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Intravenous Administration of Glial Cell Line-Derived Neurotrophic Factor Gene-Modified Human Mesenchymal Stem Cells Protects Against Injury in a Cerebral Ischemia Model in the Adult Rat

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Abstract

Intravenous administration of human mesenchymal stem cells (hMSCs) prepared from adult bone marrow has been reported to ameliorate functional deficits after cerebral artery occlusion in rats. Several hypotheses to account for these therapeutic effects have been suggested, and current thinking is that neuroprotection rather than neurogenesis is responsible. To enhance the therapeutic benefits of hMSCs potentially, we transfected hMSCs with the glial cell line-derived neurotrophic factor (GDNF) gene using a fiber-mutant F/RGD adenovirus vector and investigated whether GDNF gene-modified hMSCs (GDNF-hMSCs) could contribute to functional recovery in a rat permanent middle cerebral artery occlusion (MCAO) model. We induced MCAO by using intraluminal vascular occlusion, and GDNF-hMSCs were intravenously infused into the rats 3 hr later. MRI and behavioral analyses revealed that rats receiving GDNF-hMSCs or hMSCs exhibited increased recovery from ischemia compared with the control group, but the effect was greater in the GDNF-hMSC group. Thus, these results suggest that intravenous administration of hMSCs transfected with the GDNF gene using a fiber-mutant adenovirus vector may be useful in the cerebral ischemia and may represent a new strategy for the treatment of stroke.

Keywords

bone marrow; stroke; transplantation

Transplantation of bone marrow-derived mesenchymal stem cells (MSCs) has been reported to reduce infarction size and ameliorate functional deficits in rodent cerebral ischemia models (Chen et al., 2001; Iihoshi et al., 2004; Nomura et al., 2005; Honma et al., 2006). Indeed, MSCs derived from adult human bone marrow (hMSCs) have been shown to secrete trophic factors, including glial cell line-derived neurotrophic factor (GDNF), brain-derived

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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., 2005; Nomura et al., 2005). Moreover, transplantation of hMSCs modified to hypersecrete BDNF has a greater therapeutic effect in cerebral ischemia models than transplantation of nongene-modified hMSCs (Kurozumi et al., 2004, 2005; Nomura et al., 2005). Thus, the release of trophic factors from transplanted MSCs within the host brain may contribute to the reduction in infarction size and to the increased functional recovery following ischemia in recipient animals.

GDNF, a distantly related member of the transforming growth factor- β superfamily, is a potent neurotrophic factor that promotes neuronal survival and differentiation (Beck et al., 1995; Tomac et al., 1995; Oppenheim et al., 1995; Yan et al., 1995; Liberatore et al., 1997) and becomes expressed in the ischemic brain (Abe and Hayashi, 1997; Zhang et al., 2002). Moreover, surface application and intracerebral administration of GDNF decreased cerebral infarction volume (Wang et al., 1997; Kitagawa et al., 1998; Zhang et al., 2002). Direct intracerebral injection of hMSCs transfected with the GDNF gene (GDNF-hMSCs) resulted in therapeutic benefits in a rat middle cerebral artery occlusion (MCAO) model (Kurozumi et al., 2005).

Although direct intracerebral injection of GDNF-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 hMSCs has been reported to distribute in the ischemic lesion in the rat MCAO model and is associated with functional improvement (Nomura et al., 2005; Honma et al., 2006). To potentially enhance the therapeutic benefits of hMSCs, we transfected hMSCs with the GDNF gene by using fiber-mutant F/RGD adenovirus vector and investigated whether GDNF gene-modified hMSCs (GDNF-hMSCs) could contribute to the functional repair in a rat permanent MCAO model.

MATERIALS AND METHODS

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, plated in 150-cm² plastic tissue culture flasks, and incubated overnight. After the free cells were washed away, the adherent cells were cultured in mesenchymal stem cell basal medium (MSCBM; Cambrex) containing mesenchymal cell growth supplement (MCGS; Cambrex), 4 mM L-glutamine, in a humidified atmosphere of 5% CO₂ 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 GDNF cDNA were constructed as described previously (Kurozumi et al., 2005). Briefly, human GDNF cDNA was cloned by using the reverse-transcription polymerase chain reaction (RT-PCR) method from the total RNA extracted from primary hMSC as the template. The identity of GDNF cDNA obtained in this manner was confirmed by sequencing and comparison with the GeneBank sequence AY 052832. The human GDNF primer sequence was forward 5'-CGCAATTGCCACCATGAAGTTATGGGATGTCGTGGCTGT-3' and reverse 5'-CCAGATCTCAGATACATCCACACCTTTTAGCGG-3'. Adenoviral vectors

(AxCaHbGDNF-F/RGD) carrying the human GDNF together with the gene for RGD-mutated fiber under control of a CA promoter were constructed as described above.

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 (Nyberg-Hoffman et al., 1997). The viral titers of AxCaHbGDNF-F/RGD were 1.0×10^{12} pu/ml.

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. hMSCs were exposed to the infectious viral particles in 10 ml MSCBM containing MCGS at 37°C medium for 180 min; cells were infected with AxCaHbGDNFF/RGD at a multiplicity of infection (MOI) of 300, 1×10^3 and 3.0×10^3 pu/cell for in vitro study, and the latter was used for in vivo cell infusion studies. The medium was then removed, and the cells washed once with MSCBM containing MCGS and then recultured with normal medium for 24 hr, after which transplantation was performed.

Cerebral Ischemic Model

The rat MCAO model was used as a stroke model. This study was approved by the animal care and use committee of Sapporo Medical University, and all procedures were carried out in accordance with institutional guidelines. We induced permanent MCAO by using a previously described method of intraluminal vascular occlusion (Longa et al., 1989; Iihoshi et al., 2004). Adult female Sprague-Dawley rats weighing 250–300 g were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). 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.

Transplantation Procedures

Experiments consisted of three groups. In group 1 (control), rats were given medium alone (without donor cell administration) injected i.v. at 3 hr after MCAO (just after the initial MRI measurement). In group 2, rats were given hMSCs (1.0×10^7) in 500 μ l total fluid volume (DMEM) injected i.v. at 3 hr after MCAO. In group 3, rats were given GDNF-hMSCs (1.0×10^7) injected i.v. at 3 hr after MCAO.

In some experiments, Adex1CALacZ adenovirus was used to transduce the LacZ gene into the GDMF-hMSCs. 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 3×10^3 MOI for 3 hr and incubated at 37°C in MSCBM containing MCGS. Rats were administered cyclosporine A (10 mg/kg) i.p. daily. Table I summarizes the experimental protocol, which shows the number of animals used in the various assays.

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 by using a 7-T, 18-cm-bore superconducting magnet (Oxford Magnet Technologies) interfaced to a UNITYINOVA console (Oxford Instruments, Oxford, United Kingdom; and

Varian, Inc., Palo Alto, CA). Diffusion weighted images (DWI) were obtained from a 1.0-mm-thick coronal section with a 3-cm field of view, TR = 3,000 msec, TE = 37 msec and were reconstructed with a 128×128 image matrix, b value = 966. T₂-weighted images (T₂WI) were obtained from a 1.0-mm-thick coronal section with a 3-cm field of view, TR = 3,000 msec, TE = 30 msec and reconstructed with a 256×128 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. Both T₂WI and DWI were carried out up to 28 days, but an additional time point of 3 hr was used for DWI. The reason is that the DWI is more useful for lesion volume detection in the acute infarction phase and the T₂WI is more important for later time points. Therefore, both imaging methods were used from 6 hr to 28 days, but an additional time point (3 hr) was used for DWI. The ischemic lesion area was calculated from both DWI and T₂WI with imaging software (Scion Image, Version Beta 4.0.2; Scion Corporation), based on a previously described method (Neumann-Haefelin et al., 2000). For each slice, the higher intensity lesions in DWI and T₂WI images where the signal intensity was 1.25 times higher than the counterpart in the contralateral brain lesion were marked as the ischemic lesion area, and infarct volume was calculated taking slice thickness (1 mm/slice) into account.

Detection of GDNF In Vitro and In Vivo

Forty-eight hours after MSCs were transfected in vitro at various MOIs (pu/cell), culture supernatants were collected for analysis. Furthermore, 7 days after MCAO, rats were anesthetized with ketamine (4.4~8 mg/100 g) and xylazine (1.3 mg/100 g) i.p., their brains were removed, and coronal sections (200 mg) from -1.0~1.0 mm to bregma in the ischemic hemisphere were dissected on ice and were stored at -80°C until use. Subsequently, each tissue sample was suspended in an equal weight of homogenate buffer [1 ml; 137 mM NaCl, 20 mM Tris, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium vanadate] and homogenized with a Dounce homogenizer. The homogenate was centrifuged (10,000g) for 10 min at 4°C and the supernatant (5 µg/µl) collected for analysis. Commercial GDNF ELISA kits (Promega, Madison, WI) were used to quantify the concentration of GDNF in each of the samples.

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining and Quantitative Analysis of Infarct Volume

One week after transplantation, the rats were deeply anesthetized with ketamine (4.4~8 mg/100 g) and xylazine (1.3 mg/100 g) i.p. The brains were removed carefully and dissected into coronal 1-mm sections with a vibratome. The fresh brain slices were immersed in a 2% solution of TTC in normal saline at 37°C for 30 min. The cross-sectional area of infarction in each brain slice was examined with a dissection microscope and was measured in image analysis software (Adobe Photoshop). The total infarct volume for each brain was calculated by summation of the infarcted area of all brain slices. TTC staining was carried out only at 1 week, because metabolically active areas stain with TTC and, as gliosis occurs within the infarction zone with time, the staining resumes.

Detection of Donor MSCs and Phenotypic Analysis In Vivo

X-gal staining—One week after transplantation, brains of the deeply anesthetized rats were removed and fixed in 0.5% glutaraldehyde in phosphate buffer for 1 hr. Brains were removed, and brain slices (1.0 mm) were cut with a vibratome, and β-galactosidase-expressing cells were detected by incubating the sections at 37°C overnight with X-gal to a final concentration of 1 mg/ml in X-gal developer [35 mM K₃Fe(CN)₆/35 mM K₄Fe(CN)₆·3H₂O/2 mM MgCl₂ in phosphate-buffered saline] to form a blue reaction product within the cell.

Immunohistochemistry—One week after transplantation, analysis of the transplanted cells in vivo was carried out via laser scanning confocal microscopy. Brains of the deeply anesthetized rats were removed, fixed in 4% paraformaldehyde in phosphate buffer, dehydrated with 30% sucrose in 0.1 M PBS overnight, and frozen in powdered dry ice. Coronal cryostat sections (10 μ m) were processed for immunohistochemistry. To identify the cells derived from the donor peripheral blood, immunolabeling studies were performed with the use of antibodies to β -galactosidase (rhodamine-labeled polyclonal rabbit anti- β -galactosidase antibody; DAKO). To excite the rhodamine fluorochrome (red), a 543-nm laser line from a HeNe laser was used. Confocal images were obtained with a Zeiss laser scanning confocal microscope with Zeiss software.

Treadmill Stress Test

Rats were trained for 20 min per day, 2 days per week, to run on a motor-driven treadmill (model MK-680; Muromachi Kikai Co.) at a speed of 20 m/min with a slope of 10°. Rats were placed on a moving belt facing away from the electrified grid and induced to run in the direction opposite of the movement of the belt. Thus, to avoid foot-shocks (with intensity in 1.0 mA), the rats had to move forward. Only the rats that had learned to avoid the mild electrical shock were included in this study. The maximal speed at which the rats could run on a motor-driven treadmill was recorded. Rats were tested on 1, 3, 7, 11, 15, 19, 23, 27, and 31 days after MCAO.

Statistical Analysis

The lesion volume and the behavior scores (treadmill stress test) recorded were statistically analyzed. Data are presented as mean values $\pm$ SD. Differences among groups were assessed by ANOVA with Scheffe's post hoc test to identify individual group differences. Differences were deemed statistically significant at $P < 0.05$.

RESULTS

Morphological Characteristics of Primary and GDNF Gene-Transduced hMSCs

Both primary hMSCs and GDNF-hMSCs cultured as plastic adherent cells could be maintained in culture. A characteristic feature of MSCs is a CD34⁺, CD45⁺, SH2⁺ (CD105), SH3⁺ (CD73) CD117⁺, CD133⁺, CD166⁺, CD9⁺, CD157⁺, CD166⁺ (ALCAM) cell surface, which had been reported in our previous papers (Kobune et al., 2003; Honma et al., 2006). Figure 1A shows the morphological features of these cells. Characteristic flattened and spindle-shaped cells can be recognized. Figure 1B is a photomicrograph of GDNF-hMSCs at a similar passage as in Figure 1A. Note the similar flattened and spindle-shaped morphology of the genetically engineered cells. These data are in agreement with previous work (Kurozumi et al., 2005).

Detection of Immunoreactive Human GDNF and Quantitative Analysis In Vitro

Levels of GDNF in the supernatant of cultured hMSCs and GDNF-hMSCs with different MOI levels are shown in Figure 1C. hMSC transfected with AxCAhGDNFF/RGD (GDNF-hMSCs) at MOIs of 300, 1,000, and 3,000 pu/cell secreted GDNF at rates of 1.16 ± 0.39 , 2.31 ± 0.77 , and 4.24 ± 0.39 ng/10⁵ cells/48 hr, respectively ($n = 4$). Nontransfected hMSC also produced GDNF protein (0.04 ± 0.01 ng/10⁵ cells/48 hr; $n = 4$). The level of GDNF production from GDNF-hMSC transfected at an MOI of 1,000 pu/cell was about 58-fold greater than that seen in noninfected MSC.

Characterization of Ischemic Lesion Size by Magnetic Resonance Image Analysis

An estimate of lesion size was obtained by using in vivo MRI (see Materials and Methods). Brain images were collected from 18 experimental animals at 3 hr (DWI only), 6 hr, 1, 3, 7, 14, and 28 days (DWI and T₂WI) after MCAO. The cells were intravenously delivered

immediately after the 3-hr MRI. The upper row in Figure 2A corresponds to 3 hr DWI post-MCAO for control (Fig. 2A1), hMSCs (Fig. 2B1)-, and GDNF-hMSCs (Fig. 2C1)-injected rats. Respective images are shown at 3 and 6 hr and at 1, 3, and 7 days for each group. These coronal forebrain sections were obtained at the level of the caudato-putamen complex. Note the reduction in density in lesions on the right side of the brains that were subjected to ischemic injury. Lesion volume (mm^3) was determined by analysis of high-intensity areas on serial images collected through the cerebrum (see Materials and Methods).

At 3 and 6 hr post-MCAO, lesion volume of DWI was similar for the three groups (Fig. 2D). Lesion volume increased at 1 day, but was less for both the hMSC and the GDNF-hMSC groups. The control lesion group showed a reduced lesion volume at 3 and 7 days, but the MSC groups showed a greater reduction in lesion volume (Fig. 2D).

On T_2 WI (Fig. 3), infarction volumes were similar in the three groups at 6 hr post-MCAO (Fig. 3D). Both the hMSC- and the GDNF-hMSC-injected groups showed reduced lesion volume at 1, 3, 7, 14, and 28 days post-MCAO.

A difference between DWI and T_2 WI was observed. DWI showed that lesion volume decreased after 1 day in the three groups and detected the therapeutic effects in the treated groups only in the acute phase (within 1 week). However, on T_2 WI, lesion volume increased from 1 to 3 days, and the hMSC and GDNF-hMSC groups showed reduced lesion volumes in both acute and chronic phases (up to 28 days).

Histological Determination of Infarction Volume

One week after transplantation, the rats were perfused and stained with TTC to obtain a second independent measure of infarction volume. Normal brain (gray matter) tissue typically stains with TTC, but infarcted lesions show no or reduced staining (Bederson et al., 1986). TTC staining obtained 1 week after MCAO without cell transplantation is shown in Figure 4A. Note the reduced staining on the lesion side primarily in the corpus striatum. Lesion volume ($253.91 \pm 8.51 \text{ mm}^3$, $n = 6$) was calculated by measuring the area of reduced TTC staining in the forebrain (see Materials and Methods). As with MRI analysis, there was a progressive reduction in infarction size with MSC treatment ($198.95 \pm 13.47 \text{ mm}^3$, $n = 6$, $P < 0.05$, Fig. 4B,D). Intravenous delivery of 10^7 GDNF-hMSCs resulted in very substantial reduction in lesion volume as estimated from TTC staining ($148.04 \pm 6.91 \text{ mm}^3$, $n = 6$, $P < 0.01$, Fig. 4C,D).

GDNF Levels In Vivo

GDNF levels in local brain tissue were measured by a sandwich ELISA 7 days after MCAO (see Materials and Methods). Although GDNF levels in the nontransplant rat brain were not significantly different from those of the normal rat brain (9.49 ± 0.37 , $n = 4$; $10.51 \pm 1.31 \text{ pg/mg}$ protein, $n = 4$, respectively; $P = 0.19$), GDNF levels significantly increased in the ischemic hemisphere of MSC-treated group ($22.82 \pm 0.73 \text{ pg/mg}$ protein, $n = 4$; $P < 0.05$) compared with control group. In the GDNF-hMSC-treated rats, GDMF increased in the ischemic hemisphere ($44.62 \pm 3.32 \text{ pg/mg}$ protein, $n = 4$, $p < 0.01$) compared with control rats and hMSC-treated = rats ($P < 0.05$). These results are summarized in Figure 4E.

Identification of Donor Cells In Vivo

LacZ-transfected GDNF-hMSCs that had been administered i.v. (10^7 cells) 3 hr after MCAO were identified in vivo. The LacZ-expressing GDNF-hMSCs were found primarily in the lesion penumbra. The transmitted light image in the LacZ-transfected group is shown in Figure 5A. Note the abundance of LacZ-positive blue cellular-like elements are found predominantly in the penumbra lesion, indicating that systemic deliver of cells reached the lesion site. Immunohistochemical studies were carried out to identify LacZ-positive cells in the penumbra

lesion zone in animals transplanted with LacZ-transfected GDNF-hMSCs. Figure 5B demonstrated a large number of LacZ-positive cells in the penumbra lesion (422.5 ± 71.1 cells/mm², $n = 4$), although there are virtually no LacZ-positive cells in the nondamaged hemisphere and noninfected group.

Functional Analysis

To access behavioral performance in the lesioned and transplanted animals, the treadmill stress test was used (Fig. 6). Behavioral testing began 24 hr after lesion induction alone or with cell transplantation. In the treadmill stress test, normal animals (no lesion) reach a maximal treadmill velocity of about 70 m/min (Iihoshi et al., 2004). MCAO without transplantation maximal velocity on the treadmill test was 8.50 ± 2.68 m/min ($n = 4$). Nontreated animals showed increased treadmill velocity, with slow improvement up to 31 days (47.5 ± 4.26 m/min, $n = 4$). In the hMSC transplantation group, the improvement in velocity was greater over the time course up to 31 days. The GDNF-hMSC-treated group showed increased treadmill velocity with dramatic improvement after 7 days compare with both control and hMSC-treated group, and almost reached the maximal velocity (full recovery) after about 2 weeks. This functional improvement was maintained throughout the 31-day duration of the study indicating the stability of the functional improvement. Although the motor function continued improving in the control and hMSC groups after 2 weeks of MCAO, the rats in the GDNF-hMSC group run almost at the maximal velocity after about 2 weeks of MCAO, so we would not expect any additional improvement.

DISCUSSION

In the present study, we demonstrate that intravenous administration of either hMSCs or GDNF-hMSCs 3 hr after permanent MCAO in the rat results in reduction in infarction volume, improvement in behavioral performance, and increase in GDNF levels in the infarcted cerebral hemisphere. These results are consistent with previous studies showing beneficial effects of MSC transplantation in experimental cerebral ischemic models (Nomura et al., 2005; Honma et al., 2006) or GDNF delivery (Abe and Hayashi, 1997; Kitagawa et al., 1998; Zhang et al., 2002). Although both hMSCs and GDNF-hMSCs showed efficacy, the effects, including brain GDNF levels, were greater in the GDNF-hMSC group.

Transplanted LacZ-positive GDNF-hMSCs were identified throughout the anticipated infarcted cerebrum, predominantly in the penumbra lesion. These cells likely accessed the damaged area from the systemic circulation through adjacent nonoccluded or collateral arterial circulation, which are in agreement with previous works (Nomura et al., 2005; Honma et al., 2006). Given that the transplanted LacZ-positive GDNF-hMSCs distribute throughout the infarction zone, they likely migrated within the lesion domain from vasculature outside the lesion. LacZ-positive cells were located both in areas of severe damage and in surrounding, less damaged areas. This suggests that tissue sparing, though not complete, overlapped with deposition of transplanted cells. LacZ-positive cells were minimally observed in the contralateral noninfarcted hemisphere, suggesting that cells preferentially distributed to damaged CNS.

MSCs secrete a variety of bioactive substances such as neurotrophins (including GDNF), interleukins, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factors (Eaves et al., 1991; Majumdar et al., 1998). The neurotrophic factor BDNF increases in the ischemic brain lesion following hMSC transplantation, and transplantation of gene-modified hMSC (BDNF-hypersecreting hMSC) into the MCAO model results in better outcome (Nomura et al., 2005), suggesting that BDNF secreted by the transplanted hMSCs contributes to the therapeutic benefits. GDNF has a potent neuroprotective effect on a variety of neuronal damage both in vitro (Lin et al., 1993; Henderson et al., 1994) and in vivo (Beck et al., 1995; Li et al.,

1995; Tomac et al., 1995). Topical application of GDNF decreased ischemic brain edema and number of TUNEL-positive neurons with suppressing apoptotic pathways such as caspases-1 and -3 (Abe and Hayashi, 1997; Kitagawa et al., 1998). The placement of GDNF-pres soaked sponges in contact with the surface of cerebral cortex provided protection to the neurons within the surrounding areas (Zhang et al., 2001). Exogenous GDNF gene transfer reduced the infarct size in rat MCAO model (Hermann et al., 2001; Zhang et al., 2002). However, the effects of GDNF are transient, so repeated administration into intracerebral or intraventricular space is needed (Shingo et al., 2002). In addition, simple application of GDNF protein is difficult to administer in clinical situations because of the blood—brain barrier (Abe and Hayashi, 1997).

The present study demonstrates that intravenous administration of either hMSCs or GDNF-hMSCs 3 hr after permanent MCAO in the rat results in reduction in infarction volume and improvement in behavioral performance. Although both hMSCs and GDNF-hMSCs showed efficacy, the effects, including brain GDNF levels, were greater in the GDNF-hMSC group. In the GDNF-hMSC transplantation group, GDNF levels were nearly doubled in the lesioned hemisphere compared with the hMSC group, suggesting that GDNF production was increased in vivo in the GDNF-hMSC transplant group. Thus, intravenous administration of hMSCs might be a very useful strategy to deliver neurotrophic factors in the broad range of ischemic lesions for a prolonged period.

In a previous paper, we reported that direct injection of GDNF-hMSCs into the infarction site promoted tissue sparing and improved functional outcome, but hMSCs did not, although hMSCs can secrete GDNF in vitro (Kurozumi et al., 2005). This contrasts with results of the present study, in which transplantation of either cell type showed efficacy, but the GDNF-hMSCs response was greater. One possibility to account for this difference is that the intravenous delivery method allowed for a larger area of infarcted tissue to be reached by the transplanted cells. After direct injection, the increased GDNF secretion by the transplanted GDNF-hMSCs might have contributed to the neuroprotective effects, but the GDNF secretion of hMSCs might have been insufficient. Another difference is that the hMSCs were delivered at 3 hr postinfarction in the present study, as opposed to 24 hr in the Kurozumi et al. (2005) study. The therapeutic effect of GDNF-hMSC transplantation in the permanent rat MCAO model was observed even when applied several hours after infarction. Thus, cellular delivery of GDNF secreting hMSCs may provide a new therapeutic strategy for cerebral ischemia.

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Fig. 1. May-Giemsa staining of primary hMSCs (**A**) and GDNF-hMSCs (**B**). **C**: Secreted GDNF levels in supernatant of hMSCs (ng/10⁵ cells/48 hr) transfected with AxCaAhGDNF-F/RGD (GDNF-hMSC) at MOI 300, 1,000, and 3,000 pu/cell. Scale bars = 20 μ m.

Fig. 2.

Evaluation of the ischemic lesion volume with MRI diffusion-weighted images (DWI). hMSCs or GDNF-hMSCs were intravenously injected immediately after the initial MRI scanning (3 hr). Images obtained 3, 6, 24, 72 hr and 7 days after MCAO in medium-injected (A1–5), hMSC-treated (B1–5), and GDNF-hMSC-treated (C1–5) groups. Summary of lesion volumes evaluated with MRI diffusion-weighted images at 3, 6, 24, 72 hr and 7, 14, 28 days in each group (D). * $P < 0.05$, ** $P < 0.01$. Scale bar = 3 mm.

Fig. 3. Evaluation of the ischemic lesion volume with MRI T₂-weighted images (T₂WI). hMSCs or GDNF-hMSCs were intravenously injected immediately after the initial MRI scanning (3 hr). Images obtained 6, 24, 72 hr and 7, 14, 28 days after MCAO in medium-injected (A1–6), hMSC-treated (B1–6), and GDNF-hMSC-treated (C1–6) groups. Summary of lesion volumes evaluated with MRI T₂-weighted images in each group (D). * $P < 0.05$, ** $P < 0.01$. Scale bar = 3mm.

Fig. 4.

A—C: Brain slices stained with TTC to visualize lesions. TTC-stained brain slices from medium injection (A) and following intravenous delivery hMSCs and GDNF-hMSCs 3 hr after MCAO are shown in B and C, respectively. **D:** Lesion volumes in each group. $*P < 0.05$, $**P < 0.01$. **E:** GDNF in vivo levels assayed with ELISA. Levels of GDNF were significantly increased in the ischemic hemisphere of hMSC and GDNF-hMSC compared with nontreated rats. In addition, GDNF levels were greater in the ischemic hemisphere of GDNF-hMSC-transplanted compared with rats that received hMSC. Assays were obtained 7 days after MCAO induction. $*P < 0.01$, $**P < 0.005$. Scale bars = 3 mm.

Fig. 5.

Intravenous administered GDNF-hMSCs transfected with the reporter gene LacZ accumulated in the ischemic lesion hemisphere as observed 1 week postinjection. **A:** Vibratome section (1.0 mm) showing β -galactosidase reaction product for LacZ-positive cells (blue cells) in the ischemic hemisphere. Confocal images (**B**) demonstrating LacZ-positive cells in the penumbra in the lesion hemisphere. Scale bars = 3 mm in A; 100 μ m in B.

Fig. 6. Treadmill stress test demonstrates that the maximum speed at which the rats could run on a motor-driven treadmill was greater in the hMSC- and the GDNF-hMSC-treated rats than in control. Moreover, the greatest velocity was achieved in the GDNF-hMSC group. Velocity is plotted for 1, 3, 7, 11, 15, 19, 23, 27, and 31 days after MCAO induction. * $P < 0.05$, ** $P < 0.01$.

TABLE I
Numbers of Animals Used in the Various Assays*

LacZ						
Group	MRI (behavior)	TTC	ELISA	X-gal	Transfect (+)	
					Immun	Transfect (-) (Immun)
Control	6 (4)	6	4			
hMSC	6 (4)	6	4			
GDNF- hMSC	6 (4)	6	4	4	4	4
Normal			4			

* Immun, immunohistochemistry.