

CD44 promotes the migration of bone marrow-derived mesenchymal stem cells toward glioma

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Abstract. Previous *in vivo* and *in vitro* studies have shown that human mesenchymal stem cells (MSCs) exhibit tropism for gliomas. However, the mechanism underlying this directed migration remains unclear. The aim of the present study was to investigate the possible mechanism underlying platelet-derived growth factor-BB (PDGF-BB)-induced chemotactic migration of bone marrow-derived MSCs (BMSCs) toward glioma. Rat glioma C6 cell-conditioned medium was utilized to evaluate the chemotactic response of BMSCs toward glioma using an *in vitro* migration assay. Recombinant rat PDGF-BB was added to C6 cell-conditioned medium to assess its effect on the tropism of BMSCs. The effect of PDGF-BB on the expression levels of cluster of differentiation (CD)44 in BMSCs was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence assays. The results revealed that chemotactic migration was induced in BMSCs by rat glioma C6 cell-conditioned medium, which was enhanced by PDGF-BB treatment in a dose-dependent manner. Furthermore, RT-PCR and immunofluorescence assays showed that CD44 expression was upregulated in BMSCs following treatment with 40 ng/ml PDGF-BB for 12 h. Additionally, 3-h pretreatment with the anti-CD44 neutralizing antibody OX-50 was observed to attenuate the tropism of BMSCs toward glioma in the presence or absence of PDGF-BB. The results of the present study indicate that CD44 mediates the tropism of BMSCs toward glioma, and PDGF-BB promotes the migration of BMSCs toward glioma via the upregulation of CD44 expression in BMSCs. These

findings suggest CD44 inhibition may be a potential therapeutic target for the treatment of glioma.

Introduction

Glioma is the most common aggressive adult primary tumor of the central nervous system (1). The mortality rate associated with glioma occupies the top position among the malignant tumors worldwide (2). During the early stages (I and II) of disease, when the tumor is small, patients with glioma are usually asymptomatic, whereas grade III and IV gliomas, including glioblastoma, are aggressive and lethal malignant neoplasms (3). Glioma, in particular glioblastoma multiforme, is the most common malignant brain tumor in adults (4). The median age at diagnosis of glioblastoma patients is 65 years (5). Current treatment options include surgical resection, radiotherapy and chemotherapy (6). However, glioma carries a particularly poor prognosis, with survival measured in months rather than years (7).

The treatment approaches for malignant glioma, which is the most common type of highly aggressive primary brain tumor, are often unsuccessful due to diffuse infiltration and poor prognosis (8). A key problem regarding glioma treatment is the lack of effective tumor site-specific delivery systems available for therapeutic agents (9). Bone marrow-derived mesenchymal stem cells (BMSCs) have been shown to exhibit tropism for gliomas (10). Furthermore, these cells may be obtained easily, and may be genetically engineered and autologously transplanted, thus providing a feasible delivery vehicle for glioma-targeted therapy (11-17). Previous *in vivo* studies have demonstrated the efficacy of this delivery system (12,18). A number of cytokines, including platelet-derived growth factor-BB (PDGF-BB), have been shown to affect the migration of BMSCs (12,19-22); however, the mechanism underlying this remains to be elucidated.

It has been established that site-directed migration involves interaction between multiple adhesion molecules on migrating cells and their corresponding ligands (23,24). The cell adhesion molecule cluster of differentiation (CD)44, which is a BMSCs-specific transmembrane glycoprotein, is known to be involved in intracellular interactions that affect the motility of BMSCs (25-27). T cells migrating to inflammatory sites

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express higher levels of CD44 on their cell surface, and thus are capable of establishing more CD44-hyaluronan (HA) interactions (28,29). Therefore, CD44 may exert certain effects on the chemotactic migration of BMSCs to glioma cells. In the current study, we evaluated the role of CD44 in the tropism of BMSCs for glioma cells.

Materials and methods

Cell culture. Rat glioma C6 cells were obtained from the Key Laboratory of Cancer Prevention and Therapy (Tianjin, China) and cultured in serum-free low glucose-Dulbecco's modified Eagle's medium (L-DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO₂. Cell culture plates, including 6-well plates, 24-well plates and 60-mm dishes were purchased from Nest Biotechnology Co., Ltd. (Wuxi, China).

Ethical statement. All animal experiments were approved by the Animal Care and Use Committee of Tianjin Medical University Cancer Institute and Hospital (Tianjin, China), and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (30). A total of 20 Wistar rats were purchased from Vital River Laboratories (Beijing, China). They were housed under the specific conditions and sacrificed immediately by cervical dislocation as described previously by Yang *et al.* (31).

BMSCs isolation. The rats were housed in the animal center of Tianjin Medical University Cancer Institute and Hospital at a temperature of 20-25 °C and relative humidity of 50-70% on a 12-h dark/light cycle and provided a standard pelleted diet and water *ad libitum*. Male rats of 4 weeks old were used, and they were individually sacrificed by cervical dislocation. Four-week-old male Wistar rats were used for BMSCs isolation based on the principle of their adherence to plastic (32). Briefly, bone marrow cells collected from the bilateral tibias and femurs of sacrificed rats were cultured in L-DMEM supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). Three days later, adherent cells were passaged to fresh medium to discard non-adherent cells, and were subsequently grown to full confluence. Next, 6,000 cells/cm² cells were subcultured and grown to full confluence again prior to subculturing. Cells at fourth passage were identified as BMSCs, and used for the following experiments, as previously described (33).

Immunocytochemistry. BMSCs were collected and seeded onto 1.5% gelatin-coated coverslips. At 80% confluence, the C6 cells seeded on sterilized glass slides were allowed to attach overnight. Following fixation with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 4°C, cells were washed with phosphate-buffered saline (PBS; Sigma-Aldrich) three times for 20 min each, prior to incubation with PBS for 60 min at 4°C. Fixed cells were incubated with rabbit polyclonal anti-human anti-PDGF-BB antibody (dilution, 1:100; catalog no., ab23914; Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with goat anti-rabbit immunoglobulin G, horseradish peroxidase-conjugated secondary antibody (dilution, 1:1,000; catalog no. 7074; Cell Signaling Technology, Danvers, MA, USA) for 45 min at room temperature. Next,

the membranes were stained with 3,3'-diaminobenzidine (Sigma-Aldrich) and hematoxylin (Sigma-Aldrich), and slides were mounted with 50% glycerol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) prior to capturing images with a microscope (Eclipse ME600; Nikon Corp., Tokyo, Japan).

Immunofluorescence. BMSCs incubated in PDGF-BB-supplemented C6-conditioned medium for 12 h were fixed in 3.7% paraformaldehyde and permeabilized in pre-chilled acetone (Sinopharm Chemical Reagent Co., Ltd.). BMSCs incubated with serum-free L-DMEM were used as a negative control. Upon blocking with 5% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h, the cells were incubated with polyclonal rabbit anti-human/mouse/rat CD44 antibody (dilution, 1:100; catalog no., PA1021-2; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 4 h at room temperature, followed by incubation with rhodamine-conjugated goat anti-mouse immunoglobulin G secondary antibody (dilution, 1:100; catalog no., ZF-0313; Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) for 1 h at room temperature. Images were captured using a laser confocal microscope (TCS SP5; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed to examine the transcriptional levels of PDGF-BB in C6 cells and CD44 in PDGF-BB-treated BMSCs using a 2400 GeneAmp[®] PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). BMSCs incubated with serum-free L-DMEM served as a negative control. Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was obtained from 1 µg RNA using the ImProm-II[™] Reverse Transcription System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The primers used for PCR, synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), were as follows: Sense, 5'-CTTTAAGAAGGCCACGGTGA-3' and anti-sense, 5'-TCCAAGGGTCTCCTTCAGTG-3' for PDGF-BB; sense, 5'-AAGACATCGATGCCTCAAAC-3' and anti-sense, 5'-CTC CAGTAGGCTGTGAAGTG-3' for CD44 (34); and sense, 5'-TAT CCAGGCTGTGCTATCCC-3' and anti-sense, 5'-CCATCTCTT GCTCGAAGTCC-3' for β-actin. PCR was performed under the following conditions for PDGF-BB: Denaturation at 94°C for 46 min, followed by 40 cycles of 94°C for 15 sec, 62°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 7 min; PCR was performed under the following conditions for CD44: Denaturation for 95°C for 15 min, followed by 45 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The PCR products were separated using gel electrophoresis on a 2% agarose gel (Sigma-Aldrich). The bands were scanned using ChemiImager 5500 version 2.03 software (Alpha Innotech, San Leandro, CA, USA). Integrated density values were calculated using a computerized image analysis system (Fluor Chen 2.0; Bio-Rad, Hercules, CA, USA) and normalized to β-actin. Agarose gel, which was prepared in 1×TAE buffer containing 40 mM Tris-acetic acid (pH 8.5; Tris-base was purchased from Sigma Aldrich; acetic acid was from Sinopharm Chemical Reagent Co., Ltd.) and 2 mM ethylenediaminetetraacetic acid (Sinopharm Chemical Reagent Co., Ltd.), was supplemented with 0.5 µg/mL ethidium bromide (Sigma-Aldrich). Wide Mini-Sub Cell GT Horizontal Electrophoresis System and PowerPac[™]

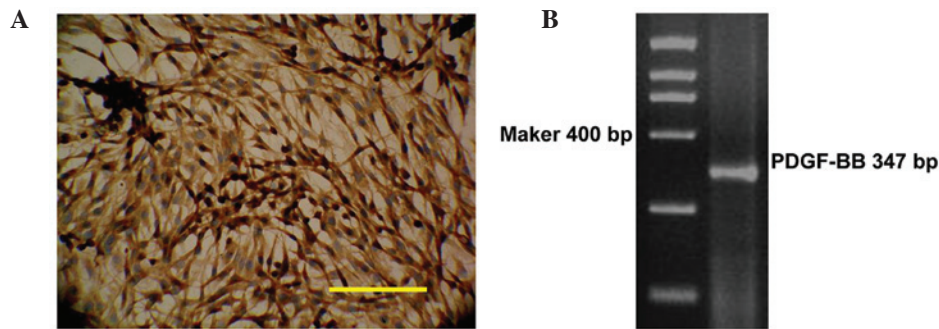


Figure 1. PDGF-BB expression in C6 glioma cells. (A) Representative image of PDGF-BB immunocytochemistry in C6 cells (magnification, x200; scale bar, 200 μ m). (B) Reverse transcription-polymerase chain reaction of PDGF-BB expression in C6 glioma cells. PDGF-BB, platelet-derived growth factor-BB.

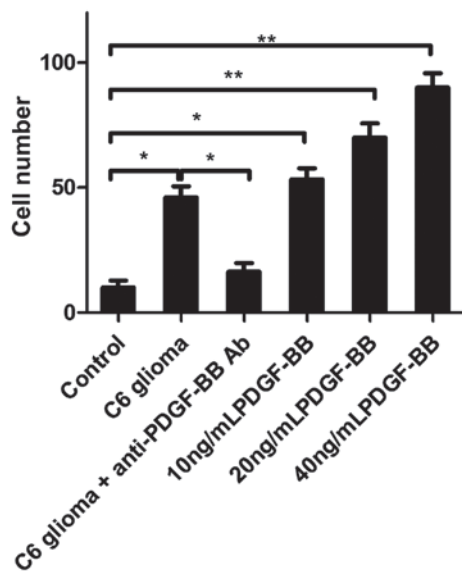


Figure 2. PDGF-BB promotes the tropism of BMSCs toward C6 glioma. *In vitro* migration assay showing the chemostatic migration of BMSCs toward C6 cell-conditioned medium supplemented with anti-PDGF-BB antibody or with 10, 20 or 40 ng/ml PDGF-BB. The results are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05, ** P <0.01 vs. control. PDGF-BB, platelet-derived growth factor-BB; BMSCs, bone marrow-derived mesenchymal stem cells; Ab, antibody.

Universal Power Supply (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were applied for gel electrophoresis, with voltage and time set at 100 V and 20 min, respectively. DNA fragments were visualized and quantified using ChemiDoc MP system (Bio-Rad Laboratories, Inc.), and relative amounts of CD44 transcripts were determined against β -actin expression.

***In vitro* migration assay.** The culture medium for rat glioma C6 cells was collected following 24-h incubation. Upon centrifugation at 1,000 \times g for 15 min at room temperature, and subsequent sterilization by 0.22-mm filtration (Thermo Fisher Scientific, Inc.), the supernatant was identified as C6 cell-conditioned medium. For the migration assay, BMSCs at a density of 2×10^5 cells/ml were seeded in the upper chamber of a Transwell plate containing an 8- μ m pore membrane (Costar; Corning Incorporated, Corning, NY, USA), and C6 cell-conditioned medium in the presence or absence of recombinant rat PDGF-BB (catalogue no. 220-BB-010; R&D Systems, Inc., Minneapolis, MN, USA) and serum-free L-DMEM containing

10, 20 or 40 μ g/l PDGF-BB was added to the lower well of the Transwell plates. Serum-free L-DMEM served as a negative control. Cells were incubated for 24 h prior to formalin fixation and hematoxylin staining. Images of nine randomly selected fields were captured, and cells were counted.

To block CD44 activity, C6-conditioned medium in the presence or absence of PDGF-BB (40 ng/ml) was incubated with mouse monoclonal anti-rat CD44 neutralizing antibody (dilution, 1:1,000; catalogue no., OX-50; Abcam) for 3 h at room temperature, prior to being added to the lower chamber of the Transwell plates. Serum-free L-DMEM served as a negative control. The subsequent procedures were performed as described above. Briefly, BMSCs were seeded in the upper chamber, followed by an incubation of 24 h at 37°C. Migrated cells were stained prior to counting. An inverted microscope (Zeiss Axio Vert A1 Inverted, Carl Zeiss Canada Ltd., North York, ON, Canada) equipped with a charge-coupled device camera (Orca ER; Hamamatsu Photonics K.K., Hamamatsu, Japan) was used to visualize and image stained cells, at x400 magnification.

Statistical analysis. All data were analyzed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Two-tailed unpaired Student's t-test was used to determine the significance of differences between groups. P <0.05 was considered to indicate a statistically significant difference. All experiments were performed at least twice, and results were expressed as the mean \pm standard deviation.

Results

Rat glioma C6 cells express PDGF-BB. The expression levels of PDGF-BB in rat glioma C6 cells were analyzed. As shown in Fig. 1A, PDGF-BB protein was highly expressed in the cytoplasm of C6 cells (Fig. 1A). In addition, a clear cDNA band corresponding to PDGF-BB was identified in C6 cells using RT-PCR (Fig. 1B).

C6 cells induce chemotactic migration of BMSCs via expression and secretion of PDGF-BB. To evaluate the effect of PDGF-BB on tropism of BMSCs toward glioma, an *in vitro* migration assay was performed. As shown in Fig. 2, increased levels of migration of BMSCs were observed in the C6 cell-conditioned medium-treated group after 24 h treatment compared with the normal medium-treated group, which was attenuated by 4-h pretreatment with

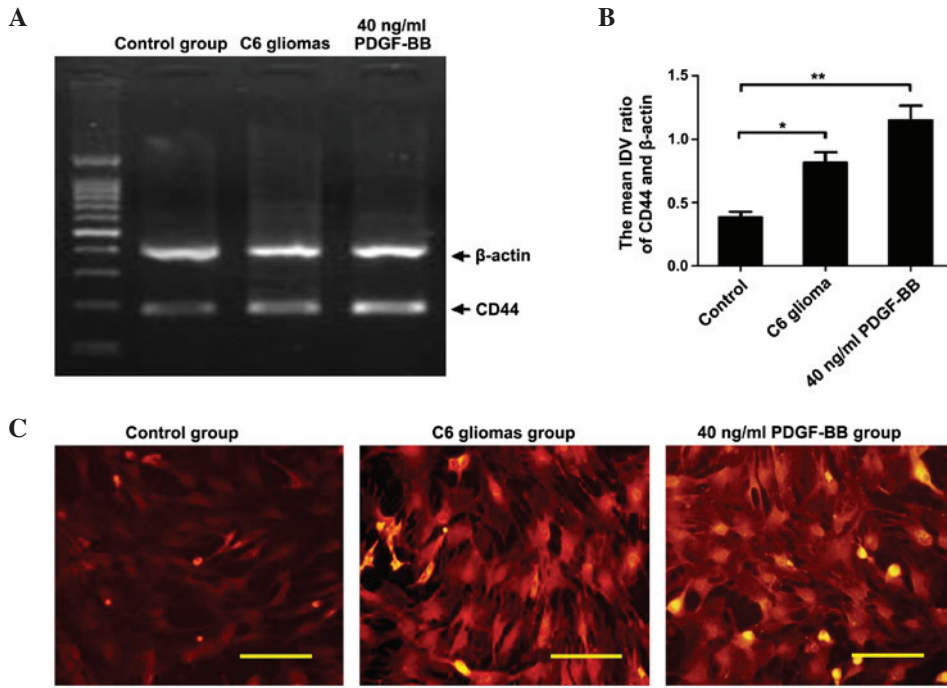


Figure 3. PDGF-BB upregulates CD44 expression in BMSCs. (A) Reverse transcription-polymerase chain reaction was performed to evaluate the changes in CD44 mRNA expression levels under PDGF-BB conditions of 40 ng/ml or C6 glioma. (B) Results demonstrated that 40 ng/ml PDGF-BB and C6 glioma significantly elevated the CD44 expression of BMSCs compared with control groups ($P<0.01$; $**P<0.001$). (C) Immunofluorescence revealing the effect of treatment with 40 ng/ml PDGF-BB on the protein levels of CD44 in BMSCs. Yellow staining indicates CD44 expression (magnification, $\times 400$; scale bar, $100 \mu\text{m}$). PDGF-BB, platelet-derived growth factor-BB; CD, cluster of differentiation; BMSCs, bone marrow-derived mesenchymal stem cells; IDV, integrated density value.

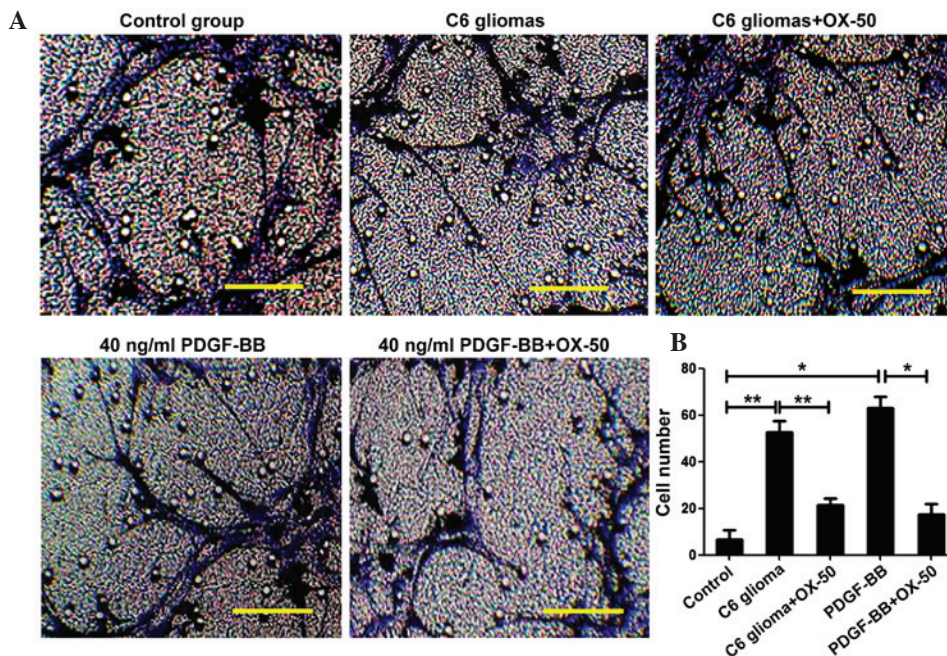


Figure 4. Anti-CD44 antibody attenuates C6 cell-induced and PDGF-BB-promoted chemotactic migration of BMSCs. (A) The effect of CD44 in chemotactic migration of BMSCs was evaluated using OX-50, an anti-CD44 antibody. Representative images of each group are presented (magnification, $\times 400$; scale bar, $100 \mu\text{m}$). (B) Quantification of the data corresponding to panel A. The graph represents the mean \pm standard deviation of three independent experiments. $*P<0.05$ and $**P<0.01$ vs. control. CD, cluster of differentiation; PDGF-BB, platelet-derived growth factor-BB; BMSCs, bone marrow-derived mesenchymal stem cells.

anti-PDGF-BB antibody, indicating that C6 cell-induced chemotactic migration of BMSCs may occur as a result of PDGF-BB secretion in the C6 cell-conditioned medium. Additionally, supplementing C6 cell-conditioned medium

with recombinant rat PDGF-BB enhanced C6 cells-induced chemotactic migration of BMSCs in a dose-dependent manner (Fig. 2), thus demonstrating that PDGF-BB promotes the tropism of BMSCs.

PDGF-BB upregulates the expression of the standard form of CD44. CD44, as a marker for BMSCs, has been reported to be involved in the mobilization and chemostatic migration of BMSCs (35). To evaluate the effect of PDGF-BB on CD44 expression in BMSCs, RT-PCR and immunofluorescence assays were performed. As shown in Fig. 3, the transcriptional and protein levels of CD44 in BMSCs were increased in the C6 cell-conditioned medium-treated group, and PDGF-BB augmented this effect, indicating that PDGF-BB promotes the chemostatic migration of BMSCs toward glioma via the upregulation of CD44 expression in BMSCs.

CD44 mediates the tropism of BMSCs for glioma. OX-50, an anti-CD44 neutralizing antibody, was used to assess the role of CD44 in the tropism of BMSCs. As shown in Fig. 4, pretreatment of C6 cell-conditioned medium with the anti-CD44 antibody OX-50 for 3 h blocked the C6 cell-induced and the PDGF-BB-promoted chemostatic migration of BMSCs, suggesting that CD44 may act as a molecular bridge between BMSCs and glioma.

Discussion

PDGF is a strong mitogen and chemoattractant for fibroblasts, myofibroblasts and smooth muscle cells (36,37). PDGF-BB, a member of the PDGF family, has been demonstrated to induce chemotactic migration of cells of mesenchymal origin (38). A number of glioma cells express and secrete PDGF, with high-grade gliomas expressing higher levels of PDGF compared with low-grade gliomas (34). In the present study, rat glioma C6 cells expressed high levels of PDGF-BB, and PDGF-BB augmented the chemostatic migration of BMSCs induced by C6 cell-conditioned medium, indicating that PDGF-BB may mediate glioma-induced tropism of BMSCs. However, further studies are required to corroborate these findings.

CD44, as a unique surface antigen of BMSCs (25,26,33,39), is involved in various cellular processes, including proliferation, differentiation, survival and migration (40). The main function of CD44 is to regulate the motility and chemotaxis of BMSCs (41). Previous studies have demonstrated that CD44 is localized on the leading edge of migrating cells (42,43), and its inhibition attenuates macrophage chemotaxis (44) and fusion (45). Additionally, loss of CD44 decreases the migratory ability of human colon cancer cells, while overexpression of CD44 promotes their migration (46), indicating the importance of CD44 in such processes. The major isoform of CD44 present in MSCs is the standard form, termed CD44s. In the present study, PDGF-BB was observed to increase the transcriptional and protein levels of CD44 in BMSCs. In addition, C6 cell-induced and PDGF-BB-promoted chemostatic migration of BMSCs was markedly attenuated by the anti-CD44 neutralizing antibody OX-50, suggesting that C6 cells may induce BMSCs tropism via the expression and secretion of PDGF-BB, which upregulates CD44 expression in BMSCs. The CD44-HA interaction presents a critical step required for cell migration (35), and has been reported to be involved in the migration of CD34⁺ stem cells to the bone marrow, as well as in the adhesion, motility and invasion of breast cancer cells (47,48). However, these mechanisms require further investigation.

In conclusion, the results of the current study revealed that CD44 mediates the tropism of BMSCs to glioma, and PDGF-BB promotes the migration of BMSCs toward glioma via upregulation of CD44 expression in BMSCs. These findings suggest CD44 inhibition may be a potential therapeutic target for the treatment of glioma.

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