Novel Therapeutic Strategy to Treat Brain Ischemia
Overexpression of Hepatocyte Growth Factor Gene Reduced Ischemic Injury Without Cerebral Edema in Rat Model

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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
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. 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, 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, HGF has a neuroprotective effect in vitro and in vivo. 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. Briefly, the virus suspension (15 000 hemagglutinating units) was inactivated by UV irradiation (99 mJ/cm²) and mixed with plasmid DNA (400 µg) and 0.5% 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. The control vector was an 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). 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. 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). 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 transcatheter 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 Fluor 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 ApoTag 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. To assess the ischemic area, we calculated the hemicraniectomy 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 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. 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. 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. Coronal sections were made at +1.0 and −0.8 mm from the bregma. To check the existence of infarction, a coronal...
section at +1.0 mm was stained with TTC as described above. Leakage of Evans blue dye was calculated as leakage \(\% = \frac{\text{LT} - (\text{RT} - \text{RB})}{\text{LT} \times 100}\), 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, 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 desiccated 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 \(\mu\)m, and mounted with a Prolong Antifade Kit (Molecular Probes Inc). Because regional variation in brain capillary density has been reported, 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).

**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 \(\mu\)m; bottom, bar=25 \(\mu\)m.

**Statistical Analysis**

All values are expressed as mean\(\pm\)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.
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. 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 ($P<0.05$). 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 area of the infarcted hemisphere in rats transfected with control vector, whereas a significant decrease in leakage was detected in rats transfected with HGF gene ($P<0.01$; Figure 7).

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 area of the infarcted hemisphere in rats transfected with control vector, whereas a significant decrease in leakage was detected in rats transfected with HGF gene ($P<0.01$; Figure 7).
area of brain in rats transfected with control vector (Figure 8a). There was no significant difference in infarcted area between rats transfected with control vector and sham-operated rats. Of importance, leakage of Evans blue dye was significantly less in rats transfected with human HGF vector than control vector, especially in the cerebral cortex ($P<0.01$; Figure 8).

**Discussion**

Disruption of blood flow to the brain initiates a cascade of events that produces neuronal death and leads to neurological dysfunction. Therefore, to prevent brain injury, numerous studies have focused on the development of neuroprotective agents that effectively prevent delayed neuronal death after transient forebrain ischemia. Recently, HGF has been the center of interest in neuroprotective substances, because HGF works as a survival factor for embryonic motor neurons. Moreover, sensory and sympathetic neurons and their precursors respond to HGF with increased differentiation, survival, and axonal outgrowth. The broad spectrum of HGF activities and its observed synergy with other neurotrophic factors suggest that the major role of HGF is to potentiate the response of developing neurons to specific signals. In addition, HGF is a well-known potent angiogenic growth factor in various models, including a brain hypoperfusion model. However, the clinical usefulness of such neuroprotective agents as HGF is quite limited because of the presence of the BBB, which makes the central nervous system relatively inaccessible to circulating proteins and peptides. One method to overcome this limitation is to use a drug delivery system into the central nervous system. In particular, we focused on gene transfer into the subarachnoid space, because intrathecal injection into the cisterna magna with a needle involves no systemic anesthesia, no burr hole, and no pain. Using the HVJ-envelope vector, the reporter gene was transferred into the widespread meninges and adventitial cells of arteries by intrathecal injection via the cisterna magna and was not transferred to other organs except the brain. In the present

**Figure 3.** a. TUNEL staining of coronal sections of cerebral cortex in boundary of infarct area after 1 day of focal cerebral ischemia. Bar=100 $\mu$m. b, Number of TUNEL-positive cells. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.01$ vs control group. n=6 for each group.

**Figure 4.** Vascular patterns in cerebral cortex at 24 hours after MCA occlusion. Region of interest was set at 2 points on surface of cerebral cortex (c). Rats transfected with human HGF gene showed a significant increase in capillary density in both intact (a) and infarcted (b) hemispheres. Dotted line in c represents infarcted region. a and b, bar=100 $\mu$m; c, bar=1 mm. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector.
study, human HGF protein was detected in the CSF after HGF gene transfer. In addition to the increase in human HGF, rat HGF concentration was increased ~10-fold compared with the nontreatment group. We speculate that the secreted human HGF in CSF from the brain surface, such as the meninges, augmented the secretion of rat endogenous HGF in an autocrine–paracrine manner. Importantly, c-met, the receptor for HGF, was increased by transfection not only at the site of injection but also in the cerebral cortex. Recently, we reported that activation of the transcription factor ets-1 plays a pivotal role in the upregulation of HGF and c-met by HGF.\textsuperscript{31} Although the present study cannot elucidate the contribution of upregulation of endogenous HGF, the positive feedback of HGF might play a role in the sustained beneficial effects of HGF.

Given the neurotrophic and angiogenic character of HGF, the present study demonstrated that (1) pretreatment with HGF gene transfer prevented ischemic injury in the rat MCA occlusion model, associated with a significant reduction in apoptotic cells; (2) overexpression of HGF reduced abnormal neurological findings, accompanied by a significant increase in capillary density; and (3) in vivo transfer of HGF gene reduced the destruction of the BBB without the exacerbation of cerebral edema. The region rescued by HGF gene transfer was primarily in the ischemic boundary region around the infarcted area, the so-called ischemic penumbra. In fact, the boundary zone in rats transfected with human HGF gene

Figure 5. Capillary density as assessed by quantitative analysis at 24 hours after MCA occlusion. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. Number of ROI is same as in Figure 4. *P<0.05, **P<0.01 vs control vector (n=7 in each group).

Figure 6. Neurological severity score at 24 hours after MCA occlusion. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector. *P<0.05 (n=7, each group).

Figure 7. Water content after 24 hours of cerebral ischemia. Water content (WC) was calculated as WC (%)=(wet wt−dry wt)/wet wt×100. *P<0.05 (n=7 in each group).
demonstrated a larger area of mixed intact and dead neurons and fewer TUNEL-positive neurons. A neuroprotective effect of HGF has been reported in vitro and in vivo.\textsuperscript{15–17,30,32,33} The mechanism of the neuroprotective effects of HGF is inhibition of apoptosis via the MAP kinase pathway\textsuperscript{32} and the phosphatidylinositol-3 kinase/Akt pathway.\textsuperscript{16,33} Another possible mechanism for the reduction of the infarct area might be the development of collateral circulation. An angiogenic effect of HGF was reported in a rat cerebral hypoperfusion model\textsuperscript{34} and rat transient focal cerebral ischemia model,\textsuperscript{9} in addition to the present study. Because the prevention of cerebral infarction was observed at 24 hours after transfection, the neuroprotective action of HGF might be contributed largely to the present results.

In the progression to human clinical gene therapy, severe side effects could be important. In particular, although recombinant VEGF is effective to reduce infarct volume when administered on the brain surface,\textsuperscript{10} early postischemic (1 hour) administration of recombinant VEGF165 to ischemic rats significantly increased BBB leakage, hemorrhage, and ischemic lesions.\textsuperscript{27} Thus, it is noteworthy to document the effects of HGF on leakage of the BBB. As a result, an increase in area and length of vessels in rats transfected with human HGF gene was observed without BBB leakage, suggesting that the collateral development induced by angiogenesis may contribute in part to the reduction of cerebral infarction volume. Moreover, it is important to examine whether HGF exacerbates cerebral edema after ischemic injury. The present study clearly demonstrated that overexpression of HGF did not exacerbate cerebral edema, in contrast to reported studies in which VEGF augmented cerebral edema and leakage of the BBB.\textsuperscript{27,28,35} Indeed, human gene therapy in patients with limb ischemia using VEGF also demonstrated lower-limb edema as a side effect,\textsuperscript{36} whereas human gene therapy using HGF plasmid DNA did not. The difference in edema formation between HGF and VEGF might be a result of the effects on vascular smooth muscle cells (VSMCs). HGF stimulates the migration of VSMC without their multiplication, but VEGF does not stimulate either the migration or the proliferation of VSMCs because of the lack of VEGF receptors on VSMCs.\textsuperscript{37} Thus, angiogenesis induced by VEGF is featured as a delay in the maturation of blood vessels. In contrast, because HGF simultaneously stimulates the migration of both endothelial cells and VSMCs, the blood vessels might mature in a well-coordinated way, thereby avoiding the release of blood-derived cells into the extracellular space. From these viewpoints, HGF might be useful to treat cerebral ischemia. Because experimental conditions may not be ideal to test the clinical usefulness, further studies are necessary in advancing toward human gene therapy.

Overall, HGF gene transfer using HVJ-envelope vector reduced ischemic injury without exacerbation of cerebral edema or BBB leakage. Although the prevention of cerebral infarction is not advanced enough for human gene therapy, proof of the improvement of stroke-related symptoms might be necessary to promote new therapeutic options to treat cerebral ischemia using HGF gene therapy. In particular, because the safety issue of HVJ-envelop vector is unknown in human trials, continuous development of systems involving vectors, promoters, or alternative routes of administration may help to achieve human gene therapy for cerebrovascular disease in the future.

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