Article

Expression of IL-10 and TNF-α in Rats with Cerebral Infarction after Transplantation with Mesenchymal Stem Cells

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We investigated the effects of bone marrow-derived mesenchymal stem cells (MSCs) transplantation on the recovery of neurological functions in rat's MCAO (middle cerebral artery occlusion) model and its mechanism. MSCs were isolated from bone marrow of male Sprague Dawley (SD) rats. Female adult SD rats were randomly assigned into 4 groups: sham-operated group, MCAO group, vehicle group and MCAO + MSCs-treated group. MSCs were injected into the lateral ventricle of rats in the MSCs-treated group and the same volume of PBS was given to the vehicle group. The expressions of IL-10 and TNF-α were assayed by RT-PCR and ELISA detections at day 1 and 4 after MCAO. The infarction volume was measured by TTC-staining. All rats underwent behavioral tests before, as well as 1, 4, and 14 days after MCAO. MSCs significantly improved functional recovery compared with the control at day 14 after transplantation. Compared with the MCAO group and the vehicle group, the expression of IL-10 mRNA and its protein level in the MSCs group significantly upregulated. However, the expression of TNF-α at day 4 after MCAO in the MSCs group significantly decreased compared with that of the MCAO group and the vehicle group. As a result, transplantation with MSCs significantly decreased infarct volume at day 1 and 4. This study strongly suggested transplantation with MSCs could reduce neuronal injury post focal cerebral ischemia in rats partly by regulating the expressions of IL-10 and TNF-α in the brain. *Cellular & Molecular Immunology*. 2009;6(3):207-213.

Key Words: Cerebral infarction, MSC, IL-10, TNF-α

Introduction

It has been proved that inflammation is important in the pathology of stroke. Cerebral ischemia induced a strong inflammatory response characterized by activation and release of cytokines, chemokines, adhesion molecules, and proteolytic enzymes that exacerbate tissue damage (1, 2). These cytokines, including tumor necrosis factor alpha (TNF)-α, interlukin (IL)-1β, and IL-8, drive inflammatory processes and accelerate additional inflammatory processes by inducing inflammatory molecules, such as intercellular adhesion molecule (ICAM), vascular cell adhesion molecule-1 (VCAM-1), and selectins. All these inflammatory modulators will induce more inflammatory leukocytes to infiltrate into ischemic region and lead to further loss of neuronal cells (3-6). Anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β1, suppress the production of pro-inflammatory cytokine in protection of damaged brain tissues after ischemic stroke (7). Bone marrow-derived mesenchymal stem cells (MSCs) have highly proliferative capacity and more potential to divide. MSCs can differentiate into neuron-like cells and nerve glial cells *in vitro* and *in vivo* (8, 9). MSCs can survive and migrate in rat brain after ischemia/reperfusion injury. Neurological function is significantly improved in rats after treatment with MSCs transplantation (10). It is of great significance to explore neural repair mechanisms in order to better use of these mechanisms in the treatment of ischemic brain damage (11). MSCs secrete a number of growth factors and cytokines, and also supply autocrine, paracrine, and juxtacrine factors that influence the cells of the microenvironment. So we hypothesize that MSCs protect cerebral ischemic by upregulating anti-inflammatory cytokines and downregulating of pro-inflammatory cytokines.

Materials and Methods

Animals

MSCs were isolated and cultured from male Sprague Dawley (SD) rats weighing 70-80 g. Adult female SD rats weighing

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Table 1. Specific primers of PCR

Primer	Sequence (5'-3')		Size (bp)
SRY	F) TCATTAGCCCTCATTT	R) CAATGGTTACCGTACC	269
IL-10	F) CTTTCACTTGCCCTCATCC	R) ACAAACAATACGCCATTCCC	265
TNF-α	F) TTGCTTCTTCCCTGTTCC	R) CTGGGCAGCGTTTATTCT	250
β-actin	F) ATTGTAACCAACTGGGACG	R) TCTCCAGGGAGGAAGAGG	490
GADPH	F) TGTGTTGTCCCTGTATGCCTCT	R) AAAACGCAGCTCAGTAACAGTCC	740

250-280 g were used as an animal model. Animals were cared for in accordance with guidelines published by the National Institutes of Health, and all study procedures were approved by the Fujian Medical University Institutional Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press (NIH, revised 1996).

Isolation, culture and characterization of MSCs

MSCs were isolated according to the Caplan method (12). In brief, we euthanized male SD rats and harvested bone marrow. Bone marrow cells were introduced into 100-mm dishes and cultured in complete medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). Culture medium was replaced every three days and the floating cells were discarded. Following two passages, the attached cells were divided into three new flasks and cultured until the cell density of the colonies grew to approximately 90% confluence. These cells were analyzed by fluorescence-activated cell sorting as described previously (13).

Animal model

Seventy-two female adult SD rats were randomly divided into 4 groups: sham-operated group, middle cerebral artery occlusion (MCAO) group, vehicle group and MCAO + MSCs-treated group (n = 18). A permanent focal cerebral ischemia model was established with the modified Longa method (14). In brief, rats were initially anesthetized with 10% chloral hydrate. Rectal temperature was controlled at 37°C with a feedback-regulated water heating system. The right common carotid artery, external carotid artery (ECA), and internal carotid artery were exposed. A 3.0 monofilament nylon suture (18.5 mm, determined by animal weight), with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the internal carotid artery until it blocked the origin of the MCA (middle cerebral artery).

MSCs transplantation

MSCs were harvested after culture for 3 or 4 passages and suspended in phosphate buffered saline (PBS) supplemented with penicillin and streptomycin. We then added sterile DAPI (Sigma, USA) to the cell suspension and incubated at 37°C for 30 min. The cells were then washed twice and re-suspended in PBS before injection in order to remove the non-binding DAPI. Cells were placed in ice bath before cell transplantation. In accordance with a stereotactic atlas, approximately 30 μl cell suspension containing 1×10^6 cells were injected into the lateral ventricle of MSCs-treated group rats and the same dose of PBS was given to the vehicle group one day after right MCAO.

Behavioral test

All animals underwent behavioral tests before MCAO and 1, 4, and 14 days after MCAO by an experimenter who was blinded to the experimental protocol. A modified neurologic severity score (mNSS) was used to measure various aspects of neurologic function. mNSS is a composite of the motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests.

Measurement of infarct size

All rats were anesthetized with 10% chloral hydrate either at day 1, 4, and 14, and the vascular system was transcardially infused with heparinized PBS followed by 4% para-formaldehyde in PBS. The brain tissues were cut into seven equally spaced (2 mm) coronal blocks. The sections were immersed in PBS containing 2% (w/v) TTC (Sigma, USA) at 37°C for 30 min, and in 10% (v/v) buffered formalin (pH 7.0) for 24 h. With TTC staining, the area without staining was determined to be the infarct area. The infarction volume is presented as a volume percentage of the infarction compared with the contralateral hemisphere. The percentage of TTC-stained tissue with respect to the whole piece of tissue was analyzed by Image J software (NIH, USA).

Histology study

At day 1, 4, and 14, rats were re-anaesthetized with 10% chloral hydrate. Rat brains were washed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde. Brains were then embedded in paraffin. The cerebral tissues were cut into 7 equally spaced (2 mm) coronal blocks. A series of adjacent 6-μm-thick sections were cut from each block in the coronal plane and were stained with hematoxylin and eosin.

Semi-quantitative RT-PCR

After 4 and 14 days, the total RNA was prepared from 0.1 mg of the brain tissues with Trizol reagents (Invitrogen, USA) according to the manufacturer's guidelines. Briefly, the

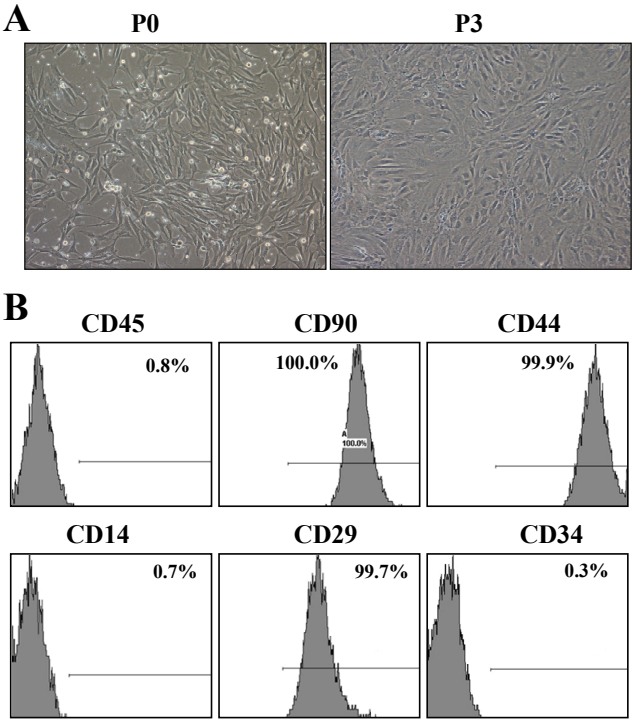

Figure 1. MSCs identification. (A) P_0, MSCs grew as a morphologically homogeneous population of fibroblast-like cells; P_3,, MSCs grew as whorls of densely packed spindle-shaped cells (×100). (B) The surface marker molecules expressed by the P_3 culture of MSCs were analyzed by flow cytometry.

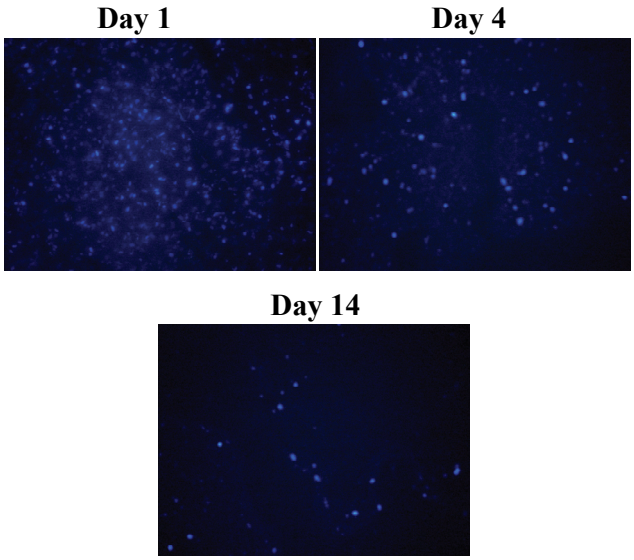

Figure 2. MSCs location after transplantation. At day 1, 4 and 14, the DAPI staining positive cells (blue fluorescence) on the side of ischemia in the MSCs group were shown.

cDNAs were synthesized with a cDNA kit (Fermentas, USA). Twenty microlitre reverse transcription mixture containing total 1 μg RNA, 1 mmol/L dNTP, 0.2 μg random primer, 20 U RNasin, and 200 U M-MuLV reverse transcriptase were incubated at 42°C for 60 min, and then the reverse transcriptase was inactivated by heating the reaction mixture at 70°C for 10 min. PCR reactions were performed on an Eppendorf Master Cycler. The reaction conditions were as follows: 2 μl of cDNA mixture was subjected to amplification in a final volume of 50 μl with 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 20 pmol of each primer (Table 1), and 2 U of Taq DNA polymerase (Fermentas, USA) in the reaction buffer. PCR reactions were as follows: 94°C, 5 min; then 94°C, 1 min, 65°C for SRY (sex determination region Y gene, SRY) and 60°C for β-actin, 1 min; 72°C, 45 sec, for 30 cycles; and 72°C 10 min to end the reaction. PCR products of 10 μl were separated by 1.8% agarose gel electrophoresis and visualized using ethidium bromide staining. The density of each band was measured with a UVP gel analysis system. This semi-quantitative measure was expressed as a ratios compared with β-actin.

Determination of the IL-10 and TNF-α protein levels

The levels of IL-10 and TNF-α in the tissues of ischemia area were measured by ELISA. Rats in all groups were euthanized at day 1 and 4 after MCAO (n = 6, per time point per group). Ischemic brain tissue was quickly removed, homogenized, and placed in an ice-cold bath. The supernatant was collected and cytokine levels measured with anti-rat IL-10 and anti-rat TNF-α ELISA kits (Jingmei Biotech., Shenzhen, China), in accordance with operations manual accompanying the testing reagents. All results were presented as means and are representative of at least 3 independent experiments.

Statistical analysis

All data are presented as mean ± SD. Significant differences were determined by using ANOVA in SPSS 13.0. The value of $p < 0.05$ was considered statistically significant.

Results

Identification of MSCs and MSCs location after transplantation

MSCs were scattered in a number of colony distributions 3 days after plated. At day 8-9, the bottle was covered with long-spindle cells. Passaged cells (mostly spindle cells) were uniformly distributed, and covered the bottom every 3-4 days (Figure 1). As shown in Figure 1, the passage 3 (P_3) culture of MSCs highly expressed the surface marker molecules CD44 (99.9%), CD90 (100%) and CD29 (99.7%), and expressed low levels of the blood cell surface molecules CD45 (0.8%), CD34 (0.3%) and CD14 (0.7%).

The DAPI-labeled MSCs showed clear nuclear and faint cytoplasmic blue fluorescence when observed under an epifluorescence microscope. DAPI labeled cells could be identified in all specimens at day 1, 4 and 14 after transplantation. After implantation numerous scattered DAPI-labeled cells (blue fluorescence) were found on the side of

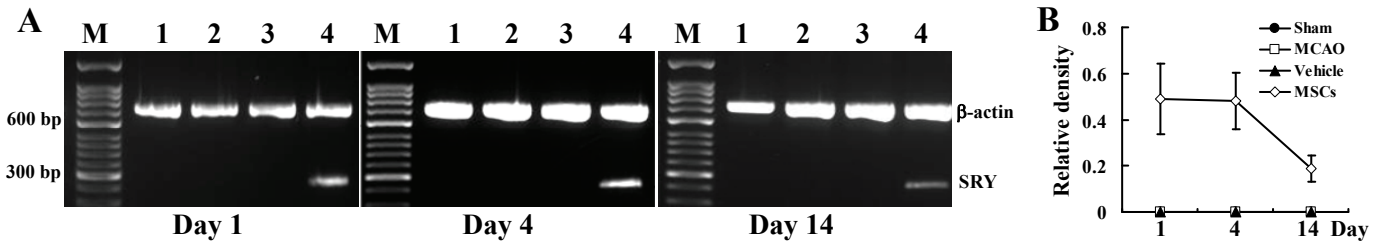

Figure 3. Detection of SRY expression by RT-PCR. (A) The expression of SRY mRNA was investigated by RT-PCR analysis at day 1, 4 and 14 post MCAO. Lane M, DNA markers; Lane 1, sham; Lane 2, MCAO; Lane 3, vehicle; Lane 4, MSCs. (B) The relative density was determined by compared with β-actin. Data were shown as mean ± SD (n = 6).

top-subcortical brain tissue (the equivalent of ischemic penumbra) in the MSCs group at day 1, and few at day 4, the least at day 14 (Figure 2).

The expression of SRY mRNA was investigated by RT-PCR analysis at day 1, 4 and 14 after MCAO. In the female rat brain tissue, there was no SRY mRNA expression in sham group, MCAO group and vehicle group. In MSCs group, the SRY gene expression peaked at day 1 post MCAO. As the days proceeded, the expression of SRY decreased, but was detectable at day 14 (Figure 3).

Effects of MSCs on neuronal cells

Four days after the success of transplantation in the MCAO group, the side of top-subcortical brain tissue was sparsely ischemic. We observed the deformation of nerve cells, nuclear condensation, nuclear fragmentation, nuclear dissolution, and the loss of structural integrity in this region. Compared with PBS group, the MSCs group showed more normal neurons and less cell death (Figure 4).

MSCs prevented cerebral ischemia injury and improved neurological function

Infarct volume and oedema occurred in ischemic brain tissue in the rats subjected to ischemia. Treatment with MSCs significantly decreased infarct volume percent ($p < 0.05$) (Figure 5). The attenuation of infarct by treatment with MSCs showed neuroprotective effect of MSCs.

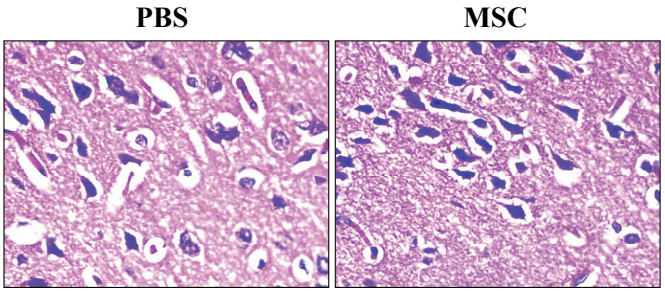

Figure 4. Effects of MSCs on neuronal cells. The right MCA was occluded and the rats were treated with MSCs or PBS. Brain sections of the cerebral cortex and striatum from the ipsilateral and contralateral sides were stained with hematoxylin-eosin.

The severity of MCAO was in parallel between the MSCs and control-treated groups ($p = 0.83$). Treatment at day 1 after MCAO with MSCs did not improve functional recovery at day 4 ($p = 0.50$), but significantly improved functional recovery, as evidenced by improved mNSS, at day 14 compared with ischemic rats treated with PBS ($p < 0.01$, Figure 6).

Effects of MSCs on expressions of IL-10 and TNF-α in the brain tissue

RT-PCR analyses showed that the expressions of TNF-α and

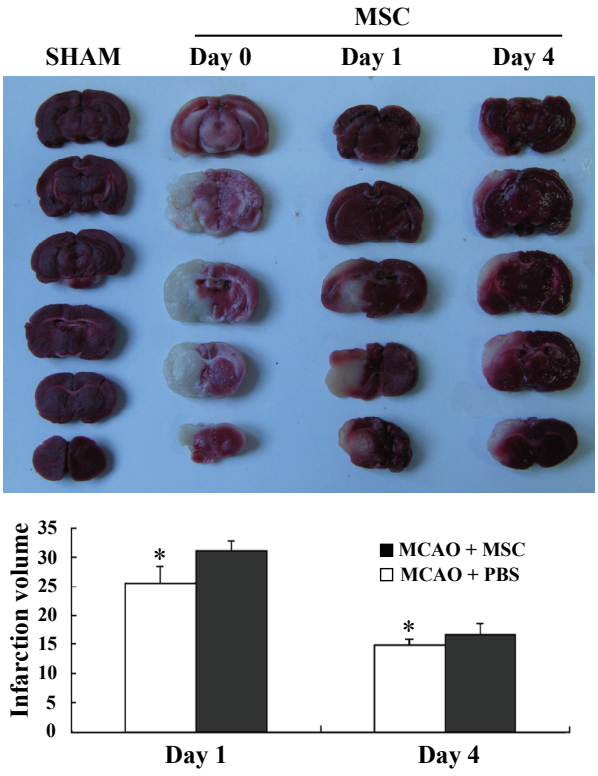

Figure 5. The protective effects of MSCs on rat cerebral ischemia injury. Illustrative TTC-stained coronal sections showing infarct area in the cerebral hemisphere as a distinct pale stained area Data are expressed as the mean ± SD (n = 6), *$p < 0.05$ *vs* PBS.

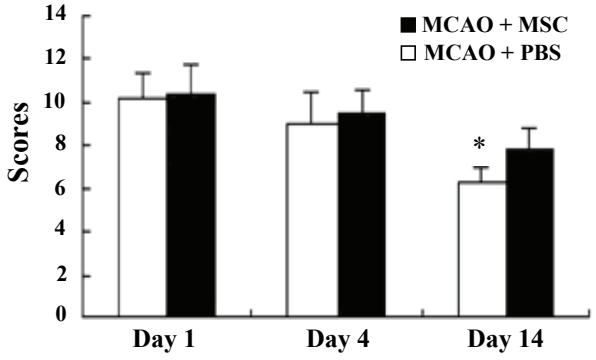

Figure 6. MSCs improves neurological function. A modified neurological severity score (mNSS) was used to measure neurologic function. Data are expressed as mean ± SD (n = 6), *$p < 0.01$ *vs* PBS.

IL-10 mRNA were seldom in the brain of sham groups and both increased in the brain of cerebral ischemia/reperfusion rats (Figures 7A and 7B); treatment with MSCs significantly decreased the expression of TNF-α mRNA, while the expression of IL-10 mRNA significantly elevated. These results from RT-PCR were next confirmed by ELISA at protein levels ($p < 0.05$) (Figure 7C). Compared to the sham operation group, TNF-α and IL-10 in ischemic brain tissue homogenate both increased after MACO. Treatment with MSCs significantly decreased the expression of TNF-α in tissue homogenate and significantly increased the expression of IL-10.

Discussion

In the present study, we showed that the transplantation of MSCs significantly improved neurological functional outcome and decreased the infarction volume after permanent MCAO. Furthermore, the present study demonstrated that MSCs transplantation could enhance the level of IL-10 and decrease the level of TNF-α in the cerebral ischemia area. The MSCs of male rats were transplanted into brains of females, and the expression of SRY was detected after the transplantation. As time proceeded, the SRY gene expression declined. These phenomena demonstrated that MSCs could survive after transplantation, but descend as time proceeded.

Many reports have demonstrated that intracerebral transplantation of MSCs after stroke significantly improves functional outcomes (15-18). Followed by the functional

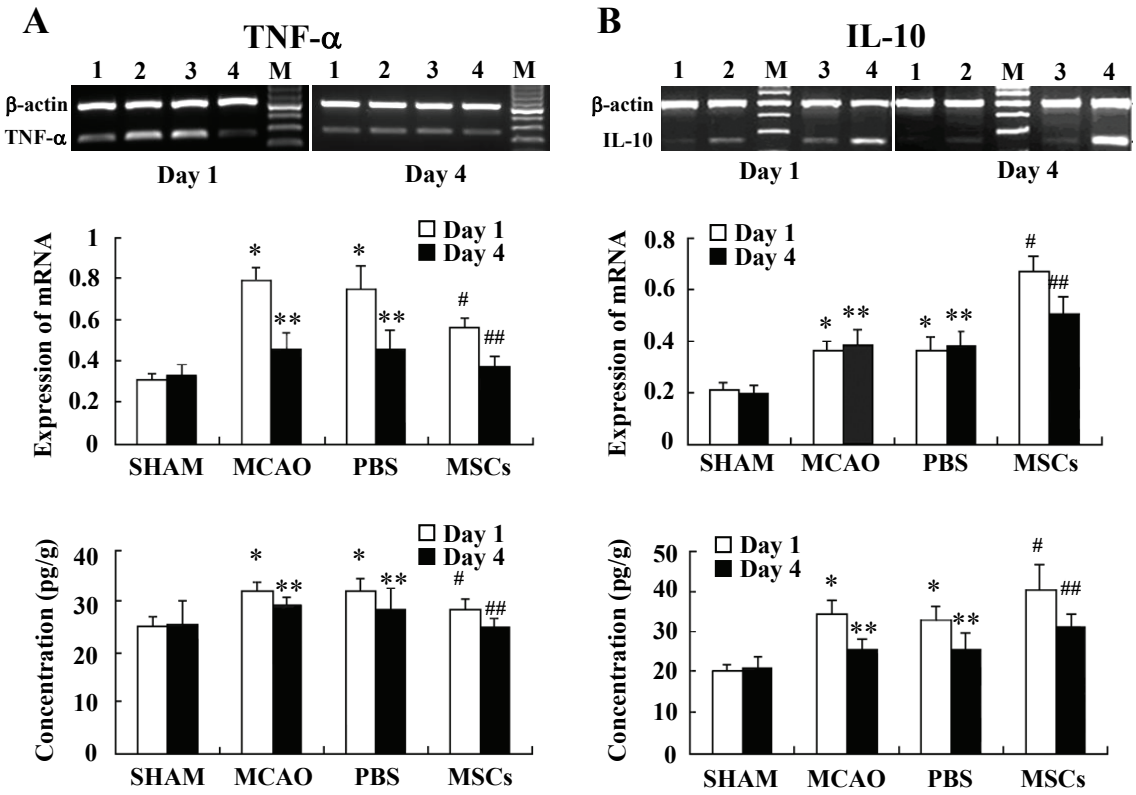

Figure 7. Effects of MSCs on the expressions of TNF-α and IL-10 in the brain tissue by RT-PCR and ELISA analyses. The expressions of TNF-α (A) and IL-10 (B) mRNA were investigated by RT-PCR analysis, and the levels of IL-10 and TNF-α in the tissues of ischemic area were measured by ELISA. Data are expressed as mean ± SD (n = 6). Lane M, DNA markers; Lane 1, sham; Lane 2, MCAO; Lane 3, vehicle; Lane 4, MSCs. * and ** *vs* sham group, $p < 0.05$; $^{\#}$ *vs* $^{\#\#}$, $p < 0.05$; $^{\#\#}$ *vs* **, $p < 0.05$.

recovery, new persistant synaptic linkages are reconstituted among the neurons. Therefore，no significant difference in functional recovery was found at day 1 and 4 after treated with MSCs, while rats had significantly lower NSS scores at day 14. The underlying mechanism of MSCs transplantation's protective role on cerebral ischemia is still uncertain. One possibility is that the MSCs integrate into the tissue, replace damaged cells, and reconstruct neural circuitry. Although multi-potential MSCs express protein phenotype of brain parenchymal cells, MSCs may have the potential to replace lost neurons. Few DAPI-labeled MSCs were observed 14 days after MCAO, which partially demonstrated this theory. But there were four possibilities in the present study. First, MSCs can integrate into brain tissue and differentiate into neurocytes, such as gliocytes, astrocytes and neurons. Second, the microenvironment in the local ischemic area could induce the necrosis of MSCs, and MSCs transplanted to the intact adult brain were rejected by an inflammatory response. Third, the MSCs could migrate out of the ischemic area. Fourth, cell division and proliferation could result in the loss of DAPI labeling intensity.

Although MSCs express protein phenotype of brain parenchymal cells, there was no evidence that these cells truly differentiate and replace brain tissue. The mechanisms by which MSCs provide therapeutic benefit may be multi-pronged. MSCs express many cytokines known to play a role in hematopoiesis and also supply many factors that influence the cells of the marrow microenvironment. MSCs within cerebral tissue can express these factors too, and it is the effect of these cytokines and trophic factors on brain tissue, which effectively promotes restoration of function (16). In this study, we focused on these two cytokines IL-10 and TNF-α after the transplantation of MSCs, in parallel with behavioral and histological assays of ischemic recovery.

IL-10 is an anti-inflammatory cytokine. It prevents downregulation of the anti-apoptotic protein Bcl-2 in ischemic brain tissue and limits excitotoxic damage to the brain through an activin-dependent mechanism (19). We and other researchers reported that ischemic insult and IL-10 administration augmented neurogenesis in the rodent brain. These effects of IL-10 were thought to contribute to infarct volume reduction and neurological improvement after experimental focal cerebral ischemia (19-22). It also reported previously that in IL-10 knockout in rats for a MCAO could augment neurological deficits and increase infarction volume (23). Recent animal experiments demonstrated that MSCs transplantation alone improved neurological deficits in cerebral infarction. TNF-α is a pro-inflammatory cytokine, also expressed in brain tissue following cerebral ischemia (24, 25). It is one of many specific molecules thought to trigger neuron death in the penumbra after a stroke. High TNF-α concentrations cause neuron apoptosis through a caspase-8-mediated pathway. TNF-α production increases after ischemia *in vivo*, and depleting TNF-α receptors can reduce neuron damage (26, 27). To maintain neural function and protect neuronal cells against neurotoxicity, it was thus important to enhance the induction of IL-10 and inhibit the induction of TNF-α under pathological conditions.

In this study, we observed following the improvement of the neural function, an increase of IL-10 and a decrease of TNF-α expression. This finding showed that MSCs were capable of inducing IL-10 expression and inhibiting TNF-α expression, which provided a possible link between these two cytokines and MSCs. Considering the biological role of IL-10 and TNF-α mentioned before, and based on data about cell therapy with MSCs after MCAO, we proposed the following model for how MSCs might be applicable to achieve the functional recovery after ischemia. The reduction of ischemic injury by MSCs may be partly caused by inhibition of TNF-α and elevation of IL-10. Although the present study was not designed to investigate mechanisms of how MSCs upregulate IL-10 and downregulate TNF-α, a possible tie between the MSCs and parenchymal cell local production of growth factors may be related to the ability of MSCs to produce a wide variety of trophic factors and cytokines, some of which activate the production of IL-10 and decrease the production of TNF-α. For instance, MSCs can secrete basic fibroblast growth factor (bFGF), and bFGF decreases expression of TNF-α (28-30). IL-10 and TNF-α were not measured at day 14 after MCAO, because the inflammation was occurred in the preliminary stage of ischemic injury.

Some MSCs could differentiate into neural cells and express neural cell phenotype, but there was no evidence that these cells develop contacts with other neurons and replace brain tissue. Further research is required to precisely demonstrate the mechanisms of how the MSCs upregulate IL-10 and downregulate TNF-α, and what the MSCs differentiate into.

In summary, the transplantation of MSCs can improve neurological function and decrease the infarction volume after cerebral ischemic injury. The possible mechanisms were the upregulation of IL-10 expression and the down-regulation of TNF-α expression.

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