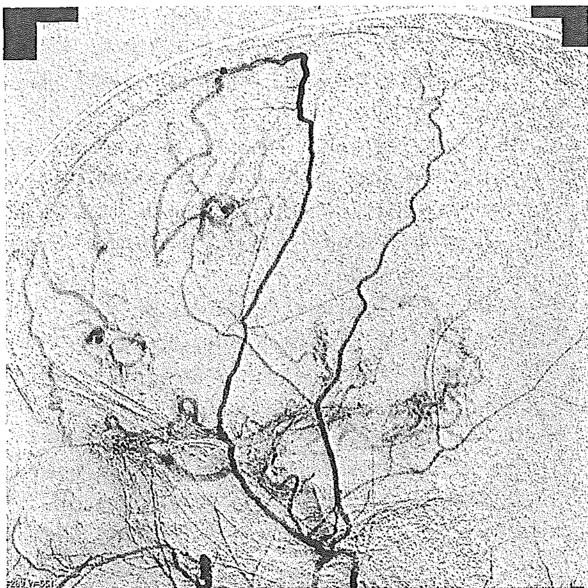


外頸動脈系の血管からの側副路を複数認める
図6　図4，5と同一症例の外頸動脈撮影

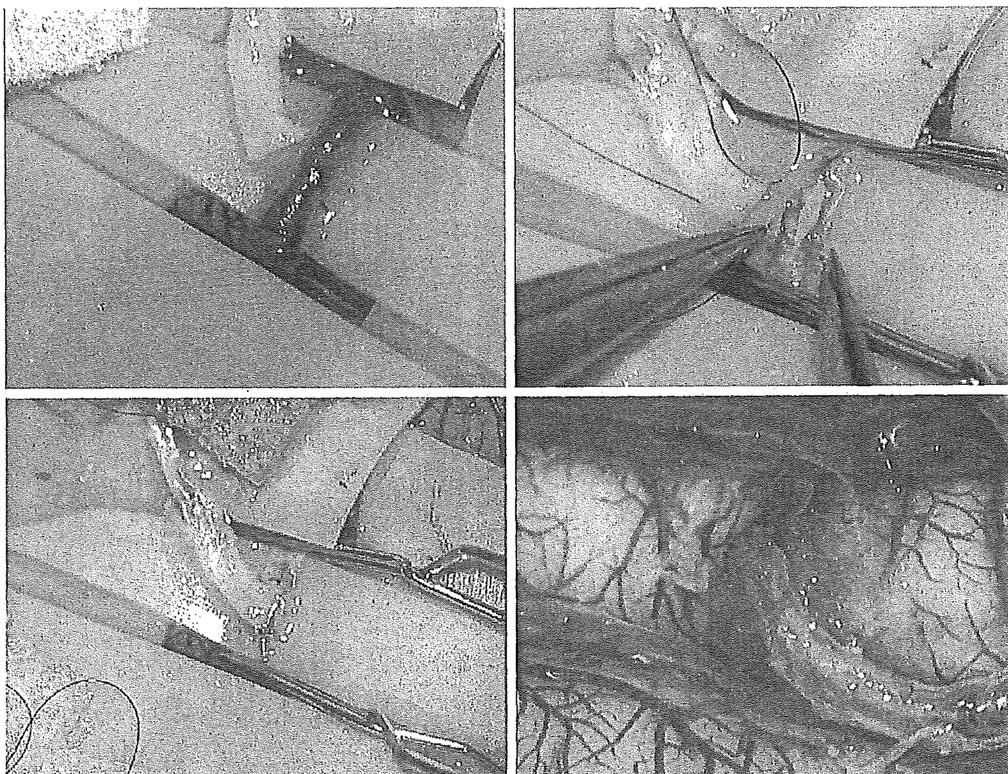
み合わせて手術をデザインする必要がある。したがって，手術前には頭蓋内外の動脈の状態と症例の虚血巣や虚血の程度を総合的に判断し，どのように新生側副路を発達させるかの検討が必要となるため，脳血管撮影および脳血流評価が必要となる（図5，6）。

一般的に中大脳動脈領域の血行再建術の際に用いられる血管，組織は頭皮表面から，①浅側頭動脈（頭皮，帽状腱膜），②眼窩上動脈（前頭部帽状腱膜），③中側頭動脈（側頭筋膜），④深側頭動脈（側頭筋，骨膜），⑤中硬膜動脈（硬膜）となる。直接的血行再建は血管径を考慮すると現実的に使用可能なものは浅側頭動脈となり，われわれは基本的に浅側頭動脈の前枝を直接吻合（図7）に，他の血管系に由来する組織を血流を温存した状態で間接的血行再建術に利用している（encephalo-duro-arterio-myo-galeo-synangiosis）。

周術期から術後の管理で注意する点は合併症の発生を未然に防ぐことであることは，あらゆる手術において変わりはないが，小児もやもや病の手術の場合の特徴としては，術後の啼泣に伴う過呼吸や貧血による脳虚血や，頭蓋外組織の虚血壊死にとくに留意する必要がある。

おわりに

小児もやもや病の治療について概説したが，救急の現場で同小児疾患が大きな虚血病変や脳出血を発症して搬入されることは少なく，比較的軽微なそして繰り返す神経症状を呈して受診することが多い。したがって，前述のような複雑な手術を緊急で行う



浅側頭動脈を脳表の中大脳動脈に吻合する
図7　直接血行再建術

ことは現実的ではなく，まずは神経症状の悪化に留意しつつ正確な診断と評価をすべきである。

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65 もやもや病

疾患概念

- 頭蓋内内頸動脈の終末部が進行性に狭窄する原因不明の疾患である.
- 6歳前後の小児と中年の成人の2つの発症頻度のピークがある.
- 病変は両側性にみられる.
- もやもや血管とよばれる細かな動脈が大脳基底核を中心に発達してくるが，これは，内頸動脈の狭窄を代償する側副血行路である.
- 日本人に特異的に多い．韓国，中国の報告が次いでいるが，欧米の患者発生はまれ.
- 発生頻度は，人口10万人1年間で0.35人
- 男女比は，1：1.8
- 原因は不明であるが，家系内発症が全体の15%以上あるとされ，遺伝子異常が関係している（すでに，第3，6，8，12，17番染色体に関連遺伝子が報告されている）.

症　状

- 小児では，脳虚血症状がほとんどである.
- 小児の典型例は，吹奏楽器演奏や激しい泣きなどの過呼吸（hyper-ventilation）によって誘発される一過性の脱力発作（transient ischemic attack: TIA）.
- 痙攣，不随意運動，頭痛なども まれではない
- 成人では，もやもや血管の末梢に発生した微小動脈瘤の破綻による脳出血，特に，大脳基底核の出血や脳室内出血が全体の半数程度にみられる.
- 成人出血例では，片麻痺，意識障害などの重篤な症状を呈するものもある.

診断の進め方

- MRIとMRAでほぼ確定診断が可能である.
- CTは，脳梗塞がない場合，診断的価値は低い.
- MRIでは，大脳基底核の通常では認められない血管のsignal voidが特徴的である.
- MRAでは，両側内頸動脈の狭窄あるいは閉塞と，大脳基底核のもやもや血管が認められる.
- 外科的治療が考えられる場合や成人の出血例では，脳血管撮影が必要である.
- 脳血流検査（SPECT）を行い，血流低下の程度と範囲を検査する.

- 脳波で，特徴的なre-built up現象が知られているが，脳虚血発作を誘発するので注意して行う.
- 家系内発症がまれではないので，家族歴の問診を行う.

治療にあたっての患者への説明のポイント

- 「もやもや」という言葉からくるnegativeなイメージを取り除き，正しい病態の理解が得られるように説明する.
- 厚労省指定の難病であることを知らせ，必要な手続きがあることを説明する.
- 脳神経外科医へのコンサルトが必要であることを説明する.
- 「難病」ではあるが，適切な治療を行えば，問題ないQOLが得られることも説明する.
- 外科的治療が必要かどうかは，脳血流検査の所見などをみて，外科医との相談が必要なことを説明する.
- 有用な薬物治療はこれまで報告されていない.

治療のグローバルスタンダード

- 小児の虚血発作のある患者は，外科的な治療の適応となる.
- 成人の脳虚血発作の患者も外科的治療の適応となる.
- 成人の出血の場合も，外科的治療が有効であるとする報告が多い．ただ，現在，無作為割付研究が進んでいる.
- 外科的治療では，
  ①直接的血行再建術
  　浅側頭動脈（STA）と中大脳動脈（MCA）を直接吻合する.
  ②間接的血行再建術
  　血行の豊富な側頭筋，硬膜，帽状腱膜などを脳に接着させる.
  という2つの基本的な術式があり，これらを組み合わせて行われる.
- 無症候例では，経過観察となる.
- 経過観察は，外来MRA/MRIにて1年に1度程度行う.

■外科的治療への時期，準備■

- 出血例では，脳出血の治療方針に準じて，急性期の外科的治療を行う.
- 虚血発症例は，できるだけ，十分な検査を行い，梗塞急性期やTIAが頻発している時期を避け，安定した時期に外科的治療を行う.
- 治療前に，脳血流検査を行う．これは，手術側の決定の目的と，虚血が強く血管反応性の障害された部位では，術後に過灌流がみられることがあるので，前もって，その危険を予知するためである.
- 一期的な治療は避け，左右，時期をおいて，治療する.

Neuroscience 136 (2005) 161–169

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. **© 2005 IBRO. Published by Elsevier Ltd. All rights reserved.**

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,

Scion Corporation), based on the previously-described method (Neumann-Haefelin et al., 2000). For each slice, the higher intensity lesions in T_2-weighted images where the signal intensity was 1.25 times higher than the counterpart in the contra-lateral brain lesion were marked as the ischemic lesion area, and infarct volume was calculated taking slice thickness (1 mm/slice) into account.

Detection of BDNF *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, seven 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 (200mg) 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 PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate) and homogenized with a Dounce homogenizer. The homogenate was centrifuged (10,000×*g*) for 10 min at 4 °C, and the supernatant (5 μg/μl) collected for analysis. Commercial BDNF ELISA kits (Promega, Madison, WI, USA) were used to quantify the concentration of BDNF in each of the samples.

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 using a vibratome. The fresh brain slices were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (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 using an image analysis software, NIH image. The total infarct volume for each brain was calculated by summation of the infarcted area of all brain slices.

Detection of donor hMSC cells and phenotypic analysis *in vivo*

One week after transplantation, phenotypic analysis of the transplanted cells *in vivo* was carried out using 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 for overnight, and frozen in powdered dry ice. Coronal cryostat sections (10 μm) were processed for immunohistochemistry. To identify the cell type derived from the donor bone marrow, double-labeling studies were performed with the use of antibodies to beta-galactosidase (rhodamine-labeled polyclonal rabbit anti-beta-galactosidase antibody, DAKO), neurons (FITC-labeled monoclonal mouse anti-NeuN, DAKO; FITC-labeled monoclonal mouse anti-neurofilament [NF], Sigma), and astrocytes (FITC-labeled monoclonal mouse anti-glial fibrillary acidic protein [GFAP], Sigma).

To excite the FITC fluorochrome (green), a 488-nm laser line generated by an argon laser was used, and for the rhodamine fluorochrome (red), a 543-nm laser line from a HeNe laser was used. Confocal images were obtained using a Zeiss laser scanning confocal microscope with the use of Zeiss software.

Treadmill stress test

Rats were trained 20 min per day for 2 days a week to run on a motor driven treadmill at a speed of 20 m/min with a slope of 0°. 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 leaned to avoid the mild electrical shock were included in this study ($n=21$). The maximum speed at which the rats could run on a motor driven treadmill was recorded.

Statistical analysis

The lesion volume and the behavior scores (treadmill stress test) recorded were statistically analyzed. Data are presented as mean values±S.D. 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 BDNF gene-transduced hMSCs

Primary 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 (Kobune et al., 2003). Fig. 1A shows the morphological features of these cells. Characteristic flattened and spindle-shaped-cells can be recognized. Fig. 1B is a photomicrograph of BDNF-hMSCs. Note the similar flattened and spindle shaped morphology of the geneti-

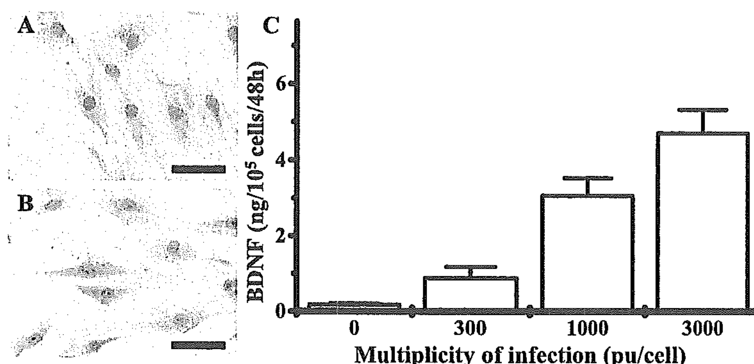

Fig. 1. May-Giemsa staining of primary hMSCs (A) and BDNF-hMSCs (B) (scale bar=20 μm). (C) Secreted BDNF levels in supernatant of hMSCs ($ng/10^5$ cells/48 h) transfected with AxCAhBDNF-F/RGD (BDNF-hMSC) at an MOI of 300, 1000, and 3000 pu/cell.

cally-engineering cells. These data are in agreement with previous work (Kurozumi et al., 2004).

Detection of immunoreactive human BDNF and quantitative analysis *in vitro*

Levels of BDNF in the supernatant of cultured hMSCs and BDNF-hMSCs with different MOI levels are shown in Fig. 1C. hMSC transfected with AxCAhBDNF-F/RGD (MSC-BDNF) at an MOI of 300, 1000, and 3000 pu/cell secreted BDNF at a rate of 0.91±0.26, 3.09±0.43, and 4.73±0.59 ng/10^5 cells/48 h, respectively (n=4). Non-transfected MSC also produced BDNF protein (0.05±0.02 ng/10^5 cells/48 h) (n=4). The level of BDNF production from BDNF-hMSC transfected at an MOI of 1000 pu/cell was 65-fold greater than that seen in non-infected MSC.

Characterization of ischemic lesion size by magnetic resonance image analysis

An estimate of lesion size was obtained using *in vivo* MRI (see Experimental Procedures). Brain images (T_2-weighted) were collected from all experimental animals 6, 24, 72 h and 7 days after MCAO. The cells were i.v. delivered immediately after the 6 h MRI. The left hand column in Fig. 2 (A1–C1) shows single brain images obtained 6 h post-injury. These coronal forebrain sections were obtained at the level of 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 Experimental Procedures). The lesion volume at 6 h post-lesion-induction was 270.30±12.10 mm^3 in the control group (Fig. 2A1) (n=7), 268.40±19.60 mm^3 in the MSC transplant-group (Fig. 2B1) (n=7), 267.20±2.84 mm^3 in the BDNF-hMSC transplant-group (Fig. 2C1) (n=7).

In order to determine the efficacy of hMSCs or BDNF-hMSCs transplantation in modulating the ischemic lesion volume, 1×10^7 cells were delivered i.v. at 6 h after lesion induction, and animals were re-imaged at 24, 72 h, and 7 days later.

In sham control (vehicle alone) (Fig. 2A1-4), MRI-estimated lesion volume reached maximum at 24 h post-MCAO (Fig. 2A2) (357.30±11.50 mm^3, n=7) and gradually decreased at 72 h and 7 days post-MCAO (326.50±2.40 mm^3, n=7; 294.50±4.10 mm^3, n=7, respectively). Although lesion volume estimated 6 h after MCAO was almost equal among three groups, lesion volume dramatically decreased with i.v. injection of hMSCs at 24 (Fig. 2B2), 72 h (Fig. 2B3) and 7 days (Fig. 2B4) post-MCAO (310.20±10.30 mm^3, n=7; 295.90±12.40 mm^3, n=7; 275.40±7.64 mm^3, n=7, respectively). Lesion volume reduction was also observed when BDNF-hMSCs were i.v. injected 6 h after MCAO. However, lesion volume in the BDNF-hMSC transplant-group was decreased to a greater degree than in the hMSC group (Fig. 2C2-4) (305.60±5.10 mm^3, n=7; 283.00±3.67 mm^3, n=7; 258.80±2.53 mm^3, n=7, at 24 h, 72 h, and 7 days, respectively). These results are summarized in Fig. 3A.

An additional sham control experiment was carried out using skin fibroblasts. No significant changes in lesion volume was observed with delivery of 10^6 skin fibroblasts at 6, 24, 72 h, and 7 days (269.90±2.23 mm^3, n=5; 340.50±7.97 mm^3, n=5; 314.50±6.51 mm^3, n=5; 293.60±2.16 mm^3, n=5, respectively; Fig. 3A).

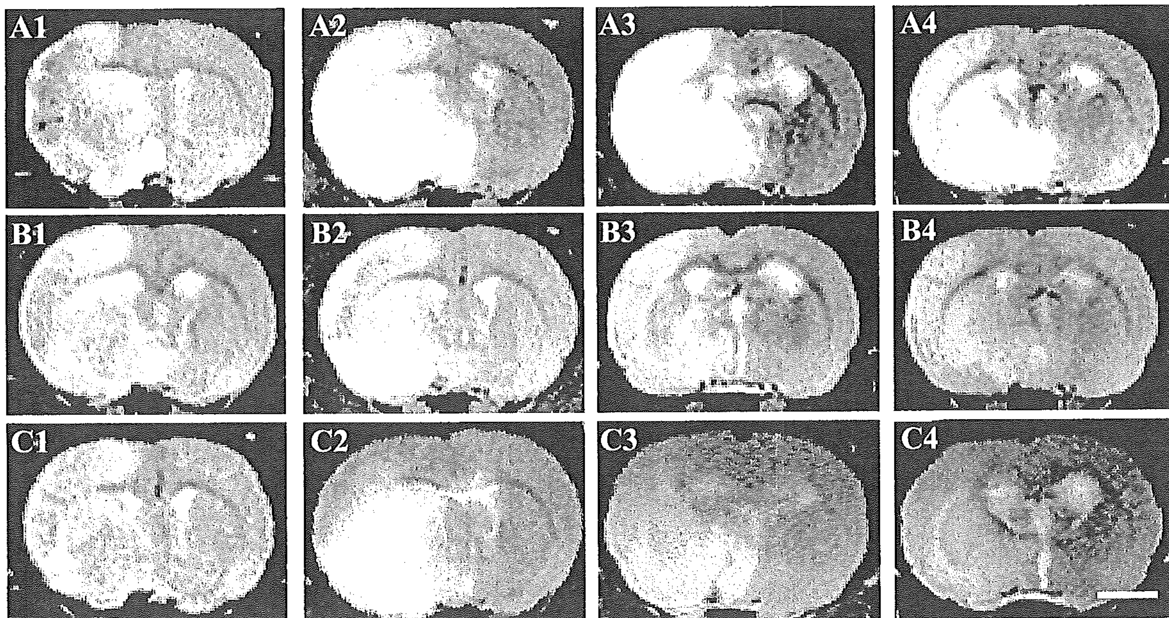

Fig. 2. Evaluation of the ischemic lesion volume with MRI T_2-weighted images. hMSCs or BDNF-hMSCs were i.v. injected immediately after the initial MRI scanning (6 h). Images obtained 6, 24, 72 h and one week after MCAO in non-treated (A1–4), hMSC-treated (B1–4), and BDNF-hMSC-treated groups (C1–4). Scale bar=3 mm.

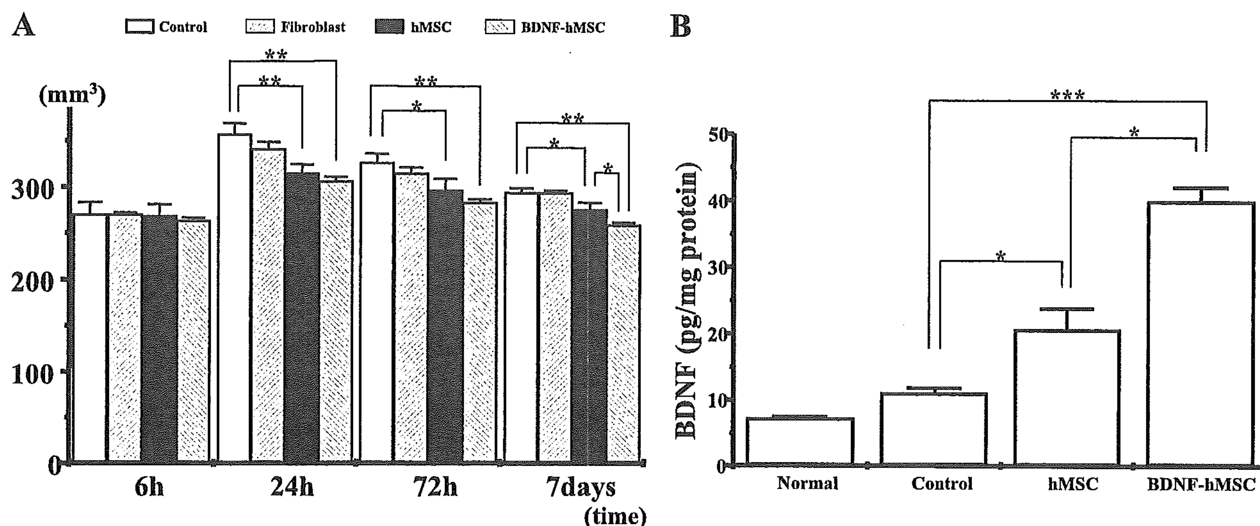

Fig. 3. (A) Summary of lesion volumes evaluated with MRI T_2-weighted images in each group. (B) BDNF *in vivo* levels assayed with ELISA. Levels of BDNF were significantly increased in the ischemic hemisphere of hMSC and BDNF-hMSC compared with non-treated rats. In addition, BDNF levels were greater in the ischemic hemisphere of BDNF-hMSC-transplanted compared with rats that received hMSC. Assays were obtained 7 days after MCAO induction. (* $P<0.05$; ** $P<0.01$, *** $P<0.0005$.)

BDNF levels *in vivo*

BDNF levels in local brain tissue were measured by a sandwich ELISA 7 days after MCAO (see Experimental Procedures). Although BDNF levels in the non-transplant rat brain were not significantly different from those of the normal rat brain (7.23±0.22, $n=4$; 11.09±0.71 pg/mg protein, $n=4$, respectively), BDNF levels significantly increased in the ischemic hemisphere of MSC-treated group (20.68±3.12 pg/mg protein, $n=4$) compared with control group. In the BDNF-hMSC treated rats, BDMF increased in the ischemic hemisphere (40.01±1.99 pg/mg protein, $n=4$) compared with control rats and hMSC-treated rats. These results are summarized in Fig. 3B.

Histological determination of infarction volume

After completion of the MRI analysis to estimate lesion volume, before and after cell delivery, the animals were perfused and stained with TTC to obtain a second inde-

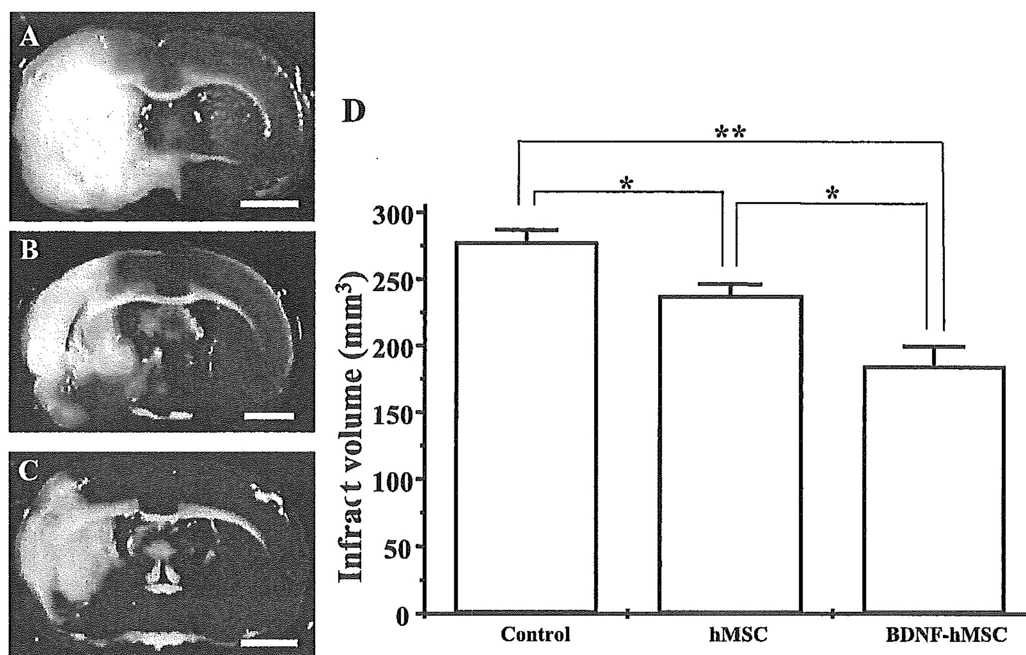

Fig. 4. (A–C) Brain slices stained with TTC to visualize lesions. TTC-stained brain slices from non-treated (A) and following i.v. delivery hMSCs or BDNF-hMSCs 6 h after MCAO are shown in B and C, respectively. (Scale bar=3 mm.) (D) Lesion volumes in each group. (* $P<0.05$; ** $P<0.0005$.)

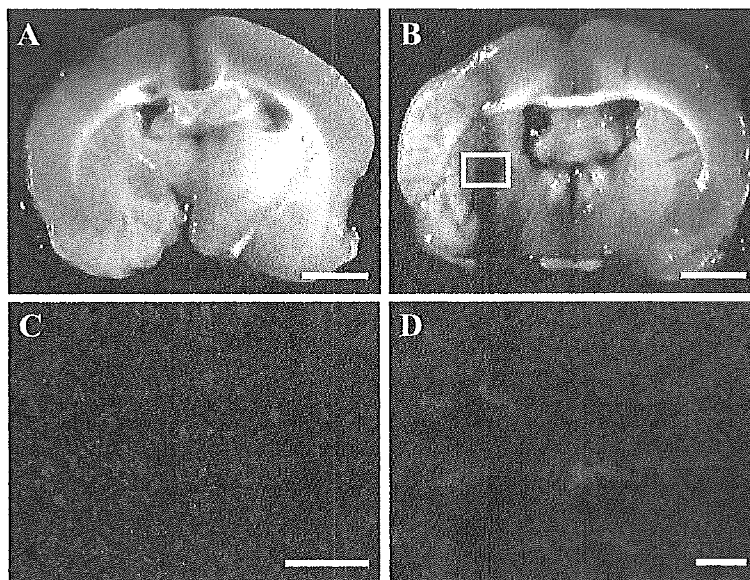

Fig. 5. I.v.-administrated BDNF-hMSCs accumulated in the ischemic lesion hemisphere. BDNF-hMSCs were transfected with the reporter gene LacZ. (B) Transplanted LacZ-positive cells (blue cells) were present in the ischemic lesion. Brain from control (BDNF-hMSCs without LacZ transfection) injected animals with comparable X-gal staining is shown in A. Confocal images (white square in panel B) at low (C) and high power (D) demonstrating a large number of LacZ-positive cells in the lesion hemisphere. Scale bar=3 mm (A, B), 100 μm (C), 10 μm (D).

pendent 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 one week after MCAO without cell transplantation is shown in Fig. 4A. Note the reduced staining on the lesion side primarily in the corpus striatum. Lesion volume was calculated by measuring the area of reduced TTC-staining in the forebrain (see Experimental Procedures). As with MRI analysis there was a progressive reduction in infarction size with MSC treatment (Fig. 4B). i.v. delivery of 10^7 BDNF-hMSCs resulted in very substantial reduction in lesion volume as estimated from TTC-staining (Fig. 4C, D).

Identification and characterization of donor cells *in vivo*

LacZ-transfected BDNF-hMSCs that had been i.v. administered (10^7 cells) 6 h after MCAO were identified *in vivo* ($n=5$). The LacZ-expressing BDNF-hMSCs were found primarily in the lesion penumbra. The transmitted light image in the LacZ-transfected group is shown in Fig. 5B. Note the abundance of LacZ-positive blue cellular-like elements in and around the affected lesion, indicating that systemic deliver of cells reached the lesion site. There was a paucity of blue staining in the non-infected group (Fig. 5A). Immunohistochemical studies were carried out to identify LacZ-positive cells in and around the lesion zone in animals transplanted with LacZ-transfected BDNF-hMSCs. Lower (C) and higher micrograph (D) demonstrated a large number of LacZ-positive cells in and around the lesion (458.6±42.1 cells/mm^2, $n=5$), although virtually there is not any LacZ-positive cells in the non-damaged hemisphere and non-infected group. Small numbers of NeuN and GFAP positive cells were co-stained with LacZ (data not shown). A small number of the LacZ-positive donor cells expressed the NeuN (7.90±0.15%, $n=5$), NF (7.32±0.52%, $n=5$), or GFAP (7.65±0.17%, $n=5$).

Functional analysis

To access behavioral performance in the lesioned and transplanted animals, the treadmill stress test was used (Fig. 6). Behavioral testing began 24 h after lesion induction alone or with cell transplantation. In the treadmill stress test control animals (no lesion) reach a maximum treadmill velocity of about 80 m/min (Iihoshi et al., 2004). Twenty-four hours after MCAO without transplantation maximum velocity on the treadmill test was 2.88±0.30 m/min ($n=7$). Non-treated animals showed increased treadmill velocity with slow improvement up to 7 days (4.13±0.23 m/min, $n=7$). In the hMSC transplantation group, the improvement in velocity was greater over the time course up to 7 days. The BDNF-hMSC-treated group showed increased treadmill velocity with dramatic improvement after 72 h compare with the MSC-treated group.

DISCUSSION

The present study demonstrates that i.v. infusion of either hMSCs or BDNF-hMSCs 6 h after permanent MCAO in the rat results in reduction in infarction volume, improvement in behavioral performance, and increases in BDNF levels in the infarcted cerebral hemisphere. These results are consistent with previous studies showing beneficial effects of bone marrow cell transplantation (Li et al., 2002; Iihoshi et al., 2004; Kurozumi et al., 2004) or BDNF delivery (Schabitz et al., 1997, 2000; Yamashita et al., 1997) in experimental cerebral ischemic models. While both

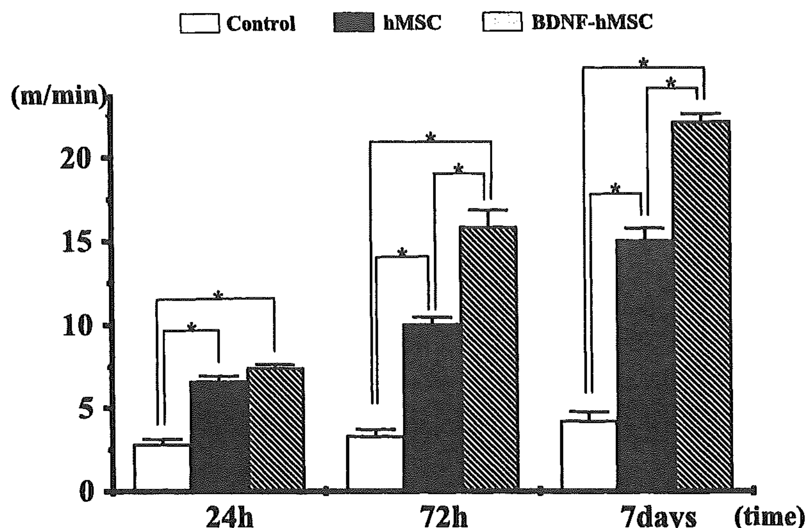

Fig. 6. The treadmill stress test demonstrates that the maximum speed at which the rats could run on a motor driven treadmill was faster in the hMSC- and the BDNF-hMSC-treated rats than control. Moreover, the greatest velocity was achieved in the BDNF-hMSC group. Velocity is plotted for three times after MCAO induction. (* $P<0.001$.)

hMSCs and BDNF-hMSCs showed efficacy, the effects, including brain BDNF levels, were greater in the BDNF-hMSC group.

Transplanted LacZ-positive BDNF-hMSCs were identified throughout the anticipated infarcted cerebrum. These cells likely accessed the damaged area from the systemic circulation (Iihoshi et al., 2004) through adjacent non-occluded or collateral arterial circulation. Given that the transplanted LacZ-positive BDNF-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, while not complete overlapped with deposition of transplanted cells. LacZ-positive cells were minimally observed in the contralateral non-infarcted hemisphere, suggesting that cells preferentially distributed to damaged CNS.

MSCs secrete a variety of bioactive substances such as neurotrophins (including BDNF), interleukins, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factors (Eaves et al., 1991; Majumdar et al., 1998). Intracranial infusion of BDNF using an osmotic mini-pump significantly reduced infarct volume following cerebral ischemia induction (Schabitz et al., 1997; Yamashita et al., 1997), suggesting that BDNF may provide a protective effect on ischemic cerebral tissue. To investigate the potential role of BDNF on the neuroprotective action of hMSC delivery in the MCAO model, we compared the effects of hMSCs to BDNF-hypersecreting hMSCs (BDNF-hMSCs). While numerous secreted trophic factors could contribute to the observed neuroprotection, we compared hMSCs that were constructed to differ only in their levels of BDNF secretion.

The BDNF gene was introduced into MSCs using Adv-F/RGD, and an elevated secretion of BDNF protein as compared with nontransgenic hMSCs was confirmed *in vitro* using ELISA. This relatively high secretory rate was achieved by using Ad-F/RGD which has a higher transfection rate (Nakamura et al., 2002; Tsuda et al., 2003). BDNF levels assayed by ELISA in the ischemic brain lesion increased after either hMSC or BDNF-MSC delivery, but levels were significantly higher in the BDNF-hMSC group. This suggests that the BDNF-MSCs could maintain high levels of BDNF during the critical post-ischemic period and that this elevated BDNF secretion contributes to the enhanced neuroprotection.

In a previous study we reported that direct injection of BDNF-hMSCs into the infarction site promoted tissue sparing and improved functional outcome, but hMSCs did not, although hMSCs can secrete BDNF *in vitro* (Kurozumi et al., 2004). This contrasts with results of the present study where transplantation of either cell type showed efficacy, but the BDNF-hMSCs response was greater. One possibility to account for this difference is that the i.v. delivery method allowed for a larger area of infarcted tissue to be reached by the transplanted cells. Following direct injection, the increased BDNF secretion by the transplanted BDNF-hMSCs may have contributed to the neuroprotective effects, but the BDNF secretion of hMSCs may have been insufficient. Another difference is that the hMSCs were delivered at 6 h post-infarction in the present study, as opposed to 24 h in the Kurozumi et al. (2004) study.

I.v. injection is less invasive, and can be more readily carried out in the clinic compared with the intracerebral injection. Certainly, many more cells would be needed to treat patients via i.v. injection as compared with the intracranial transplantation. It has been estimated that about 1% of i.v.-injected cells reach and survive in the lesion of rats (Chen et al., 2001). Targeting infarction lesions by intracerebral injection could cause significant surgical risks. For example, insertion of an injection needle for cell transplantation into the ischemic brain lesion in the acute

phase would result in increased risk of the intracerebral hemorrhage. I.v. delivery of these cells could lead to a more global neuroprotection over the entire ischemia-related brain lesion with subsequent repair.

CONCLUSION

In summary, the i.v. injection of either hMSCs or BDNF-hMSCs transfected with the BDNF gene using a fiber-mutant adenovirus vector resulted in reduced ischemic damage and improved function in a rat MCAO model, but the BDNF-hMSCs showed greater efficacy. The therapeutic effect of BDNF-hMSC transplantation in the permanent rat MCAO model was observed even when applied six hours after infarction. Thus, cellular delivery of BDNF secreting hMSCs may have a therapeutic effect following cerebral ischemia.

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Mesenchymal Stem Cells That Produce Neurotrophic Factors Reduce Ischemic Damage in the Rat Middle Cerebral Artery Occlusion Model

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Mesenchymal stem cells (MSC) were reported to ameliorate functional deficits after stroke in rats, with some of this improvement possibly resulting from the action of cytokines secreted by these cells. To enhance such cytokine effects, we previously transfected the telomerized human MSC with the BDNF gene using a fiber-mutant adenovirus vector and reported that such treatment contributed to improved ischemic recovery in a rat transient middle cerebral artery occlusion (MCAO) model. In the present study, we investigated whether other cytokines in addition to BDNF, i.e., GDNF, CNTF, or NT3, might have a similar or greater effect in this model. Rats that received MSC-BDNF ($P < 0.05$) or MSC-GDNF ($P < 0.05$) showed significantly more functional recovery as demonstrated by improved behavioral test results and reduced ischemic damage on MRI than did control rats 7 and 14 days following MCAO. On the other hand, rats that received MSC-CNTF or MSC-NT3 showed neither functional recovery nor ischemic damage reduction compared to control rats. Thus, MSC transfected with the BDNF or GDNF gene resulted in improved function and reduced ischemic damage in a rat model of MCAO. These data suggest that gene-modified cell therapy may be a useful approach for the treatment of stroke.

Key Words: cerebral infarction, mesenchymal stem cell, gene therapy, adenoviral vector, BDNF, GDNF, CNTF, NT3, MRI

INTRODUCTION

Mesenchymal stem cells (MSC) have recently been investigated for their efficacy as a clinical therapeutic tool. They exist primarily in the bone marrow and can differentiate into osteoblasts, chondrocytes, adipocytes [1,2], and hepatocytes [3]. It has also been suggested that they might be able to differentiate into other phenotypes as well, including neuronal cells [4]. MSC were reported to ameliorate functional deficits after stroke in rats, with some of this improvement possibly resulting from the action of cytokines secreted by these cells [5–8]. We reported that these cytokine effects could be enhanced by the intracerebral injection of MSC transfected with the brain-derived neurotrophic factor (BDNF) gene, using a fiber-mutant adenovirus vector. Specifically, such treatment resulted in improved function and reduced ischemic damage in the rat transient middle cerebral artery occlusion (MCAO) model [9].

Recent studies showed that the expression of several neurotrophic factors in the brain was influenced by ischemia [10–12], including BDNF, glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and neurotrophin-3 (NT3). BDNF promotes the survival and differentiation of neuronal tissue by acting on receptor kinases [13–15]. Intraventricular administration of BDNF prior to the induction of focal cerebral ischemia [16] or intraparenchymal infusion of BDNF after the ischemia [17] significantly reduced infarct volume in the cortex, and intravenous BDNF also reduced infarct size after temporary focal cerebral ischemia [13].

GDNF, a member of the transforming growth factor-β superfamily, is a potent neurotrophic factor that promotes the survival and morphological differentiation of

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dopaminergic neurons [18] and motoneurons [19]. Shingo *et al.* [20] reported that encapsulated GDNF-producing cells stably produced GDNF and had protective and restorative effects on host dopaminergic neurons. Zhang *et al.* showed that the topical application of GDNF and adenovirus-mediated GDNF gene transfer significantly reduced infarct size in a time-dependent manner [21,22].

CNTF was initially identified as a factor that supported the *in vitro* survival of parasympathetic neurons from the chick ciliary ganglion and has been purified and molecularly cloned [23]. CNTF has also been shown to promote the development of neurons and glial cells [24] and the differentiation of O2A progenitor cells to type 2 astrocytes [25]. More recently, *in vitro* and *in vivo* studies showed that CNTF supported neurons from several brain regions, including the hippocampus [26], and that it prevented ischemia-induced learning disability and neuronal loss in gerbils [27].

NTF3 is a member of the nerve growth factor family. Topical application of NT3 was shown to attenuate ischemic brain injury after transient MCAO in rats [28].

Recently, we established a human hTERT-transduced MSC line [29], the cells of which have a differentiation potential that is similar to that of primary MSC. These cells can also be more easily expanded *ex vivo* than primary MSC. In this study, we investigated whether BDNF, GDNF, CNTF, and NT3 could enhance the therapeutic effects of MSC and whether the injection of MSC transfected with these cytokine genes could contribute to functional recovery in the rat transient MCAO model.

RESULTS

Detection of Immunoreactive Human BDNF, GDNF, CNTF, and NT3 and Their Quantitative Analysis *in Vitro*

MSC transfected with AxCAhBDNF-F/RGD (MSC-BDNF) at an m.o.i. of 300, 1000, or 3000 particle unit (pu)/cell secreted BDNF at a rate of 0.434 ± 0.122, 0.931 ± 0.101, and 1.860 ± 0.410 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced BDNF protein (0.0407 ± 0.0059 ng/10^5 cells/48 h). The level of BDNF production from MSC-BDNF transfected at an m.o.i. of 1000 pu/cell was 23-fold greater than that seen in uninfected MSC.

MSC transfected with AxCAhGDNF-F/RGD (MSC-GDNF) at an m.o.i. of 300, 1000, or 3000 pu/cell secreted GDNF at a rate of 1.05 ± 0.20, 2.26 ± 0.41, and 4.15 ± 0.54 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced GDNF protein (0.044 ± 0.034 ng/10^5 cells/48 h). The level of GDNF production from MSC-GDNF transfected at an m.o.i. of 1000 pu/cell was 51-fold greater than that seen in uninfected MSC.

MSC transfected with AxCAhCNTF-F/RGD (MSC-CNTF) at an m.o.i. of 300, 1000, or 3000 pu/cell secreted CNTF at a rate of 0.136 ± 0.028, 0.854 ± 0.145, and 3.58 ± 0.43 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced CNTF protein (0.0520 ± 0.0150 ng/10^5 cells/48 h). The level of CNTF production from MSC-CNTF transfected at an m.o.i. of 1000 pu/cell was 16-fold greater than that seen in uninfected MSC.

MSC transfected with AxCAhNT3-F/RGD (MSC-NT3) at an m.o.i. of 300, 1000, or 3000 pu/cell secreted NTF3 at a rate of 2.67 ± 0.09, 4.24 ± 0.16, and 6.88 ± 0.07 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced NT3 protein (0.012 ± 0.001 ng/10^5 cells/48 h). The level of NT3 production from MSC-NT3 transfected at an m.o.i. of 1000 pu/cell was 35-fold greater than that seen in uninfected MSC (Fig. 1).

Correlation between Contralateral Hemispheric Lesion Volume (%HLV) and 2,3,5-Triphenyltetrazolium Chloride (TTC)-Derived %HLV

Lesion volume was expressed as a percentage of contralateral hemispheric volume (%HLV). Two weeks after MCAO, regression analysis revealed a significant correlation (r = 0.914, P = 0.0001, n = 10) between the T2-weighted image-derived %HLV (30.1 ± 7.2%) and the %HLV (30.5 ± 6.6%) determined with the aid of TTC staining (Figs. 2A and 2B).

Therapeutic Effects of MSC-BDNF and MSC-GDNF as Determined by the Performance of Limb Placement Tests

Prior to MCAO, neurological scores were similar among all animals. One day after MCAO but prior to the intracerebral injection of MSC, there was still no statistical difference in limb placement score among the six ischemic groups. However, 8 days after MCAO, rats that received MSC-BDNF and MSC-GDNF achieved significantly higher limb placement scores (8.43 ± 1.52, $P < 0.05$; 8.71 ± 2.2, $P < 0.05$; respectively) compared to DMEM rats (3.71 ± 0.49). MSC-BDNF- and MSC-GDNF-grafted animals also showed relatively better recovery from ischemia compared to animals in the EGFP-transduced MSC group (6.83 ± 2.04), though this difference did not reach statistical significance. On the other hand, on day 8, rats that received MSC-CNTF (7.57 ± 2.82) or MSC-NT3 (7.14 ± 2.6) did not achieve significantly higher scores than control rats (Fig. 3). Similarly, 15 days after MCAO, the scores of rats that received MSC-BDNF or MSC-GDNF were 9.14 ± 2.61 ($P < 0.05$) and 9.57 ± 2.9 ($P < 0.05$), respectively, which were significantly higher than that seen in the DMEM group (5.00 ± 1.73). MSC-BDNF- or MSC-GDNF-grafted animals also displayed a relatively, but statistically insignificantly, better recovery from ischemia compared to animals in the EGFP-transduced MSC group (8.50 ± 3.14). On the other hand, on day 15, the scores of rats that received MSC-CNTF (8.57 ± 3.87) or MSC-NT3 (8.50 ± 2.64) were not significantly higher than those of rats in the control groups (Fig. 3).

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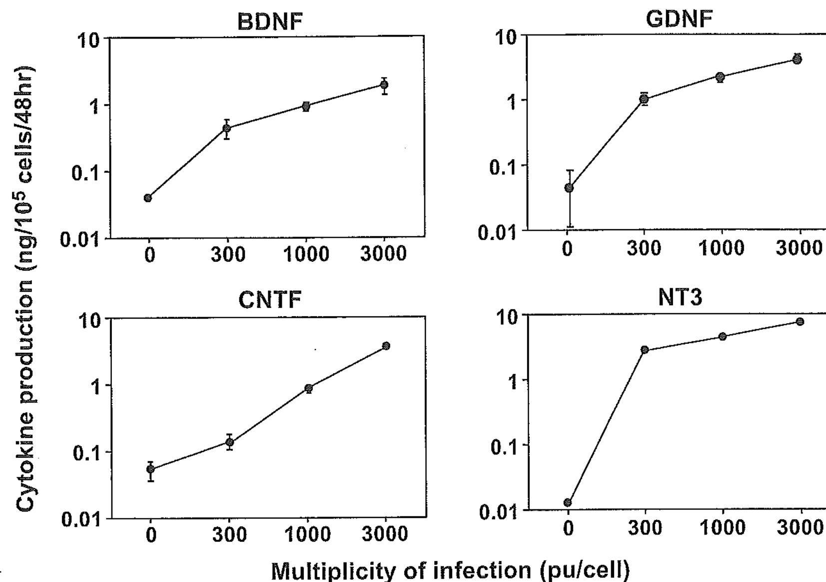

FIG. 1. BDNF, GDNF, CNTF, and NT3 (NT3) production by cultured MSC. MSC transfected with AxCAhBDNF-F/RGD at an m.o.i. of 300, 1000, or 3000 pu/cell secreted BDNF at a rate of 0.434 ± 0.122, 0.931 ± 0.101, and 1.860 ± 0.41 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced BDNF (0.0407 ± 0.0059 ng/10^5 cells/48 h). For GDNF, MSC transfected with AxCAhGDNF-F/RGD at an m.o.i. of 300, 1000, or 3000 pu/cell secreted GDNF at a rate of 1.05 ± 0.20, 2.26 ± 0.41, and 4.15 ± 0.54 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced GDNF protein (0.044 ± 0.034 ng/10^5 cells/48 h). For CNTF, MSC transfected with AxCAhCNTF-F/RGD at an m.o.i. of 300, 1000, or 3000 pu/cell secreted CNTF at 0.136 ± 0.028, 0.854 ± 0.145, and 3.58 ± 0.43 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced CNTF protein (0.0520 ± 0.0150 ng/10^5 cells/48 h). Finally, for NT3, MSC transfected with AxCAhNT3-F/RGD at an m.o.i. of 300, 1000, or 3000 pu/cell secreted NT3 at a rate of 2.67 ± 0.09, 4.24 ± 0.16, and 6.88 ± 0.07 ng/10^5 cells/48 h, respectively. Nontransfected MSC also produced NT3 protein (0.12 ± 0.001 ng/10^5 cells/48 h).

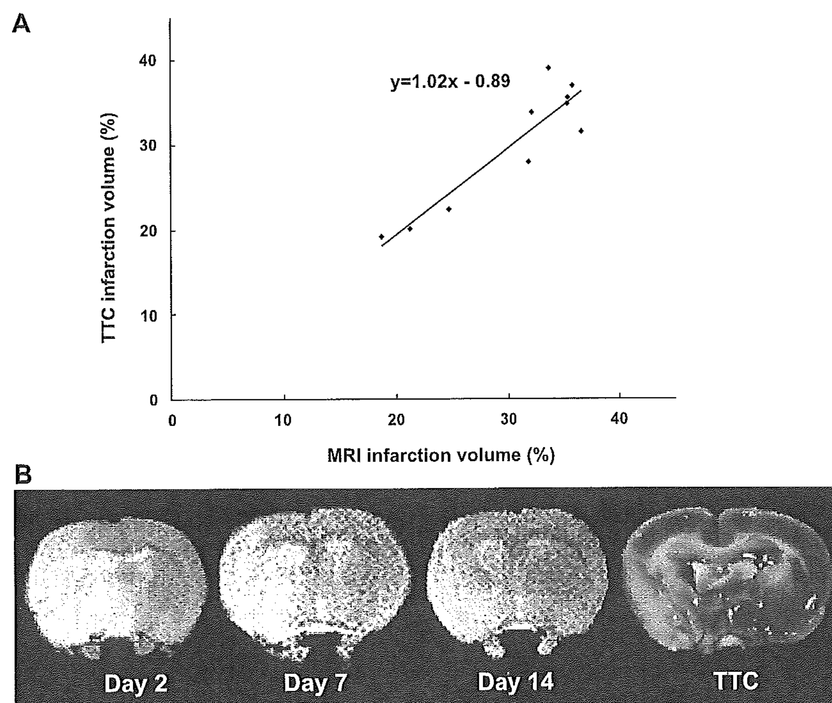

FIG. 2. (A) Correlation between the MRI and the TTC infarction volumes ($r = 0.914$, $P = 0.0001$, $n = 10$). (B) Representative T2W images obtained 2, 7, and 14 days after MCAO and TTC staining 14 days after MCAO.

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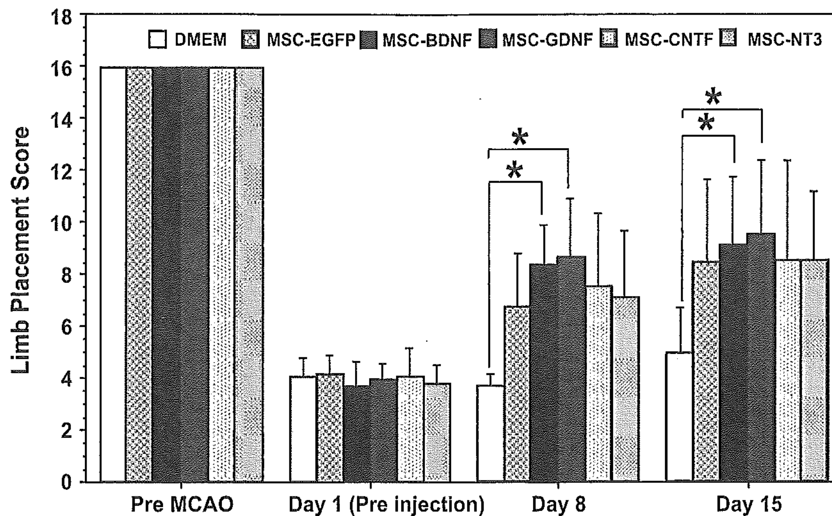

FIG. 3. Assessment of brain ischemia-induced neurological deficits. Deficits in limb placement were evaluated using the following scale: 0, severe neurological deficits; 16, no neurological deficits. One day after MCAO but prior to intracerebral MSC injection, there was no statistical difference in limb placement score among the six ischemic groups. Eight days after MCAO, rats that received MSC-BDNF (*$P < 0.05$) and MSC-GDNF (*$P < 0.05$) achieved significantly higher limb placement scores, compared to DMEM rats. Fifteen days after MCAO, the score of rats that received MSC-BDNF (*$P < 0.05$) and MSC-GDNF (*$P < 0.05$) were significantly higher than that seen in the DMEM group. On the other hand, neither on day 8 nor on day 15 did rats that received MSC-CNTF or MSC-NT3 achieve higher scores than control rats that received DMEM.

Reduction of Infarct Area by MSC-BDNF and MSC-GDNF Injection

We summed the areas of hyperintensity on T2-weighted images over the central six MR images and expressed lesion volume as %HLV. In all groups, the %HLV decreased from day 2 to day 14. Two days after MCAO, there were no significant differences in %HLV between the MSC-BDNF (35.0 ± 4.8%), the MSC-GDNF (36.2 ± 3.1%), the MSC-CNTF (36.5 ± 3.1%), the MSC-NT3 (38.0 ± 2.1%), the MSC-EGFP (38.5 ± 1.6%), and the DMEM (38.7 ± 4.9%) groups. However, 7 days after MCAO, there was a significant reduction in %HLV in rats in the MSC-BDNF (25.4 ± 2.8%; $P < 0.05$) and MSC-GDNF groups (25.3 ± 2.8%; $P < 0.05$) compared to rats in the control DMEM (32.8 ± 4.9%) group. After 14 days, there was a significant reduction in %HLV in rats in the MSC-BDNF (23.7 ± 3.6%; $P < 0.05$) and MSC-GDNF groups (23.5 ± 1.3%; $P < 0.05$) compared to the DMEM controls (29.6 ± 3.6%). Rats treated with MSC-CNTF or -NT3 did not show any significant recovery in %HLV on either day 7 (26.7 ± 5.8, 26.6 ± 3.8%) or day 14 (24.1 ± 3.6, 24.6 ± 1.8%), respectively, compared to the control DMEM and MSC-EGFP groups (Figs. 4A and 4B).

Hematoxylin and Eosin (HE) Staining

Two weeks after MCAO, coronal sections stained with HE showed the presence of dark and red neurons in the ischemic core of rats from all groups. We detected damaged and relatively intact cells in the ischemic boundary area (data not shown).

Engraftment Levels of Genetically Modified MSC

We detected numerous Discosoma red fluorescent protein (DsR)-positive MSC (red) within less than 2 mm of the injection site. DsR-positive cells were more numerous in the injection site of animals treated with MSC-BDNF-DsR or MSC-GDNF-DsR compared to the MSC-DsR group (Fig. 5A). There were significantly more DsR-positive cells in animals injected with MSC-BDNF-DsR (254 ± 45.3; $P < 0.05$) or MSC-GDNF-DsR (252 ± 40.5; $P < 0.05$) 14 days after MCAO compared to those injected with MSC-DsR (157 ± 16.7). On the other hand, the DsR-positive cells did not achieve significantly higher amounts in animals injected with MSC-CNTF-DsR (220 ± 20.0) or MSC-NT3-DsR (209 ± 20.0) compared to those that were injected with MSC-DsR (Fig. 5B).

Discussion

In our study, we transfected MSC with cytokine genes using Adv-F/RGD and confirmed their production of cytokine protein using an ELISA. MSC-BDNF, MSC-GDNF, MSC-CNTF, and MSC-NT3 transfected at an m.o.i. of 1000 pu/cell secreted BDNF, GDNF, CNTF, and NT3 at rates of 2.3, 5.7, 2.1, and 11 ng/5 × 10^5 cells/24 h, respectively. These secretion rates were quite high and reflected the efficiency of transfection using Ad-F/RGD [30,31]. Fiber-modified Adv (Adv-F/RGD) [32,33], which has an RGD-containing peptide in the HI loop of the fiber knob domain of the adenovirus type 5 (Ad5), was reported to enhance the effectiveness of BDNF and bone morphogenetic protein 2 gene transfer into MSC. MSC express low levels of coxsackie–adenovirus receptor but high levels of integrin αv [31]; this latter finding probably accounts for the high effectiveness of transfection using this vector, since Adv-F/RGD is known to infect cells by interacting with their cell surface integrins [34]. In our study, regression analysis showed that there was a good correlation between the T2-weighted imaging-derived %HLV recorded 2 weeks after MCAO and the histologically (TTC staining) derived %HLV. Doerfler *et al.* [35] demonstrated that T2-weighted imaging was a suitable

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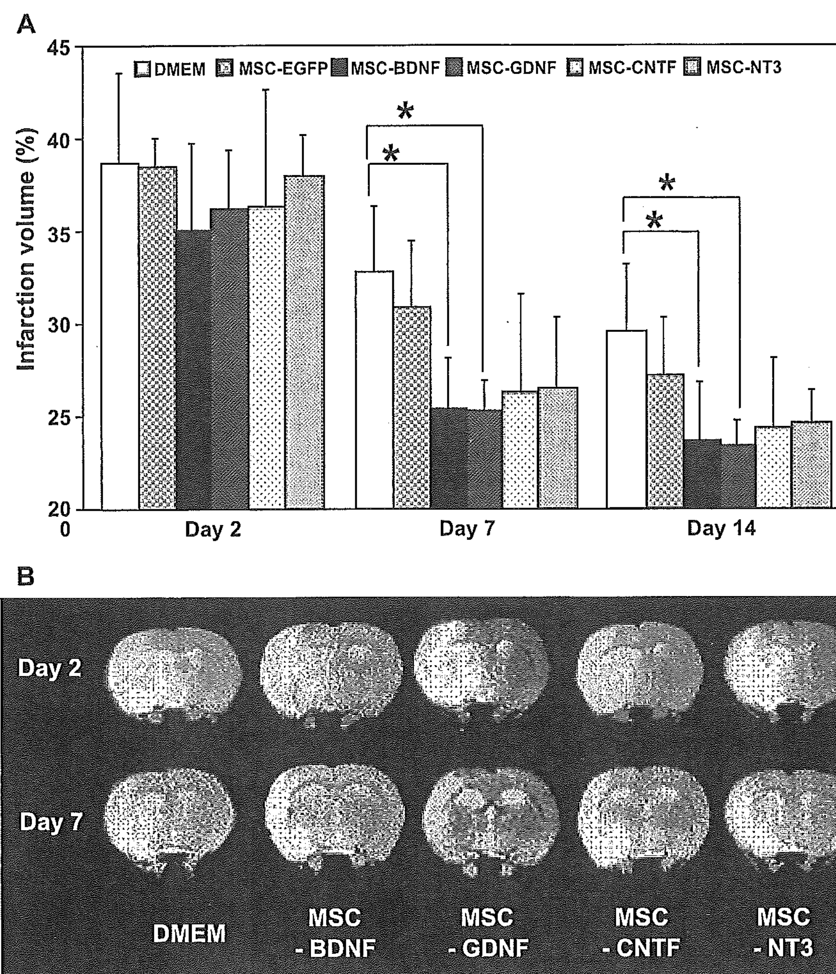

FIG. 4. (A) Seven days after MCAO, there was a significant reduction in %HLV in rats in the MSC-BDNF (*$P < 0.05$) and MSC-GDNF groups (*$P < 0.05$) compared to rats in the control DMEM group. After 14 days, there was similarly a significant reduction in %HLV in rats in the MSC-BDNF (*$P < 0.05$) and MSC-GDNF groups (*$P < 0.05$) compared to DMEM control. Rats treated with MSC-CNTF or -NT3 did not show any significant recovery in %HLV on either day 7 or day 14 compared to the control DMEM and MSC-EGFP groups. (B) Representative T2W images obtained 2 and 7 days after MCAO in rats injected with DMEM, MSC-BDNF, MSC-GDNF, MSC-CNTF, or MSC-NT3. A reduction in the volume of ischemic damage was detected in the MSC-BDNF and MSC-GDNF groups compared to the other groups on day 7.

follow-up monitoring parameter of infarct size during the subacute phase, and their regression analysis revealed a good correlation between the two 24 to 168 h after MCAO. Palmer *et al.* [36] reported that there were highly significant correlations between lesion size determined by MRI and histopathology at 2 and 7 days post-transient MCAO. We similarly demonstrated a correlation between our MRI-derived %HLV and TTC staining results 14 days after MCAO. The use of MRI allowed us to track the infarcted volume and investigate which cytokine most enhanced the effects of the MSC.

In a previous study, we demonstrated that MSC-BDNF promoted functional recovery and reduced ischemic damage following stroke [9]. In the current study, rats that were intracerebrally injected with MSC-GDNF also exhibited significantly more functional recovery and reduced infarct volume following stroke than did rats in the control groups. Zhang *et al.* [22] reported that adenovirus-mediated GDNF gene expression reduced ischemic damage after MCAO in rats. Since they intracranially administered Ad-GDNF (1×10^8 pfu/10 μl), it took several hours for GDNF to be expressed and its effect was transient. On the other hand, in our study, the MSC were transfected at an m.o.i. of 1000 pu/cell, which did not appear to be a more toxic approach than that of direct intracranial injection of the adenovirus vector. In another report [21], the placement of GDNF-presoaked sponges (8 mm^3 each; 2.5 μg/9 μl) in contact with the surface of the cerebral cortex provided protection to the neurons within the surrounding areas. The production of cytokine using this latter approach would likely diminish far sooner than would its production using the MSC gene-engineered approach. In our study, as with MSC-BDNF, there was enhanced survival of MSC-GDNF in the rat brain 14 days after MCAO compared to MSC-DsR. Higher engraftment of MSC transduced with the BDNF or GDNF gene thus appears to improve function and reduce ischemic damage in the rat model of MCAO.

Our results showed that there was no effect of CNTF on infarct volume in rats 7 and 14 days after MCAO.

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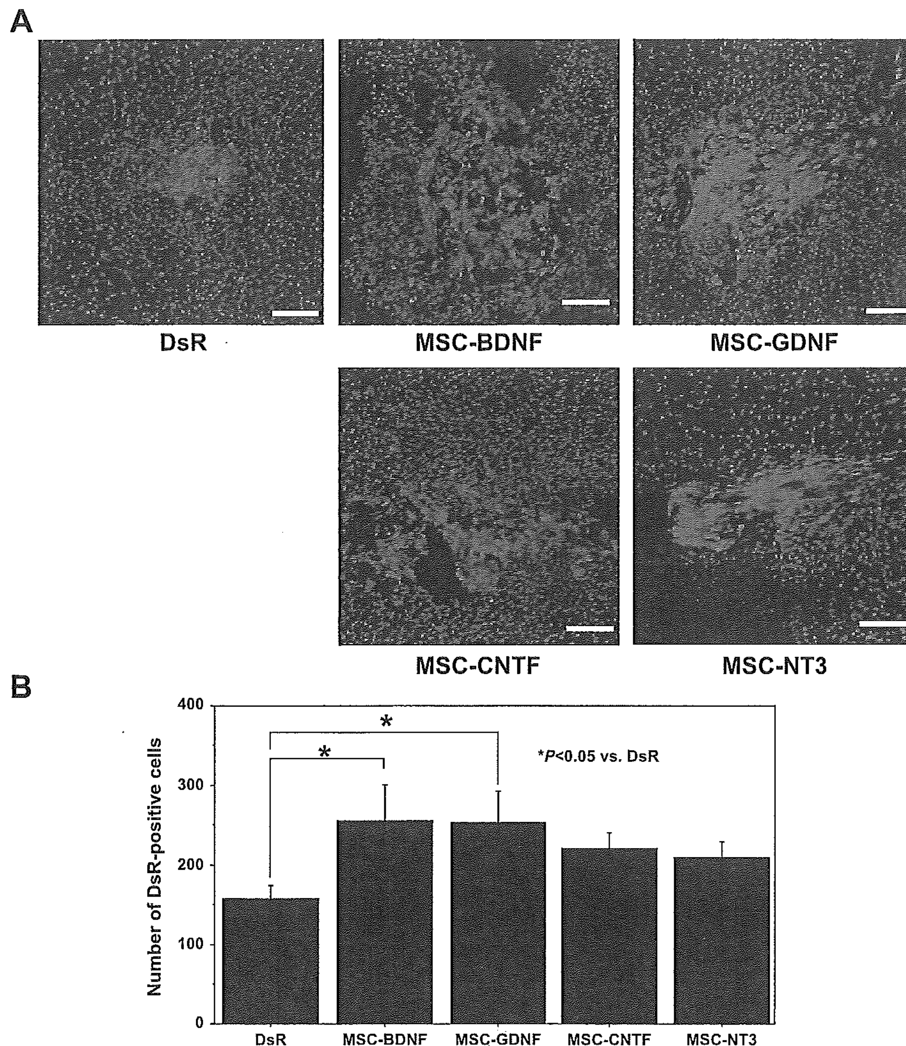

FIG. 5. (A) The engraftment levels of genetically modified MSCs. More positive cells existed in the injection area of rats administered MSC-BDNF-DsR or MSC-GDNF-DsR compared to those that received MSC-DsR. DsR (red), DAPI (blue). Scale bar, 100 μm. (B) The number of DsR-positive cells detected was significantly more in animals treated with MSC-BDNF or MSC-GDNF compared to animals injected with MSC-DsR (* $P < 0.05$).

Ogata *et al.* [37] reported that the protective effect of CNTF in the rat forebrain ischemia model may be limited compared with other neurotrophic factors. On the other hand, Hermann [38] reported that the preinjection of adenovirus-transfected GDNF and CNTF into the dorsolateral striatum protected against injury following MCAO in mice. In their experiment, the administration of CNTF before MCAO may have facilitated neuroprotection. Interestingly, Ad-CNTF showed pronounced rescue effects in the area immediately surrounding the ischemic area, but not in regions that were farther away.

As with CNTF, we were unable to show any effect of NT3 on infarct volume in rats 7 and 14 days after MCAO. Numerous studies have implicated NT3 and neurotrophin signaling as important factors in neuronal cell death [39–41]. A recent report by Zhang and co-workers [28] demonstrated that the cortical application of NT3 after MCAO in rats had a neuroprotective effect, as demonstrated by the significant reduction in infarct size. Bates *et al.* [42] noted that while this observation was relevant to neuroprotection, it did not represent the physiological role of endogenous NT3. What has yet to be demonstrated is how this neuroprotective effect is mediated and if it involves TrkC, the physiological NT3 receptor, since high concentrations of NT3 have been shown to activate both TrkA and TrkB receptors [43,44]. Interestingly, recent reports demonstrated that cortical infusion of NT3 led to the induction of BDNF expression and phosphorylation of the BDNF receptor, TrkB [41,45], suggesting that its neuroprotective effects were mediated

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through nonphysiological signaling through the BDNF pathway.

On the other hand, endogenous NT3 has been shown to promote cell death in other neuronal injury paradigms. For example, inactivation of endogenous NT3 was found to improve the survival of axotomized corticospinal neurons, an effect that was seen in mutant mice lacking a single copy of the NT3 gene [41]. Bates *et al.* [42] demonstrated that NT3 enhanced neuronal cell death and their production of reactive oxygen species induced by oxidative damage. In our study, although the secretion rate of NT3 was very high in MSC-NT3, its protein levels were no greater than those of the exogenous protein.

Thrombolytic therapy, administered within 3 h after the onset of symptoms, is the treatment of choice for acute stroke [46]. Zhang *et al.* [22] demonstrated a time-dependent ameliorative effect of GDNF after transient cerebral ischemia. They also reported that the infarction volumes in GDNF-treated groups were significantly reduced when GDNF was applied immediately or 1 h, but not 3 h, after the onset of ischemia. Further studies will be necessary to evaluate the effectiveness of MSC treatment in the early phase of stroke.

In conclusion, the intracerebral injection of MSC transfected with the BDNF or GDNF gene using a fiber-mutant adenovirus vector resulted in improved function and reduced ischemic damage in a rat model of MCAO. These data indicate a possible usefulness of this gene-modified cell therapy as an approach for the treatment of stroke.

Materials and Methods

Cell preparations. Human bone marrow from three healthy adult volunteers was obtained as previously described [47]. The adherent cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (Gibco BRL, Rockville, MD, USA) in a humidified atmosphere of 5% CO_2 at 37°C. After reaching confluence, the cells were harvested and used for gene transfection with a retroviral vector, BABE-hygro-hTERT [47]. MSC that experienced no greater than 40 population doublings were used in this study. The morphological features of the MSC were the same as those previously described by Kobune *et al.* [29].

Adenoviral vectors. Adenoviral vectors (AxCAEGFP-F/RGD) carrying a humanized variant of *Aequoria victoria* green fluorescent protein were constructed as described previously [30,48]. The cosmid vector pWEAx-CAEGFP-F/RGD generated together with *Cla*I- and *Eco*T22I-digested DNA-TPC from Ad5*dlx*-F/RGD was cotransfected into human embryonic kidney 293 cells. The adenoviral EGFP expression vector AxCAEGFP-F/RGD, obtained from isolated plaques, was expanded in these cells and purified by cesium chloride ultracentrifugation [49].

Adenoviral vectors (AxCADsR-F/RGD) carrying humanized DsR together with the gene for RGD-mutated fiber under the control of a CA promoter were constructed as described above.

Human GDNF cDNA was cloned using the reverse transcription polymerase chain reaction (RT-PCR) method using the total RNA extracted from primary MSC as the template. The identity of GDNF cDNA obtained in this manner was confirmed by sequencing and was compared to GenBank sequence AY052832. The human GDNF primer sequence was Forward, 5′-CGCAATTGCCACCATGAAGTTATGG-GATGTCGTGGCTGT-3′, and Reverse, 5′-CCAGATCTCAGATACATCCA-CACCTTTTAGCGG-3′.

Adenoviral vectors (AxCAhGDNF-F/RGD) carrying human GDNF together with the gene for RGD-mutated fiber under the control of a CA promoter were constructed as described above. Adenoviral vectors (AxCAhBDNF-F/RGD) carrying human BDNF together with the gene for RGD-mutated fiber under the control of a CA promoter were constructed as described previously [9]. Briefly, the BDNF cDNA was inserted between the *Eco*RI 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 BDNF expression unit was cloned into the adenoviral expression cosmid, resulting in pWEAxCAhBDNF-F/RGD. The recombinant adenovirus, AxCAhBDNF-F/RGD, was generated by transfection with *Pac*I-digested pWEAxCAhBDNF-F/RGD.

Human CNTF cDNA was cloned using the RT-PCR method using the total RNA extracted from primary MSC as the template. The identity of CNTF cDNA obtained in this manner was confirmed by sequencing and was compared to GenBank sequence NM_000614. The human CNTF primer sequence was Forward, 5′-CGGAATTCCACCATGGCTTTCACA-GAGCATTCACCGC-3′, and Reverse, 5′-CCAGATCTACATTTTCTTGTTGT-TAGCAATATAATG-3′.

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

Human NT3 cDNA was cloned using the RT-PCR method using the total RNA extracted from primary MSC as the template. The identity of NT3 cDNA obtained in this manner was confirmed by sequencing and was compared to GenBank sequence M37763. The human NT3 primer sequence was Forward, 5′-CGGAATTCCACCATGTCCATCTTGTTTTATGT-GATATTTCTC-3′, and Reverse, 5′-CCAGATCTCATGTTCTTCC-GATTTTTCTCGACAAG-3′.

Adenoviral vectors (AxCAhNT3-F/RGD) carrying human NTF3 together with the gene for RGD-mutated fiber under the 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 (pu/ml), the viral solution was incubated in 0.1% sodium dodecyl sulfate and A_{260} was measured [50]. The titer of each virus is described in Table 1.

Adenovirus infection. Adenovirus-mediated gene transfection was performed as previously described [31]. Briefly, the cells were seeded at a density of 2×10^6 cells per 15-cm plate. MSC were exposed to the infectious viral particles in 7.5 ml of DMEM at 37°C for 60 min; cells were infected with AxCAEGFP-F/RGD, AxCADsR-F/RGD, AxCAhBDNF-F/RGD, AxCAhGDNF-F/RGD, AxCAhCNTF-F/RGD, and AxCAhNT3-F/RGD at m.o.i. of 1×10^3, 4×10^3, 1×10^3, 1×10^3, 1×10^3, and 1×10^3, respectively. The media were then removed, and the cells were washed once with DMEM and then recultured with normal medium for 24 h, after which intracerebral transplantation was performed.

Cytokine ELISAs. Forty-eight hours after MSC were transfected *in vitro* at various m.o.i. (pu/cell), culture supernatants were collected for analysis. The following commercial ELISA kits were used to quantify the concentration of cytokine in each of the samples: BDNF, GDNF, NT3 (Promega, Madison, WI, USA), and CNTF (R&D Systems, Inc., Minneapolis, MN, USA). All samples were run in triplicate.

Induction of transient MCAO and intracerebral transplantation. Focal cerebral ischemia was induced in male Wistar rats (250–300 g each) by endovascular MCAO as previously described [9]. A No. 5-O monofilament nylon suture with a silicone-coated tip was inserted through an arteriotomy in the right common carotid artery and gently advanced into the internal carotid artery to a point approximately 18 mm distal to the bifurcation of the carotid artery. After 90 min of transient occlusion, cerebral blood flow was restored by withdrawal of the nylon thread.

Intracerebral transplantation of donor MSC was carried out as previously described [9]. After the induction of ischemic brain injury

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TABLE 1: Infectious titers of Ad preparation

	Particle units (pu)/ml[a]	pfu/ml[b]	pu/pfu ratio
AxCAEGFP-F/RGD	3.61×10^{11}	8.32×10^{9}	43.39
AxCADsR-F/RGD	1.03×10^{12}	2.27×10^{10}	45.37
AxCAhBDNF-F/RGD	4.43×10^{11}	2.61×10^{10}	16.97
AxCAhGDNF-F/RGD	5.21×10^{11}	3.32×10^{10}	15.69
AxCAhNT3-F/RGD	3.21×10^{11}	9.66×10^{9}	33.23
AxCAhCNTF-F/RGD	5.61×10^{11}	5.18×10^{10}	10.83

[a] Calculated from virion total protein and DNA (1.1×10^{12} virions per unit OD_{260}).
[b] Analyzed by plaquing on 293 cells.

was confirmed using the behavioral tests described below, the animals were randomized for transplantation. They were anesthetized with an intraperitoneal (ip) injection of ketamine (2.7–3 mg/100 g) and xylazine (0.36–0.4 mg/100 g) and positioned in a Narishige stereotaxic frame (Model SR-6N, Narishige Co., Japan). With a 26-gauge Hamilton syringe, 5×10^5 MSC in 5 μl of serum-free DMEM were then injected into the right dorsolateral striatum 4 mm beneath the skull surface and 3 mm lateral to the bregma level over 2.5 min; this position approximated the ischemic boundary zone. Rats were administered cyclosporin A (10 mg/kg daily) ip to prevent them from rejecting their human MSC implants.

MRI studies and measurement of infarct volume. MRIs were performed on all animals on days 2, 7, and 14 after MCAO as previously described [9]. The MRI machine consisted of a 7 T, 18-cm-bore superconducting magnet interfaced to a UNITYINOVA console (Oxford Instruments, UK, and Varian, Inc., Palo Alto, CA, USA). The animals were kept in the same position during imaging. Multislice T2-weighted spin-echo MR images (TR 3000 ms, TE 40 ms, field of view 40 × 30 mm, slice thickness 2 mm, gapless) were obtained.

The disposition of the ischemic area was evaluated by calculating hemispheric lesion volumes (%HLV) from T2-weighted images using imaging software (Scion Image, Version Beta 4.0.2, Scion Corp.). For each slice, ischemic tissue was marked and infarct volume calculated taking slice thickness (2 mm/slice) into account. To avoid overestimation of the infarct volume, the corrected infarct volume, or CIV, was calculated as previously described [9], i.e., CIV = $(L_T - (R_T - R_I)) \times d$, where L_T was the area of the left hemisphere in square millimeters, R_T was the area of the right hemisphere in square millimeters, R_I was the infarcted area in square millimeters, and d was the thickness of the slices (2 mm). Relative infarct volumes were expressed as a percentage of right hemispheric volume.

TTC, HE staining, and assessment of engraftment levels of genetically modified MSC. Fourteen days after MCAO, rats were anesthetized and decapitated. Their brains were removed and coronally sectioned into six 2-mm coronal slices, incubated for 30 min in a 2% solution of TTC at 37°C, and fixed with 4% paraformaldehyde (PFA) in PBS. Infarcted volume was calculated as described above.

Fifteen days after MCAO, the brains of other rats were perfused with PBS followed by 4% paraformaldehyde under deep anesthesia. The excised brains were postfixed with 4% paraformaldehyde overnight and then equilibrated in PBS containing 30% sucrose for 48 h. The blocks were fixed with 10% neutral-buffered formalin and stained with HE for light-microscopic examination.

Finally, the engraftment levels of genetically modified MSC were examined. A separate cohort of rats was anesthetized and transcardially perfused with PBS followed by 4% PFA in PBS, 14 days after MCAO. Their brains were then harvested and immersed in 4% PFA in PBS for 2 days, after which 30-μm frozen sections (coronal coordinates bregma from −1.0 to 1.0 mm) were cut with a cryostat at −20°C. MSC transfected with AxCADsR-F/RGD stained red. Sections were counterstained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI), which stains blue. The number of red-stained cells was counted in three 1 × 1 mm^2 regions in the injection area.

Behavioral testing. Limb placement tests (LPTs) included eight subtests and were performed 24 h after ischemia as previously described [9]. Briefly, the rat's four limbs were evaluated using the top and edges of a countertop. For each subtest, the animals received a score of 0 if they were unable to place their limbs, 1 if they displayed partial and/or delayed (more than 2 s) placement of their limbs, and 2 if they exhibited immediate and correct limb placement.

Experimental groups. Experiment 1 was designed to test the correlation between the MRI infarcted and the TTC volumes. MRIs were performed on days 2, 7, and 14. Fourteen days after MCAO, TTC staining was performed.

Experiment 2 was designed to test the therapeutic effectiveness of cytokines over the course of 2 weeks following MCAO. The experimental groups included group 1 (control)—rats injected with DMEM (n = 7), group 2—rats transplanted with MSC-EGFP (n = 6), group 3—rats transplanted with MSC-GDNF (n = 7), group 4—rats transplanted with MSC-BDNF (n = 7), group 5—rats transplanted with MSC-CNTF (n = 7), and group 6—rats transplanted with MSC-NT3 (n = 7). LPTs were performed on days 1, 8, and 15 after MCAO. MRIs were performed on days 2, 7, and 14. HE staining was carried out 15 days after MCAO.

Experiment 3 was performed to assess the engraftment levels of the genetically modified MSCs. Rats were sacrificed 14 days after MCAO and brain tissue was examined from animals transplanted with MSC-DsR (n = 3), MSC-BDNF-DsR (n = 3), MSC-GDNF-DsR (n = 3), MSC-CNTF-DsR (n = 3), or MSC-NT3-DsR (n = 3).

Data analysis. Data were presented as the mean ± standard deviation. Data from the LPTs were analyzed using a one-way ANOVA followed by a Games Howell post hoc test. %HLV data and the number of DsR-positive cells were analyzed using a one-way ANOVA followed by a Scheffe post hoc test. Data between %HLV of MRI and of TTC staining were paired and analyzed for correlations. Significance was assumed if the P value was <0.05.

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