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HVJ-envelope vector for gene transfer into central nervous system

Munehisa Shimamura,[a] Ryuichi Morishita,[a,*] Masayuki Endoh,[a] Kazuo Oshima,[a] Motokuni Aoki,[a] Satoshi Waguri,[b] Yasuo Uchiyama,[b] and Yasufumi Kaneda[a]

[a] *Division of Gene Therapy Science, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Osaka, Japan*
[b] *Department of Cell Biology and Neuroscience (A1), Osaka University Graduate School of Medicine, Yamada-oka 2-2, Suita 565-0871, Osaka, Japan*

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Abstract

To overcome some problems of virus vectors, we developed a novel non-viral vector system, the HVJ-envelope vector (HVJ-E). In this study, we investigated the feasibility of gene transfer into the CNS using the HVJ-E both in vitro and in vivo. Using the Venus reporter gene, fluorescence could be detected in cultured rat cerebral cortex neurons and glial cells. In vivo, the reporter gene (Venus) was successfully transfected into the rat brain by direct injection into the thalamus, intraventricular injection, or intrathecal injection, without inducing immunological change. When the vector was injected after transient occlusion of the middle cerebral artery, fluorescence due to EGFP gene or luciferase activity could be detected only in the injured hemisphere. Finally, luciferase activity was markedly enhanced by the addition of 50 U/ml heparin ($P < 0.01$). Development of efficient HVJ-E for gene transfer into the CNS will be useful for research and clinical gene therapy.
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Cerebral occlusive disease caused by atherosclerosis of the cerebral arteries or Moyamoya disease often causes chronic hypoperfusion of the brain. Although such a condition leads not only to cerebral ischemic events, but also to neuropathological changes including dementia [1–4], an effective treatment to improve hypoperfusion has not yet been established. Recently, preclinical studies have demonstrated that angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) can stimulate the development of collateral arteries in animal brain ischemia model [5–7], a concept called therapeutic angiogenesis. As the efficacy of therapeutic angiogenesis using gene transfer of angiogenic growth factors has been reported in human patients with critical limb ischemia or myocardial infarction [8–11], the strategy for therapeutic angiogenesis should be realm for the treatment of patients with cerebral ischemia, if the safe and effective gene transfer methods would be developed for human treatment. From this viewpoint, the current gene transfer techniques may not be ideal. Gene transfer for central nervous system (CNS) could be achieved by various virus vectors including adeno-associated virus (AAV) [12], retrovirus [13], adenovirus [14], and herpes simplex virus 1 [15]. These vector systems have advantages and disadvantages for human gene therapy. Although these methods are efficient for in vivo gene transfer into CNS, numerous problems such as safety and production are yet to be resolved toward human gene therapy.

Previously, we have reported the efficacy of HVJ-liposome method for CNS [16,17] in rats and in primates [18]. HVJ-liposome method utilized the combination of liposome and fusion activities of proteins derived from HVJ envelope [19] for various cells and tissues. However, it takes a long time and is a complexed procedure to make HVJ-liposome vesicle. In addition, the fusion activity of HVJ-liposome is much lower (~2%) than that of native HVJ because the density of fusion proteins of particle surface becomes smaller than that of native HVJ. These issues might affect the usage of HVJ-liposome complex for human gene therapy. To overcome these problems, we have recently developed the second

* Corresponding author. Fax: +81-6-6879-3909.
E-mail address: morishit@gts.med.osaka-u.ac.jp (R. Morishita).

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generation of HVJ vector, so-called HVJ-envelope vector (HVJ-E) [20]. The advantages of HVJ-E vector as compared to those of HVJ-liposome vector are: (1) it is easy to make, (2) the production does not take much time (less than 90 min), and (3) fusion activity of HVJ-E is as much as that of native HVJ. Although it was reported that some reporter genes and oligodeoxynucleotides (ODN) could be transfected into various tissue and cells without a complicated procedure [20], in the present study, we have investigated the possibility of gene transfer into CNS using HVJ-E vector both in vivo and in vitro.

Materials and methods

Preparation of HVJ envelope vector. HVJ envelope vector was prepared as described before [20]. Briefly, the virus suspension (10,000 hemagglutinating unit) was inactivated by UV irradiation (99 mJ/cm^2) and mixed with plasmid DNA (200 μg) and 0.3% Triton-X. After centrifuging, it was washed with 1 ml balanced salt solution (BSS; 10 mM Tris–Cl, pH 7.5, 137 mM NaCl, and 5.4 mM KCl) to remove the detergent and unincorporated DNA. Following centrifuge, the envelope vector was suspended in 100 μl phosphate-buffered saline (PBS). The vector was stored at 4 °C until use. For in vitro study, protamine sulfate (Nakalai tesque, Japan) was added to the culture plate at the rate of 10 μg/well before the treatment with the vector, to enhance the transfection efficiency [20]. In the case of co-injection with heparin (Aventis, Japan), low molecular weight heparin (Fragmin, Kissei, Japan), or argatroban (Slonnon, Daiichi, Japan), they were mixed with PBS and added to the vector.

Plasmid DNA. pEGFP-C1 was purchased from Clontech (CA, USA). pCMV-luciferase-GL3 (pcLuc-GL3: 7.4 kb) was constructed by cloning the luciferase gene from the pGL3-Promoter Vector (Promega, Madison, WI, USA) into pcDNA3 (5.4 kb) (Invitrogen, San Diego, CA, USA) at the *Hin*dIII and *Bam*HI sites. Plasmids were purified with the Qiagen plasmid isolation kit (Hilden, Germany). Venus/pCS2 [21] was provided by Dr. Miyawaki (Laboratory for Cell Function and Dynamics, Advanced Technology Development Center, Brain Science Institute, RIKEN, Japan).

Determination of fluorescence due to Venus. Expression of Venus was examined under a fluorescent stereomicroscope 72 h after injection. More precise images were obtained with a confocal laser microscope (Bio-Rad, Hercules, CA, USA).

Assay for luciferase activity. Rats transfected with luciferase gene were sacrificed under anesthesia at 24 h after transfection. Organs (brain, lung, spleen, liver, kidney, and testis) were harvested and placed individually in FALCON 50 ml tubes. Luciferase activity assay was performed as described previously [22]. Luciferase levels were normalized by determining the protein concentrations of the tissue extracts [22]. Luciferase units were expressed as relative light units (RLU) per gram of tissue protein.

In vitro gene transfer. Rat embryonic cerebral cortex neurons were obtained from pregnant Wistar rats at 19 days' gestation (Charles River Japan, Atsugi, Japan) and cultured [23]. Briefly, the cerebral cortex was dissected and individual cells were isolated by treatment with papain and triturated in Leibovitz's L-15 medium (Invitrogen, CA, USA). Cells were plated on poly-D-lysine-coated 24-well plastic culture dishes with DMEM (Invitrogen)/B-27 (Invitrogen) at 37 °C in a humidified atmosphere of 95% air–5% CO_2. The medium was changed on the first and fourth days. The rate of immuno-positive cells for MAP_2 (microtubule-associated protein) on the seventh day was 92.9%. Before transfection, the medium was changed to fresh 500 μl DMEM/well. HVJ-E vector (250 HAU) containing the Venus gene was added to each well and left for 10 min at 37 °C. After transfection, the medium was changed to fresh DMEM/B-27 and the dishes were incubated at 37 °C. The expression of Venus was observed at 2 days after transfection using laser scanning confocal microscopic images. Transfection efficiency was calculated as (the number of cells expressing Venus/the number of MAP_2 immunoreactive cells) ×100(%). To average the efficiency, five visual fields were randomly selected and the number of cells was counted.

Immunohistochemistry. In vitro cultured cells were fixed with 4% paraformaldehyde at 37 °C for 15 min and treated with 0.5% Triton X-100 for 10 min. The cells were blocked with PBS containing 2% goat serum, bovine serum albumin (5 mg/ml), and glycine (50 mM). Then, the cells were incubated with a mouse monoclonal antibody against MAP_2 (1:1000, Sigma–Aldrich, Saint Louis, MO, USA) or a mouse monoclonal antibody against GFAP (Glial Fibrillary Acidic Protein, 1:1000, Sigma–Aldrich) overnight at 4 °C. After washing with PBS, Alexa Fluor 546-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, Oregon, USA) was applied as a secondary antibody and the dishes were incubated for 1 h at room temperature. The image was analyzed with a confocal laser microscope.

In vivo gene transfer in normal rats. Wistar male rats (270–300 g; Charles River Japan, Atsugi, Japan) were used in this study. All procedures were conducted in accordance with Osaka University guideline. Rats were anesthetized with ketamine (Sankyo, Japan) and placed in a stereotactic frame (Narishige Scientific Instrument Laboratory, Tokyo, Japan), with the skull exposed. A stainless steel canula (30 gauge; Becton–Dickinson, Franklin Lakes, NJ) with a specially designed Teflon connector (FEP tube, Bioanalytical Systems, West Lafayette, IN) was introduced into the thalamus (3.8 mm posterior to the bregma, 2.4 mm lateral to the midline, and 5.0 mm below the skull surface) or lateral ventricle (0.48 mm anterior to the bregma, 0.8 mm lateral to the midline, and 3.8 mm below the skull surface). The HVJ-E vector containing the Venus gene was injected at a speed of 1.0 μl/min. The total volume was 5.0 μl in the case of direct injection into thalamus and 20 μl in the case of injection into lateral ventricle. After infusion, the infusion cannula was removed. No behavioral change such as convulsion or abnormal movement of extremities was observed in any animal.

For infusion into the subarachnoid space, the head of each animal was fixed in the prone position and the atlanto-occipital membrane was exposed through an occipitocerebral midline incision. A stainless cannula (27 gauge; Becton–Dickinson) was introduced into cisterna magna (subarachnoid space). HVJ-E vector (100 μl) containing luciferase or Venus gene was infused at a speed of 50 μl/min after removing 100 μl CSF. Then, the animals were placed head down for 30 min.

For infusion into the common carotid artery, the left common carotid artery, the left external carotid artery, and the left internal carotid artery were isolated via a midline incision under an operating microscope (Konan, Japan). The left common carotid artery and internal carotid artery were ligated temporally and PE-50 catheter (Clay Adams, Parsippany, NY, USA) was introduced into the left common carotid artery via a cutdown in the left external carotid artery. HVJ-E vector (100 μl) containing EGFP or luciferase gene was injected at a speed of 25 μl/min. After injection, the cannula was removed and blood flow to the common carotid artery was restored by release of the ligatures. In each procedure, EGFP or Venus was observed 3 days after transfection and luciferase activity was measured at 1 day after transfection. Luciferase activity in spleen, lung, liver, kidney, and testis was also measured 1 day after intrathecal injection. All rats showed no weight loss or loss of activity after administration. To clarify the histological change after administration of vector, HE staining of coronal section was performed at 14 days after intraventricle and intrathecal injections. The coronal sections were made at +1.0, −3.30, −5.30, −11.30, and −14.60 mm from the bregma.

In vivo gene transfer after transient middle cerebral artery occlusion. To make the middle cerebral artery occlusion model, the left middle cerebral artery was occluded by placement of poly-L-lysine coated 4-0 nylon at the origin of MCA as described before [24]. Briefly, animals

were anesthetized with halothane (1–3.5% in a mixture of 70% N_2O and 30% O_2) using a face mask. The rectal temperature was maintained at 37 ± 1°C throughout the surgical procedure using a feedback regulated heating pad. Under the operating microscope the left common carotid artery, the left external carotid artery, and the left internal carotid artery were isolated via a midline incision. 4-0 Nylon was advanced from the left external carotid artery and advanced 20 mm. After 60 min, common carotid artery and internal carotid artery were transiently ligated and the 4-0 nylon was removed. After 10 min, the common carotid artery and the internal carotid artery were transiently ligated again. The PE-50 catheter was placed at the common carotid artery from external carotid artery as described above, and the vector was injected at the speed of 10 μl/min after the release of ligation. After injection, PE-50 catheter was removed and the external carotid artery was ligated by 6-0 nylon. The expression of luciferase or EGFP was observed at 1 day or at 3 days after the infusion. Coronal sections were stained by triphenyltetrazolium chloride to confirm the existence of infarction.

Results and discussion

Transfection into the cultured rat cerebral cortex cells

With non-viral vector, mitotic cells are transfected well, but non-mitotic cells such as quiescent (G_0) neurons are transfected so poorly [25]. The rate of gene transfer into cultured neurons was also low by HVJ-liposome vector that was previously used in vivo gene transfer into CNS. To investigate the feasibility of HVJ-E for gene transfer into cultured neuron, we initially transfected the reporter gene (Venus gene) into cultured rat cerebral cortex cells (E19), since Venus reporter gene has been reported as the easily detected transfection method. It is reported that the use of Venus as an acceptor allowed early detection of reliable signals of fluorescence in brain slices [21]. At 2 days after transfection, the cultured rat cerebral cortex cells demonstrated the readily detectable fluorescence in cultured cells (Figs. 1A and D). Using immunohistochemical staining, the cells were immuno-positive for MAP_2 (the neuronal marker) or GFAP (the astrocytic marker) (Figs. 1B and E). The merged image showed successful gene transfection into neurons (Fig. 1C) and astrocytes (Fig. 1F). The efficiency of transfection into neuronal cells as calculated from the number of Venus positive cells/the number of MAP_2 positive cells was 28.2 ± 5.8%, while no positive cells could be detected in cells transfected with control vector. Cell death could not be observed after transfection using HVJ-E vector. Thus, the reporter gene was well transfected into non-mitotic neurons using HVJ-E vector. Efficient transfection might be achieved due to the existence of the virus receptor for sialic acid, which was involved abundantly with the surface of neurons.

In vivo gene transfer into brain

Then, we examined the transfection of Venus gene into the brain. Initially, we injected HVJ-E vector containing Venus gene into the thalamus and lateral ventricle. Stereotactic injection of Venus gene into thalamus showed the limited expression at the site of injection

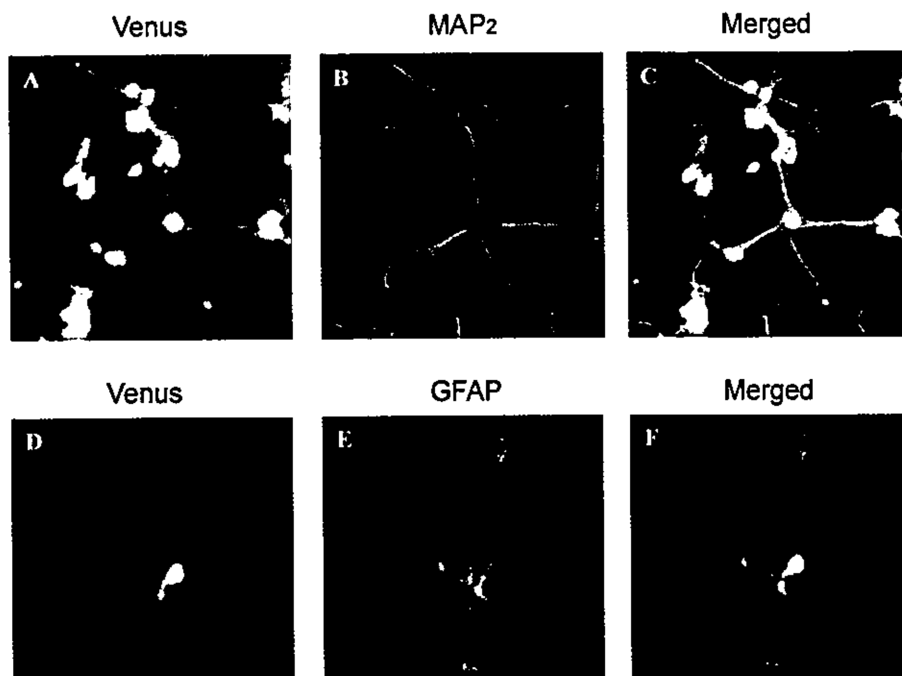

Fig. 1. Typical example of the cultured rat cerebral cortex neurons and glial cells transfected with Venus gene using HVJ-E vector. Laser scanning confocal microscopy images of Venus (A, D), immunofluorescent staining for MAP_2 (B), GFAP (E), and merged image (C, F). Most of the cells expressing Venus were immuno-positive for MAP_2 (C). This experiment was repeated at least five times (magnification 400×).

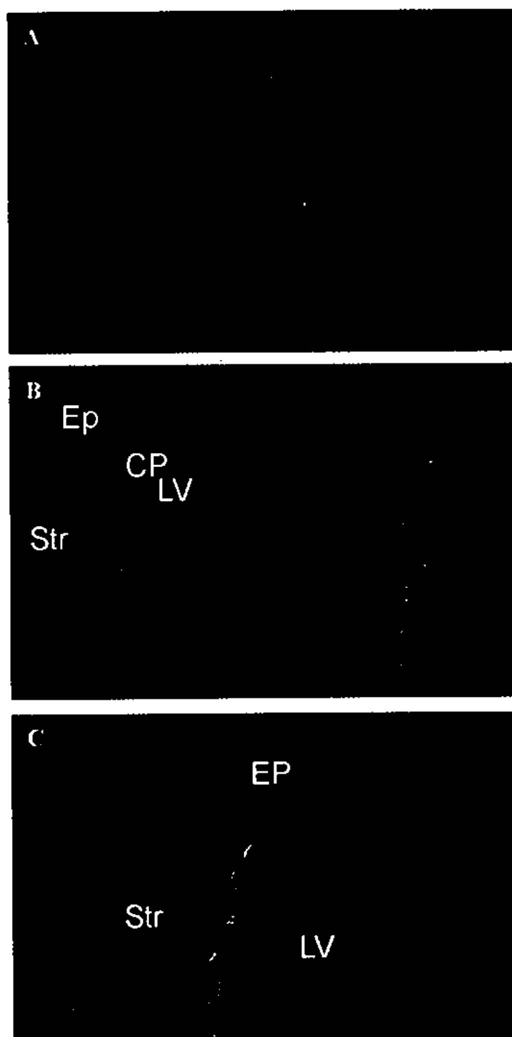

Fig. 2. In vivo gene transfer of Venus plasmid into brain using stereotactic injection. A fluorescent stereomicroscope image (A, B) and laser scanning confocal microscopy images of fluorescence (C). Stereotactic injection into thalamus (A) showed the limited expression at the site of injection. Stereotactic injection into lateral ventricle (B) revealed the expression at the choroid plexus and ependymal cells (C). CP, choroid plexus; LV, lateral ventricle; Str, striatum; Ep, ependymal cell layer. This experiment was repeated at least three times (magnification 25× (A, B) and 100× (C)).

(Fig. 2A). By the stereotactic injection into lateral ventricle, fluorescence could be detected at the choroid plexus and ependymal cells (Figs. 2B and C). Unexpectedly, no fluorescent signal was detected in neurons. No positive staining for fluorescence could be detected in brain transfected with control vector or untransfected brain.

Next, we employed the injection into the subarachnoid space. In this experiment, luciferase or Venus reporter gene was injected into CSF via cisterna magna to be circulated in the brain. Although widespread expression of luciferase or Venus was detected (Table 1 and Figs. 3A–C), coronal sections showed the limited expression of Venus only in the meningotheles (Fig. 3D)

Table 1
Luciferase activity at 24 h after transfection via cisterna magna

Site	RLU/g protein ($\times 10^3$)
Olfactory bulb	5809.5 ± 2229.5
Frontal cortex	312.0 ± 141.0
Parietal cortex	42.0 ± 22.0
Occipital cortex	269.3 ± 220.0
Brain stem	276.0 ± 76.5

After intrathecal injection of luciferase gene via cisterna magna, luciferase activity was measured from the tissue dissected from the olfactory bulb, frontal cortex, parietal cortex, occipital cortex, and brainstem. Widespread gene expression was observed.

and adventitial cells of artery (Fig. 3E). As the harvested materials for luciferase assay included meningotheles and arteries, those cells might mainly show luciferase activity. These data suggest that widespread gene transfection was successfully achieved in the brain surface adjacent to CSF. Importantly, no luciferase gene transfection was observed in other organs except brain (Table 2). HE staining of coronal section at 14 days after intrathecal injection showed no inflammatory change (data not shown).

Compared with HVJ-liposome method, the distribution of gene expression was different in vivo gene transfer into CNS. By intrathecal injection, β-galactosidase gene expression was observed in cerebral parenchyma by HVJ-liposome method [17,18], while gene expression was detected only in the meningotheles and adventitial cells of artery using HVJ-E vector. Besides, intraventicle cationic-liposome-mediated gene transfer showed the expression in cerebral parenchyma [26], whereas HVJ-E vector-mediated gene transfection revealed the transgene expression only at the choroid plexus and ependymal cells. Considering the fact that the direct injection of HVJ-E vector into thalamus resulted in the successful transfection into cerebral parenchyma, the presence of liposome might be important to cross over the meninx or ependymal cells. The limited expression of reporter gene in the brain and no histological change seem to be advantages of HVJ-E vector from the viewpoint of safety.

Gene transfer into CNS after transient middle cerebral artery occlusion

Considering the treatment of cerebral ischemic disease in the clinical setting, it seems best to employ infusion into the subarachnoid space rather than injection into the lateral ventricle using a stereotactic frame. To further explore the feasibility of gene therapy using HVJ-E vector in cerebral ischemia, we examined gene transfer into CNS via carotid artery. However, intraarterial infusion into the carotid artery produced little expression of transgene in the brain and microvascular endothelial cells at 3 and 7 days after injection (data not shown). To overcome this issue, we hypothesized that

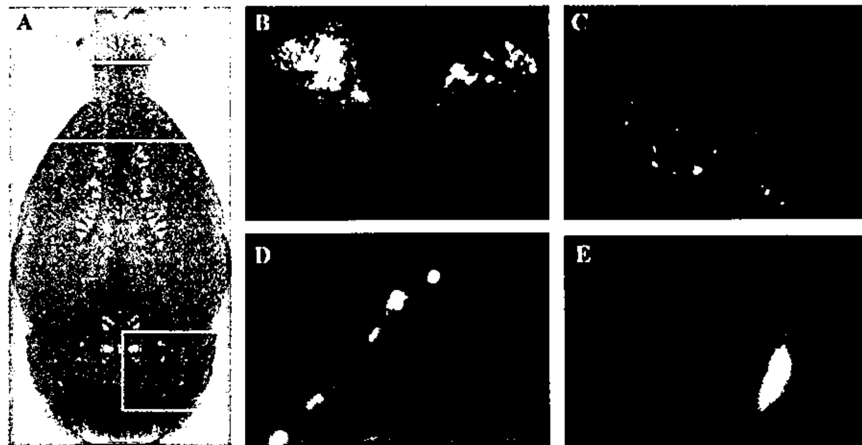

Fig. 3. In vivo gene transfer of Venus plasmid into brain by the injection via cisterna magna. An image of whole brain (A), a fluorescent stereomicroscope image of the yellow area in (A) (B), the green area in (A) (C), and a fluorescent microscope image of coronal slice (D, E). Intrathecal injection via cisterna magna showed *the gene expression in the brain, especially in the surface* of olfactory bulb, frontal lobe, and brainstem (B, C). Coronal section revealed the expression at the meningotheles (D) and adventitial cells of artery (E). This experiment was repeated at least three times (magnification 12.5× (B), 25× (C), 200× (D), and 200× (E)).

Table 2
Luciferase activity at 24 h after intrathecal injection of luciferase gene

Organ	RLU/g protein ($\times 10^3$)
Brain	187.7 ± 32.9
Lung	0
Liver	0
Kidney	0
Spleen	0
Testis	0

Luciferase activity was measured in the brain, lung, liver, kidney, spleen, and testis after intrathecal injection of luciferase gene via cisterna magna. Luciferase activity was not observed except the brain.

gene transfer after brain ischemia might show the different transfection efficiencies into CNS, since brain ischemia caused the change in blood–brain barrier [27–29]. Thus, we infused HVJ-E vector including EGFP gene into the carotid artery after transient middle cerebral artery occlusion for 60 min. Interestingly, fluorescence due to EGFP could be detected at the infarcted cerebral cortex at 3 days after transient occlusion (Fig. 4). The feasibility of transfection into CNS was confirmed by the experiments using luciferase gene. Luciferase activity at 24 h after injection was much higher in infarcted hemisphere than that in contra lateral hemisphere (Fig. 5, $P < 0.05$).

Although the destruction of blood–brain barrier after transient middle cerebral artery occlusion was controversial [27–29], Kuroiwa et al. [28] demonstrated a biphasic opening of the blood–brain barrier after 1 h of transient middle cerebral artery occlusion, occurring first at 15 min after the release of occlusion then at 5 and 72 h of reperfusion. Infusion of HVJ-E vector during this limited time period would allow us to transfect the therapeutic genes into cerebral parenchyma via intra-arterial approach.

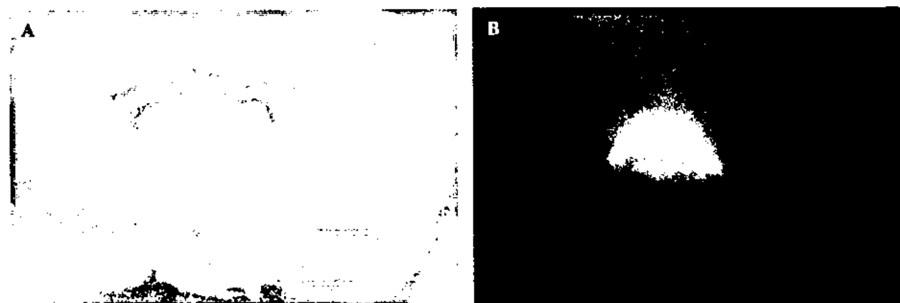

Fig. 4. Fluorescence due to EGFP gene transfer via carotid artery after transient occlusion of middle cerebral artery. Triphenyltetrazolium chloride stained coronal section at 3 days after left transient occlusion of middle cerebral artery for 60 min (A) and a fluorescent stereomicroscope image (B). HVJ-E vector having EGFP gene was infused in the left carotid artery during the reperfusion. The white region in (A) indicates infarction. Gene expression was observed only at the injured lesion, while no fluorescence could be detected at the intact hemisphere. This experiment was repeated at least three times (magnification 25× (B)).

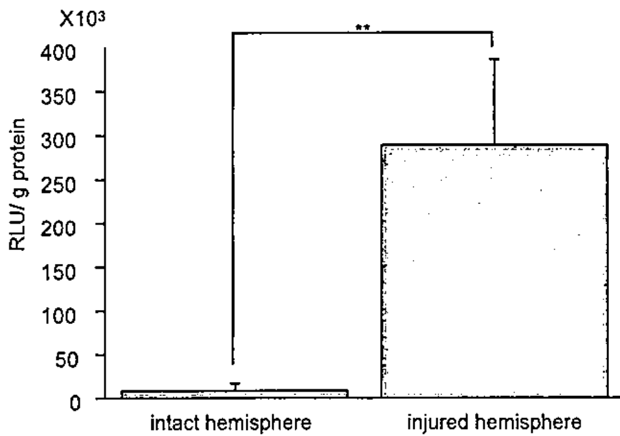

Fig. 5. Luciferase activity in injured hemisphere and the contra lateral hemisphere after gene transfer into carotid artery after transient occlusion of middle cerebral artery. Luciferase activity was measured at day 1 after left transient occlusion of middle cerebral artery for 60 min. HVJ-E vector containing luciferase gene was infused in the left carotid artery during the reperfusion. $n = 5$ for each group. $^{**}p < 0.05$.

Gene transfection with heparin

Finally, we investigated whether co-administration of heparin would increase the transfection efficiency. After the making of HVJ-E vector containing luciferase gene, heparin was added into the vector at the concentrations of 10, 50, 100, and 200 U/ml. The HVJ-E vector was injected into CSF via cisterna magna. As shown in Fig. 6, luciferase activity was dramatically enhanced on the addition of 50 U/ml heparin ($P < 0.01$). This result is similar to the report in which co-infusion with heparin using AAV vector induced higher and more homogeneous gene expression [30,31]. To clarify the mechanism of the increase in transfection efficiency with heparin, we examined the effects of low molecular weight heparin (LMWH) at the concentrations of 10, 50, and 100 U/ml, or argatroban at a concentration of 0.1 or 0.2 mg/ml. However, neither low molecular weight heparin nor argatroban affected the luciferase activity (Fig. 6). The difference between conventional heparin (12,000–15,000 kDa) and LMWH (5000 kDa) is only the molecular size and the degree of sulfation [32]. Probably, these factors may influence the interaction between HVJ-E vector and targeted cell surface.

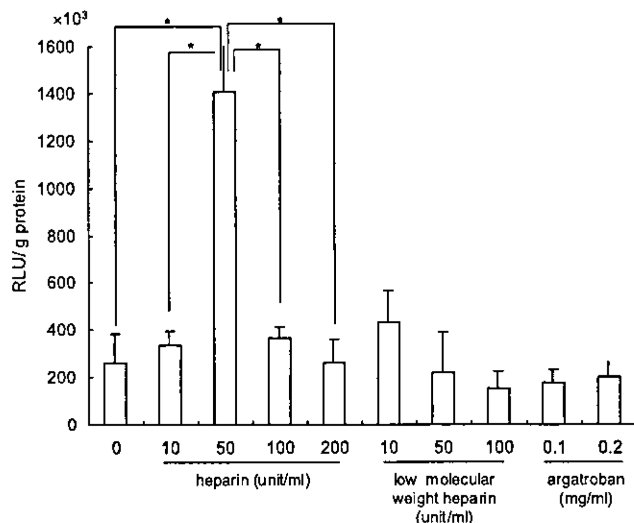

Fig. 6. Effect of heparin on luciferase activity. Luciferase activity was measured at day 1 after transfection of pGL3 luciferase gene via cisterna magna. Heparin was added to the vector at the concentration of 0, 10, 50, or 100, 200 (U/ml), low molecular weight heparin at 10, 50, or 100 (U/ml), and argatroban at 0.1 or 0.2 (mg/ml). The transfection rate was increased about fivefold by adding 50 U/ml heparin. $^{*}P < 0.01$ vs. 0, 10, 100, and 200. $n = 3$ for each group.

Here, the present study demonstrated the potent transfection efficiency using HVJ-E vector into CNS in vivo as well as in vitro without any apparent toxicity, while the site of gene expression was different among various administration routes. Successful gene transfection by intra-arterial injection after transient arterial occlusion will provide the promising approach for treatment of cerebral ischemia. In addition, to our knowledge, there has been no evidence of side effects. HVJ is not pathogenic to humans [33,34] and is completely inactivated by appropriate chemical modification without losing the fusion activity. In the present study, intrathecal or intraventricular administration showed no loss of weight, no neurological deficits, and no inflammatory change. Additionally, no luciferase activity was observed at any other organs after intrathecal injection. Therefore, the HVJ-E vector seems to be safe for the transfection into brain. Moreover, low antigenicity of inactivated HVJ has already been demonstrated because repeated administration of plasmid DNA and antisense ODN by HVJ-liposome method over five times did not result in any loss of biological effects or production of antibody against HVJ [35], and no cytotoxic T lymphocyte against HVJ were generated by repeated injection of HVJ-liposome into rat liver [36]. For these reasons, HVJ-E vector may be suitable gene transfer method for the treatment of cerebrovascular disease.

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No influence of tumor growth by intramuscular injection of hepatocyte growth factor plasmid DNA: safety evaluation of therapeutic angiogenesis gene therapy in mice

Atsushi Matsuki,[a,b,c] Seiji Yamamoto,[b] Hironori Nakagami,[a] Motokuni Aoki,[d] Katsuto Tamai,[b] Kunio Matsumoto,[e] Toshikazu Nakamura,[e] Toshio Ogihara,[d] Yasufumi Kaneda,[b] and Ryuichi Morishita[a,*]

[a] *Division of Clinical Gene Therapy, Biomedical Research Center, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan*
[b] *Division of Gene therapy Science, Biomedical Research Center, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan*
[c] *Digestive and General Surgery, Graduate School of Medical and Dental Science, Niigata University, Asahimachi, Niigata 951-8122, Japan*
[d] *Department of Geriatric Medicine, Biomedical Research Center, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan*
[e] *Division of Biochemistry, Department of Oncology, Biomedical Research Center, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan*

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Abstract

Recently, a novel therapeutic treatment for ischemic diseases using angiogenic growth factors to augment collateral artery development has been proposed. As intramuscular injection of naked human hepatocyte growth factor (HGF) plasmid DNA induced therapeutic angiogenesis in several animal test subjects, we have started a clinical trial to treat peripheral arterial disease. However, one might assume that over-expression of angiogenic growth factors could enhance tumor growth. To resolve this issue, we examined the over-expression of HGF in tumor bearing mice. Tumors on their backs were prepared with an intradermal inoculation of A431, human epidermoid cancer cells expressing c-Met. These mice were intramuscularly injected with human HGF plasmid or control plasmid into the femoral muscle. Human HGF concentration was increased only in the femoral muscle, but not in blood. Although recombinant HGF stimulated the growth of A431 cells in vitro, temporally and locally HGF elevation in hindlimb had no effect on tumor growth in mice.

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Critical limb ischemia is estimated to develop in 500–1000 individuals per million per year [1]. In a large proportion of these patients, the anatomical extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Most importantly, there is no optimal medical therapy for critical limb ischemia. Therefore, novel therapeutic modalities are needed to treat these patients. Recently, the efficacy of therapeutic angiogenesis using gene transfer of angiogenic growth factors such as VEGF (vascular endothelial growth factor) has been tested in human patients with critical limb ischemia and myocardial ischemia [2–4]. Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia or myocardial infarction. In addition to VEGF, hepatocyte growth factor (HGF) originally cloned as hepatocyte mitogen [5] is also of particular interest, as HGF exclusively stimulated the growth of endothelial cells without replication of vascular smooth muscle cells (VSMC) [6–8]. Indeed, others and we have previously reported that HGF is a potent angiogenic growth factor that is useful for effective therapeutic angiogenesis in several animal tests [9,10]. Based upon the preclinical study, we have started human clinical trials of gene

* Corresponding author. Fax: +81-6-6879-3409.
E-mail address: morishit@gts.med.osaka-u.ac.jp (R. Morishita).

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therapy to treat peripheral arterial disease using naked plasmid DNA encoding the HGF gene by intramuscular injection into ischemic legs. Although human clinical trials using angiogenic growth factors including HGF do not exhibit severe adverse effects at the present time, one might assume that the potential risks such as the promotion of tumor growth might exist. As HGF is well known as a pleiotropic effector of cells expressing the c-Met tyrosine kinase receptor [11–13], HGF produced by mesenchymal cells may act predominantly on cells of epithelial origin in an endocrine and/or paracrine fashion [14,15]. HGF especially regulates cell growth, cell motility, and morphogenesis of various types of cells [5]. HGF-Met signaling clearly plays a role in tumor development and progression, as c-Met was originally isolated as the product of human oncogene, trp-met, encodes an altered Met protein possessing constitutive, ligand-independent tyrosine kinase activity, and transforming ability [16,17]. The oncogenic capability of HGF-Met signaling is likely due to the inappropriate use of mitogenic and angiogenic signals. HGF-Met signaling also induces the invasiveness and metastatic potential [18–20]. To answer this safety question, we examined the effects of local over-expression of HGF by intramuscularly injection of plasmid DNA on tumor growth. The present study documented no influence of HGF plasmid DNA gene therapy on tumor growth despite the increase in local HGF concentration in mice.

Materials and methods

Construction of plasmids. To produce a HGF expression vector, human HGF cDNA (2.2 kb) was inserted into pVAX1 (3.0 kb) (Invitrogen, Carlsbad, CA, USA) at the *Bam*HI and *Not*I sites. pVAX1 not containing HGF cDNA was used as a control. pCMV-luciferase-GL3 (7.4 kb) was constructed by cloning the luciferase gene from the pGL3-Promoter Vector (Promega, Madison, WI, USA) into pcDNA3 (5.4 kb) (Invitrogen, USA) at the *Hin*dIII and *Bam*HI sites. Plasmids were purified with the Qiagen plasmid isolation kit (Hilden, Germany).

Human recombinant HGF. Human recombinant HGF was purified from a culture medium of Chinese hamster ovary cells or C-127 cells transfected with an expression plasmid containing human HGF cDNA [9,21].

Cell scattering and proliferation assay. A431 cells were cultured in DMEM supplemented with 10% FBS. Then the cells were plated in 6-well tissue culture dishes at 1×10^5 cells/well in 3.0 ml DMEM with 1% FBS and cultured for 48 h in the absence or presence of various concentrations of HGF [21]. For proliferation assay, A431 cells were cultured in DMEM supplemented with 10% FBS. Then the cells were plated in 96-well tissue culture dishes at 3×10^3 cells/well in 200 μl DMEM with 1% FBS and cultured for 24 h. After washing with DMEM, the cells were cultured for 48 h in DMEM with 1% FBS in the absence or presence of various concentrations of HGF [21]. The number of cells was counted with MTS colorimetric assay system (Promega, USA).

In vivo gene transfer using direct intramuscular injection approach. All procedures were approved by the Osaka University Committee on Animal Research. Six-week-old male BALB/cA nu/nu mice bearing tumor on their back were prepared by an intradermal inoculation of 5×10^6 A431 cells in 0.1 ml PBS [21,22]. A431 human epidermoid carcinoma cells express c-Met/HGF receptor but scarcely produce HGF. A431 cells produce IL-1, one of the HGF-inducing soluble factors [21,23]. One week later, the mice received an intramuscular injection of 200 μg human HGF plasmid DNA in 0.1 ml saline on hindlimb [24,25]. After 4 weeks observation, mice were killed under anesthesia and examined for body weight, tumor weight, tumor invasion, central necrosis, and metastasis. HGF concentrations in blood and tumor tissues were measured by enzyme-immunoassay (EIA).

In vivo gene transfer into the tumor tissues. A431 cells were intradermally implanted into nude mice same as above and one week later, the direct injection of 200 μg human HGF plasmid DNA or empty vector was injected into tumor tissues. After 4 weeks observation, mice were killed under anesthesia and examined for tumor properties.

Measurement of HGF concentration in hindlimb and other tissues. To document the successful transfection of HGF vector into the hind limb, we examined the production of human immunoreactive HGF [8,26]. Before and at 1, 4, 7, and 14 days after transfection, the hindlimb of the mouse transfected with HGF vector, opposite leg, back skin, liver, and blood serum were removed and stored at −70 °C until use. On the day of extraction, the tissue was thawed at 4 °C, weighed, and homogenized by polytron in assay solution. Each specimen was centrifuged at 20,000*g* for 30 min at 4 °C, to remove the lysate. The concentration of HGF in the hindlimb was determined by EIA using anti-human HGF antibody [8,26]. In brief, rabbit anti-rat or anti-human HGF IgG was coated on 96-well plates (Corning, NY) at 4 °C for 15 h. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), the conditioned medium was added to each well and the preparation was incubated for 2 h at 25 °C. The wells were washed three times with PBS containing 0.025% Tween 20 (PBS–Tween), biotinylated rabbit anti-human HGF IgG was added, and the preparation was incubated for 2 h at 25 °C. After washing with PBS–Tween, the wells were incubated with horseradish peroxidase-conjugated streptavidin–biotin complex in PBS–Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/ml *o*-phenylenediamine, 100 mM sodium phosphate, 50 mM citric acid, and 0.015% H_2O_2. The enzyme reaction was stopped by adding 1 M H_2SO_4 and absorbance at 490 nm was measured. The antibody against human HGF reacts with only human HGF and not with rat HGF [27]. Mouse endogenous immunoreactive HGF was also measured by EIA using anti-mouse HGF antibody, as the antibody against mouse HGF reacts with only mouse HGF, and not with human HGF [26]. The lower detection limit of this method was 0.1 ng/ml.

Assay for luciferase activity. The mice transfected with luciferase gene were killed under anesthesia at 24 h post-injection. The tissues (tumor, left hindlimb, and right hindlimb) were harvested and placed individually in flat-bottomed 1.5 ml Eppendorf microfuge tubes. Luciferase activity assay was performed as described previously [28]. Luciferase levels were normalized by determining the protein concentrations of the tissue extracts [28]. Luciferase units were expressed as relative light units (RLU) per microgram of tissue protein.

Statistical analysis. All statistical analyses were done with the Stat View, release 4.11 (Abacus Concepts, CA, USA). The results were compared with Student's *t* test. All data are expressed as means ± SD. The differences were considered statistically significant at a *P* value of <0.05.

Results

As co-expression of c-Met/HGF receptor and HGF molecules in the same cell, which generates an autocrine stimulatory loop, is reported to be oncogenic [18,29], we initially identified the in vitro effects of HGF on the growth cells expressing c-Met. We chose A431 human epidermoid carcinoma cells, since A431 cells express

c-Met, but rarely produce HGF. Initially, we examined the effects of HGF on tumor scattering. As shown in Fig. 1, addition of rHGF induced the scattering of A431 cells in a dose-dependent manner. In addition to scattering, a significant increase in the number of A431 cells was observed under rHGF stimulation in a dose-dependent manner (Fig. 2, $P < 0.01$).

Using these A431 cells, we next examined the effects of local over-expression of HGF on tumor growth in mice harboring A431 tumors. Initially, we measured tissue HGF concentration in tumor induced by A431 cells implantation. As reported previously, A431 cells themselves rarely produced HGF, but secreted the HGF inducers for fibroblasts such as interleukin 1 [21,23]. Indeed, as shown in Fig. 3A, human HGF concentration both in A431 cells and the medium of A431 cells in vitro could not be detected. Similarly, neither could mouse HGF concentration be detected in either the A431 cells or the medium of A431 cells in vitro. In contrast, human HGF concentration was readily detected in tumor tissues in vivo. Tumors induced by A431 cells most likely secrete human HGF locally. In addition, mouse endogenous HGF concentration was also readily detectable by tumor harboring. As A431 cells secreted inducers for HGF such as interleukin 1 [21,23], endogenous HGF levels would be induced. In this

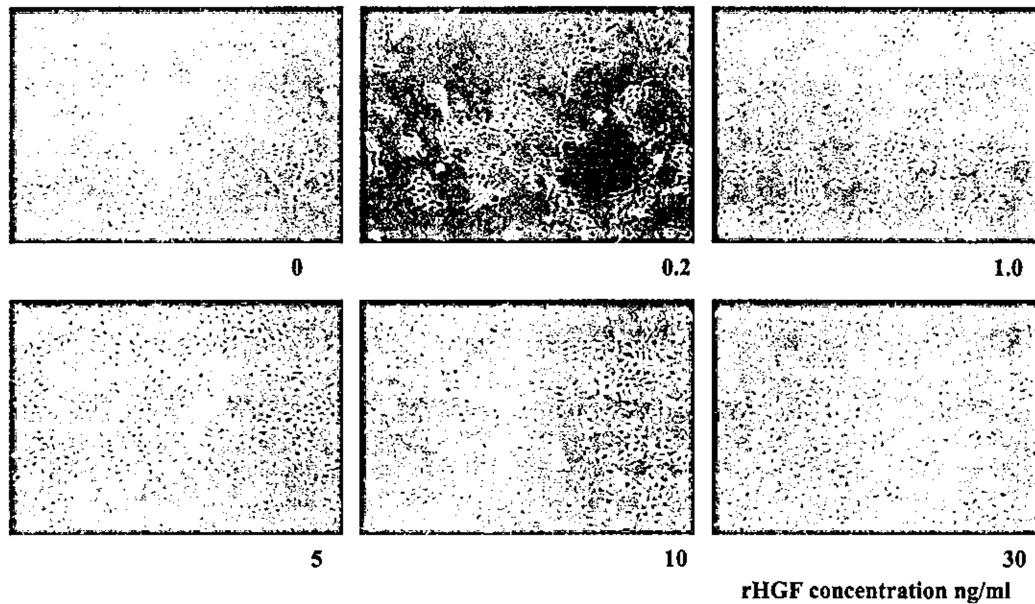

Fig. 1. Effects of human recombinant HGF protein (rHGF) on cell scattering. A431 cells were plated at a density of 1×10^5 cells/well on 6-well plates and cultured for 48 h in various concentrations of rHGF.

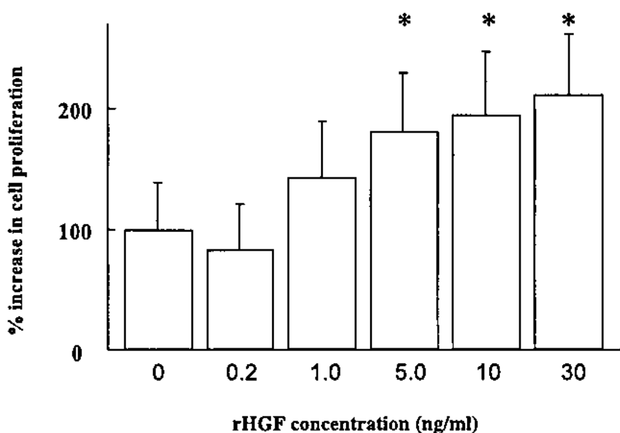

Fig. 2. Effects of rHGF on cell proliferation. A431 cells were plated at a density of 3×10^3 cells/well on 96-well plates and cultured for 48 h in various concentrations of rHGF for every 16 wells. Cell numbers of each well were measured using MTS assay.

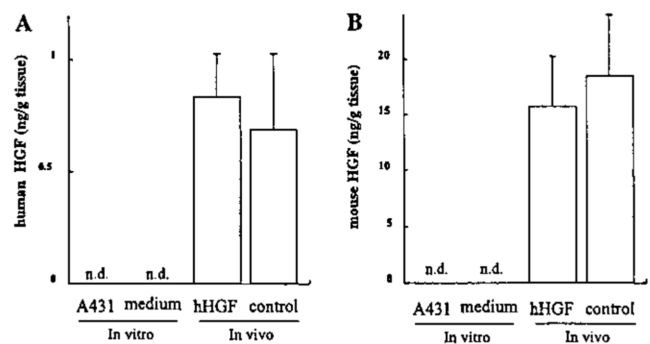

Fig. 3. HGF concentration in vitro and in vivo. (A) Human HGF concentration in A431 cells and medium in vitro and tumor tissues in vivo. The mice received intradermal injections of A431 cells (5×10^6 cells) on their back on day 0, intramuscularly injections of human HGF plasmid (hHGF, $n = 3$) or empty vector (pVAX1, $n = 3$) on hindlimb on day 7. Their tumors were resected on day 28 and HGF concentration was measured by EIA. (B) Mouse HGF concentration in same objects.

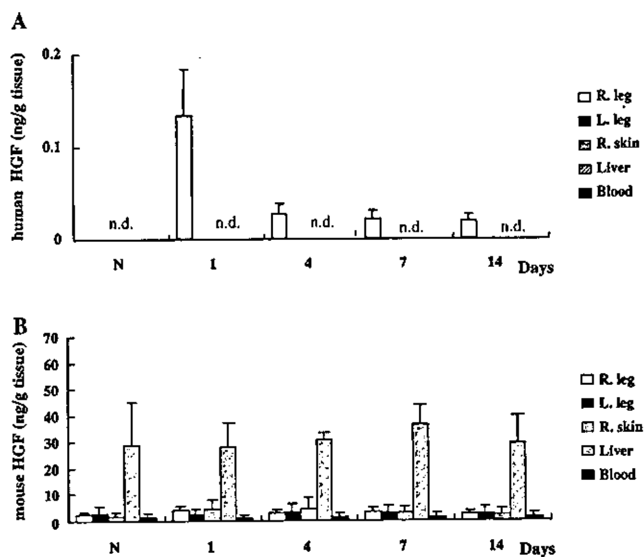

Fig. 4. HGF concentration in various tissues. (A) Change of human HGF concentration in right femoral muscle (R. leg), left femoral muscle (L. leg), right back skin (R. skin), liver, and blood of BALB/cA nu/nu mice before (N), and 1, 4, 7, and 14 days after intramuscularly injection of 200 μg naked human HGF plasmid on right hindlimb $n = 4$. (B) Change of mouse HGF concentration in the same tissues of BALB/cA nu/nu mice before and after intramuscular injection of 200 μg naked human HGF plasmid on right hindlimb $n = 4$.

model, naked plasmid DNA was intramuscularly injected into hindlimb. As shown in Fig. 4A, human HGF concentrations were only detected in the right hindlimb directly transfected with human HGF plasmid. Other organs such as contra-lateral left hindlimb, skin, and liver did not exhibit a detectable HGF level. In addition, neither could serum HGF concentration be detected. In contrast, mouse endogenous HGF concentration could be detected in all tissues. However, HGF level in the liver was extremely high as compared to other organs. Local transfection of HGF plasmid DNA did not affect mouse endogenous HGF level (Fig. 4B). Interestingly, the human and mouse HGF levels in the tumors were also not affected by intramuscular injection of HGF plasmid DNA (Figs. 3A and B).

Based upon the increase in local HGF concentration, we examined the effects of over-expression of HGF in hindlimb on tumor growth. Transfection of human HGF plasmid DNA into peripheral arterial disease mouse model at same dosage resulted in a significant increase in blood flow and the capillary density (data not shown). However, as expected, there was no significant difference in tumor size, weight, invasion, and central necrosis between the mice intramuscularly transfected with human HGF plasmid and control vector (Fig. 5). In addition, no significant difference in tumor size, weight, invasion, and central necrosis could be detected between the mice intramuscularly transfected with control vector and the untreated mice (Fig. 5). Metastasis was not detected in all mice. None of the mice transfected with HGF gene died during the observation period.

Since mice were bearing tumors on their right back, we examined the site effects of direct injection of HGF plasmid DNA. There was no significant difference in tumor properties between the mice intramuscularly injected with HGF plasmid on the right hindlimb and the left hindlimb (data not shown). We next examined whether intratumor injection of HGF plasmid DNA accumulates tumor invasion and growth. There was no significant difference in tumor size between the mice injected with empty vector into tumor tissues and the untransfected mice. In addition, no significant difference in tumor size could be detected between the mice injected with HGF plasmid DNA and empty vector

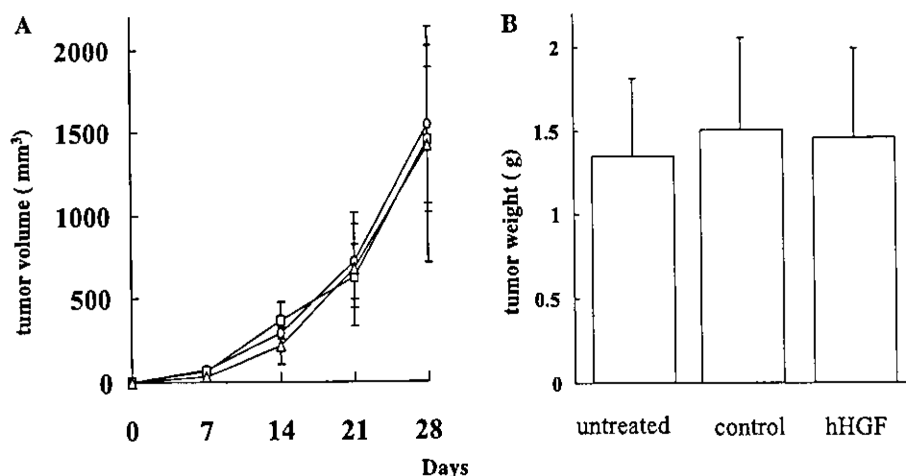

Fig. 5. Growth of tumor cells in vivo. (A) Effect of intramuscular injection of naked human HGF plasmid on the growth of tumor cells in vivo. Mice received intradermal injections of A431 cells (5×10^6 cells) on their right back on day 0 and intramuscular injections of human HGF plasmid (hHGF; ○, $n = 9$) or empty vector (pVAX1; △, $n = 9$) on left hindlimb on day 7 or not (N; □, $n = 9$). Tumor volume on their back was calculated as $1/2 \times \text{length} \times \text{width}^2$ (length > width). (B) Tumor weight on day 28. Tumors were dissected on day 28 and measured by weight.

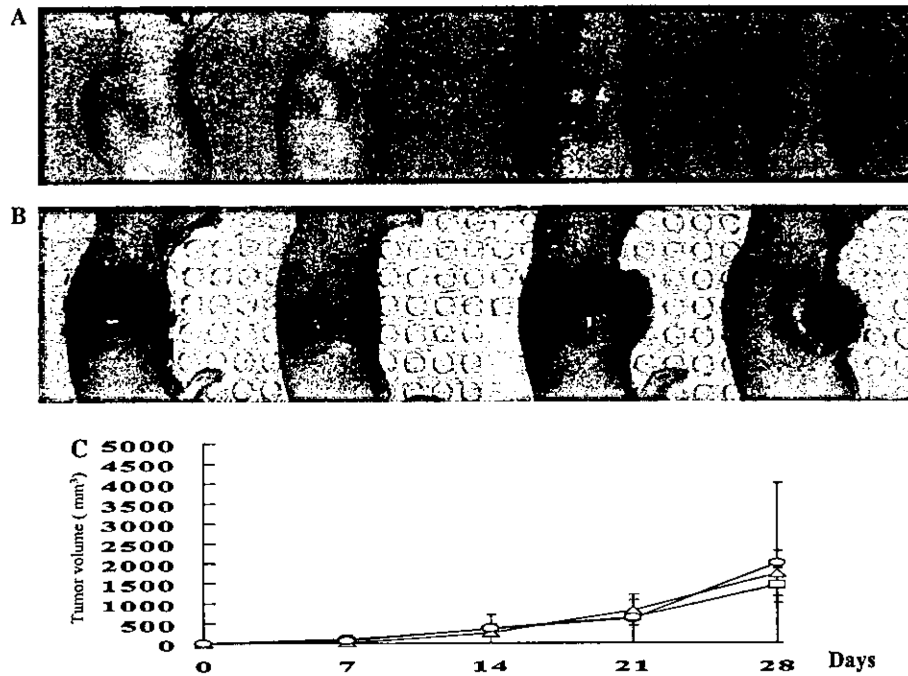

Fig. 6. Effect of direct injection of naked human HGF into the tumor on the growth of tumor. Mice were intradermally injected with A431 cells (5×10^6 cells) on their right back on day 0. Direct injection of human HGF plasmid (O, $n = 4$) or empty vector (Δ, $n = 4$) into tumor tissues was performed on day 7, whereas control mice were not transfected (□, $n = 9$). (A) Mice transfected with human HGF plasmid DNA (O, $n = 4$). (B) Mice transfected with empty control vector (Δ, $n = 4$). (C) Effect of direct injection of naked human HGF into the tumor on the growth of tumor.

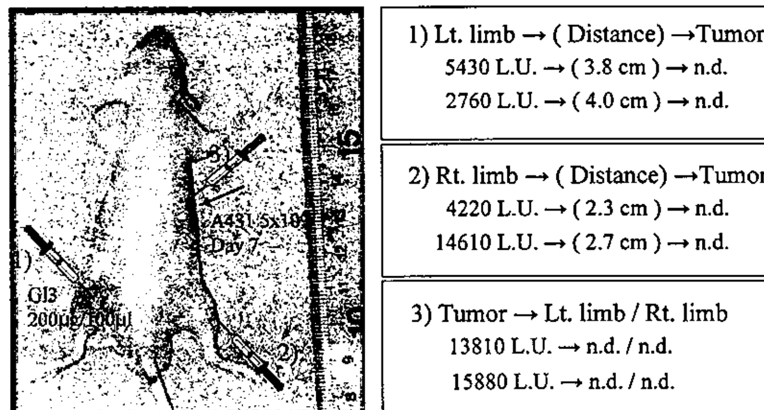

Fig. 7. Measurement of luciferase activity after injection of plasmid DNA: (1) Intramuscular injection into left hindlimb, (2) intramuscular injection into right hindlimb, and (3) injection into tumor. n.d., not detected.

(Fig. 6). Distant metastasis could not be detected in all mice. Finally, we tested the possibility that plasmid DNA should occur within the tumor by leakage of plasmid DNA from the hindlimb to the circulating system. As shown in Fig. 7, our present study denied this possibility. Intramuscular injection of pCMV-luciferase-GL3 plasmid DNA into the hindlimb resulted in the detection of luciferase activity only in hindlimb, while no luciferase activity could be detected in the tumor. In contrast, even with an injection into the tumor, luciferase activity was only detected in the tumor and not the hindlimb. These results demonstrate that the transfection of the injection of plasmid DNA results in the limited expression within the injection site.

Discussion

As the feasibility of therapeutic angiogenesis using angiogenic growth factors such as VEGF, FGF, and HGF has been reported in experimental models [9], in vivo transfer of these genes into the muscle is currently being tested as gene therapy for untreatable peripheral arterial disease. In this study, we evaluated the most

important safety issue regarding the use of plasmid vector encoding angiogenic growth factors in the patients who have tumors. The two important questions are: (1) the promotion of tumor by the intramuscular injection of naked plasmid DNA and (2) the expression of transgene in tumors by the leakage of plasmid DNA from the injected sites. In this study, we chose HGF as a model of safety evaluation, since the previous reports demonstrated the oncogenic capability of HGF-Met signaling, the invasiveness, and metastatic potential [18–22]. Indeed, we confirmed the stimulatory effects of human rHGF on scattering and proliferation using A431 cells in vitro. Although HGF transgenic mice develop a remarkably broad array of histologically distinct tumors of both mesenchymal and epithelial origin [30], in this study, no change was detected in tumor growth in mice intramuscularly transfected with HGF plasmid DNA into the hindlimb of tumor bearing mice. In addition, no evidence of metastasis and death could be detected throughout the observation period. Although human and mouse HGF concentrations were significantly increased in tumor tissues in vivo than in A431cells in vitro, there was no significant difference in local HGF concentration in tumor tissue between the mice intramuscularly injected with human HGF plasmid and control vector, while local human HGF was over-expressed in the hindlimbs. In fact, human HGF concentration was significantly elevated only in femoral muscle where human HGF plasmid was injected, but not elevated in other tissues and blood. In addition, despite an extremely high concentration of HGF in tumor tissues on mice, there was no significant difference in circulating HGF concentration between the tumor bearing mice and no treatment mice. How about the comparability of the present study with human clinical trial? In this study, we employed 200 μg human HGF plasmid DNA by the intramuscular injection into the hindlimb, while the body weights of the mice were pretty small about 30 g/body. Even at this dose, we confirmed that transfection of HGF plasmid DNA is enough to induce blood flow and the number of capillary density. That amount per body weight is equal to about 400–600 mg in a human case. In a human clinical trial, a total of 4 or 8 mg of human HGF plasmid DNA was used in trial. Even in extremely large amounts of human HGF plasmid, no promotion of the tumor was detected. Regarding the expression of plasmid DNA within the tumor from the leakage of plasmid DNA from the hindlimb to the circulating system, our present study denies this possibility. Intramuscular injection of plasmid DNA resulted in the detection of luciferase activity only in the hindlimb, while no luciferase activity could be detected in the tumor. These results denied the potential side effects of therapeutic angiogenesis using plasmid DNA in cancer patients with peripheral arterial disease.

However, there are still some elements of this series of safety evaluations to limit the conclusions. In this study, we used the mice bearing the tumors on their backs, but in clinical situation there are many different types of tumors that arise from variable tissues. In addition, other angiogenic growth factors such as VEGF may not be same as HGF, since serum concentration of VEGF, but not HGF, was increased after gene transfer into the human legs using naked plasmid DNA [31]. Nevertheless, the present study clearly demonstrated that local over-expression of HGF did not induce tumor promotion. HGF gene therapy is safe for mice tumor and further studies have to be done in the case of human diseases. The present information is important to consider the stimulation of new vessel formation by HGF as a new therapeutic option in angiogenesis-dependent conditions such as wound healing, inflammatory diseases, ischemic heart disease, myocardial infarction, and peripheral arterial disease.

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Novel Therapeutic Strategy to Treat Brain Ischemia

Overexpression of Hepatocyte Growth Factor Gene Reduced Ischemic Injury Without Cerebral Edema in Rat Model

Munehisa Shimamura, MD; Naoyuki Sato, MD, PhD; Kazuo Oshima, MD; Motokuni Aoki, MD, PhD; Hitomi Kurinami, MD, PhD; Satoshi Waguri, MD, PhD; Yasuo Uchiyama, MD, PhD; Toshio Ogihara, MD, PhD; Yasufumi Kaneda, MD, PhD; Ryuichi Morishita MD, PhD

Background—Although cerebral occlusive disease leads to cerebral ischemic events, an effective treatment has not yet been established. An ideal therapeutic approach to treat ischemia might have both aspects of enhancement of collateral formation and prevention of neuronal death. Hepatocyte growth factor (HGF) is a potent angiogenic factor that also acts as a neurotrophic factor. Thus, in this study, we examined the therapeutic effects of HGF on brain injury in a rat permanent middle cerebral artery occlusion model.

Methods and Results—Gene transfer into the brain was performed by injection of human HGF gene with hemagglutinating virus of Japan–envelope vector into the cerebrospinal fluid via the cisterna magna. Overexpression of the HGF gene resulted in a significant decrease in the infarcted brain area as assessed by triphenyltetrazolium chloride staining, whereas rats transfected with control vector exhibited a wide area of brain death after 24 hours of ischemia. Consistently, the decrease in neurological deficit was significantly attenuated in rats transfected with the HGF gene at 24 hours after the ischemic event. Stimulation of angiogenesis was also detected in rats transfected with the HGF gene compared with controls. Of importance, no cerebral edema or destruction of the blood-brain barrier was observed in rats transfected with the HGF gene.

Conclusions—Overall, the present study demonstrated that overexpression of the HGF gene attenuated brain ischemic injury in a rat model, without cerebral edema, through angiogenic and neuroprotective actions. In particular, the reduction of brain injury by HGF may provide a new therapeutic option to treat cerebrovascular disease. (***Circulation*. 2004;109:424-431.**)

Key Words: gene therapy ■ nervous system ■ stroke ■ cerebral ischemia ■ angiogenesis

Cerebral occlusive disease caused by atherosclerosis of the cerebral arteries or Moyamoya disease often causes global ischemia of the brain. Although such a condition leads to not only cerebral ischemic events but also neuropathological changes, including dementia,[1,2] an effective treatment to improve brain ischemic injury has not yet been established. Ischemic stroke induces active angiogenesis, particularly in the ischemic penumbra, which correlates with longer survival in humans.[3] However, the natural course of angiogenesis is not sufficient to compensate for the hypoperfusion state. Therefore, novel therapeutics are needed to treat these patients. Because angiogenic growth factors stimulated the development of collateral arteries in animal models of peripheral and myocardial ischemia, a concept called therapeutic angiogenesis,[4,5] the therapeutic implications of angiogenic growth factors to treat cardiovascular disease were recently described. The efficacy of therapeutic angiogenesis using vascular endothelial growth factor (VEGF) gene transfer has been reported in human patients with critical limb ischemia or myocardial infarction.[6,7] Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with ischemia. From this viewpoint, therapeutic angiogenesis must be an effective therapy for cerebral ischemia, resulting in the prevention of future stroke. Indeed, several angiogenic growth factors, such as fibroblast growth factor, hepatocyte growth factor (HGF), and VEGF were applied to prevent the extension of focal ischemic injury in animal models.[8–10]

However, the simple effects of stimulation of angiogenesis might not be enough to treat brain ischemia, because neurons are highly sensitive to hypoxia–ischemia. Given this susceptibility and their postmitotic nature, the development of

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From the Division of Clinical Gene Therapy (M.S., N.S., H.K., R.M.), Division of Gene Therapy Science (M.S., K.O., Y.K.), Department of Geriatric Medicine (M.A., T.O.), and Department of Cell Biology and Neuroscience (A1) (S.W., Y.U.), Graduate School of Medicine, Osaka University, Suita, Japan.

Dr Morishita is a board member of AnGes MG.

Correspondence to Ryuichi Morishita, MD, PhD, Professor, Division of Clinical Gene Therapy, Osaka University, Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan. E-mail morishit@cgt.med.osaka-u.ac.jp

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effective protective therapeutic strategies is also essential. In particular, pyramidal neurons in the CA1 subfield of the hippocampus are known to be the most vulnerable to cerebral ischemia.[11] After transient occlusion of the bilateral common carotid arteries in the gerbil, delayed neuronal death begins in CA1 pyramidal neurons a few days after recirculation, during which time no energy crisis or morphological change is observed. Therefore, prevention of delayed neuronal death might be of therapeutic value. Thus, several neurotrophic growth factors, such as brain-derived neurotrophic factor,[12,13] were reported to prevent the extension of focal ischemic injury in animal models. To consider both aspects of brain ischemic injury, the ideal growth factors should have both functions of angiogenesis and neurotrophic actions. Because HGF is a well-known potent pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activities in a variety of cells,[14] HGF has a neuroprotective effect in vitro and in vivo.[15–17] Here, we demonstrated that gene transfer of HGF into the subarachnoid space could cause beneficial effects on neurological symptoms through the prevention of brain injury and stimulation of angiogenesis without any apparent toxicity in a rat model.

Methods

Preparation of HVJ-Envelope Vector

A hemagglutinating virus of Japan (HVJ)-envelope vector was prepared as described previously.[18,19] Briefly, the virus suspension (15 000 hemagglutinating units) was inactivated by UV irradiation (99 mJ/cm^2) and mixed with plasmid DNA (400 μg) and 0.3% Triton-X. After centrifugation, it was washed with 1 mL balanced salt solution (10 mmol/L Tris-Cl, pH 7.5, 137 mmol/L NaCl, 5.4 mmol/L KCl) to remove the detergent and unincorporated DNA. After centrifugation, the envelope vector was suspended in 100 μL PBS. The vector was stored at 4°C until use. To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eukaryotic expression plasmid that uses the cytomegalovirus promoter/enhancer.[20] The control vector was expression vector plasmid with the same structure, including the promoter, but not containing HGF cDNA.

In Vivo Gene Transfer Into Subarachnoid Space in Normal Rats

Injection of the HVJ-envelope vector into the cisterna magna was performed for gene transfer to the brain of Wistar male rats (270 to 300 g; Charles River Japan, Atsugi, Japan).[19] The head of each animal was fixed in the prone position, and the atlanto-occipital membrane was exposed through an occipitocerebral midline incision. A stainless cannula (27 gauge; Becton Dickinson) was introduced into the cisterna magna (subarachnoid space). HVJ-envelope vector (100 μL) containing human HGF gene was infused at a speed of 50 μL/min after removal of 100 μL of cerebrospinal fluid (CSF). Then, the animals were placed head down for 30 minutes. No behavioral change, such as convulsion or abnormal movement, was observed. All procedures were conducted in accordance with Osaka University guidelines.

To investigate the effects of HGF gene transfer on cerebral ischemia, a rat permanent middle cerebral artery (MCA) occlusion model was used in the present study. To generate the MCA occlusion model, the right MCA was occluded by placement of poly-L-lysine–coated 4-0 nylon around the origin of the MCA.[21] The right common carotid artery, right external carotid artery, and right internal carotid artery were isolated via a midline incision. Then, 4-0 nylon was inserted from the right external carotid artery and advanced 20 mm. The right external carotid artery was ligated with 6-0 nylon. To examine transfection of the HGF gene in the CSF, 100 μL CSF was collected 5 and 12 days after gene transfer. The concentration of HGF was determined by enzyme immunoassay using anti-human or anti-rat HGF antibody (Institute of Immunology, Tokyo, Japan).[17] The antibody against human HGF reacts with only human HGF, and not with rat HGF.

Histological Examination

For immunohistochemical staining for c-met, rats were killed 5 days after gene transfer by transcardial perfusion fixation with normal saline followed by 4% paraformaldehyde. The brain was removed, postfixed, and cut on a vibratome at 40 μm. After blocking, free-floating sections were incubated in 3% normal goat serum and anti–c-met antibody (SP 260, 1:250; Santa Cruz), followed by anti-rabbit fluorescent antibody (1:1000, Alexa Flour 488, Molecular Probes). For in situ end-labeling of fragmented DNA, brain at 1 day after MCA occlusion was fixed with 10% formalin and processed for paraffin embedding. Terminal dUTP nick end-labeling (TUNEL) of apoptotic cells was measured with an ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Integren Inc). Counterstaining was performed by immersing slides in methyl green in 0.1 mol/L sodium acetate solution (pH 4.0) for 5 minutes at room temperature.

Evaluation of Effect of HGF Gene Transfer on Infarcted Area

The right MCA was occluded at 5 days after gene transfer into the subarachnoid space. Rats were killed 24 hours after occlusion, and the brain was removed within 3 minutes of death. Coronal sections were made at +3.7, +1.0, −0.8, −3.3, and −5.3 mm from the bregma, and brain slices were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Nakalai tesque) in normal saline at 37°C. This procedure can be used as a reliable marker of ischemic damage.[22] To assess the ischemic area, we calculated the hemispheric lesion area (HLA) in coronal sections. The corrected HLA was calculated as HLA (%)=[LT−(RT−RI)]/LT×100, where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarcted area.

Behavior Examination

For behavior assessment, we used a simple protocol[23] to evaluate neuromuscular function that uses the following categories (maximum score is 4). Forelimb flexion: Rats were held by the tail on a flat surface. Paralysis of the forelimbs was evaluated by the degree of left forelimb flexion. Torso twisting: Rats were held by the tail on a flat surface. The degree of body rotation was checked. Lateral push: Rats were pushed either left or right. Rats with right MCA occlusion showed weak or no resistance against a left push. Hindlimb placement: One hindlimb was removed from the surface. Rats with right MCA occlusion showed delayed or no placement of the hindlimb when it was removed from the surface.

Evaluation of Cerebral Edema After Permanent MCA Occlusion

The brain was removed within 3 minutes of death after 24 hours of MCA occlusion. The brain was divided into the intact hemisphere and the infarcted hemisphere. The wet weight was measured quickly, and the brain was dried in an oven at 110°C for 24 hours.[24] Then the dry weight was measured. The water content of these samples was calculated as water content (%)=(wet wt−dry wt)×100/wet wt.

Evaluation of Blood-Brain Barrier Permeability With Evans Blue Dye

To evaluate the effect of HGF on blood-brain barrier (BBB) permeability, Evans blue dye was used as a marker of albumin extravasation.[25] Evans blue dye (2% in saline, 3 mL/kg) was injected via the femoral vein under halothane anesthesia at 6 hours after MCA occlusion. Three hours after Evans blue dye injection, the rats were anesthetized with sodium pentobarbital and perfused with physiological saline.[24] Coronal sections were made at +1.0 and −0.8 mm from the bregma. To check the existence of infarction, a coronal

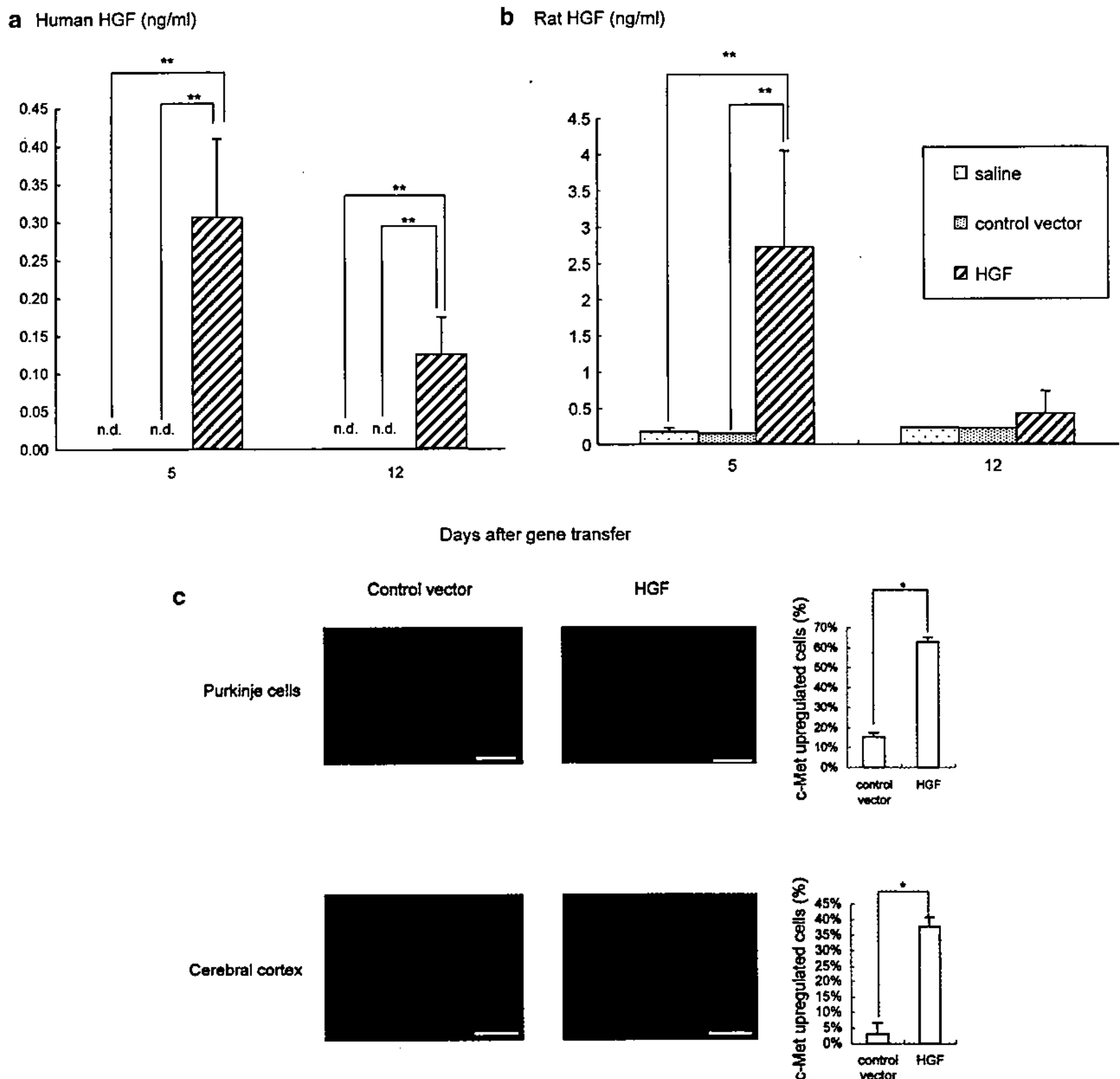

Figure 1. Concentrations of human (a) and rat HGF (b) in CSF at 5 and 12 days after gene transfer. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector; n.d., not detected; $^{**}P<0.01$. c, Immunohistochemical staining for c-met at 5 days after gene transfer in cerebral cortex and injected site (cerebellum). Top, bar=50 μm; bottom, bar=25 μm.

section at +1.0 mm was stained with TTC as described above. Leakage of Evans blue dye was calculated as leakage (%)=[LT−(RT−RB)]/LT×100, where LT is the left hemisphere, RT the right hemisphere, and RB the area stained blue.

Evaluation of Capillary Density

By use of a recently developed microangiographic technique,[26] capillary density and blood-brain leakage were evaluated in the cerebral cortex after MCA occlusion. This technique allows evaluation of BBB function as well as vascular pattern. Briefly, fluorescent albumin solution was prepared by reconstituting 500 mg bovine desiccate albumin-fluorescein isothiocyanate (Sigma-Aldrich) in 50 mL PBS. The solution was injected via the jugular vein at a rate of 1 mL/min (10 mL/kg) 24 hours after MCA occlusion. The same amount of blood was withdrawn before the injection to avoid systematic blood pressure elevation. Brain was fixed in 10% formalin solution, cut in the coronal plane at 100 μm, and mounted with a Prolong Antifade Kit (Molecular Probes Inc). Because regional variation in brain capillary density has been reported,[26] we set the region of interest at the surface of the cerebral cortex (width, 0.625 mm; depth, 0.8 mm). The region of interest was set as the region supplied by the anterior cerebral artery, because the area was adjacent to the area supplied by the MCA. Five consecutive sections in each rat were observed with a confocal laser microscope (Bio-Rad). The acquired images were imported into Adobe Photoshop (version 7.0, Adobe System), and the color of the image was inverted. Then, the area or length of vessels was analyzed with an Angiogenesis Image Analyzer (version 1.0, Kurabo).

Statistical Analysis

All values are expressed as mean±SEM. ANOVA with subsequent Duncan's test was used to determine the significance of differences in multiple comparisons. Differences with a probability value of $P<0.05$ were considered significant.

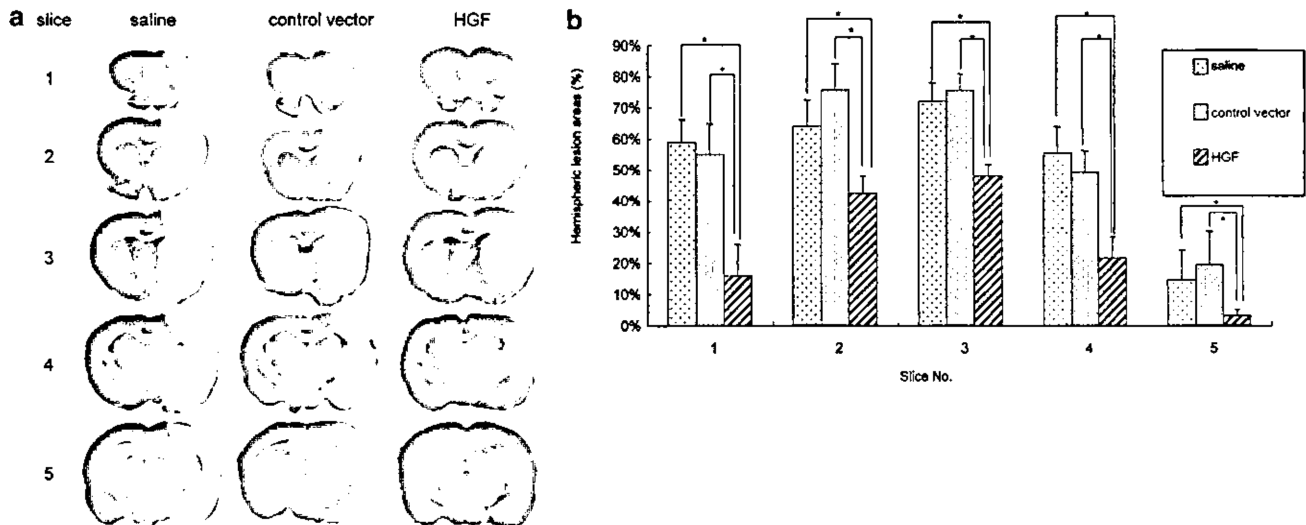

Figure 2. a, Reduction of infarcted area by HGF gene transfer: coronal sections stained with TTC at 24 hours after MCA occlusion. Red region shows intact area; white region shows infarcted area. b, Quantification of hemispheric lesion area 24 hours after ischemia. Corrected HLA was calculated as HLA (%)=[LT−(RT−RI)]/LT×100. LT indicates left hemisphere; RT, area of right hemisphere; RI, infarcted area; Saline, rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector. Number of slice is consistent with that in Figure 3. *$P<0.05$. n=6 for each group.

Results

Reduction of Infarcted Area by In Vivo Transfer of Human HGF Gene Into Subarachnoid Space

To test for successful gene transfer via the subarachnoid space, the concentrations of human HGF and rat HGF in CSF were measured by ELISA at 5 and 12 days after gene transfer (Figure 1, a and b). On day 5, human HGF could be detected in the CSF of rats transfected with human HGF vector, whereas human HGF protein could not be detected in control rats. The increase in human HGF protein in CSF continued up to 12 days after transfection. Interestingly, an increase in rat endogenous HGF was also observed in rats transfected with human HGF vector compared with control ($P<0.01$). Because upregulation of the receptor of HGF, c-met, has been reported in the central nervous system after human HGF gene transfer,[17] immunohistochemical staining for c-met was also examined. Consistently, upregulation of c-met was observed in the cerebral cortex as well as the brainstem and cerebellum of rats transfected with human HGF vector (Figure 1c). During the experimental periods, there was no abnormal activity, such as convulsion, after gene transfer into the subarachnoid space.

Given the successful gene transfer, we also investigated whether HGF could reduce ischemic injury in the right MCA occlusion model. The infarcted area in coronal sections was clearly detected by staining with TTC at 24 hours after MCA occlusion in rats transfected with control vector. Importantly, the ischemic area was reduced significantly in rats transfected with human HGF gene compared with control vector ($P<0.01$; Figure 2). There was no significant difference in infarcted area between rats transfected with control vector and sham-operated rats. Histological examination demonstrated a vague ischemic boundary in rats transfected with human HGF gene compared with control vector, consistent with the findings in coronal sections stained with TTC. Interestingly, numerous TUNEL-positive cells were observed in rats transfected with control vector, whereas a significant decrease in TUNEL-positive cells was detected in rats transfected with HGF gene ($P<0.01$; Figure 3). There was no significant difference in mean blood pressure and rectal temperature among the groups.

In addition, we investigated the effect of overexpression of HGF on capillary density in the cerebral cortex. As expected, the capillary density in rats transfected with human HGF vector showed more complex patterns than with control vector (Figure 4). As shown in Figure 5, in each region, the scores of area and length of vessels were significantly higher in rats transfected with human HGF vector compared with control vector ($P<0.01$). Importantly, there was no leakage through the BBB in rats transfected with human HGF vector, whereas destruction of the BBB was reported previously in the ischemic brain. To assess functional activity, we measured neurological severity score. As shown in Figure 6, neurological score was decreased significantly in rats transfected with human HGF vector compared with control vector ($P<0.01$). There was no significant difference in neurological severity score between rats transfected with control vector and sham-operated rats.

Inhibition of Destruction of BBB by Transfer of HGF Gene

Finally, we studied the side effects of overexpression of HGF, because overexpression of VEGF was reported to stimulate cerebral edema.[27,28] The infarcted hemisphere at 24 hours after MCA occlusion contained more water than the intact hemisphere ($P<0.01$; Figure 7). Unlike those with VEGF, the water content in the brain of rats transfected with human HGF was significantly decreased compared with control vector ($P<0.05$; Figure 7). Finally, we further checked the leakage of Evans blue dye to assess the extent of BBB destruction. Leakage of Evans blue dye was clearly detected in a wide