VEGF treatment promotes bone marrow-derived CXCR4+ mesenchymal stromal stem cell differentiation into vessel endothelial cells

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Abstract. Stem/progenitor cells serve an important role in the process of blood vessel repair. However, the mechanism of vascular repair mediated by C-X-C chemokine receptor type 4-positive (CXCR4+) bone marrow-derived mesenchymal stem cells (BMSCs) following myocardial infarction remains unclear. The aim of the present study was to investigate the effects of vascular endothelial growth factor (VEGF) on vessel endothelial differentiation from BMSCs. CXCR4+ BMSCs were isolated from the femoral bone marrow of 2-month-old mice and the cells were treated with VEGF. Expression of endothelial cell markers and the functional properties were assessed by reverse transcription-quantitative polymerase chain reaction, flow cytometry and vascular formation analyses. The results indicated that the CXCR4+ BMSCs from femoral bone marrow cells expressed putative cell surface markers of mesenchymal stem cells. Treatment with VEGF induced platelet/endothelial cell adhesion molecule-1 (PECAM-1) and von Willebrand factor (vWF) expression at the transcriptional and translational levels, compared with untreated controls. Moreover, VEGF treatment induced CXCR4+ BMSCs to form hollow tube-like structures on Matrigel, suggesting that the differentiated endothelial cells had the functional properties of blood vessels. The results demonstrate that the CXCR4+ BMSCs were able to differentiate into vessel endothelial cells following VEGF treatment. For cell transplantation in vascular disease, it may be concluded that CXCR4+ BMSCs are a novel source of endothelial progenitor cells with high potential for application in vascular repair.

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Introduction

Myocardial infarction is a leading cause of heart failure and mortality in developed countries. In recent years, bone marrow-derived mesenchymal stem cells (BMSCs) and endothelial progenitor cells (EPCs) have been reported to stimulate angiogenesis and collateral vessel formation in models of myocardial infarction and limb ischemia (1-3).

Williams and Hare reported that BMSCs and EPCs are able to integrate into vascular structures and differentiate into blood endothelial cells to participate in angiogenesis and tissue repair following ischemic injury (4). This may be favorable for physiological function and improve the patient survival rate (4), suggesting that BMSCs and EPCs serve an important role in angiogenesis (5).

Previous studies have found that the stem/progenitor cells can be recruited to areas of myocardial ischemia for vascular repair and this process may require chemokine and chemokine receptors (6,7). Chemokine receptor-expressing cells directly respond to chemokine stimulation, and are recruited to areas of injury or inflammation (8). The movement of chemotactic recruitment has been shown to be associated with the chemokine concentration (9). When myocardial infarction occurs, the transcription of stromal cell-derived factor (SDF-1) is significantly increased in the ischemic tissue. SDF-1 activates the expression of C-X-C chemokine receptor type 4-positive (CXCR4+) BMSCs to the myocardial infarction area, and their involvement in vessel repair and/or angiogenesis processes following myocardial infarction (10,11).

Studies have indicated that BMSCs expressing CXCR4 are able to promote myocardial repair when administered by cell transplantation following myocardial infarction (12,13). In the present study, the mechanisms by which CXCR4+ BMSCs differentiate into endothelial cells *in vitro* were investigated. The results may provide new insights providing a fundamental basis for the therapy of myocardial infarction.

Materials and methods

Antibodies and reagents. Anti-CXCR4 (cat no. sc-9046), anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1;

cat. no. sc-52713) and anti-von Willebrand factor (vWF; cat. no. sc-8068) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The secondary goat anti-mouse immunoglobulin (Ig) G (SA00007-1), goat anti-rabbit IgG (SA00007-2) and rabbit anti-goat IgG (SA00007-4) antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA). Matrigel was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) while cluster of differentiation (CD) 117 (cat. no. 553355), CD54 (cat no. 561605), Flt-1, also known as vascular endothelial growth factor receptor 1 (VEGFR-1; cat. no. 561252) and CD107 (cat. no. 641581) antibodies were purchased from BD Pharmingen™, BD Biosciences (San Diego, CA, USA).

Isolation and culture of mouse CXCR4+ BMSCs. Six 2-month-old C57/BL6 female mice were obtained from Shanghai SLAC laboratory Animal Co., Ltd. (Shanghai, China) and allowed to acclimate for 1 week. Mice were housed at 20-22˚C and 50-60% humidity with a 12:12-h light-dark cycle and *ad libitum* access to rodent chow and tap water. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Zhejiang University (Zhejiang, China). BMSCs were isolated from the tibia and femur of the mice and $2x10^5$ cells/well were seeded in wells pretreated with 0.5% gelatin (cat. no. 9963; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Cells were then incubated with Dulbecco's modified Eagle's medium (DMEM; cat. no. 11995065, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; cat. no. 10082147, Gibco; Thermo Fisher Scientific, Inc.) at 5% CO_2, 37˚C and 100% humidity. The culture medium was changed to remove suspended cells after 24 h. Following this, the medium was changed every 3 days. The cells were harvested by digestion when the BMSCs were passaged to the third generation. The cells were washed with PBS and then incubated with FBS containing CXCR4 antibody (10 μg/ml). Following incubation with anti-rabbit IgG magnetic beads (cat. no. 11203D, Thermo Fisher Scientific, Inc.), CXCR4+ cells were isolated using the DynaMag™-15 Magnet (cat. no. 12301D, Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and the cell surface markers CD117, CD54, VEGFR-1 and CD107 were analyzed by flow cytometry using the BD FACS Calibur™ Operator (cat no. 337662, BD Biosciences) (14). The purity of the CXCR4+ BMSCs was ≥95%.

Gene expression analysis. The sixth generation of CXCR4+ BMSCs ($1x10^6$) were treated with 0, 10 or 20 ng/ml VEGF (cat no. 19003, Proteintech Group, Inc.) for 24 h. There were three replicates per group. Total RNA was isolated from these cells using Trizol Reagent® (Invitrogen, Thermo Fisher Scientific, Inc.) and reverse transcribed using the SuperScript® III First-Strand Synthesis system for RT-PCR (cat. no. 18080-051, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The cDNA was amplified by quantitative PCR using the SYBR®-Green PCR Master mix (cat no. 4309155; Thermo Fisher Scientific, Inc.) using a CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences used were as follows: PECAM-1 forward, 5'-GAGAAGAGCAGCCGATTCCT-3' and reverse, 5'-AACCTCCTTTCACCCCCC-3'; vWF forward, 5'-TGTACCATGAGGTTCTCAATGC-3' and reverse, 5'-TTATTGTGGGCTCAGAAGGG-3'; GAPDH forward, 5'-CCAATCAGCTTGGGCTAGAG-3' and reverse, 5'-CCTGGGAAAGGTGTCCTGTA-3'. The cycling conditions were as follows: Initial denaturation at 95˚C for 20 sec, 40 cycles amplication at 95˚C for 3 sec and 60˚C for 30 sec. All quantifications were normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method (15).

Detection of PECAM-1 and vWF expression levels. CXCR4+ BMSCs were treated with 0, 10 or 20 ng/ml VEGF for 24 h, as described in the aforementioned paragraph. The cells were then collected and incubated with anti-PECAM-1 (1:100) and anti-vWF (1:100) antibodies for 1 h at room temperature, and subsequently incubated with the secondary goat anti-mouse IgG (1:150) and goat anti-rabbit IgG (1:200) antibodies for 1 h at room temperature. The isotype antibodies, normal rat IgG2a (1:150, cat. no. sc-3883) and normal goat IgG (1:150, cat no. sc-3887), were purchased from Santa Cruz Biotechnology, Inc., and used as controls. The levels of PECAM-1 and vWF expression were analyzed by fluorescence-activated cell sorting (FACS) following a previously described protocol (14).

Capillary formation analysis. The procedure followed was as previously described (16). Firstly, 200 μl Matrigel (5 mg/ml) was added to 24-well plates, which were incubated at 37˚C for 30 min for 1 h for polymerization. CXCR4+ BMSCs (400 μl, $3x10^4$ cells/ml) were then added to the Matrigel in the wells. Approximately $1.2x10^4$ cells were pre-treated with 20 ng/ml VEGF in the culture medium containing 1% FBS for 1 h. Following incubation of the cells for 3 and 6 h, angiogenesis phenomena were monitored under a light microscope and the length and size of tube-like structures were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experimental data are reported as the mean ± standard deviation. Statistical analyses were performed with a two-tailed unpaired t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of CXCR4+ BMSCs. CXCR4+ BMSCs isolated from BMSCs exhibited diverse morphology, for example, a round, spindle, long-spindle and spiral appearance (Fig. 1A). CXCR4+ BMSC-specific markers, namely CD117, CD54, Flt-1 (VEGFR-1) and CD107, were detected by FACS (Fig. 1B).

VEGF induces PECAM1 and vWF expression in CXCR4+ BMSCs. PECAM-1 is a glycoprotein, expressed on endothelial cells, which can induce cell-cell adhesion in a calcium-independent manner. PECAM-1 is considered as a specific marker of mature endothelial cells (17) and vWF is a glycoprotein subunit of endothelial cells, which promotes platelet cell adhesion (18). Treatment with 10 and 20 ng/ml VEGF significantly upregulated the expression level of PECAM-1 in CXCR4+ BMSCs (11.9±3.5 and 25.5±9.4-fold, respectively; $P<0.05$) compared with that of the control group, and the induction

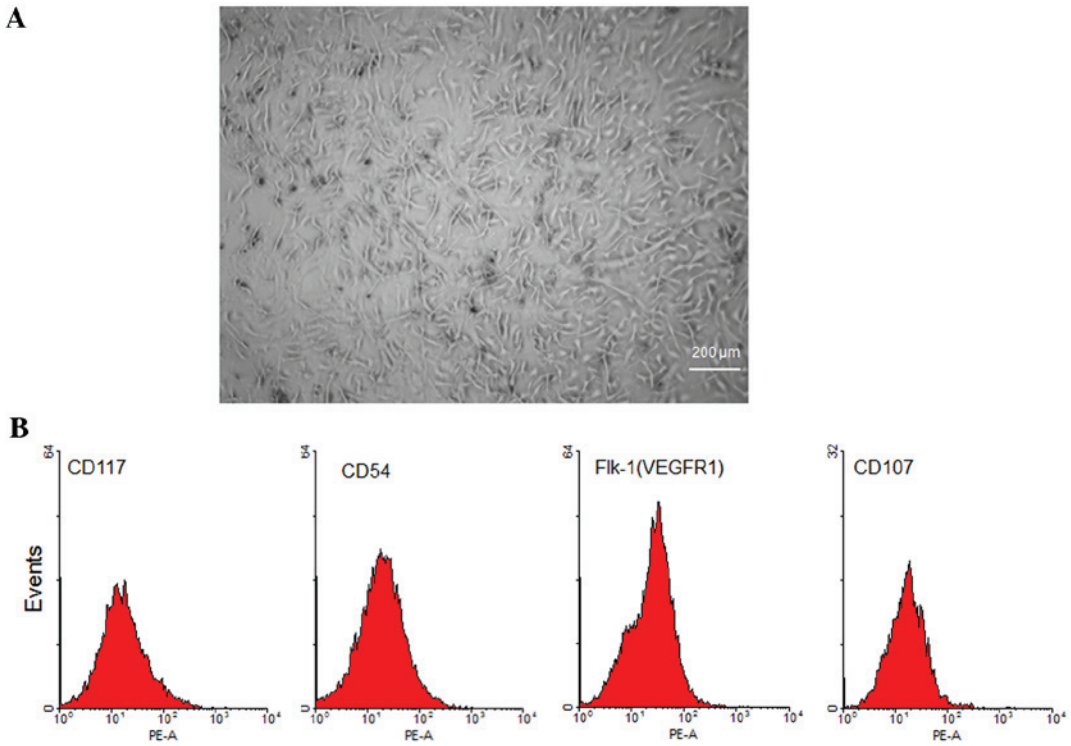

Figure 1. Characterization of CXCR4+ BMSCs. (A) CXCR4+ BMSC morphology (scale bar=200 μm, magnification, x10). (B) Analysis of CXCR4+ BMSC-specific cell markers by fluorescence-activated cell sorting analysis. CXCR4+ BMSCs, C-X-C chemokine receptor type 4-positive bone marrow-derived mesenchymal stem cells; CD, cluster of differentiation; VEGFR-1, vascular endothelial growth factor receptor 1.

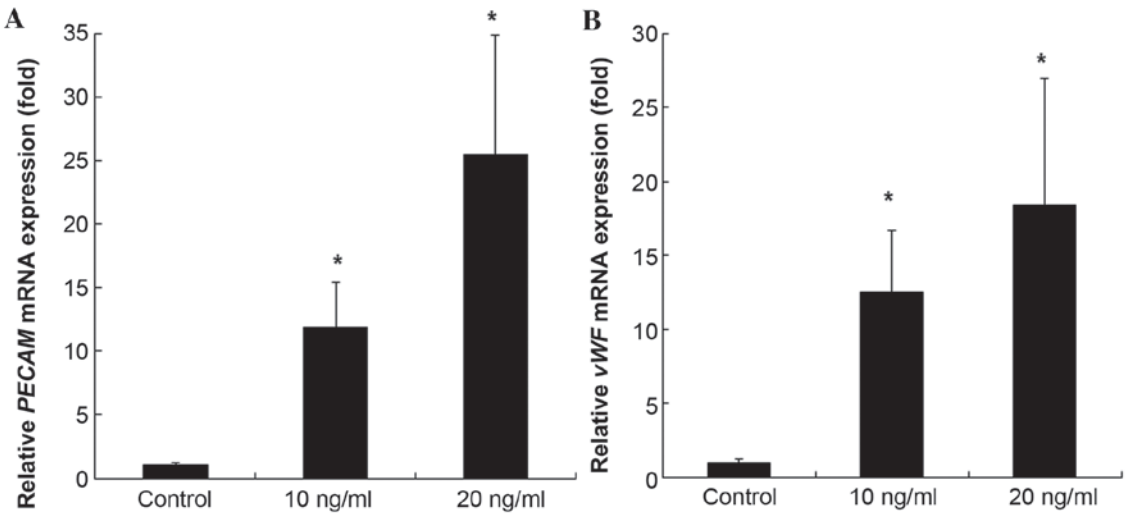

Figure 2. Expression levels of PECAM-1 and vWF mRNA induced by vascular endothelial growth factor in CXCR4+ BMSCs. (A) PECAM-1 and (B) vWF mRNA expression levels in the CXCR4+ BMSCs. *P<0.05 vs. the control group. PECAM-1, platelet/endothelial cell adhesion molecule-1; vWF, von Willebrand factor; CXCR4+ BMSCs, C-X-C chemokine receptor type 4-positive bone marrow-derived mesenchymal stem cells.

occurred in a concentration-dependent manner (Fig. 2A). The vWF transcription level was also increased (12.5±4.2 and 18.4±8.6-fold, respectively; P<0.05) compared with that of the control group (Fig. 2B). These results indicate that VEGF treatment induced the expression of PECAM-1 and vWF in CXCR4+ BMSCs.

Protein levels in CXCR4+ BMSCs analyzed by FACS. FACS results showed that 10 and 20 ng/ml VEGF stimulation increased PECAM-1 levels in CXCR4+ BMSCs (8.32±2.54 and 17.86±3.86%) compared with those in the control group after 24 h (P<0.05; Fig. 3A). As shown in Fig. 3B, the vWF levels in CXCR4+ BMSCs were increased (10.45±2.58 and 25.56±7.98%; P<0.05) compared with those in the control group following stimulation with 10 and 20 ng/ml VEGF, respectively. These results indicate that VEGF stimulation is able to increase the levels of PECAM-1 and vWF in CXCR4+ BMSCs.

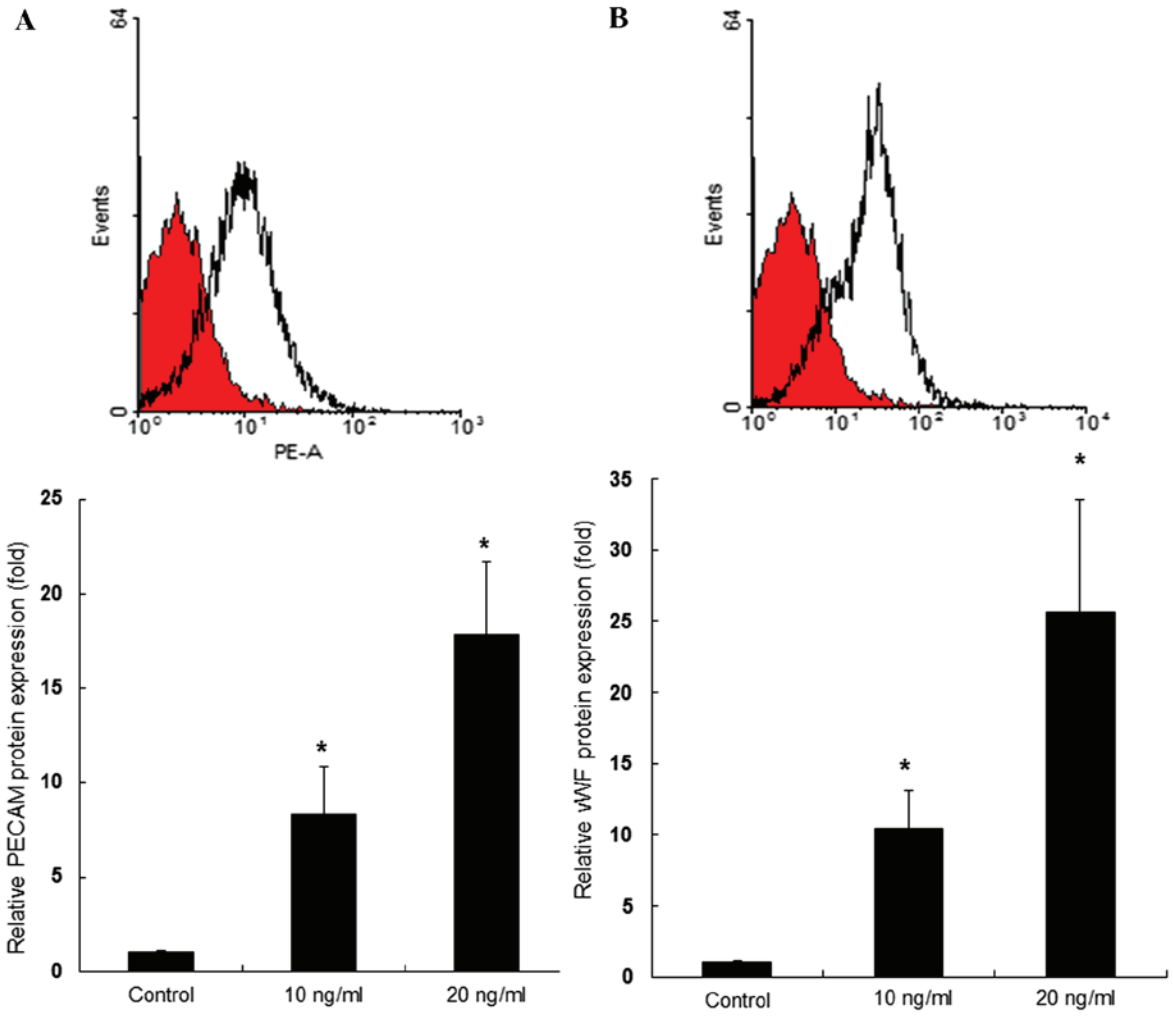

Figure 3. VEGF-mediated alteration of PECAM-1 and vWF levels in CXCR4+ BMSCs. (A) PECAM-1 and (B) vWF protein levels were analyzed by fluorescence-activated cell sorting and quantified in the control and VEGF-stimulated CXCR4+ BMSCs. *P<0.05 vs. the control group. VEGF, vascular endothelial growth factor; PECAM-1, platelet/endothelial cell adhesion molecule-1; vWF, von Willebrand factor; CXCR4+ BMSCs, C-X-C chemokine receptor type 4-positive bone marrow-derived mesenchymal stem cells.

CXCR4+ BMSCs trigger the generation of vessel tube-like structures. Endothelial cells possess the ability to form vessel tube-like structures (19). CXCR4+ BMSCs were treated with VEGF for 1 h prior to being grown on Matrigel for 12 h, and then the morphology of the CXCR4+ BMSCs was observed under a light microscope. Some of the CXCR4+ BMSCs formed net- and tube-like structures (Fig. 4A). The tube length was increased to 1.54±0.24 and 1.87±0.34 mm/mm^2 for the CXCR4+ BMSCs stimulated with 10 and 20 ng/ml VEGF, respectively, which were higher than the tube length in the control group (0.46±0.14 mm/mm^2). Compared with the control group, 20 ng/ml VEGF treatment increased the total tube length by up to 5-fold on average (P<0.05; Fig. 4B). These results indicate that CXCR4+ BMSCs promote the formation of capillary tube-like structures on Matrigel under VEGF stimulation.

Discussion

In the present study, it was identified that stem cell markers were expressed in CXCR4+ BMSCs and the mature endothelial cell markers PECAM-1 and vWF were induced following VEGF stimulation of the cells. PECAM-1, a glycoprotein of endothelial cells regarded as a specific marker of mature endothelial cells, is able to undergo intercellular adhesion in a non-calcium-dependent manner (17). vWF, a multimeric glycoprotein involved in endothelial cell-matrix interactions, is able to promote platelet adhesion (18). In the present study, it was found that VEGF stimulation triggered the formation of pseudopodia and tube-like structures by the CXCR4+ BMSCs on Matrigel. The formation of pseudopodia may be responsible for initiating the formation of vascular structures. These findings suggest that CXCR4+ BMSCs can differentiate into mature endothelial cells following stimulation with VEGF and could potentially be used as a source for cell transplantation. In addition, the observation that CXCR4+ BMSCs are capable of forming vessel-like structures, suggests that CXCR4+ BMSCs may act as endothelial progenitor cells and serve an important role in angiogenesis, with potential for use in the treatment of diseases of associated with vasculature, and vascular engineering and repair.

The goal of stem cell biology is to clinically utilize these cells for therapy. There is accumulating evidence that

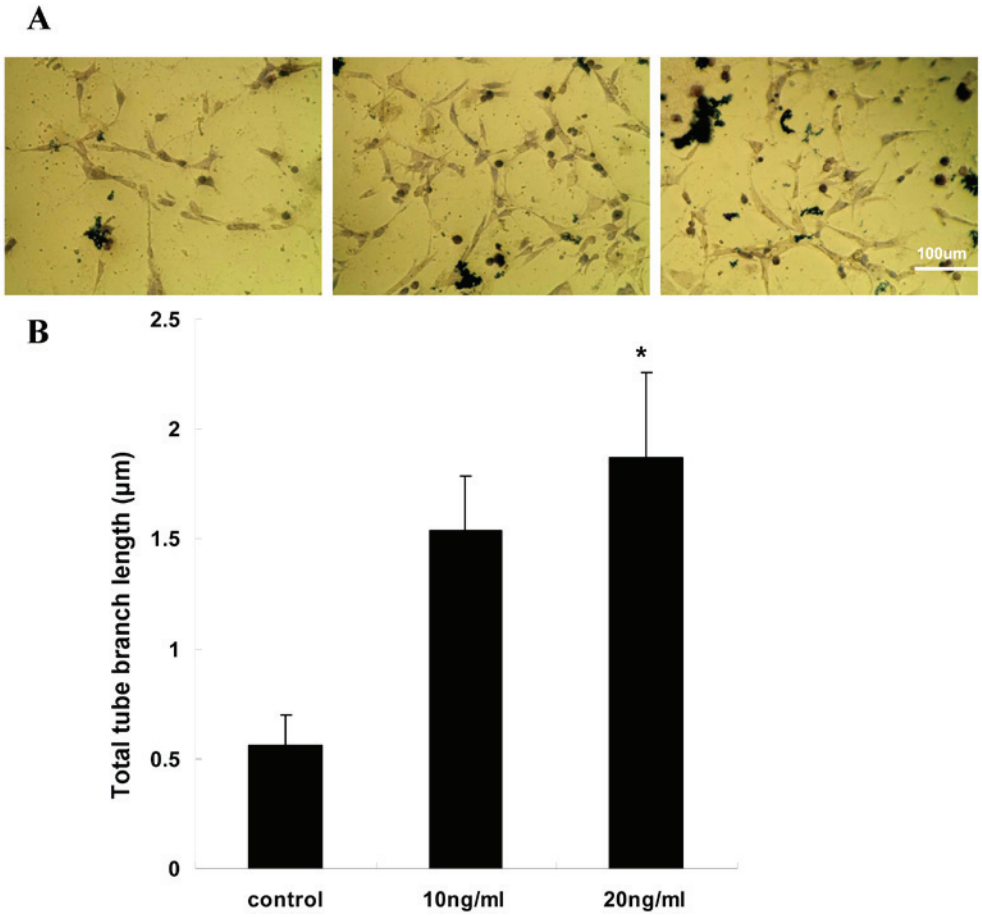

Figure 4. VEGF stimulation induces the formation of tube-like structures by CXCR4+ BMSCs. (A) CXCR4+ BMSC morphologies were observed after 12 h of growth on Matrigel with indicated concentrations of VEGF stimulus for 1 h. (magnification, x400). (B) Capillary tube-like structure formation (length) was semi-quantitatively analyzed. *P<0.05 vs. the control group. VEGF, vascular endothelial growth factor; CXCR4+ BMSCs, C-X-C chemokine receptor type 4-positive bone marrow-derived mesenchymal stem cells.

progenitor cells have important roles in vascular development, hematopoiesis and reconstruction, bone marrow generation and blood circulation (20-22).

CXCR4+ mesenchymal stem cells (MSCs) possess the ability to differentiate into multiple types of cells, including osteocyte, adipose and endothelial cells (23,24). Previous studies reported that somatic cell and vascular endothelial progenitor cells are important for new vascular formation following vascular damage (25,26); for example, shortly after injury, endothelial progenitor cells derived from bone marrow have been found to promote the re-endothelialization of denuded arteries, and are able to repair the vasculature (27,28). Therefore, progenitor cells from bone marrow or circulating blood provide potential novel therapeutic techniques for certain diseases, such as the treatment of atherosclerosis and vascular stenosis, and the prevention of graft failure and cerebrovascular diseases (29). Stem/progenitor cell migration may be associated with the induction and effects of cytokines. Chemotactic factor SDF-1 interacts with its receptor CXCR4 and serves an important role in progenitor cell migration and differentiation (10,30,31). Previous studies have shown that tissue injury such as hypoxia and ischemia induces the production of a high amount of SDF-1 and activates CXCR4+ in endothelial progenitor cells (32,33), which is important for the generation of a SDF-1/CXCR4 interaction, cell mobilization and homing, and ischemia tissue repair. Shiba *et al* (34) and Seeger *et al* (35) reported that CXCR4+ MSCs enhanced therapeutic angiogenesis in cardiovascular disease and have increased potential for use in therapy.

In the present study, CXCR4+ BMSCs were isolated and it was identified that these cells express greater amounts of PECAM-1 and vWF proteins following VEGF stimulation. In addition, VEGF stimulation triggered the formation of tube-like structures from CXCR4+ BMSCs on Matrigel. These results indicate that CXCR4+ BMSCs have endothelial cell features and are able to differentiate into endothelial-like cells under appropriate induction conditions. Therefore, CXCR4 can be used as a selection marker for endothelial progenitor cells and MSCs, which is useful for obtaining highly pure endothelial progenitor cells. In conclusion, a method for the production of endothelial progenitor cells was established, in the present study and direct evidence of the differentiation and activation of BMSCs was provided, with potential use as a human stem cell-based targeted therapy.

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