GLIA 57:13–23 (2009)

Neuroprotective Effects of Human Mesenchymal Stem Cells on Dopaminergic Neurons Through Anti-Inflammatory Action

YOU-JOUNG KIM,[1] HYUN-JUNG PARK,[1] GWANG LEE,[1] OH YOUNG BANG,[2] YOUNG HWAN AHN,[1] EUNHYE JOE,[3] HYUN OK KIM,[4] AND PHIL HYU LEE[5]*

[1]*Center for Neuroregeneration and Stem Cell Research, Ajou University School of Medicine, Suwon, South Korea*
[2]*Department of Neurology, Sungkyunkwan University School of Medicine, Seoul, South Korea*
[3]*Neuroscience Graduate Program, Ajou University School of Medicine, Suwon, South Korea*
[4]*Department of Laboratory Medicine, Yonsei Cell Therapy Center, Yonsei University College of Medicine, Seoul, South Korea*
[5]*Department of Neurology, Yonsei University College of Medicine, Seoul, South Korea*

KEY WORDS
mesenchymal stem cells; neuroprotective effect; anti-inflammatory; Parkinson's disease

ABSTRACT
Parkinson's disease (PD) is a common, progressive neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra (SN). Numerous studies have provided evidence suggesting that neuroinflammation plays an important role in the pathogenesis of PD. In this study, we used lipopolysaccharide (LPS)-induced *in vitro* and *in vivo* inflammation models to investigate whether human mesenchymal stem cells (hMSCs) have a protective effect on the dopaminergic system through anti-inflammatory mechanisms. The hMSC treatment significantly decreased LPS-induced microglial activation, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS) mRNA expression, and production of NO and TNF-α compared with the LPS-only treatment group. In co-cultures of microglia and mesencephalic dopaminergic neurons, hMSC treatment significantly decreased the loss of tyrosine hydroxylase-immunopositive (TH-ip) cells. The hMSC treatment in rats showed that TH-ip neuronal loss induced by LPS stimulation in the SN was considerably decreased and was clearly accompanied by a decrease in activation of microglia, as well as TNF-α and iNOS mRNA expression and production of TNF-α. These data suggest that hMSCs have a neuroprotective effect on dopaminergic neurons through anti-inflammatory actions mediated by the modulation of microglial activation. Along with various trophic effects and trans-differentiational potency, the anti-inflammatory properties of MSCs could have major therapeutic implications in the treatment of PD.

INTRODUCTION

Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies, proteinaceous inclusions that contain α-synuclein, synphilin-1, components of the ubiquitin proteasomal pathway, and parkin in the substantia nigra (SN) (Moore et al., 2005). Recent advances have revealed that the pathogenesis of neuronal degeneration in PD may involve several molecular and cellular events, including oxidative stress, proapoptotic mechanisms, mitochondrial dysfunction, and the accumulation of toxic proteins resulting from dysfunction of the protein degradation system (Moore et al., 2005; von Bohlen und Halbach et al., 2004). However, the relationships between these biochemical changes and their temporal context remain unresolved.

A glial reaction and inflammatory processes may participate in the cascade of neuronal degeneration in PD. A postmortem study described extensive proliferation of reactive amoeboid microglia in the SN of PD patients (McGeer et al., 1988), indicating that activated microglia may lead to dopaminergic neurodegeneration. An additional pathological study demonstrated the conspicuous presence of activated microglia in the SN of PD patients who were exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1999), suggesting that an ongoing stimulus could lead to disease progression long after the initial toxic insult. A positron emission tomography study using a radiotracer for activated microglia revealed that microglial activation occurred in patients with early PD and was closely linked to the degree of dopaminergic neuronal loss (Ouchi et al., 2005). Furthermore, increased levels of cytokines such as tumor necrosis factor (TNF)-α, interleukin-1β, and interferon-γ have been demonstrated in the SN of PD patients (Boka et al., 1994; Hunot et al., 1999; Nagatsu et al., 2000). Evidence of inflammation in dopaminergic neuronal death has also been documented in animal models of PD, created by numerous neurotoxins such as MPTP, 6-hydroxydopamine, and rotenone (Cicchetti et al., 2002; Gao et al., 2002; Liberatore et al., 1999).

Mesenchymal stem cells (MSCs) are present in adult bone marrow and represent <0.01% of all nucleated

Conflict of interest: Nothing to report.

The first two authors contributed equally to this work.

Grant sponsor: Ministry of Science and Technology, Republic of Korea [Stem Cell Research Center of the 21st Century Frontier Research Program (SC-4111)].

*Correspondence to: Phil Hyu Lee, Department of Neurology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea. E-mail: phisland@chol.com

Received 29 January 2008; Accepted 4 June 2008

DOI 10.1002/glia.20731

Published online 25 July 2008 in Wiley InterScience (www.interscience.wiley.com).

bone marrow cells. MSCs are themselves capable of multipotency, with differentiation under appropriate conditions into chondrocytes, skeletal myocytes, and neurons (Makino et al., 1999; Pittenger et al., 1999; Woodbury et al., 2000). Recent reports have shown that the neuroprotective effect of MSCs may be mediated not only by their differentiation into neuron-like cells, but also by their ability to produce various trophic factors that may contribute to functional recovery, neuronal cell survival, and stimulation of endogenous regeneration (Barry and Murphy, 2004). Of these, the anti-inflammatory role of MSCs in cell transplantation as a protective mechanism has been demonstrated *in vitro* and in animal models of ischemic heart disease and experimental autoimmune encephalomyelitis (Gerdoni et al., 2007; Guo et al., 2007; Zappia et al., 2005).

Although a few studies have reported effects of MSC transplantation in animal models of PD, the application of MSCs in neurodegenerative diseases from the viewpoint of their neuroprotective effects, via anti-inflammatory actions, has not been studied. Thus, we used lipopolysaccharide (LPS)-induced *in vitro* and *in vivo* inflammation models to investigate whether MSCs have a protective effect on the dopaminergic system through an anti-inflammatory mechanism.

MATERIALS AND METHODS

Isolation and Maintenance of Human MSCs

We obtained written informed consent from all persons who agreed to the use of their cells for research purposes. Bone marrow aspirates were prepared and mononuclear cells were isolated by Ficoll density centrifugation. Mononuclear cells were placed in 10-cm dishes. These cells were cultivated in low-glucose Dulbecco modified Eagles' medium (DMEM, Gibco-BRL, Grand. Island, NY), containing 10% FBS and 1% penicillin/streptomycin, and incubated in a humidified incubator at 37°C in 5% CO_2. Medium containing nonadherent cells was replaced every 3 days of culture. When the cells reached 70–80% confluence, they were trypsinized and subcultured. At passage 6 of the human mesenchymal stem cells (hMSCs), cells were co-cultured with microglia in a Transwell and then injected into animals via the tail vein.

Cortical Microglial Cultures

Microglia were cultured from the cerebral cortices of 1-day-old Sprague-Dawley (SD) rats (Ryu et al., 2000). The cortices were rinsed twice in minimum essential medium (MEM; Sigma, St Louis, MO) containing 10% fetal bovine serum and were mechanically triturated. The dissociated cells were plated in 75-cm^2 T-flasks. After 13–15 days, the microglia were detached from the flasks, applied to a nylon mesh to remove astrocytes, and then seeded into 24-well plates (1×10^5 cells/well) or 6-well plates (1×10^6 cells/well). After 30 min to 1 h, the culture medium was replaced with MEM containing 5% fetal bovine serum; 24 h later, they were treated with LPS (100 ng/mL; Sigma) for 4 h to induce inflammation. The microglia were then used in immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR) of TNF-α and nitric oxide synthase (iNOS).

Co-Cultures of Microglia and Mesencephalic Neurons

Mesencephalic neurons were cultured from the SN of 14-day embryonic SD rats (Chung et al., 2001). The tissue was incubated in Ca^{2+}-free, Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS) for 10 min and in 0.01% trypsin solution in CMF-HBSS for 9 min at 37°C. The cultures were rinsed twice in RF (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 6 mg/mL glucose, 204 μg/mL L-glutamine, and 100 U/mL penicillin/streptomycin [P/S]) for trypsin inhibition and then dissociated into single cells by trituration. Dissociated cells were plated on 12-mm round aclar plastic coverslips or culture slides precoated with 0.1 mg/mL poly-D-lysine and 4 μg/mL laminin, and seeded in 24-well culture plates at a density of 1×10^5 cells/coverslip or slide. The cells were incubated in a humidified incubator at 37°C and 5% CO_2 for 24 h. In 2-day-old *in vitro* cultures (DIV 2), the culture medium was replaced with chemically defined serum-free medium composed of Ham's nutrient mixture (F12-DMEM) and with 1% ITS (insulin, transferrin, selenium), glucose, L-glutamine, and P/S. At DIV 3, the cells were co-cultured with 1×10^5 cortical microglia/well (Lee et al., 2005; Liu et al., 2000); at DIV 4, the cells were treated with LPS for 4 h to induce inflammation.

Co-Cultures of LPS-Stimulated Microglia or Microglia and Mesencephalic Neurons with hMSCs

To test the effects of co-cultures of LPS-stimulated microglia and hMSCs without cell contact, cultured hMSCs were maintained on the bottom side of a Costar Transwell insert (0.4-μm pore size; Corning, NY 14831) in a humidified incubator at 37°C and 5% CO_2 for 24 h. After LPS-treatment for 4 h in a microglia culture alone or in co-cultures of microglia and mesencephalic neurons, each Transwell inserted with cultured hMSCs (3×10^5 cells/well) or DMEM was dipped in the basal plate of the LPS-treated culture system. hMSCs in a Transwell were maintained for 24, 48, and 72 h in a plate of microglia culture alone and for 6 and 24 h in a plate of co-cultures of microglia and mesencephalic neurons. The cells and supernatants were then collected to assay the levels of TNF-α, nitrate, iNOS, and immunocytochemistry. To identify soluble factors associated with modulation of microglial activation, each Transwell inserted with cultured LPS-stimulated microglia or DMEM was dipped in the basal plate of the cultured hMSCs ($3 \times$

10^5 cells/well). hMSCs in a Transwell were maintained for 3 and 12 h, and then hMSCs were collected to assay the level of interleukin (IL)-6, IL-10, and transforming growth factor (TGF)-β.

Animal Study

SD rats were allowed to acclimate for 3 days before the experiments. All experiments were conducted using male rats (weighing 240–270 g). The rats were anesthetized with chloral hydrate (400 mg/kg) and positioned in a stereotaxic apparatus. For injection of LPS (5 μg/μL) into the left SN, the following coordinates were used: 5.3 mm posterior, 2.3 mm lateral, 7.7 mm ventral from bregma, and injected at a rate of 1 μL/5 min using a 26-gauge Hamilton syringe attached to an automated microinjector. The needle was then left in place for an additional 10 min before slow retraction. The control group was injected with 1 M PBS into the same position and by the same method as for the LPS injection group. At 8 h following LPS stimulation, hMSCs (1×10^6 cells/1 mL PBS) or vehicle was injected into rats via the tail vein. For the MPTP-treated PD model, 8-week-old male C57BL/6 mice (weighing 20–25 g) were administrated MPTP intraperitoneally (20 mg/kg) five times for 1 day. At 8 h following last injection of MPTP, hMSCs (1×10^5 cells/1 mL PBS) or vehicle was injected into mice via the tail vein, and immunohistochemical analysis was performed 3 days later (Hamre et al., 1999).

RT-PCR

Total RNA was extracted from dissected tissue using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and was quantified spectrophotometrically. Reverse transcription (RT) was performed using Superscript reverse transcription (Invitrogen), with total RNA (2 μg) as the template. The PCR reaction was performed using 10 pmol each of the 5′ and 3′ primers for TNF-α (sense 5′-GTA GCC CAC GTC GTA GCA AA-3′ and antisense 5′-CCC TTCTCC AGC TGG GAG AC-3′), iNOS (sense 5′-GCA GAA TGT GAC CAT CAT GG-3′ and antisense 5′-ACA ACC TTG GTG TTG AAG GC-3′), and GAPDH (sense 5′-TCC CTC AAG ATT GTC AGC AA-3′ and antisense 5′-AGA TCC ACA ACG GAT ACA TT-3′), and anti-inflammatory cytokines the hMSCs; human GAPDH (hGAPDH sense 5′-CAC CAC ACC TTC TAC AAT GAG CTG C-3′ and antisense 5′-ACA GCC TGG ATA GCA ACG TAC ATG G-3′), hIL-6 (sense 5′-GCG CCT TCG GTC CAG TTG-3′ antisense 5′-CTC CTT TCT CAG GGC TGA G-3′), hIL-10 (sense 5′-CCT AGG TCA CAG TGA CGT GG-3′), hTGF-β (sense 5′-CCT TTC CTG CTT CTC ATG GC-3′, antisense 5′ACT TCC AGC CGA GGT CCT TG-3′). After an initial denaturation at 94°C for 5 min, 30 cycles of PCR were performed, consisting of denaturation (30 s, 95°C), annealing (1 min, 56°C [TNF-α, GAPDH], 52°C [iNOS], 60°C [hGAPDH], 55°C [hIL-6], 51°C [hIL-10], 57°C [TGF-β]) extension (1 min, 72°C), followed by a final extension (10 min, 72°C). The PCR products were separated by electrophoresis on 2% agarose gel and stained with ethidium bromide. Gels were examined under UV illumination.

Quantification of TNF-α Release

Supernatant in co-cultures of LPS-stimulated microglia with vehicle or hMSCs using a Transwell was collected for quantification of TNF-α release. Brain tissues from the ipsilateral SN were dissected and homogenized in MEM containing 1% P/S with sea sand. Tissue homogenates were centrifuged (20 min, 15,000 rpm, 4°C), and the supernatant was transferred to a fresh tube. The release of TNF-α into the culture supernatant and the supernatant of the tissue homogenates was determined using enzyme-linked immunosorbent assay (ELISA, Pyo et al., 1998) kit (Biosource, Camarillo, CA). The assay was carried out according to the manufacturer's protocol with same volume of supernatants (50 μL). The sensitivity of this assay was 15.6 pg/mL.

Determination of NO Release

The amount of NO in the conditioned microglia medium was determined at 24, 48, and 72 h following LPS treatment. The amount of nitrite converted from NO (Lee et al., 2005) was measured by mixing the culture medium (50 μL) with an equal volume of Griess reagent (0.1% naphthlethylene diamine, 1% sulfanilamide, and 2.5% H_3PO_4). The optical density was measured at 540 nm.

Tissue Preparation

For immunohistochemistry, the animals, anesthetized with 10% chloral hydrate, were perfused with saline solution containing 0.5% sodium nitrate and heparin (10 U/mL) and fixed with 4% paraformaldehyde dissolved in 0.1 M PB (~200 mL/rat of both) after 4 h, 3, 7, and 14 days. The brain was removed from the skull, postfixed for 3 days in buffered 4% paraformaldehyde at 4°C, and stored in 30% sucrose solution for 1–2 days at 4°C until it sank. The brains were then sectioned on a sliding microtome to obtain 40-μm thick coronal sections. All sections were stored in tissue stock solution (30% glycerol, 30% ethylene glycol, 30% 3× DW, 10% 0.2 M PB, pH 7.2) at 4°C until required. For ELISA and RNA extraction, the SN area was rapidly removed from the brain and frozen at −70°C.

Immunocytochemistry and Immunohistochemistry

The brain sections and co-cultured cells were rinsed twice in PBS and incubated in 0.2% Triton X-100 for 30 min at room temperature. They were rinsed three times with 0.5% bovine serum albumin (BSA) in 1× PBS for blocking. After blocking, they were incubated

overnight at 4°C with primary antibodies; the primary antibodies for OX-42, Iba-1, and tyrosine hydroxylase (TH) were OX-42 (1:200 for immunohistochemistry, 1:500 for immunocytochemistry; Serotec, Raleigh, NC), Iba-1 (1:1,000; Wako Pure Chemical Industries, Osaka, Japan) and TH (1:2,000 for immunohistochemistry, 1:7,500 for immunocytochemistry; Pel-freez, St. Rogers, AR), respectively. After overnight, the cultures and brain sections were rinsed three times in 0.5% BSA in 1× PBS (10 min/rinse) and incubated with an appropriate biotinylated secondary antibody and avidin-biotin complex (Elite Kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The bound antibodies were visualized by incubating with 0.05% diaminobenzidine-HCL (DAB) and 0.003% hydrogen peroxide in 0.1 M PB. The cultures and brain sections were rinsed with 0.1 M PB for DAB inhibition. The immunostained cells were analyzed under bright-field microscopy. The number of activated microglia in cortical microglial cultures was determined by counting process-bearing microglia in all microscopic fields because LPS treatment induced dramatic changes in cell morphology from a spherical shape into process-bearing cells (Nakamura et al., 1999).

Stereological Cell Counts

An unbiased stereological estimation of the total number of TH-immunopositive (TH-ip) cells in the SN was made using an optical fractionator as previously described (Kirik et al., 1998, 2000), with some modifications. This sampling technique is not affected by tissue volume changes and does not require reference volume determinations (West et al., 1991). The sections used for counts covered the entire SN from the rostral tip of the pars compacta back to the caudal end of the pars reticulate. This generally yielded eight to nine sections in a series. Sampling was performed using the Olympus C.A.S.T.-Grid system (Olympus Denmark A/S, Denmark), using an Olympus BX51 microscope connected to the stage and feeding the computer with distance information in the *z*-axis. The SN was delineated with a 1.25× objective. A counting frame (60%, 35,650 μm^2) was placed randomly on the first counting area and systemically moved through all counting areas until the entire delineated area was sampled. Actual counting was performed using a 40× oil objective. Guard volumes (4 μm from the top and 4–6 μm from the bottom of the section) were excluded from both the surfaces to avoid the problem of lost caps, and only the profiles that came into focus within the counting volume (with a depth of 10 μm) were counted. The total number of TH-ip cells was calculated according to the optical fractionator formula (West et al., 1991).

Statistical Analysis

The group means were compared using the Mann-Whitney *U*-test for pairs and the Kruskal-Wallis analysis for multiple groups. Statistical significance was deemed at $P < 0.05$. Statistical analyses were performed using commercially available software (SPSS version 10.0; SPSS, Chicago, IL).

RESULTS

hMSCs Co-Cultured with LPS-Stimulated Microglia in a Transwell Decreased Microglial Activation and Increased Expression of IL-6, IL-10, and TGF-β

We analyzed the morphological changes in microglia using OX-42 to examine the effect of hMSCs on microglial activation. With the introduction of LPS, the morphology of microglia changed dramatically from spherical-shaped to process-bearing cells at 6 and 24 h after co-culture with vehicle. However, co-culture with hMSCs (3×10^5 cells/well, using a Transwell) significantly decreased the number of process-bearing activated microglia induced by LPS stimulation at 6 and 24 h following the introduction of hMSCs (Fig. 1A,B). To identify soluble factors associated with modulation of microglial activation, we analyzed expression of IL-6, IL-10, and TGF-β in hMSCs co-cultured with LPS-stimulated microglia and hMSCs alone. IL-6 expression was significantly increased at 3 and 12 h, and IL-10 and TGF-β expressions were significantly increased at 12 h in hMSCs co-cultured with LPS-stimulated microglia compared with hMSCs alone (Fig. 1C,D).

The Presence of hMSCs in a Transwell Downregulated the Production of TNF-α and iNOS by LPS-Stimulated Microglia

To investigate the effect of hMSC co-culture on the LPS-induced production of TNF-α and iNOS and their gene expression in microglia, microglia-enriched cultures were treated with LPS for 4 h and then co-cultured with vehicle or hMSCs using a Transwell. After 24, 48, and 72 h, culture supernatants and cells were collected for RT-PCR and protein assays. LPS treatment significantly induced mRNA expression of TNF-α and iNOS and accumulation of NO and TNF-α, compared with the control group, whereas co-culture with hMSCs resulted in a significant reduction in TNF-α and iNOS mRNA expression and TNF-α and NO production compared with treatment with LPS alone at 24, 48, and 72 h (Fig. 2A–E).

Co-Culture with hMSCs Significantly Decreased Dopaminergic Neuronal Loss Induced by LPS Stimulation in Co-Culture System of Microglia and Mesencephalic Tissue

Co-cultures of microglia and mesencephalic neurons were treated with LPS for 4 h and then co-cultured with vehicle or hMSCs in a Transwell to determine the effect of hMSCs-induced antimicroglial activation on dopami-

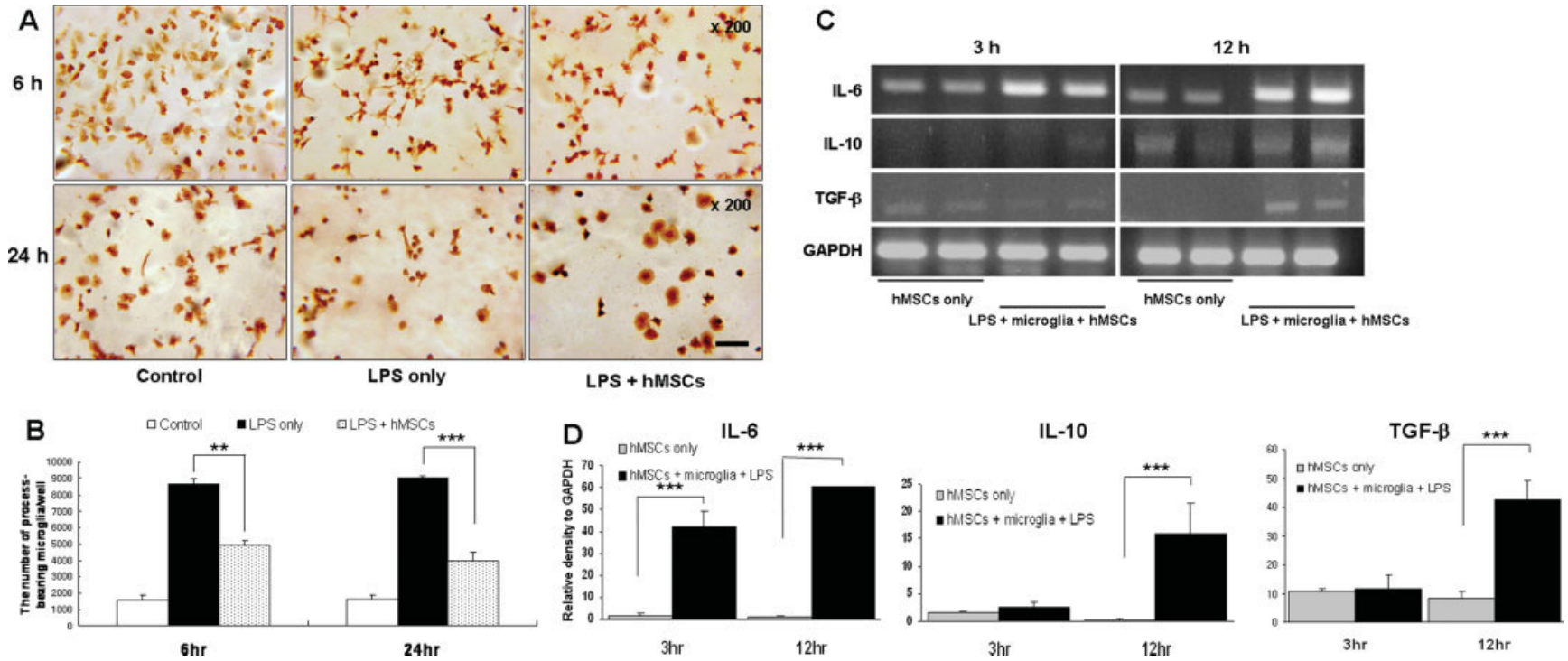

Fig. 1. Human mesenchymal stem cells (hMSCs) co-cultured with LPS-stimulated microglia in a Transwell decreased microglial activation and increased expression of IL-6, IL-10, and TGF-β. To identify soluble factors associated with modulation of microglial activation, we analyzed expression of IL-6, IL-10, and TGF-β in hMSCs co-cultured with LPS-stimulated microglia and hMSCs alone. The introduction of hMSCs significantly decreased the number of process-bearing activated microglia at 6 and 24 h following hMSC treatment (**A**, **B**). When hMSCs were co-cultured with LPS-stimulated microglia, IL-6 expression was significantly increased at 3 and 12 h, and IL-10 and TGF-β expressions were significantly increased at 12 h compared with hMSCs alone (**C**,**D**).The data are displayed as the mean (column) ± SEM (bar). The results are representative of five replications in each group. $^{**}P < 0.01$. $^{***}P < 0.001$. Scale bar, 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

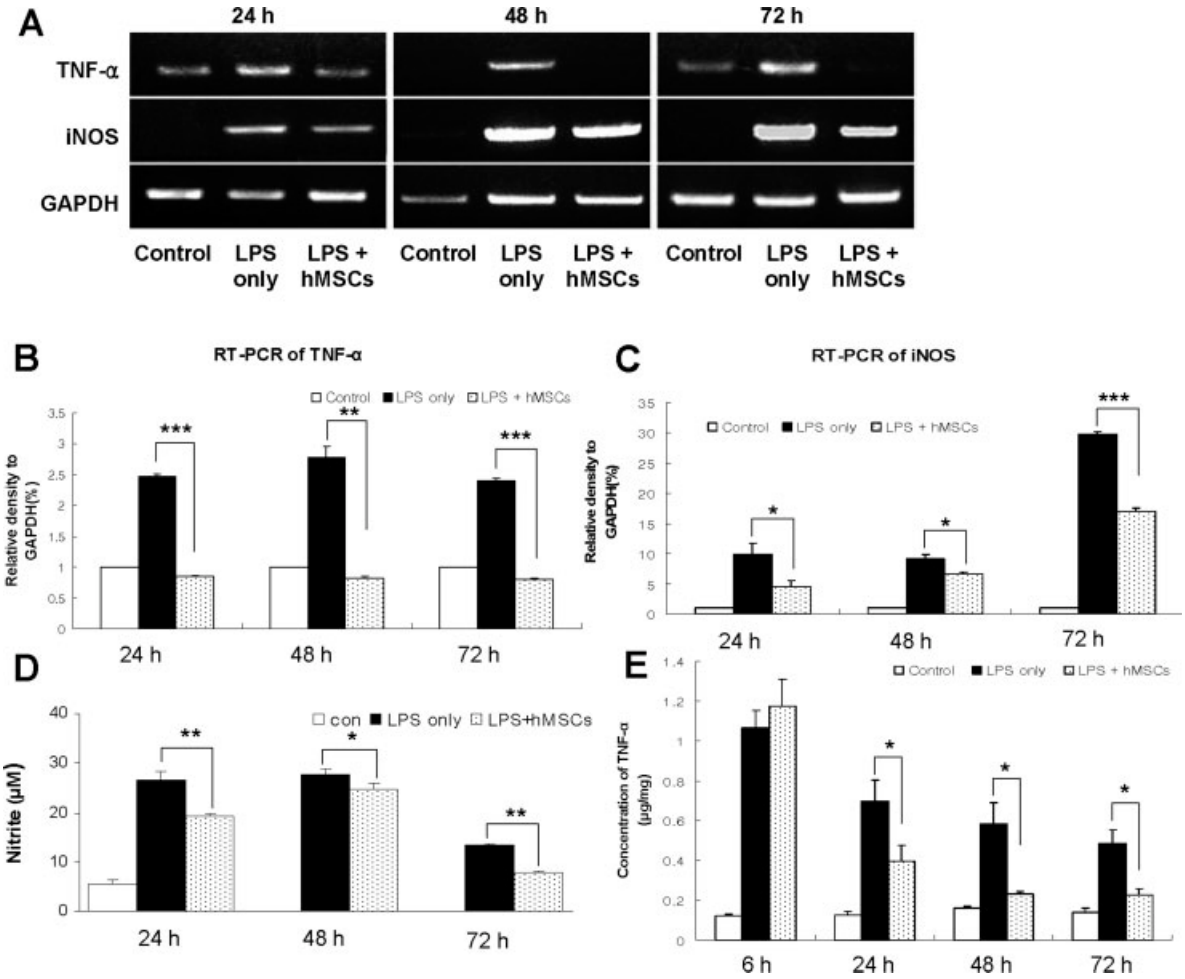

Fig. 2. Effect of co-culture with human mesenchymal stem cells (hMSCs) on lipopolysaccharide (LPS)-induced production of TNF-α and iNOS. Microglia from primary cultures were treated with LPS for 4 h and then co-cultured with vehicle or hMSCs in a Transwell. After 6, 24, 48, and 72 h, culture supernatants and cells were collected for RT-PCR and ELISA. LPS treatment significantly induced mRNA expression of TNF-α and iNOS and accumulation of NO and TNF-α, compared with the control group, whereas co-culture with hMSCs showed significant reductions in TNF-α and iNOS mRNA expression (**A**–**C**) and production of NO and TNF-α (**D**,**E**), when compared with those treated with LPS alone at 24, 48, and 72 h. The data are displayed as the mean (column) ± SEM (bar). The results of RT-PCR and ELISA are indicative of three and five replications, respectively. *$P < 0.05$. ** $P < 0.01$. ***$P < 0.001$.

nergic neurons. After 6 or 24 h, the cultures were immunostained with anti-TH antibody. LPS treatment resulted in a significant loss of TH-ip cells, whereas co-culture with hMSCs significantly decreased the loss of TH-ip cells (Fig. 3A). A cell counting analysis showed that co-culture with hMSCs resulted in significant incremental survival of TH-ip cells at 6 and 24 h following hMSC introduction (Fig. 3B).

hMSC Treatment Significantly Decreased Dopaminergic Neuronal Loss and Microglial Activation in the SN Induced by LPS and MPTP Administration

In the animal study, hMSCs were infused via the tail vein 8 h after the injection of LPS to examine the effects of hMSCs on LPS-induced microglial activation and dopaminergic neuronal loss in the rat SN. LPS stimulation in the SN resulted in a considerable loss of TH-ip cells and activation of microglia (Fig. 4A). The hMSC treatment considerably reduced the loss of TH-ip cells induced by LPS stimulation in the SN and clearly attenuated microglial activation, detected by OX-42 staining (Fig. 4A). On stereological analysis, hMSC treatment significantly decreased the loss of TH-ip cells at 7 and 14 days following LPS stimulation (Fig. 4B). The effect of hMSCs on survival of TH-ip cells and microglial activation in LPS-treated animals was also recapitulated in MPTP-treated animals. In MPTP-treated animals, there was a significant decrease in the number of TH-ip cells with increased microglial activation in the SN compared with saline-injected controls. However, hMSCs treatment group showed that the loss of TH-ip cells in the SN was significantly reduced, which was accompanied by considerable decrease in microglial activation when compared with MPTP-only treatment group (Fig. 4C,D). Additionally, hMSC treatment significantly

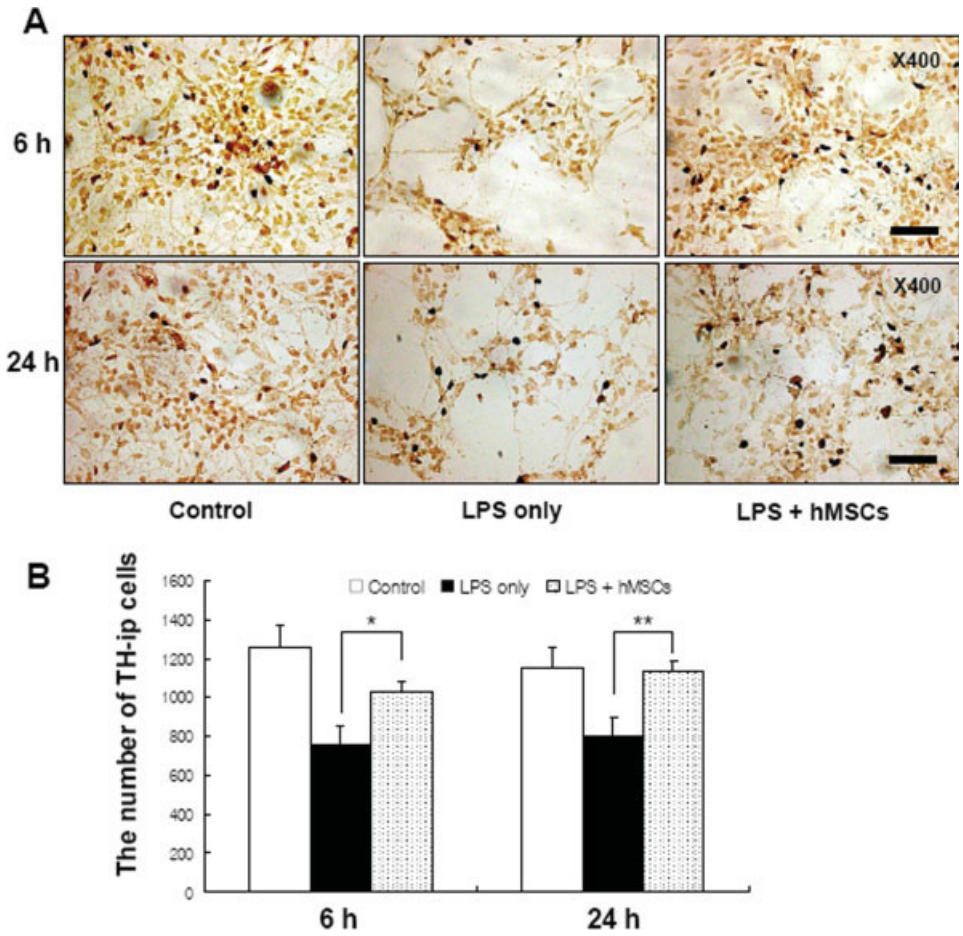

Fig. 3. Effect of human mesenchymal stem cells (hMSCs) against lipopolysaccharide (LPS)-induced damage to tyrosine hydroxylase-immunopositive (TH-ip) cells in mesencephalic neuron cultures. Co-cultures of microglia and mesencephalic neurons were treated with LPS for 4 h and then co-cultured with vehicle or hMSCs in a Transwell. LPS treatment resulted in a significant loss of TH-ip cells, whereas co-culture with hMSCs significantly decreased the loss of TH-ip cells (**A**). The total number of TH-ip cells in each well was counted under a converted microscope, and the data are presented as the mean ± SEM (**B**). The results are representative of five replications in each group. $^{*}P < 0.05$. $^{**}P < 0.01$. Scale bar, 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

downregulated the LPS-induced increase in the expression of TNF-α and iNOS mRNA at 3 days following LPS stimulation (Fig. 5A–C). TNF-α production was significantly decreased in the hMSC group compared with the LPS-only treatment group at 4 h and 3 days following LPS stimulation (Fig. 5D).

DISCUSSION

Our study showed that hMSCs have a protective effect on dopaminergic neurons through anti-inflammatory actions. In enriched microglial cultures, hMSC treatment prevented dopaminergic neuronal death by reducing LPS-induced release of proinflammatory cytokines. In animal study, hMSC injection significantly reduced LPS-induced dopaminergic neuronal loss in the SN.

In addition to the regenerative capacity of MSCs, they possess immunoregulatory properties. Although the exact mechanism of MSC-mediated immunoregulation is not understood, *in vitro* studies suggest that MSCs can not only inhibit nearly all cells participating in the immune response cell–cell contact-dependant mechanism, but can also release a variety of soluble factors that may be involved in the immunosuppressive activity of MSCs (Karussis et al., 2007; Krampera et al., 2006; Nauta and Fibbe, 2007). Recent animal studies in a model of experimental autoimmune encephalomyelitis demonstrated that MSC treatment results in a significantly milder disease and fewer relapses compared with control mice, with a decreased number of inflammatory infiltrates and reduced demyelination and axonal loss (Gerdoni et al., 2007; Zappia et al., 2005). Additionally, Guo et al. (2007) reported that MSC transplantation decreases protein production and gene expression of inflammation cytokines and increases functional recovery from myocardial infarcts. These studies suggest that the anti-inflammatory actions of MSCs may be one underlying mechanism of the tissue-protective effect.

In co-culture experiments with LPS-stimulated microglia and hMSCs using a Transwell to physically separate the microglia and hMSCs and thereby inhibit cell–cell

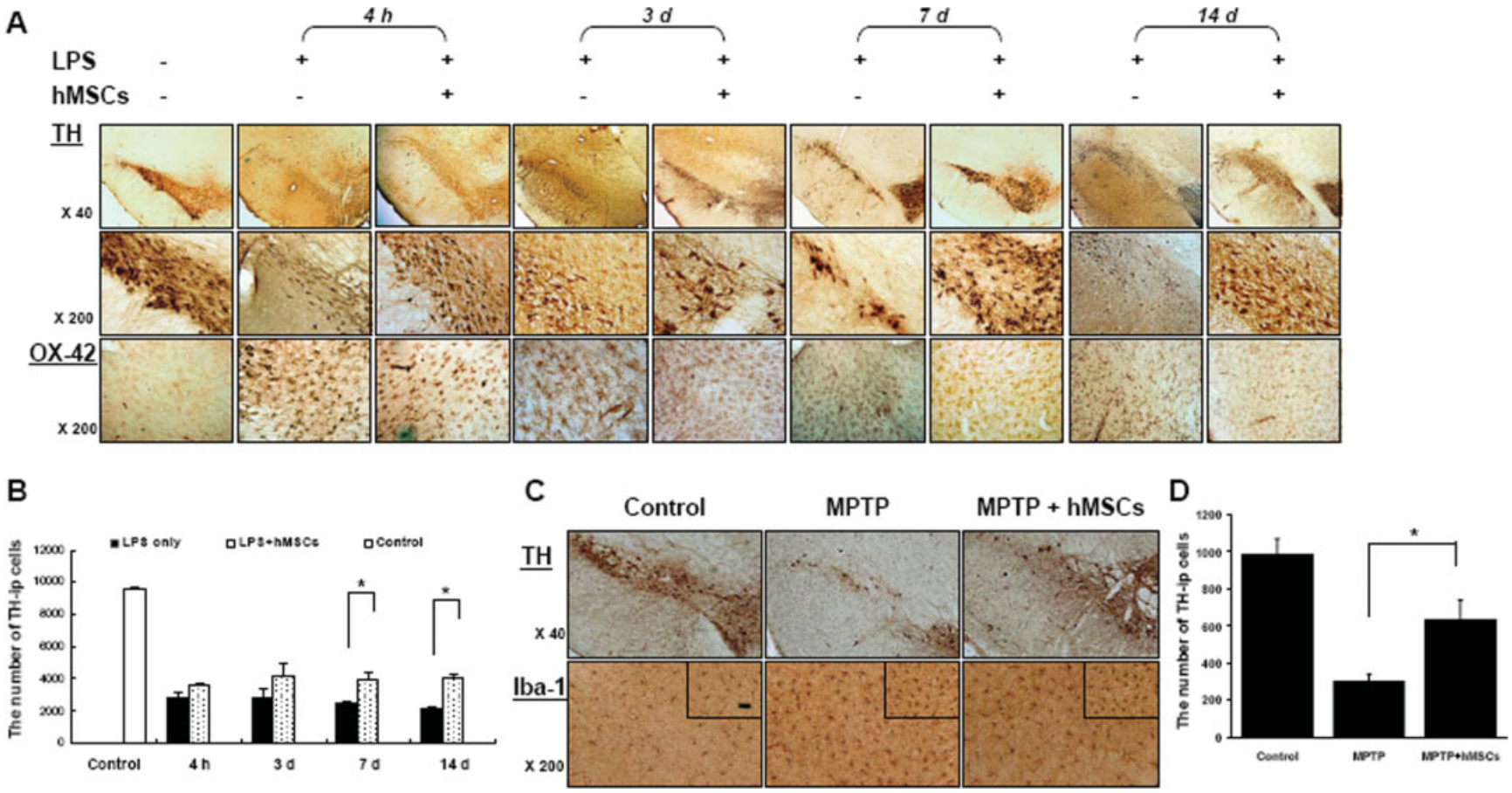

Fig. 4. Immunohistological evaluation of protective effect of human mesenchymal stem cells (hMSCs) against lipopolysaccharide (LPS) and MPTP-induced damage to dopaminergic neurons in the substantia nigra (SN). The hMSCs treatment considerably reduced the loss of TH-ip cells and microglial activation induced by LPS stimulation in the SN (**A**). On stereological analysis, hMSC treatment significantly decreased the loss of TH-ip cells at 7 and 14 days following LPS stimulation (**B**). In MPTP-treated animals, hMSCs treatment showed that the loss of TH-ip cells in the SN was significantly reduced, which was accompanied by considerably decrease in microglial activation when compared with MPTP-only treatment group (**C**,**D**). The data are displayed as the mean (column) ± SEM (bar). The results are representative of five rats in each group. $^{*}P < 0.05$. Scale bar: 100 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

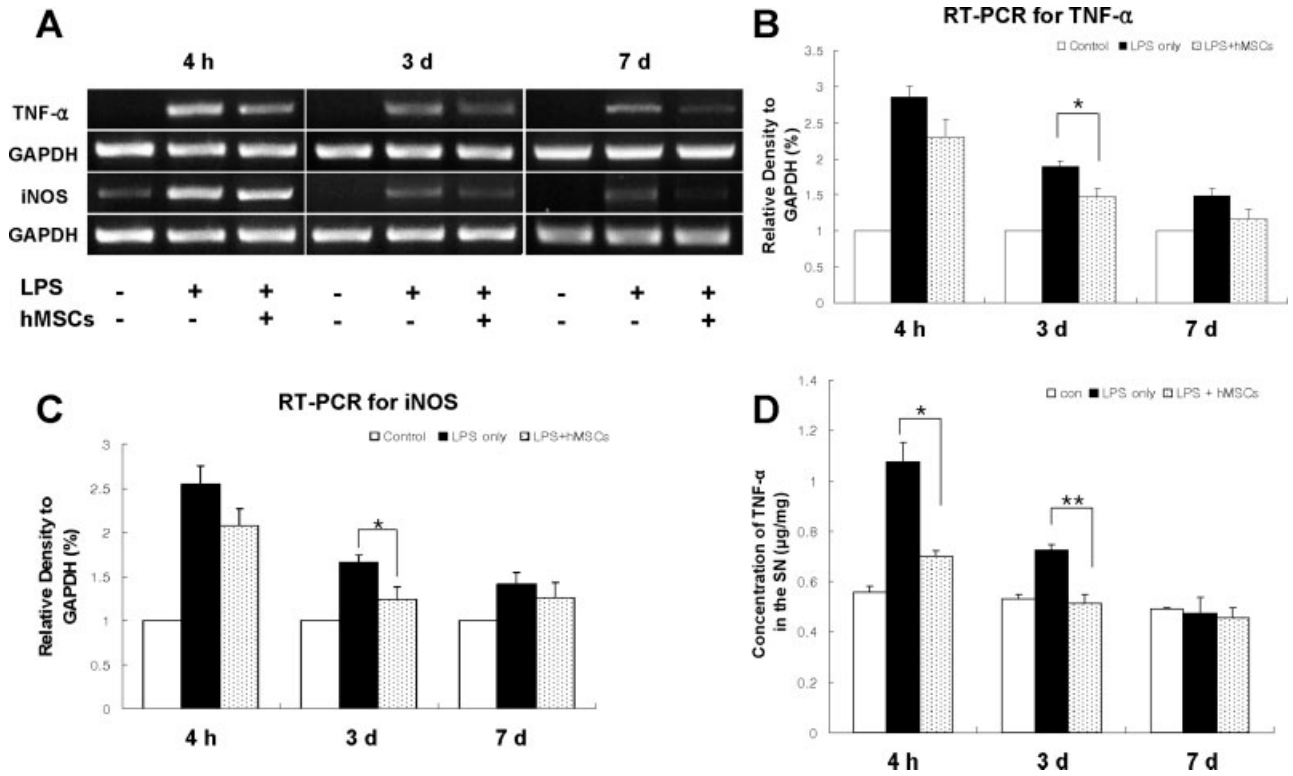

Fig. 5. TNF-α and iNOS in the substantia nigra (SN) following human mesenchymal stem cell (hMSC) administration. The hMSC administration significantly downregulated the lipopolysaccharide (LPS)-induced increase in the expression of TNF-α and iNOS mRNA at 3 days after LPS stimulation (**A–C**). TNF-α production was significantly decreased in the hMSC group compared with the LPS-only treatment group at 4 h and 3 days after LPS stimulation (**D**). The data are displayed as the mean (column) ± SEM (bar). The results are representative of five rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

contact, we found that the hMSCs decreased the number of activated form of microglia and had increased expression of anti-inflammatory cytokines (IL-6, IL-10, and TGF-β). Furthermore, hMSCs decreased production of TNF-α and iNOS from microglia stimulated by LPS in a contact-independent manner. Although the precise mechanism of the inhibitory effect of MSCs on microglial activation is unknown, there is ample evidence that microglial activation can be modulated by various cytokines and neurotrophic factors (Hanisch and Kettenmann, 2007; Nakamura, 2002; Oh et al., 2008). Therefore, it is speculated that soluble factors released from MSCs, such as IL-6, IL-10, and TGF-β may regulate the microglia response to LPS in our experiment.

In this study, we found that hMSCs significantly decreased the release of TNF-α and iNOS and dopaminergic neuronal loss induced by LPS stimulation. In cocultures of microglia and mesencephalic neurons, these anti-inflammatory actions of hMSCs actually resulted in a significant decrease (up to ~50%) in dopaminergic neuronal death induced by LPS stimulation. Furthermore, hMSC administration dramatically decreased the dopaminergic neuronal loss in the SN induced by LPS stimulation and MPTP treatment, which was clearly accompanied by the attenuation of microglial activation. Interestingly, increased survival of TH-ip cells after hMSC treatment was clearly evident in the later period (7 and 14 days) following LPS stimulation by approximately two times more than in the LPS-only treatment. In contrast, the inhibitory effect of hMSCs on the production of TNF-α and iNOS occurred in the early period (4 h and 3 days) following LPS stimulation. According to the animal studies, microglial activation appears to occur before the death of dopaminergic neurons, and microglia, once activated, continue to promote the degeneration of dopaminergic neurons (Cho et al., 2006; Gao et al., 2002). Thus, the neuroprotective effect of hMSCs on dopaminergic neurons that we observed may be a consequence of the inhibition of inflammatory actions that occur in the early stages of the inflammatory process in the SN. In addition to a variety of pleiotrophic mechanisms of MSCs as trophic mediators (Caplan and Dennis, 2006), our data suggest that the neuroprotective properties of hMSCs via anti-inflammatory effects were also evident in an animal model of PD.

A large body of *in vivo* studies suggests that the inhibition of the inflammatory response can prevent the degeneration of nigrostriatal dopaminergic neurons. For example, sodium salicylate, a COX-2 inhibitor, and minocycline have been shown to significantly reduce dopaminergic neuronal loss induced by MPTP or LPS (Aubin et al., 1998; Du et al., 2001; Teismann et al., 2003). Additionally, recent epidemiological studies showed the beneficial effect of NSAIDs in the development or progression of PD (Chen et al., 2003; Wahner et al., 2007).

Thus, these studies have raised the possibility that the inhibition of inflammation may be a viable neuroprotective strategy in the treatment of PD patients.

MSCs express several specific neuronal markers and transcription factors, with a large proportion of these genes being involved in the neuro-dopaminergic system, suggesting that the expression of neural gene and genes associated with the dopaminergic system is a widespread phenomenon in MSCs (Blondheim et al., 2006). Regarding the application of MSCs in animal models of PD, Li et al. (2001) and Blondheim et al. (2006) reported that intrastriatal injections of MSCs exhibited the phenotype of dopaminergic neurons. Along with the possible transdifferentiation potency of MSCs into a dopaminergic phenotype, the neuroprotective properties of MSCs on dopaminergic neurons via anti-inflammatory action raises the possibility of the clinical application of MSCs as a therapeutic strategy in PD. In addition to the molecular and cellular benefits of MSCs, cell therapy with MSCs has a major advantage in clinical applications; specifically, MSCs can be readily harvested from the patient's bone marrow, cultured *in vitro*, and administered to the patient via various routes, including intravenous, intraarterial, intrathecal, and intralesional infusion. In contrast with embryonic stem cell therapy, there is no immunological rejection, and cell therapy with MSCs is free from ethical issues. Importantly, regarding the safety of MSCs in clinical applications, our group has demonstrated that cell therapy with hMSCs in patients with multiple system atrophy and ischemic stroke was feasible and safe (Bang et al., 2005; Lee et al., 2008).

In summary, we demonstrated that hMSCs have neuroprotective effects on dopaminergic neurons via an anti-inflammatory mechanism mediated by the modulation of microglial activation. Along with various trophic effects and their transdifferentiational potency, these anti-inflammatory properties of hMSCs may have major therapeutic implications in the treatment of PD.

REFERENCES

Aubin N, Curet O, Deffois A, Carter C. 1998. Aspirin and salicylate protect against MPTP-induced dopamine depletion in mice. J Neurochem 71:1635–1642.

Bang OY, Lee JS, Lee PH, Lee G. 2005. Autologous mesenchymal stem cell transplantation in stroke patients. Ann Neurol 57:874–882.

Barry FP, Murphy JM. 2004. Mesenchymal stem cells: Clinical applications and biological characterization. Int J Biochem Cell Biol 36:568–584.

Blondheim NR, Levy YS, Ben-Zur T, Burshtein A, Cherlow T, Kan I, Barzilai R, Bahat-Stromza M, Barhum Y, Bulvik S, Melamed E, Offen D. 2006. Human mesenchymal stem cells express neural genes, suggesting a neural predisposition. Stem Cells Dev 15:141–164.

Boka G, Anglade P, Wallach D, Javoy-Agid F, Agid Y, Hirsch EC. 1994. Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. Neurosci Lett 172:151–154.

Caplan AI, Dennis JE. 2006. Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076–1084.

Chen H, Zhang SM, Hernan MA, Schwarzschild MA, Willett WC, Colditz GA, Speizer FE, Ascherio A. 2003. Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. Arch Neurol 60:1059–1064.

Cho BP, Song DY, Sugama S, Shin DH, Shimizu Y, Kim SS, Kim YS, Joh TH. 2006. Pathological dynamics of activated microglia following medial forebrain bundle transection. Glia 53:92–102.

Chung ES, Joe EH, Ryu JK, Kim J, Lee YB, Cho KG, Oh YJ, Maeng SH, Baik HH, Kim SU, Jin BK. 2001. GT1b ganglioside induces death of dopaminergic neurons in rat mesencephalic cultures. Neuroreport 12:611–614.

Cicchetti F, Brownell AL, Williams K, Chen YI, Livni E, Isacson O. 2002. Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. Eur J Neurosci 15:991–998.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, Triarhou LC, Chernet E, Perry KW, Nelson DL, Luecke S, Phebus LA, Bymaster FP, Paul SM. 2001. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 98:14669–14674.

Gao HM, Hong JS, Zhang W, Liu B. 2002. Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. J Neurosci 22:782–790.

Gerdoni E, Gallo B, Casazza S, Musio S, Bonanni I, Pedemonte E, Mantegazza R, Frassoni F, Mancardi G, Pedotti R, Uccelli A. 2007. Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. Ann Neurol 61:219–227.

Guo J, Lin GS, Bao CY, Hu ZM, Hu MY. 2007. Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30:97–104.

Hamre K, Tharp R, Poon K, Xiong X, Smeyne RJ. 1999. Differential strain susceptibility following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration acts in an autosomal dominant fashion: Quantitative analysis in seven strains of Mus musculus. Brain Res 828:91–103.

Hanisch UK, Kettenmann H. 2007. Microglia: Active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10:1387–1394.

Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debre P, Agid Y, Dugas B, Hirsch EC. 1999. FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-α in glial cells. J Neurosci 19:3440–3447.

Karussis D, Kassis I, Basan GS, Slavin S. 2007. Immunomodulation and neuroprotection with mesenchymal bone marrow stem cells (MSCs): A proposed treatment for multiple sclerosis and other neuroimmunological/neurodegenerative diseases. J Neurol Sci 265:131–135.

Kirik D, Rosenblad C, Bjorklund A. 1998. Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. Exp Neurol 152:259–277.

Kirik D, Rosenblad C, Bjorklund A, Mandel RJ. 2000. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: Intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J Neurosci 20:4686–4700.

Krampera M, Pasini A, Pizzolo G, Cosmi L, Romagnani S, Annunziato F. 2006. Regenerative and immunomodulatory potential of mesenchymal stem cells. Curr Opin Pharmacol 6:435–441.

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D. 1999. Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol 46:598–605.

Lee DY, Oh YJ, Jin BK. 2005. Thrombin-activated microglia contribute to death of dopaminergic neurons in rat mesencephalic cultures: Dual roles of mitogen-activated protein kinase signaling pathways. Glia 51:98–110.

Lee PH, Kim JW, Bang OY, Ahn YH, Joo IS, Huh K. 2008. Autologous mesenchymal stem cell therapy delays the progression of neurological deficits in patients with multiple system atrophy. Clin Pharmacol Ther 83:723–730.

Li Y, Chen J, Wang L, Zhang L, Lu M, Chopp M. 2001. Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Neurosci Lett 316:67–70.

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S. 1999. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med 5:1403–1409.

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705.

McGeer PL, Itagaki S, Boyes BE, McGeer EG. 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38:1285–1291.

Moore DJ, West AB, Dawson VL, Dawson TM. 2005. Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci 28:57–87.

Nagatsu T, Mogi M, Ichinose H, Togari A. 2000. Cytokines in Parkinson's disease. J Neural Transm Suppl 172:143–151.

Nakamura Y. 2002. Regulating factors for microglial activation. Biol Pharm Bull 25:945–953.

Nakamura Y, Si QS, Kataoka K. 1999. Lipopolysaccharide-induced microglial activation in culture: Temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci Res 35:95–100.

Nauta AJ, Fibbe WE. 2007. Immunomodulatory properties of mesenchymal stromal cells. Blood 110:3499–3506.

Oh JY, Kim MK, Shin MS, Lee HJ, Ko JH, Wee WR, Lee JH. 2008. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. Stem Cell 26:1047–1055. DOI: 10.1634/stemcells. 2007-0737.

Ouchi Y, Yoshikawa E, Sekine Y, Futatsubashi M, Kanno T, Ogusu T, Torizuka T. 2005. Microglial activation and dopamine terminal loss in early Parkinson's disease. Ann Neurol 57:168–175.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Pyo H, Jou I, Jung S, Hong S, Joe EH. 1998. Mitogen-activated protein kinases activated by lipopolysaccharide and β-amyloid in cultured rat microglia. Neuroreport 9:871–874.

Ryu J, Pyo H, Jou I, Joe E. 2000. Thrombin induces NO release from cultured rat microglia via protein kinase C, mitogen-activated protein kinase, and NF-κ B. J Biol Chem 275:29955–29959.

Teismann P, Tieu K, Choi DK, Wu DC, Naini A, Hunot S, Vila M, Jackson-Lewis V, Przedborski S. 2003. Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. Proc Natl Acad Sci USA 100:5473–5478.

von Bohlen und Halbach O, Schober A, Krieglstein K. 2004. Genes, proteins, and neurotoxins involved in Parkinson's disease. Prog Neurobiol 73:151–177.

Wahner AD, Bronstein JM, Bordelon YM, Ritz B. 2007. Nonsteroidal anti-inflammatory drugs may protect against Parkinson disease. Neurology 69:1836–1842.

West MJ, Slomianka L, Gundersen HJ. 1991. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231:482–497.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370.

Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A. 2005. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 106:1755–1761.