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I.V. INFUSION OF BRAIN-DERIVED NEUROTROPHIC FACTOR GENE-MODIFIED HUMAN MESENCHYMAL STEM CELLS PROTECTS AGAINST INJURY IN A CEREBRAL ISCHEMIA MODEL IN ADULT RAT

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Abstract—I.v. delivery of mesenchymal stem cells prepared from adult bone marrow reduces infarction size and ameliorates functional deficits in rat cerebral ischemia models. Administration of the brain-derived neurotrophic factor to the infarction site has also been demonstrated to be neuroprotective. To test the hypothesis that brain-derived neurotrophic factor contributes to the therapeutic benefits of mesenchymal stem cell delivery, we compared the efficacy of systemic delivery of human mesenchymal stem cells and human mesenchymal stem cells transfected with a fiber-mutant F/RGD adenovirus vector with a brain-derived neurotrophic factor gene (brain-derived neurotrophic factor–human mesenchymal stem cells). A permanent middle cerebral artery occlusion was induced by intraluminal vascular occlusion with a microfilament. Human mesenchymal stem cells and brain-derived neurotrophic factor–human mesenchymal stem cells were i.v. injected into the rats 6 h after middle cerebral artery occlusion. Lesion size was assessed at 6 h, 1, 3 and 7 days using MR imaging, and histological methods. Functional outcome was assessed using the treadmill stress test. Both human mesenchymal stem cells and brain-derived neurotrophic factor–human mesenchymal stem cells reduced lesion volume and elicited functional improvement compared with the control sham group, but the effect was greater in the brain-derived neurotrophic factor–human mesenchymal stem cell group. ELISA analysis of the infarcted hemisphere revealed an increase in brain-derived neurotrophic factor in the human mesenchymal stem cell groups, but a greater increase in the brain-derived neurotrophic factor–human mesenchymal stem cell group. These data support the hypothesis that brain-derived neurotrophic factor contributes to neuroprotection in cerebral ischemia and cellular delivery of brain-derived neurotrophic factor can be achieved by i.v. delivery of human mesenchymal stem cells.

Key words: bone marrow, neuroprotection, regeneration, stroke, transplantation.

Transplantation of bone marrow-derived cells several hours after ischemia onset has been shown to reduce infarction size and improve functional outcome in rodent cerebral ischemia models (Chen et al., 2001; Iihoshi et al., 2004). The post-injury time window for cell delivery and the prospect of preparation of large numbers of autologous cells from bone marrow aspirates, suggest the potential utility of this approach as a treatment in stroke.

The precise cell type within bone marrow responsible for the neuroprotection is not known, but is thought to reside within the marrow stromal or mesenchymal stem cell (MSC) population (Li et al., 2002; Iihoshi et al., 2004). MSCs can be isolated and expanded as plastic adherent cells having a flattened fibroblast-like morphology (Friedenstein, 1976; Woodbury et al., 2000) that are $CD34^-$, $CD45^-$, $SH2^+$, and $SH3^+$ (Majumdar et al., 1998; Kobune et al., 2003). MSCs have been suggested to differentiate into osteoblasts, chondrocytes, adipocytes and hepatocytes (Prockop, 1997; Pittenger et al., 1999; Sanchez-Ramos et al., 2000; Krause et al., 2001; Kobune et al., 2003). It has also been suggested that they can differentiate into cells of neuronal and glial lineage (Azizi et al., 1998; Kopen et al., 1999; Brazelton et al., 2000; Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Sasaki et al., 2001; Iihoshi et al., 2004). Recent work indicates that under some conditions MSCs can fuse with other cells thereby making the distinction between cell fusion and transdifferentiation difficult (Castro et al., 2002; Alvarez-Dolado et al., 2003).

Independent of the cell fusion vs transdifferentiation issue, isolated cultured bone marrow-derived MSCs have been shown to secrete trophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), vascular endothelial growth factors (VEGF), and hepatocyte growth factor (HGF) (Hamano et al., 2000; Chen et al., 2002; Iihoshi et al., 2004; Kurozumi et al., 2004). Indeed, BDNF administration has neuroprotective effects in a rat ischemia model (Schabitz et al., 1997, 2000; Yamashita et al., 1997). The release of trophic factors from transplanted MSCs within the host brain may contribute to the reduction in infarction size and to the recovery of function following ischemia in recipient animals (Iihoshi et

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Abbreviations: BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; hMSC, human mesenchymal stem cell; MCAO, middle cerebral artery occlusion; MOI, multiplicity of infection; MSC, mesenchymal stem cell; MSCBM, mesenchymal stem cell basal medium; NF, neurofilament; pu, particle unit; TTC, 2,3,5-triphenyltetrazolium chloride.

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al., 2004). Additionally, direct intracranial injection of human mesenchymal stem cells transfected with the BDNF gene (BDNF-hMSCs) resulted in therapeutic benefits in the transient rat middle cerebral artery occlusion (MCAO) model (Kurozumi et al., 2004).

Although direct intracerebral injection of BDNF-hMSCs in the rat MCAO model showed efficacy, this approach would be limited in clinical applications because of the surgical requirement and the difficulty in distributing cells to large areas of brain by focal injection. Systemic delivery of bone marrow cells has been reported to distribute in the ischemic lesion in the rat MCAO model and is associated with functional improvement (Iihoshi et al., 2004). In this study, hMSCs and hypersecreting BDNF-hMSCs were i.v. delivered at 6 h after induction of unilateral permanent cerebral ischemia to investigate if cellular delivery of BDNF by MSCs could influence lesion volume and functional outcome.

EXPERIMENTAL PROCEDURES

Preparation of hMSCs

Human bone marrow from healthy adult volunteers was obtained by aspiration from the posterior iliac crest after informed consent was obtained; this study was approved by the institutional review board at our university (Kobune et al., 2003). Bone marrow mononuclear cells were isolated, and were plated in 150-cm^2 plastic tissue culture flasks and incubated overnight. After washing away the free cells, the adherent cells were cultured in mesenchymal stem cell basal medium (MSCBM, Cambrex, Walkersville, MD, USA) containing mesenchymal cell growth supplement (MCGS, Cambrex, Walkersville, MD, USA), 4 mM L-glutamine, in a humidified atmosphere of 5% CO_2 at 37 °C. After reaching confluence, they were harvested and cryopreserved as primary MSCs or used for gene transduction.

Adenoviral vectors

Adenoviral vectors carrying a human BDNF cDNA were constructed as described previously (Kurozumi et al., 2004). Briefly, human BDNF cDNA was cloned using the reverse-transcription polymerase chain reaction (RT-PCR) method from the total RNA extracted from primary hMSC as the template. The identity of BDNF cDNA obtained in this manner was confirmed by sequencing and comparing it to the GeneBank sequence XM_006027.

The human BDNF primer sequence was forward, 5′-CGGAATTCCACCATGACCATCCTTTTCCTTACTATGGTTA-3′, and reverse, 5′-CCAGATCTATCTTCCCCTTTTAATGGTCAATGTA-3′.

The BDNF cDNA was inserted between the *Eco*R I site and the *Bgl*II site in the pCAcc vector and the resulting plasmid was designated pCAhBDNF. The plasmid pCAhBDNF was digested with *Cla*I, and the fragment containing the BDNF cDNA expression unit was isolated by agarose gel electrophoresis. The adenoviral BDNF expression vector, pWEAxCAhBDNF-F/RGD, was prepared using LipofectAMINE 2000 (Invitrogen, Tokyo, Japan).

Before being used, the above viral vectors were evaluated for their viral concentration and titer, and viral stocks were examined for potential contamination with replication-competent viruses. To determine viral concentration (particle unit (pu)/ml), the viral solution was incubated in 0.1% sodium dodecyl sulfate and A_{260} was measured. The viral titers of AxCAhBDNF-F/RGD were 1.0×10^{12} pu/ml, respectively.

Adenovirus infection

Adenovirus-mediated gene transfection was performed as previously described (Tsuda et al., 2003; Kurozumi et al., 2004). Briefly, the cells were seeded at a density of 2×10^6 cells per 15 cm plate. MSCs were exposed to the infectious viral particles in 7.5 ml DMEM at 37 °C medium for 60 min; cells were infected with AxCAhBDNF-F/RGD at a multiplicity of infection (MOI) of 3.0×10^3 pu/cell. The medium was then removed, and the cells washed once with DMEM and then recultured with normal medium for 12 h, after which transplantation was performed.

Cerebral ischemic model

The rat MCAO model was used as a stroke model. We induced permanent MCAO by using a previously described method of intraluminal vascular occlusion (Longa et al., 1989; Iihoshi et al., 2004). Adult male Sprague–Dawley rats ($n=80$) weighing 250–300 g were initially anesthetized with 5% isoflurane and maintained under anesthesia with 1.5% isoflurane in a mixture of 70% N_2O and 30% O_2 with mechanical ventilation. Rectal temperature was maintained at 37° with an infrared heat lamp. The left femoral artery was cannulated for measuring blood pH, pO_2, and pCO_2 throughout the surgery. A length of 20.0–22.0 mm 4-0 surgical Dermalon suture with the tip rounded by heating near a flame was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA.

This study was approved by the Institutional Review Board at our university, and all experiments conformed to international guidelines on the ethical use of animals, minimizing the number of animals used and their suffering.

Transplantation procedures

Experiments consisted of three groups ($n=66$). In group 1 (control), rats were given medium alone (without donor cell administration) injected i.v. at 6 h after MCAO (just after the initial MRI measurement) ($n=22$). In group 2, rats were given hMSCs (1.0×10^7) in 1 ml total fluid volume (MSCBM) injected i.v. at 6 h after MCAO ($n=22$). In group 3, rats were given BDNF-hMSCs (1.0×10^7) injected i.v. at 6 h after MCAO ($n=22$). Seven rats in each group were used to calculate the infarct lesion volume, and the remaining rats were used for the additional histological analysis.

In some experiments, Adex1CAlacZ adenovirus was used to transduce the LacZ gene into the MSCs. Details of the construction procedures are described elsewhere (Nakamura et al., 1994; Nakagawa et al., 1998; Takiguchi et al., 2000; Iihoshi et al., 2004). For *in vitro* adenoviral infection, 1.0×10^7 hMSCs were placed with Adex1CAlacZ at 50 MOI for 1 h and incubated at 37 °C in DMEM containing 10% fetal calf serum.

MRI

Rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) i.p. Each rat was placed in an animal holder/MRI probe apparatus and positioned inside the magnet. The animal's head was held in place inside the imaging coil. All MRI measurements were performed using a 7-T, 18-cm-bore superconducting magnet (Oxford Magnet Technologies) interfaced to a UNITYINOVA console (Oxford Instruments, UK and Varian, Inc., Palo Alto, CA, USA). T_2-weighted images were obtained from a 1.0-mm-thick coronal section using a 3 cm field of view, TR=3000 ms, TE=35 ms, and reconstructed using a 512×512 image matrix. Accurate positioning of the brain was performed to center the image slice 5 mm posterior to the rhinal fissure with the head of the rat held in a flat skull position. MRI measurements were obtained 6, 24, 72 h and 1 week after MCAO.

The ischemic lesion area was calculated from T_2-weighted images using imaging software (Scion Image, Version Beta 4.0.2,