Original Paper

Intravenously Delivered Allogeneic Mesenchymal Stem Cells Bidirectionally Regulate Inflammation and Induce Neurotrophic Effects in Distal Middle Cerebral Artery Occlusion Rats Within the First 7 Days After Stroke

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Key Words

Cerebral ischemia • Mesenchymal stem cell • Microglia • Macrophages • Transplantation

Abstract

Background/Aims: Neurotrophic effects and immunosuppression are the main therapeutic mechanisms of mesenchymal stem cells (MSCs) in stroke treatment. Neurotrophins are produced by graft cells, host neurons, astrocytes, and even microglia/macrophages. Meanwhile, MSCs can increase inflammation if they are not sufficiently induced by pro-inflammatory cytokines. We examined whether intravenously transplanted bone marrow MSCs (BM-MSCs) increase inflammation in distal middle cerebral artery occlusion (dMCAO) rats, how long the increased inflammation effect persists for, and what the final therapeutic outcomes will be. We also tested the neurotrophic role of BM-MSCs and attempted to identify the neurotrophin-producing cells. **Methods:** At 1 h after dMCAO was performed on Sprague-Dawley rats, allogeneic BM-MSCs were transplanted intravenously. The infarct volume was examined by Tetrazolium Red staining at 2 days (day 2), and the behavioral tests (cylinder test and grid walking test) were performed at 2, 4 (day 4) and 7 days (day 7) after transplantation. The concentrations of inflammation related cytokines and neurotrophins in the ischemic cortex, ipsilateral striatum, and serum, were measured using ELISA at days 2-7. The cell source of neurotrophins was observed by immunohistochemistry. **Results:** The transplanted cells were mainly found in the infarct border

zone (IBZ) of the brain. Infarct volume was reduced and behavioral outcomes were improved at 2 days after ischemia. In the striatum and circulation, BM-MSC transplantation increased inflammation at day 2 and decreased it at day 7. At days 2-7, insulin-like growth factor-1 (IGF-1) and brain-derived neurotrophic factor (BDNF) concentrations in the ischemic core of the cortex were significantly higher in the BM-MSC group than in the ischemia vehicle group. IGF-1 and BDNF were derived mainly from host microglia/macrophages in the ischemic core, and transplanted cells in the IBZ. At day 2, BM-MSC transplantation significantly increased the number of IGF-1⁺CD68⁺ and BDNF⁺Iba-1⁺ double positive cells in the ischemic core cortex.

Conclusions: Although increased inflammation, BM-MSCs were still beneficial to dMCAO recovery at day 2. The immunopromoting effect of MSCs was transient and shifted to an immunosuppressive action at day 7. The neurotrophic factors IGF-1 and BDNF, which were mainly derived from transplanted BM-MSCs and host microglia/macrophages, contributed to the therapeutic effects from day 2 to day 7.

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Introduction

Cell therapy has developed quickly and may potentially promote the functional recovery of many central nervous system disorders, including ischemic stroke. Among the cell candidates, bone marrow mesenchymal stem/stromal cells (BM-MSCs) have unique advantages in that they can be used for allogeneic or autologous transplantation without raising ethical or immunological problems [1].

Most of the preclinical studies using the systemic delivery of stem cells in stroke models have chosen the acute phase as the transplantation time (ie, 1 h to 2-3 days after stroke), and the therapeutic outcomes are usually measured at 2 weeks, 4 weeks, or longer after cell transplantation. To date, the therapeutic effects and mechanisms in the acute phase (within 48 h) and sub-acute phase (within 4-7 days after ischemia) still remain to be addressed. On the basis of current knowledge, the neuro-protective effects of intravenous mesenchymal stem/stromal cell (MSC) transplantation have mainly been ascribed to anti-inflammatory and neurotrophic effects, but not differentiation into neurons or astrocytes [2].

MSCs are capable of inhibiting the proliferation of lymphocytes *in vitro* and attenuating graft-versus-host disease (GVHD) *in vivo*. However, a number of studies have found that instead of alleviating GVHD, MSC transplantation exaggerated it in some cases [3]. Wei et al. suggested that this is because the immunosuppressive capacity of MSCs is not innate but must be induced under inflammatory conditions [3]. If the concentration of pro-inflammatory cytokines in the microenvironment is not sufficient to induce MSCs to become immunosuppressive, MSCs may increase the proliferation of lymphocytes *in vitro* and prompt inflammatory reactions *in vivo* [3]. Furthermore, it usually takes several days to induce MSCs to become immunosuppressive [4]. These results suggest that in the acute phase of non-severe ischemic stroke, such as in the distal middle cerebral artery occlusion (dMCAO) model, pro-inflammatory cytokines may not induce transplanted MSCs to suppress inflammation, but may actually increase inflammation. This is the first issue we examined in the present study.

The second issue is how long the pro-inflammatory effects of MSCs persist for. Do BM-MSCs shift from immunopromotion to immunosuppression? *In vitro* experiments found that 400 pg/mL is the threshold for pro-inflammatory cytokines to induce MSCs to be immunosuppressive [4]. Whether pro-inflammatory cytokines in the microenvironment of the dMCAO model can induce MSCs to become immunosuppressive is not known.

The third issue is the involvement of neurotrophic factors secreted from BM-MSCs and evoked host cells in neuroprotection after MSC transplantation in the dMCAO model. In addition to their anti-inflammation function, MSCs are capable of producing neurotrophic cytokines. MSCs also induce the production of many trophic cytokines, including BDNF [5], glial cell line-derived neurotrophic factor (GDNF) [6], and IGF-1, by host parenchymal cells [7, 8]. These neurotrophic cytokines, no matter whether they are derived from transplanted

MSCs or evoked host cells, have been suggested to play an important role in neuroprotection, and finally may facilitate functional recovery. Many studies have compared the neurotrophic and growth factor-producing ability of BM-MSCs, adipose MSCs, and umbilical cord MSCs, with the aim of identifying the best choice for cell therapy [9-13]. If neurotrophic factors from host cells are more directly related to functional recovery after ischemia, the ability of MSCs to induce host cells to produce neurotrophic factors should also be taken into consideration when choosing the best candidate for MSC therapy.

We are also interested in the sources of neurotrophic factors, especially the role played by microglia/macrophages. In the dMCAO model, resident neurons and microglia cells, which do not completely die, and macrophages that migrate into the ischemic core may be responsible for the recovery of the damaged brain. Microglia and macrophages are the main components of neuro-inflammation and are believed to elicit secondary injury of stroke [14, 15]. However, a growing body of research has provided support for dual roles for microglia and macrophages, both beneficial and harmful. Microglia/macrophage activation is thought to benefit the injured brain by removing cellular debris and producing neurotrophic factors [16].

Two phenotypes have been used to identify activated microglia/macrophages. The M1 phenotype represents a detrimental state, characterized by the high expression of pro-inflammatory mediators, which propels the pathological process of cerebral ischemia. The M2 phenotype can increase neuronal survival and restricts brain damage after ischemic injury, and M2 is usually associated with the high expression of BDNF, interleukin-10 (IL-10), transforming growth factor-beta (TGF- β), IGF-1 [17, 18], and so on. Hu et al. described the temporal kinetics of microglia/macrophage polarization in a mouse model of middle cerebral artery occlusion (MCAO). Usually within 7 days after the onset of ischemia, cells with the M2 microglia/macrophage phenotype are recruited to the infarct area, which may represent an endogenous effort to remove injured tissue and restrict brain damage. Then, M1 microglia/macrophages, which are characterized by reduced phagocytosis and the increased secretion of pro-inflammatory mediators, begin to dominate the injured brain region [19]. A similar M2-to-M1 shift has also been reported in models of brain trauma and spinal cord injury, suggesting that the phenotypic changes in microglia/macrophages may be a common pathologic mechanism underlying multiple types of central nervous system injuries [20]. On the basis of this evidence, we hypothesized that microglia/macrophages in the ischemic core of dMCAO rats have a role in the protection of the brain during the acute and sub-acute phases.

Materials and Methods

Isolation and characterization of rat BM-MSCs

BM aspirate was isolated from adult male Sprague-Dawley rats. The cells were seeded at a density of 2.0×10^5 cells/cm² in low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin (Invitrogen, Carlsbad, CA). The medium was changed every 3 to 4 days until the adherent fibroblast-like cells reached 70-80% confluence. Plastic-adherent MSCs were collected and re-suspended in fresh culture medium and then sub-cultured in new flasks.

Cell-surface markers (CD44, CD73, CD90, and CD105) (BD Bioscience, Sparks, MD) were used to characterize the MSCs at 3-5 passages. To assess adipogenic, osteogenic, and chondrogenic differentiation, rat BM-MSCs at the 3rd to 4th passages were used. Induction was performed according to previously published protocols [21, 22].

Animals

All animal procedures were approved by the Laboratory Animal Ethics Committee of Xuanwu Hospital and performed in accordance with the Laboratory Animal Guide. All animals were maintained in a specific-pathogen-free facility. Ninety adult male Sprague-Dawley rats (8 weeks old, 280-320g) were divided into 3 cohorts. In each cohort, 30 rats were randomly allocated to sham, ischemia vehicle, and transplantation

(ischemia+rMSCs) groups (n = 10 per group). The 3 cohorts were used for infarct volume measurement, cytokine level analysis, and immunofluorescent staining, respectively.

dMCAO model surgery

The rats were initially anesthetized with 3.5% isoflurane and maintained with 1.0-2.0% isoflurane in 2:1 N₂O:O₂ using a face mask. Permanent occlusion was induced by electrocauterization of the distal portion of the MCA in conjunction with ligation of both common carotid arteries for 30 min [23, 24]. During surgery, body temperature was maintained at 37°C using a heating pad. This procedure resulted in a consistent infarction in the area of the sensorimotor cortex governing forelimb and hind limb function and allowed for the assessment of neurological outcomes.

MSC labeling and transplantation

Half of the animals (sham, ischemia vehicle, and ischemia+rMSC groups, n = 5 per group) used for immunofluorescent staining were transplanted with MSCs that were labeled with CM-DiI, a lipophilic carbocyanine dye with red fluorescence. The labeling procedure was in accordance with the manufacturer's instructions. In brief, at 1 day before transplantation, the MSCs (80% confluence) were incubated with CM-DiI (1 µg/mL) for 5 min at 37°C and then for an additional 15 min at 4°C, and finally they were washed with phosphate-buffered saline and cultured in fresh medium. The next day, approximately 1.0×10⁶ MSCs in 1000 µL of 0.9% saline were administered via intravenous injection at 1 h after ischemia in the MSC groups. Saline (0.9%, 1 mL) was given as an injection to the ischemia vehicle group (n = 10).

Cylinder test

The cylinder test provides a way to evaluate spontaneous forelimb use in rodents and has been used in a number of motor system injury models of stroke [25]. To evaluate forelimb deficits, the animal is placed in a transparent plexiglas cylinder and observed. Animals with unilateral brain damage will display asymmetry in forelimb use during vertical exploration. The test procedure was in accordance with the method of Hua et al. [26].

Grid walking

The grid walking task has been found to be an objective measure of motor coordination deficits and rehabilitation effects after stroke [25]. The animal was placed on an elevated grid with a frame size of 3.3 × 3.3 cm. Animals without brain damage will typically place their paws precisely on the wire frame to hold themselves while moving along the grid. Each time a paw slips through an open grid, a "foot fault" was recorded. The number of both contralateral and ipsilateral faults for each limb was compared to the total number of steps and then scored using a foot fault index. Intact animals will generally demonstrate few to no foot faults, and when faults occur, they do so symmetrically. Ischemic animals typically make significantly more contralateral foot faults than do intact animals.

Evaluation of infarct volume

The rats used for the determination of infarct volume were decapitated after deep anesthesia at 48 h after dMCAO. The brains were removed and rapidly frozen in liquid nitrogen vapor. Cryostat sectioning was performed, and six 2-mm-thick sections for each rat were analyzed. Triphenyltetrazolium chloride (TTC) dye staining was used to confirm the presence of infarction sections, which were imaged and measured using Image J 3.0 (National Institutes of Health, Bethesda, MD) software. To minimize the error introduced by edema, which distorts and enlarges the infarcted tissue and surrounding white matter, infarct areas for each section were calculated by subtracting the area of intact tissue in the ipsilateral hemisphere from the corresponding area of the contralateral hemisphere. Infarct volumes were calculated by multiplying the summarized infarct areas (all 6 sections) by the interval distance between the sections (2 mm in this experiment) [27].

Immunohistochemistry and cell counting

Under deep anesthesia with a lethal dose of sodium pentobarbital (0.1 g/kg), the rats were perfused with 0.9% saline and 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Then, the brains were dissected out and post-fixed in the same fixative for 1 day. Coronal sections (40 µm) were prepared using

a vibratome (Leica, Wetzlar, Germany) and immunostaining according to the standard procedures with antibodies to CD68 (Chemicon, Temecula, CA; 1:200), Iba-1 (Wako Pure Chemical Industries, Osaka, Japan; 1:300) NeuN (Abcam, Cambridge, MA; 1:500), BDNF (Boster, Wuhan, China), GDNF (Boster, Wuhan, China), and IGF-1 (Boster, Wuhan, China) was performed. An FITC- or Cy3-conjugated secondary antibody (Jackson ImmunoResearch, Inc., West Grove, PA; 1:300) was applied for 2 h at room temperature, followed by DAPI treatment for 20 min to stain nuclei. After being washed, double labeling of neurotrophins (BDNF or IGF-1) and neurons or microglia/macrophages (NeuN, CD68, and Iba-1) was also performed.

After being mounted onto slides, digital images were acquired on a confocal laser scanning microscope (TCS SP5 II; Leica, Wetzlar, Germany) at 200 × magnification. The confocal settings, such as gain and offset, were maintained to ensure all images were collected with the same parameters.

Single labeled BDNF, IGF-1, NeuN, CD68, and Iba-1-positive cells, together with double labeled cells were counted in the infarct area. The estimated cell numbers were determined using the method of Gelosa et al. based on view field size and section thickness [27]. The number of labeled cells was calculated in 5 coronal sections from each rat, located between -2.0 mm and 2.0 mm to the bregma, and expressed as the mean number of cells per 0.0256 mm³. The counting areas were located 400 μm from the imaginary border between the ischemic core and peri-core area, so the counting area was in the infarct. For each section, the cells were counted in 2 squares of the dorsal infarct area and averaged. Each square was 0.64 mm² (0.8 mm × 0.8 mm=0.64 mm²), so the calculated volume was 2 × 0.0256 mm³ (0.64 mm² × 0.04 mm=0.0256 mm³) for each square, and 2 × 0.0256 mm³ for each slide.

All analyses were performed by investigators blinded to sample identity and treatment groups.

Analysis of cytokine levels by enzyme-linked immunosorbent assays

On days 2, 4, and 7 after the induction of stroke, a total of 10 rats in each group were sacrificed by anesthetization with 10% chloral hydrate solution (0.3 mL/100 g, i.p.).

Brain tissue samples were obtained from the infarct cortex and peri-infarct striatum, as shown in the rectangles in Fig. 2A. Proteins were extracted with a lysis buffer containing 1% NP-40, 1% Triton-X 100, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

Blood was obtained from the abdominal aorta. Subsequently, the blood samples were allowed to clot at room temperature. The clots were removed by centrifuging at 1000 × g for 10 min to obtain serum. The serum was apportioned into 0.5-mL aliquots, and stored at -80°C for further use.

The levels of TNF-α, IL-1β, interferon (IFN)-γ, IL-6 (Peprotech, Rocky Hill, NJ), IL-4, and IL-10 (eBioscience, San Diego, CA) were measured using enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The levels of BDNF, GDNF, and IGF-1 (eBioscience) were also evaluated by ELISAs. The loading amount of each well was 40 μg brain total protein and the loading volume for each well was 100 μL.

Statistical analysis

All data from functional tests, infarct volume assays, and ELISAs are expressed as the mean ± standard deviation. Data were tested for normal distribution by the Kolmogorov-Smirnov test, and then analyzed by repeated-measures of variance and unpaired Student's t-test in SPSS version 16. Two-tailed probability values P<0.05 were considered statistically significant.

Results

Culture and identification of rat MSCs

By passages 3–5, rat BM-MSCs became a nearly homogeneous population of cells (Fig.1A), and were found to be CD73⁺, CD90⁺, CD105⁺, and CD29⁺, but CD34⁻ (Fig.1B–G). The rat BM-MSCs were capable of adipogenic, osteogenic, and chondrogenic differentiation (Fig. 1H–J).

Infarct volume and behavioral tests

We investigated the effects of rat MSC (rMSC) transplantation on infarct volume at 48 h post-transplantation (49 h post-dMCAO) by TTC staining. The dMCAO induced ischemic

lesions mainly in the cortex. Both the caudo-putamen and hippocampus were marginally involved (Fig. 2A).

Compared to non-transplanted dMCAO rats, infarct size in the brains of MSC transplanted rats was significantly reduced. The mean infarct volume (white area) in the ipsilesional hemisphere of dMCAO rats was $29.46 \pm 5.76\%$ and $22.68 \pm 5.51\%$ in MSC transplanted rats ($P < 0.05$; Fig. 2B, 2E).

The cylinder and grid walking tests showed that motor and sensory functions were impaired by the ischemic insult. At 1 day after treatment, there was significant functional recovery in the cylinder test ($P < 0.01$) and grid walking test ($P < 0.05$) between the MSC group and ischemia vehicle group (Fig. 2C-2D). At day 3, the MSC transplantation group showed a significant improvement compared with the ischemia vehicle group in both tests (Fig. 2C-2D). However, the treated groups showed no significant improvement at 5 and 7 days after ischemia.

Migration of transplanted MSCs into the brain

Half of the animals received CM-DiI labeled MSC transplantation. At 1 day before transplantation, the MSCs were labeled with CM-DiI. After incubation, $99.9 \pm 5.07\%$ MSCs were labeled successfully with CM-DiI and showed bright red fluorescence (Fig. 2F-H).

At 48 h after transplantation, CM-DiI-labeled cells were found in the brain. After cell counting, the total number of surviving cells in the brain was estimated as $1.2-1.5 \times 10^5$ cells. The majority (70-80%) of these cells were located in the infarct boundary zone (IBZ) (Fig. 2I-2K), and a subset of these cells (20-30%) migrated into and were scattered throughout the infarct area.

Fig. 1. Typical cytometric marker test and differentiation of rat MSCs in passages 3-5. A: Rat MSCs at passage 5. B: Negative control. C-F: Rat MSCs at passages 3-5 were CD44-, CD73-, CD90-, and CD105-positive. G: CD34-negative. At passages 3-5, the MSCs derived from rat bone marrow were investigated for their in vitro multilineage differentiation ability. H: Adipogenesis was detected by the formation of neutral lipid vacuoles and were stained red with Oil Red O. I: Osteogenesis was demonstrated by the formation of the mineralized matrix and Alizarin Red staining. J: Cells after chondrogenic differentiation were stained with Alcian Blue. Scale bar = $100 \mu\text{m}$. $n = 5$.

Increment and decrement of inflammatory cytokines after MSC transplantation in the ischemic core

At day 2, except for a reduction in the level of IL-10 after the onset of ischemia, the other cytokines were not changed significantly by the ischemic injury. This finding indicated that, at least at this time, inflammation in the core area was not a significantly detrimental factor (Fig.3A-3F).

At day 4, possibly due to the ischemic injury in the core and consequent cell apoptosis in this area, except for no change in IFN- γ , all of the cytokines tested were lower in the ischemia vehicle group than in the sham group (Fig.3A-3F). At day 7, IL-6 and IL-10 were lowered by ischemia as compared with the sham group (Fig.3A-3F).

Given the effects of MSC transplantation on the levels of inflammatory cytokines at days 2 and 4, the concentrations of TNF- α and IL-1 β were increased significantly in the MSC transplantation group (TNF- α at days 2 and 4: 425.75 ± 50.45 pg/mL and 405.18 ± 122.68 pg/mL; IL-1 β at days 2 and 4: 131.76 ± 8.35 pg/mL and 109.68 ± 20.84 pg/mL, respectively) compared to the ischemia vehicle group (TNF- α at days 2 and 4: 314.11 ± 26.84 pg/mL and 252.80 ± 55.03 pg/mL; IL-1 β at days 2 and 4: 86.39 ± 6.32 pg/mL and 78.31 ± 16.75 pg/mL, respectively).

As an anti-inflammatory cytokine, IL-4, was decreased by MSC transplantation on days 4 and 7 (Fig.3A-3F). The IL-4 concentrations at days 4 and 7 were 128.435 ± 14.27 pg/mL and 159.98 ± 28.81 pg/mL, respectively, and BM-MSC transplantation significantly decreased them to 105.49 ± 11.03 pg/mL and 119.85 ± 14.51 pg/mL, respectively.

These results indicate that instead of decreasing the levels of pro-inflammatory cytokines, MSC

Fig. 2. Therapeutic effects of rat MSC transplantation and the migration of MSCs into the brain. The day of ischemia onset and transplantation was set as day 0. The day before ischemia onset was day -1. Infarct volume was observed at day 2, while behavioral outcome tests were performed at days 1, 3, 5, and 7. A: Representative infarct of the ischemia vehicle group. B: Representative infarct of the ischemia and rMSC transplantation groups. "□" in the third slice: the upper left rectangle represents the border between the infarct and peri-infarct areas, where images I-K were photographed. The upper right and lower rectangles represent the infarcted cortex and ipsilateral striatum area, respectively, where brain tissues for ELISA were sampled. C: The cylinder test found that the rMSC transplantation group showed a significant improvement compared with the ischemia vehicle group at days 1 and 3 (* $P < 0.05$, ** $P < 0.01$). D: Similarly, the rMSC transplantation group showed a significant improvement in the grid-walking test as compared with the ischemia vehicle group (* $P < 0.05$, ** $P < 0.01$). E: Infarct volume was significantly reduced by MSC transplantation (* $P < 0.05$). $n = 10$. F-H: More than 95% BM-MSCs are CM-Dil-positive before transplantation. I-K: At 48 h after transplantation, most of the transplanted cells were located in the infarct boundary zone. Scale bar = $50 \mu\text{m}$.

Fig. 3. Inflammation-related cytokines change after MSC transplantation in the ischemic core at days 2, 4 and 7. A-F: TNF- α , IL-1 β , IL-4, IL-6, and IL-10 in the ischemic core cortex were down-regulated by ischemia at day 4, while IL-6 and IL-10 were up-regulated by ischemia at day 7 (# P < 0.05, ## P < 0.01, compared with the sham group). At days 2 and 4, TNF- α and IL-1 β increased significantly after the administration of BM-MSCs. At days 4 and 7, IL-4 levels were still significantly lowered by BM-MSCs in the transplantation group compared with the ischemia vehicle group (* P < 0.05, ** P < 0.01, compared with the ischemia group). n = 10.

transplantation increased their levels in the ischemic core area at days 2 and 4. However, the immunosuppressing effect of MSCs co-existed with an immunopromoting effect in the ischemic core of the cortex. At day 2, IL-6 was significantly lower in the transplantation group (120.40 ± 18.30 pg/mL) than in the ischemia control group (161.94 ± 40.22 pg/mL). After MSC

transplantation, IL-10 was increased from 259.13 ± 42.84 pg/mL to 384.40 ± 45.22 pg/mL at day 2. In addition, at day 7, IL-6 was decreased by MSC transplantation from 143.01 ± 38.33 pg/mL in the ischemia vehicle group to 105.70 ± 17.14 pg/mL in the MSC transplantation group.

Fig. 4. Inflammatory cytokines are up-regulated at days 2-4 and down-regulated later in the serum by MSC transplantation. In the serum, ischemia increased TNF-α at days 2 and 7, increased IL-1β at days 2, 4, and 7, and lowered IL-6 at day 4 (#P < 0.05, ##P < 0.01, compared with the sham group). MSC infusion increased IL-6 at day 2. TNF-α and IL-6 were both increased at day 4 by BM-MSCs. The anti-inflammatory effect of MSCs was also found for IL-1β and TNF-α at day 7 (*P < 0.05, **P < 0.01, compared with the ischemia group). n=10.

Inflammatory cytokines are increased early and decreased later in serum after MSC transplantation

A growing body of evidence shows strong correlations between periphery lymphoid organs, such as the spleen, and the therapeutic effects of systemically administrated MSCs in cerebral ischemia. Splenectomy studies demonstrated a causal link between the spleen and cerebral ischemia damage, providing direct evidence that key inflammatory mediators

Fig. 5. Inflammatory cytokines are increased early and decreased later in the striatum after MSC transplantation. In the striatum, ischemia increased TNF-α, IL-1β, and IL-4 at day 4, and increased IL-10 at days 2 and 4 (#P < 0.05, ##P < 0.01, compared with the sham group). IL-10 decreased after MSC transplantation at day 2 and could be considered immunopromoting. TNF-α was reduced by the transplantation of BM-MSCs at days 4 and 7 (*P < 0.05, **P < 0.01, compared with the ischemia group). n=10.

(cytokines, chemokines, and inflammatory cells) released by the spleen contribute directly to ischemic brain injury [28]. This is the case for severe stroke; however, the relative importance of systemic infused MSCs in changing the peripheral inflammatory status remains to be determined in the dMCAO model.

We found that the levels of many pro-inflammatory cytokines were not increased and some were even lowered by ischemia in the ischemia-damaged cortex. Inflammation in the peripheral circulation caused by dMCAO did not follow the same trend (Fig.4A-4F). Among the inflammatory cytokines tested, IL-1 β at 2, 4, and 7 days after ischemia onset, together with TNF- α at days 2 and 7, were significantly higher in the ischemia vehicle group than in the sham group. This finding suggests that, different to that observed in the ischemic cortex, inflammation was enhanced in the blood after the induction of ischemia. In contrast, IL-6 levels were decreased at days 2 and 4 in the ischemia vehicle group compared with the sham group (Fig.4A-4F).

Given the immuno-modulatory function of MSCs in serum, up-regulation in the early phase (days 2-4) and later down-regulation (days 4-7) were both found. The levels of IL-6 at day 2, together with TNF- α and IL-6 at day 4, were increased following the infusion of MSCs. The concentrations of IL-6 at days 2 and 4 were increased from 53.34 $\pm$ 6.83 pg/mL and 50.81 $\pm$ 2.98 pg/mL to 67.77 $\pm$ 5.49 pg/mL ($P < 0.01$) and 69.79 $\pm$ 7.15 pg/mL ($P < 0.01$), respectively. TNF- α at day 4 was increased from 57.37 $\pm$ 17.13 to 83.53 $\pm$ 20.96 pg/mL ($P < 0.01$).

The anti-inflammatory effect of MSCs was also found at day 7 (Fig.4A-4F). TNF- α was decreased by MSC transplantation from 62.79 $\pm$ 10.17 pg/mL to 54.41 $\pm$ 6.08 pg/mL ($P < 0.01$). IL-1 β was decreased by MSC transplantation from 85.34 $\pm$ 23.48 pg/mL to 54.05 $\pm$ 18.45 pg/mL at day 7 ($P < 0.05$).

Inflammation increases early and decreases later in the striatum after MSC transplantation

As compared with the sham group at day 4, TNF- α and IL-1 β were increased in the ischemia vehicle group. IL-10 was also increased after ischemia in the striatum (Fig.5A-5F).

The immuno-modulatory effect of MSC transplantation in the striatum was complicated. As an anti-inflammatory cytokine, IL-10 was decreased in the striatum from 193.21 $\pm$ 19.95 pg/mL in the ischemia vehicle group to 155.16 $\pm$ 38.89 pg/mL after MSC transplantation at day 2 ($P < 0.05$), which could be considered as an immunopromoting effect. The levels of TNF- α at days 4 and 7 in the ischemia vehicle group were 638.31 $\pm$ 124.85 pg/mL and 533.95 $\pm$ 149.98 pg/mL, and were reduced by MSC transplantation to 412.54 $\pm$ 64.52 pg/mL ($P < 0.01$) and 405.35 $\pm$ 85.38 pg/mL ($P < 0.05$), respectively (Fig.5A-5F). These changes in the levels of TNF- α could be considered as MSC-induced immunosuppression.

BDNF and IGF-1 are increased in the ischemic core and striatum after MSC transplantation

The concentrations of BDNF at days 2 and 4 in the ischemia vehicle group (522.68 $\pm$ 151.67 pg/mL and 466.95 $\pm$ 89.66 pg/mL, respectively) were enhanced significantly by MSC transplantation to 724.13 $\pm$ 215.30 pg/mL ($P < 0.05$) and 562.52 $\pm$ 87.26 pg/mL ($P < 0.05$), respectively (Fig.6A).

The expression levels of the neurotrophic factor IGF-1 in the ischemia cortex were 394.13 $\pm$ 206.65 pg/mL, 796.48 $\pm$ 169.57 pg/mL, and 268.85 $\pm$ 105.41 pg/mL at days 2, 4, and 7, respectively, and were increased significantly by MSC transplantation to 571.04 $\pm$ 188.93 pg/mL ($P < 0.05$), 967.09 $\pm$ 212.81 pg/mL ($P < 0.05$), and 448.39 $\pm$ 153.53 pg/mL ($P < 0.05$), respectively (Fig.6B). However, for GDNF, its levels were not significantly higher in the MSC transplantation group than those in the ischemia vehicle group at all 3 time points (Fig.6C).

The concentrations of BDNF, GDNF, and IGF-1 were also evaluated in the striatum. BDNF and GDNF concentrations were not significantly different between the ischemia vehicle and MSC transplanted rats. For IGF-1, its expression was significantly higher in the MSC group (99.35 $\pm$ 30.99 pg/mL) than in the ischemia vehicle group (64.24 $\pm$ 27.55 pg/mL) at day 2 ($P < 0.05$) (Fig.6D-6F).

Fig. 6. BDNF and IGF-1 are increased in the ischemic core and striatum after MSC transplantation A-C: In the ischemic core cortex, BDNF and IGF-1 were both enhanced by transplanted BM-MSCs in the core area at days 2 and 4. At day 7, IGF-1 was still significantly higher than in the ischemia vehicle group. GDNF was not found to be higher than in the ischemia vehicle group at all 3 time points. D-F: BDNF, GDNF, and IGF-1 concentrations were also evaluated in the striatum. BDNF and GDNF concentrations were not different between the ischemia vehicle and MSC transplanted rats. The IGF-1 levels of the MSC transplantation group were significantly higher than in the ischemia vehicle group at day 2 (* $P < 0.05$, ** $P < 0.01$). $n = 10$.

BDNF and IGF-1 are partly derived from transplanted MSCs in the IBZ at day 2

Another finding was that the neurotrophic factors were mainly derived from both host and grafted cells. By immunostaining in the IBZ, we found CM-DiI-labeled MSCs overlapped with a subset of cells expressing BDNF (Fig. 7A-7D) or IGF-1 (Fig. 7E-7H) at 2 days after transplantation. Approximately 20-30% of CM-DiI-labeled MSCs were positive for IGF-1 and 10-20% were positive for BDNF.

Fig. 7. Transplanted MSCs express BDNF and IGF-1 in the infarct border zone at 2 days after transplantation. A: BDNF immunostaining. B: Transplanted cells maintained the red fluorescence of CM-DiI. C: Merged image of BDNF, CM-DiI, and DAPI. D: High magnification of the rectangle shown in C. E: IGF-1. F: The red fluorescence of transplanted MSCs. G: Merged image of IGF-1, CM-DiI, and DAPI. H: High magnification of the rectangle shown in G. Arrow: Double labeled cells. Scale bar=50 μ m.

Fig. 8. Host neurons and microglia/macrophages express IGF-1 in the infarct area at 2 days after transplantation. A: IGF-1. B: NeuN. C: Merged image of IGF-1, NeuN, and DAPI. D: Amplified image of the square in C. E: IGF-1. F: CD68. G: Merged image of IGF-1, CD68, and DAPI. H: Magnified image of the square in G. Arrow: Double labeled cells. Scale bar=50 μ m.

Host microglia/macrophages are the main sources of IGF-1 and BDNF in the infarct area

By immunostaining, we found that IGF-1 was mainly located in the infarct area. A small portion of IGF-1 cells overlapped with cells expressing NeuN (Fig.8A-8D), while many IGF-1-positive cells co-localized with cells expressing CD68 (Fig.8E-8H), which is a marker of activated microglia/macrophages.

The number of CD68⁺ cells in the infarct area was not changed by MSC transplantation (Table 1). The number of IGF-1⁺ single and IGF-1⁺/CD68⁺ double positive cells in the infarct area of the ischemia vehicle group was 28.54 ± 19.24 cells and 11.86 ± 8.30 cells (per 0.0256

mm³), respectively. MSC transplantation significantly ($P < 0.01$) increased the number of IGF-1⁺ cells to 36.51 ± 13.08 cells and IGF-1⁺/CD68⁺ cells to 21.12 ± 7.17 cells per 0.0256 mm³. The percentage of IGF-1⁺/CD68⁺ double positive cells in the total number of CD68⁺ cells was significantly increased by MSC transplantation from $19.33 \pm 13.96\%$ to $35.68 \pm 12.89\%$ ($P < 0.01$).

After transplantation, the number of NeuN⁺ neurons was increased from 124.5 ± 31.33 to 155.16 ± 35.21 cells per 0.0256 mm³. However, the number of IGF-1⁺NeuN⁺ double positive cells and the percentage of these double positive cells in the total number of NeuN⁺ neurons were not changed (Table 1).

BDNF plays a crucial role in neuronal survival and plasticity. It is mainly synthesized by neurons and secondarily by microglia, and is distributed in substantial amounts in the peri-ischemic core cortex, ipsilateral striatum, and contralateral

Table 1. Number of IGF-1-expressing cells in the infarct core area of the dMCAO cortex. Cell density is shown as the number of cells per 0.0256 mm³. ** $P < 0.01$ as compared to the ischemia group. n=10

Cell marker	Ischemia	Ischemia+rMSC
IGF-1 ⁺	28.54 ± 19.24	$36.51 \pm 13.08^*$
CD68 ⁺	61.74 ± 35.21	65.36 ± 18.87
NeuN ⁺	124.5 ± 31.33	$155.16 \pm 35.21^{**}$
IGF-1 ⁺ CD68 ⁺	11.86 ± 8.30	$21.12 \pm 7.17^{**}$
(IGF-1 ⁺ CD68 ⁺)/CD68 ⁺	$19.33 \pm 13.96\%$	$35.68 \pm 12.89\%^{**}$
IGF-1 ⁺ NeuN ⁺	10.13 ± 5.93	14.42 ± 8.31
(IGF-1 ⁺ NeuN ⁺)/NeuN	$8.68 \pm 6.21\%$	$9.03 \pm 3.72\%$

Fig. 9. Host neurons and microglia/macrophages express BDNF in the infarct area at 2 days after transplantation. A, E, and I: BDNF-positive cells in the infarct area. White line in A is the border of the infarct cortex and peri-infarct area. BDNF distributed both in the infarct area and peri-infarct area. B, F, and J: Immunostaining of CD68, Iba-1, and NeuN, respectively. C: Merged image of A, B, and nuclear staining with DAPI. G: Merged image of E, F, and DAPI. K: Merged image of I, J, and DAPI. D, H, and L: High magnification of the rectangles shown in C, G, and K. Arrow: Double staining of BDNF-positive cells with CD68, Iba-1, and NeuN. Scale bar=50 μ m.

hemisphere [5, 29]. In our experiments, the distribution of BDNF was not restricted within the infarct area; however, we counted the cells in only the area of cortical infarction.

In the ischemic core cortex, BDNF was expressed in NeuN-positive neurons (Fig.9A-9D) and CD68-positive microglia/macrophages (Fig.9E-9H), but mainly in Iba-1-positive microglia/macrophages (Fig.9I-9L).

The quantity of BDNF⁺, Iba-1⁺, BDNF⁺Iba-1⁺ double positive cells, together with the percentage of BDNF⁺Iba-1⁺ cells in the total number of Iba-1⁺ cells (40.86±18.70/0.0256 mm³, 36.83±13.75/0.0256 mm³, 19.92±6.55/0.0256 mm³, and 60.15±28.20%, respectively) in the ischemic core were all increased after MSC transplantation to 50.91±20.68/0.0256 mm³, 62.83±21.20/0.0256 mm³, 58.92±23.37/0.0256 mm³, and 89.21±12.27%, respectively (Table 2).

Neither the BDNF⁺CD68⁺, BDNF⁺NeuN⁺ double positive cells nor the percentage of these double positive cells in the total number of CD68 microglia/macrophages or NeuN⁺ neurons were significantly changed by MSC transplantation (Table 2).

Discussion

The overall objective of this study was to assess the mechanisms underlying the therapeutic effects of the intravenous allogeneic transplantation of MSCs in the dMCAO rat model.

Immunomodulatory and neurotrophic effects are the two major functions by which transplanted MSCs exert their therapeutic effects. In many studies, the cells are transplanted stereologically into the brain or intravenously into the body at 7 days or more after ischemia. The mechanisms that are responsible for the protection or recovery of the ischemia-damaged brain in the acute (2 days) or subacute phase (4-7 days) after dMCAO are still not clear.

Our first concern was to explore whether intravenously transplanted MSCs increase cerebral or peripheral inflammation in the dMCAO model. There is strong evidence that the immunosuppressive capacity of MSCs is induced under inflammatory conditions. The pre-stimulation of MSCs with the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-1β not only increases their potency to inhibit lymphocyte proliferation but also inhibits the production of inflammatory cytokines [30]. Cytokine priming, such as IFN-γ pretreatment, have been reported in the therapeutic effect of MSCs to avoid GVHD-induced death [31]. Previous *in vitro* reports have demonstrated that MSC activation requires a threshold concentration of TNF-α and IFN-γ as high as 400 pg/mL [32]. If not fully activated, MSCs may increase the inflammatory reaction and exacerbate disease outcome [33].

Post-stroke inflammation, which is largely represented by pro-inflammatory cytokines, was not changed dramatically in the dMCAO model in our experiments. In the ipsilateral cortex and striatum, ischemia had no effect on the majority of pro-inflammatory cytokines at days 2 and 7. At day 4 in the ischemic cortex, instead of increasing after ischemia, the levels of TNF-α, IL-1β, IL-4, IL-6, and IL-10 were all decreased. In the ipsilateral striatum, some signs of increased inflammation were found at day 4 only. TNF-α, IL-1β, and IL-4 were increased in the ischemia group as compared with the sham group at this time.

Table 2. Number of BDNF-expressing cells in the infarct core area of the dMCAO cortex. Cell density is shown as the number of cells per 0.0256 mm³. **P<0.01 as compared to the ischemia group. n=10

Cell marker	Ischemia	Ischemia+rMSC
BDNF ⁺	40.86±18.70	50.91±20.68**
Iba-1 ⁺	36.83±13.75	62.83±21.20**
BDNF ⁺ Iba-1 ⁺	19.92±6.55	58.92±23.37**
(BDNF ⁺ Iba-1)/Iba-1 ⁺	60.15±28.20%	89.21±12.27%**
BDNF ⁺ CD68 ⁺	26.25±12.30	29.61±12.17
(BDNF ⁺ CD68 ⁺)/CD68 ⁺	42.30±31.20%	48.46±15.59%
BDNF ⁺ NeuN ⁺	11.50±6.20	12.68±4.41
(BDNF ⁺ NeuN ⁺)/NeuN ⁺	11.66±7.92%	10.80±6.62%

In the serum after ischemia, although TNF- α increased at days 2 and 7, and IL-1 β was elevated by ischemia at days 2, 4, and 7, their concentrations were lower than 400 pg/mL, the threshold required for inducing MSCs to become immunosuppressive [33]. These results suggest that shortly after ischemia, such as at days 2 and 4, BM-MSCs were not effectively activated or induced to be immunosuppressive in either the ischemic core cortex or in the peripheral circulation. Conversely, BM-MSCs promoted immune responses at these time points. After MSC transplantation, TNF- α and IL-1 β were increased at days 2 and 4, while IL-4 was decreased at days 4 and 7 in the ischemic core area. In addition, IL-10 was decreased in the striatum at day 2. As IL-4 and IL-10 are usually considered as anti-inflammatory cytokines [34], their decrease after MSC transplantation was also considered to promote inflammation [32].

In the serum, TNF- α at day 4 and IL-6 at days 2 and 4 were also significantly increased by MSC transplantation. IL-6 appears to have pleiotropic roles [35]. Usually, it is considered as a pro-inflammatory cytokine. There is evidence that IL-6, which is released by many hematogenous cells in response to injury and infection, plays a role in promoting T cell and B cell growth and is a predictive marker of stroke outcome [36]. Elevated levels of circulating IL-6 are found after stroke [37] and are correlated with infarct size [38] and poor outcome [39].

We can conclude that the immunomodulation of MSCs at 2 days after dMCAO is not immunosuppressive but more likely immunopromoting. The following issues about the immunomodulatory ability of the MSCs in our experiments are worth mentioning.

First, although MSC transplantation decreased the pro-inflammatory cytokine IL-6 and increased the anti-inflammatory cytokine IL-10 in the ischemic core at day 2, the concentrations of TNF- α and IL-1 β were increased. The reason we concluded that the inflammation at day 2 in the core was enhanced by MSC transplantation was as follows: pro-inflammatory cytokines, such as TNF- α and IL-1 β , are usually considered to contribute to ischemic damage, and the increase of Iba-1⁺ microglia/macrophages (Table 2) is usually considered to represent an increase of inflammation [40].

Second, IFN- γ is a very important mediator of MSC immunosuppression because IFN- γ synergizes with TNF- α , IL-1 α , or IL-1 β to induce MSCs to inhibit T cell proliferation. IFN- γ is necessary but not sufficient; either TNF- α , IL-1 α , or IL-1 β is also required [4], and these three cytokines act interchangeably. As in the work of Wakabayashi et al. [41], the IFN- γ levels in our experiments were almost completely negligible in the ischemic core, striatum, and serum. This may be partially responsible for the insufficient activation of MSCs.

Third, inflammation is usually considered to contribute to damage after stroke; however, inflammation may also contribute to recovery after stroke [40]. We are unsure whether the MSC-induced increase of inflammation is beneficial or harmful to the recovery of rats at day 2. This determination becomes more difficult because the effects of inflammation at this time in dMCAO rats may be both time- and region-dependent.

The second concern is, under MSC-increased inflammation, will the transplantation of MSCs in the acute phase still be beneficial to the animals? In addition, how long will the immunopromoting function of BM-MSCs last? We found that intravenously transplanted allogeneic BM-MSCs still induced a functional improvement and reduced infarct volume in dMCAO ischemic rats at day 2. The MSCs used in our experiments were capable of immunosuppression. Although the pro-inflammatory cytokines in the brain and serum did not reach the threshold required for the activation of MSCs *in vitro* [4], the immunoregulating function of intravenously transplanted allogeneic BM-MSCs shifted from immunopromotion at the acute phase (day 2) to immunosuppression at the sub-acute phase (day 7) in the dMCAO rat model. This shift is interesting and the underlying mechanisms are worthy of further study.

Similarly, many reports have concluded that chronic inflammation usually exacerbates neurodegeneration, while some studies suggested the main therapeutic effect of MSCs shifts from neurotrophic in the acute or sub-acute phase of ischemia stroke to immunosuppressive in the chronic phase [35, 42]. For example, Acosta et al. transplanted MSCs at 30 days after

stroke and found a therapeutic effect due to immunosuppression over the next 14 and 30 days [43]. How these MSCs were induced to be immunosuppressive during the chronic phase of stroke remains to be addressed too.

The third concern is the involvement of neurotrophins in the recovery of the dMCAO model, and the cell sources of these neurotrophins. The concentrations of BDNF and IGF-1 in the ischemic core were obviously increased by MSC transplantation at 48h after the onset of ischemia. The importance of BDNF in the protection against cerebral ischemia has been well documented [2, 44, 45]. Direct intracerebral, intraventricular, and intravenous administration of BDNF has been shown to reduce infarct volume in several stroke models [46]. IGF-1 is a growth factor that is known to reduce ischemic injury [47, 48] and to promote neuronal survival and angiogenesis [49-51]. We found not only that CM-DiI-labeled BM-MSCs coexpressed BDNF and IGF-1 in the ischemic border zone but also that BM-MSCs induced these two trophic factors within parenchymal microglia/macrophages.

In the ischemic core area, the majority of BDNF immunostaining co-localized with Iba-1, a marker of microglia/macrophages, and most of the IGF-1⁺ cells co-localized with CD68⁺ microglia/macrophages. These results suggest that the beneficial effects of MSCs may not be restricted to paracrine actions but may be due to their induction of host microglia/macrophages to produce neurotrophic cytokines.

Although the neurotrophic effects of BM-MSCs in the dMCAO model are promising, it was reported that these neurotrophic effects are not very long-lasting. In some reports, it lasted almost 14 days. Wakabayashi et al. transplanted 3.0×10^6 immortalized human MSCs (line: B10) through the jugular vein into MCAO rats. Several rat growth factors, such as VEGF, EGF, bFGF, BDNF, and GDNF, were found to be increased in B10-transplanted rats in particular at 7 days. At 14 days, however, these cytokines all decreased dramatically [41]. Steinberg's group transplanted BM-MSCs intra-arterially into ischemic rats and found that BDNF was diminished at 14 days [52]. In our MSC transplanted groups, the expression patterns of rat BDNF and IGF-1 were similar; most showed a peak at day 4 and declined at day 7. In all of these experiments, the transient neurotrophic effects of MSCs were in accordance with the observation of activated microglia showing the M2 phenotype in the brain for a period of time after cerebral ischemia, and then a shift from the beneficial M2 phenotype to the detrimental M1 phenotype occurs [15]. It seems that the transplantation of MSCs slowed down but could not stop the shift of microglia/macrophages from the M2 to M1 phenotype.

In conclusion, our findings suggest that BM-MSCs increase inflammation during the acute phase of ischemia (within the first 2 days after onset). The allogeneic transplantation of MSCs is still feasible due to its improvement of behavioral function and reduction of infarct volume. The neurotrophic effect of these cells is most likely responsible for the therapeutic effect at this time. Both transplanted MSCs and endogenous microglia/macrophages in the ischemic core cortex area are involved in the neurotrophic effect of MSCs.

In future work, the IBZ and peri-ischemic core area in the cortex should be examined to determine which area is more important for the protection of the ischemic brain in MSC therapy. Additionally, other neurotrophic cytokines, such as bFGF and TGF- β , should be studied. More importantly, we are eager to understand how to maintain or prolong the neurotrophin-producing status of MSCs and microglia/macrophages in the acute and sub-acute phase of stroke, and to identify the mechanism underlying the induction of immunosuppression by MSCs at the chronic stage of ischemia.

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Disclosure Statement

The authors declare no competing financial interests.

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