Overexpression of sialidase NEU3 increases the cellular radioresistance potential of U87MG glioblastoma cells

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1. Introduction

Sialidases or neuraminidases (E.C. 3.2.1.18) are exoglycosidases that play a pivotal role in sialoglycoconjugates metabolism and in cell growth and cellular differentiation [1]. These enzymes remove sialic acid residues from the terminal ends of the oligosaccharide chains of glycoconjugates. Among the four different mammalian sialidases (NEU1–NEU4) identified [2–4], NEU3 is the plasma membrane-associated form of the enzyme, present at the cell surface and associated to the external leaflet of the plasma membrane [5] and exosomes [6]. Gangliosides, particularly GM3 and GD1α, are the preferential substrates of the enzyme [7–9]. By modifying the cellular ganglioside composition, NEU3 regulates different physiological phenomena like proliferation, apoptosis but also tumor transformation [10,11]. Up-regulation of NEU3 has been correlated to different human tumors such as colon, renal, prostate and ovarian cancers [12]. Overexpression of NEU3 in HeLa cells induces the phosphorylation of Akt even in the absence EGF [13] and a recent study has demonstrated that NEU3 can also directly act on EGFR glycans(s), resulting in the activation of the receptor [14]. In the last few years many scientific papers demonstrated the involvement of the enzyme in oncogenic transformation mediated by EGF [10,15]. Amplification of EGFR gene, that results in EGFR overexpression, is the most frequent alteration in Glioblastoma Multiforme (GBM) [16], that represents the most common and aggressive primary brain tumor [17]. To date, standard therapy for GBM includes maximal resection followed by radiotherapy and concomitant adjuvant chemotherapy [18]. Nevertheless, survival after diagnosis varies from 12 to 15 months, with less than 3–5% of patients surviving beyond five years [19]. In this study, we analyzed whether expression of sialidase NEU3 could influence the radioresistance characteristics of the GBM cell line U87MG.
2. Material and methods

2.1. Chemicals and reagents

All chemicals were molecular biology-grade (SIGMA-Aldrich) unless specified. Cetuximab (MerckSerono) was kindly provided by Dr. L. Triggiani from Istituto del Radio “O. Alberti”, Department of Radiation Oncology, University of Brescia and Spedali Civili Hospital, Brescia.

2.2. Cell culture, transfection and treatments

U87MG cells were obtained from IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, Brescia, Italy) and cultured in DMEM (GIBCO) containing 4 mM l-Glutamine, 100 Units/ml Penicillin, 100 μg/ml Streptomycin and 10% (v/v) Fetal Bovine Serum (FBS) (GIBCO). Cells were stably transfected with pcDNA3.1-mNEU3–HA [13], while Mock cells were obtained by transfection with pcDNA3.1 empty vector. Transfectants were grown in complete medium supplemented with 0.5 mg/ml G418. Treatment with inhibitors was performed by adding to the medium 5 μM Erlotinib (Cell Signaling) for 1 h; 60 nM Cetuximab (Merck-Serono) for 1 h; 50 μM LY-2094002 (Cell Signaling) for 1 h; 5 μM KU-60019 (Selleckem) for 24 h; 5 μM KU-0063794 (Selleckem) for 24 h.

2.3. Protein determination and sialidase activity assay

Protein determination was performed using the Bredford Method Coomassie Protein Assay Reagent. The enzymatic activity was determined as described previously [9] using 0.1 mM 4-MU-C0 and 1 mM MgCl2 and 1 mM CaCl2 (PBS þþ) for 15 min at room temperature. Indirect immunofluorescence was performed as already described [5]. Rabbit anti-H2AX (1:100; Cell Signaling) was used as primary antibody and donkey anti-rabbit Alexa Fluor-555 (1:400; Invitrogen) as secondary antibody. Cell nuclei were stained with 0.1 μg/ml DAPI for 1 min. Analysis was performed with LSM 510 META confocal laser scanning microscope (Carl Zeiss) and images was processed using ImageJ Software.

2.4. Ionizing radiation

Cells were irradiated at the Istituto del Radio “O. Alberti” - Department of Radiation Oncology, University and Spedali Civili Hospital, Brescia - using a Linac Accelerator (Elekta SL75), nominal Energy 6 MV. Irradiation was performed with gantry angle 180°/C0/C0 and cultured in DMEM (GIBCO) containing 4 mM l-Glutamine, 100 Units/ml Penicillin, 100 μg/ml Streptomycin and 10% (v/v) Fetal Bovine Serum (FBS) (GIBCO). Cells were stably transfected with pcDNA3.1-mNEU3–HA [13], while Mock cells were obtained by transfection with pcDNA3.1 empty vector. Transfectants were grown in complete medium supplemented with 0.5 mg/ml G418. Treatment with inhibitors was performed by adding to the medium 5 μM Erlotinib (Cell Signaling) for 1 h; 60 nM Cetuximab (Merck-Serono) for 1 h; 50 μM LY-2094002 (Cell Signaling) for 1 h; 5 μM KU-60019 (Selleckem) for 24 h; 5 μM KU-0063794 (Selleckem) for 24 h.

2.5. Clonogenic assay

Cells were grown in complete DMEM and treated with different radiation doses. After 30 min cells were collected by scraping, resuspended in cold culture medium and seeded for clonogenic cell survival assay and the remaining cells were used for Western-blot analysis. Cells were grown in complete DMEM for 14 days. Surviving colonies were fixed with 80% ice cold ethanol and stained with 0.1% Crystal Violet in 20% methanol. Only colonies of 50 cells or more were counted. Surviving fractions were calculated as the ratio between the colonies formed by irradiated cells and those formed by non-irradiated cells.

2.6. Immunoblotting

Proteins were separated by SDS/10% PAGE and transferred to a Hybond-P PVDF membrane (GE Healthcare). Membranes were then blocked with 5% (w/v) BSA C0 and fraction V in PBS, washed with PBS containing 0.1% Tween 20 (PBST) and incubated with primary antibody diluted in PBST containing 1% (w/v) BSA for 1 h at room temperature. After washes with PBST, membranes were incubated with HRP-conjugated secondary antibody diluted in PBST. Immunocomplexes were detected using the Western-blot Luminol Reagent (Santa Cruz). The following primary antibodies were used: rabbit anti-HA (1:1000; Sigma); mouse anti-GAPDH (1:8000; Millipore); rabbit anti-phospho-ERK1/2 (1:500; Cell Signaling); rabbit anti-EKR1/2 (1:500; Cell Signaling); rabbit anti-phospho-AKT Ser473 (1:1000; Cell Signaling); rabbit anti-phospho-AKT Thr308 (1:1000; Cell Signaling); rabbit anti-AKT (1:1000; Cell Signaling). The following secondary antibodies were used: goat anti-rabbit (1:5000; Cell Signaling) and donkey ant-mouse (1:10000; Sigma).

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3.2. The increased survival capacity of U87MG cells overexpressing sialidase NEU3 is driven by PI3K

Ionizing radiations are known to activate EGFR [20,21] resulting in the further activation of down-stream survival pathways, including ERK1/2 and Akt pathways [22]. We analyzed this aspect in our cellular model and found that at 0 Gy U87MG cells expressing NEU3 showed a higher phosphorylation of ERK1/2, Akt-Thr308 and Akt-Ser473 compared to Mock cells (Suppl. Fig. 1). Irradiation increased the phosphorylation of ERK1/2 in both cells types, with a stronger effect in 1D1 cells. A similar behavior was observed for Akt-Thr308 while no significant changes were observed for Akt-Ser473. Of note, the phosphorylation state of Akt-Ser473 in cells overexpressing NEU3 was always higher compared to Mock cells. These data demonstrate that overexpression of NEU3 in U87MG cells results in an increased basal activation of ERK1/2 and Akt, and irradiation enhances this effect. Since ERK1/2 and Akt are downstream targets of EGFR, Mock and 1D1 cells were pre-treated or not with the Tyrosine Kinase inhibitor Erlotinib or with the chimeric EGFR monoclonal antibody Cetuximab before irradiation and subjected to clonogenic assay. Treatment with the two EGFR inhibitors alone resulted in a slight decrease in the colony formation potential in both cell lines (Fig. 2A). Pre-treatment with Erlotinib or Cetuximab before irradiation did not modify the survival potential of 1D1 cells. On the contrary, the survival potential of Mock cells resulted significantly decreased after pre-treatment with the two EGFR inhibitors. These data demonstrate that EGFR is not involved in the increased radioresistance features observed in U87MG cells expressing NEU3.

We then decided to inhibit the Akt pathway focusing on the two essential phosphorylated positions of the protein, i.e. Serine 473 and Threonine 308. As first, we blocked the Akt activation on Seine 473 by inhibiting mTORC2 and ATM protein kinase. Cells were pre-treated or not with the mTORC1/2 inhibitor KU-0063794 or with the ATM inhibitor KU-60019 before irradiation and subjected to clonogenic assay. Pre-treatment with the inhibitors alone resulted in a similar decrease in the colony formation potential in both cell lines and compared to the corresponding untreated cells (Fig. 2B). After irradiation, the surviving potential of 1D1 cells after pre-treatment with KU-0063794 and KU-60019 was statistically significant higher ($p < 0.05$) compared to Mock cells. These data point out that inhibition of mTORC and ATM is not sufficient to abolish the radioresistance capacity observed in NEU3 expressing cells. We then blocked the activation of Atk at Threonine 308 by inhibiting PI3K, another key molecule in this signaling pathway. Interestingly, no statistically significant differences in the surviving fraction between Mock and 1D1 cells could be observed after irradiation in presence of the PI3K inhibitor (Fig. 2B). In detail, the surviving fraction observed in Mock and 1D1 cells after 2 Gy irradiation in presence of LY-294002 was 30% and 38%, respectively, and compared to untreated and non-irradiated corresponding cells.

Fig. 1. Overexpression of sialidase NEU3 in U87MG cells increases cell survival after irradiation. (A) Analysis of the expression of sialidase NEU3 by means of Western blot (upper panel) and enzymatic activity (lower panel) in cell extracts deriving from Mock transfected (Mock) and NEU3 transfected U87MG cells (1D1). Values represent the average of five independent determinations. SD: Standard deviation. (B) Histogram showing the surviving fraction of Mock and 1D1 cells subjected to the indicated irradiation doses. Values represent the number of colonies counted relative to the corresponding not irradiated cells. Values represent the average of three independent experiments ($**p < 0.01; ***p < 0.001$).

Fig. 2. The survival capacity of U87MG cells overexpressing sialidase NEU3 after irradiation is driven by PI3K. Mock and 1D1 cells were treated or not with (A) Cetuximab or Erlotinib as EGFR inhibitors, (B) KU-0063794 as mTORC1/2 inhibitor, KU-60019 as ATM inhibitor, LY-294002 as PI3K inhibitor before irradiation. Values represent the number of colonies counted relative to the corresponding not irradiated cells. Values represent the average of three independent experiments ($*p < 0.05; **p < 0.01; ***p < 0.001$).
These results demonstrate that PI3K is deeply involved in the higher surviving potential observed in U87MG cells expressing NEU3.

3.3. Overexpression of sialidase NEU3 in U87MG cells increases the DNA-repair capacity

An immediate effect of ionizing radiations is represented by the DNA damage, to which cells respond activating different DNA-repair mechanisms. Among these, phosphorylation of histone H2AX (γH2AX) is a commonly used parameter to bring to evidence DNA double strand brakes (DNA-DSB) [23]. We investigated whether overexpression of NEU3 could influence the mechanisms of DNA-DSB after irradiation by the detection of γH2AX-positive nuclei after irradiation. Without irradiation, no significant differences in the number of γH2AX-positive nuclei between Mock and 1D1 cells were found (Fig. 3). After 30 min from irradiation with 2 and 4 Gy, both cell lines showed a significant increase in the relative number of γH2AX-positive nuclei compared to the corresponding non-irradiated cells. Although the relative number of γH2AX-positive nuclei increased in both cell lines in relation to the different radiation doses, a statistically significant lower number was observed in 1D1 cells. After 4 h from irradiation, the relative number of γH2AX-positive nuclei in 1D1 was statistically lower compared to Mock cells. After 24 h from irradiation the relative number of γH2AX-positive nuclei in 1D1 cells further decreased and remained significantly lower compared to Mock cell, reaching values comparable to non-irradiated 1D1 control cells. These data demonstrate that overexpression of NEU3 accelerates the DNA-DSB repair mechanisms of U87MG cells.

We then analyzed whether ATM, a protein with a key role in DNA-DSB repair, could be responsible for the different response to radiation-induced DNA damage observed in cells overexpressing NEU3. Mock and 1D1 cells were pre-treated or not with the ATM inhibitor KU-60019 before irradiation and after 24 h γH2AX-positive nuclei were counted. In Mock cells pre-treated with KU-60019 the number of γH2AX-positive nuclei was 3-fold and 4-fold higher after 2 Gy and 4 Gy irradiation, compared to non-irradiated cells. The difference in the number of γH2AX-positive nuclei between Mock and 1D1 cells in presence of ATM inhibitor was not statistically significant at 2 Gy and slightly significant at 4 Gy (Fig. 4). These results demonstrate that inhibition of ATM could counteract the protective effect on DNA-DSB repair induced by NEU3.

4. Discussion

In the last years, many reports demonstrated that upregulation of sialidase NEU3 occurs in different tumors [10–12]. GBM
represents the most common and most aggressive variant among primary brain tumors and radiotherapy is an important component of multimodality GBM treatment that comprehends also surgery and chemotherapy [18].

Irradiations have been shown to induce EGFR activation [21] and Akt activation in multiple cell types, including some human GBM cells [24]. Both pathways are related to cell proliferation and cell survival features of tumor cells. Moreover, Akt regulates also DNA repair [25], and modifications in PI3K/Akt pathway can lead to radioresistance [26]. Akt is fully activated through phosphorylation of two key residues, i.e. Threonine 308 and Serine 473. In the cytosol, mTORC2 mediates phosphorylation at Serine 473 while PDK1 phosphorylates Akt at Threonine 308. The completely active Akt can translocate to the nucleus [27,28]. On the other hand, partially activated Akt can translocate to the nucleus after irradiation and become fully activated by nuclear PDK1 acting on Threonine 308 or by nuclear DNA-PKcs, including ATM, at Serine 473 in response to DNA damage [29,30]. Moreover, beside phosphorylation of Akt at Serine 473, ATM is responsible for the phosphorylation of histone H2AX by ATM on Serine 139 (γH2AX), one of the earliest events involved in DNA Damage Response (DDR) after irradiation [23,29,31].

Little is known about the possible role played by NEU3 in relation to brain tumors such as GBM. A recent report demonstrated that overexpression of NEU3 in different GBM cell models, namely U251, A172 and T89G, reduced invasion and migration properties by promoting the assembly of focal adhesions, although the molecular mechanism remains unclear at present [32]. We investigated the possible role played by NEU3 in relation to radioresistance features in a GBM cell model represented by U87MG cells. Overexpression of NEU3 resulted in a significant increased capacity of U87MG to resist to irradiation. The radioresistance capacity of NEU3 expressing cells resulted to be independent from EGFR. Instead, by selective inhibition of mTORC, ATM and PDK1 we found that the PI3K/Akt pathway is deeply involved in the GBM radioresistance. The PI3K/Akt pathway is deeply involved in survival and death in cancer biology, Nat. Rev. Canc. 16 (2016) 201–214, https://doi.org/10.1038/nrncanc.2016.1. Akt is fully activated through phosphorylation of two key residues, i.e. Threonine 308 and Serine 473. In the cytosol, mTORC2 mediates phosphorylation at Serine 473 while PDK1 phosphorylates Akt at Threonine 308. The completely active Akt can translocate to the nucleus [27,28]. On the other hand, partially activated Akt can translocate to the nucleus after irradiation and become fully activated by nuclear PDK1 acting on Threonine 308 or by nuclear DNA-PKcs, including ATM, at Serine 473 in response to DNA damage [29,30]. Moreover, beside phosphorylation of Akt at Serine 473, ATM is responsible for the phosphorylation of histone H2AX by ATM on Serine 139 (γH2AX), one of the earliest events involved in DNA Damage Response (DDR) after irradiation [23,29,31].

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.11.086.

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References
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