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Full Length Research Paper

Increased vascular endothelial growth factor (VEGF) expression in rats with spinal cord injury by transplantation of bone marrow stromal cells

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Vascular endothelial growth factor (VEGF), a well known angiogenic factor, has been shown to have direct and/or indirect influence on spinal cord injury (SCI). The purpose of this study is to observe VEGF expression changes in rats with SCI by bone marrow stromal cells (BMSCs) treatment. The mRNA expression of VEGF in rats was analyzed by semi-quantitative RT- PCR and the protein expression level was quantified by means of western blot and immunohistochemistry technology. It was found that VEGF was significantly up-regulated in BMSCs treatment group in comparison to the sham group and DMEM group on mRNA level. On protein level, VEGF was also highly increased in BMSCs treatment group, which reached the highest level after 14 days treatment. The present study suggests a potential local role for VEGF as mediators of SCI which might provide a certain reasonable clue to clarify molecular mechanisms of BMSCs treatment in SCI.

Key words: Vascular endothelial growth factor (VEGF), spinal cord injury, bone marrow stromal cells, reverse transcription-polymerase chain reaction (RT-PCR), western blot, immunohistochemistry.

INTRODUCTION

Each year, many people worldwide suffer from spinal cord injury (SCI). These injuries cause death of neural cells, severance and demyelination of descending and ascending axons, and consequently, loss of motor and sensory function. Endogenous repair efforts fail to repair the spinal cord and, as a result, the functional impairments are permanent (Wrigley et al., 2009; Wollaars et al., 2007). Potential treatments for SCI are being tested in the clinic but so far, none of these have emerged as one that reverses the devastating functional consequences of SCI, however, several studied demonstrated that bone marrow stromal cells (BMSCs) may also have therapeutic promise for SCI (Shields et al., 2010; Bunge., 2008). Survival, migration and differentiation of BMSCs after transplantation have been widely researched in recent years (Shields et al., 2010; Bunge, 2008; Someya et al., 2008). The underlying molecular mechanisms of repairing injured spinal cord and improving functional recovery with BMSCs have not, however, been largely understood. Better understanding of the molecular mechanism of BMSCs treatment for SCI may contribute significantly to the development of BMSCs reagents as well as help in

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Abbreviations: SCI, Spinal cord injury; **BMSCs,** bone marrow stromal cells; **DMEM,** Dulbecco's modified Eagle's medium; **FBS,** fetal bovine serum; **RT,** room temperature; **DEPC,** diethylpyrocarbonate; **VEGF,** vascular endothelial growth factor; **PCR,** polymerase chain reaction; **IDV,** integrated densities value; **EDTA,** ethylenediaminetetraacetic acid; **DAB,** diaminobenzidine; **COX-2,** cyclooxygenase-2.

SCI control. Consequently, it is of vital importance to define the molecular mechanisms of BMSCs treatment for successful treatment of SCI.

Angiogenesis plays an important role in increasing blood flow at the lesioned site, where hypoxia occurs after SCI and is an essential component of nerve regeneration across a gap of injured spinal cord (Carmeliet et al., 2002). Similarly, neo-angiogenesis is also essential for trauma repair, which requires nutrients and oxygen to overcome hypoxia (Carmeliet et al., 2002). It has been reported that SCI may induce angiogenesis (Casella et al., 2002), however, under normal circumstances after SCI, the angiogenesis of the injured spinal cord is insufficient to support the spinal cord plasticity required for functional recovery. Recent data from the medical literature have also shown a positive correlation between the density of blood vessels and functional improvement following SCI (Blight., 1991; Widenfalk et al., 2003). These findings suggest that stimulating angiogenesis may improve recovery of function after SCI. BMSCs have the ability to stimulate angiogenesis, which had been reported in recent medical literature (Tang et al., 2006; Chen et al., 2003; Kayo et al., 2005). BMSC may promote angiogenesis through VEGF expression in the spinal cord, according to the results of the present study, which may contributes to shed more light on the molecular mechanism of BMSCs treatment in SCI.

In this study, we investigated whether BMSCs transplantation could induce the expression of VEGF in the injured spinal cord and provided a certain reasonable clue that shed more light on the molecular mechanisms of BMSCs treatment in SCI.

MATERIALS AND METHODS

The adult male Wistar rats (12 weeks, 250 to 300 g) were purchased from the Center for Experimental Animals of China Medical University. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Culture and identification of MSCs

BMSCs were isolated from rat bone marrow and cultured as described by previous literature (Yano et al., 2005). Briefly, fresh whole bone marrow was harvested aseptically from tibias and femurs by 5 ml syringe needle. The marrow was extruded with 5 ml of Dulbecco's modified Eagle's medium (DMEM)-low glucose medium. Bone marrow was mechanically dissociated to obtain a homogeneous cell suspension. The cells were placed in a 25 cm^2 plastic flask for cell culture with 5 ml DMEM-low glucose medium containing 10% fetal bovine serum (FBS). The cells were incubated at 37°C in 95% humidity and 5% CO_2 for 24 h. At this time, non-adherent cells were removed by replacing the medium. The culture medium was replaced three times a week. After the cultures have reached 80 ~ 90% confluency, they were lifted by incubation in a solution containing 0.25% trypsin for 5 min at 37°C for passage. Cultured BMSCs were indentified with antibodies against CD34 and CD44 according to previous literature (Yano et al., 2005).

Spinal cord injury model preparation and intramedullary spinal cord injection

The adult male Wistar rats (12 weeks, 250 to 300 g) were anesthetized with pentobarbital and laminectomies were performed at the T8 to T10 level. Spinal cords were injured using the weight drop technique according to Allen's method (Allen, 1914) with a slight modification. A plastic impounder (2-mm diameter) was placed gently on the exposed dura and a 10-giron weight was dropped from a height of 10 cm onto the impounder. The weight and impounder were immediately removed after impact and paravertebral muscle and skin were closed. Rats were randomly divided into three groups: Sham group, DMEM group and BMSCs group.

Thirty minutes after injury, rats received four injections of a suspension of BMSCs (passage 3 ~ 4 cells, 75000 in 3μl of DMEM each) into peri-lesion area of the exposed spinal cord (BMSCs group). Two injections were applied 1 mm rostral and two injections 1 mm caudal to the lesion epicenter, and one injection at each side 0.5 mm lateral to the cord midline. The microneedler was carefully inserted from the dorsal surface 1.75, 1.25 and 0.75 mm deep into the spinal cord. Each rat received a total of 3.0×10^5 BMSCs. In DMEM group, the rats received four injections of an equal volume of DMEM alone at the same position and depth. Sham controls underwent the same operations but without spinal cord insult. Rats used in this study were treated strictly according to the NIH Guide for Care and Use of Laboratory Animals.

RNA preparation and semi-quantitative RT-PCR

Total RNA was isolated from frozen spinal tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol and as described in the online supplement. In short, the spinal tissue was ground with mortar and pestle, cooled by liquid nitrogen of the ground tissue, and 100 mg was incubated with 1 ml TRIzol for 5 min at room temperature (RT). Cell debris was removed by centrifugation (12,000 ×g at 4°C for 10 min) and 0.4 ml chloroform was added. After vortexing, the mix was incubated for 5 min at RT. The phases were separated by centrifugation (12,000 ×g at 4°C for 15 min) and the aqueous phase was transferred to a new tube. 0.6× volume of isopropyl alcohol and a 0.1× volume of 3 M sodium acetate were added to this aqueous phase and incubated for 10 min at 4°C. The precipitated RNA was pelleted by centrifugation (12,000 ×g at 4°C for 15 min) and after the removal of the supernatant, the RNA pellet was washed twice with 70% ethanol. After drying, the RNA was resuspended in 30 μl diethylpyrocarbonate (DEPC)-treated water. The quality and quantity of the RNA was verified by the presence of two discrete electropherogram peaks corresponding to the 28S and 18S rRNA at a ratio approaching 2:1. Using mRNA as template, single-stranded cDNAs were generated by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's directions. The vascular endothelial growth factor (VEGF) primer sequences were as follows: Sense prime: 5′-GAGTATATCT TCAAGCCGTCCTGT-3′; anti-sense prime: 5′- ATCTGCATAG TGACGTTGCTCTC -3′. GAPDH (Applied Biosystems) served as the internal control. The polymerase chain reaction (PCR) conditions were 94°C for 3 min, followed by 30 cycles of DNA amplification (45 s at 94°C, 1 min at 61°C and 1 min 30 s at 72°C) and 8 min incubation at 72°C. PCR products were separated by electrophoresis at a constant voltage (2 V/cm) in a 1.2% (w/v) agarose gel. Images were captured using a Gel Print 2000i/VGA (Bio Image), and the integrated densities value (IDV) was analyzed with computerized image analysis system (Motic Images Advanced 3.2). All DNA manipulations were performed as described by Sambrook and Russell, (2001).

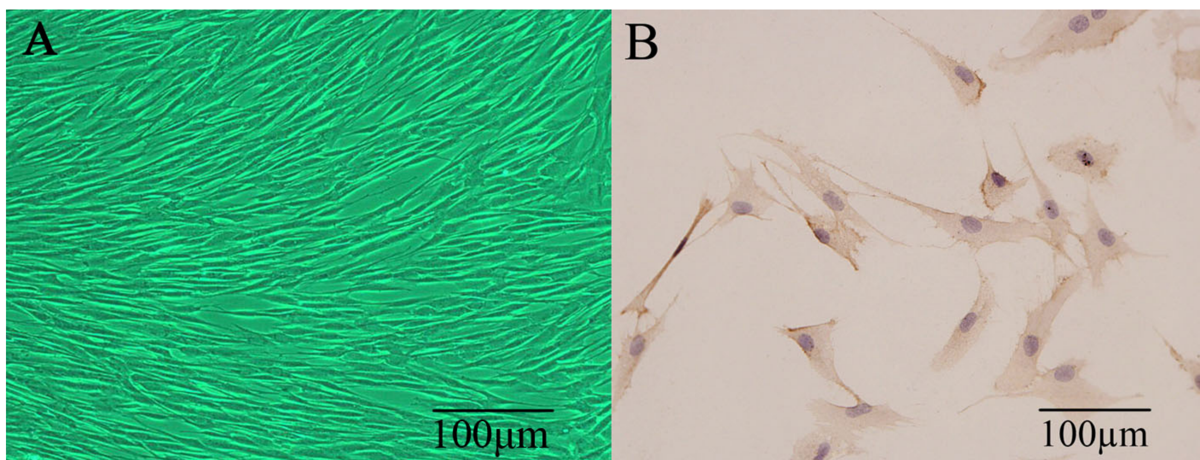

Figure 1. Bone marrow stromal cells, culture and detect. A, Third passage of bone marrow mesenchymal stem cells was observed by inverted phase contrast microscope; B, the expression of CD44 in bone marrow stromal cells was observed by phase contrast the inverted microscope

Western blot

In order to detect protein expression level of VEGF, western blot was performed as described in the online supplement. The spinal cord tissues were obtained from the peri-lesion region at days 3, 7 and 14 post-surgery. Protein homogenates of spinal cord samples were prepared by rapid homogenization in 10 volumes of lysis buffer (2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethylene glycol tetraacetic acid (EGTA), 0.4% NaF, 20 mM Tris-HCl, pH7.5). Tissue homogenate were centrifuged at 17,000 xg for 1 h at 4℃ and the protein concentration in the supernatant was determined by the Coomassie (G250) binding method. Equal amounts of protein (20 μg) from each sample were loaded and separated into a 4 to 7.5% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Electroblotting proteins were transferred onto nitrocellulose membranes (Santa Cruz Biotechnology, Inc). After blocking with 5% nonfat dry milk overnight at 4℃, membranes were incubated for 2 h at room temperature in agitation with the following antibodies: rabbit polyclonal anti-VEGF (dilution 1:400; Santa Cruz Biotechnology, Inc), and rabbit polyclonal anti-β-actin (dilution 1:400; Santa Cruz Biotechnology, Inc). Secondary horseradish peroxidase conjugated rabbit anti-goat/goat anti-rabbit antibodies (Santa Cruz Biotechnology, Inc) were used at 1:5000 dilution for 2 h at room temperature in agitation. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology, Inc) and scanned using Chemi Imager 5500 V2.03 software. The integrated densities value (IDV) was analyzed with computerized image analysis system (Fluor Chen 2.0) and normalized with that of β-actin.

Immunohistochemistry

To detect expression and localization of VEGF in spinal cord tissue, immunohistochemistry was performed. The cryostat sections were thawed, air-dried and then blocked with goat serum for 30 min at room temperature, and then incubated with rabbit polyclonal anti-VEGF antibody (dilution 1:150, Santa Cruz Biotechnology, USA) at 4℃ over night. Following reaction with primary antibodies, the sections were stained using the ABC Kit (Santa Cruz Biotechnology, USA), and the color was developed with diaminobenzidine (DAB). Negative controls were conducted by exchange of primary antibody for phosphate buffered saline (PBS).

Statistics analysis

To calculate the statistical differences among the sham, DMEM and BMSCs groups, the statistical package SPSS13.0 (SPSS Incorporated, USA) was used for all analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test were utilized to determine the significant difference among multiple groups. Student's *t* test was used to determine the significance of differences between the groups. All values were expressed as mean ±SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

BMSC cell culture and detection

Most cells suspended in culture medium, non-adherent cells were removed by replacing the medium. Adherent cells began to proliferate and form cell colony at about 72 h later. Cells surrounded the cell colony center to distribute toward circumference. Cell morphology was homogeneous, which appeared spindle-shaped with serial sub-cultivation (Figure 1A). Most cultured adherent passage 3 cells expressed CD44 (Figure 1B). In contrast, a majority of adherent passage 3 cells were negative for CD34.

Semi-quantitative RT-PCR analysis of VEGF expression

In order to detect the mRNA expression of VEGF in rat with SCI by BMSC treatment, reverse transcription PCR was conducted. As shown in Figure 2, VEGF mRNA levels in BMSCs group were significantly increased at 1,

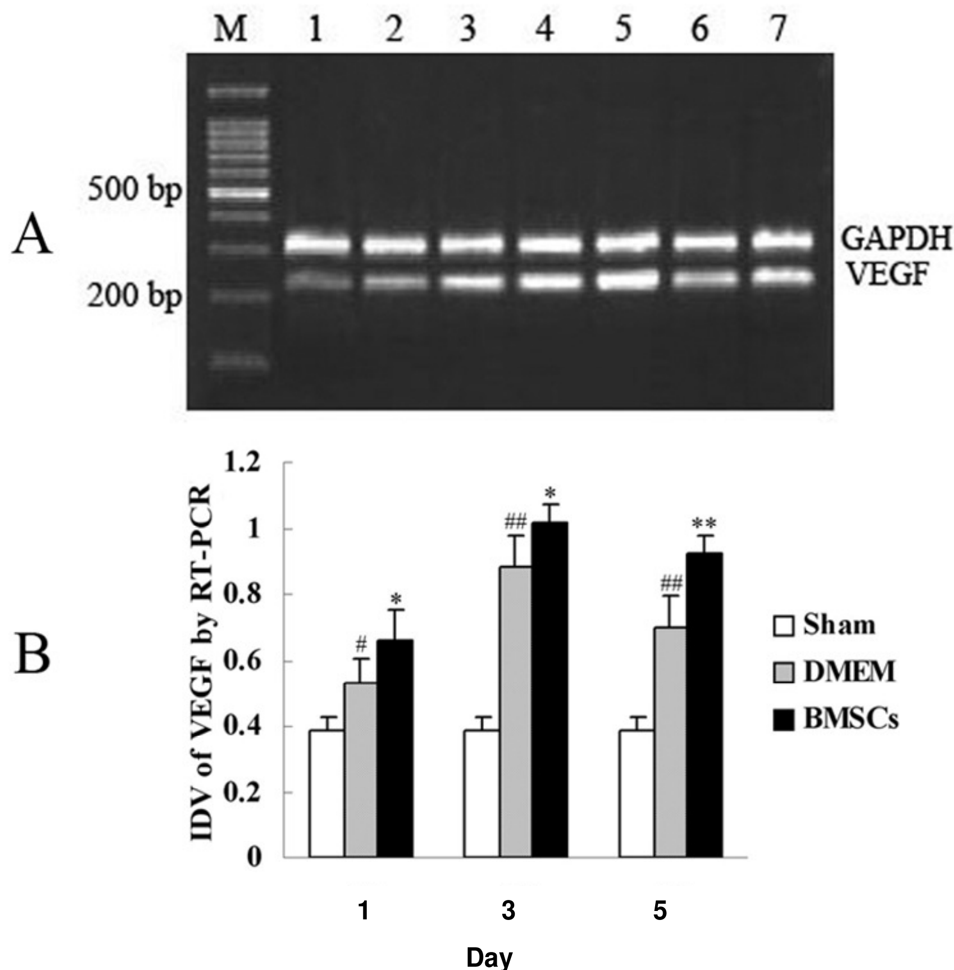

Figure 2. The mRNA expression of VEGF at different time points in rats from each group after transplantation. A, The expression of VEGF in different group by RT-PCR; M: marker; 1: sham operation group; 2, 4, 6: DMEM group at transplantation after 1, 3, 5 days, respectively; 3, 5, 7: BMSCs group at transplantation after 1, 3, 5 days, respectively. B: The integrated density value of vascular endothelial growth factor mRNA at different time points in rats from different group after transplantation. Different mark represent the significant difference at $p < 0.05$

3 and 5 days after transplantation of BMSC as compared to DMEM group ($P < 0.05$), moreover, DMEM group were significantly higher than sham group ($P < 0.05$) in different time. These results showed that transplantation of BMSC could up-regulate VEGF mRNA expression.

Western blot analysis of VEGF expression

The western blot was performed to detect protein expression of VEGF. The result showed that VEGF expression was at a low level in the normal group rats. The expression level of VEGF protein in the DMEM group rats increased and peaked at 3 days ($P < 0.01$), then decreased quickly with the extension of time, approaching the expression levels of the sham group rats at 7 and 14 days ($P < 0.05$) after spinal cord injury. In the BMSCs group rats, the up-regulation of VEGF protein expression was observed at 7 and 14 days after SCI as compared with that in DMEM group rats ($P < 0.05$), which were time-dependent and reached the highest level at 14 days after spinal cord injury (Figure 3).

Immunohistochemistry analysis of VEGF expression

In order to further confirm VEGF protein expression, immunohistochemistry was conducted. The results showed that there were no obvious differences ($P < 0.05$) in VEGF expression between BMSCs and DMEM groups rats at 3 day after transplantation (Table 1). The expression level of VEGF in BMSCs group rats were increased

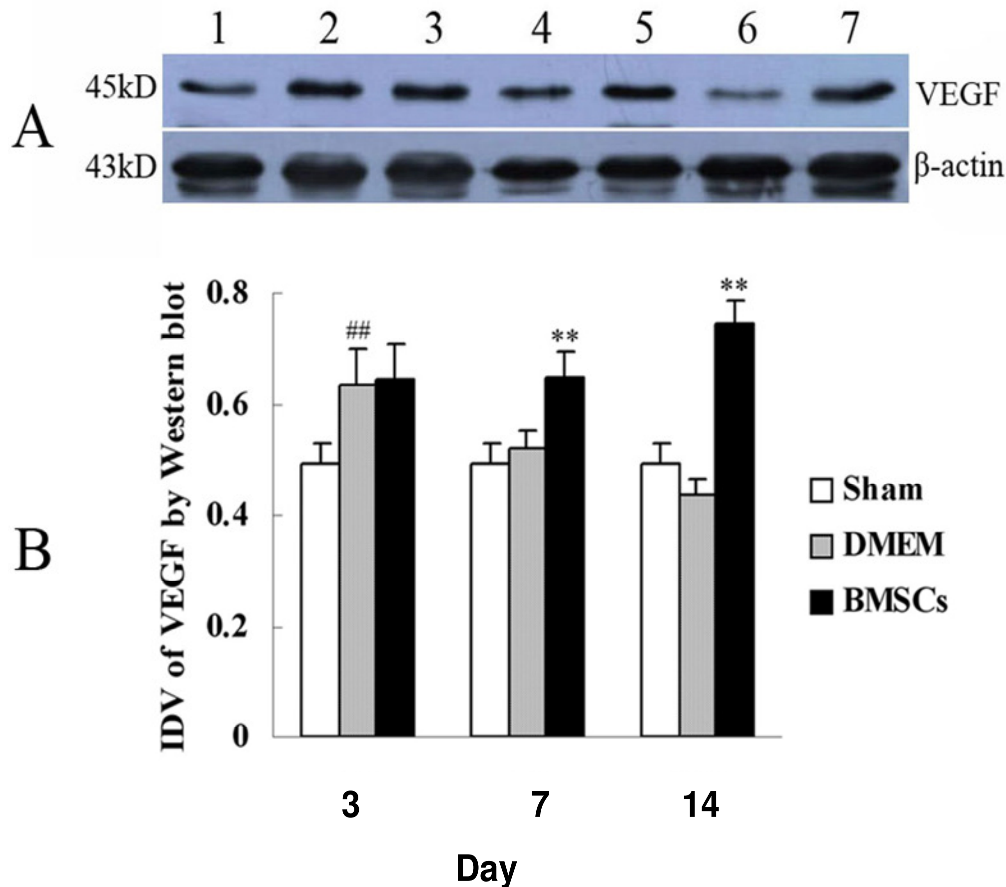

Figure 3. The expression of VEGF at different time points in rats from each group after transplantation in protein level. A: The expression of VEGF in different group by western blot; 1: sham operation group; 2, 4, 6: DMEM group at transplantation after 3, 7, 14 days, respectively; 3, 5, 7: BMSCs group at transplantation after 3, 7, 14 days, respectively. B: The integrated density value of vascular endothelial growth factor protein at different time points in rats from different group after transplantation. Different mark represent the significant difference at $p < 0.05$.

Table 1. The mean optical density of VEGF at different time points in rat in all groups after the transplantation by immunohistochemical staining ($n = 6$, $x \pm s$). Different mark represent the significant difference at $p < 0.05$.

Time point after the transplantation (day)	3	7	14
Sham group	0.1800±0.0061	0.1811±0.0089	0.1823±0.0063
DMEM group	0.3153±0.0098##	0.2381±0.0095##	0.1954±0.0038
BMSCs group	0.3150±0.0097	0.2520±0.0052*	0.2281±0.0079**

significantly at 7 and 14 days ($P < 0.05$) after transplantation when compared with that in DMEM group (Figure 4 and Table 1).

DISCUSSION

VEGF is essential for developmental angiogenesis and plays important roles in adult animals to control vascular permeability and homeostasis, blood pressure, and pathological angiogenesis associated with wound healing (Ku et al., 1993; Shibuya, 2006; Lee et al., 2007). Based on its major role as an angiogenic factor and autocrine growth factor for many diseases, therapeutic angiogenesis drugs have been developed for clinical use by improvement of VEGF expression. Previous studies

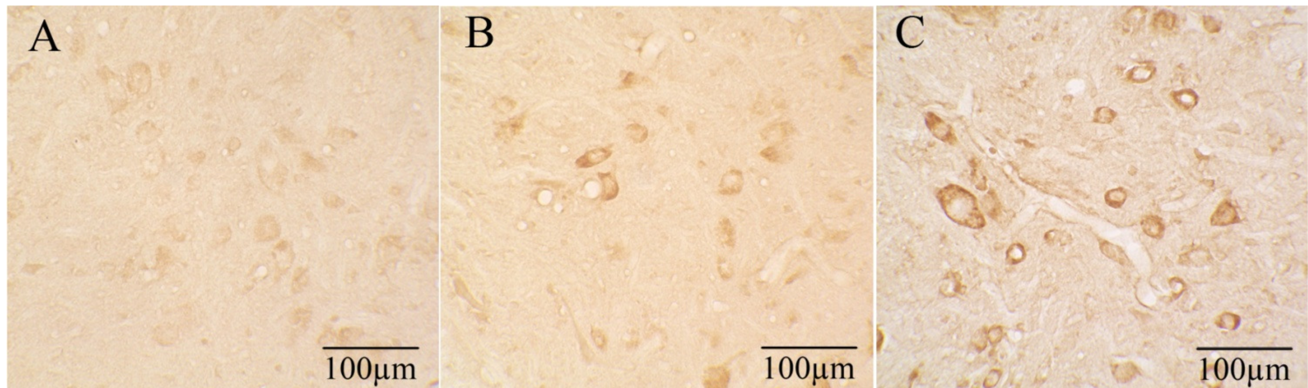

Figure 4. The expression of VEGF by immunohistochemical staining at 7 days after transplantation. A, Sham operation group; B, DMEM group; C, BMSCs group.

had shown that administration of VEGF could significantly increase the microvascular density in central nervous system (Blight., 1991; Widenfalk et al., 2003). In this study, we found that BMSCs treatment not only increased the level of VEGF expression, but also extended the time of VEGF expression in SCI, which would strengthen the biological effects of VEGF in promoting angiogenesis of SCI, and this seems to be compatible with the reported results that BMSCs implantation could induce angiogenesis in a rat model of chronic hind limb ischemia (Chen et al., 2003), acute myocardial infarction and stroke (Rubén et al., 2006). The present study suggests that VEGF probably plays a pivotal role in the treatment of SCI by BMSC.

BMSCs are mesenchymal elements normally providing structural and functional support for hemopoiesis (Pittenger et al., 1999), which have the potential to differentiate into other kinds of cells such as osteoblasts, adipocytes, and chondrocytes (Pittenger et al., 1999; Prockop, 1997), and produce a variety of neurotrophic factors, cytokines, cell adhesion molecules, and growth factor such as VEGF, thereby providing the pathway for regenerating axons. BMSCs transplanted into a rat model of chronic hind limb ischemia (Al-Khaldi et al., 2003), acute myocardial infarction (Tang et al., 2006) and ischemic brain (Chen et al., 2003) have provided their beneficial effects by enhancing angiogenesis. The angiogenesis stimulated by the BMSCs transplantation is considered to at least in part, result from angiogenic factors secreted by BMSCs and/or stimulating endogenous parenchymal cells (Chen et al., 2003). VEGF is one of the most potent angiogenic factors, capable of promoting proliferation, migration and survival of endothelial cells and plays the most important role in the initiation of new blood vessel formation (Kayo et al., 2005; Breen., 2007; Leung et al., 1989), therefore, VEGF may play important role in the treatment of SCI by BMSC.

In conclusion, we have demonstrated that VEGF expression was significantly up-regulated in BMSC group in comparison to the normal group and DMEM group. The current study provides a new approach for studying the molecular mechanism underlying BMSCs treatment in SCI. However, this molecular mechanism is largely unknown; elucidation of these questions will depend on further experiments. The next proposal of our study will be to analyze whether VEGF expression could be accompanied by cyclooxygenase-2 (COX-2), a protein relevant in regulating pro-inflammatory processes, which have been demonstrated to play a relevant role in VEGF regulation (Siner et al., 2007). These data have to be confirmed in future experiments, which would provide further valuable clues to clarify molecular mechanisms of BMSCs treatment in SCI.

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