Glioma cells enhance endothelial progenitor cell angiogenesis via VEGFR-2, not VEGFR-1

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Abstract. Although potential contribution of endothelial progenitor cells (EPCs) to angiogenesis in glioma has been proposed, the molecular mechanisms of EPCs recruitment to vasculature have not been fully elucidated. Here, we show that the supernatant from glioma cells promotes EPCs angiogenesis via VEGFR-2, not VEGFR-1. Moreover, VEGFR-2 siRNA inhibits VEGFR-2 expression in EPCs, tube formation on matrigel and cell migration. MMP-9 activity and expression and the Akt and ERK phosphorylations are decreased by VEGFR-2 siRNA. Thus, these results indicate that glioma cells enhance EPC angiogenesis via VEGFR-2, not VEGFR-1, mediated by the MMP-9, Akt and ERK signal pathways.

Introduction

Glioma remains one of the human tumors most refractory to treatment, despite continuing advances in radiotherapy, chemotherapy, and surgical techniques. The treatment failure is due to diffuse infiltration of the surrounding 'normal brain tissues'. The current wisdom is that tumor growth, invasion and metastasis are angiogenesis-dependent. Glioma is one of the best vascularized tumors and its proliferation is hallmarked by a distinct proliferative vascular component. Therefore, developing angiogenesis-targeted therapeutic strategies for glioma would be a top priority.

Recent studies have provided ample evidence that angiogenesis is required for the mobilization and functional incorporation of endothelial progenitor cells (EPCs). The

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recruitment of EPCs to angiogenesis represents a multistep process, including: active arrest and homing of the circulating cells within the angiogenic microvasculature; transendothelial extravasation into the interstitial space; extravascular formation of cellular clusters; creation of vascular sprouts and cellular networks; incorporation into a functional microvasculature (1). Their contribution to the formation of new blood vessels has drawn attention to their potential for therapeutic application. Recent data suggested that significant number of EPCs migrate to glioma and incorporate into vasculature with high specificity (2,3). However, the molecular mechanisms responsible for EPCs angiogenesis are not fully understood. Several growth factors, including vascular endothelial growth factor, stem cell growth factor, and granulocyte-macrophage colony-stimulating factor, have been reported to be involved in the regulation of endothelial differentiation and migration to form functional vessels (4-6).

VEGFR-1 and VEGFR-2 have been considered to be the major signal transducers for both physiological and pathological angiogenesis. VEGFR-1 and VEGFR-2 have distinct functions in vascular development in embryos. VEGFR-1 knockout mice, which die around embryonic day 8.5 (E8.5), have abundant ECs, which do not assemble in functional vessels (7). In contrast, VEGFR-2 knockout mice, which also die by E8.5, lack both ECs and hematopoietic cells (8). Recent studies have emphasized the effects of VEGFR-1 on the regulation of tumor angiogenesis (9). In mature endothelial cells, VEGFR-2 functions to promote angiogenesis by regulating endothelial cell attachment, proliferation, migration and growth. However, little is understood about the role of VEGFR-1 and VEGFR-2 during glioma-associated angiogenesis of EPCs. These studies lead us to speculate that glioma cells may promote angiogenesis of EPCs via regulating the expression of VEGFR-1 or/and VEGFR-2. We therefore try to clarify the above hypothesis in the present study.

In this study we characterize that glioma cells augment angio-genesis of EPCs through up-regulating the expression of VEGFR-2 but not VEGFR-1. Then we used RNA interference method to block the endogenous VEGFR-2, and investigated the mechanism responsible for EPCs contribution to angiogenesis. We demonstrated that inhibition of migration and tube formation are detected in EPCs after RNA interference against VEGFR-2 expression. We also showed

that VEGFR-2 siRNA inhibits MMP-9 expression and Akt and ERK phosphorylations.

Materials and methods

Cell culture. Under informed consent, mononuclear cells were isolated from human cord blood by density gradient centrifugation. Then CD133+ cells were purified by magnetic activated cell sorting columns (MACS; Miltenyi Biotech), suspended in EGM-2 MV media (Cambrex) containing endothelial basal medium (EBM), 5% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, and heparin, then seeded on fibronectin-coated (Chemicon) culture dishes. After 72 h, non-adherent cells were removed. The Ethics Committee of the Nanjing Medical University approved the experiments for this study.

Human glioma U87 cells (kindly provided by Dr Jianhong Zhu, Neurosurgical Department of Huashan Hospital, Shanghai, P.R. China) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (penicillin at 100 U/ml and streptomycin at 100 μg/ml) in a humidified atmosphere of 5% carbon dioxide and 95% air at 37˚C. The supernatant from glioma cells was prepared by culturing cells in serum-free medium (DMEM) for 48 h, and a centrifugal filter device (Millipore) was utilized to concentrate the supernatant from glioma cells.

Immunofluorescence staining. After 14 days of culture, cells were seeded on glass coverslips coated with collagen in 6-well plates. To detect acetylated low-density lipoprotein (Ac-LDL) uptake and ulex europaeus agglutinin-1 (UEA-1) binding, they were fixed with 4% paraformaldehyde at room temperature for 30 min prior to incubation with 10 μg/ml DiI-Ac-LDL (Molecular Probes). After fixation, cells were reacted with FITC-UEA-1 (Sigma). After the staining, cells were examined under confocal microscopy. Cells which both take up Dil-ac-LDL and bind FITC-UEA-1, were judged to be EPCs (10,11). To detect CD31 expression, cells were incubated with goat anti-CD31 (dilution 1:100, Santa Cruz) and rabbit anti-ß-tubulin (dilution 1:200, Sigma) for 1 h at 37˚C, then incubated with Rhodamine-conjugated donkey anti-goat antibody (dilution 1:500, Santa Cruz) and FITC-conjugated goat anti-rabbit antibody (dilution 1:500, Invitrogen) for 30 min at 37˚C. Nuclei were stained with DAPI (dilution 1:1000, Molecular Probes). Images were acquired with fluorescence microscopy.

Fluorescence-activated cell sorter analysis. To assess the change of surface antigen of cells, we performed fluorescence-activated cell sorter (FACS) analysis. Labeling was carried out using CD133/2-PE (Miltenyi Biotec) and CD31-FITC (eBioscience) in 50 μl of phosphate-buffered saline (PBS) at room temperature for 30 min. The number of positive cells was compared with the number of cells positive in staining with the immunoglobulin G isotype controls (BD Pharmingen) and determined with FACS flow cytometer (BD Biosciences). Three separate experiments were performed.

siRNA transfection. The sequences for VEGFR-2 silencing have been reported previously (12). The sense sequence is 5'-AAUACUUGUCGUCUGAUUCUCCAGG-3' and the anti-

sense sequence is 5'-CCUGGAGAAUCAGACGACAAGUA UU-3'. The negative control siRNA: the sense sequence (5'-UUCUCCGAACGUGUCACGUTT-3'), the antisense sequence (5'-ACGUGACACGUUCGGAGAATT-3'). VEGFR-2 siRNA and negative control siRNA were both synthesized by Shanghai Genepharma Co. Inc. (Shanghai, P.R. China). EPCs at 70% confluency were transfected with VEGFR-2 siRNA, negative control siRNA and no siRNA using Lipofectamine 2000 (Invitrogen), which indicated the siRNA group, the siRNAnc group and the no siRNA group, respectively. After 4 h, the transfection medium was removed; the cells were washed twice with PBS, and then maintained in EGM-2 MV media for 72 h before performing the experiments.

In vitro tube formation assay. *In vitro* tube formation assay was performed by plating EPCs or transfected cells (5x10³ cells) suspended in the supernatant from glioma cells or EGM-2 MV media onto 96-well plates coated with matrigel (BD PharMingen). After 24 h of incubation, images of tubular structures were taken, and the total number of tube formation in three independent 200x fields for each well were calculated. Three separate experiments were performed.

In vitro cell migration assay. EPC migration was evaluated using matrigel-coated Transwell (Costar) with 8-μm pore size filter. Cells were starved for 4 h in EBM (serum-free medium). EPCs (200 μl) or transfected cells suspensions in EBM (10^6/ml) were applied to the upper chamber. The supernatant from glioma cells or EGM-2 MV media was added to the lower chamber. Migration was allowed to proceed for 20 h at 37˚C. Cells remaining on the upper surface of the filters were carefully removed with cotton swabs. Migrated cells were stained with crystal violet and examined by light microscopy. After microscopic documentation, stained cells were extracted and measured for optical density at 560 nm in a microplate reader. Three separate experiments were performed.

Western blot analysis. Three hours after treatment of the supernatant from glioma cells or 72 h after transfection, cells were washed, harvested, and lysed in RIPA lysis buffer (Pierce) on ice for 10 min. For phosphoprotein analysis, cell lysis buffer was supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM NaF, and 10 mM μ-glycerophosphate). The supernatants were collected and quantified for protein concentration by the Bradford assay. Equal amounts of protein per lane were separated by 5-10% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked in 5% skim milk for 1 h and then incubated with a specific antibody for 2 h. The antibodies used for Western blot analysis in this study were: antibodies to VEGFR-1, VEGFR-2, MMP-9, Akt, phospho-Akt[(Ser473)], ERK, and phospho-ERK[(Thr202/Tyr204)] (Santa Cruz). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as protein loading control. Antibody binding was detected using a chemiluminescence detection system (Pierce), according to the manufacturer's recommendations. Western blot film was digitized, and band net intensities were quantified using the Quantity One software (Bio-Rad).

Gelatin zymography. Three hours after treatment of the supernatant from glioma cells or 72 h after transfection, total
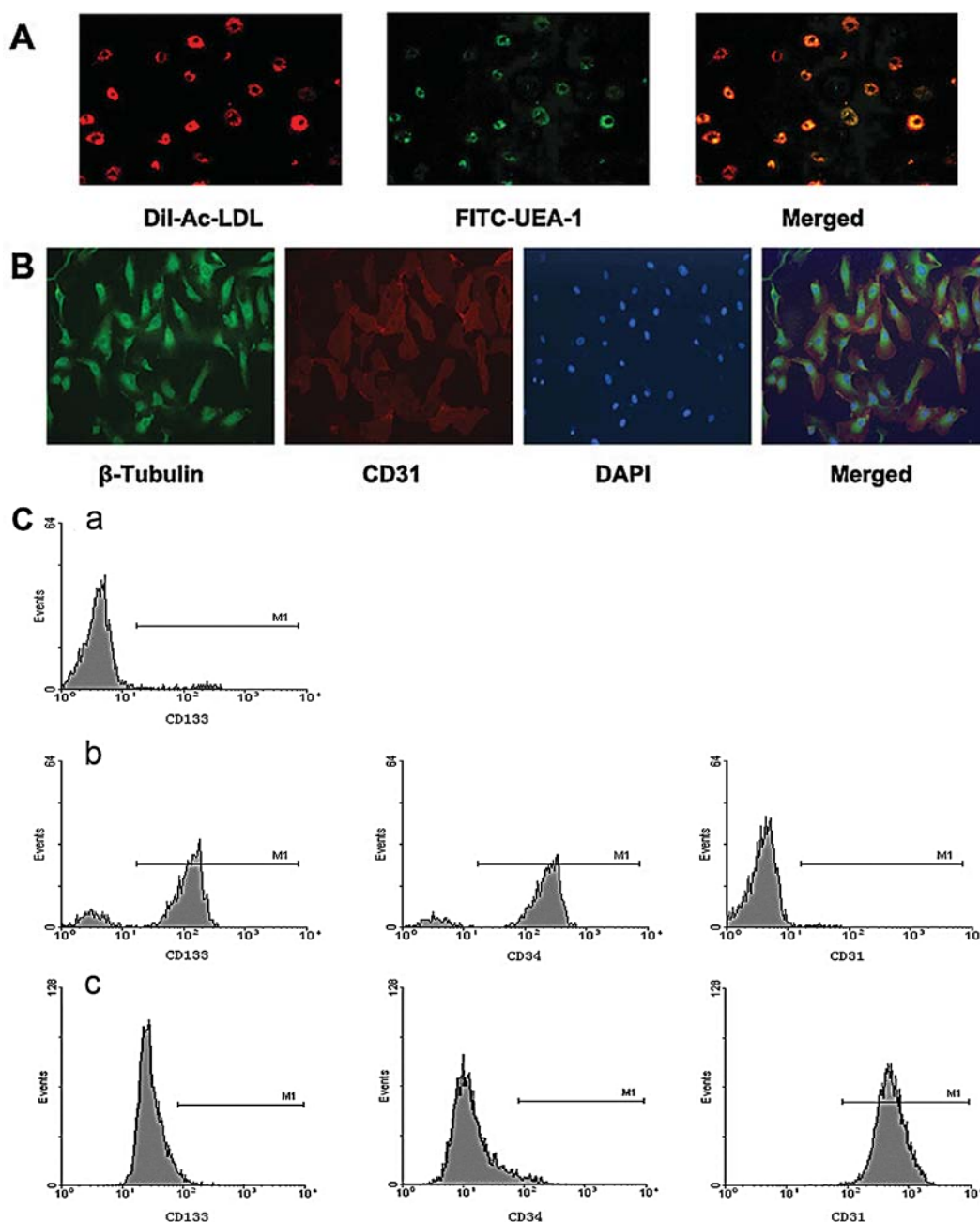
Figure 1. Phenotypic characterization of EPCs from human cord blood. (A), Two-color confocal microscopic image indicating Dil-Ac-LDL incorporation (red) and FITC-UEA-1 binding (green). Cells which both take up Dil-ac-LDL and bind FITC-UEA-1, are EPCs. (B), Three-color fluorescence microscopic image indicating ß-tubulin staining (red) and CD31 staining (green). Nuclei (DAPI) are dark blue. (C), Fluorescence-activated cell sorter analysis indicating the change of surface antigen of cells (a, before CD133 immunomagnetic sorting; b, after CD133 immunomagnetic sorting; c, cultured at day 14).

cellular protein was extracted with lysis buffer (1% NP-40 in 50 mM Tris-HCl buffer, pH 8.0 containing 150 mM NaCl). Equal amounts of protein from the treatment groups were electrophoresed on a gelatin containing 8% SDS-polyacryl-amide gel. Following electrophoresis, the gel was incubated in the washing buffer (50 mM Tris-HCl, pH 7.5, 2.5% Triton X-100). Then the gel was incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 150 mM NaCl, 0.02% NaN_3) at 37°C. Twenty-four hours later the gel was stained with 0.05% Coomassie Blue. Gels were visualized by Gel Doc™ XR gel documentation system (Bio-Rad).

Statistical analysis. Data were analyzed with Stata 7.0. Statistical significance (P<0.05) was determined by One-Way ANOVA and independent sample two-tailed t-tests followed by assessment of differences.

Results

EPCs culture and identification. After 7 days of culture, the CD133+ cells isolated from human cord blood mononuclear cells exhibited small colonial morphology; then, the colonies expanded, confluenced, and displayed a typical 'cobblestone' morphology. More than 90% attached cells took up Dil-ac-LDL and bound FITC-UEA-1 (Fig. 1A), and were judged to be EPCs. The endothelial phenotype of expanded EPCs was further characterized by expression of endothelial markers such as CD31 (Fig. 1B). Compared with the original, cell
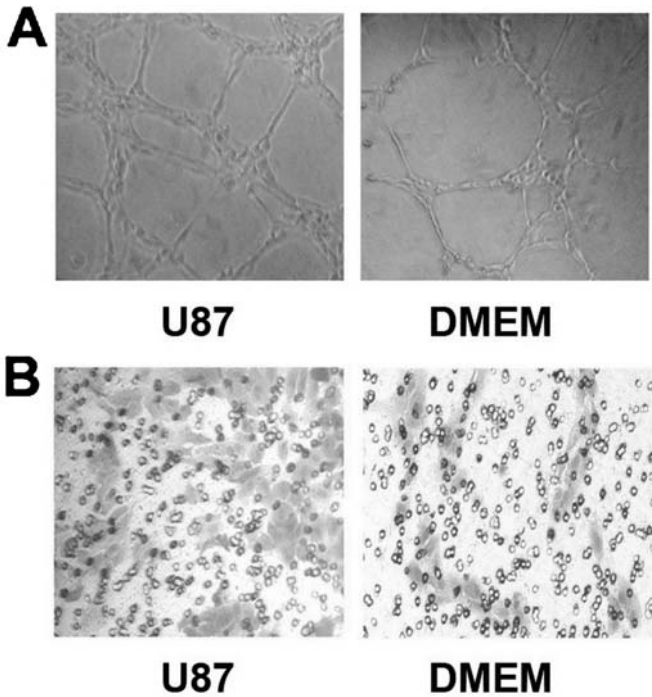
Figure 2. The supernatant from glioma cells increases EPC tube formation on matrigel and migration of EPCs. (A), Tubes were visualized by phase contrast microscopy at a magnification of x200. The supernatant from glioma cells significantly induces formation of tube structures, compared with DMEM (40.7±3.3 versus 21±2.3, P<0.05). (B), Migrated cells were stained with crystal violet and examined with light microscopy at a magnification of x200. The OD values were 0.60±0.04, 0.27±0.02, respectively for the supernatant from glioma cells and DMEM (P<0.05).
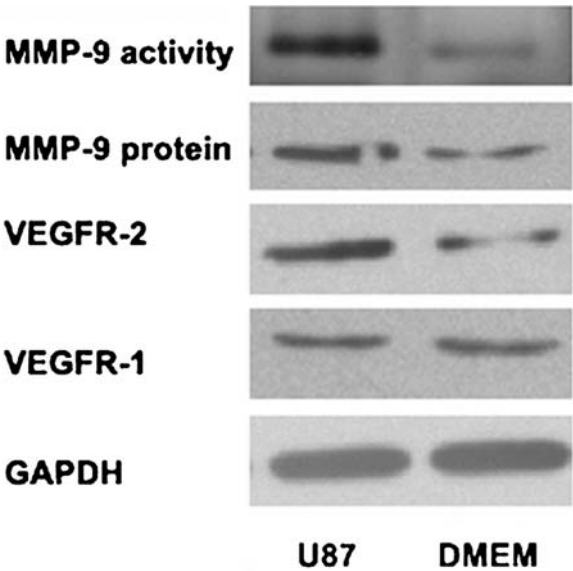

Figure 3. The supernatant from glioma cells up-regulates expression of VEGFR-2 and MMP-9, not VEGFR-1 in EPCs. Effects of the supernatant from glioma cells on VEGFR-1, VEGFR-2 and MMP-9 expression were determined by Western blot analysis. The quantitative data show relative protein levels of VEGFR-1, VEGFR-2 and MMP-9 (normalized to GAPDH level) in the no siRNA group. Cell lysates were evaluated for MMP-9 activity by zymography analysis and protein expression by Western blot analysis as described in Materials and methods.

markers CD133 and CD34 decreased from 86.04±3.36% to 2.96±0.45% and from 90.88±2.86% to 2.99±0.11%, respectively, while CD31 increased from 1.12±0.04% to 99.88±0.04% (Fig. 1C). EPCs had high potential of proliferation and expanded for at least 15 PDs without obvious signs of senescence. Based on our data, EPCs we cultured were similar to late EPCs, which were first proposed by Hur *et al* (13).

The supernatant from glioma cells increases EPC tube formation on matrigel and migration. To explore the effect of the supernatant from glioma cells on angiogenic function of EPCs, we performed tube formation on matrigel and migration assay. In the absence of the supernatant from glioma cells, capillary morphogenesis of EPCs was clearly induced, compared with DMEM (40.7±3.3 versus 21±2.3, P<0.05) (Fig. 2A). In the migration assay, the OD values were 0.60±0.04 and 0.27±0.02, respectively for the supernatant from glioma cells and DMEM (P<0.05) (Fig. 2B). These results suggest that the supernatant from glioma cells augmented the ability of EPCs angiogenesis.

The supernatant from glioma cells up-regulates VEGFR-2 and MMP-9 expression of EPCs not VEGFR-1. In order to examine whether VEGFR-1 and VEGFR-2 are involved in EPC angiogenesis regulated by the supernatant from glioma cells, Western blot analysis was performed. A strong induction of VEGFR-2 protein was observed when EPCs were cultured with the supernatant from glioma cells. VEGFR-2 expression

significantly increased by 2.6±0.1-fold compared with the treatment of DMEM, while expression levels of VEGFR-1 were almost unchanged (Fig. 3). Additionally, we investigated the MMP-9 activity and expression of EPCs after the treatment of the supernatant from glioma cells by gelatin zymography and Western blot analysis. As shown in Fig. 3, the induction of MMP-9 expression was detected (2.5±0.2-fold) with the regulation of the supernatant from glioma cells. A similar change was observed in MMP-9 activity. These results indicated that the supernatant from glioma cells enhanced VEGFR-2 and MMP-9 expression of EPCs, not VEGFR-1.

Reduction of VEGFR-2 expression in EPCs by siRNA. Seventy-two hours after transfection, the cell lysates were analyzed by Western blot analysis for VEGFR-2 expression. As shown in Fig. 4, VEGFR-2 duplexes triggered a marked decrease in the protein levels for the target proteins in EPCs, with minimal effect on the expression of VEGFR-1 and GAPDH. These results indicated that VEGFR-2 siRNA effectively and specifically down-regulated VEGFR-2 protein expression in EPCs.

VEGFR-2 siRNA inhibits EPC tube formation on matrigel and the migration. To evaluate the impact of VEGFR-2 knock-down on EPC tube formation, we employed a matrigel tube formation assay using the VEGFR-2 siRNA transfected EPCs. When compared with the siRNAnc group and the no siRNA group, VEGFR-2 siRNA transfected cells lost the ability to form tubular networks on matrigel, failed to form tube-like networks (Fig. 5A), suggesting that EPCs tube formation on matrigel is regulated by the expression of VEGFR-2. As observed in the migration assays, we found that the migration capacity of the cells transfected with
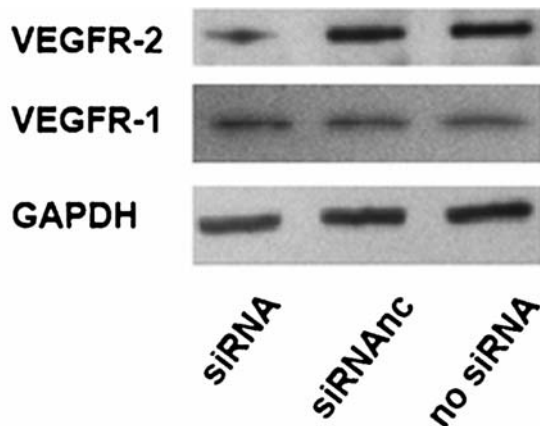
Figure 4. VEGFR-2 siRNA down-regulates expression of VEGFR-2 in EPCs. EPCs were treated with VEGFR-2 siRNA, siRNAnc or no siRNA as described in Materials and methods. VEGFR-2, VEGFR-1 and GAPDH expressions were determined by Western blot analysis. The quantitative data show relative protein levels of VEGFR-2, VEGFR-1 (normalized to GAPDH level) in the no siRNA group.

VEGFR-2 siRNA was significantly decreased compared with that of the cells transfected with control siRNA (Fig. 5B). The OD values were 0.24±0.06, 0.56±0.06 and 0.59±0.08, respectively for the VEGFR-2 siRNA, siRNAnc and no siRNA group (P<0.05). These experiments demonstrated that VEGFR-2 was required for EPC tube formation and migration.

VEGFR-2 siRNA inhibits MMP-9 protein expression and MMP-9 activity of EPCs. In order to explore the role of

VEGFR-2 in the MMP-9 activity and expression of EPCs, we used gelatin zymography and Western blot analysis, respectively. MMP-9 activity of VEGFR-2 siRNA was significantly decreased 72 h after transfection (Fig. 6A). Consistently, Western blot analysis demonstrated a pronounced reduction in MMP-9 protein expression as compared to the siRNAnc group and the no siRNA group, suggesting that decreased activity of MMP-9 in VEGFR-2 siRNA transfected cells is due to an inhibition of protein expression. Thus, VEGFR-2 appeared to be an important factor in the regulation of MMP-9 expression.

VEGFR-2 inhibits the Akt and ERK signaling pathways in EPCs. To investigate whether the Akt and ERK pathways were down-regulated by VEGFR-2 siRNA transfected EPCs, phosphorylations of Akt and ERK were measured (Fig. 6B). Activation of Akt by VEGFR-2 siRNA was significantly reduced by ~50% compared with the siRNAnc group and the no siRNA group, while almost a 30% reduction of phosphorylation of ERK was detected. No obvious changes were observed for total Akt and ERK protein expressions. The results indicated that the Akt and ERK pathways were major signaling pathways in VEGFR-2 regulation.

Discussion

In this study, we evaluated the function of glioma cells in EPC angiogenesis. Our data show that the supernatant from glioma cells can enhance EPC tube formation and migration via up-regulating VEGFR-2 expression, not VEGFR-1. Furthermore, VEGFR-2 siRNA silences endogenous VEGFR-2
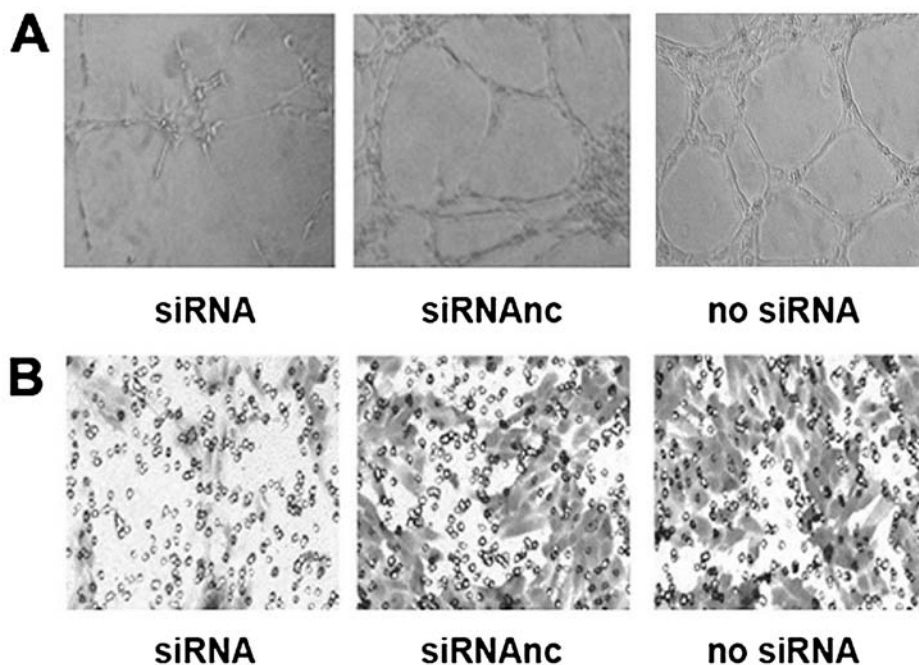

Figure 5. VEGFR-2 siRNA inhibits EPC tube formation on matrigel and migration of EPCs. (A), Tubes were visualized by phase contrast microscopy at a magnification of x200. VEGFR-2 siRNA transfected cells lost the ability to form tubular networks on matrigel, and the number of tube are 15.0±6.0 (P<0.05), compared with the siRNAnc group (45.7±4.9) and the no siRNA group (40.3±4.2). (B), Migrated cells were stained with crystal violet and examined with light microscopy at a magnification of x200. The OD values were 0.24±0.06, 0.56±0.06, 0.59±0.08, respectively for the VEGFR-2 siRNA, siRNAnc and no siRNA group (P<0.05).
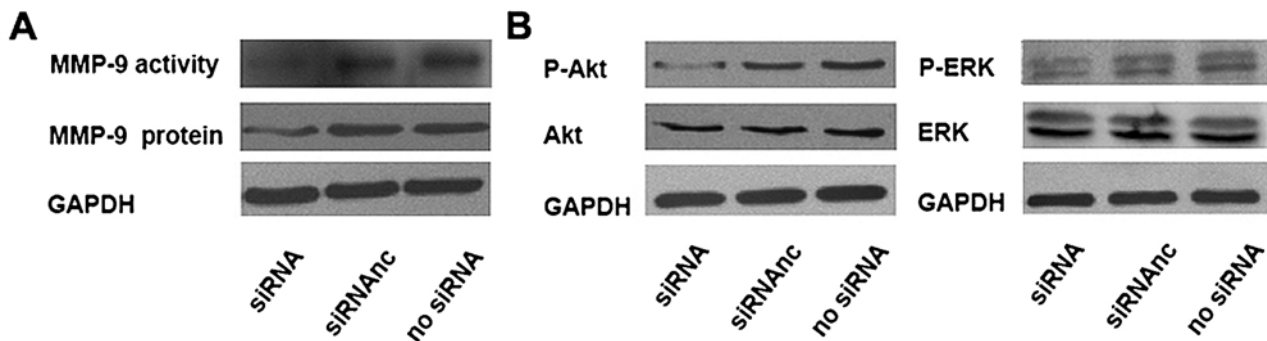
Figure 6. VEGFR-2 siRNA inhibits activity and expression of MMP-9 through Akt and ERK pathways in EPCs. (A), Cell lysates were evaluated for MMP-9 activity by zymography analysis and protein expression by Western blot analysis as described in Materials and methods. (B), Effects of VEGFR-2 siRNA on MMP-9, Akt, phospho-Akt, ERK and phospho-ERK expression were determined by Western blot analysis. The quantitative data show relative protein levels of MMP-9, Akt, phospho-Akt, ERK and phospho-ERK (normalized to GAPDH level) in the no siRNA group.

expression in EPCs, and inhibits EPC angiogenesis by down-regulating the MMP-9, Akt and ERK pathways.

Although a great deal is known about the mechanisms and key regulators of VEGFR-1 and VEGFR-2 involved in angiogenesis, reports on the effect of VEGFR-1 and VEGFR-2 on glioma-associated angiogenesis of EPCs are few. VEGFR-1 and VEGFR-2 are key proteins modulating vascular development during angiogenesis. VEGFR-1 has a structure very similar to VEGFR-2 and binds to VEGF with a higher affinity than VEGFR-2. The role of VEGFR-1 has been debated and its exact function is still unclear (9). On the contrary, VEGFR-2 is a major positive signal transducer and it shows a higher kinase activity than VEGFR-1. Based on analyses of protein level, our data reveal that the supernatant from glioma cells up-regulates EPC angiogenesis via induction of VEGFR-2 expression, while no significant change in VEGFR-1 protein expression was observed.

In order to investigate the further mechanism responsible for glioma cell contribution to EPCs angiogenesis, we use RNA interference method to block the endogenous VEGFR-2. Most of the studies on the involvement of VEGFR-2 during angiogenesis were either carried out in the presence of serum or growth factors, which are inducers of VEGFR-2, or in the presence of VEGFR-2 inhibitors. The use of natural or synthetic inhibitors against VEGFR-2 might not completely explain the role of VEGFR-2, as these inhibitors can also affect various other components of angiogenic process. This is evidenced by the report where SU5416 inhibited angiogenesis by binding to VEGFR-1, VEGFR-2 and EGF receptors (14). The advantages of RNA interference over other modalities include its high specificity (in some cases, a single point mutation can abolish silencing effect), versatility (interfering RNA can be designed against virtually any gene), and efficiency (in many cases, genes can be silenced by over 90%) (15,16). Thus, the relatively new technique of RNA interference with short double-stranded RNA molecules that are complementary to the target mRNA is a potentially powerful tool to silence endogenous VEGFR-2 and evaluate its function. We chose the small interfering RNAs technique to explore how VEGFR-2 exerts its activity in EPCs. VEGFR-2 protein expression is significantly decreased 72 h after transfection of VEGFR-2 siRNA. In contrast, non-silencing RNA duplexes have no effect on VEGFR-2 expression. Expression

levels of VEGFR-1 and GAPDH are almost unchanged in the two different siRNA groups, indicating that the transfection procedure has no effect on the expression of these genes. The functional consequences of silencing VEGFR-2 expression in EPCs through VEGFR-2 siRNA were investigated. EPCs with diminished VEGFR-2 expression are less angiogenic and migrative as compared to the siRNAnc and no siRNA group.

Moreover, in our study, the supernatant from glioma cells obviously enhances the MMP-9 activity and expression of EPCs. MMP-9, a key enzyme that functions to promote angiogenesis by regulating endothelial cell attachment, proliferation, migration and growth (17-19). MMP-9, which leads to the release of soluble KIT ligand, promotes the proliferation and motility within the bone marrow microenvironment, thereby laying the framework for EPC mobilization to the peripheral circulation, and homing to the vasculature (20,21). It is observed that reducing the expression of VEGFR-2 inhibits the activity and expression of MMP-9. Thus, our data indicate that VEGFR-2 siRNA inhibits EPCs angiogenesis via down-regulating the activity and expression of MMP-9.

Additionally, the Akt and ERK pathways have been documented to contribute to signal transduction pathways in regulation of VEGFR-2 and MMP-9 (12,22-24). Therefore, we hypothesize that VEGFR-2 is a key modulator of angiogenesis by modulating Akt and ERK pathways in EPCs. As is well-known, the Akt and ERK pathways are involved in angiogenesis. Akt, also known as protein kinase B (PKB), regulates essential cellular functions such as migration, proliferation, differentiation, apoptosis, and metabolism. In endothelial cells, the majority of growth factor-induced angiogenic responses are mediated by the activation of the Akt signaling cascade (25). In HUVEC angiogenesis, VEGFR-2 protein expression is associated with phosphorylation of Akt (24). Additionally, Hur *et al* (26) have reported that Akt plays a critical role in the homing of EPCs to the ischemic muscle through increased attachment and transendothelial migration, and its modulation enhances the homing of EPCs and new vessel formation in target organs. ERK is a member of the family of mitogen-activated protein kinases and has been demonstrated to play an important role in transmitting extracellular signals into various cellular

responses such as proliferation and migration. VEGFR-2 acts via the ERK pathway which is known to lead to endothelial cell modulation (27,28). Consistent with these premises, VEGFR-2 siRNA blocks both the Akt and ERK pathways, although inhibition of other pathways by VEGFR-2 siRNA cannot be ruled out. Our data strongly show that the Akt and ERK pathways are both involved in VEGFR-2 signal transduction, however, the further mechanism is still under investigation.

In summary, we have demonstrated that glioma cells enhance EPCs angiogenesis via VEGFR-2, not VEGFR-1. Further, VEGFR-2 siRNA effectively and specifically reduces the expression of VEGFR-2 in EPCs. Down-regulation of VEGFR-2 inhibits the MMP-9, Akt and ERK pathways in EPC angiogenesis. Therefore, the results of the present study suggest that the modulation of the mechanism responsible for EPC-mediated glioma angiogenesis may be used as a therapeutic strategy to affect pathological angiogenesis and thus warrants further investigation.

Acknowledgements

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