Original Article

Transplantation of bone marrow mesenchymal stem cells inhibits apoptosis in injured rat spinal cord

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Abstract: Many studies have showed potential therapeutic effects of bone marrow mesenchymal stem cells (BMSCs) to spinal cord injury (SCI) in animal model. However, the exact mechanism is still uncertain. The authors hypothesized that spinal neurons might be lost by apoptosis and investigated a potential anti-apoptotic mechanism of BMSCs transplantation for rat SCI. A total of 48 adult male SD rats were divided into 3 groups randomly: control, model and treatment group, n=16 in every group. Rats in model and treatment group were performed a partial low thoracic SCI by modified Allen’s method at T10. Rats in control group received only laminectomy. At day 7 After SCI, the dura at L4-L5 intervertebral space was exposed; 100 μl of Hank’s buffered saline solution contained 1 million rat BMSCs or the same amount of Hank’s buffered saline solution was injected into the subarachnoid space. Evaluated hind limb motor function by Basso, Beattie and Bresnahan (BBB) scale pre-injection and at day 7, 14, 21, 28 post-injection. Injured rat spinal cords were harvested at day 14, 28 post-injection, fixed and then embedded in paraffin for terminal deoxynucleotidyl transferase-mediated DUTP-biotin nick end labeling (TUNEL) staining, and for immunohistochemical staining of Bax and Bcl-2. At day 3, 7 days after surgery, BBB scale score of rats in model group decreased progressively, higher than those in control group (P<0.01). After transplantation of BMSCs, BBB scale score of rats in treatment group increased progressively, higher than those in model group (P<0.01), but still lower than control group (P<0.01) at day 14, 28 post-injection. TUNEL staining showed that the apoptotic cell ratio in model group was lower than those in control group (P<0.01). After transplantation of BMSCs, the apoptotic cell ratio in treatment group increased progressively, higher than those in model group (P<0.01), but still lower than control group (P<0.01) at day 14, 28 post-injection. Immunostaining demonstrated that model group showed higher Bax positive expression than the other two groups (P<0.05) at day 14 post-injection, but there was no significant difference in Bcl-2; at day 28 post-injection, the trends were similar, but without significant difference. The results showed that BMSCs transplantation could improve the hind limb motor function of SCI rats, reduce spinal neuronal apoptosis and the expression of Bax protein, which implied a potential anti-apoptotic mechanism of transplanted BMSCs for rat SCI.

Keywords: Bone marrow mesenchymal stem cells, spinal cord injury, apoptosis, Bax

Introduction

Severe spinal cord injury (SCI) is a devastating condition which is associated with permanent disability and decreased life expectancy and can be costly in human and social terms. Despite much research, the development of new strategies to severe SCI is still a major clinical challenge. Bone marrow mesenchymal stem cells (BMSCs) is capable of transdifferentiating to neural cell types under appropriate experimental conditions in vitro [1-3], BMSCs even could spontaneously differentiate into neural precursor cells after long-term culture [4]. The marrow is readily accessible, overcoming the risks of obtaining neural stem cells from the brain, BMSCs can be used as autologous cell grafts that easily can expand from relatively small amounts of bone marrow aspirates, along with their inflammatory and immune-modulating function, overcomes the ethical concerns associated with the use of fetal tissue [5, 6]. Therefore, it may be an attractive source for cellular transplantation in the treatment of a wide variety of neurologic diseases and trauma. Many studies have demonstrated potential therapeutic effects of BMSCs to SCI in animal models [7-18]. However, the exact mechanism is still uncertain. The authors hypothesized that spinal neurons might be lost by apoptosis and...
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investigated a potential anti-apoptotic mechanism of BMSCs transplantation for rat SCI.

Materials and methods

Animals

Six male Sprague-Dawley (SD) rats (6 week old, body weight 90-103 g) and 48 adult male SD rats (body weight 260-300 g) were supplied by the Medical Experimental Animal Center, Soochow University, China (License No. SYXX (Su) 2002-0037). Ethical approval was obtained from the Ethical Committee of Soochow University. Protocols were in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985).

Rat BMSCs isolation and culture

Primary rat BMSCs were isolated as previously described by Jendelová [19] and the experience of Jiangsu Institute of Hematology. Briefly, six male 6-week-old SD rats were sacrificed by overdose chloral hydrate injection (1600 mg/kg body weight). Tibias and femurs were dissected free and the proximal and distal ends were removed to reveal the marrow cavity which was aspirated with 10 ml of BMSCs growth medium: (LG-DMEM+10% FBS) (all from Gibco, USA) through a 21G needle. The aspirate was centrifuged at 1,000 rpm for 5 minutes twice. The cell pellet obtained (containing both haematopoietic cells and BMSCs) was then suspended in BMSCs growth medium. The suspension were then cultured in 25 cm² plastic culture flasks and incubated at 37°C with 5% humidified carbon dioxide (CO₂) for 24 hours. Flasks were washed with Phosphate Buffered Saline (PBS) to leave an adherent layer of cells containing rBMSCs.

Fresh culture medium was replaced every 3-4 days. When the cultures approached near confluency, the cells were harvested and digested with 0.25% trypsin/1 mM EDTA (Gibco) and diluted 1:2 or 1:3 per passage for further expansion. After this cycle was repeated three times, BMSCs were obtained to perform Wright-Giemsa staining (fast Wright-Giemsa Stain Kit, Baso, Taiwan, China) for morphological observation: removed medium, washed with PBS, added solution A incubated for 5 min, then added solution B incubated for 5 min, washed with PBS, cleared water with absorbent paper.

Flow cytometric analysis

The third passages of BMSCs were characterized by flow cytometry. In brief, cells were trypsinized and washed with FACS buffer, consisting of Ca²⁺/Mg²⁺-free PBS, 13.6 mM tri-sodium citrate and 1% BSA (Sigma, USA). After centrifugation at 2000 rpm for 5 min at 4°C, cells were suspended in FACS buffer and divided into 5 tubes with 100 μl suspension at a concentration of 1×10⁶/100 μl for each antibody tested. Cells were then labeled with 10 μl of fluorescein-conjugated monoclonal antibody or standard control at 4°C for 30 min: the first tube, standard control (BD Biosciences, USA); the second tube, FITC CD29 (BD Biosciences); the third tube, PE CD34 (Abcam, USA); the fourth tube, PE/CY5 CD45 (BD Biosciences); the fifth tube, PE/CY7 CD90 (BD Biosciences). The labeled cells were analyzed by flow cytometry (Beckman Coulter, USA) within 2 hours. FITC colouration is yellow-green, the wave length of excitation light is 488 nm, the maximum wave length is 518 nm; PE colouration is yellow, the wave length of excitation light is 488 nm, the maximum wave length is 578 nm; PE/Cy5 colouration is red, the wave length of excitation light is 488 nm, the maximum wave length is 667 nm; PE/Cy7 colouration is red, the wave length of excitation light is 488 nm, the maximum wave length is 785 nm.

Spinal cord injury model

A total of 48 adult male SD rats were divided into 3 groups, and every group were divided into 2 subgroups depending on the timing of tissue harvest: group A1 (control group, 14 days after BMSCs transplantation); group A2 (control group, 28 days after BMSCs transplantation); group B1 (model group, 14 days after BMSCs transplantation); group B2 (model group, 28 days after BMSCs transplantation); group C1 (treatment group, 14 days after BMSCs transplantation); group C2 (treatment group, 28 days after BMSCs transplantation), n=8 in every subgroup. The rats in model and treatment groups were performed a partial low thoracic spinal cord injury (SCI) by modified Allen’s method [20] (weight drop method) at T10 under chloral hydrate anesthesia (350 mg/kg body weight). The 10 g impact rod was centered above T10 and dropped through a hollow transparent tube from a height of 5.0 cm to induce a consistent partial, incomplete SCI. Dural tear was never
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Hank's buffered saline solution contained $1.0 \times 10^6$ BMSCs (control and treatment group) or the same amount of Hank's buffered saline solution (model group) were injected into the subarachnoid space by single shot with a micro-injector (Hamilton, Switzerland) via a tip-bent 31G needle (Hamilton). Muscle and skin were sutured by layer after operation. Then observed the hind limb motor function of all animals, and evaluated by BBB scale. Postoperative management was the same to previously described method.

**Tissue harvest and histologic preparation**

Rats were anesthetized by chloral hydrate injection (350 mg/kg body weight) at day 14, 28 after BMSCs transplantation according to the grouping. 50 ml of PBS followed 200 ml of 4% paraformaldehyde were perfused intracardially for each rat, the spinal cord segments containing the injured sites or counterparts were removed from the spinal column. Spinal cords were postfixed in 4% paraformaldehyde overnight then were paraffin-embedded and sectioned at 4 μm thickness.

**TUNEL staining**

Neurocyte apoptosis was examined by the TUNEL staining with TUNEL Staining Kit (Merck Millipore, Germany): Slides were incubated with 0.2% proteinase K at room temperature for 15 minutes, followed by incubated with TdT-labeling buffered solution in 37°C wet box for 1 hour, then incubated with streptavidin-HRP solution at room temperature for 10 minutes. DAB as color developing reagent, counterstaining with methyl green. Prepared slides were evaluated by a light microscopy, positive cells exhibited brown nuclear staining. Negative control received the same steps except incubated without TdT-labeled buffered solution, indicated the level of background labeling (DAB) associated with non-specific binding of the streptavidin-HRP and didn’t have any brown staining.

**Immunohistochemistry**

The expression of Bax and Bc1-2 protein were tested with immunohistochemical staining: Slides were washed with PBS, incubated with 3% H$_2$O$_2$ deionized water at room temperature for 30 minutes, followed by incubated with goat serum working fluid at 30°C for 40 minutes,
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then incubated with primary antibodies of rabbit anti-Bax monoclonal antibody (1:100, Bioworld Technology, USA) and rabbit anti-Bcl-2 monoclonal antibody (1:50, Bioworld Technology) at 4°C for 24 hours. Slides were washed with PBS, incubated with biotin conjugated goat anti rabbit second antibody at 37°C for 40 minutes. Slides were washed with PBS, incubated with streptavidin-HRP at 37°C for 30 minutes. Slides were washed with PBS, then added DAB for colour development. Prepared slides were evaluated by a light microscopy, positive cells exhibited brown nuclear staining.

Image processing
All images taken were analyzed by using image analysis software Image-pro Plus 5.0. Under the same conditions, 100 μm×100 μm were selected respectively for counting positive staining cells.

Statistical analysis
All data were statistically processed using SPSS 18.0 Package through Repeated Measures Analysis and One-Way ANOVA. Comparisons between groups were made by Least Significant Difference (LSD) method. The data of each group were expressed as means ± standard deviation (X±S). P<0.05 was considered to indicate statistical significance.

Results

Rat BMSCs isolation and culture
The primary rat BMSCs were uneven in shape, but with excellent reproductive activity. After 3 passages, the cells got relative purification. The BMSCs were good in reproductive activity and relatively uniform in shape (Figure 1). After Wright-Giemsa staining, BMSCs displayed typical spindle-shaped cells, presented layered and whirlpool-like growth (Figure 2).

Flow cytometric analysis
Using flow cytometric analysis, the BMSCs were positive for the surface antigens CD29 and CD90, but negative for CD34, CD45 and standard control (Figure 3).

Figure 3. The flow cytometry of BMSCs showed: CD29(+), CD90(+), CD34(-), CD45(-).

Figure 4. BBB scale score pre- and post-spinal cord injury.
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Hind Limb motor function of animals

Before surgery, all animals’ BBB scale score was 21 (the maximum value). At day 1 after surgery, the animals of control group reached 21 BBB scale score. Three, seven days after surgery, BBB scale score of the animals in model group and treatment group were lower than control (P<0.01), but without significant difference (P>0.05) between this two groups (Figure 4). All animals’ urinary function recovered within 3 days after surgery.

After transplantation of BMSCs following a subarachnoid injection, BBB scale score of the animals in treatment group increased progressively. 14 days after grafting procedure, BBB score of model group and treatment group reached a mean value of 5.88±1.46 (group B1), 5.75±1.39 (group B2) and 9.38±2.00 (group C1), 9.50±2.20 (group C2) respectively, still lower than control group (P<0.01), treatment group’s BBB score higher than those in model group (P<0.05). 28 days after grafting procedure, BBB score of model group and treatment group reached a mean value of 6.50±1.60 (group B2) and 13.50±3.70 (group C2) respectively, lower than control group (P<0.01), treatment group’s BBB score higher than those in model group (P<0.01) (Figure 5).

TUNEL staining

TUNEL staining showed that the apoptotic cell ratio in model group were higher than those in control and treatment group (P<0.01), treatment group were higher than those in control group (P<0.01) at day 14 post-injection (Figures 6, 7), the trend was similar at day 28 post-injection, but without significant difference (P>0.05) (Figure 7).

Figure 5. BBB scale score after BMSCs injection.

Figure 6. TUNEL staining at day 14 post-injection: A. control group; B. model group; C. treatment group (×400).
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Immunohistochemistry

Immunohistochemical staining demonstrated that model group showed higher Bax positive expression than the other two groups (P<0.05) at day 14 post-injection (Figures 8, 9), but there was no significant difference in Bcl-2; the trend was similar at day 28 post-injection, but without significant difference (P>0.05) (Figure 9).

Discussion

According to previous studies, CD29 and CD90 were regarded as positive cell-surface markers for MSCs, while CD34 and CD45 was regarded as negative surface markers [22-25]. The cells used in this study were positive for CD29, CD90 and negative for CD34, CD45, indicated that the isolated BMSCs showed typical MSC characteristics.

Many studies have demonstrated that BMSCs transplantation is one of the most promising therapeutic approaches to SCI. In most animal studies, BMSCs have been directly injected into the injured spinal cord [7, 10-13, 16, 17]. However, this transplantation method has many potential problems. For example, inflammatory and cytotoxic chemokines that released by injured spinal cord tissue could make the local environment hostile to the transplanted cells [26]. In addition, direct injection of BMSCs into the lesion would be unrealistic clinically when the lesion is widely spread or multifocal [27]. Even if proven to be efficacious for the fear of causing further deterioration or procedure-related complications in an already compromised individual. Moreover, the procedure itself is potentially harmful to the spinal cord. Furthermore, the technique of direct parenchymal cell transplantation does not allow delivery of multiple dosages of therapeutic cells. In addition, although the procedure is acceptable in animal experiments, its extrapolation to humans may be difficult because an operation will be required.

Some groups have evaluated intravascular delivery of BMSCs [9, 14, 15, 18]. Of course, intravenous stem cell delivery is comparatively the least invasive approach. However, the procedure is limited by the multisegmental arterial
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**Figure 9.** A. Bax immunostaining results; B. Bcl-2 immunostaining results. **P<0.01.

Supply of the spinal cord, which requires highly selective and technically challenging cannulation of the spinal arteries; and sometimes required irradiation, which might damage the function of the blood brain barrier [28]. Additionally, the reproducibility of this procedure has been a question [8, 14].

Subarachnoid space injection of cells at lumbar level in animal is similar to lumbar puncture (LP) in human. In humans, LP is performed usually at the L3-4 or L4-5 level, far away from the cervical or thoracic spinal cord, which is most commonly affected by SCI. This makes LP delivery of BMSCs relatively safe. Additional advantages of LP delivery are related to several factors [8]: 1) the cells are delivered across the blood-brain barrier into the CSF, making it far more efficient than intravenous delivery; 2) CSF circulates within the central nervous system, allowing transplanted cells to home into the injured tissues, and cells are preserved in a relatively immune-privileged environment; and 3) because the transplanted cells are delivered away from the hostile environment of the injured tissue, they are given a greater opportunity to survive and migrate to the injury site. Several clinical trials of SCI treatment applied BMSCs by LP delivery [29-31]. Therefore, in this study, we chose subarachnoid space injection at lumbar level as BMSCs transplantation method.

Studies have demonstrated potential therapeutic effects of BMSCs to SCI in animal models [7-18]. However, the exact mechanism is still uncertain. The possible mechanism include: neural differentiation and regeneration [32, 33], neurotrophic effect and microcirculation improvement [34-36], induction effect [37, 38], bridging effect [39], anti-inflammation [16, 40, 41].

Apoptosis, or programmed cell death (PCD), is an important process which is necessary for normal embryological development and the maintenance of homeostasis. After SCI in the rat, typical post-traumatic necrosis occurred, but in addition, apoptotic cells were found from 6 hours to 3 weeks after injury, especially in the spinal white matter [42]. Apoptosis is a very important mechanism of secondary injury after SCI [43] that is triggered by a number of mechanisms. Members of the bcl-2 gene family play a central role in regulating the relative sensitivity and resistance of cells to a wide variety of apoptotic stimuli. Bax and bcl-2 play a most important role in the apoptosis pathway related to mitochondria. The pro-apoptosis factor bax is a nuclear-encoded protein residing in the cytosol that adopts a globular alpha-helical structure, seemingly as monomers. Following a variety of stress signals, it converts into pore-forming proteins by changing conformation and assembling into oligomeric complexes in the mitochondrial outer membrane. Cytochrome c from the mitochondrial inter membrane space then empties into the cytosol [44]. Release of cytochrome c triggers active caspase-3 and ultimately induces cell apoptosis. On the contrary, the anti-apoptosis factor bcl-2 presents in the outer mitochondrial membrane which block cytochrome c release from mitochondria to the cytosol [45].
In this study, we have demonstrated that transplantation of BMSCs by lumbar level subarachnoid space injection 1 week after contusion injury significantly improves hind limb motor functional outcome, both gait and coordination, as measured on the BBB test. Significant improvement of function persists till to 4 weeks at least. Meanwhile, transplantation of BMSCs reduced neuronal apoptosis in injured spinal cord as determined by TUNEL method. In fact, apoptosis is a dynamic process, in the present study, we just observed the spinal neuronal apoptosis at specific time points. Compared with previous studies [32, 33], the results of this study implied a potential anti-apoptotic mechanism that reduced deficits with BMSCs transplantation after spinal cord injury is that the transplanted cells trigger endogenous survival signaling pathways in neurons that mediate protection against apoptotic insults, which alleviated secondary spinal cord injury, besides BMSCs integrate into the tissue and replace damaged cells. This potential anti-apoptotic effect may achieve by down regulation of the Bax protein, which served as a stimulus to apoptosis. However, we have no clear evidence to determine the exact apoptotic regulation pathway after BMSCs transplantation to SCI rats.

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Disclosure of conflict of interest

None.

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