Effects of SEMA3G on migration and invasion of glioma cells

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Received January 19, 2012; Accepted April 6, 2012

DOI: 10.3892/or.2012.1796

Abstract. Glioblastoma multiforme is the most aggressive type of brain tumor with a strong ability to invade and migrate into surrounding normal brain tissues, leading to high tumor recurrence and mortality. Most of class-3 semaphorins, especially SEMA3A, SEMA3B and SEMA3F, have been reported to have strong tumor inhibition ability, but the role of SEMA3G in tumor biology is largely unknown. We report here that SEMA3G possesses anti-migration and anti-invasion ability. To determine the potential effects of SEMA3G on migratory and invasive ability, we generated stable SEMA3G expression U251MG cells. We found that stably overexpressed SEMA3G inhibited the migratory and invasive behavior of U251MG cells. In addition, treatment with SEMA3G conditioned media also decreased the migratory and invasive ability of parental U251MG cells. Furthermore, SEMA3G also inhibited the activity of MMP2, an index of tumor invasion ability. Thus, our results suggest that SEMA3G inhibited tumor cell migration and invasion, which may be obtained through cell autonomous or paracrine mechanisms, and SEMA3G is a potential target for antitumor migration and invasion.

Introduction

Glioblastoma multiforme is the most common and aggressive type of brain tumor characterized by dissemination and infiltration of tumor cells into the brain stroma (1). As the infiltrating tumor cells are the source of tumor recurrence, the invasive nature of tumor cells makes a critical contribution to the ineffectiveness of current treatment modalities, suggesting the urgent need to understand in more detail the mechanisms of tumor invasiveness (2).

Class-3 semaphorins (SEMA3s) are a group of secreted glycoproteins of ~100 kDa consisting of seven subtypes (designated by the letters A-G), which are initially implicated in the development of the nervous system and in axon guidance (3). More recently, SEMA3s have been found to act as pleiotropic signals that are able to control multiple functions in tumor cells, ranging from survival, proliferation and apoptosis to cell adhesion and migration (4-7). Of the SEMA3 family members, SEMA3A, SEMA3B, and SEMA3F have the best-described roles in tumor growth and metastasis. For example, SEMA3A can dramatically inhibit the proliferation of breast cancer cells both in soft agar *in vitro* and in tumor models *in vivo* (8). In addition, SEMA3A is also found to reduce both the migratory and invasive behavior of breast tumor cells (9,10). The expression level of SEMA3B in tumor tissues is correlated with poor prognosis and metastatic progression (11,12). Consistently, SEMA3B overexpression in tumor cell lines induces apoptosis (13,14) and inhibits cell proliferation and colony formation in soft agar (5,15,16). SEMA3F expression also inversely correlates with lung-cancer tumor grading and staging (17). In addition, overexpression of SEMA3F in tumor cells inhibits proliferation, prevents anchorage-independent growth (18-20) and potently inhibits tumorigenesis *in vivo* (16). Moreover, SEMA3F inhibits cell-spreading and migration in breast carcinoma (6), melanoma cells (21) and colorectal cancer cells (22). Taken together, these findings suggest that SEMA3A, SEMA3B, and SEMA3F inhibit tumor growth. Although most members of the SEMA3s have been shown to be inhibitory, some promote tumor growth and metastasis (23). For instance, SEMA 3C promotes glomerular endothelial cell proliferation, adhesion, directional migration, and tube formation *in vitro* (24). Therefore, the role and mechanism of SEMA3 family proteins in tumor biological behavior need more extensive and intensive study.

Among SEMA3 family proteins, there are few studies on SEMA3G, and its role and mechanism in tumor biology are largely unknown. Although it is reported that overexpression of SEMA3G can inhibit breast cancer and melanoma growth and angiogenesis (8,25), its role in the inhibition of invasion and migration has not been reported. Because SEMA3s exert its function by interacting with its receptors neuropilins (NRP) and plexins (23) and SEMA3G receptor NRP2 participates in human glioma progression (26), we designed this study to determine the role of SEMA3G in glioma cell migration and invasion ability. In this study, we generated the SEMA3G stable expressing human glioblastoma U251 (U251MG) cell line and then examined the migratory and invasive behavior. We found that SEMA3G inhibited the migration and invasion abilities of U251 MG cells dramatically. The culture media from stable

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Key words: glioma, SEMA3G, migration, invasion, MMP2

SEMA3G expression U251MG cell (SEMA3G conditioned media) treatment also inhibited the migration and invasion abilities of naïve U251MG cells. Thus, our results indicated that SEMA3G acted in a cell autonomous or a paracrine fashion to affect tumor cell migration and invasion.

Materials and methods

Stable SEMA 3G overexpressing U251 MG cell generation. U251MG cells (purchased from Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences) were maintained in DMEM supplemented with 10% fetal bovine serum and transfected with pEGFP-N1-SEMA3G or pEGFP-N1 respectively using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The SEMA 3G-GFP construct was purchased from Shanghai Minghong Biotech, which was generated by cloning the human SEMA 3G cDNA into the expression vector pEGFP-N1 at the *Eco*RI and *Kpn*I restriction sites. After 24-h transfection, cells were maintained in DMEM containing G418 (1000 μg/ml) for ~2 weeks and the living GFP positive clones were picked up, digested with trypsin and living cells were counted via trypan blue staining. The isolated cells were plated into a 96-well plate with only one cell per well using limiting dilution and cultured with DMEM containing G418 (500 μg/ml) for maintaining selection pressure. About 2 weeks later, the big single cell clone was obtained, digested with trypsin and the isolated cells were plated into a 12-well plate. After the cells were grown to 90% confluence, they were transferred to a flask for more cells. The culture media and the cell lysates were collected and examined by RT-PCR or western blotting to analyze the expression and secretion of SEMA 3G-GFP. The media of positive cells were collected and the cells were frozed for future use. The cells were cultured under G418 selection pressure at all times.

Reverse transcription-polymerase chain reaction. Total RNA of U251MG cells was extracted using the TRIzol reagent (Tiangen Biotech Co.). First-strand cDNA was synthesized in a 20-μl reaction volume using the reverse transcription reagents [Takara RNA PCR Kit (AMV) Ver.3.0] according to the manufacturer's instructions. Primers (Sangon Biotech Co.) for SEMA 3G were as follows: sense: 5'-GGG TCT GTG CTC AAA GTC ATC G-3'; antisense: 5'-AAG TCC CAC TGC CTC TTC TTC C-3'. Primers for GAPDH were: sense: 5'-AGA AGG CTG GGG CTC ATT TG-3'; antisense: 5'-AGG GGC CAT CCA CAG TCT TC-3' yielding a product as a reference. Polymerase chain reaction conditions were: the first-strand DNA template was heated at 94˚C for 3 min before amplification, a total of 30 cycles were used including at 94˚C for 30 sec (denaturation), at 55˚C for 30 sec (annealing) and at 72˚C for 1 min (extension) and another 72˚C for 5 min (extension).

Western blot analysis. Cells were lysed in western blot lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 2 mM EDTA (pH 8.0), 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, aprotinin and leupeptin each at 10 pg/ml, 0.5% deoxycholic acid, and 0.1% SDS] for 30 min on ice. Protein lysates were concentrated using the BCA Protein Assay kit (Thermo Scientific) and equal amount of protein lysates or equal volume of media were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride membrane of 0.45-μm pore size (Millipore), and probed with primary antibodies (GFP or β-actin) at 4˚C overnight and horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Bound antibodies were detected by the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc.) and exposed to X-ray film. Band densities were quantified by ImageJ software, and the densitometric results were shown. The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the internal loading control. Western blot experiments were carried out in three biological replicates and average fold changes were reported.

Cell migration assay. The migration behavior of cells was evaluated using a wound healing assay. U251MG cells were grown to confluence in 6-well plates. Twenty-four hours after stable SEMA 3G expression cell plating or SEMA 3G conditioned media treatment, linear wounds were created by scraping confluent cell monolayer with a sterile blade in a definite array. The monolayer was rinsed twice with PBS and incubated in serum-free media or SEMA 3G conditioned media. At the initiation of the experiment (0 h), five randomly selected fields at the lesion border were acquired using a CCD camera on an inverted microscope (Olympus, IX71) and 24 or 48 h later the same region was imaged again. The total migratory cells into the wound area and the farthest distance migrated were quantified as the cell mobility ability index. Experiments were done at least three times and measurements were made in triplicate (27,28).

Cell invasion assay. The transwell matrigel invasion assays were used to assess the effect of SEMA3G on the invasiveness of cancer cells. Briefly, cell invasion assays were performed using insert membranes (8.0-μm pore size with polycarbonate membrane; Corning Incorporated) precoated with diluted matrigel (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. A cell suspension ($1x10^5$) in serum-free culture media was added to the inserts, and each insert was placed in the lower chamber filled with culture media containing 10% FBS. To test the invasion ability of SEMA 3G conditioned media treated cells, after treated with SEMA 3G conditioned media for 24 h, cell suspension in SEMA 3G conditioned media were plated onto the top of the inserts which was placed in the lower chamber filled with culture media containing 10% FBS. After 24-h incubation, the non-invading cells remaining on the upper surface of the membrane were removed by wiping with cotton-tipped swabs. Cells on the lower surface of the membrane were stained with crystal violet and 5 fields of adherent cells in each well were randomly photographed under an inverted microscope and counted. Experiments were repeated at least three times (27,29).

Gelatin substrate gel zymography. Gelatin substrate gel zymography was performed to examine the gelatinase MMP2 activity which was an index of cell invasion ability. After 24-h incubation with serum-free DMEM, the conditioned media was collected and centrifuged for 5 min at 10,000 x g to discard unsoluble materials. Equal volume of conditioned media was mixed with SDS loading buffer in the absence of sulfhydryl reducing agent and electrophoresed in 10% SDS polyacrylamide gel containing

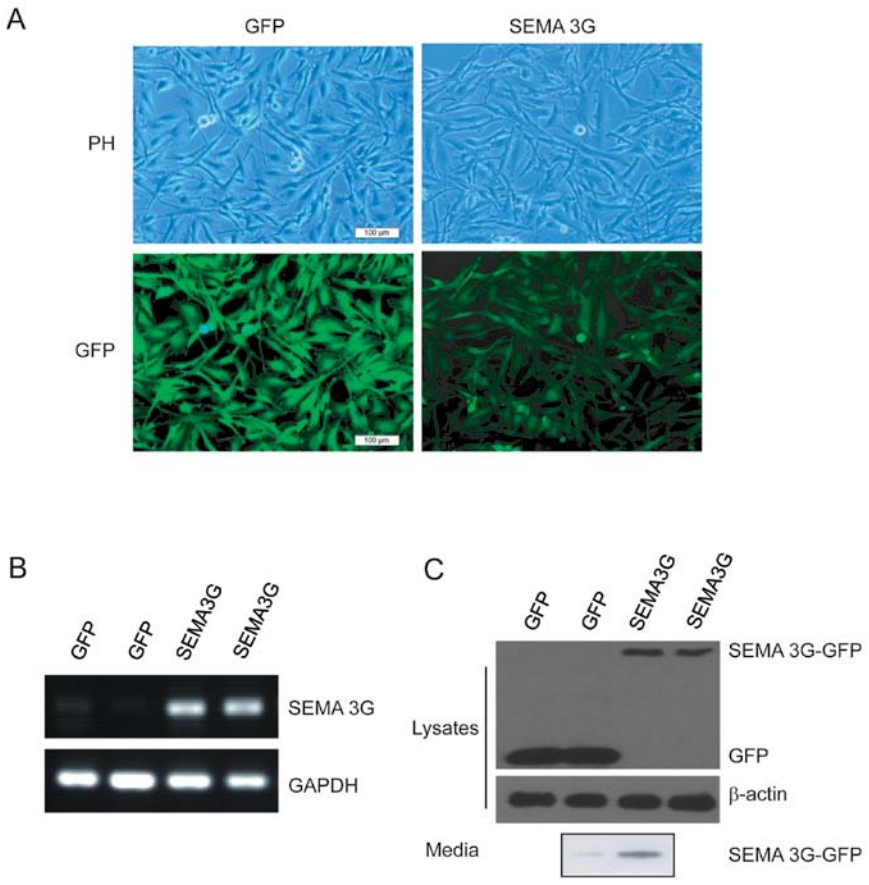

Figure 1. Stable expression of SEMA 3G in U251MG cells. (A) Morphological features of SEMA 3G overexpressing glioma cells. U251MG cells were stably infected with GFP or SEMA 3G-GFP as indicated. PH, phase contrast. Scale bar, 100 μm. (B) The mRNA level of SEMA 3G in GFP or SEMA 3G-GFP overexpressing glioma cells. After G418 selection for 2 months, total RNA of U251 cells transfected with GFP or SEMA 3G-GFP were extracted and analyzed by using RT-PCR. GAPDH served as a control. (C) The protein level of SEMA 3G in GFP or SEMA 3G-GFP overexpressing glioma cell lysates and culture media. Equal amount of protein from each cell line or equal amount of media were immunoblotted with antibody against GFP. β-actin was the loading control.

1 mg/ml gelatin. After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1.5 h to remove SDS, then incubated in reaction buffer (50 mmol/l Tris-HCl pH 7.5, 10 mmol/l $CaCl_2$, 1 μmol/l $ZnCl_2$ and 1% Triton X-100) for 18 h at 37˚C to promote activity of proteinases. Gels were stained for 2 h with 0.25% coomassie blue and destained with 45% methanol and 10% acetic acid. Proteolytic activity was visualized as clear bands (zones of gelatin degradation, ~ 65 kDa corresponded to MMP2) against the blue background of stained gelatin (30).

Statistical analysis. Statistical analysis for protein and mRNA levels, cell migration and invasion was performed using a two-sided Student's t-test. In all analysis, quantitative data were obtained from at least three independent experiments and expressed as means ± SEM. P-values <0.05 were considered statistically significant (*P<0.05, **P<0.01). All statistical analyses were performed using Office Excel 2004 (Microsoft Corp.) or SPSS software (SPSS version 17.0).

Results

Stable expression of SEMA 3G in U251MG cells. To exam the possible effect of SEMA 3G on glioma cell migration and invasion ability, we firstly generated the stable SEMA 3G expression U251MG cells by using SEMA 3G transfection combined with the classic G418 selection method. As shown in Fig. 1A, after G418 selection for ~2 months, each cell stably expressing SEMA 3G-GFP (SEMA 3G group) or GFP (GFP group) showed GFP positive, although the fluorescence intensity of SEMA 3G group was weaker than that of GFP group. This phenomenon may be due to the fact that the nucleotide sequence of SEMA 3G-GFP (>3 kb) was far longer than that of GFP (~700 bp), which led to difficult expression. Furthermore, the mRNA and protein levels of SEMA 3G was tested with RT-PCR and western blot assay, respectively. As Fig. 1B and C show, compared with the GFP group, the mRNA and protein levels of SEMA 3G group increased dramatically. In addition, because SEMA3G is the ligand protein for NPR2, it means SEMA3G should be secreted out of cells. Therefore, we checked whether the stable SEMA3G overexpression cell line could secrete SEMA 3G-GFP. To test whether the SEMA 3G-GFP could be secreted by SEMA 3G overexpressing U251MG cells, the conditioned media were collected and tested by western blotting. The results showed that SEMA 3G-GFP could be secreted into culture media (Fig. 1C), indicating the stable SEMA 3G expression U251MG cell line was generated successfully.

SEMA 3G decreases motility of U251MG cells. Next, we tested the effects of SEMA 3G on cell migration ability by wound healing assay. The repopulation of cells into a cell-free region

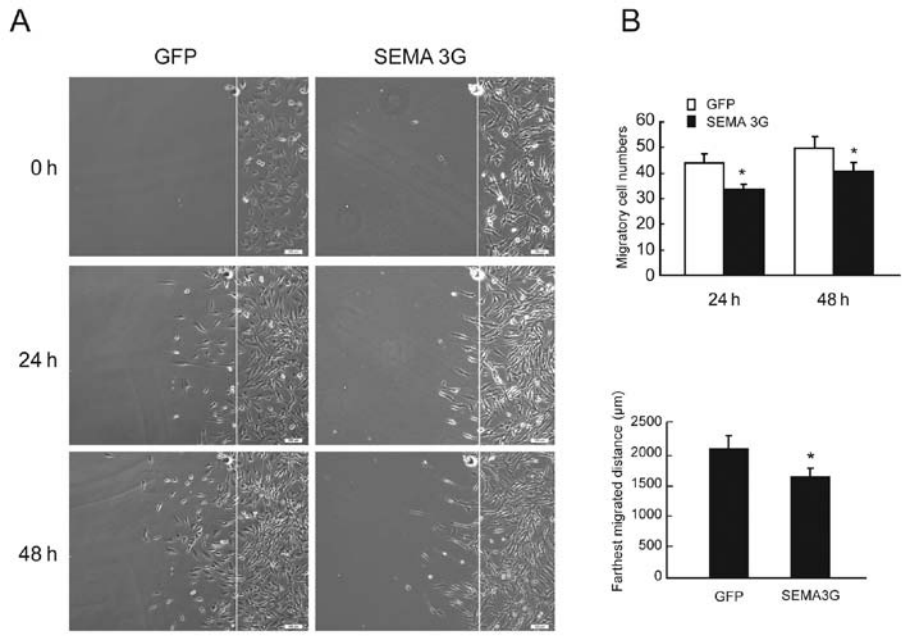

Figure 2. Effect of stable SEMA 3G overexpression on cell migration ability examined by wound healing assay. (A) Wound healing assays indicated that cells overexpressing SEMA 3G exhibited a strong reduction in migratory potential when compared with GFP-transfected cells. Representative digital images were taken at 0, 24 and 48 h. (B) Quantitative results of the stable cell lines overexpressing SEMA 3G-GFP or GFP using wound healing assays. Up, quantitative migratory cell numbers. Down, quantitative farthest migrated distance taken at 48 h.

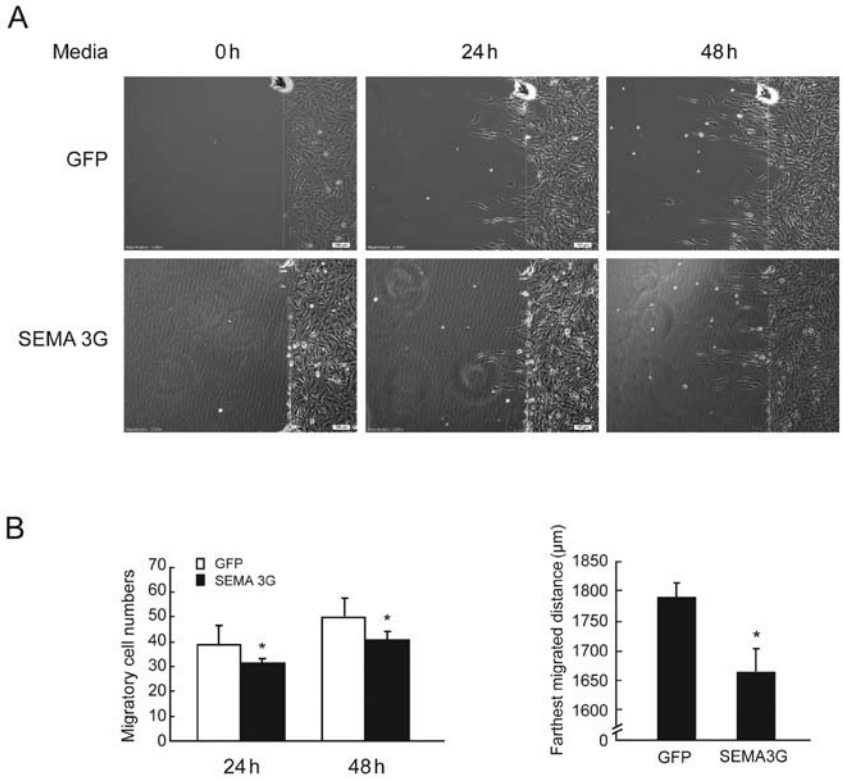

Figure 3. Effect of SEMA 3G conditioned media on cell migration ability examined by wound healing assay. (A) Wound healing assays indicated that cells treated with SEMA 3G conditioned media exhibited a strong reduction in migratory potential compared with GFP group media treatment. Representative digital images were taken at 0, 24 and 48 h. (B) Quantitative results of the SEMA 3G conditioned media or GFP group media treatment using wound healing assays. Left, quantitative migratory cell numbers. Right, quantitative farthest migrated distance taken at 48 h.

(scar) was examined quantitatively. Compared with the GFP group, SEMA 3G group showed decreased migration ability both at 24 and 48 h after being scraped. Fig. 2A is a digital image of the scarred region at 0, 24 and 48 h after being scraped from a typical experiment and the same experiment is quantified in Fig. 2B. The number of migratory cells in the scar for GFP group

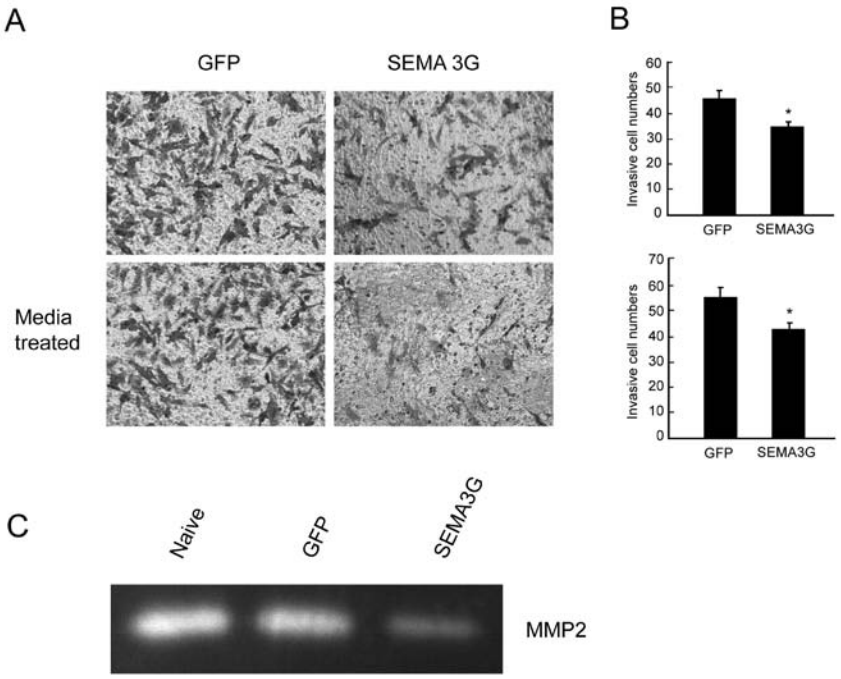

Figure 4. Effect of SEMA 3G on cell invasion ability examined by matrigel precoated transwell chambers (A) Stable SEMA 3G overexpressing cells or conditioned media treated glioma cells that invaded through matrigel-coated transwell inserts were stained with crystal violet. Stable SEMA 3G overexpressing or SEMA 3G conditioned media treatment inhibited the invasion ability of glioma cells. Upper panel, stable overexpression. Lower panel, conditioned media treatment. (B) Quantitative results of invasion assay. (C) Gelatin zymographic analysis of MMP-2 in conditioned media of GFP or SEMA 3G groups. The conditioned media of untransfected U251 cells (naive) was the negative control. Equal amount of samples were electrophoresed on SDS-PAGE gels containing gelatin as substrate to detect enzyme activity.

was 44.83±8.24 (24 h) and 54.41±8.31 (48 h), whereas the number of SEMA 3G group was 35.75±5.67 (24 h) and 46.91±7.05 (48 h). Compared with the GFP group, the migratory cell numbers of SEMA 3G group decreased by 20% (24 h, P<0.01) and 14% (48 h, P<0.05) respectively. The farthest distance migrated was quantified at the same time and the farthest distance of GFP group was 2030.91±257.12 μm (48 h), whereas the distance of SEMA 3G group was 1833.91±168.88 μm (48 h) (P<0.05).

In addition, we treated the naïve U251MG cells with SEMA 3G conditioned media and repeated the above experiment. As Fig. 3 shows the cell migratory ability decreased significantly by SEMA 3G conditioned media treatment. The results were very similar to those of stable SEMA 3G overexpression. The migratory cell numbers in the scar for GFP group media treatment were 37.16±4.40 (24 h) and 48.66±5.05 (48 h), whereas the number of SEMA 3G conditioned media treatment was 31.50±4.18 (24 h) and 41.75±4.93 (48 h) (P<0.01). The farthest distance migrated was quantified at the same time and the distance of GFP group media treatment was 1786.91±93.41 μm (48 h), whereas the distance of SEMA 3G conditioned media treatment was 1669.41±70.44 μm (48 h) (P<0.05).

SEMA 3G decreases invasion of U251MG cells. As migration and invasion have close inter-communication and SEMA 3G decreases the migration of human glioma cells, we then examined the effect of SEMA 3G on invasion of U251MG cells using matrigel precoated transwell chambers. Fig. 4A showed representative digital image of the cells on the lower surface of the membrane from a typical experiment and the same experiment is quantified in Fig. 4B. The results showed that SEMA 3G decreased the invasion ability of human glioma cells significantly both in SEMA 3G group and SEMA 3G conditioned media treatment group. Compared with the GFP group, the invasive cell numbers of SEMA 3G group decreased by 23% (P<0.05). The number of invasive cells for GFP group media treatment was 53.90±7.24 (24 h), whereas the number of SEMA 3G conditioned media treatment was 43.0±5.52 (24 h) (P<0.05).

Glioblastoma multiforme is a highly malignant brain tumor that is extremely refractory to therapy, partly because of aggressive tendency of the tumor cells to invade the surrounding tissues (1,2). Numerous studies *in vitro* and *in vivo* have documented a direct correlation between high levels of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, and increased invasive capacity of a large number of glioma cell lines (30). Thus, the MMPs activity is considered as an index of tumor invasion ability. Because the above data showed that SEMA 3G decreased the invasive ability of human glioma cells, we examined the MMPs activity by gelatin substrate gel zymography. As shown in Fig. 4C, a clear band at ~65 kDa corresponding to MMP2 was visualized and the band intensity of SEMA 3G group was weaker than that of naïve and GFP overexpression cells, indicating SEMA 3G decreased the invasion ability of human glioma cells.

Discussion

In this study, we have shown that SEMA3G plays an important role in migratory and invasive behavior of glioma cells. We found that not only SEMA3G stably overexpressing U251MG cells showed decreased migration and invasion ability, but also SEMA3G conditioned media treatment inhibited naïve U251 cell migratory and invasive behavior. Our results thus suggest

for the first time that SEMA3G possesses anti-migration and anti-invasion properties similar to those displayed by the previously identified tumor suppressor SEMA3s.

SEMA3s were firstly described as negative regulators of axonal guidance that repel axons and collapse growth cones (31,32). Later, SEMA3s were found to play important roles in cell migration, development, tumorigenesis, and tumor angiogenesis. Several studies showed that Sema3A, Sema3B, SEMA3E, Sema3F, SEMA3G and their known receptors and coreceptors Plexin-A1, Plexin-A3, NRP1 and NRP2 decreased and have some relationship with disease progression (11,33). SEMA3A can dramatically inhibit the proliferation and reduce both the migratory and invasive behavior of breast tumor cells (9,10). SEMA3F is the most studied semaphorin and acts as a tumor suppressor gene by reducing angiogenesis and metastasis, probably through the inhibition of integrin mediated adhesion and VEGF expression (19,20,34-38). SEMA3B, like SEMA3F and SEMA3A, is also described as a tumor suppressor gene (5,15).

As a recently identified semaphorin (39), scarce data have been published to our knowledge on the function of SEMA3G in tumors. Recently, it was reported that SEMA3G was the only significant prognostic marker in gliomas when a multivariate Cox analysis was performed (25). Because local invasion and migration of tumor cells are pivotal mechanisms in glioma progression, proteins involved in these processes are most likely important. To clarify the potential function of SEMA3 G in migratory and invasive behavior of glioma cells characterized by high migration potential, we generated SEMA3G stable expression U251MG cell line by using SEMA 3G transfection combined with the classic G418 selection method and analyzed its migratory and invasive behavior. We found that overexpressing SEMA3G inhibited the migration and invasion ability of U251MG cells either using the wound healing assay or with matrigel precoated transwell chambers. These results suggested that the effect of SEMA3G might be obtained through cell autonomous mechanism. Furthermore, we found that GFP tagged SEMA3G could be secreted into the culture media and SEMA3G conditioned media treatment also inhibited the migration and invasion ability of U251 parental cells, which indicated that SEMA 3G might exert its effect in a paracrine manner.

During tumor progression, cancer cells secrete various extracellular matrix degrading enzymes, including MMPs to facilitate their migration and invasion (30,40). It is well established that MMPs are closely associated with tumor invasion and metastasis in a variety of human tumors. In particular, MMPs are highly expressed in gliomas compared with normal brain tissues, and their mRNA and protein levels further increased upon tumor progression. As is shown in our present study, overexpression of SEMA3G significantly inhibited MMP-2 activity, suggesting that this could be one mechanism by which SEMAs may attenuate tumor progression, including migration and cell invasion.

In conclusion, we reported for the first time that SEMA3G could reduce the migratory and invasive ability of U251MG cells and this effect might be obtained through cell autonomous or paracrine mechanism. Since U251MG cells have NRP2, the receptor for SEMA 3G and SEMA 3F, and SEMA3F blocked tumor formation by associating with loss of activated $\alpha v\beta 3$ integrin, impaired cell adhesion to extracellular matrix components (36,41,42), it could be informative to determine the mechanism of SEMA3G in tumorigenesis and invasive progression.

Acknowledgements

This study was supported by the project of National Natural Science Foundation of China (no. 81072072), the grant from Xuzhou Medical College (no. 09KJZ18), and the grant from State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (no. SKLN-2011A01).

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