Li PF, Dietz R, von Harsdorf R (1999) p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome *c*-independent apoptosis blocked by Bcl-2. *EMBO J* 18:6027–36

Matsumori Y, Hong SM, Aoyama K, Fan Y, Kayama T, Sheldon RA *et al* (2005) Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. *J Cereb Blood Flow Metab* 25:899–910

Matsumoto K, Nakamura T (1996) Emerging multipotent aspects of hepatocyte growth factor. *J Biochem (Tokyo)* 119:591–600

McGahan L, Hakim AM, Robertson GS (1998) Hippocampal Myc and p53 expression following transient global ischemia. *Brain Res Mol Brain Res* 56:133–45

Meli E, Pangallo M, Baronti R, Chiarugi A, Cozzi A, Pellegrini-Giampietro DE *et al* (2003) Poly(ADP-ribose) polymerase as a key player in excitotoxicity and post-ischemic brain damage. *Toxicol Lett* 139:153–62

Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, Korsmeyer SJ *et al* (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. *J Cell Biol* 139:205–17

Miyazawa T, Matsumoto K, Ohmichi H, Katoh H, Yamashima T, Nakamura T (1998) Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor—a novel neurotrophic factor. *J Cereb Blood Flow Metab* 18:345–8

Nagayama T, Simon RP, Chen D, Henshall DC, Pei W, Stetler RA *et al* (2000) Activation of poly(ADP-ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. *J Neurochem* 74:1636–45

Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T (2000) Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J Clin Invest* 106:1511–9

Nakamura T, Nawa K, Ichihara A (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 122:1450–9

Nakamura T, Nawa K, Ichihara A, Kaise N, Nishino T (1987) Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS Lett* 224:311–6

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A *et al* (1989) Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342:440–3

Niwa M, Hara A, Iwai T, Wang S, Hotta K, Mori H *et al* (2001) Caspase activation as an apoptotic evidence in the gerbil hippocampal CA1 pyramidal cells following transient forebrain ischemia. *Neurosci Lett* 300:103–6

Ostrakhovitch EA, Cherian MG (2005) Inhibition of extracellular signal regulated kinase (ERK) leads to apoptosis inducing factor (AIF) mediated apoptosis in epithelial breast cancer cells—the lack of effect of ERK in p53 mediated copper induced apoptosis. *J Cell Biochem* 95:1120–34

Ozaki M, Haga S, Zhang HQ, Irani K, Suzuki S (2003) Inhibition of hypoxia/reoxygenation-induced oxidative stress in HGF-stimulated antiapoptotic signaling—role of PI3-K and Akt kinase upon rac1. *Cell Death Differ* 10:508–15

Pastoriza Gallego M, Sarasin A (2003) Transcription-coupled repair of 8-oxoguanine in human cells and its deficiency in some DNA repair diseases. *Biochimie* 85:1073–82

Plesnila N, Zhu C, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. *J Cereb Blood Flow Metab* 24:458–66

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N *et al* (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 3:839–43

Renolleau S, Benjelloun N, Ben-Ari Y, Charriaut-Marlangue C (1997) Regulation of apoptosis-associated proteins in cell death followingtransient focal ischemia in rat pups. *Apoptosis* 2:368–76

Rideout HJ, Stefanis L (2001) Caspase inhibition—a potential therapeutic strategy in neurological diseases. *Histol Histopathol* 16:895–908

Selznick LA, Zheng TS, Flavell RA, Rakic P, Roth KA (2000) Amyloid beta-induced neuronal death is bax-dependent but caspase-independent. *J Neuropathol Exp Neurol* 59:271–9

Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M *et al* (2000) Two distinct pathways leading to nuclear apoptosis. *J Exp Med* 192: 571–80

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM *et al* (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397:441–6

Takagi N, Sasakawa K, Besshoh S, Miyake-Takagi K, Takeo S (2003) Transient ischemia enhances tyrosine phosphorylation and binding of the NMDA receptor to the Src homology 2 domain of phosphatidylinositol 3-kinase in the rat hippocampus. *J Neurochem* 84: 67–76

Tomasevic G, Kamme F, Stubberod P, Wieloch M, Wieloch T (1999) The tumor suppressor p53 and its response gene p21WAF1/Cip1 are not markers of neuronal death following transient global cerebral ischemia. *Neuroscience* 90:781–92

Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Yoshida A, Uchida K *et al* (1997) Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1—its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab Invest* 76:365–74

Tsuzuki N, Miyazawa T, Matsumoto K, Nakamura T, Shima K (2001) Hepatocyte growth factor reduces the infarct volume after transient focal cerebral ischemia in rats. *Neurol Res* 23:417–24

Wang YT, Salter MW (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* 369:233–5

Wick A, Wick W, Hirrlinger J, Gerhardt E, Dringen R, Dichgans J *et al* (2004) Chemotherapy-induced cell death in primary cerebellar granule neurons but not in astrocytes—*in vitro* paradigm of differential neurotoxicity. *J Neurochem* 91:1067–74

Won MH, Kang T, Park S, Jeon G, Kim Y, Seo JH *et al* (2001) The alterations of *N*-methyl-D-aspartate receptor expressions and oxidative DNA damage in the CA1 area at the early time after ischemia-reperfusion insult. *Neurosci Lett* 301:139–42

Yu SW, Wang H, Dawson TM, Dawson VL (2003) Poly(ADP-ribose) polymerase-1 and apoptosis inducing factor in neurotoxicity. *Neurobiol Dis* 14:303–17

Zhan RZ, Wu C, Fujihara H, Taga K, Qi S, Naito M *et al* (2001) Both caspase-dependent and caspase-independent pathways maybe involved in hippocampal CA1

neuronal death because of loss of cytochrome *c* from mitochondria in a rat forebrain ischemia model. *J Cereb Blood Flow Metab* 21:529–40

Zhang Y, Zhang X, Park TS, Gidday JM (2005) Cerebral endothelial cell apoptosis after ischemia–reperfusion—role of PARP activation and AIF translocation. *J Cereb Blood Flow Metab* 25:868–77

Zhao H, Yenari MA, Cheng D, Barreto-Chang OL, Sapolsky RM, Steinberg GK (2004) Bcl-2 transfection via herpes simplex virus blocks apoptosis-inducing factor translocation after focal ischemia in the rat. *J Cereb Blood Flow Metab* 24:681–92

Zheng F, Gingrich MB, Traynelis SF, Conn PJ (1998) Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. *Nat Neurosci* 1: 185–191

Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G *et al* (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia–ischemia in the neonatal rat brain. *J Neurochem* 86:306–17

Journal of Cerebral Blood Flow & Metabolism (2006) 26, 1176–1188

www.jcbfm.com

Novel therapeutic strategy for stroke in rats by bone marrow stromal cells and *ex vivo* HGF gene transfer with HSV-1 vector

Ming-Zhu Zhao[1,2], Naosuke Nonoguchi[1], Naokado Ikeda[1], Takuji Watanabe[1], Daisuke Furutama[3], Daisuke Miyazawa[4], Hiroshi Funakoshi[4], Yoshinaga Kajimoto[1], Toshikazu Nakamura[4], Mari Dezawa[5], Masa-Aki Shibata[6], Yoshinori Otsuki[6], Robert S Coffin[7], Wei-Dong Liu[2], Toshihiko Kuroiwa[1] and Shin-Ichi Miyatake[1]

[1]*Department of Neurosurgery, Osaka Medical College, Takatsuki, Osaka, Japan;* [2]*Department of Neurosurgery, Pu Nan Hospital, Shanghai, People's Republic of China;* [3]*First Department of Internal Medicine, Osaka Medical College, Takatsuki, Osaka, Japan;* [4]*Division of Molecular Regenerative Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan;* [5]*Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, Kyoto, Japan;* [6]*Department of Anatomy and Biology, Osaka Medical College, Takatsuki, Osaka, Japan;* [7]*Department of Molecular Pathology in Windeyer Institute of Medical Sciences of University College, London, UK*

Occlusive cerebrovascular disease leads to brain ischemia that causes neurological deficits. Here we introduce a new strategy combining mesenchymal stromal cells (MSCs) and *ex vivo* hepatocyte growth factor (HGF) gene transferring with a multimutated herpes simplex virus type-1 vector in a rat transient middle cerebral artery occlusion (MCAO) model. Gene-transferred MSCs were intracerebrally transplanted into the rats' ischemic brains at 2 h (superacute) or 24 h (acute) after MCAO. Behavioral tests showed significant improvement of neurological deficits in the HGF-transferred MSCs (MSC-HGF)-treated group compared with the phosphate-buffered saline (PBS)-treated and MSCs-only-treated group. The significant difference of infarction areas on day 3 was detected only between the MSC-HGF group and the PBS group with the superacute treatment, but was detected among each group on day 14 with both transplantations. After the superacute transplantation, we detected abundant expression of HGF protein in the ischemic brain of the MSC-HGF group compared with others on day 1 after treatment, and it was maintained for at least 2 weeks. Furthermore, we determined that the increased expression of HGF was derived from the transferred *HGF* gene in gene-modified MSCs. The percentage of apoptosis-positive cells in the ischemic boundary zone (IBZ) was significantly decreased, while that of remaining neurons in the cortex of the IBZ was significantly increased in the MSC-HGF group compared with others. The present study shows that combined therapy is more therapeutically efficient than MSC cell therapy alone, and it may extend the therapeutic time window from superacute to acute phase.
Journal of Cerebral Blood Flow & Metabolism (2006) **26,** 1176–1188. doi:10.1038/sj.jcbfm.9600273; published online 18 January 2006

Keywords: gene transfer; hepatocyte growth factor; herpes simplex virus; intracerebral transplantation; mesenchymal stromal cell; transient cerebral ischemia

Correspondence: Dr S-I Miyatake and Dr T Kuroiwa, Department of Neurosurgery, Graduate School of Medicine, Osaka Medical College, 2-7 Daigakumachi, Takatsuki City, Osaka 569-8686, Japan. E-mail: neu070@poh.osaka-med.ac.jp
This work was supported by Grants-in-Aid for Scientific Research (B) (14370448) and (C) (12671353), and by a Grant-in-Aid for Exploratory Research (14657350) from the Japanese Ministry of Education, Science and Culture, Japan to Shin-Ichi Miyatake, MD, PhD. Additional support was provided in the form of a grant from the Special Assistance for Promoting the Advancement of the Education & Research of the Private University, Promotion and Mutual Aid Corporation for Private Schools of Japan and the Science Research Promotion Fund to Shin-Ichi Miyatake, MD, PhD, and by the High-Tech Research Program of Osaka Medical College. This work was also supported in part by Grants-in-Aid 17790989 from the Ministry of Education, Science and Culture, Japan to Naosuke Nonoguchi, MD.
Received 22 August 2005; revised 29 November 2005; accepted 5 December 2005; published online 18 January 2006

Introduction

Occlusive cerebrovascular disease often causes global ischemia of the brain and results in neuropathological changes. Several methods have been proposed to augment brain reorganization, including the stimulation of endogenous processes through pharmacologic or molecular manipulation, gene therapy, behavioral and rehabilitation strategies, and the provision of new substrates for recovery through cell therapy.

Bone marrow contains the precursors of nonhematopoietic tissues that are referred to as mesenchymal stem cells or marrow stromal cells (MSCs) (Friedenstein *et al*, 1978). Marrow stromal cells are characterized by the ability to self-renew in a

number of nonhematopoietic tissues, and by their multipotentiality for differentiation into various tissues, such as fibroblasts, bone, muscle, and cartilage (Caplan and Bruder, 2001; Phinney, 2002). Additionally, they share some characteristics of neurons and astrocytes when cultured *in vitro* (Kim *et al*, 2002) or after being implanted into the central nervous system *in vivo* (Chopp *et al*, 2000; Nakano *et al*, 2001; Li *et al*, 2001, 2002; Chen *et al*, 2002*a*, *b*). Marrow stromal cells can also secrete growth factors and cytokines into the soluble stromal and neurochemicals into the brain (Li *et al*, 2002; Chen *et al*, 2002*a*, *b*), cross the blood–brain barrier (BBB) and migrate throughout the brain preferentially to areas that have suffered damage (Chen *et al*, 2000; Li *et al*, 2000, 2001; Damme *et al*, 2002). Many previous researchers have reported on mesenchymal stromal cell (MSC) transplantation as a source for autoplastic therapies and improvement in functional recovery after stroke (Chen *et al*, 2000; Li *et al*, 2000, 2001, 2002; Rempe and Kent, 2002; Kurozumi *et al*, 2004).

Hepatocyte growth factor (HGF) is a disulfide-linked heterodimeric protein that was initially purified and cloned as a potent mitogen for hepatocytes and a natural ligand for the c-met proto-oncogene product (Nakamura *et al*, 1984; Matsumoto and Nakamura, 1996). Subsequently, several functions have been ascribed to HGF, including antiapoptosis, angiogenesis, motogenesis, morphogenesis, hematopoiesis, tissue regeneration in a variety of organs, and the enhancement of neurite outgrowth (Matsumoto and Nakamura, 1997; Hayashi *et al*, 2001; Sun *et al*, 2002*a*, *b*; Jin *et al*, 2003). It has also been reported that HGF administration could inhibit the BBB destruction, decrease brain edema, and provide a neuroprotective effect after brain ischemia (Miyazawa *et al*, 1998; Hayashi *et al*, 2001; Shimamura *et al*, 2004).

Recent experimental studies suggest the possibility that gene transduction into MSCs could enhance their existing therapeutic potential (Chen *et al*, 2000; Kurozumi *et al*, 2004). Here, we evaluate the efficiency and effects of gene transduction into MSCs using a replication-incompetent herpes simplex virus type-1 (HSV1764/4-/pR19) vector disabled by the deletion of three critical genes for viral replication encoding infected cell polypeptide (ICP)4, ICP34.5, and virion protein (VP16) (vmw65). This vector contains HSV latency-associated transcript (LAT) promoter and two kinds of enhancer elements: cytomegalovirus (CMV) enhancer and Woodchuck posttranscriptional regulatory elements (WPRE). The availability of this vector has already been examined in the nervous system (Coffin *et al*, 1998; Palmer *et al*, 2000; Lilley *et al*, 2001).

In the present study, we intracerebrally transplanted MSCs in which a gene of interest was transferred with this HSV-1 vector *ex vivo* into a rat transient middle cerebral artery occlusion (MCAO) model under superacute and acute therapeutic time phase, and investigated whether such combined therapy could improve the effects of ischemia.

Materials and methods

Donor Cell Preparation

Marrow stromal cells of adult Wistar rats were prepared following the method described by Azizi *et al* (1998). In brief, the marrow of rat tibias and femurs was extruded with 10 mL of alpha-MEM (Sigma Chemical Co., St Louis, MO, USA) and cultured in the same medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and antibiotic–antimycotic 1 mL/100 mL (GIBCO Invitrogen, Carlsbad, CA, USA) at 37°C, 98% humidity and 5% CO_2. After 48 h, the nonadherent cells were removed by replacing the medium, and the adherent cells were continuously subcultured as MSCs. The fifth to seventh passages were used for the following experiments.

HSV1764/-4/pR19-Hepatocyte Growth Factor Virus and Propagation

One of the authors of the current study (Coffin) constructed the prototype HSV1764/4/pR19GFP virus and has previously described this vector's characteristics (Palmer *et al*, 2000; Lilley *et al*, 2001), which are also described briefly in the Introduction. In the present study, the green fluorescent protein (*GFP*) gene was replaced with a full-length rat HGF complementary DNA (cDNA) tagged with the KT3 (SV (simian virus)40 large, T antigen) epitope (ratHGFKT3) (Sun *et al*, 2002*b*), and the authenticity of this vector (pR19ratHGFKT3WPRE) was confirmed by sequence analysis. Homologous recombination was performed in M49 cells by cotransfection of plasmid pR19ratHGFKT3WPRE DNA and HSV1764/-4/pR19GFP viral DNA. White plaques were selected and purified three times, and replication-incompetent viruses were propagated as described previously (Palmer *et al*, 2000). We ultimately obtained the HSV1764/-4/pR19-HGF virus (HSV-HGF) with a titer of 2×10^8 pfu/mL for use in the present experiments.

Ex Vivo Gene Delivery to MSCs

The cultured MSCs from the fifth to seventh passages were infected with the virus suspension by incubation for 1 h. After infection, the virus suspension was changed to normal culture medium for MSCs and continuously cultured for the subsequent 24 h before transplantation.

Our previous experiments show that the transduction efficiency of the *GFP* gene into the MSCs with our HSV-1 vector is more than 50% even with a multiplicity of infection (MOI) of 5. Here we set the MOI at 5 for the desired gene transfer to MSCs *ex vivo*.

Hepatocyte Growth Factor Detection with Enzyme-Linked Immunosorbent Assay (ELISA) *In Vitro*

We prepared 1.6×10^5 MSCs in each well of a six-well dish. The MSCs were transferred with *HGF* gene by infection with HSV-HGF at MOIs of 0, 0.1, 1, 5, and 10. At 1 h after infection, the infected MSCs were successively incubated with normal culture medium for another 24 h. The culture supernatant and cells were then individually collected through centrifugation. The HGF protein concentrations in MSC culture supernatant and in MSC extracts prepared using 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride (PMSF) (Wako, Osaka, Japan), 2 μg/ml antipain (Peptide Institute Inc., Osaka, Japan), 2 μg/ml leupeptin (Peptide Institute), and 2 μg/ml pepstatin (Peptide Institute) were determined by ELISA using an anti-rat HGF polyclonal antibody (Tokushu Meneki, Tokyo, Japan) as described (Sun *et al*, 2002*b*).

Transient Middle Cerebral Artery Occlusion Animal Model

Experiments were performed on 8-week-old male Wistar rats weighing 250 to 280 g. We induced transient MCAO using the previously described method of intraluminal vascular occlusion (Longa *et al*, 1988). In brief, a length (18.5 to 19.0 mm, determined according to the animal's weight) of 4-0 surgical nylon suture was gently advanced from the external carotid artery into the lumen of the internal carotid artery until it reached the proximal segment of the anterior cerebral artery. After 2 h of MCAO the animals were reanesthetized, and reperfusion was achieved by withdrawing the nylon suture.

The rats were subjected to transient MCAO for 2 h to produce a consistent and reproducible ischemic lesion in the unilateral striatum and cortex.

Intracerebral Transplantation of MSCs

At 2 or 24 h after the onset of MCAO (i.e., on reperfusion), the animals were placed in a stereotactic head holder (model 900, David Kopf Instruments, Tujunga, CA, USA) under inhalation anesthesia. MSCs were intracerebrally transplanted by inserting a 26-gauge needle with a Hamilton syringe into the right striatum (anteroposterior (AP) = 0 mm; lateral to midline (ML) = 2.0 mm; vertical to dura (DV) = 4.5 mm) from bregma, based on the atlas given by Paxinos *et al* (1985). There were 1×10^6 cells in total 10-μl fluid volumes that transplanted into each animal over a 10-min period. No immunosuppressive drugs were used in any animal.

Experimental Groups

In this study, there were seven experimental groups: groups 1 and 5 were treated with phosphate-buffered saline (PBS); groups 2 and 6 were treated with untreated MSCs only; group 3 was treated with the GFP-transferred MSCs (MSC-GFP); and groups 4 and 7 were treated with *HGF* gene-transferred MSCs (MSC-HGF).

Groups 1 to 4 were treated 2 h after MCAO (superacute phase) and groups 5 to 7 were treated 24 h after MCAO (acute phase).

Behavioral Testing

The rats of groups 1 to 4 ($n = 6$) were subjected to a modified neurological severity score (mNSS) test (Schallert *et al*, 1997) to evaluate neurological function before MCAO, at 2 h after MCAO, and at 1, 4, 7, 14, 21, 28, and 35 days after MCAO. The rats of groups 5 to 7 ($n = 6$) were subjected to mNSS before MCAO and at 0, 1, 4, 7, and 14 days after MCAO. These tests are battery of motor, sensory, reflex, and balance tests, which are similar to the contralateral neglect tests in humans. The higher the score, the more severe the neurological deficit (Chen *et al*, 2001).

Infarction Volume

We stained the brains of groups 1, 2, and 4 ($n = 6$) and groups 5 to 7 ($n = 5$) with 2,3,5-triphenyltetrazolium chloride (TTC) (Wako Pure Chemical Industries, Osaka, Japan) to detect the infarction volume of each group at 3 and 14 days after treatment. Briefly, the rats' brains were removed and cut into seven equally spaced (2 mm) coronal sections. These sections were immersed in a 2% solution of TTC at 37°C for 20 mins to reveal the infarcted areas. This procedure is known to reliably mark ischemic damage even at 14 days after MCAO (Bederson *et al*, 1986; Kurozumi *et al*, 2005).

The disposition of the ischemic area was evaluated by calculating the hemispheric lesion area using imaging software (Scion Image, version Beta 4.0.2; Scion Corp., Frederick, MD, USA). To avoid overestimation of the infarct volume, the corrected infarct volume (CIV) was calculated as $CIV = [LT - (RT - RI)] \times d$, where LT is the area of the left hemisphere, RT is the area of the right hemisphere, RI is the infarcted area, and d is the slice thickness (2 mm) (Raymond *et al*, 1990). Relative infarct volumes are expressed as a percentage of contralateral hemispheric volume.

Terminal Deoxynucleotidyltransferase (dUTP) Nick End-Labeling (TUNEL) Staining and Immunohistochemical Assessment

Sample Preparation: At different time points, rats of groups 1, 2, and 4 were reanesthetized and transcardially perfused with saline, followed by 4% paraformaldehyde in PBS. The brain tissues were cut into seven equally spaced coronal blocks. The tissues were processed and 10-μm cryosections were cut.

Immunohistochemical Staining: We can detect three kinds of HGF in this study: the endogenous HGF secreted by the rat ischemic brain tissue after stoke (en-HGF), the exogenous HGF secreted by the transplanted MSCs (ex-HGF-1), and the exogenous HGF delivered from the

HSV-HGF (ex-HGF-2). For the immunohistochemical staining of HGF, the whole rats' brain sections of groups 1, 2, and 4 were prepared on days 2 and 14 after treatment. Rabbit anti-rat HGF primary antibody (prepared by some of the authors of this article, and belonging to the Division of Molecular Regenerative Medicine, Osaka University Graduate School of Medicine, Japan) was used to detect the three kinds of HGF (mixed); a KT3 primary monoclonal antibody (1:1000) (Covance Research Products, Berkeley, CA, USA) was used to detect the ex-HGF-2; a biotinylated universal secondary antibody (VECSTAIN Elite ABC Kit, PK-6200, Vector Laboratories, Burlingame, CA, USA) and a goat anti-rabbit IgG affinity-purified rhodamine-conjugated secondary antibody (1:200) (Chemicon International, Temecula, CA, USA) were also used here. Reaction products were visualized with the VECSTAIN Elite ABC Kit (PK-6200) and a DAB Substrate Kit (Vector Laboratories, Burlingame, CA, USA). To detect the donor MSCs, bisbenzimide (Hoechst 33258; Polysciences, Eppelheim, Germany) was used to fluorescently label cell nuclei *in vitro*. Some sections were counterstained with hematoxylin and observed under a normal light microscope (VB-S20 Multiviewer System, Keyence, Osaka, Japan and Microphot-FXA, Nikon Corp., Tokyo, Japan), and some were directly observed by a fluorescence microscope (BX-50-34-FLAD1, Olympus). The donor MSCs could be detected under ultraviolet (UV) light with blue fluorescence as marked by Hoechst 33258.

To visualize the remaining neurons in the cortex of the ischemic boundary zone (IBZ) of groups 1, 2, and 4 ($n=3$), 7 days after treatment, microtubule-associated protein 2 (MAP-2) was used as the first antibody (1:500) (Chemicon International Inc., CA, USA). Negative control slides for each animal received identical preparation for immunohistochemical staining, except that primary antibodies were omitted.

Terminal Deoxynucleotidyltransferase Nick End-Labeling Staining: At 7 days after treatment, coronal cryosections (10-μm thick) of each rat of groups 1, 2, and 4 ($n=3$) were stained by the TUNEL method for *in situ* apoptosis detection (ApopTag kit, Chemicon International, USA). Specifically, after postfix slides were incubated in a mixture containing terminal deoxynucleotidyl transferase and anti-digoxigenin-rhodamine (Red). Then, they were counterstained with bisbenzimide (Hoechst 33258), which stains blue for each nucleus. The total numbers of TUNEL-positive cells and Hoechst counter-staining positive cells were individually counted in 2 slides from each brain, with each slide containing five random fields from the IBZ, under an $\times$ 20 objective of the fluorescence microscope system (BX-50-34-FLAD1, Olympus), using a 3-CCD color video camera (Keyence VB-7010, Keyence, Osaka, Japan).

Statistical Analysis

Data are presented as means$\pm$standard deviations (s.d.). Data from the behavior test (mNSS) were evaluated with repeated-measures analysis of variance (ANOVA), with subsequent Fisher's protected least significant difference (PLSD) test. StatView 5.0 software (SAS Institute, Cary, NC, USA) performing the Student's *t*-test was used to test the CIV data and the difference in means of percentage of the apoptosis-positive cells and the remaining neurons. A difference with a probability value of $P\leq 0.05$ was considered to be statistically significant.

Results

Quantification of Hepatocyte Growth Factor Analysis with Enzyme-Linked Immunosorbent Assay *In Vitro*

As a result, the HGF concentration was approximately 15 times higher in the culture supernatant than in the cell extract at the same MOI, and its increase was correlated with an increase in MOI. Although normal MSCs can produce HGF protein at 0.4 ng/1.6 $\times$ 10^5 cells/24 h, after the MSCs were infected with HSV-HGF at an MOI of 5, they were found to produce HGF protein at 2.4 ng/1.6 $\times$ 10^5 cells/24 h (Figure 1).

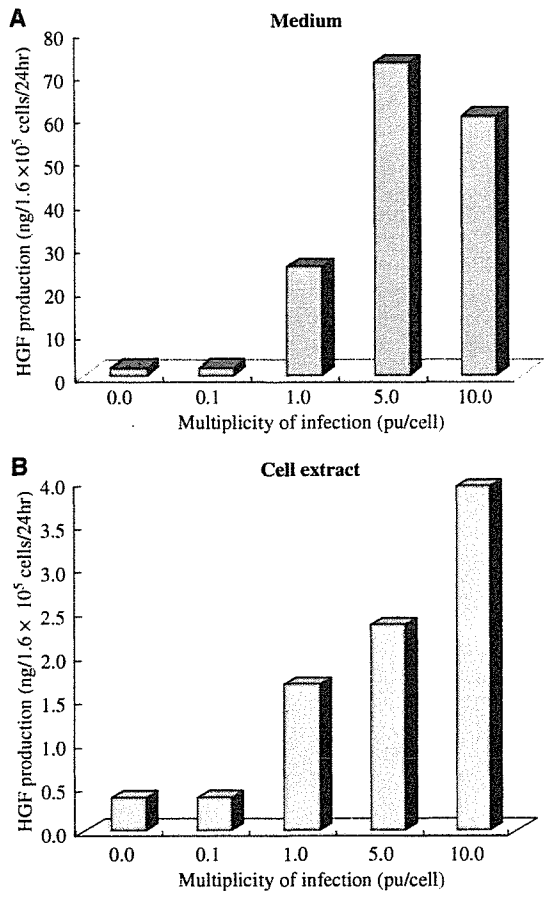

Figure 1 Enzyme-linked immunosorbent assay to determine HGF concentration *in vitro*. Hepatocyte growth factor concentrations were detected in MSC culture supernatant (**A**) and in MSC cell extract. (**B**) After 24 h, 1.6 $\times$ 10^5 MSCs were transfected with HSV-HGF at MOIs of 0, 0.1, 1, 5, and 10.

Neurological Outcome

No significant difference in neurological function was detected among all the groups just before cell transplantation. Significant differences of functional recovery were found in group 1 individually compared with group 2 (days 14 to 35, $P<0.05$), with group 3 (days 21 to 35, $P<0.05$), and with group 4 (days 4 and 7, $P<0.05$; days 14 to 35, $P<0.01$) during the observation periods after the superacute transplantation (Figure 2A), and in group 5 individually compared with group 6 (day 14, $P<0.05$), with group 7 (day 7, $P<0.01$ and day 14, $P<0.01$) after the acute transplantation (Figure 2B). Interestingly, we observed significant differences of functional recovery on day 14 among all the superacute treated groups including the MSC-GFP group, which served as a control for *ex vivo* nontherapeutic gene transduction ($P<0.05$). Exceptionally, there was no significant difference only between the MSC-only and the MSC-GFP groups at that time point (Figure 2A). We also found significant neurological recovery on day 14 in the combined therapy group treated even in the acute phase, compared with the MSC-only group treated in the superacute phase (Figure 2C). Also, significant difference of functional recovery on day 14 was found among the groups treated in the acute phase (Figure 2B).

Quantitative Analysis of Infarct Volume

We compared the infarction areas in coronal sections of groups 1, 2, and 4 on day 3 (Figure 3A) and day 14 (Figure 3B), and compared those of groups 5 to 7 on the same time points by TTC staining, and expressed lesion volume as a percentage of contralateral hemispheric volume. At 3 days after treatment, significant difference of %CIV was only detected in the MSC-HGF group compared with the PBS group ($34.52\% \pm 3.44\%$ versus $41.83\% \pm 6.25\%$, $P<0.05$), both of which were treated in the superacute phase (Figure 3C). However, on day 3 there was no significant difference of %CIV among any group that was treated in the acute phase (Figure 3C), while on day 14 there were significant reductions of %CIV in the rats of the MSC-HGF group compared with not only the PBS group but also the MSC-only group treated in the both therapeutic phases (Figure 3D). Also on day 14, the rats treated with MSC-only showed significant reduction in %CIV compared with the PBS group that was treated in the superacute phase (Figure 3D).

Hepatocyte Growth Factor and herpes simplex virus type Gene-Transferred Hepatocyte Growth Factor Detection *In Vivo*

The macrographs presented in Figure 4 showed that mixed HGF protein was diffusely overexpressed in almost the whole ipsilateral brain in the MSC-HGF group compared with other groups, not only on day 2 (column A) but throughout at least the first 2 weeks (column C) after treatment. The microphotographs presented in column B of Figure 4 showed that high HGF expression in the MSC-HGF group could be detected in both the ipsilateral cortex and the ipsilateral basal ganglia at 2 days after treatment. Nevertheless, almost no HGF expression could be detected on the contralateral hemisphere in any treatment group (Figure 4).

Fluorescent staining of groups 1, 2, and 4 on day 14 (Figure 5, column C) also showed higher mixed HGF expression in the MSC-HGF group than that of the other groups. Also, we could detect donor MSCs with blue fluorescence expression by direct observation under UV light (Figure 5, column B). We could identify the HGF expression with red fluorescence in both the transplanted cells and the intercellular space in the transplantation area.

Furthermore, we detected ex-HGF-2 expression, which was transferred from HSV-HGF by anti-KT3 staining (Figures 6D to 6F) of the implantation area. As a result, we had identified ex-HGF-2 expression both in the *HGF* gene-transferred MSCs (arrows in Figure 6G) and in the intercellular space of the transplantation area (arrowheads in Figure 6G) only in the MSC-HGF group (Figure 6F) even 14 days after transplantation. Additionally, we confirmed that MSC itself can also secrete HGF *in vivo* (Figure 6B).

Antiapoptosis

Using TUNEL staining (Figure 7, columns B and C), apoptotic cells with red fluorescence were counted in the IBZ 7 days after treatment, while cells were counted in the same area with blue fluorescence by Hoechst 33258 nuclei marking. In this area we could not detect transferred MSCs; therefore, counterstained cells seemed to be host-derived. The percentage of apoptotic host cells was significantly decreased in the MSC-HGF group ($4.92\% \pm 2.15\%$) compared with the PBS group ($22.12\% \pm 4.28\%$, $P<0.01$) and MSC-only group ($10.73\% \pm 5.64\%$, $P<0.01$). However, there was also significant decrease of apoptotic cells between the MSC-only group ($10.73\% \pm 5.64\%$) and the PBS group ($22.12\% \pm 4.28\%$, $P<0.01$) (Figure 7C).

Neuroprotection

Immunohistochemical staining revealed the remaining neurons of the host with MAP-2 neuronal marker 7 days after treatment (Figure 8A). The percentage of remaining neurons in the cortex of IBZ significantly increased in the MSC-HGF group ($20.73\% \pm 2.38\%$) compared with the PBS group ($7.75\% \pm 1.58\%$, $P<0.01$) and the MSC-only group ($12.13\% \pm 3.05\%$, $P<0.01$). Also, the significant

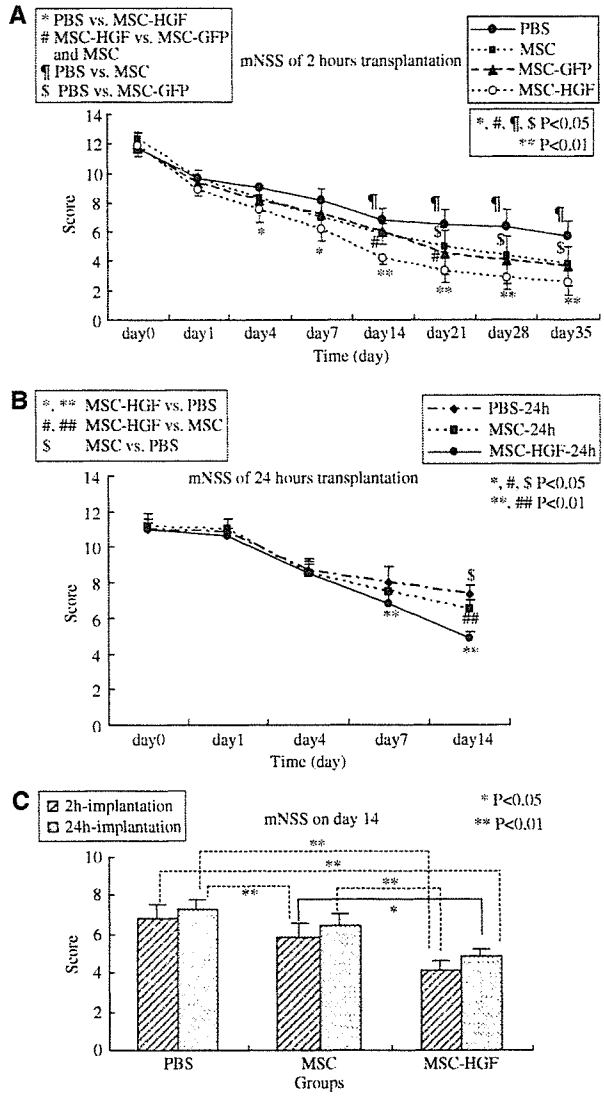

Figure 2 Behavioral functional test (mNSS) before and after MCAO. Groups 1 and 5: treated with PBS; groups 2 and 6: treated with MSC-only; group 3: treated with MSC-GFP; groups 4 and 7: treated with MSC-HGF ($n = 6$ per group). The rats of groups 2 to 7 received 1.0×10^6 cells via intracerebral transplantion in 10 μl PBS. (**A**) Groups 2 to 4 received transplantation 2 h after MCAO (superacute phase); (**B**) groups 4 to 7 received transplantation 24 h after MCAO (acute phase). (**C**) Lists the mNSS on day 14 of groups 1, 2, 4, 5, 6, and 7, showing that the significant neurological recovery among 6 groups while under the comparing condition is only the different therapeutic time phase. Significant functional recovery was detected in the MSC-HGF group compared with the other groups. Data are presented as means $\pm$ s.d.

increase of remaining neurons was found in the MSC-only group (12.13% $\pm$ 3.05%), in comparison with the PBS group (7.75% $\pm$ 1.58%, $P < 0.01$) (Figure 8B).

Discussion

Brain ischemia initiates a cascade of events that produces neuronal death and leads to neurological deficits. To prevent brain injury after ischemia, some studies have focused on cell therapies by using embryonic stem cell. But ethical and logistical problems make it unlikely that such therapy could serve as a source of material for therapeutic transplants. Recently, MSC transplantation was reported as a source of autoplastic therapies which not only improve functional recovery after stroke but also have a low risk of tumorigenesis and do not provoke immune reactions (McIntosh and Bartholomew, 2000; Li *et al*, 2002). In the present study, rats of the MSC-only and MSC-HGF groups also showed more significant neurological functional recovery than those of the PBS group.

It is well known that the efficiency of gene transduction to such MSC populations is low, even with virus vectors such as an adenovirus (Ad) (Conget and Minguell, 2000). To date, Kurozumi *et al* (2004) have reported the relatively high efficiency of gene transduction using fiber mutant Ad vector, but the peak level of expression was transient because the Ad vector would not integrate the gene of interest into the genome of the host cells. Lentivirus could express a high efficiency of gene transduction into MSC, but its biosafety remains uncertain because of its origin, the human immunodeficiency virus (Trono, 2000). Retroviruses, which have the ability to integrate the gene of interest into the chromosomes of the host cells, also show a relatively high efficiency of gene transduction to MSC. However, a note of warning was stressed against the potential rise of a neoplasm with a retrovirus-based vector (Pages and Bru, 2004).

In the present study, by the *in vitro* HGF ELISA data and histological detection, we showed that our HSV-1 vector had successfully transferred the gene of interest to the MSC population with high efficiency *in vitro*, and gene-transferred MSCs had successfully functioned *in vivo* to express and maintain a high level of the gene of interest. We confirmed that the increased HGF expression on day 14 was primarily due to the ex-HGF-2 expression that was proven by anti-KT3 staining, as the HSV-1 vector-transferred HGF cDNA was tagged with KT3 epitope. Also, such ex-HGF-2 protein was produced within the *HGF* gene-transferred MSCs and secreted in the intercellular space diffusely in the combined therapy group.

Furthermore, there were no significant differences in functional recovery between the MSC-only group and the MSC-GFP group during the whole detection time course. Also, no obvious difference of apoptosis and the dividing ability was observed between naive MSCs and the *HGF* gene-transferred MSCs in the current study in the first 2 weeks after transplantation (data not shown). It may indicate that gene transfer with HSV-1 vector *ex vivo* would

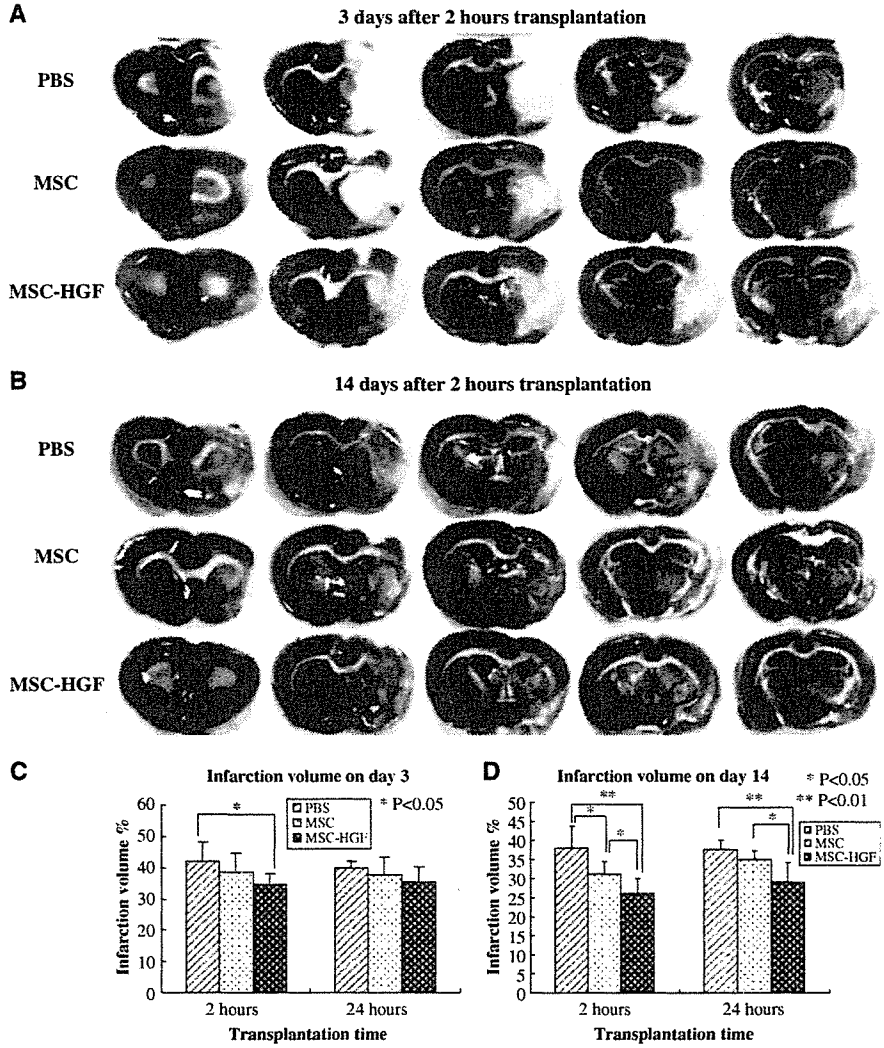

Figure 3 Infarction volume detected by TTC staining. (**A**, **B**) Reduction of infarction areas on days 3 and 14 of groups 1, 2, and 4, which received transplantation 2 h after MCAO occurred: coronal sections stained with TTC. The red region shows intact area; white region shows infarction area. (**C**, **D**) Individually presents the quantification of % CIV in the hemispheric lesion area on days 3 and 14, while being treated at 2 and 24 h after ischemia occurred. Data are presented as means ± s.d. ($P < 0.05$; < 0.01). $n = 6$ for groups 1, 2, 4, and $n = 5$ for groups 5 to 7 at each time point.

not influence the survival and dividing abilities and the therapeutic efficiency of MSCs after transplantation.

So far, to reduce the disability resulting from stroke, some studies have focused on the development of neuroprotective agents such as brain-derived neurotrophic factor, the fibroblast growth factor that effectively prevents delayed neuronal death after transient brain ischemia (Kurozumi *et al*, 2004; Watanabe *et al*, 2004). Recently, overexpression of HGF that can improve the neurological sequalae by neuroprotection, reduce the infarct volume, and the likelihood of brain edema after stroke was reported (Miyazawa *et al*, 1998; Tsuzuki *et al*, 2000; Hayashi *et al*, 2001; Shimamura *et al*, 2004). It suggested that HGF should be one of the most potent growth factors for treating brain ischemia.

To detect the therapeutic efficiency of combined therapy, we tried to treat brain ischemia in the superacute and acute therapeutic phases. Both of them showed significant improvement of neurological deficits compared with MSC-only cell therapy. We got the same result as that Shimamura *et al* (2004) had reported, that HGF had the therapeutic efficiency of reducing the infarction volume after transient MCAO. We also found on day 14 that the MSC-only treated group could significantly reduce the infarction volume under the superacute treatment compared with the PBS-treated group, but not

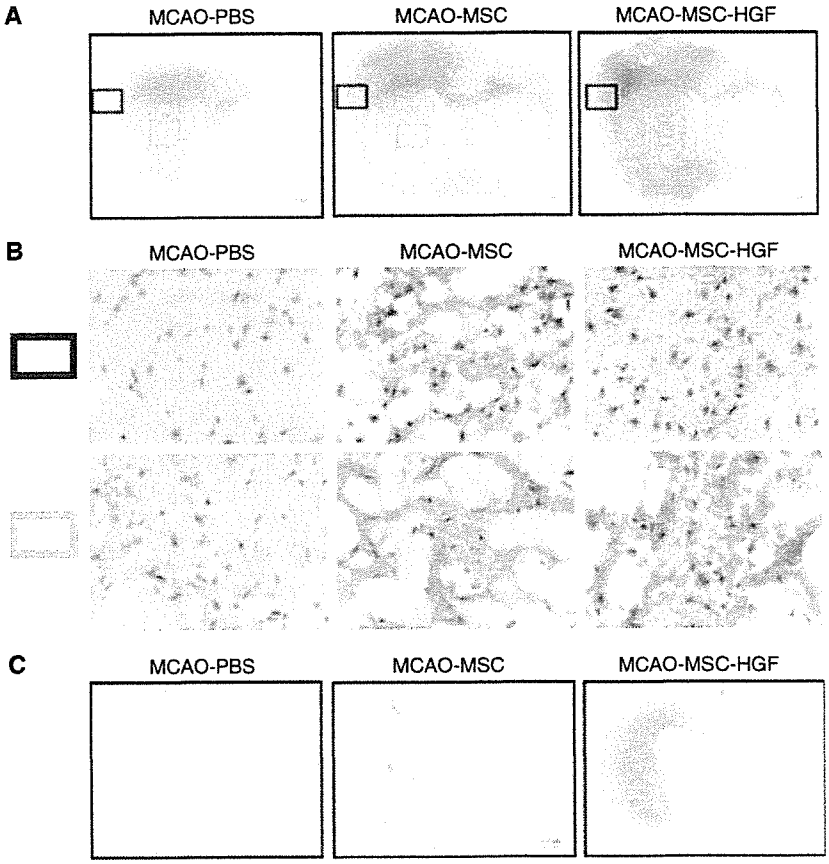

Figure 4 Immunohistochemistry for HGF expression *in vivo*. Mixed HGF expression (brown color) in the ischemic brains of groups 1, 2, and 4 detected by immunohistochemistry on days 2 (row A) and 14 (row C). Scale bar: 1.0 mm. The upper and lower rows identified in B (original magnification, × 400) show the cortex and basal ganglia, respectively, of the images shown in row A.

under the acute treatment. This was the same as the Chopp's group had reported, that transplanted MSCs to the transient MCAO model 24 h after ischemia occurred had improved neurological function recovery, but not significantly decreased infarction area (Chen *et al*, 2000, 2001; Li *et al*, 2000, 2001), while our data of the superacute treatment showed the contrast result. We thought that it might have been caused by the different therapeutic time window. Furthermore, we found significant neurological recovery of the rats treated with combined therapy on day 14, 24 h after MCAO occurred (acuter phase), than the rats of the MSC-only group treated even 2 h after MCAO occurred (superacute phase). It indicates that our combined therapeutic method may extend the therapeutic time window for treating brain ischemia at least until 24 h after the onset of MCAO, while compared with the MSC-only cell therapy. To treat transient ischemia, both the combined therapeutic method and superacute therapeutic time window might be important.

Mesenchymal stromal cell-only therapy also showed significant improvement of functional outcome and decrease of infarction volume when cells were administered 2 h after stroke. A more likely mediator of short-term benefit may reflect increased production of growth factors, including neurotrophins adjusted to the needs of the compromised tissue with an array of reducing host cells' apoptosis in the IBZ, including neurons, and promoting functional recovery of the remaining neurons (David and Thomas, 2002; Chopp and Li, 2002). After stroke, cerebral tissue reverts to an earlier stage of development and thus becomes highly responsive to stimulation by cytokines, trophins, and growth factors from the invading MSCs (Chopp and Li, 2002). The MSCs may simply provide the resources required by the ontogenous cerebral tissue to stimulate cerebral remodeling.

In the present study, the combined therapy group showed more therapeutic benefit than the MSC-only cell therapy. Hepatocyte growth factor gene-modified MSCs may also behave as small molecular factories, secrete an array of cytokines and trophic factors over an extended period and not in a single bolus dose, directly involved in promoting plasticity of the ischemic damaged neurons or in stimulating

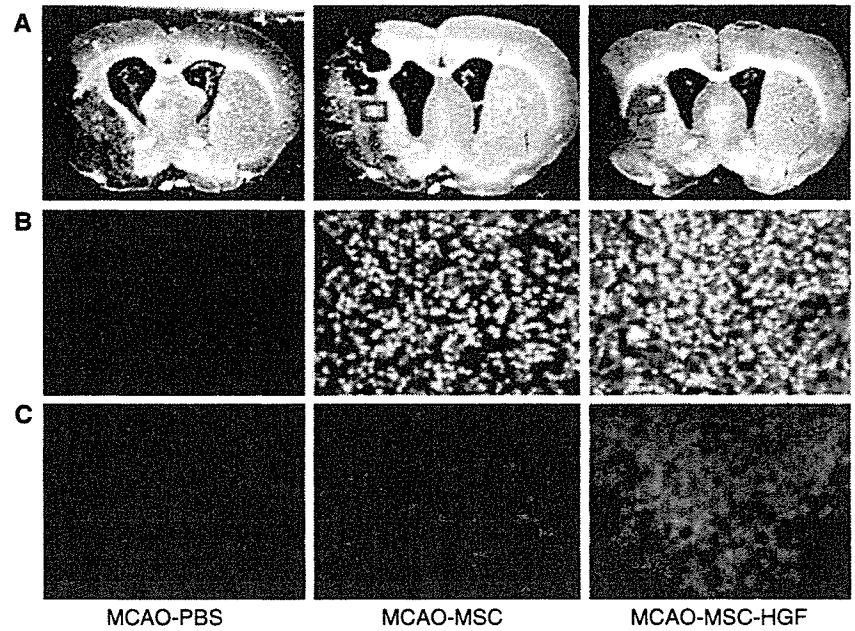

Figure 5 Expression of HGF and identification of transplanted MSCs. Photographs in row C show mixed HGF expression in the ipsilateral brain of groups 1, 2, and 4 with red fluorescence, and photographs in row B show transplanted donor MSCs of groups 2 and 4 with blue fluorescence, at 2 weeks after treatment. The microphotographs shown in rows B and C have the same size and higher power magnification than the blue squares in row A. Original magnification, × 200.

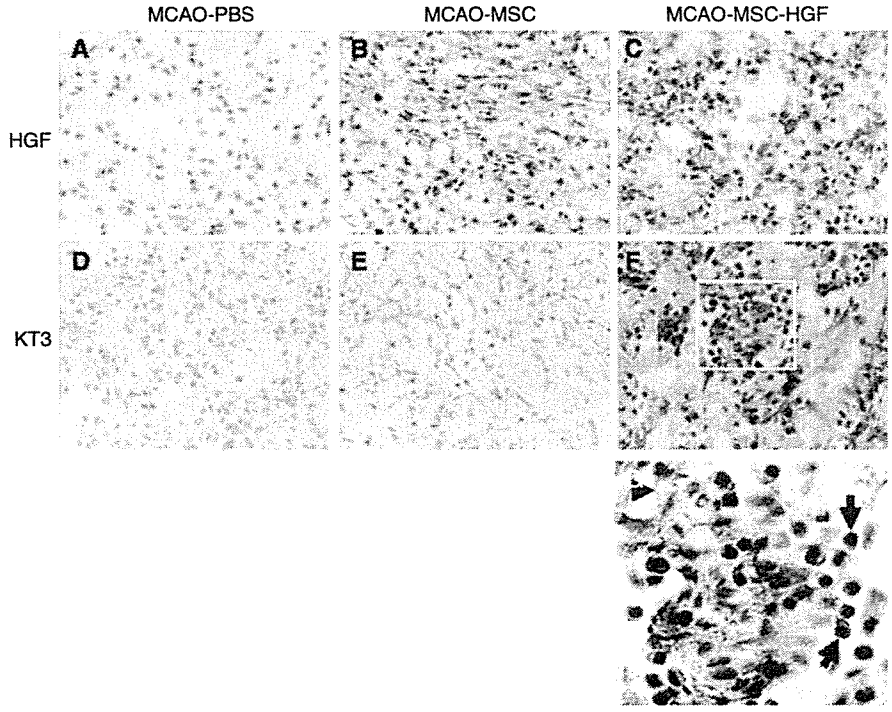

Figure 6 Immunohistochemistry for HSV-1 vector-transferred exogenous HGF and mixed HGF expression. The upper column (**A–C**) shows mixed HGF expression in groups 1, 2, and 4 with anti-rat HGF immunostaining, and the lower column (**D–F**) shows ex-HGF-2 expression with anti-ratHGFKT3 immunostaining at 2 weeks after treatment. Original magnification, × 200. (**G**) is the enlarged white square in (**F**), arrows mark HGF expression in the transplanted MSCs and an arrowhead marks HGF expression in the intracellular space.

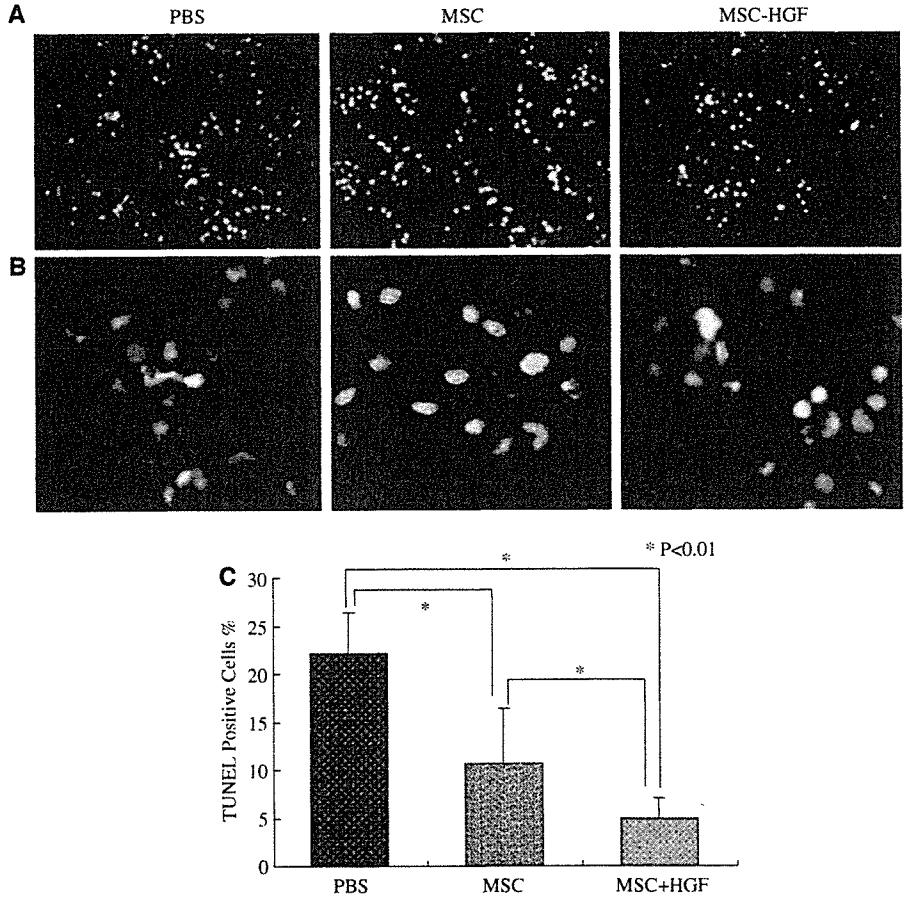

Figure 7 Apoptotic cells in the IBZ with TUNEL staining. (**A**) Column, fewer TUNEL-positive cells were detected in rats treated with MSC-HGF than others treated either with PBS or MSC-only (Rhodamine, red, TUNEL positive; Hoechst, blue, nuclear; original magnification with × 40 object). (**B**) Column, 4 times enlarged magnification of × 40 object. (**C**) The percentage of TUNEL-positive cells in the IBZ was significantly reduced in the MSC-HGF group compared with the other groups 7 days after treatment.

glial cells to secrete neurophins. Marrow stromal cells secrete many cytokines known to play a role in hematopoiesis (Dormady *et al*, 2001), and also supply autocrine, paracrine, and juxtacrine factors that influence the cells of the marrow microenvironment themselves (Haynesworth *et al*, 1996). The interaction of MSCs with the host brain may lead MSCs and parenchymal cells also to produce abundant trophic factors, which may contribute to recovery of function lost as a result of a lesion too (Williams *et al*, 1986).We speculate that *HGF* gene-modified MSCs also had carried out such ways not only to produce extended and abundant exogenous HGF, but also a variety of other cytokines and trophic factors, and interact with each other in an anatomically distributed, tissue-sensitive, and temporally ongoing way.

Other functions of HGF include reducing the BBB destruction without exacerbating cerebral edema, decreasing intracranial pressure, inducing angiogenesis, and interacting with other kinds of neurotrophic factors; cytokines that are secreted by MSCs themselves may also take part in improving the neurological recovery after stroke. We also speculate that the various cytokines secreted from MSCs or MSC-HGF activate the proliferation and differentiation of endogenous neural stem and progenitor cells in the subventricular zone, such as Chopp and Li (2002) had reported. Also, transplanted MSCs themselves might differentiate into some kinds of central never system cells (Woodbury *et al*, 2000). Actually, we also found some MSCs expressing glial phenotype 4 weeks after transplantation only in the combined therapy group (data not shown), which might suggest that *HGF* gene transduction could influence transplanted MSC differentiation. But tissue regeneration might be another part of the mechanisms that induced recovery after stroke mainly occurs in the chronic therapeutic time course.

Anyhow, our MSC-HGF combined therapy enhanced the therapeutic efficiency than the MSC-only

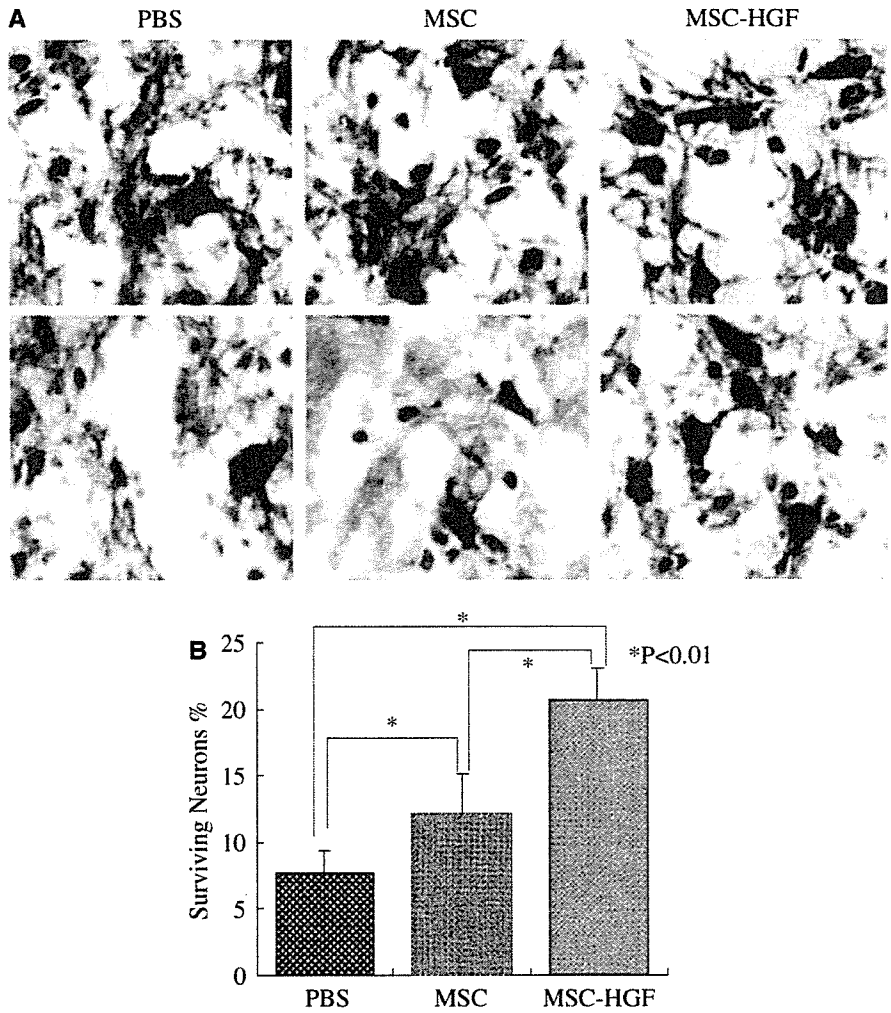

Figure 8 Remaining neurons in the cortex of IBZ with MAP-2 immunostaining. (**A**) Column, more neurons could be detected in rats treated with MSC-HGF than others treated either with PBS or MSC-only (MAP-2, dark brown, neurons; hematoxylin, blue, nuclear; original magnification, × 600). (**B**) The percentage of neurons in the cortex of IBZ was significantly increased in the MSC-HGF group compared with the other groups 7 days after treatment.

cell therapy for stroke in rats treated in both the superacute and acute phases. The target gene was successfully transferred to MSCs with the HSV-1 virus vector *in vitro*, and later the gene-modified MSCs served as both a therapeutic material and a vector platform that continuously carried the target gene into the brain and functioned *in vivo*. This method might be safer than direct gene transfer with viral vectors for *in vivo* treatments, more therapeutically efficient than MSC-only cell therapy, extend the therapeutic time window from superacute to at least 24 h after ischemia happened, and also could be used as a post-treatment method for stroke. Although the best therapeutic time schedule, the administration route of MSCs and best cytokine gene (or cocktail of the genes) should be explored for better clinical application. It may require a broad array of treatments to prevent neurological disorders in brain ischemia, which may offer a promise for human clinical treatment in future.

References

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—similarities to astrocyte grafts. *Proc Natl Acad Sci USA* 95:3908–13

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM (1986) Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* 17:1304–8

Caplan AI, Bruder SP (2001) Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* 7:259–64

Chen J, Li Y, Chopp M (2000) Intracerebral transplantation of bone marrow with BDNF after MCAO in rat. *Neuropharmacology* 39:711–6

Chen JL, Li Y, Wang L, Zhang ZG, Lu DY, Lu M *et al* (2001) Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 32:1005–11

Chen X, Katakowski M, Li Y, Lu D, Wang L, Zhang L *et al* (2002*a*) Human bone marrow stromal cell cultures conditioned by traumatic brain tissue extracts: growth factor production. *J Neurosci Res* 69:687–91

Chen X, Li Y, Wang L, Katakowski M, Zhang L, Chen J *et al* (2002*b*) Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology* 22:275–9

Chopp M, Li Y (2002) Treatment of neural injury with marrow stromal cells. *Lancet Neurol* 1:92–100

Chopp M, Zhang XH, Li Y, Wang L, Chen J, Lu D *et al* (2000) Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. *Neuroreport* 11:3001–5

Coffin RS, Thomas SK, Thomas DP, Latchman DS (1998) The herpes simplex virus 2 kb latency associated transcript (LAT) leader sequence allows efficient expression of downstream proteins which is enhanced in neuronal cells: possible function of LAT ORFs. *J Virol* 79:3019–26

Conget PA, Minguell JJ (2000) Adenoviral-mediated gene transfer into *ex vivo* expanded human bone marrow mesenchymal progenitor cells. *Exp Hematol* 28:382–90

Damme AV, Driessche TV, Collen D, Chuah MKL (2002) Bone marrow stromal cells as targets for gene therapy. *Curr Gene Ther* 2:195–209

David AR, Thomas AK (2002) Using bone marrow stromal cells for treatment of stroke. *Neurology* 59:486–7

Dormady SP, Bashayan O, Dougherty R, Zhang XM, Basch RS (2001) Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. *J Hematother Stem Cell Res* 10:125–40

Friedenstein AJ, Ivanov-Smolenski AA, Chajlakjan RK, Gorskays UF, Kuralesova AI, Latzinik NW *et al* (1978) Origin of bone marrow stromal mechanocytes in radiochimeras and heterotopic transplants. *Exp Hematol* 6:440–4

Hayashi K, Morishita R, Nakagami H, Yoshimura S, Hara A, Matsumoto K *et al* (2001) Gene therapy for preventing neuronal death using hepatocyte growth factor: *in vivo* gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. *Gene Therapy* 8:1167–73

Haynesworth SE, Baber MA, Caplan AI (1996) Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: effects of dexamethasone and IL-1 alpha. *J Cell Physiol* 166:585–92

Jin H, Yang R, Li W, Ogasawara AK, Schwall R, Eberhard DA *et al* (2003) Early treatment with hepatocyte growth factor improves cardiac function in experimental heart failure induced by myocardial infarction. *J Pharmacol Exp Ther* 304:654–60

Kim BJ, Seo JH, Bubien JK, Oh YS (2002) Differentiation of adult bone marrow stem cells into neuroprogenitor cells *in vitro*. *Neuroreport* 13:1185–8

Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Ishii K, Kobune M *et al* (2005) Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Mol Ther* 11:96–104

Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Kobune M, Hirai S *et al* (2004) BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. *Mol Ther* 9:189–97

Li Y, Chen J, Chen XG, Wang L, Gautam SC, Xu YX *et al* (2002) Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology* 59:514–23

Li Y, Chen J, Wang L, Lu M, Chopp M (2001) Treatment of stoke in rat with intracarotid administration of marrow stromal cells. *Neurology* 56:1666–72

Li Y, Chopp M, Chen J, Wang L, Gautam SC, Xu YX *et al* (2000) Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. *J Cerebr Blood Flow Metab* 20:1311–9

Lilley CE, Groutsi F, Han Z, Palmer JA, Anderson PN, Latchman DS *et al* (2001) Multiple immediate-early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in culture and widespread gene delivery to the central nervous system *in vivo*. *J Virol* 75:4343–56

Longa EZ, Weinstein PR, Carlson S, Cummins R (1988) Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20:84–91

Matsumoto K, Nakamura T (1996) Emerging multipotent aspects of hepatocyte growth factor. *J Biochem (Tokyo)* 119:591–600

Matsumoto K, Nakamura T (1997) Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochem Biophys Res Commun* 239: 639–44

McIntosh K, Bartholomew A (2000) Stromal cell modulation of the immune system. *Graft* 3:324–8

Miyazawa T, Matsumoto K, Ohmichi H, Katoh H, Yamashima T, Nakamura T (1998) Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: a novel neurotrophic factor. *J Cereb Blood Flow Metab* 18:345–8

Nakamura T, Nawa K, Ichihara A (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 122:1450–9

Nakano K, Migita M, Mochizuki H, Shimada T (2001) Differentiation of transplanted bone marrow cells in the adult mouse brain. *Transplantation* 71:1735–40

Pages JC, Bru T (2004) Toolbox for retrovectorologists. *J Gene Med* 6(Suppl 1):S67–82

Palmer JA, Branston RH, Lilley CE, Robinson MJ, Groutsi F, Smith J *et al* (2000) Development and optimization of herpes simplex virus vectors for multiple long-term gene delivery to the peripheral nervous system. *J Virol* 74:5604–18

Paxinos G, Watson C, Pennisi M, Topple A (1985) Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. *J Neurosci Methods* 13:139–43

Phinney DG (2002) Building a consensus regarding the nature and origin of mesenchymal stem cells. *J Cell Biochem* 38:7–12

Raymond AS, Matthew TM, George TW, Robert AS, Charisse D, Frank RS (1990) A semiautomated method for measuring brain infarct volume. *J Cerebr Blood Flow Metab* 10:290–3

Rempe DA, Kent TA (2002) Using bone marrow stromal cells for treatment of stroke. *Neurology* 59:486–7

Schallert T, Kozlowski DA, Humm JL, Cocke RR (1997) Use-dependent structural events in recovery of function. *Adv Neurol* 73:229–38

Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S *et al* (2004) Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. *J Circ* 109:424–31

Sun W, Funakoshi H, Nakamura T (2002*a*) Localization and functional role of hepatocyte growth factor (HGF) and its receptor c-met in the rat developing cerebral cortex. *Mol Brain Res* 103:36–48

Sun W, Funakoshi H, Nakamura T (2002*b*) Overexpression of HGF retards disease progression and prolongs life span in a transgenic mouse model of ALS. *J Neurosci* 22:6537–48

Trono D (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Therapy* 7:20–3

Tsuzuki N, Miyazawa T, Matsumoto K, Nakamura T, Shima K, Chigasaki H (2000) Hepatocyte growth factor reduces infarct volume after transient focal cerebral ischemia in rats. *Acta Neurochir Suppl* 76:311–6

Watanabe T, Okuda Y, Nonoguchi N, Zhao MZ, Kajimoto Y, Furutama D *et al* (2004) Post-ischemic intraventricular administration of FGF-2 expressing adenoviral vectors improves outcome and reduces infarct volume after transient focal cerebral ischemia in rats. *J Cerebr Blood Flow Metab* 24:1205–13

Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, Bjorklund A *et al* (1986) Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transaction. *Proc Natl Acad Sci USA* 83:9231–5

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 61:364–70

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
European Journal of Pharmacology 535 (2006) 114–124
ejp
www.elsevier.com/locate/ejphar

Effects of hepatocyte growth factor on phosphorylation of extracellular signal-regulated kinase and hippocampal cell death in rats with transient forebrain ischemia

Makiko Niimura [a], Norio Takagi [a], Keiko Takagi [a], Hiroshi Funakoshi [b], Toshikazu Nakamura [b], Satoshi Takeo [a,*]

[a] *Department of Molecular and Cellular Pharmacology, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*
[b] *Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, 2-2-B7 Yamadaoka, Suita, Osaka 565-0871, Japan*

Received 6 October 2005; received in revised form 12 January 2006; accepted 20 January 2006
Available online 3 March 2006

Abstract

Hepatocyte growth factor (HGF) has been implicated in protection against several types of cell injuries. We investigated the effects of human recombinant HGF (hrHGF) on the selective neuronal cell death in the hippocampal CA1 region after transient forebrain ischemia in rats and explored the nature of the intracellular signaling pathway for the protection against this neuronal injury. hrHGF was injected continuously into the hippocampal CA1 region directly using an osmotic pump from 10 min to 72 h after the start of reperfusion. The marked increase in the number of TUNEL-positive cells found in the CA1 region after ischemia was almost completely abolished by the hrHGF treatment. Akt phosphorylation as well as IκB phosphorylation, which has been implicated in events downstream of the Akt, was not affected by hrHGF treatment. Extracellular signal-regulated kinase (ERK) phosphorylation was decreased in the CA1 region with time after ischemia. hrHGF increased or recovered ERK phosphorylation without changing the total amount of ERK protein. Immunohistochemical analysis demonstrated that phosphorylated ERK was colocalized with a neuronal nucleus marker NeuN in the hippocampal CA1 region of ischemic rats with hrHGF treatment at the early period after reperfusion. These results suggest that the protective effects of hrHGF against neuronal death in the hippocampal CA1 after transient forebrain ischemia could be related to an ERK-dependent pathway.

Keywords: Cerebral ischemia; Hippocampus; Hepatocyte growth factor; Extracellular signal-regulated kinase

1. Introduction

Cerebral ischemia, a pathological condition in which brain tissue experiences a shortage of glucose and oxygen, is associated with cerebrovascular disease, brain trauma, epilepsy, and cardiac arrest. Many biochemical changes, which may lead to neuronal cell death, thereby induce dysfunction of the central nervous system. Several neurotrophic factors have been challenged to protect cerebral cells from ischemic injury or to restore the neuronal function. The hepatocyte growth factor (HGF) was purified and cloned as a potent mitogen for hepatocytes (Nakamura et al., 1984, 1989). The physiological activities of HGF as an organotrophic factor for regeneration and protection were demonstrated in various organs (Zarnegar and Michalopoulos, 1995; Matsumoto and Nakamura, 1996, 2001; Balkovetz and Lipschutz, 1999). Furthermore, HGF has been shown to exert motogenic, morphogenic, angiogenic, and anti-apoptotic activities in various types of cells (Nakamura et al., 1984, 1989; Matsumoto and Nakamura, 1996). These findings suggest that HGF has an ability to exert the multipotent activities under pathophysiological conditions. Such multipotent activities of HGF can be mediated by its binding to a transmembrane tyrosine kinase receptor c-Met (Bottaro et al., 1991; Higuchi and Nakamura, 1991). Recent studies showed that HGF and c-Met

* Corresponding author. Faculty of Pharmaceutical Sciences, Department of Pharmacology, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Tel.: +81 426 76 4584; fax: +81 426 76 4584.
E-mail address: takeos@ps.toyaku.ac.jp (S. Takeo).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.01.037

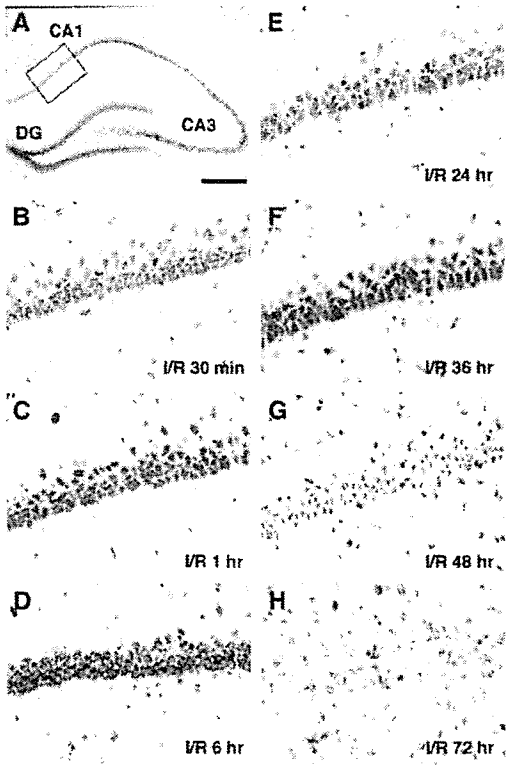

Fig. 1. Representative photomicrographs of cresyl violet-stained hippocampus and hippocampal CA1 region. (Panel A) Photomicrograph of cresyl violet-stained hippocampus of non-operated naïve rat. (Panels B and H) Photomicrographs of cresyl violet-stained hippocampal CA1 region of four-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 30 min (B), 1 (C), 6 (D), 24 (E), 36 (F), 48 (G), and 72 (H) h of reperfusion. Scale bar represents 500 μm in (A), and 50 μm in (B–H). DG, dentate gyrus.

were expressed in various regions of the brain (Honda et al., 1995; Achim et al., 1997) and that HGF enhanced the survival of hippocampal and cortical neurons in vitro (Honda et al., 1995; Hamanoue et al., 1996; Machide et al., 1998). In addition, the administration of human recombinant HGF (hrHGF) was shown to protect hippocampal neurons from transient global ischemia and to reduce infarct size after transient focal cerebral ischemia (Miyazawa et al., 1998; Tsuzuki et al., 2001), suggesting that HGF has the ability to prevent ischemic brain injuries. However, the intracellular signaling pathways for these protective effects of HGF on the brain remain to be determined. It has been well demonstrated that the hippocampus is one of the brain regions most vulnerable to ischemia. With respect to this, transient forebrain ischemia leads to the selective degeneration of vulnerable neurons in the hippocampal CA1 region. It is thus an important objective to explore the nature of the intracellular signal transduction pathways operating via c-Met and underlying the protective effects of HGF against ischemic brain injuries.

In the present study, we investigated the effects of hrHGF on the selective neuronal cell death in the hippocampal CA1 region after transient forebrain ischemia and also explored the intracellular signaling pathways triggered by hrHGF treatment under in vivo pathophysiological conditions. Our findings demonstrate that treatment with hrHGF prevents neuronal cell damage, which may be related to an ERK-dependent pathway.

2. Materials and methods

2.1. Recombinant HGF

Human recombinant HGF (hrHGF) was purified from culture media of Chinese hamster ovary cells transfected with

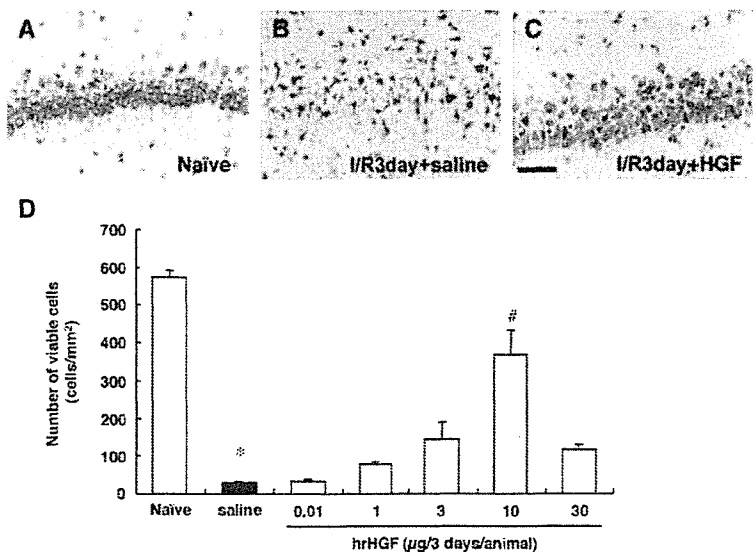

Fig. 2. Effects of hrHGF on neuronal cell death in the hippocampal CA1 region of the 4 vessel-occluded rat after 15 min of transient forebrain ischemia followed by 72 h of reperfusion. (Panels A–C) Photomicrographs of cresyl violet-stained hippocampal CA1 region from non-operated naïve rat (A), 4-vessel-occluded rat without hrHGF (B), and 4-vessel-occluded rat with 10 μg/3 days/animal of hrHGF (C). Scale bar represents 50 μm. (Panel D) Effects of hrHGF (0.01–30 μg/3 days/animal) on the number of neurons in the hippocampal CA1 region of the 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 72 h of reperfusion. The number of viable cells in the hippocampal CA1 region was counted. Values represent the means±S.E.M. ($n=3$). * indicates a significant difference from the naïve rats ($P<0.05$). # indicates a significant difference from the vehicle (saline)-treated ischemic rats ($P<0.05$).

an expression vector containing human HGF cDNA as described (Nakamura et al., 1989). The purity of hrHGF was >98%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2. Animal model

Male Wistar rats weighing 200–250 g (Charles River Japan Inc., Atsugi, Japan) were used in the present study. The animals were housed in a cage and maintained on a 12-h light/12-h dark cycle at a temperature of 23±1 °C with a humidity of 55±5% throughout the experiment. The animals had free access to food and water according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guideline of Experimental Animal Care issued by the Prime Minister's Office of Japan. All efforts were made to minimize the animals' suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Science. Transient (15 min) forebrain ischemia was produced by the 4-vessel occlusion procedure for rats previously described (Takagi et al., 2003). In brief, rats were anesthetized intraperitoneally with 40 mg/kg sodium pentobarbital. The right and left second cervical vertebras were exposed, and both visible vertebral arteries were permanently electrocauterized. Two silk threads were placed around both common carotid arteries without interrupting the blood flow. Twenty-four hours after electrocauterization, anesthesia was induced with 3% enflurane and maintained with 1.5% enflurane in a mixture of oxygen/nitrous oxide (25/75%). Bitemporal subdermal electroencephalogram (EEG) needle electrodes were placed in reference to a frontal subdermal electrode. After a baseline EEG level had been established, both common carotid arteries were exposed and occluded with aneurysm clips for 15 min, after which time the clips were removed and the rat allowed to recover. Rectal temperature was continuously monitored during ischemia and was maintained at 37.0 to 37.5 °C with a heating pad. During the carotid artery occlusion, anesthesia was removed, and only rats that showed a completely flat EEG, a dilated pupil, and a loss of righting reflex were chosen for use in the present study. Sham-operated animals received exactly the same surgical procedure, but without the arterial occlusion. Each set of animals received the same degree of surgical preparation and the same recovery paradigms to minimize variations that could result from surgical procedures.

2.3. HGF treatment in vivo

hrHGF was diluted in a physiological saline and infused into the right hippocampal CA1 region using an osmotic pump (Alzet model 1003D; DURECT Corp., Cupertino, CA, USA) attached to a 30-gauge needle using polyvinylchloride tube. To start infusion just after needle implantation, each osmotic pump was pre-incubated in physiological saline at 37 °C according to the instructions for use of the Alzet. A 30-gauge needle connected to osmotic pump was implanted at 3.5 mm posterior and 2.5 mm lateral to the bregma, and with a depth of 2.4 mm from the cortical surface and secured to the skull with dental cements and stainless steel screws at 10 min after the start of reperfusion. The connecting tube and osmotic pump were placed subcutaneously in the neck and shoulder area. The infusion of hrHGF was begun at 10 min after the start of reperfusion at a flow rate of 1.0 μl/h with a concentration of 0.01–30 μg/3 days/animal. As a control, physiological saline was infused using osmotic pump.

2.4. Tissue preparation

At various times after the start of reperfusion, animals were sacrificed by decapitation, and their heads were quickly near-frozen in liquid nitrogen. The hippocampi were removed on ice, and hippocampal slices (730 μm) were prepared with a McIlwain tissue chopper (Brinkmann, Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). The hippocampal CA1 regions were dissected on ice in ice-cold 125 mM Tris–HCl, pH 7.4, containing 320 mM sucrose, 2 mM sodium orthovanadate, 20 mM sodium diphosphate decahydrate, 20 mM DL-α-glycerophosphate, 0.1 mM phenylmethylsulfonyl

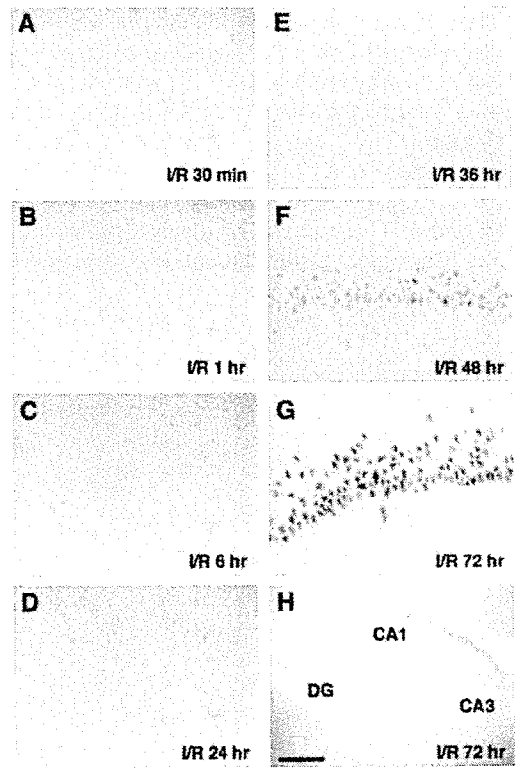

Fig. 3. Representative photomicrographs of TUNEL-stained hippocampal CA1 region and hippocampus of 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by reperfusion. (Panels A–G) Photomicrograph of TUNEL-stained hippocampal CA1 region of four-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 30 min (A), 1 (B), 6 (C), 24 (D), 36 (E), 48 (F), and 72 (G) h of reperfusion. (Panel H) Photomicrographs of TUNEL-stained hippocampus of 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 72 h of reperfusion. Scale bar represents 50 μm in (A–G) and 500 μm in (H). DG, dentate gyrus.

fluoride, and 5 μg/ml each of antipain, aprotinin, and leupeptin (homogenization buffer). The dissected CA1 region was homogenized in the ice-cold homogenization buffer. The samples were stored at −80 °C until used and were thawed only once.

2.5. Immunoblotting

Hippocampal CA1 homogenates that had been solubilized by boiling for 5 min in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, and 2% SDS, in 62.5 mM Tris–HCl, pH 6.8) were separated on 8.5% or 10% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Protein blots were incubated with the appropriate antibodies, and the bound antibody was detected by the enhanced chemiluminescence method (Amersham Biosciences Inc., Piscataway, NJ, USA) as described by the manufacturer. Quantification was performed using computerized densitometry and an image analyzer (ATTO Co., Tokyo, Japan). Care was taken to ensure that bands to be semiquantified were in the linear range of response. For removal of bound antibodies, immunoblots were heated for 30 min at 65 °C in 62.5 mM Tris–HCl buffer, pH 6.8, containing 2% SDS and 0.1 M β-mercaptoethanol. The efficacy of the stripping procedure was confirmed by reacting the stripped blot with secondary antibody alone to ensure that no bound antibodies had remained. Antibodies used were anti-phospho-p44/42 mitogen-activated protein (MAP) kinase (Thr^{202}/Tyr^{204}), anti-p44/42 MAP kinase, anti-phospho-Akt (Ser^{473}), anti-Akt, anti-phospho-IκB-α (Ser^{32}) (Cell Signaling Technology, Inc., Beverly, MA), anti-c-Met, anti-IκB-α, anti-Bcl-x_L (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-Met (Y1234, Y1235), and anti-phosphotyrosine (clone 4G10, Upstate Biotechnology, Inc., Lake Placid, NY).

2.6. Immunoprecipitation

For immunoprecipitation of c-Met, hippocampal CA1 tissues were lysed in a buffer comprising 10 mM Tris–HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 2 mM sodium orthovanadate, 0.1 mM PMSF, 5 μg/ml each of antipain, aprotinin, and leupeptin. Lysates were preincubated for 1 h with protein G-agarose beads and then centrifuged to remove any proteins that adhered nonspecifically to the protein G-agarose beads. The supernatant was then incubated with anti-c-Met antibody for 2 h or overnight at 4 °C. Next, protein G-agarose beads were added, and the incubation was continued at 4 °C for 2 h. The immune complexes were isolated by centrifugation and washed, and the bound proteins were eluted by boiling in SDS sample buffer.

2.7. Histological analysis

Animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia. Their brains were quickly removed, cut into approximately 5-mm-thick coronal slabs, and post-fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Coronal slabs were embedded in paraffin and cut serially at 7 μm with a microtome. The coronal sections were then stained with cresyl violet acetate to assess neuronal damage. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were detected by using an in situ Apoptosis Detection Kit (MK500; Takara Bio Inc., Shiga,

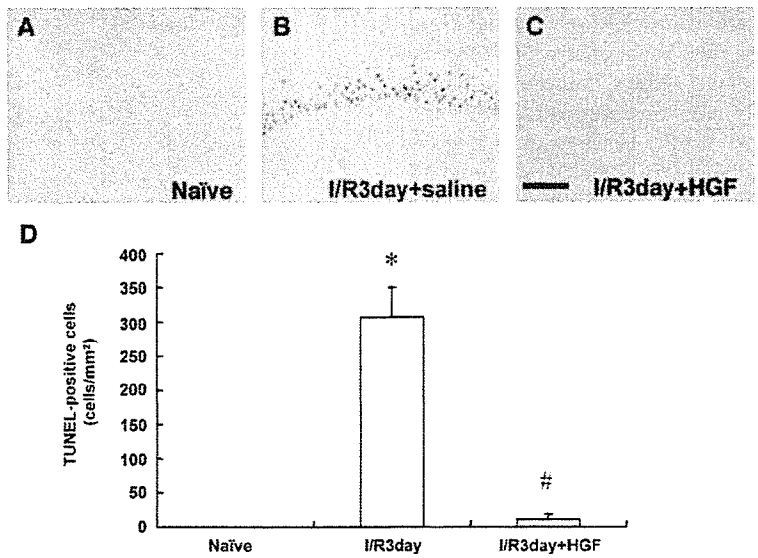

Fig. 4. Effects of hrHGF on apoptotic cells in the hippocampal CA1 region of 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 72 h of reperfusion. (Panels A–C) Photomicrographs of TUNEL-stained hippocampal CA1 region of non-operated naïve rat (A), 4-vessel-occluded rat without hrHGF (B), and 4-vessel-occluded rat with 10 μg/3 days/animal of hrHGF (C). Scale bar represents 50 μm. (Panel D) Effect of hrHGF (10 μg/3 days/animal) on the number of TUNEL-positive cells in the hippocampal CA1 region of the 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 72 h of reperfusion (I/R 3 day). The number of TUNEL-positive cells in the hippocampal CA1 region was counted. Values represent the means±S.E.M. (n=3). * indicates a significant difference from the naïve rats (P<0.05). # indicates a significant difference from the vehicle (saline)-treated ischemic rats (P<0.05).

Japan). Surviving pyramidal cells and TUNEL-positive cells in the hippocampal CA1 region were counted under ×400 magnification (Olympus BX-51) in 5 to 7 sections per animal. Results were expressed as the average number of cells per mm^2 in the areas which consisted of the hippocampal CA1 pyramidal cell layer. For immunohistochemical detection of c-Met protein, sections were incubated with anti-c-Met antibody, and then incubated with biotinylated rabbit anti-goat IgG. They were further incubated in streptavidin–biotin–peroxidase solution (Vector Laboratories, Burlingame, CA, USA). The peroxidase reaction was carried out by incubating with diaminobenzidine and hydrogen peroxidase (Vector Laboratories). For double immunostaining, sections were boiled by microwaves in 10 mM citrate buffer for 5 min to retrieve antigen. After blocking, sections were incubated overnight with rabbit anti-phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}) at 4 °C and then incubated for 1 h with TRITC-conjugated swine anti-rabbit IgG (1:200, DAKO, Carpinteria, CA, USA) at room temperature. Thereafter, sections were incubated with mouse anti-NeuN (Chemicon, Temecula, CA, USA), mouse anti-MAP-2ab (Sigma-Aldrich, St. Louis, MO, U.S.A.), or mouse anti-glial fibrillary acidic protein (GFAP) (1:400, Chemicon) for 2 h at room temperature. These sections were incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (ICN Pharmaceuticals, Inc., Aurora, OH, USA). Fluorescence was detected using an Olympus fluorescence microscopy (BX-52) or a Bio-Rad MRC 1024 confocal imaging system equipped with a krypton–argon laser and Nikon Diapot microscope. Images were processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). The microscopic observations were performed by a person unaware of the study group.

2.8. Statistics

The results were expressed as the means±S.E.M. Statistical comparison among multiple groups was evaluated by analysis of variance followed by Fisher's protected least significant difference test.

3. Results

At first, we examined the in vivo effects of hrHGF treatment on neuronal cell death using a rat model of transient forebrain ischemia. This model induces selective neuronal cell death in the hippocampal CA1 region from day 2 to day 3 (Fig. 1). In vivo treatment with hrHGF dose-dependently prevented neuronal cell death in the hippocampal CA1 region on day 3 (Fig. 2A–D). Treatment with 10 μg/3 days/animal of hrHGF exerted the maximal protective effect (Fig. 2C and D). Based on these results, we used 10 μg/3 days/animal of hrHGF in the subsequent studies. We next counted the number of TUNEL-positive cells in the hippocampal CA1 region. TUNEL-positive cells in the CA1 region appeared from 48 h after the reperfusion and a marked increase in the number of TUNEL-positive cells was detected at 72 h after the reperfusion (Fig. 3). Treatment with hrHGF almost completely

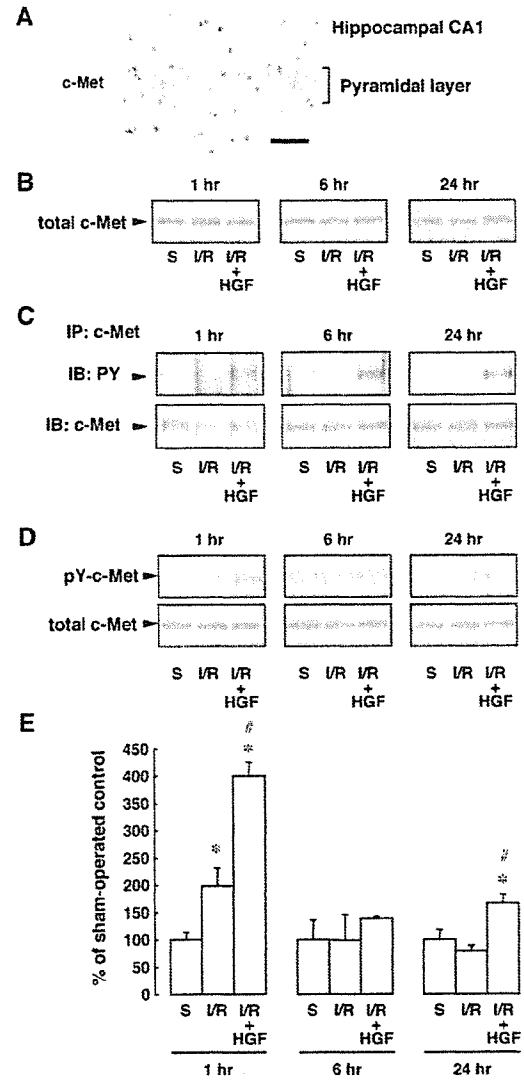

Fig. 5. Effects of hrHGF on tyrosine phosphorylation of c-Met in the hippocampal CA1 region after transient forebrain ischemia. (Panel A) Photomicrograph of immunohistochemical staining of hippocampal CA1 region of non-operated naïve rat. Scale bar represents 50 μm. (Panel B) Proteins (50 μg) from sham-operated (S) and 4-vessel-occluded rats at 1, 6, and 24 h of reperfusion without (I/R) or with hrHGF (I/R+HGF) were separated by SDS-PAGE and subjected to immunoblotting with anti-c-Met antibody. (Panel C) Proteins (500 μg) from sham-operated (S) and 4-vessel-occluded rats at 1, 6, and 24 h of reperfusion without (I/R) or with hrHGF (I/R+HGF) were immunoprecipitated (IP) with anti-c-Met antibody, and the precipitates were analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody (PY). The blots were stripped and re-probed with anti-c-Met antibody. (Panel D) Proteins (50 μg) were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-c-Met (Y1234, Y1235) antibody (pY-c-Met). The blots were then stripped and re-probed with anti-c-Met antibody (total c-Met). (Panel E) Bands corresponding to phospho-c-Met (Y1234, Y1235) were scanned, and the scanned bands were normalized by total c-Met on the same blot. The results are expressed as the mean percentages of values for sham-operated group (S)±S.E.M. (n=3). * indicates a significant difference from sham-operated group (P<0.05). # indicates a significant difference from the hrHGF-untreated ischemic group (I/R) (P<0.05).