Immune following suppression mesenchymal stem cell transplantation in the ischemic brain is mediated by TGF-β

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Transplantation of mesenchymal stem cells (MSCs) has been shown to enhance the recovery of brain functions following ischemic injury. Although immune modulation has been suggested to be one of the mechanisms, the molecular mechanisms underlying improved recovery has not been clearly identified. Here, we report that MSCs secrete transforming growth factor-beta (TGF-β) to suppress immune propagation in the ischemic rat brain. Ischemic stroke caused global death of resident cells in the infarcted area, elevated the monocyte chemoattractant protein-1 (MCP-1) level, and evoked massive infiltration of circulating CD68+ immune cells through the impaired blood–brain barrier. Transplantation of MSCs at day 3 post-ischemia blocked the subsequent upregulation of MCP-1 in the ischemic area and the infiltration of additional CD68+ immune cells. MSC-conditioned media decreased the migration and MCP-1 production of freshly isolated immune cells in vitro, and this effect was blocked by an inhibitor of TGF-β signaling or an anti-TGF-β neutralizing antibody. Finally, transplantation of TGF-β1-silenced MSCs failed to attenuate the infiltration of CD68+ cells into the ischemic brain, and was associated with only minor improvements in motor function. These results indicate that TGF-β is key to the ability of MSCs to beneficially attenuate immune reactions in the ischemic brain. Our findings offer insight into the interactions between allogeneic MSCs and the host immune system, reinforcing the prospective clinical value of using MSCs in the treatment of neurological disorders involving inflammation-mediated secondary damage.

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Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be isolated from various tissues including bone marrow, adipose tissue, and umbilical cord blood (Kern et al., 2006; Pittenger et al., 1999; Wagner et al., 2005). MSCs have been administered to patients with diverse neurological diseases, including ischemic stroke (Parr et al., 2007). Despite their potential to differentiate into adipocytes, osteocytes and chondrocytes, ex vivo cultured autologous MSCs are safe when injected intravenously in stroke patients, and the grafted groups showed higher frequencies of functional recovery (Honmou et al., 2011; Li et al., 2008). Additional clinical studies are currently ongoing or planned (http://www.clinicaltrials.gov), making it increasingly important to use preclinical animal studies to understand the biological mechanisms underlying MSC-mediated tissue restoration.

Ischemic stroke impairs cellular energy metabolism, leading to failure of the energy-dependent processes necessary for cell survival. Brain cells undergo cell death and release their cytoplasmic contents into the extracellular space, evoking inflammatory cascades and amplifying the tissue damage. Reactive macrophages and leukocytes are recruited into ischemic regions, where they cooperate with resident neurons, astrocytes, and microglia to generate proinflammatory mediators; these include enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-1 (COX-2), as well as cytokines and chemokines, such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis...
factor (TNF)-α, and monocyte chemoattractant protein-1 (MCP-1) (Lakhan et al., 2009). Together, these inflammatory mediators increase the permeability of the blood–brain barrier (BBB), provoking a massive invasion of peripheral immune cells and causing inflammatory cascades that further amplify tissue damage (Pan and Kastin, 2007).

Preclinical studies by our group and others have indicated that MSCs exert pleiotropic influences in rodent stroke models (Castellanos and Serena, 2007; Chopp and Li, 2002; Lee et al., 2008; Yoo et al., 2008). MSCs have been shown to increase host cell survival, promote the proliferation of endogenous neural progenitor cells in the neurogenic niche, and reduce brain inflammation. However, although numerous studies have suggested that the beneficial effects of MSCs are mediated by trophic factors and cytokines (Parr et al., 2007), the underlying molecular mechanisms have not yet been clarified.

In this study, we demonstrate that MSC-generated TGF-β is a key player in suppressing immune propagation during the delayed period post-ischemia. We demonstrate that ischemic damage-induced MCP-1 expression causes peripheral immune cells to infiltrate through the impaired BBB, but MSC-secreted TGF-β decreases the production of MCP-1 in immune cells and blocks the subsequent migration of additional immune cells. In contrast, TGF-β-knockdown MSCs fail to block immune responses, leading to poor recovery in a rat stroke model. This study provides preclinical evidence that TGF-β may be a key molecule governing the beneficial effects of MSCs in stroke patients.

Materials and methods

Cell culture

All experimental protocols using MSCs were approved by the Institutional Review Board of the Ajou University Medical Center (Suwon, South Korea). Human MSCs were isolated from bone marrow aspirates and cultured in vitro, as previously described (Kim et al., 2005). Cultured MSCs were positive for surface antigens STRO-1, CD29, CD49a, CD73, CD90, and CD105 and were negative for HLA-DR, CD45, CD34, and CD11b, and CD117 and displayed the capacity for multi-lineage differentiation into adipocytes, osteoblasts, and chondrocytes (Sung Park et al., 2013). MSCs at passages 4–6 were used in this study.

Induction of MCAo, transplantation of MSCs, and measurement of infarct volume

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Ajou University Medical School. Adult male Sprague–Dawley rats weighing 250–270 g were utilized. Transient middle cerebral artery occlusion (MCAo) was induced for 2 h by introducing a 4-0 monofilament nylon suture with a rounded tip into the common carotid artery (CCA) lumen until it blocked the bifurcating orifice of the middle cerebral artery (MCA). As previously described (Kim et al., 2008). Animals showing similar behavioral symptoms and comparable infarct volumes (tested with MRI at day 2) were selected and randomly grouped. At day 3, 5.0 × 10^5 of MSCs or MSCs/shTGF-β1 in 7 μl of PBS were injected into the striatum (AP, 0.5; ML, 2.5; DV, 5.0) and cortex (AP, −0.5; ML, 2.0; DV, 2.5) in the penumbra ipsilateral to the injury; the injection was performed over 10 min with the aid of a stereotactic apparatus. A total of 86 rats were used in this study, including those in the sham-operated group (n = 11), PBS group (n = 18), MSC group (n = 18), MSC/shTGF-β1 group (n = 15) and MCAo-only group (n = 24) (Supplementary Table S1). The ischemic area from each T2-weighted image was manually marked, and the infarct volume was calculated as previously described (Kim et al., 2008). Independent investigators were double-blinded to the animal’s pre- and post-transplant status to avoid subjective bias in all experiments. Additionally, independent investigators were blinded to the assessment of infarct volumes.

Validation of BBB breakdown

Animals were injected with 4 ml/kg of 2% Evans blue dye (Sigma-Aldrich Corporation, St. Louis, MO) in normal saline through the tail vein (n = 3 per group) and intracardially perfused with PBS and 4% paraformaldehyde (PFA) 4 h later. The brains were isolated and photographed.

Immunohistochemical analysis

For immunohistochemistry, animals were intracardially perfused with PBS and then fixed with 4% PFA at the indicated times after perfusion. Brains were embedded in paraffin and sectioned to 5-μm thickness. Antigens were retrieved by boiling in 10 mM sodium citrate (pH 6.0) in a microwave oven, the sections were blocked in PBS with 1% bovine serum albumin and 5% normal serum, and probed with primary antibodies against ionized calcium binding protein-1 (iba1, rabbit, 1:500; Sigma-Aldrich Corporation), CD68 (mouse, 1:200; Serotech, Kidlington, UK), myeloperoxidase (MPO, rabbit, 1:100; Abcam, Cambridge, UK) and MCP-1 (rabbit, 1:100; Novus Biologicals, Littleton, CO). The sections were then incubated with Alexa Fluor 488– or -594-conjugated anti-IgG secondary antibodies (Molecular Probes, Eugene, OR) and counterstained with Hoechst 33258 (Molecular Probes). For measurement of microglial death, the sections were subjected to staining with an in situ cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany), and fluorescent images were acquired using a Zeiss LSM510 confocal microscope (Carl Zeiss AG, Jena, Germany). Alternatively, the sections were incubated with a biotin-conjugated secondary antibody (1:500) and an avidin–biotin complex, using an ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer’s protocol. Bright-field images were acquired using a Zeiss Axiopt microscope (Zeiss).

Quantification

Four 5-μm-thick coronal sections spaced 400 μm apart from the ischemic central region (AP, + 1.2 mm to −0.8 mm based on bregma) were prepared from five animals per group. Light microscopic images were acquired using a ScanScope CS digital slide scanner (Aperio, Vista, CA), and the total number of immunoreactive cells in the ischemic penumbra were calculated using the Quantity One image analysis software (Bio–Rad Laboratories, San Diego, CA). To measure the number of CD68+ /MCP-1+ cells in ischemic penumbra regions, five con focal images were generated for each of the four sections, and the immunoreactive cells that had distinct Hoechst-positive nuclei were counted using NIH imaging software (Image, http://rsbweb.nih.gov/ij). Independent investigators were blinded to the counting of immune positive cells. The results from the five animals are presented as mean ± SD.

In vitro transwell migration assay

To induce enrichment of macrophages, 10 ml of 10% thioglycollate (Sigma-Aldrich Corporation) was injected into the peritoneal cavities of Sprague–Dawley rats. Four days later, immune cells were harvested from the peritoneal cavity and red blood cells were removed with hemolysis buffer (Invitrogen, Grand Island, NY). Peritoneal immune cells (1 × 10^6 cells) were suspended in 300 μl DMEM and plated in the upper chamber of a transwell apparatus (pore size, 8 μm; Corning Costar, Corning Inc., NY). The lower chamber was loaded with 600 μl DMEM, 100 μg/ml of brain ischemic extract (IE), and 100 ng/ml of recombinant MCP-1 (R&D Systems, Minneapolis, MN), 1 μg/ml of anti-MCP-1 neutralizing antibody (R&D Systems), or 1 × 10^5 MSCs. For preparation of IE, the ipsilateral hemisphere of day-3 ischemic brain was homogenized in PBS and centrifuged at 12,000 ×g, and the
supernatant was used as IE. After incubation of the transwell apparatus for 4 h at 37 °C in 5% CO2, the cells adhering to the upper side of the membrane were removed with a cotton swab, and transmigrated cells at the bottom side of the membrane were visualized by cresyl violet or Hoechst dye staining. The average cell number from four random fields of view was calculated from each well. The results from three independent experiments are presented as mean ± SD.

Knockdown of TGF-β1 with shRNA

To produce a lentiviral vector encoding a short hairpin RNA for TGF-β1 (Lentilox-shTGF-β1), a double-stranded DNA (5′-TCAAT TCTGGCCATACCTCGAACCTCGTGTGAGGATCAGGAAATTGTGTTTTC-3′) was inserted into pLentilox 3.7 (Addgene, Cambridge, MA). The resulting pLentilox-shTGF-β1 was cotransfected into 293 T cells along with pCMV-VSV-G and pCMVd8.9 (Addgene). Virus-containing media were collected 48 h after transfection and filtered through a 0.45-μm filter. MSCs were transduced with Lentilox-shTGF-β1 at 10 MOI and sorted with Lentilox-shTGF-β1. The resulting cells (MSC/shTGF-β1) were enriched for GFP expression using BS FACSAria III (BD Biosciences, San Jose, CA). Knockdown of TGF-β1 expression was verified by measurement of the level of secreted TGF-β1 (Supplementary Fig. S1). Finally, the TGF-β1-knockdown cells were expanded in growth medium and used for transplantation.

RNA isolation and RT-PCR

The ischemic penumbra region at day 4 (9.0 mm3, 1.0 × 1.5 × 6.0 mm) was dissected from AP, 0.5 mm to AP, -0.5 mm (Supplementary Fig. S2) and total RNA was isolated using RNAzol B (Tel-Test Inc., Friendswood, TX) according to the manufacturer’s protocol. Peritoneal immune cells were isolated as described above and plated in 60-mm culture dishes at a density of 1.0 × 10⁶ cells/well. Cells were allowed to adhere to the plates, and unattached cells were removed 3 h later. MSC or NIH3T3 cell cultures were grown in DMEM containing 10% FBS, 1% penicillin and streptomycin for 2 days, and media were collected and filtered through a 0.2-μm filter. Peritoneal immune cells were treated with the filtrates with or without recombinant human TGF-β1 (20 ng/ml, rTGF-β1; Millipore, Billerica, MA), TGF-β receptor I kinase [3-(pyridine-2yl)-4-(quinonyl)]-1H-pyrazone (100 nM, TGF-β-RI; Millipore), or anti-TGF-β1-neutralizing antibody (1 μg/ml; R&D Systems). After 10 min, 100 μg/ml ischemic extract (IE) was added. Six hours after treatment, total RNA was isolated and 1 μg of RNA was reverse transcribed in a 20-μl reaction using a 1st Strand cDNA synthesis kit (Roche Diagnostics). PCR analysis was carried out on 1 μl of the first-strand cDNA (primer sequences and PCR conditions are summarized in Supplementary Table S2). The products were subjected to semi-quantitative analysis using the Quantity One image analysis software (Bio-Rad Laboratories).

Statistics

Results were analyzed using one-way or repeated measures ANOVA with treatment groups and days of testing as the independent variables, followed by Scheffe’s post-hoc test for multiple comparisons by measurement day. Student’s t-tests were used to analyze between-group differences in mRNA expression and in vitro transwell migration. The level of statistical significance was set at p < 0.05. All values are presented as the mean ± SD.
Results

Inflammatory responses in the ischemic brain

To assess immune responses in the ischemic brain, we determined when the BBB started to disintegrate under our experimental conditions. We injected Evans blue dye into the tail veins of ischemic animals at various time points after induction of ischemia and sacrificed the animals 4 h later. The leakage of Evans blue dye was found specifically near the occlusion point (i.e., the branching point of the MCA and anterior cerebral artery) as early as post-ischemic day 1, but progressively spread out to the entire hemisphere between day 3 and day 7 (Fig. 1A). Concurrently, the number of CD68+ cells with amoeboid morphologies and thick processes increased massively in the ischemic region between day 3 and day 7 (Fig. 1B). These cells were not found in the sham-operated animals, where the resident microglia were Iba1+ cells with ramified morphology and branched processes. In the ischemic brain, CD68-immunoreactivity colocalized with Iba1+ cells (Fig. 1C), suggesting that most of the CD68+ cells with amoeboid morphology emerging from day 1 were phagocytic cells, such as activated microglia and/or infiltrating peripheral immune cells. However, TUNEL+ assays indicated that a majority of the Iba1+ cells were undergoing cell death in the ischemic region at day 1 (arrows in Fig. 1D). Furthermore, between days 3 and 7, TUNEL+ nuclei (arrowheads) appeared to be adjacent to round-shaped Iba1+ cells, as if the dying cells had been engulfed by phagocytic Iba1+ cells. Notably, the emergence rates of round-shaped Iba1+ or CD68+ cells correlated well with the extent of BBB breakage between days 3 and 7. Collectively, these data suggest that, similar to neuronal and glial cells, most resident Iba1+ microglia in the ischemic area undergo cell death. Furthermore, the Iba1+/CD68+ cells those repopulate the area between days 3 and 7 appear to be phagocytic macrophages that mobilize from the blood circulation through the impaired BBB.

To further verify the peripheral origin of CD68+ cells, we freshly isolated immune cells from the peritoneal cavity of GFP-expressing transgenic rats (Supplementary Fig. S3A) and injected 5 × 10⁶ cells through the tail veins of MCAo-induced ischemic rats at day 1. When the animals were sacrificed at day 3, transplanted GFP+ cells were found in the ischemic region, where they constituted up to 24% of the total CD68+ cells (Supplementary Fig. S3B).

Although it was not practical to measure the total number of CD68+ cells in the entire body, the observed migration of substantial numbers of exogenous GFP+ immune cells to the ischemic area reinforced our hypothesis that the majority of CD68+ inflammatory cells found between days 3 and 7 represent monocytes/macrophages recruited from the peripheral blood.

MCP-1 as a cytokine responsible for recruiting peripheral immune cells

Transplantation of MSCs in the ischemic penumbra at day 3 dramatically inhibited the massive infiltration of CD68+ cells. Between days 4 and 7, the total number of CD68+ cells dramatically increased (by 4.7-fold) in the PBS-injected control group, whereas it increased by only 2.2-fold in the MSC-injected group (Figs. 2A, B). Transplantation
of MSCs also decreased MCP-1 expression in the CD68+ cells (Figs. 2C, D). These results suggest that MSCs may directly downregulate the expression of MCP-1 in the CD68+ immune cells that moved into the ischemic region prior to MSC transplantation, thereby decreasing the subsequent infiltration of additional CD68+ immune cells.

Indeed, RT-PCR analysis of mRNA indicated that the expression of a variety of proinflammatory cytokines and signaling molecules, including iNOS, TNF-α, IL-1β and MCP-1, were enhanced at day 4 (Figs. 3A, B). Importantly, the induction of these proinflammatory cytokines was not seen in the MSC-treated group. This in vivo finding was reinforced by in vitro assays with freshly isolated peritoneal immune cells. Addition of ischemic brain extracts (IE) to the cell culture induced the expression of MCP-1 in peritoneal immune cells. This was blocked by 67% in the presence of MSC-conditioned medium (Fig. 4A); in contrast, NIH3T3-conditioned medium had only a minor effect, suggesting that MSCs specifically secrete anti-MCP-1 molecule(s). Likewise, transwell migration assays indicated that the MCP-1 present in IE was a key chemoattractant for the migration of immune cells (Fig. 4B). The presence of IE in the lower chamber induced migration of peritoneal immune cells from the upper chamber. MCP-1 (positive control) also induced a similar extent of immune cell migration. However, these effects were blocked by the presence of cultured MSCs in the lower chamber (Fig. 4C). Pretreatment of IE with an anti-MCP-1-neutralizing antibody decreased the immune cell migration by 65% (Fig. 4B). Collectively, these data suggest that ischemic injury induces MCP-1 to recruit peripheral monocyte/macrophages, and that soluble factor(s) secreted by MSCs can suppress MCP-1 expression and the subsequent infiltration of peripheral immune cells.

MCP-1 is known to be downregulated by immunomodulatory cytokines, such as IL10 (Li and Flavell, 2008) and TGF-β (Wolf et al., 2002). A cytokine array analysis indicated that MSCs expressed high levels of TGF-β but very low levels of IL-10 (Supplementary Fig. S1). Thus, we tested whether TGF-β mediated the MSC-induced downregulation of MCP-1. We found that the introduction of an anti-TGF-β neutralizing antibody negated the suppressive functions of MSC-conditioned medium and restored MCP-1 expression in peripheral immune cells recruited by simple exposure to IE (Fig. 4A). To further show the anti-MCP-1 functions of TGF-β, we knocked down TGF-β1 in MSCs using a lentiviral vector encoding a TGF-β1-specific shRNA. Conditioned medium from TGF-β1-knockdown MSCs (MSCs/shTGF-β1) failed to suppress MCP-1 expression (Fig. 4D). Furthermore, a TGF-β1 receptor I kinase inhibitor (TGF-β-RI) partially restored MCP-1 expression, and the addition of recombinant TGF-β1 reversed the effect of TGF-β1 knockdown (Fig. 4D). Either anti-TGF-β1-neutralizing antibody or TGF-β1-RI did not affect the basal MCP-1 expression in the absence of IE (data not shown). Consistent with these findings, co-culture of MSCs/shTGF-β1 in the lower chamber of the transwell apparatus failed to block the IE-induced migration of immune cells (Fig. 4C). Together, these data clearly indicate that MSC-secreted TGF-β1 suppresses MCP-1 expression in inflammatory cells.

**The therapeutic functions of MSCs are mediated by TGF-β1**

To verify the in vivo role of TGF-β1, MSCs/shTGF-β1 were transplanted into the ischemic penumbra at day 3, when we observed CD68+ macrophages starting to massively infiltrate the ischemic area through the broken BBB (Fig. 1). Decreased expression of TGF-β1 in MSCs/shTGF-β1 transplantation was confirmed through confocal analysis with hMT and hTGF-β1 specific antibodies 4 days after into the ischemic rat brain (Fig. 5A). As we previously reported (Kim et al., 2008), transplantation of MSCs prevented the infiltration of CD68+ cells at day 7 (Fig. 5B), reduced the infarct volume (34% and 25% in the control and MSC-treated groups, respectively, on day 28) (Fig. 5C), and improved the functional recovery from ischemic injury (Fig. 5D). In contrast, TGF-β1-knockdown MSCs failed to prevent the infiltration of CD68+ cells into the ischemic area at day 7 and had only minimal (non-significant) effects on infarct volume and functional recovery (Figs. 5B–D). Collectively, these in vitro and in vivo findings indicate that the anti-inflammatory functions of MSCs are mediated via TGF-β1 in an animal stroke model.

**Discussion**

In the present study, we elucidated the molecular mechanisms underlying the immune modulatory functions of MSCs in an animal stroke model. We report that MCP-1, a chemoattractant for monocytes/macrophages, is highly elevated in the ischemic rat brain, where it increases the production of MCP-1 and attenuates the subsequent infiltration of additional CD68+ immune cells.

Microglia, which comprise a major immune cell population in the central nervous system, occur as Iba1+ cells with ramified processes in the resting brain. When activated by brain damage or infection, microglia increase in number and transform to amoeboid forms with shorter processes (Benveniste et al., 2001; Hemmer et al., 2002; Yrjanheikki et al., 1998). Activated microglia secrete a variety of proinflammatory cytokines that are known to aggravate brain inflammation. In the ischemic brain, the number of Iba1+ cells with amoeboid morphologies greatly increased in number between days 3 and 7 (Fig. 1B). In contrast to observations in chronic brain diseases, most of the Iba1+ cells that newly emerge in the ischemic area between days 3 and 7 do not appear to be the activated, resident microglia. Instead, we propose that these cells represent circulatory macrophages that have infiltrated though the impaired BBB. This is supported by several lines of evidence. Blockage of the blood supply caused massive cell...
death of all cell types, including the resident Iba1+ microglia, in our experimental models (Fig. 1D). The repopulation rate of Iba1+/CD68+ cells was proportional to the extent of BBB breakage (compare Figs. 1A and C). A substantial proportion of the CD68+ cells in the ischemic area were found to be GFP+ peripheral immune cells when such cells were exogenously injected into the ischemic animals (Supplementary Fig. S3). We also observed that MPO+/CD68+ neutrophils had infiltrated into the ischemic area at day 1 but had disappeared by day 7 (Supplementary Fig. S4). Our findings are consistent with previous reports that CD11b+ resident microglia die following lipopolysaccharide injection and brain damage, sequentially evoking the infiltration of neutrophils followed by monocytes/macrophages (Ji et al., 2007; Matsumoto et al., 2007). However, our results could not completely eliminate the possibility that a minor subpopulation of CD68-positive cells were reactive microglia. It is possible that a subpopulation of the observed CD68+ cells came from the proliferation or relocation of surviving microglia from adjacent intact brain areas.

Following ischemia, the recruitment of immune cells toward the injured area is specifically guided by chemoattractants, including MCP-1 and SDF-1. It has been reported that the level of SDF-1β is markedly enhanced by ischemic stroke, whereas the expression of SDF-1α remains unchanged (Stumm et al., 2002). Elevated SDF-1β can recruit exogenously transplanted neuronal progenitor cells (Imitola et al., 2004) and endogenous neuroblasts newly generated from the subventricular zone (Thored et al., 2006). Here, we found that MCP-1 expression was markedly increased by ischemic injury, whereas SDF-1α expression was unaltered (Fig. 3A). The pathological role of MCP-1 has been well demonstrated in stroke, and the protein is known to be upregulated in ischemic stroke patients (Arakelyan et al., 2005) and animal models (Wang et al., 1995; Yamagami et al., 1999). Immediately after brain injury, MCP-1 is primarily secreted by neurons, astrocytes, and microglia (Banisadr et al., 2005). This secreted MCP-1 propagates immune responses by recruiting monocytes/macrophages, which subsequently produce more MCP-1. Thus, increased MCP-1 levels mirror the development of inflammatory responses in vivo. In murine models, genetic ablation of MCP-1 (Hughes et al., 2002) or CCR2 (Dimitrijevic et al., 2007), the primary receptor for MCP-1, were shown to reduce ischemic infarcts, BBB permeability, brain edema, and macrophage infiltration.

Fig. 4. MSC-secreted TGF-β1 downregulates MCP-1 expression and attenuates the migration of immune cells. (A) RT-PCR analysis of IE-stimulated peritoneal immune cells in the presence of conditioned medium (CM) from MSCs or NIH3T3 cells. MSC-CM suppressed MCP-1 expression, and an anti-TGF-β1 antibody reversed this effect. (B) Transwell analysis with peritoneal immune cells in the upper chamber. The presence of recombinant MCP-1 (rMCP-1) or IE in the lower chamber evoked the migration of immune cells, but the additional presence of an anti-MCP-1 neutralizing antibody (α-MCP1) in the lower chamber blocked this migration. (C) MSC-knockdown cells in the lower chamber failed to suppress the migration of immune cells. The migratory cells (those on the lower side of the membrane) were counted from four random fields. (D) RT-PCR analysis of peritoneal immune cells treated with CM from MSCs/shTGF-β1 or MSCs expressing a control scrambled RNA (shScr), with or without 100 nM of recombinant TGF-β1 protein (rTGF-β1) or an inhibitor of TGF-β1 signaling (TGF-β-RI). MCP-1 mRNA levels were normalized with respect to that of GAPDH. (A–D) Data from at least 3 independent experiments are presented as means ± SD (t-test; *, p < 0.05 and **, p < 0.01).
Importantly, we found that MCP-1 expression and macrophage infiltration following ischemia could be prevented by MSC transplantation. MSC-secreted TGF-β1 could directly suppress the production of MCP-1 in inflammatory cells, impeding the subsequent migration/infiltration of macrophages. The presence of an anti-TGF-β1 neutralizing antibody or an inhibitor of TGF-β1 signaling abolished the suppressive functions of MSC. When TGF-β1 was depleted, MSCs could no longer decrease the expression of MCP-1 in immune cells or attenuate their recruitment to the ischemic area. From this, a model emerges: Following ischemia, the damaged tissues release MCP-1, prompting CD68+ cells to infiltrate through the impaired BBB; these cells produce additional MCP-1, leading to the recruitment of more peripheral immune cells. This vicious cycle of immune propagation, however, can be blocked by MSC-secreted TGF-β1 (Fig. 6).

TGF-β1 is a regulatory cytokine that plays pleiotropic roles in the immune system, where its functions are determined by the presence of various cytokines at the location of T-cell stimulation (Becker, 2010). Although early studies suggested that TGF-β1 has a proinflammatory function in recruiting monocytes (Wahl et al., 1987), accumulating evidence suggests that TGF-β1 is generally an anti-inflammatory cytokine (Li et al., 2006). In animal stroke models, depletion of TGF-β1 has been shown to increase inflammatory responses (Kulkarni et al., 1993), whereas administration of TGF-β1 protein or adenoviral transfer of the TGF-β1-encoding gene reduced the inflammatory response via downregulation of MCP-1 (Pang et al., 2001). Transcription of MCP-1 was regulated by an AP1 complex composed of a c-jun/c-fos heterodimer (Shyy et al., 1995) and this AP1-dependent MCP-1 expression was...
suppressed by sequestration of c-jun with Smad3 (Feinberg et al., 2004). Smad3 is a well-known downstream effector of TGF-β1. Targeted mutation of Smad3 increased the infiltration of MCP-1-expressing macrophages following cardiac allografting (Feinberg et al., 2004). Thus, it is very likely that the anti-inflammatory effect of MSCs in this study was mediated through Smad3 activation by TGF-β1. It should be noted that the recovery scores (infarct volume, rotated test, and adhesive removal tests) of animals treated with TGF-β1-knockdown MSCs were intermediate between those of the MSC- and PBS-treated groups (Fig. 6). Therefore, it is possible that other beneficial factors in addition to TGF-β1, such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), or nerve growth factor (NGF), may help confer the full benefits of MSC therapy (Chang et al., 2008; Kinnaird et al., 2004; Labouyrie et al., 1999; Mahmood et al., 2004).

Stem cell therapy with autograftable MSCs has recently emerged as a prominent clinical therapy in regenerative medicine. Numerous studies (including our own) have suggested that the therapeutic effects of MSCs in animal stroke models arise via the paracrine functions of MSCs rather than their transdifferentiation into neurons (Capellan and Dennis, 2006; Chopp and Li, 2002; Kim et al., 2008; Yoo et al., 2008). Recent reports have suggested that MSCs are not only immune privileged due to their low (or undetectable) expression of MHC class II, CD40, CD80, and/or CD86 co-stimulatory molecules, but also (and more importantly) display immune-modulating properties (Nauta and Fibbe, 2007; Uccelli et al., 2006a). Based on the immune-modulating property of MSCs, various trials have sought to use them as a therapeutic agent to block pathogenic immune responses, such as autoimmune encephalomyelitis (EAE), inflammation after chemical burning of the cornea (Oh et al., 2008; Uccelli et al., 2006b; Zappia et al., 2005), graft-versus-host diseases (Le Blanc et al., 2002; Ringden et al., 2006), and Crohn’s disease (Duijvestein et al., 2010). These recent reports have indicated that MSCs and MSC-secreted factors definitely interfere with the recruitment of immune cells. However, the mechanisms underlying the immune-suppressive effects of MSCs have not yet been clearly defined.

In sum, we herein demonstrate that MSCs exert therapeutic effects by producing TGF-β1 in animal stroke models. Our findings also suggest that the immune-modulatory functions of MSCs support their potential use for the treatment of neurological dysfunctions accompanied by brain inflammation. We also propose that transplantation of MSCs for the treatment of stroke patients may be a more plausible strategy than administration of anti-inflammatory drugs, including TGF-β1. This is because MSCs can supply additional neurotrophic factors that protect the neurons from ischemic injury and promote endogenous neurogenesis, while also providing TGF-β1 to make the brain environment less hostile and allow more time for spontaneous recovery.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.06.001.

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Fig. 6. Schematic model showing how MSCs interrupt immune propagation in the ischemic brain. Ischemic damage sequentially induces MCP-1 expression, massive cell death of brain cells, and increased BBB permeability. MCP-1 chemoattracts CD68+ monocytes/macrophages from the circulatory blood to the ischemic region through the impaired BBB. These extravasated CD68+ cells produce MCP-1, which induces the subsequent infiltration of more CD68+ cells. Transplanted MSCs downregulate this MCP-1 expression by secreting TGF-β1, thereby attenuating the infiltration of additional immune cells from the blood. Thus, MSCs interrupt the propagation of immune responses. Furthermore, MSCs may suppress the production of NO from the extravasated CD68+ cells through a similar mechanism, and appear to block the inductions of TNF-α and IL-1β.
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