bone marrow stromal and endothelial cells significantly increase in the presence of rhSDF-1. To show that this phenomenon was CXCR4/SDF-1 system dependent, we blocked the receptor on SJSA cells and showed that the adhesion to HUVEC and to bone marrow stromal cells decreased at about 25% (Fig. 6*B*).

Although SDF-1 regulates migration/adhesion processes of SJSA cells, MTT assay showed that rhSDF-1 treatment did not significantly modify osteosarcoma cell proliferation (data not shown).

T134 Inhibits Osteosarcoma Cell Metastases to the Lung

To further investigate whether CXCR4 is involved in *in vivo* osteosarcoma progression, we blocked the chemokine receptor with T134, a 14-amino-acid peptide, which has been shown to specifically block CXCR4 receptor (24, 25).

Injection of SJSA cells into a tail vein of the mice determined lung metastasis formation in 5 of 5 mice. Figure 7*B* shows a representative lung section of a mouse given an injection of SJSA cells alone in which we can observe an extensive metastasis of osteosarcoma cells. On the contrary, coinjection of T134 with SJSA cells and the following peptide treatment completely prevent lung metastasis formation, both macroscopically and microscopically, in 6 of 6 mice (Fig. 7*C*; $P < 0.001$).

DISCUSSION

Expression of the chemokine receptor CXCR4 can drive the migration of tumor cells across lymphatic and vascular system

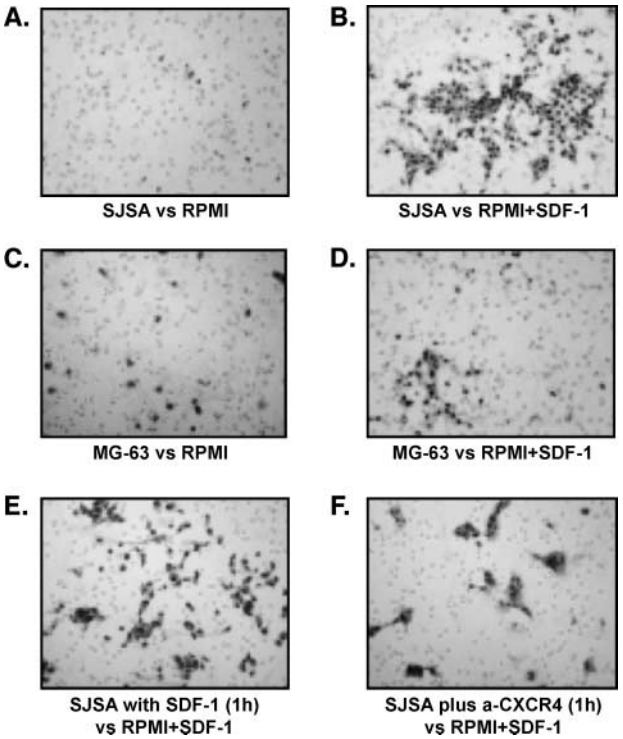

Fig. 5 Osteosarcoma cell migration toward medium with or without rhSDF-1. *A,* SJSA cell migration toward medium. *B,* migration of SJSA cells toward medium with rhSDF-1 (100 ng/mL). *C,* MG-63 cell migration toward medium. *D,* MG-63 cell migration in response to rhSDF-1. *E,* rhSDF-1-pretreated SJSA cell migration toward medium plus rhSDF-1. *F,* migration of SJSA cells pretreated with α-CXCR4 mAb toward medium with rhSDF-1.

and the arrest in SDF-1-rich organs. Osteosarcoma cells metastasize to organs that produce high levels of SDF-1 such as lung (5).

In the present study, we analyzed the possibility that the metastatic process in osteosarcoma involves the CXCR4/SDF-1 axis.

In vitro studies on osteosarcoma cell lines showed an inverse correlation between ligand and receptor expression, suggesting that CXCR4 expression is regulated by autocrine production of SDF-1. The exposure of osteosarcoma $CXCR4^+$ cells to exogenous rhSDF-1 caused a down-regulation of the receptor. This down-regulation induced by SDF-1 is the result of CXCR4 internalization, as occurs in hematopoietic cells (36).

MMPs have extracellular matrix–degrading activity and they are involved during cancer progression; the increase of MMPs activity contributes to acquisition of malignant properties (30, 31). It was previously shown that endothelial, epithelial, and hematopoietic cells express MMPs and that growth factors and cytokines could induce MMP-2 and MMP-9 (gelatinase A and B) secretion (33, 34). We detected pro-MMP-9 (92 kDa) and MMP-9 (83 kDa), but not MMP-2 in osteosarcoma cell line supernatants. We observed that rhSDF-1 treatment increased the pro-MMP-9 release in the supernatant of the MG-63 cell line but not in the SJSA cell line. Nevertheless, rhSDF-1 treatment increased the gelatinolytic activity of MMP-9 (83 kDa) corresponding to an active form of MMP-9, as described by other authors (32), but had no effects on the MMP-2 secretion.

Chemoinvasion of SJSA cell line, which expresses high levels of CXCR4, occurs following SDF-1 ligand gradient. On the contrary, MG-63 cell migration toward rhSDF-1 was significantly lower according to the level of CXCR4 expression. We showed that migration is CXCR4 dependent: by blocking CXCR4/SDF-1 interactions with a monoclonal neutralizing antibody α-CXCR4 before assay, cell migration was significantly impaired.

Different levels of CXCR4 on osteosarcoma cells might explain the different behavior of tumor cells. Autocrine regulation of CXCR4 by endogenous SDF-1, as occurs on MG-63, might limit their metastatic potential but support local invasion (30, 31), as shown by low migration capacity and high MMP-9 production.

SDF-1 production might be involved in the localization of CXCR4-positive cells to tissue compartments. It has been shown that SDF-1 binding on cell membrane heparin and glycosaminoglycans produces a biological active form of SDF-1 (37). Adhesion of SJSA cells on a monolayer of endothelial or bone marrow stromal cells was increased after rhSDF-1 treatment. The blockade of the receptor with a mAb α-CXCR4 caused a

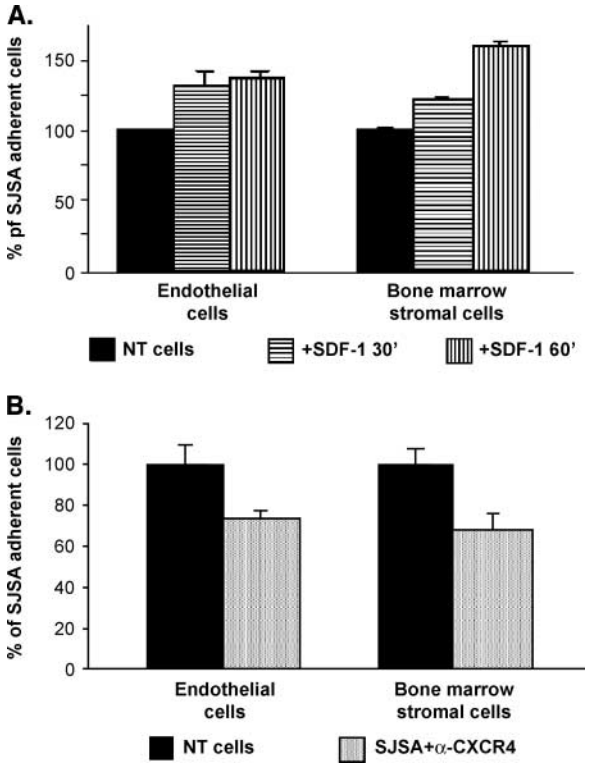

Fig. 6 Adhesion of SJSA cells to endothelial (HUVEC) or to bone marrow stromal cells. *A,* labeled SJSA cells were added to a SDF-1-stimulated and unstimulated (*NT cells*) monolayer of HUVEC or bone marrow stromal cells (100 ng/mL SDF-1 for 1 hour at 37°C), and incubated for 30 (+ *SDF-1 30′*) and 60 (+ *SDF-1 60′*) minutes. *B,* SJSA cell adhesion, after 1-hour incubation with a neutralizing mAb α-CXCR4, to HUVEC or bone marrow stromal cells. The adhesion of SJSA to the cell monolayer not treated with rhSDF-1 (*NT cells*) was normalized as 100%.

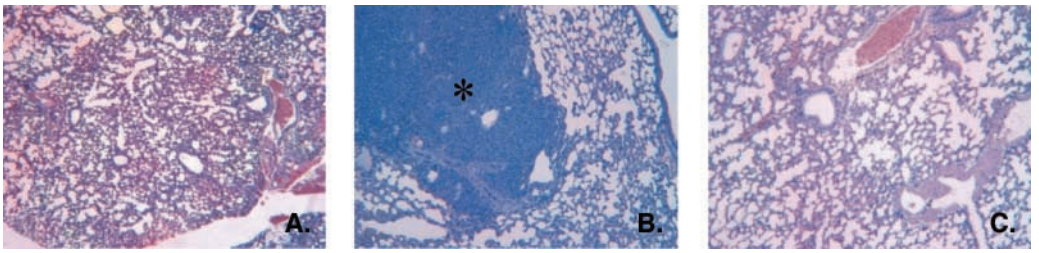

Fig. 7 Effect of CXCR4-neutralization on osteosarcoma metastases *in vivo*. Representative H&E staining of lungs from BALB/c *nu/nu* mice. *A,* negative control, mouse not injected. *B,* lung section with osteosarcoma colony formation after i.v. injection of SJSA cells. *C,* lung section after SJSA and T134 coinjection. *, tumor cells. Magnification ×20.

reduction of cell adhesion. The SDF-1 ligand promotes migration and adhesion processes of osteosarcoma cells, but it does not influence their proliferation and survival as described in rhabdomyosarcoma and in lymphohematopoietic cells (19, 38); however, SDF-1 might synergize *in vivo* with other growth factors as previously described (39).

Osteosarcoma is characterized by a high local aggressiveness with a tendency to metastasize to the lung, one of the preferential sites for SDF-1 production, so a specific regulation of CXCR4 can occur at this level. These *in vitro* experiments with stromal and endothelial cells cannot address the question of tissue specificity; therefore we set up an *in vivo* model with T134 peptide that mimics the region of SDF-1 involved in binding to CXCR4 and prevents receptor activation. It was shown that daily treatment with T22 reduces pulmonary metastases in mice after inoculation of CXCR4-transduced melanoma cells (22).

Here we reported that coinjection of osteosarcoma cells and T134 peptide into BALB/c *nu/nu* mice strongly prevents lung metastasis formation. Because T134 is CXCR4 specific and other receptors might be involved in osteosarcoma progression, such as CCR7 or CCR5 (40, 41), blockage of the CXCR4/SDF-1 pathway could not completely prevent metastasis formation. In our model, T134 inhibits the osteosarcoma cell adhesion to the pulmonary epithelium because at this level there is a stronger interaction of T134 with CXCR4 than with SDF-1; this peptide competes with SDF-1 at much lower concentration and its smaller size confers higher affinity for CXCR4.

In conclusion, our results strongly suggest that the CXCR4/SDF-1 pathway might have a role in osteosarcoma tumor progression, supporting some of the sequential events that are involved in metastasis formation. Small CXCR4 antagonists such as T134 could represent the starting point for innovative therapeutic strategies in osteosarcoma treatment.

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