Human bone marrow mesenchymal stem cell transplantation attenuates axonal injury in stroke rats

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Abstract

Previous studies have shown that transplantation of human bone marrow mesenchymal stem cells promotes neural functional recovery after stroke, but the neurorestorative mechanisms remain largely unknown. We hypothesized that functional recovery of myelinated axons may be one of underlying mechanisms. In this study, an ischemia/reperfusion rat model was established using the middle cerebral artery occlusion method. Rats were used to test the hypothesis that intravenous transplantation of human bone marrow mesenchymal stem cells through the femoral vein could exert neuroprotective effects against cerebral ischemia via a mechanism associated with the ability to attenuate axonal injury. The results of behavioral tests, infarction volume analysis and immunohistochemistry showed that cerebral ischemia caused severe damage to the myelin sheath and axons. After rats were intravenously transplanted with human bone marrow mesenchymal stem cells, the levels of axon and myelin sheath-related proteins, including microtubule-associated protein 2, myelin basic protein, and growth-associated protein 43, were elevated, infarct volume was decreased and neural function was improved in cerebral ischemic rats. These findings suggest that intravenously transplanted human bone marrow mesenchymal stem cells promote neural function. Possible mechanisms underlying these beneficial effects include resistance to demyelination after cerebral ischemia, prevention of axonal degeneration, and promotion of axonal regeneration.

Key Words: nerve regeneration; human bone marrow mesenchymal stem cells; ischemic stroke; neural function; neuroprotection; microtubule-associated protein 2; myelin basic protein; growth-associated protein 43; neuraxon; myelin sheath; demyelination; axon regeneration; neural regeneration

Introduction

Cerebral ischemia is an important clinical neurological disorder, and there is considerable interest in protecting neurological function following cerebral ischemia. To date, the best method for treating such patients has been thrombolytic therapy in the acute period; however, few patients can receive this therapy because of the limited time window.

Mesenchymal stem cells have the potential to differentiate into various cell types, including osteoblasts, chondrocytes, adipocytes, and hepatocytes, as well as other tissue lineages including neurons, neural stem cells, microglial cells, and astrocytes (Deng et al., 2006). Mesenchymal stem cells are regarded as one of the most promising types of stem cells used for the treatment of experimental cerebral ischemic stroke to promote neurological function (Tang et al., 2007; Li et al., 2010; Bao et al., 2011). However, the neurorestorative mechanisms by which mesenchymal stem cells improve functional recovery remain largely unknown.

Myelinated axons are essential for the normal functioning of the brain. Increasing evidence using a variety of markers targeting different structures within axons has demonstrated the acute vulnerability of axons to cerebral ischemia in vivo (Dewar et al., 1997; Yam et al., 1998). Myelinated axons are susceptible to anoxia, and functional and structural impairment occurs within the axon itself after focal cerebral ischemia (Philippa et al., 2000).

In the present study, we assessed neurological status in ischemic model rats generated by transient middle cerebral artery occlusion (MCAO) following intravenous injection of human bone marrow mesenchymal stem cells (hBMSCs). Furthermore, we analyzed the alteration of axons and myelin sheath-associated proteins, such as microtubule-associated protein 2 (MAP-2), myelin basic protein (MBP), and growth-associated protein 43 (GAP-43), in these animals. This was done in an effort to show whether the mechanism by which transplantation of hBMSCs protects against cerebral ischemia is associated with the ability to attenuate axonal injury, such as demyelination.

Materials and Methods

Animals
Twenty adult male Sprague-Dawley rats, weighing 250–300 g, were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China; certificate
No. SCXK (Jing) 2012-0001). All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were maintained on a 12-hour light/dark cycle with free access to food and water.

**hBMSC preparation and culture in vitro**

Bone marrow aspirates were obtained by puncture of the posterior iliac crest of healthy human donors (Fang et al., 2003) after informed consent was obtained. Mononuclear cells in bone marrow specimens were separated on Percoll-Paque Hydroxypropylmethylcellulose (Dow, Beijing, China). Flk1+, CD34+, CD31− hBMSCs (the cell line was obtained under phase one clinical approval; approval No. 2004L04792, Food and Drug Administration, USA) were purified using immunomagnetic beads. Mononuclear cells were plated at a density of $1 \times 10^6$ cells/25 cm$^2$ cell culture flask in 20 mL of culture medium containing 58% DMEM/F12 (Gibco, Grand Island, NY, USA) + 40% MCDB-201 (Sigma, St. Louis, MO, USA), 2% fetal bovine serum (HyClone, Grand Island, NY, USA), 10 ng/mL epidermal growth factor, 10 ng/mL platelet-derived growth factor, 2 mmol/L L-glutamine (HyClone, USA) and 100 μg/mL penicillin, and cultured at 37°C in a humidified atmosphere containing 5% CO2. After 1 day, nonadherent cells were washed and used for experiments. The medium was replaced with the same fresh medium every third day. Plastic-adherent hBMSCs were digested with 0.125% trypsinase and 0.01% EDTA upon reaching 80% confluence. hBMSCs at passage 3 were collected and used for transplantation. Nucleated marrow cells were counted using a flow cytometer to ensure adequate cell number for transplantation. hBMSCs were analyzed by flow cytometry (eFluor, eBioscience, San Diego, CA, USA) for phycoerythrin anti-human phycoerythrin-Flk-1, CD31 and CD34.

**Establishing MCAO and reperfusion models**

Sprague-Dawley rats ($n = 20$) were initially anesthetized with 3.5% chloral hydrate. We induced transient MCAO using an intraluminal vascular occlusion method (Koizumi et al., 1986; Nagasawa et al., 1989). In brief, rats were anesthetized with 3.5% chloral hydrate. Rectal temperature was maintained at 37°C throughout the surgical procedure using a heating lamp. The right common carotid artery, external carotid artery, and internal carotid artery were exposed. A length of 40 mm of monofilament nylon suture (0.24 mm diameter) was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery. At 2 hours after MCAO, the nylon suture was withdrawn until the suture tip cleared the lumen of the external carotid artery to realize reperfusion.

**Grouping**

At 24 hours after MCAO, rats were evaluated for modified neurological severity score (mNSS), which is based on a series of motor (Borlongan et al., 1995), sensory (Markgraf et al., 1992), balance and reflex tests (Germano et al., 1994). Scores of 1–6 indicate mild injury, scores of 7–12 indicate moderate injury, and scores of 13–18 indicate severe injury. MCAO rats with an mNSS of 10–14 were randomly assigned to two groups: an MCAO + saline group ($n = 6$) and an MCAO + hBMSCs group ($n = 6$).

**hBMSC transplantation**

At 24 hours after MCAO and neurological assessment, rats were anesthetized as described above and received transplantation of hBMSCs or saline. The femoral vein was punctured under a microscope to ensure the needle was properly inserted. Approximately $1 \times 10^6$ hMSCs in 10 μL of saline (Mahmood et al., 2003) were injected into the animals in the MCAO + hBMSCs group at a rate of 1 μL/min; an equal volume of saline was injected into animals in the MCAO + saline group at the same rate. The wounds were stitched up immediately after transplantation.

**Behavioral testing**

After MCAO surgery, all rats underwent two behavioral tests, an adhesive-removal somatosensory test and the mNSS test, before reperfusion, and at 1 day, 3 days, 1 week, 2 weeks, 3 weeks, and 4 weeks after reperfusion, by an investigator who was blinded to the experimental groups.

The adhesive-removal somatosensory test was applied to measure forelimb somatosensory asymmetries (Schallert et al., 1984). Small adhesive-backed paper dots ($63.62 \text{ mm}^2$) were used as bilateral tactile stimuli and applied to the radial aspect of the wrist of each forelimb on three trials per day in the home cage. The number of seconds at which the rat contacted and removed the stimuli was recorded. Individual trials were separated by at least 10 minutes. The animals were trained in the adhesive-removal dot test for 3 days prior to MCAO surgery. Once the rats were able to remove the dots within 10 seconds, they were subjected to MCAO.

The mNSS test is a composite of motor, sensory, balance, and reflex tests, used to grade various aspects of neurological function (Borlongan et al., 1995). Neurological function is graded on a scale of 0–18 (normal score = 0, maximal deficit score = 18). A single point is awarded for a specific abnormal behavior or for the lack of a tested reflex. The higher the score is, the more severe the injury is.

**Slice preparation**

All rats were allowed to survive for 4 weeks after MCAO and reperfusion, and were used for morphologic analysis. Rats were deeply anesthetized with 10% chloral hydrate. The vascular system was transcardially perfused with heparinized PBS, followed by 4% paraformaldehyde. The brains were immersed in 4% paraformaldehyde in PBS for 24 hours. Then, the brain tissues were dissected into six 2-mm coronal blocks and embedded in paraffin. A series of adjacent 3-μm-thick slices were cut from each block.

**Measurement of infarct volume**

The brain sections were stained with hematoxylin and eosin and photographed under a microscope (XDS-1B, Chongqing Optical Instrument Factory, Chongqing, China). Rela-
Immunohistochemical staining
Sections were processed for double-immunofluorescence staining for MAP-2/GAP-43 or MAP-2/MBP. Briefly, after blocking in 5% normal goat serum for 30 minutes at 37°C, slides were incubated at 4°C overnight in mouse anti-MAP-2 polyclonal antibody (1:10,000; Boehringer, Ingelheim am Rhein, Rhineland-Palatinate, Germany), and then treated with Cy3-conjugated sheep anti-mouse IgG (1:500; Chemicon, Temecula, CA, USA) or rabbit anti-MBP (1:500; polyclonal, Chemicon), followed by FITC-conjugated sheep anti-rabbit IgG (1:50; Chemicon) staining.

To visualize the nuclei, the sections were counterstained with 6-diamidino-2-phenyl-indole (DAPI; Dojindo, Kumamoto, Japan).

Negative control sections from each rat received identical preparation for immunohistochemical staining, except that the primary antibodies were omitted.

Immunofluorescence-labeled slides were examined under a confocal laser-scanning microscope (Nikon, Eclipse-80i, Tokyo, Japan); green (FITC) and red (Cy3) fluorochromes on the slides were excited by the laser beam at 488 nm and 647 nm, and emissions were acquired sequentially with a photomultiplier tube through 522 nm and 670 nm emission filters.

tive infarct volume was analyzed using the Image Pro Plus 6.0 analysis system (Media Cybernetics, Silver Spring, MD, USA). The indirect lesion area, in which the infarct area in the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Relative infarct volume is presented as percent volume of the indirect lesion compared with the contralateral hemisphere (Swanson et al., 1990).

Statistical analysis
Data are presented as the mean ± SD and were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used for statistical comparisons among groups. A P value of less than 0.05 was considered statistically significant.

Results
Growth of hBMSCs in vitro
The results of human BMSC characterization and karyotype analysis showed that the hBMSCs used in this study were positive for fetal liver kinase-1 (Flk-1) and negative for CD31 and CD34. hBMSCs at passage 5 were used for karyotype analysis, which was conducted to check chromosome stability. No trisomy, tetraploidy, or chromosome rearrangement was observed.

Neurological functional testing after hBMSC transplantation
The mNSS test and adhesive-removal dot test were applied to compare behavioral functions of rats between the hBMSC-treated and control groups. There was no difference in neurological functions before cell transplantation. At 1–4 weeks after MCAO, significant functional recovery was observed in the adhesive-removal dot test in the hBMSC-treated group (P < 0.05 or P < 0.01; Figure 1B), while neurological severity scores in mNSS test were obviously lower in the hBMSC-treated group compared with the control group (P < 0.01; Figure 1A).

Infarct volume after hBMSC transplantation
By hematoxylin and eosin staining, the infarct was mainly seen in the cortex and striatum at 4 weeks after MCAO. The relative infarct volume in hBMSC-treated rats was significantly less than that in the saline-treated group (P < 0.01;
Double immunohistochemistry staining for MAP-2/GAP-43, MAP-2/MBP in the infarct area of cerebral ischemic rats after hBMSC transplantation

MAP-2 fluorescence showed the density and courses of axons around the infarct-affected cortex. GAP-43 was distributed in the neuropil. At 4 weeks after 2 hours of MCAO, MAP-2 and GAP-43 levels were decreased more significantly in the ischemic core of MCAO rats. MAP-2 was strongly expressed in the infarct area and colocalized with the higher levels of GAP-43 in hBMSC-transplantation rats, being distributed in the neuropil throughout axons. hBMSCs: Human bone marrow mesenchymal stem cells; MCAO: middle cerebral artery occlusion; MAP-2: microtubule-associated protein 2; MBP: myelin basic protein; GAP-43: growth-associated protein 43.
around the infarction area displayed fasciculation at equal pace with axon course. The fasciculation distribution of MAP-2 and MBP were successive and integrated relatively than saline-treated group, which indicated that hBMSC transplantation could possibly prevent demyelination after cerebral ischemia (Figure 3).

Discussion
In the present study, we demonstrated that transplantation of hBMSCs into MCAO rats improved neurological function and reduced infarct volume. This was accompanied by elevated levels of the axon- and myelin sheath-related proteins MAP-2, MBP and GAP-43. Possible mechanisms underlying these beneficial effects include resistance to demyelination after cerebral ischemia, prevention of axonal degeneration, and promotion of axon regeneration.

Cerebral ischemia is a major cause of death and disability worldwide. Thrombolytic treatment is effective when administered within 4.5 hours after stroke (Shinozuka et al., 2013). However, only a few patients are able to receive treatment within this limited therapeutic window (Shinozuka et al., 2013). Furthermore, thrombolytic treatment may increase the risk of intracranial hemorrhage (Hacket et al., 2008).

There is increasing evidence that transplantation of BMSCs could represent an alternative therapy for ischemic stroke. The transplanted BMSCs significantly promote functional recovery after ischemic stroke in animal models (Ding et al., 2013) and in the clinic (Lee et al., 2010; Honmou et al., 2011). However, it is unclear what brings about the purported benefit from BMSC transplantation.

Previous studies have reported that BMSCs could differentiate into brain parenchymal cells and replace the injured neurons in the infarct area (Woodbury et al., 2000; Chen et al., 2001), which was regarded as the main goal of cell therapy (Woodbury et al., 2000; Chen et al., 2001). However, very few transplanted cells were found in the brain and, of these, only a small percentage of cells expressed neuronal cell markers (Chen et al., 2002; Shen et al., 2007). Moreover, after BMSC transplantation, these cells are very unlikely to have truly integrated into the parenchymal tissue and to have formed the complex connections that promote functional recovery (Chen et al., 2002).

Other studies have shown that BMSC transplantation can reduce cell apoptosis (Huang et al., 2013), induce angiogenesis (Guo et al., 2012), promote proliferation of endogenous cells (Bao et al., 2011), and induce the production of trophic and angiogenic factors (Chen et al., 2002; Zhang et al., 2005). We studied the anti-demyelination effects of hBMSC transplantation, and examined the effects of hBMSC transplantation on promoting the regeneration of axons and myelin sheaths.

MAP-2, a marker of dendrosomatic neuronal injury, has been perceived primarily as a static, structural protein, being necessary along with other intracellular components, e.g., actin and neurofilaments, to maintain the neuroarchitecture. Several studies have reported decreases in MAP-2 levels in the gerbil brain after ischemia (Matesic et al., 1994; Dawson et al., 1996). Loss of MAP-2 may participate in the initial phase of neuronal dysfunction, and dendritic breakdown may be a first sign of neurodegeneration as early as 1 hour after cerebral ischemia (Dawson et al., 1996). Moreover, MAP-2 has roles in the growth, differentiation, and plasticity of neurons, with key roles in neuronal responses to growth factors, neurotransmitters, synaptic activity, and neurotoxins (Johnson et al., 1992). The results of these studies indicate that modification and rearrangement of MAP-2 is an early obligatory step in many processes that modify neuronal function.

GAP-43 (a 43-kDa growth-associated protein) is a nervous tissue-specific protein, synthesized at high levels during axonal outgrowth during neuronal development and regeneration (Jacobson et al., 1986; Goslin et al., 1988; Stoemer et al., 1995). Axonal sprouting, a component of anatomic plasticity, can be identified by the elevated expression of GAP-43 (Skene et al., 1989; Buffo et al., 1997). An acute increase in the level of expression of GAP-43 in the cortex is observed after cortical ischemia (Stoemer et al., 1993; Stoemer et al., 1995) and in the substantia nigra after striatal ischemic injury in adult rats (Goto et al., 1994). These reports suggest that GAP-43 is a sensitive marker for the assessment of axonal damage and the regenerative response in the ischemic area.

MBP is a major myelin-associated protein located with the membranes of neurons. Changes in MBP levels are used as a diagnostic indicator of demyelination (Yam et al., 2000).

Our data indicate that treatment with hBMSCs decreased infarct volume and improved functional recovery relative to the saline-treated group 4 weeks after MCAO. In an effort to show the role of axon- and myelin sheath-associated proteins in cerebral ischemia, we assessed the levels and colocalization of MAP-2/MBP and MAP-2/GAP-43 using double-staining immunohistochemistry. The results of the present study demonstrated that these proteins are preferentially expressed in the cerebral infarct zone in hBMSC-treated rats 4 weeks after MCAO, relative to the levels in saline-treated animals, suggesting a compensatory role for these proteins in this region. MAP-2 is sensitive to ischemia. The modification and rearrangement of MAP-2 contributed to the improvement of neuronal function (Li et al., 1998). The increase in the levels of MAP-2 in the transplantation group was associated with recovery of neuronal function, combined with the expression of MBP, an indicator of demyelination in cerebral ischemia, suggesting that neuronal functional recovery is related to this anti-demyelinating effect of hBMSCs. In the present study, the level of GAP-43, a sensitive marker for the assessment of axonal damage and regeneration, was also higher in the ischemic area in the hBMSC-treated group compared with the saline-treated group, suggesting a possible role for hBMSCs in axonal protection and regeneration.

Author contributions: Xu Y, Du SW and Yu XG designed the study and provided critical revision of the manuscript for intellectual content. Xu Y performed research, analyzed data and wrote the paper. Han X, Hou JC, Gao H gave technical and material support. All authors approved the final version of the manuscript.

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