Semaphorin3B modulates radiosensitivity of human glioma U-87MG cells postprint

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Abstract

This study aimed to investigate the role of Semaphorin3B (SEMA3B) in the response of glioma cells to irradiation. Two glioma cell lines were employed: U-87MG, which harbors wild-type p53, and U-251, which carries mutated p53. SEMA3B mRNA was detectable in both cell lines. The expression level of SEMA3B mRNA was higher in U-87MG cells than in U-251 cells and increased in a time-dependent manner in U-87MG cells following irradiation. Knockdown of SEMA3B expression by shRNA decreased the radiosensitivity of U-87MG cells, which may be associated with increased G2 accumulation following irradiation. Furthermore, G2 accumulation following irradiation was enhanced in U-87MG cells with low SEMA3B expression. These results demonstrate that SEMA3B is implicated in the response of glioma cells to irradiation.

Full Text

Preamble

Semaphorin3B modulates radiosensitivity of human glioma U-87MG cells

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Abstract

This study investigated the role of Semaphorin3B (SEMA3B) in glioma cell response to irradiation. Two glioma cell lines were used: wild-type p53 (U-87MG) and mutated p53 (U-251). SEMA3B mRNA was detectable in both cell lines, with higher expression in U-87MG cells than in U-251 cells. In U-87MG cells, SEMA3B expression increased with time after irradiation. Knockdown of SEMA3B expression by shRNA decreased the radiosensitivity of U-87MG cells, which may be associated with increased G2 accumulation after irradiation. Additionally, G2 accumulation after irradiation was enhanced in SEMA3B low-expressing U-87MG cells. These results demonstrate that SEMA3B is implicated in glioma cell response to irradiation.

Key words: SEMA3B, RNA interference, Ionizing radiation, Radiosensitivity, Glioma

Introduction

Semaphorin3B (SEMA3B), a member of the semaphorin family located at chromosome region 3p21.3 in humans, encodes a secreted protein belonging to the class 3 semaphorins. Beyond axon guidance, semaphorin3 proteins are implicated in organogenesis, immune modulation, and drug resistance. SEMA3B is frequently inactivated by allele loss and promoter methylation at relatively early stages of tumor progression and is considered a tumor suppressor. The function of SEMA3B is mediated by two types of cell surface receptors (neuropilins and plexins). Neuropilins provide binding sites for SEMA3B, while plexins are responsible for signal transduction. The conditioned medium of recombinant SEMA3B-expressing cells can reduce cell growth and induce apoptosis in several lung and breast cancer cell lines. As an ovarian cancer cell line, HEY cells expressing diminished tumorigenicity in BALB/C nu/nu mice indicate SEMA3B's role in tumor suppression. However, SEMA3B is reportedly expressed at high levels in many invasive and metastatic human tumors. Experimental tumor models have confirmed that SEMA3B expression in tumor cells promotes metastatic dissemination, although this may delay tumor growth. To our knowledge, the role of SEMA3B in radiotherapy has not yet been studied.

Glioma is one of the most common malignancies in the central nervous system, accounting for about 50% of brain tumors. Its high potential for invasion makes complete surgical resection impossible. Radiotherapy is another treatment modality for glioma, but due to its radioresistance, all patients suffer tumor recurrence despite aggressive irradiation. The median survival for glioma patients is approximately one year. Research based on Affymetrix gene chip analysis reported that SEMA3B expression was associated with poorer overall survival in glioma patients. To date, the role of SEMA3B in human gliomas remains ambiguous.

In this study, we investigated the correlation between SEMA3B and radiosensitivity in glioma cells harboring wild-type p53 (U-87MG) and mutated p53

(U-251). Furthermore, the possible mechanisms were discussed.

2.1 Cell culture

Human malignant glioma cell lines U-87MG and U-251 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were incubated in 5% CO_2 at 37°C.

2.2 Real-time reverse transcription-PCR

Total RNA was extracted from cells with Trizol (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed using Random Primer, dNTP, and PrimeScript Reverse Transcriptase (TaKaRa, Japan). Quantitative real-time PCR was performed in triplicate using SYBR Green Master mix (ABI, USA) on ABI Prism 7000 System. The PCR conditions were 50°C for 2 min, 95°C for 10 min, and 42 cycles at 95°C for 15 s and 60°C for 1 min. SEMA3B and 18S primers included: sense 5 -AGACTTTCAGCCTGGAGCGAAC-3 and antisense 5 -GCAAATGGGTGCGGTTGTAG-3 for SEMA3B; sense 5 -ACGACCCATTCGAACGTCTG-3 and antisense 5 -CCGTTTCTCAGGCTCCCTC-3 for 18S. The 18S was used as an endogenous control. Relative SEMA3B mRNA levels were calculated as follows: ΔCt (sample) = Ct (SEMA3B) − Ct (18S); ΔΔCT = ΔCt (radiation dose point or post-irradiation time point) − CT (0 Gy or 0 h), and relative expression = 2^(−ΔΔCt).

2.3 Construction and transfection of short hairpin RNA (shRNA) plasmid expression vector

19-mer hairpin sequences were designed, and the sequences of small interfering RNA constructs targeting SEMA3B (GenBank ID: NM_{001005914}.1) were as follows: sense, GCUCAGUAUAAUCCCAAAUUG and antisense, CAAUUUGGGAUUAUACUGAGC. A scrambled sequence of siRNA with no homology to human sequences was used as a control. The oligonucleotides were synthesized, annealed, and ligated into linearized pGPU6/Neo siRNA Expression Vector (GenePharma, China). To generate SEMA3B shRNA-transfected U-87MG cells, U-87MG cells were seeded into 6-well plates at 3$×10^{5}$ per well. When cells reached 80% confluence, plasmid DNA (4 g) was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, cells were placed in selection medium containing 200 g/mL G418 (Amresco, USA) for two weeks. The stably transfected cells, designated as U-87MG/SEMA3BshRNA or U-87MG/controlshRNA, were cultured in medium containing 100 g/mL G418.

2.4 Western blot analysis for expression of SEMA3B protein

Cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer for 40 min. After centrifugation, supernatants containing proteins were collected. Proteins were electrophoresed on a 5–10% gradient SDS-PAGE gel and transferred to PVDF membranes. After blocking with 5% nonfat dry milk in PBS containing 0.2% Tween-20, the membrane was probed with anti-SEMA3B antibody (Lifespan, USA) at 1:1000, followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime, China). Immunoblot signals were detected using ECL reagent (Beyotime). As a loading control, β-actin expression level was determined using mouse anti-β-actin and HRP-labeled goat anti-mouse IgG (Beyotime).

2.5 Cell irradiation

Irradiation equipment (Soochow University) was used for all radiation-related experiments. Exponentially growing cells were irradiated by a ^{60}Co gamma-radiation source at a dose rate of 0.6 Gy/min.

2.6 Clonogenic survival assay

Cells irradiated with different doses were trypsinized and appropriately diluted, then replated in 6-well plates for colony formation assay. Cells were incubated in routine medium at 37°C for 10 days. All cells were fixed with 10% methanol and stained with Giemsa. Colonies of 50 cells or more were counted. Plating efficiency (PE) and survival fraction (SF) were calculated as follows: PE = (number of colonies counted)/(number of unirradiated cells seeded) × 100%, and SF = (number of colonies counted)/(number of cells seeded × PE). The normalized survival fraction relative to unirradiated cells was calculated. Survival curves were obtained and analyzed by SigmaPlot to obtain D_0, Dq, and SF_2.

2.7 Cell cycle analysis by flow cytometry

For cell cycle analysis, exponentially growing cells were synchronously cultured by serum deprivation, irradiated with a dose of 4 Gy, and incubated for 24 h at 37°C. Cells were then harvested, washed, and fixed with 70% ice-cold ethanol overnight. Cells were incubated in propidium iodide (50 mg/mL in PBS containing 100 mg/mL RNase A) at 4°C for 1 h and analyzed by flow cytometry.

2.8 Statistical analysis

Data were expressed as mean ± standard deviation (±SD) and analyzed by SAS statistical software. Student's t-test was used to measure statistical significance between groups. Multiple comparisons were performed with one-way analysis

of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

3.1 Expression of SEMA3B mRNA in U-87MG and U-251 cell lines

To study the role of SEMA3B in glioma cells exposed to irradiation, SEMA3B expression in U-87MG and U-251 cells was compared using real-time PCR. SEMA3B mRNA was detectable in both glioma cell lines, with significantly higher levels in U-87MG cells than in U-251 cells ($p<0.05$) (Fig.1).

3.2 Effect of irradiation on SEMA3B mRNA expression in glioma cell lines

In U-87MG cells, SEMA3B mRNA levels showed an irradiation-induced increase (Fig.2A), with the highest level observed in cells irradiated with 4 Gy, approximately 2.41-fold higher than in unirradiated U-87MG cells. However, SEMA3B mRNA expression in U-251 cells showed a radiation-induced decrease, with the lowest level in cells treated with 10 Gy. Comparing U-87MG with U-251, a statistically significant increase in SEMA3B expression was observed at each dose point ($p<0.05$). As shown in Fig.2B, following a single irradiation dose of 4 Gy, SEMA3B mRNA levels in U-87MG cells showed a time-dependent increase, with maximal increase at 72 h post-irradiation.

3.3 Knockdown of SEMA3B expression by shRNA transfection

Real-time RT-PCR and Western blotting were performed to assess the effect of SEMA3B shRNA on SEMA3B expression. The inhibition rate of SEMA3B mRNA after stable transfection with SEMA3B shRNA was 97% (Fig.3A). Compared with U-87MG/controlshRNA, SEMA3B protein expression was significantly downregulated in U-87MG/SEMA3BshRNA cells as determined by Western blotting (Fig.3B). These results confirm that the cells were stably transfected with inhibited SEMA3B expression.

3.4 Knockdown of SEMA3B decreased radiosensitivity of U-87MG cells

As radiation enhanced SEMA3B mRNA expression in U-87MG cells, we examined the radiosensitivity of cells with downregulated SEMA3B expression. Radiosensitivity was evaluated by clonogenic survival assay. Compared with control cells, our data demonstrated that the survival fraction of U-87MG/SEMA3BshRNA cells significantly increased at each dose point ($p<0.05$). The survival curves showed that downregulation of SEMA3B

enhanced the colony-forming ability of U-87MG cells (Fig.4). Values of D_0, Dq, and SF_2 were calculated by SigmaPlot (Fig.4B), indicating that SEMA3B knockdown decreased the radiosensitivity of U-87MG cells.

3.5 Knockdown of SEMA3B expression enhanced G2 accumulation of U-87MG after irradiation

To explore the mechanism by which SEMA3B modulates the radiosensitivity of U-87MG cells, cell cycle distribution after exposure to 0 or 4 Gy was analyzed. After irradiation, the G2 phase percentage increased in both U-87MG/SEMA3BshRNA and U-87MG/controlshRNA cells, which was more evident in U-87MG/SEMA3BshRNA than in U-87MG/controlshRNA cells (Fig.5). Moreover, there was G1 accumulation in U-87MG/controlshRNA but not in U-87MG/SEMA3BshRNA cells. This may be the mechanism of SEMA3BshRNA-induced radioresistance in U-87MG cells.

Fig.5. Cell cycle analysis of U-87MG/SEMA3BshRNA and U-87MG/controlshRNA cells by flow cytometry. (A) Representative figures of cell cycle distribution. (B) The cell cycle distribution of cells exposed to 0 or 4 Gy and harvested at 24 h post-irradiation. Data are means ±SD (n=3). *Statistical significance compared with U-87MG/controlshRNA irradiated with 0 Gy. **Statistical significance compared with U-87MG/SEMA3BshRNA irradiated with 0 Gy ($p<0.05$).

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4 Discussion

This study investigated the role of SEMA3B in irradiated glioma cell lines. SEMA3B mRNA expression was higher in U-87MG cells than in U-251 cells. Irradiation enhanced SEMA3B expression in U-87MG in a time-dependent manner, with the highest mRNA level when cells were irradiated with 4 Gy. However, irradiation induced a decrease in SEMA3B mRNA in U-251 cells. The differential response of SEMA3B to radiation may be due to the genetic nature of the glioma cell lines, possibly ascribed to p53 status. U-87MG is a glioma cell line with wild-type p53, while U-251 harbors mutated p53. In axon guidance, SEMA3B is a direct target of p53 and inducible by p53. Introduction of exogenous p53 into a glioma cell line lacking wild-type p53 can dramatically induce SEMA3B mRNA expression, and endogenous SEMA3B expression was induced in response to UV irradiation in a p53-dependent manner, consistent with our results using gamma irradiation.

To further examine radiation-enhanced SEMA3B expression in U-87MG, we downregulated SEMA3B expression by RNA interference to obtain stably transfected U-87MG cells with suppressed SEMA3B expression. RNA interference is a technique in which double-stranded RNA (dsRNA) is used to target specific mRNAs for degradation, thereby silencing specific gene expression. Although

dsRNA's ability to silence gene expression was only discovered a few years ago, it has become an important tool for analyzing gene function across a broad diversity of organisms, including human cells. Using this method, we found that SEMA3B may be correlated with the radiosensitivity of U-87MG cells, as U-87MG cells transfected with SEMA3B shRNA were more resistant to irradiation than control cells. Cell cycle distribution analysis showed that knockdown of SEMA3B expression in U-87MG enhanced G2 accumulation after irradiation. Cellular radiosensitivity is determined by a number of fundamental processes, and cell cycle distribution is one of the factors affecting cell radiosensitivity. Cell cycle delay at G2 is considered a process during which DNA damage is repaired. Therefore, increased G2 cells and the promotion of DNA damage repair may be the mechanism by which SEMA3B modulates radiosensitivity in U-87MG cells.

Additionally, SEMA3B could inhibit the phosphatidylinositol 3-kinase (PI3K)/Akt pathway through neuropilin-1 in lung and breast cancer cells. Activation of the PI3K/AKT pathway is implicated in radioresistance and associated with three major factors: radiation sensitivity, tumor cell proliferation, and hypoxia. The PI3K/AKT pathway may represent another mechanism by which SEMA3B modulates cell radiosensitivity. Also, SEMA3B competes with vascular endothelial growth factor (VEGF) for binding to the neuropilin receptor, and VEGF signaling is related to radioresistance. Inhibition of VEGF enhances radiation sensitivity. These results may provide insight into the mechanism by which SEMA3B modulates cell radiosensitivity.

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5 Conclusions

SEMA3B is implicated in glioma cell response to irradiation. Radiation enhanced SEMA3B mRNA expression in U-87MG in a time-dependent manner. Knockdown of SEMA3B expression increased the radioresistance of U-87MG cells, which may be associated with increased G2 accumulation after irradiation. Our study may help to understand the role of SEMA3B in glioma radiotherapy.

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Note: Figure translations are in progress. See original paper for figures.