

Chemokines mediate mesenchymal stem cell migration toward gliomas *in vitro*

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Received December 21, 2009; Accepted February 26, 2010

DOI: 10.3892/or_00000796

Abstract. Previous studies have demonstrated the tremendous tropism of mesenchymal stem cells (MSCs) for malignant gliomas, making these cells a potential vehicle for delivery of therapeutic genes to disseminated glioma cells. However, the mechanisms underlying the tropism of MSCs for gliomas remain poorly defined. It has been suggested that malignant gliomas secrete a variety of chemokines, including macrophage chemoattractant protein-1 (MCP-1) and stromal cell-derived factor-1 α (SDF-1 α). We isolated and cultured MSCs from rat bone marrow and found that these cells express CCR2 and CXCR4, the respective receptors for MCP-1 and SDF-1 α . *In vitro* analysis revealed that MCP-1 and SDF-1 α induce the migration of MSCs. Furthermore, neutralization data suggest that MCP-1 and SDF-1 α play a role in the mediation of MSC migration toward gliomas. These results highlight the potential of these cells as a tumor targeting strategy for glioma gene therapy.

Introduction

Malignant gliomas are the most prevalent type of primary brain tumor. Despite extensive surgical excision and adjuvant radio- and chemotherapy, the prognoses of patients with malignant gliomas, such as glioblastoma multiforme (GBM) or anaplastic astrocytoma, remain extremely poor (1-3). The median survival is 1 year or less for patients diagnosed with

GBM and ~3 years for those diagnosed with anaplastic astrocytoma (4). This treatment resistance arises, in part, from tumor infiltration of and invasion into the surrounding brain architecture. Surgical tumor resection is almost always followed by regrowth of tumor cells residing in adjacent regions of normal brain tissue because it is impossible to eliminate successfully all tumor cells using current technologies (5-7). Single tumor cells deeply infiltrate the surrounding tissue, and are thought to be responsible for tumor relapse. New therapies should target these single tumor cells, especially those that have escaped the main tumor mass (8-10).

Neural stem cells (NSCs) possess extensive tropism for experimental gliomas when administered intracranially (11,12). This characteristic of NSCs has been exploited as a tumor targeting strategy for glioma gene therapy (11-20). Unfortunately, the acquisition of sufficient therapeutic NSCs is challenging technically, and their practical application is problematic due to ethical concerns and immunological rejection. Previously, it has been suggested that mesenchymal stem cells (MSCs) may represent an alternative source of therapeutic stem cells (21,22). In experimental brain glioma models, intracranially implanted or intravenously injected MSCs can migrate away from the injection site toward tumor beds (23-27). Additionally, gene modification of MSCs with therapeutic cytokines clearly prolongs the survival of tumor-bearing animals (23,25,28,29).

A better understanding of the molecular events that govern MSC homing is necessary for the development of a clinically-applicable tumor targeting strategy for glioma gene therapy. Certain chemokines and growth factors, such as vascular endothelial cell growth factor (VEGF), interleukin-8 (IL-8), transforming growth factor- β (TGF- β), and neurotrophin-3 (NT-3), released from glioma cells have been reported to mediate the tropism of MSCs for gliomas (30-32). In addition to IL-8, several other chemokines are secreted by glioma cells, including macrophage chemoattractant protein-1 (MCP-1) (33,34) and stromal cell-derived factor-1 α (SDF-1 α) (35-37). Therefore, in this study we analyzed the chemotactic effect of MCP-1 and SDF-1 α on MSCs. In addition, we investigated the role of these chemokines in mediating the migration of MSCs toward gliomas.

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Key words: macrophage chemoattractant protein-1, stromal cell-derived factor-1 α , mesenchymal stem cells, migration, glioma

Table I. Gene specific primers for PCR.

Gene	GenBank accession no.	Oligonucleotide (5'→3')	Size (bp)
MCP-1	NM_031530.1	Forward: TGTCACGCTTCTGGGCCTGTTG Reverse: CAGAAGTGCTTGAGGTGGTTGTGGAA	407
SDF-1 α	AF189724.1	Forward: CTGTGCTGGCCCTGGTGCTG Reverse: CGGGTCAATGCACACTTGTCTGTTGT	203
CCR2	NM_021866.1	Forward: TGATCCTGCCCCTACTTGTTCATGG Reverse: GAGCTCACTCGGTCTGCTGTCTCC	403
CXCR4	NM_022205.3	Forward: AGCCAGGGGGACGGCAGGTA Reverse: TGCTGCGCGGAGCTCTTGAA	413

Materials and methods

MSC preparation. According to institutional guidelines and an approved protocol, bone marrow aspirates from Fischer 344 rats (9 weeks old, male) were plated and cultured in Dulbecco's minimal essential medium (DMEM; Gibco/BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (Gibco/BRL) as described previously (25). Rat MSCs (rMSCs) were adherent, elongated, and spindle-shaped in the primary culture after 24 h of plating. After 24 h, non-adherent cells were removed by changing the medium; thereafter, the medium was changed twice a week. rMSCs reached 80% confluence after 12-14 days. Cells were harvested by trypsinization and passaged for expansion purposes. rMSCs were used at passage 2 in all experiments (flow cytometry analysis, RT-PCR, and migration assays).

Glioma cell conditioned medium. Rat glioma C6 cells (American Type Culture Collection, Rockville, MD) were cultured in 45% DMEM, 45% Ham's F-12 medium, and 10% FBS with an antibiotic supplement in 75 cm² culture flasks. Confluent cultures were washed with medium without the addition of FBS and incubated with serum-free medium for 4 days. After conditioning, the medium was aspirated from the cells, centrifuged at 1,000 \times g for 5 min, and filtered through 0.22 μ m-diameter pore Millipore filters. Aliquots of the conditioned medium (CM) were stored frozen until required.

Flow cytometry analysis. rMSCs were treated with 0.25% trypsin-EDTA, harvested, and washed twice with DMEM. Before staining, cells were allowed 2 h in suspension to recover expression of surface markers. Cell staining was performed using primary antibodies followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG or FITC-conjugated goat anti-mouse secondary antibodies (Sigma). The following primary antibodies were used in this study: goat polyclonal CCR2 and CXCR4 (Santa Cruz Biotechnology, CA, USA), mouse anti-rat CD11b (Serotec, Oxford, UK), mouse anti-rat CD45, CD44, and CD73 and isotype-specific mouse IgG (Pharmingen, San Diego, CA).

Stained cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA).

RT-PCR. Total RNA was extracted from C6 glioma cells and rMSCs using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNAs were reverse transcribed using the SuperScript III First-Strand synthesis system (Invitrogen) with oligo(dT) as primers. PCR reactions were performed in a DNA Thermal Cycler 480 (PerkinElmer Life Sciences, Boston, MA) and the amplifications were carried out in a volume of 12.5 μ l containing 1 μ g cDNA, 10 mM Tris-HCl, 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 10 pmol of each primer, and 0.1 U Taq polymerase, for 5 min at 94°C for initial denaturing, followed by 32 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and a final incubation at 72°C for 7 min. PCR products were sized fractionated by electrophoresis on 2% agarose gels and visualized with ethidium bromide. The specific primers used are shown in Table I.

Migration assays. rMSC migration was performed in Transwell dishes (Costar) 6.5-mm in diameter, with 8- μ m pore filters. rMSCs (4 \times 10⁵/ml) in 200 μ l of serum-free DMEM were added to the upper chamber and 600 μ l of tested samples containing chemokines, glioma cell CM, or CM supplemented with specific neutralized antibodies were placed in the lower chambers. Recombinant rat MCP-1 (rrMCP-1; Perprotech, NJ, USA) and recombinant human SDF-1 α (rhSDF-1 α ; R&D Systems, Wiesbaden, Germany) were diluted in serum-free DMEM to different concentrations ranging from 4 ng/ml to 500 ng/ml. Medium incubated in the absence of cells served as a negative control. After overnight incubation in 5% CO₂ at 37°C, cells remaining on the upper face of the filters were removed with a cotton wool swab. Chambers were fixed for 20 min at room temperature with 4% formaldehyde in PBS, stained in 0.5% cresyl violet for 20 min, and rinsed in water. Cells that had migrated through the pores and adhered to the lower surface of the membrane were analyzed under high-power (x400) light microscopy and counted in five random high-power fields. For neutralization studies, glioma cell CM was incubated with rabbit anti-rat MCP-1 antibody (Cedarlane Laboratories Ltd., Ontario, Canada) or rMSCs

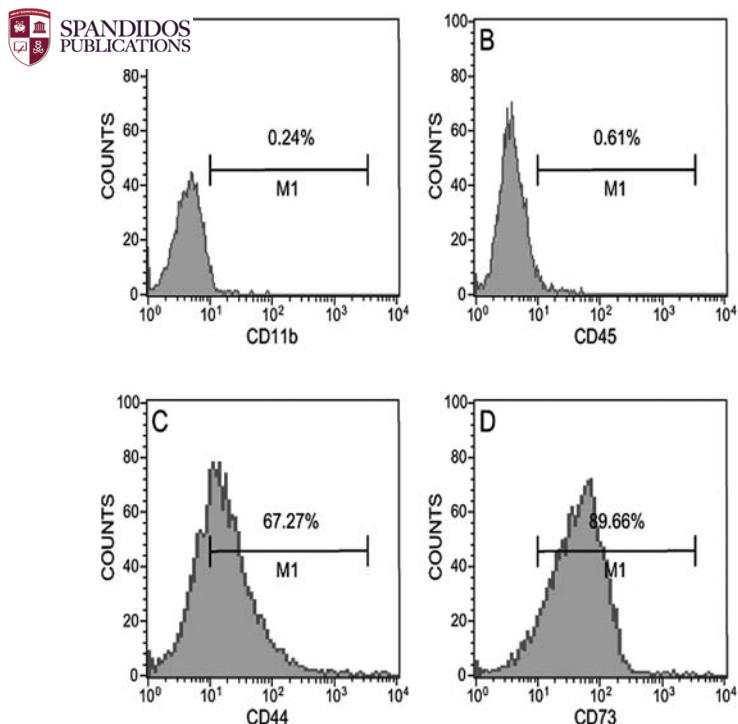


Figure 1. Graphs summarize FACS analysis of rMSC expression of cell surface markers. (A) rMSCs (0.24%) reacted with the anti-CD11b antibody; (B) 0.61% of rMSCs reacted with the anti-CD45 antibody; (C) 67.27% of rMSCs reacted with the anti-CD44 antibody; (D) 89.66% of rMSCs reacted with the anti-CD73 antibody.

were incubated with anti-CXCR4 polyclonal antibody (MBL, Nagoya, Japan).

Statistical methods. Each experiment was performed a minimum of 3 times. For migration assays, data are expressed as the mean number of cells per high-power field (cells/HPF) \pm standard error (SE). Statistical analysis was performed using Student's t-tests. Statistical significance was set at $p < 0.05$.

Results

Before performing specific studies, we verified that the cultured cells were negative for CD11b and CD45 surface markers and positive for CD44 and CD73 surface markers (Fig. 1). We also confirmed that the cultured cells demonstrated multipotentiality by giving rise to osteoblasts and adipocytes when exposed to adequate differentiating conditions (data not shown).

C6 glioma cells produce MCP-1 and SDF-1 α . It has been shown that MCP-1 and SDF-1 α are produced by glioma cells *in vitro* and *in vivo* (33-37). We hypothesized that these chemokines released by glioma cells may be potential mediators of MSC migration. To test this hypothesis, we examined their expression in C6 glioma cells. Using RT-PCR, we observed that C6 glioma cells express the transcripts for MCP-1 and SDF-1 α (Fig. 2A).

MSCs express the chemokine receptors CCR2 and CXCR4. To study the role of chemokine receptors in cell migration

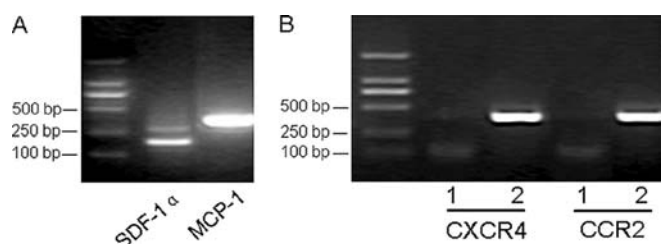


Figure 2. RT-PCR analysis of MCP-1, SDF-1 α , CCR2, and CXCR4 mRNA levels. (A) MCP-1 and SDF-1 α mRNA were expressed in rat C6 glioma cells. (B) CCR2 and CXCR4 mRNA were expressed in rMSCs, but not in fibroblasts (non-migratory cells as control). Lane 1: fibroblasts; lane 2: rMSCs.

toward gliomas, we then examined the expression of CCR2 and CXCR4 (the receptors for MCP-1 and SDF-1 α , respectively) in rMSCs. Considering of non-migratory properties of fibroblasts toward glioma cells *in vitro* (27), we used fibroblasts as control cells in RT-PCR assays. It showed that CCR2 and CXCR4 mRNA were expressed in rMSCs, but not in fibroblasts (Fig. 2B).

To confirm RT-PCR data, we further examined protein expression of CCR2 and CXCR4 in rMSCs by fluorescence-activated cell sorter (FACS) analysis. It demonstrated that a small percentage of rMSCs (14.85%) react with the anti-CXCR4 antibody and a high percentage of rMSCs (32.15%) react with the anti-CCR2 antibody (Fig. 3).

MSCs migrate in response to MCP-1 and SDF-1 α . In light of the observation that rMSCs express CCR2 and CXCR4, an *in vitro* chemotaxis assay was performed using rrMCP-1 and rhSDF-1 α . We found a significant increase in rMSCs migration with rrMCP-1 at concentrations of 4 and 20 ng/ml ($p < 0.01$) (Fig. 4A). Interestingly, higher concentrations of rrMCP-1 (100 and 500 ng/ml) did not result in significant rMSCs migration. However, rhSDF-1 α induced migration of rMSCs in a dose-dependent manner; the maximum migration of rMSCs was observed at 100 ng/ml of rhSDF-1 α (Fig. 4B). Pretreatment of rMSCs with a blocking anti-MCP-1 antibody (10 μ g/ml) or with anti-CXCR4 antibody (10 μ g/ml) abrogated cell migration in response to rrMCP-1 and rhSDF-1 α , respectively, confirming the specificity of the migration (data not shown).

MCP-1 and SDF-1 α play a role in mediating MSC migration toward gliomas. To determine whether glioma-secreted chemokines contribute to MSC chemotaxis, we incubated C6 glioma cell CM with anti-MCP-1 antibody (10 μ g/ml), or/and incubated rMSCs with anti-CXCR4 antibody (10 μ g/ml). Whereas C6 glioma cell CM resulted in a significant increase in rMSC migration, addition of the anti-MCP-1 neutralizing antibody significantly attenuated the migration of rMSCs (Fig. 5). Similarly, following incubation with an anti-CXCR4 blocking antibody, a significant decrease was found in rMSC migration toward C6 glioma cell CM. Importantly, this inhibition was even more pronounced when both antibodies were added. These results suggest that MCP-1 and SDF-1 α are important for the mediation of MSC migration toward gliomas.

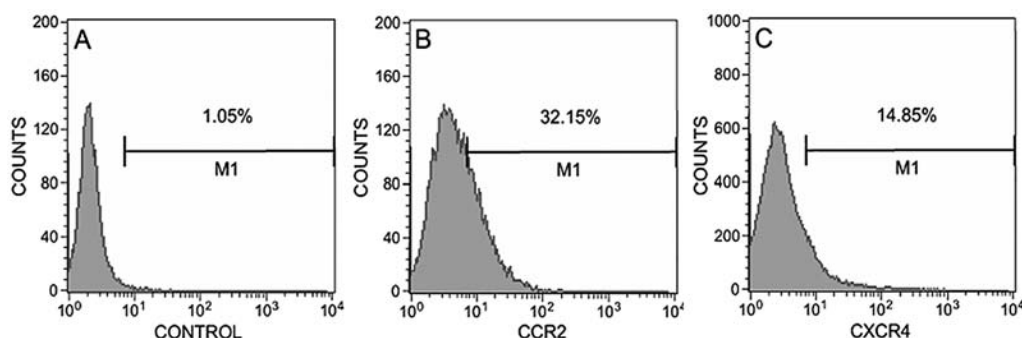


Figure 3. Graphs summarize FACS analysis of rMSC expression of CCR2 and CXCR4. (A) rMSCs (1.05%) reacted with the isotype-specific Ig and FITC-labeled secondary antibody; (B) 32.15% of rMSCs reacted with the anti-CCR2 antibody; (C) 14.85% of rMSCs reacted with the anti-CXCR4 antibody.

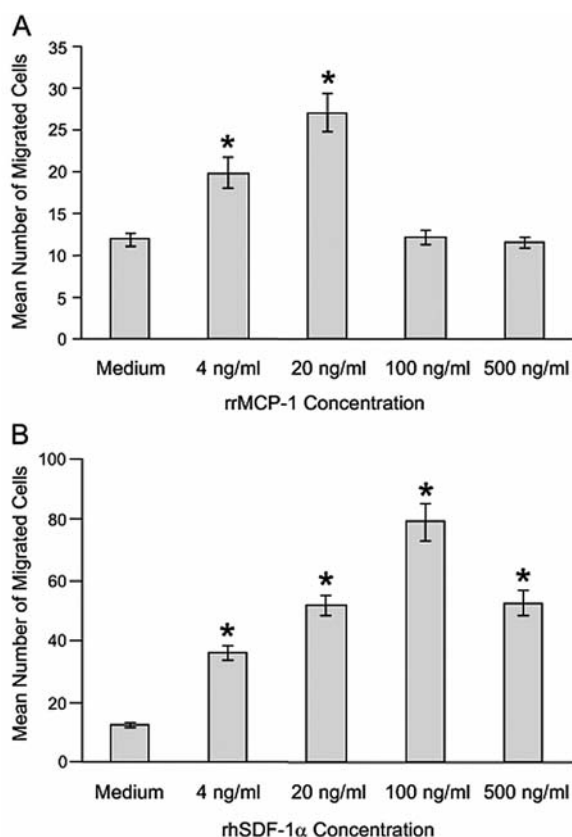


Figure 4. Graphs summarize chemokine-mediated migration of MSCs *in vitro*. (A) Effect of rrMCP-1 on MSC migration. A significant increase in the number of migrated MSCs was found at rrMCP-1 concentrations of 4 and 20 ng/ml (* $p < 0.01$), when compared with control. (B) Effect of rhSDF-1 α on MSC migration. The number of migrated MSCs increased dose-dependently at rhSDF-1 α concentrations of 4-500 ng/ml (* $p < 0.01$), when compared with control. The maximum effect of rhSDF-1 α was observed at a concentration of 100 ng/ml.

Discussion

The robust tropism of stem cells for intracranial gliomas makes them highly attractive as vehicles for the delivery of a wide variety of therapeutic gene products directly to tumor cells. Understanding the mechanisms underlying the migration of stem cells toward malignant gliomas is crucial to the success of clinical implementation of a tumor-targeting strategy

involving MSCs. Herein, we demonstrate that specific chemokines such as MCP-1 and SDF-1 α mediate the migration of MSCs toward gliomas *in vitro*.

MCP-1, a member of the CC chemokine family with chemoattractant activity for major inflammatory cells, is produced by malignant gliomas *in vitro* as well as *in vivo* (33,34). MCP-1 expression is associated with a higher astrocytoma tumor grade (34,38). Moreover, MCP-1 can play a role in macrophage recruitment into gliomas *in vivo* (38,39). Previously it has been suggested that MCP-1 might be responsible for MSC migration toward ischemic cerebral tissue (40). In our study, we demonstrate MCP-1 receptor CCR2 expression in rMSCs, and the chemotaxis of rMSCs in response to MCP-1. Furthermore, addition of the anti-MCP-1 neutralizing antibody significantly attenuated the migration of MSCs toward gliomas. Therefore, MCP-1 might play an important role in MSC migration toward gliomas. This is consistent with a recent study demonstrating that MCP-1 secreted by primary breast tumors stimulates migration of MSCs (41). Interestingly, MCP-1 was found to be upregulated in rat MSCs exposed to tumor environment (42). However, there are conflicting reports showing that human MSCs do not migrate in response to MCP-1 (43). These contradictory findings may be explained by varying culture homogeneity.

The CXC chemokine SDF-1 α , which binds to its receptor CXCR4, plays an important and unique role in regulation of stem/progenitor cell trafficking (44). Previous studies have demonstrated that high-grade gliomas secrete significant levels of SDF-1 α , and that the expression of this protein and the CXCR4 receptor correlates with the histological grade and invasive capacity of these tumors, as well as tumor cell survival (35-37). SDF-1 α expression by tumor-derived endothelium serves to attract the migration of NSCs (45,46). Ehtesam *et al* (47) demonstrated that SDF-1 α /CXCR4 interactions play a functional role in gliomatropic migration of NSCs. Tabatabai *et al* (48) also documented that SDF-1 α /CXCR4 interactions appear to be essential for the gliomatropism of circulating adult haematopoietic progenitor cells. Similarly, we found that SDF-1 α induces the migration of CXCR4-expressing MSCs *in vitro*. Moreover, neutralization data suggest that SDF-1 α might be involved in the migration of MSCs toward gliomas.

In conclusion, we demonstrated that MSCs express the chemokine receptors CCR2 and CXCR4, and consistent with

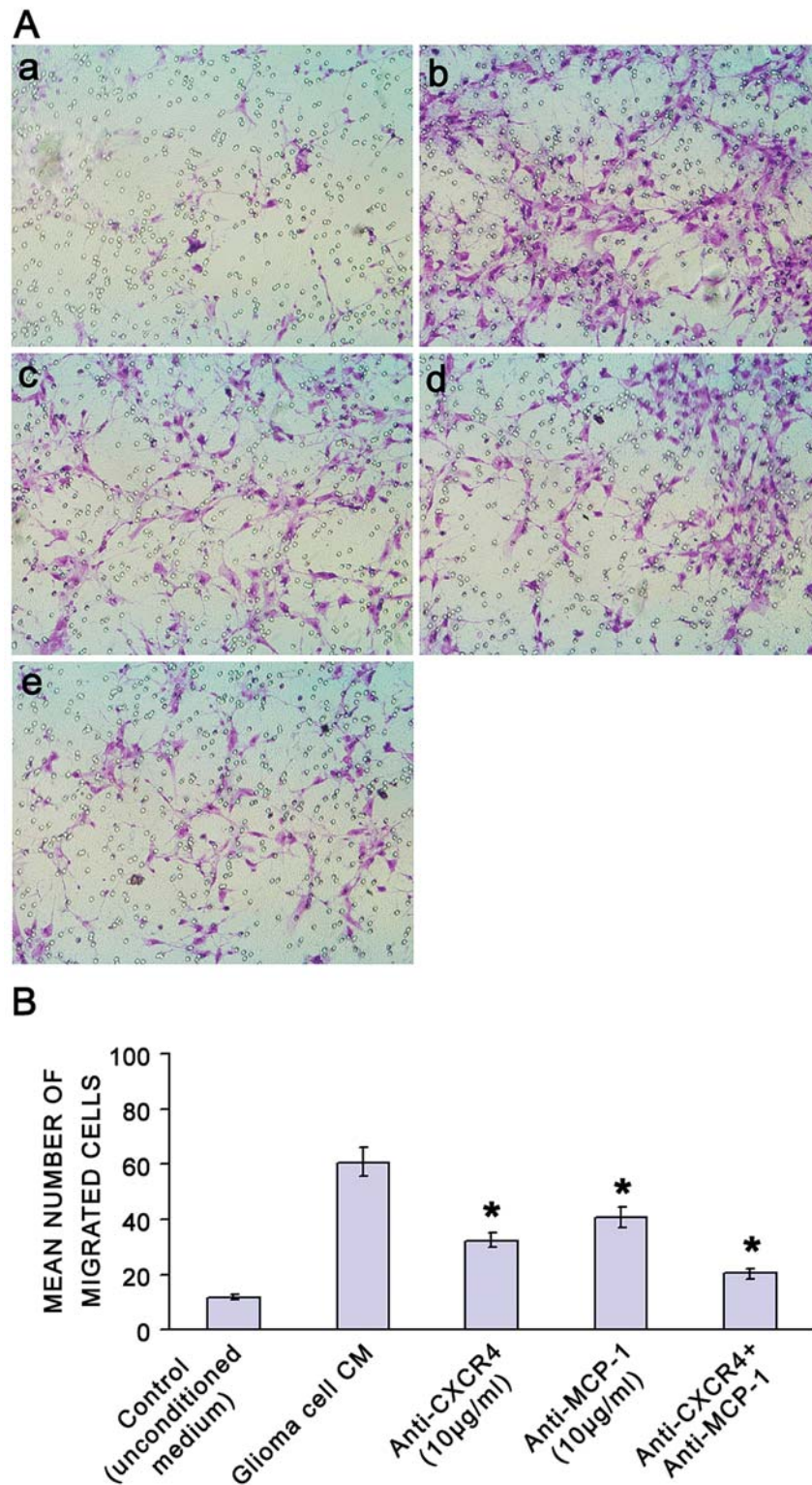


Figure 5. Panel A, photographs of cresyl violet-stained membranes. (a) MSC migration toward normal unconditioned medium. (b) MSC migration toward glioma cell CM. (c) MSC migration toward glioma cell CM after addition of anti-CXCR4 blocking antibody. (d) MSCs migration toward glioma cell CM after addition of anti-MCP-1 neutralizing antibody. (e) MSCs migration toward glioma cell CM after combination of antibodies. Panel B, graph summarizes migration of MSCs toward gliomas after neutralization. Results indicated that C6 glioma cell CM significantly induced the migration of MSCs compared with normal unconditioned medium. Glioma cell CM was incubated with 10 µg/ml anti-MCP-1, rMSCs were preincubated with 10 µg/ml anti-CXCR4, or both conditions were observed. (* $p < 0.01$).

this they migrate in response to the chemokines MCP-1 and SDF-1 α . Moreover, we have shown that MCP-1 and SDF-1 α mediate the migration of MSCs toward gliomas. However,

the *in vitro* migration assay employed in this study may not directly mimic the *in vivo* conditions necessary for migration of MSCs from the vasculature to the tumor. Further elucidation

of the mechanisms underlying the migration of MSCs toward gliomas may provide insights into methods for increasing the effectiveness of cell engraftment.

Acknowledgements

The authors thank Dr Hongshan Chen and Dr Xiongfei Xu for their skillful technical assistance.

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