Vascular endothelial growth factor participates in modulating the C6 glioma-induced migration of rat bone marrow-derived mesenchymal stem cells and upregulates their vascular cell adhesion molecule-1 expression

ZHIQIANG GAO[1*], PENG CHENG[2*], YIXUE XUE[3] and YUNHUI LIU[1]

[1]Department of Neurosurgery, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004; [2]Department of Neurosurgery, First Affiliated Hospital of China Medical University; [3]Department of Neurobiology, College of Basic Medicine, China Medical University, Shenyang, Liaoning 110001, P.R. China

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Abstract. Bone marrow-derived mesenchymal stem cells (BMSCs) have been shown to be able to migrate towards glioma, but the molecular mechanisms responsible for this migratory behavior still require further elucidation. This study aimed to test the role of vascular endothelial growth factor (VEGF) in the C6 glioma-induced migration of BMSCs, evaluate the effect of VEGF on the migratory capacity and vascular cell adhesion molecule-1 (VCAM-1) expression of BMSCs and explore the role of VCAM-1 in the VEGF-induced migration of BMSCs. The results showed that C6 glioma cells significantly increased the migration of BMSCs *in vitro*, which was partially blocked by a VEGF neutralizing antibody, and 20 ng/ml recombinant rat $VEGF_{164}$ incubation enhanced the migration of BMSCs. Moreover, 12 h of 20 ng/ml $VEGF_{164}$ incubation upregulated the VCAM-1 expression of BMSCs and the blocking of VCAM-1 reduced the $VEGF_{164}$-induced migration of BMSCs. The data also revealed that LY294002, an inhibitor of phosphoinositide-3-kinase (PI3K), decreased the VEGF-induced migration and VCAM-1 expression of BMSCs. These findings indicate that VEGF participates in mediating the C6 glioma-induced migration of BMSCs by upregulating their VCAM-1 expression, and that PI3K is involved in the signal transduction of $VEGF_{164}$-induced migration and VCAM-1 expression of BMSCs.

Correspondence to: Dr Yunhui Liu, Department of Neurosurgery, Shengjing Hospital of China Medical University, Sanhao Street No. 36, Heping, Shenyang, Liaoning 110004, P.R. China
E-mail: liuyunhuicmu@yahoo.com.cn

*Contributed equally

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Introduction

Malignant gliomas are the most common primary malignant tumors of the central nervous system. Despite the improvement in conventional treatment such as surgery, radiotherapy and chemotherapy, the prognosis for patients with high-grade gliomas is still very poor (1). In recent years, more and more researchers have shown interest in targeted gene therapies against gliomas (2,3) and there is a great need to develop appropriate carriers for these treatment programs to deliver therapeutic agents efficiently into glioma tissues. As a type of adult multipotent stem cell, bone marrow-derived mesenchymal stem cells (BMSCs) are characterized by their easy isolation and high *ex vivo* expansion potential (4,5). Previous studies have proven that BMSCs can migrate towards gliomas after intracerebral or systemic injection, which has made these cells an attractive delivery vehicle in targeted gene therapy of gliomas (2,3). Elucidating the molecular signaling pathway of this migratory behavior will help us to further improve the efficiency of targeted gene therapy mediated by BMSCs.

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic cytokines expressed in glioma tissues and is involved in the progression of malignant brain tumors (6,7). Previous studies have proven the correlation of VEGF expression and glioma grade (8). In addition, the chemotactic effects of VEGF on various cell types have been widely reported (9,10). As a cell surface glycoprotein, vascular cell adhesion molecule-1 (VCAM-1) mediates the adhesion and migration of leukocytes and T lymphocytes through brain microvascular endothelial cells, according to binding to its receptors, the α4β1 and α4β7 integrins (11,12). In a previous study, we proved that VCAM-1 plays an important role in the migration of BMSCs induced by C6 and U87 glioma cells (13). Analyzing the role of VEGF in regulating the glioma-induced migration and VCAM-1 expression of BMSCs may help us understand the mechanism of BMSCs migrating towards gliomas after intravascular delivery or intracerebral transplantation. Furthermore, previous data have demonstrated that phosphoinositide-3-kinase (PI3K) plays a crucial role in

the signaling pathways linking extracellular signals to crucial cellular processes and contributes to growth factor stimulating signal transduction from cell membrane to cytoplasm. It has also been confirmed that PI3K participates in the intracellular signal transduction of VEGF-induced cell migration (14,15).

Therefore, in this study, we attempted to evaluate the role of VEGF in the C6 glioma-induced migration of BMSCs, whether VEGF upregulates the VCAM-1 expression of BMSCs, the relation between VCAM-1 and the VEGF-induced migration of BMSCs and whether PI3K is involved in the signal transduction of VEGF-induced migration and VCAM-1 upregulation of BMSCs.

Materials and methods

Isolation and culture of BMSCs. Four-week-old male Wistar rats (80-100 g) were used in our study. The rats were purchased from the Laboratory Animal Center of China Medical University. All experiments using animals were approved by the Animal Care and Use Committee of China Medical University and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. BMSCs were prepared as previously described with some modifications, according to their adherence to plastic (16,17). In brief, the rats were sacrificed by cervical dislocation after they were anesthetized with 10% chloral hydrate (0.3 ml/100 g) by intraperitoneal injection. Bilateral tibias and femurs were dissected aseptically, and bone marrow was collected and suspended in low glucose Dulbecco's modified Eagle's medium (L-DMEM; Gibco, Invitrogen Corp., Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco). The cell suspension was then transferred into 25-cm^2 culture flasks. After 48 h of cultivation, the culture medium was replaced to remove the non-adherent hematopoietic lineage cells, and adherent cells were further cultured and expanded. BMSCs at passage 3 were used for the studies.

Culture of C6 glioma cells. Rat C6 glioma cells (American Type Culture Collection, Rockville, MD) were maintained in L-DMEM supplemented with 10% FBS.

In vitro migration assay. In our experiments, 24-well Transwell inserts with an 8-μm pore size (Corning Costar) were used to evaluate the migration of BMSCs under different conditions. C6 glioma cells and BMSCs were trypsinized and resuspended in serum-free L-DMEM at $5x10^5$/ml and $1x10^6$/ml, respectively. To explore the migration of BMSCs toward C6 glioma cells, 1 ml of C6 glioma cell suspension was added into the lower chambers and 6 h later, a 200-μl BMSC suspension was added into the upper chambers. Moreover, to ascertain whether VEGF promotes the migration of BMSCs towards C6 glioma, we examined the migration of BMSCs in response to a suspension of C6 glioma cells supplemented with or without a VEGF neutralizing antibody (1 μg/ml, Abcam, Cambridge, UK), which were placed in the lower chambers, respectively. In addition, we applied $VEGF_{164}$, one major isoform of rat VEGF, to test the chemotactic effect of VEGF on BMSCs. Serum-free L-DMEM with recombinant rat $VEGF_{164}$ (R&D Systems, Minneapolis, MN, USA) at concentrations of 20 ng/ml was added into the lower wells. VCAM-1 blocking antibody (Covance, Princeton, NJ, USA) was added to the suspension of BMSCs at 10 μg/ml to evaluate the role of VCAM-1 in the $VEGF_{164}$-induced migration of BMSCs. Furthermore, we used the PI3K selective inhibitor, LY294002, to assess the role of PI3K in the VEGF-induced migration of BMSCs. BMSCs were treated with LY294002 (20 μM; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min prior to, and for the duration of, the stimulation of 20 ng/ml $VEGF_{164}$. The contents of the upper and lower wells were separated by a polycarbonate membrane (8-μm pore size). Cell migration was allowed for 36 h at 37˚C. Following incubation, the media were aspirated, and the cells remaining on the upper surface of the polycarbonate membrane were removed with a cotton swab. The cells migrating to the lower surface were stained with Giemsa stain for 15 min. Cell counting was performed under an inverted microscope by two researchers separately. The average numbers of migrated cells were determined by counting the cells in 6 random high-power fields (x400). Serum-free L-DMEM alone served as negative controls.

RT-PCR. To investigate the change in VEGF-induced VCAM-1 expression of BMSCs and the relevance of PI3K to this process, the cells were incubated with L-DMEM containing 10% FBS in the presence of $VEGF_{164}$ (20 ng/ml) with or without LY294002 (20 μmol/l) for 12 h. The expression of VCAM-1 mRNA was evaluated by reverse transcriptase-polymerase chain reaction analysis (RT-PCR). The cells incubated with L-DMEM containing 10% FBS served as a negative control. Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen) and cDNA was generated from 1 μg of total RNA from each sample. The primers used were as follows: VCAM-1, 5'-ACACCTCCCCCAAGAATACAG-3' (forward) and 5'-GCT CATCCTCAACACCCACAG-3' (reverse) (18); β-actin, 5'-TCA GGTCATCACTATCGGCAAT-3' (forward) and 5'-AAAGA AAGGGTGTAAAACGCA-3' (reverse). PCR conditions for both were as follows: 32 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 2 min. The PCR products were subjected to electrophoresis on 1.5% agarose gels. All of the cDNA bands were scanned using Chemi Imager 5500 V2.03 software, and the integrated density values (IDV) were calculated by computerized image analysis system (Fluor Chen 2.0) and normalized with that of β-actin.

Immunofluorescence assays. For immunofluorescence assays, BMSCs were cultured on glass coverslips coated with 0.1% gelatin. After incubation was performed as mentioned above, the cells were fixed with acetone and immunostained with mouse monoclonal anti-VCAM-1 antibody (diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4˚C overnight. Subsequent visualization was performed with anti-mouse IgG conjugated with rhodamine (TRITC) (diluted 1:200; Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C in darkness. Coverslips were mounted in mounting media, and images were captured with an Olympus BX60 upright fluorescence microscope with appropriate filters and objectives, using identical acquisition parameters per experiment. L-DMEM containing 10% FBS served as a control.

Statistical analysis. All results are described as mean ± SD for each group. A Student's t-test was used to assess a significant

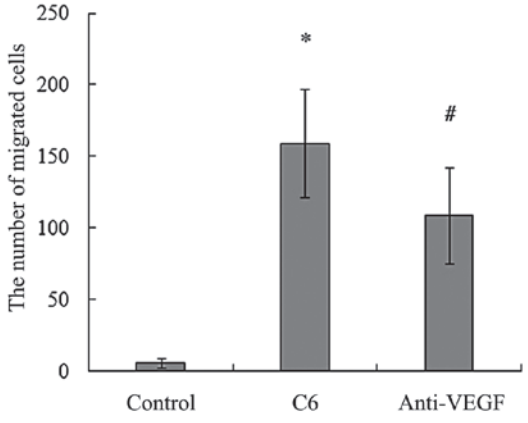

Figure 1. Role of VEGF in the migration of BMSCs induced by C6 glioma cells. Compared to the control, the coculture of BMSCs with C6 glioma cells led to a significant increase in BMSC migration. The application of VEGF neutralizing antibody significantly reduced the number of migrating BMSCs. C6 glioma cells and BMSCs were trypsinized and resuspended in serum-free L-DMEM at $5x10^5$/ml and $1x10^6$/ml, separately. For each group, 200 μl of a BMSC suspension was added into the upper chamber. Control group, 1 ml of serum-free L-DMEM was added into the lower chamber; C6 group, 1 ml of C6 cell suspension was added into the lower chamber; anti-VEGF group, 1 ml of C6 cell suspension supplemented with a VEGF neutralizing antibody (1 μg/ml) was added into the lower chamber. *P<0.05 vs. control group and #P<0.05 vs. C6 group.

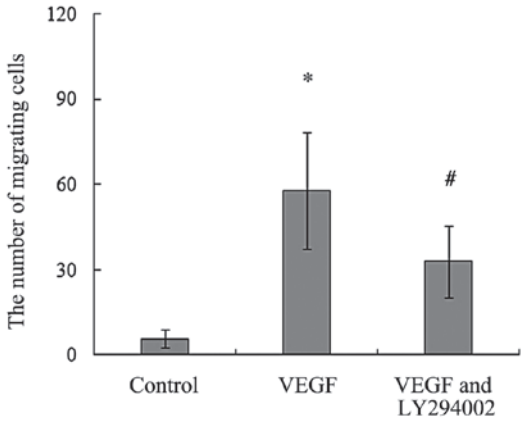

Figure 2. Recombinant rat $VEGF_{164}$ is chemotactic for BMSCs and LY294002 inhibit the VEGF-induced migration of BMSCs. The number of migrating BMSCs increased significantly with the stimulation of $VEGF_{164}$ at the concentration of 20 ng/ml. In the presence of LY294002, the number of migrating BMSCs decreased. C6 glioma cells and BMSCs were trypsinized and resuspended in serum-free L-DMEM at $5x10^5$/ml and $1x10^6$/ml, respectively. For each group, 200 μl of the BMSC suspension was added into the upper chamber. Control group, 1 ml serum-free L-DMEM was added into the lower chamber. VEGF group, 1 ml serum-free L-DMEM with 20 ng/ml $VEGF_{164}$ was added into the lower chamber. VEGF and LY294002 group, 1 ml serum-free L-DMEM with $VEGF_{164}$ (20 ng/ml) and LY294002 (20 μM) were added into the lower chamber. *P<0.05 vs. control group; #P<0.05 vs. VEGF group.

difference between two groups. One-way analysis of variance (ANOVA) was performed to determine significant differences between multiple groups. P<0.05 was considered to indicate a statistically significant result.

Results

BMSCs exhibit the capacity to migrate towards C6 glioma in vitro. We used an *in vitro* migration asssay to evaluate the migratory ability of BMSCs towards C6 glioma cells. Consistent with previous studies, in our experiment, we found that BMSCs migrated directionally towards glioma (2). As shown in Fig. 1, the average number of migrating cells towards C6 glioma cells was significantly higher than that in the control.

Neutralization of VEGF decreases the number of BMSCs migrating towards C6 glioma. To determine whether VEGF has a role in the BMSC migration toward gliomas, a VEGF neutralizing antibody was added into the lower chamber together with the C6 glioma cells. We found that the neutralization of VEGF significantly reduced the migration-enhancing effect of C6 glioma cells and the number of migrating cells decreased compared with the control (Fig. 1). These results suggest that VEGF participates in mediating the migration of BMSCs toward C6 glioma *in vitro*.

Recombinant rat $VEGF_{164}$ promotes the migration of BMSCs. To evaluate the chemotactic effect of VEGF on rat BMSCs, we analyzed the effect of recombinant rat $VEGF_{164}$ on the migration of BMSCs. Our data showed that the addition of recombinant rat $VEGF_{164}$ caused chemotaxis activity of BMSCs and induced them to migrate towards the lower chambers. When compared to the control, $VEGF_{164}$, at a concentration of 20 ng/ml, led to a significant increase in the number of migrating BMSCs (Fig. 2).

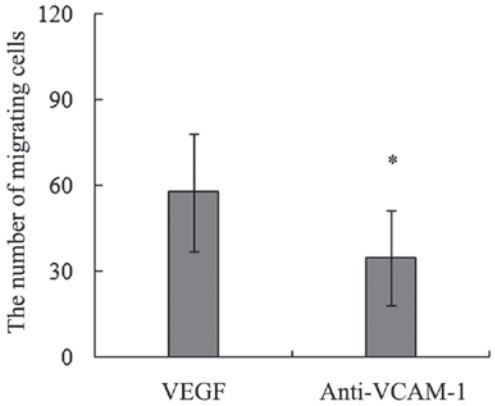

Figure 3. VCAM-1 is an important adhesion molecule mediating the migration of BMSCs induced by $VEGF_{164}$. Compared with the $VEGF_{164}$ group, the addition of VCAM-1 neutralizing antibody in the suspension of BMSCs obviously reduced the migration of BMSCs towards $VEGF_{164}$. BMSCs were trypsinized and resuspended in serum-free L-DMEM at $1x10^6$/ml. For each group, 200 μl of a BMSC suspension was added into the upper chamber. VEGF group, $VEGF_{164}$ was added into the lower chamber at 20 ng/ml. Anti-VCAM-1 group, $VEGF_{164}$ was added into the lower chamber at 20 ng/ml and VCAM-1 neutralizing antibody was added into the upper chamber at 10 μg/ml. *P<0.05 vs. VEGF group.

Blocking of VCAM-1 decreases the migration of BMSCs induced by $VEGF_{164}$. Our data of the *in vitro* migration assays demonstrated that the addition of a VCAM-1 neutralizing antibody significiantly decreased the number of migrating BMSCs towards $VEGF_{164}$ (Fig. 3). These results imply that VCAM-1 is a key adhesion molecule mediating the migration of BMSCs induced by $VEGF_{164}$.

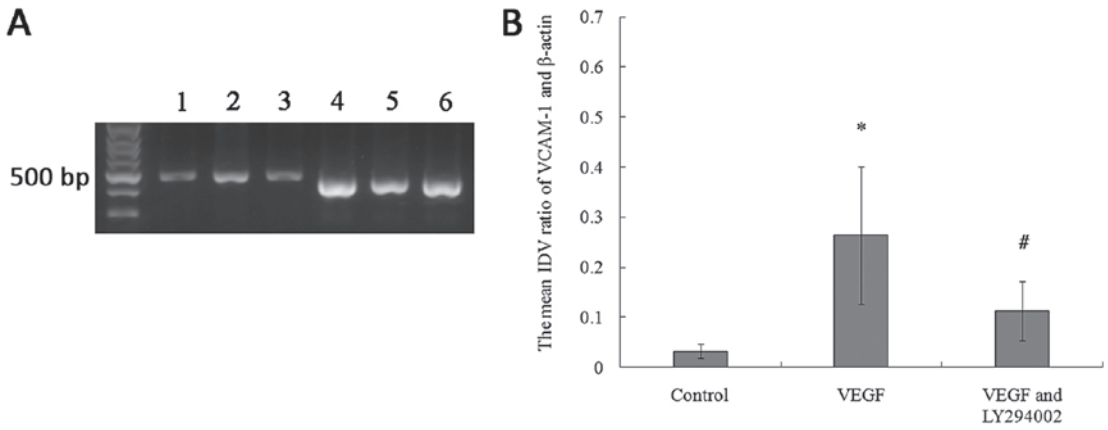

Figure 4. RT-PCR analysis reveals that VCAM-1 mRNA expression of the BMSCs is upregulated by the incubation with 20 ng/ml $VEGF_{164}$, which was inhibited by LY294002. (A) Representative results of the RT-PCR illustrating differences in the 477-bp band of VCAM-1; (Lane 1, VCAM-1 in the control group; Lane 2, VCAM-1 in the VEGF group; Lane 3, VCAM-1 in the VEGF and LY294002 group; Lane 4, β-actin in the control group; Lane 5, β-actin in the VEGF group; Lane 6, β-actin in the VEGF and LY294002 group). (B) Relative integrated density value (IDV) analysis of VCAM-1 mRNA. VCAM-1 mRNA expression of BMSCs (n=8, each; $^{*}P<0.05$ vs. control group and $^{\#}P<0.05$ vs. VEGF group). Control group, the cells were incubated with L-DMEM containing 10% FBS for 12 h; VEGF group, the cells were incubated with L-DMEM containing 10% FBS in the presence of VEGF (20 ng/ml) for 12 h; VEGF and LY294002 group, the cells were incubated with L-DMEM containing 10% FBS in the presence of VEGF (20 ng/ml) and LY294002 (20 μM) for 12 h.

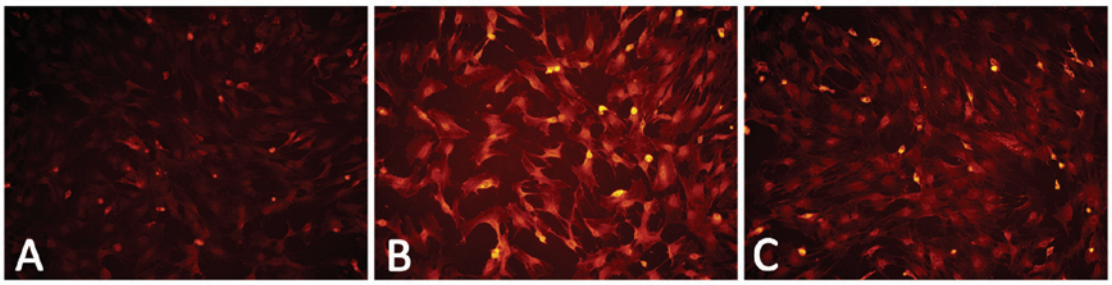

Figure 5. Immunofluorescence analysis reveals that VEGF enhances the VCAM-1 expression of BMSCs, which is blocked by LY294002. Representative results of immunofluorescence illustrating differences in the expression level of VCAM-1. Imaged at x200. (A) Control group; (B) VEGF group; (C) VEGF and LY294002 group. Control group, cells were incubated with L-DMEM containing 10% FBS for 12 h; VEGF group, cells were incubated with L-DMEM containing 10% FBS in the presence of VEGF (20 ng/ml) for 12 h; VEGF and LY294002 group, cells were incubated with L-DMEM containing 10% FBS in the presence of VEGF (20 ng/ml) and LY294002 (20 μM) for 12 h.

Recombinant rat $VEGF_{164}$ upregulates the VCAM-1 expression of BMSCs. The results of RT-PCR and immunofluorescence assays revealed that the BMSCs expressed a low level of VCAM-1 in the culture without $VEGF_{164}$, while the cells treated with 20 ng/ml $VEGF_{164}$ for 12 h exhibited a higher VCAM-1 expression than the cells without the stimulation of $VEGF_{164}$ (Figs. 4 and 5).

Inhibition of PI3K reduces the migratory response and VCAM-1 expression of BMSCs to recombinant rat $VEGF_{164}$. The results of the *in vitro* migration assays showed that the migration of BMSCs toward recombinant rat $VEGF_{164}$ was found to be partially blocked and the number of migrating BMSCs significantly decreased with the addition of LY294002. This indicates that $VEGF_{164}$-mediated PI3K activation may correlate with the migration of BMSCs induced by $VEGF_{164}$ (Fig. 2). We also found that the upregulation of VCAM-1 mRNA and protein expression decreased following treatment with LY294002, which suggests that PI3K activation may play an active role in VCAM-1 upregulation of BMSCs stimulated by $VEGF_{164}$ (Figs. 4 and 5).

Discussion

In the present study, we demonstrated that VEGF participated in modulating C6 glioma-induced migration and in promoting VCAM-1 expression of BMSCs, VCAM-1 plays an important role in VEGF-induced migration of BMSCs and that PI3K was involved in the signal transduction of this regulatory process.

It has been reported that BMSCs have the capacity of migrating to gliomas (2,19). Consistent with these findings, our *in vitro* migration results demonstrated that rat BMSCs migrated directionally to C6 glioma cells. After being co-cultured 36 h with C6 glioma cells, the average number of migrating cells was significantly higher than that in the control. This directional migratory behavior may be due to the soluble factors released from C6 glioma cells.

VEGF is one of the strongest and most specific angiogenesis cytokines. VEGF is not only closely related to the invasiveness of glioma, but is also proportional to the grade of malignancy of glioma (20,21). The expression of VEGF mRNA and protein has been reported to increase in C6 glioma (22). Moreover, it has been shown that VEGF is expressed in glioma tissues and

acts as an angiogenic factor for tumor vessels (6,23). Therefore, we added a VEGF neutralizing antibody into the lower wells to block the effect of VEGF on the C6 glioma-induced migration of BMSCs. We found that the number of migrating BMSCs decreased obviously, which suggests the important role of VEGF in this process. Our results also showed that the number of migrating BMSCs was still higher than the control after blocking VEGF, which indicates that there may be other soluble active factors released from C6 glioma cells which participate in inducing the migration of BMSCs. These results were further supported by analyzing the effect of recombinant rat $VEGF_{164}$, one major isoform of rat VEGF, on the migration of BMSCs using an *in vitro* migration assay. The data demonstrated that recombinant rat $VEGF_{164}$ at a concentration of 20 ng/ml in the lower wells led to a significant increase in migrating BMSCs.

As a transmembrane glycoprotein, VCAM-1 is a member of the immunoglobulin superfamily. VCAM-1 and its receptor intergrin α4β1 are not only expressed on BMSCs, but are also expressed on glioma microvascular endothelia (24,25). Previous data showed that the interaction of VCAM-1 and integrin α4β1 enhances the migration of human melanoma cells across activated endothelial cell layers (26). In addition, VCAM-1 contributes to neutrophil trafficking into the central nervous system (27). Our previous data also demonstrated the important role of VCAM-1 in the migration towards gliomas (13). Since $VEGF_{164}$ is one of the most abundant forms of VEGF found in C6 glioma cells and rat brain, we investigated the effect of recombinant rat $VEGF_{164}$ on the VCAM-1 expression of BMSCs (28). The RT-PCR and immunofluorescence assay revealed that the incubation with 20 ng/ml $VEGF_{164}$ elevated the expression of VCAM-1 mRNA and protein in BMSCs. Our data also showed that the neutralization of VCAM-1 decreased the number of migrating BMSCs towards $VEGF_{164}$, which indicates that VCAM-1 plays an important role in the $VEGF_{164}$-induced migration of BMSCs. Collectively we conclude that VEGF induces the migration of BMSCs by increasing the VCAM-1 expression in BMSCs.

Initially defined as an important intracellular signal transduction pathway, PI3K extensively takes part in a series of intracellular physiological and pathological responses induced by VEGF, including migration (29,30). We utilized LY294002, a PI3K-specific inhibitor, to explore the effect of PI3K on the VEGF-induced migration of BMSCs. The data showed that after blocking PI3K, the migration of BMSCs induced by $VEGF_{164}$ was inhibited. We also found that the upregulated expression of VCAM-1 mRNA and protein decreased following the treatment with LY294002. These results provide evidence that the PI3K signaling pathway is correlated with the intracellular signal transduction of this directional migration. Since LY294002 cannot completely block VEGF-induced migration of BMSCs, there may be other signaling transduction pathways participating in the regulation of the migratory capacity of BMSCs and VCAM-1 upregulation.

In summary, our results demonstrate that VEGF plays an important role in C6 glioma-induced migration, and recombinant rat $VEGF_{164}$ promotes the migration of BMSCs by elevating the VCAM-1 expression of BMSCs, and that PI3K is one of the important signaling molecules mediating the signal transduction of $VEGF_{164}$-induced migration and VCAM-1 expression of BMSCs.

Acknowledgements

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