Gene Transfer of Hepatocyte Growth Factor to Subarachnoid Space in Cerebral Hypoperfusion Model

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Abstract—Although cerebral hypoperfusion caused by cerebral occlusive disease leads to cerebral ischemic events, an effective treatment has not yet been established. Recently, a novel therapeutic strategy for ischemic disease using angiogenic growth factors to expedite and/or augment collateral artery development has been proposed. Therapeutic angiogenesis might be useful for the treatment of cerebral occlusive disease. Hepatocyte growth factor (HGF) is a potent angiogenic factor, in addition to vascular endothelial growth factor (VEGF), whereas in the nervous system HGF also acts as a neurotrophic factor. Therefore, we hypothesized that gene transfer of these angiogenic growth factors could induce angiogenesis, thus providing an effective therapy for cerebral hypoperfusion or stroke. In this study, we employed a highly efficient gene transfer method, the viral envelop (Hemagglutinating Virus of Japan [HVJ]-liposome) method, because we previously documented that β-galactosidase gene could be transfected into the brain by the HVJ-liposome method. Indeed, we confirmed wide distribution of transgene expression using β-galactosidase via injection into the subarachnoid space. Of importance, transfection of HGF or VEGF gene into the subarachnoid space 7 days before occlusion induced angiogenesis on the brain surface as assessed by alkaline phosphatase staining (P<0.01). In addition, significant improvement of cerebral blood flow (CBF) was observed by laser Doppler imaging (LDI) 7 days after occlusion (P<0.01). Unexpectedly, transfection of HGF or VEGF gene into the subarachnoid space immediately after occlusion of the bilateral carotid arteries also induced angiogenesis on the brain surface and had a significant protective effect on the impairment of CBF by carotid occlusion (P<0.01). Interestingly, coinjection of recombinant HGF with HGF gene transfer revealed a further increase in CBF (P<0.01). Here, we demonstrated successful therapeutic angiogenesis using HGF or VEGF gene transfer into the subarachnoid space to improve cerebral hypoperfusion, thus providing a new therapeutic strategy for cerebral ischemic disease. (Hypertension. 2002;39:1028-1034.)

Key Words: endothelial growth factors ■ cerebral ischemia ■ stroke ■ endothelium ■ DNA

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 to not only cerebral ischemic events, but also neuropathological changes including dementia,1–4 an effective treatment for hypoperfusion has not yet been established. Ischemic stroke induces active angiogenesis, particularly in the ischemic penumbra, which correlates with longer survival in humans.5 However, the natural course of angiogenesis is not sufficient to compensate for the damage caused by the hypoperfusion. Recently, preclinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia,6–11 a concept called therapeutic angiogenesis. Most of the studies have used endothelial growth factor (VEGF), also known as vascular permeability factor, as well as a secreted endothelial-cell mitogen. The endothelial cell specificity of VEGF has been considered to be an important advantage for therapeutic angiogenesis, because endothelial cells represent the critical cellular element responsible for new vessel formation.12,13 More recently, the efficacy of therapeutic angiogenesis using VEGF gene transfer has been reported in human patients with critical limb ischemia or myocardial infarction.14–17 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. However, to date, it is not known whether this strategy is effective in cerebral ischemia.

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In addition, hepatocyte growth factor (HGF) is a potent angiogenic growth factor, although it was originally described as a pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activities toward a variety of cells. Both HGF and the c-Met/HGF receptor of membrane-spanning tyrosine kinase are expressed in various regions of the brain. More importantly, functional coupling between HGF and c-Met enhances the survival of hippocampal neurons in primary culture and induces neurite outgrowth in neuronal development in vitro. Recently, it was reported that HGF is induced in neurons during ischemia and that HGF has a neuroprotective effect against postischemic delayed neuronal death in the hippocampus. In this study, we tested the possibility that gene transfer of HGF or VEGF into the subarachnoid space could cause angiogenesis on the brain surface and improve vessel function by forming anastomoses around the ischemic area, resulting in improvement of cerebral hypoperfusion induced by cerebral artery occlusion.

**Methods**

**Construction of Plasmids**

To produce a HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eucaryotic expression plasmid that uses the cytomegalovirus (CMV) promoter/enhancer. The control vector was pUC-SRox expression vector plasmid, which has the same structure as the promoter, but does not contain HGF cDNA. We obtained β-galactosidase gene expression vector driven by SV 40 promoter from a commercially available source (Promega Corporation, Madison, Wis).

**Ligation of Bilateral Carotid Arteries**

The bilateral carotid arteries of male Sprague-Dawley rats (350 to 400 g; Charles River Japan, Atsugi, Japan) were ligated. The procedures used for the preparation of Hemagglutinating Virus of Japan (HVJ)-liposomes have been described previously. To establish an efficient method of gene transfer into the central nervous system (CNS) in vivo, we examined three different approaches for delivering plasmid using the HVJ-liposome method: (1) direct infusion into the internal carotid artery, (2) infusion into the lateral ventricle, and (3) infusion into the cisterna magna. For infusion into the internal carotid artery, a polyethylene catheter (PE-50) was introduced into the left external carotid artery via a cutdown into the left common carotid artery. The distal external segment was transiently isolated with temporary ligatures. HVJ-liposome complex (1 mL) containing a reporter gene was infused into the external segment using a stereotactic frame (Narisihge Scientific Instrument Laboratory). A stainless steel cannula (30 gauge, Becton Dickinson) with a Teflon connector (FEP tube, Bioanalytical Systems) was introduced into the left lateral ventricle. The stereotactic coordinates were as follows: 1.3 mm posterior to the bregma, 2.1 mm lateral to the midline, and 3.6 mm below the skull surface. HVJ-liposome complex was infused into the lateral ventricle (20 μL). No behavioral change, such as convulsions or abnormal movement of the extremities, was observed in any animal undergoing injection. 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 occipitocervical midline incision. A stainless steel cannula (27 gauge) was introduced into the cisterna magna. After withdrawal of 100 μL cerebrospinal fluid for confirmation of the cannula position, and to avoid increased intracerebral pressure, HVJ-liposome solution (100 μL: 10 μg/μL) was carefully injected over 1 minute into the cisterna magna (subarachnoid space). Therefore, the animals were placed head down for 30 minutes. In the preliminary experiments, the blood pressure was not apparently changed after gene transfer of HGF or VEGF.

**Blood Flow as Assessed by Laser Doppler Imaging**

Laser Doppler imaging (LDI) was used to record serial blood flow measurements over the course of 2 weeks postoperatively. The LDI system (Moore Instruments, Ltd) incorporates a 2 mW helium-neon laser to generate a beam of light that sequentially scans a 12×12-cm tissue surface area to a depth of 600 μm. Low or no perfusion was displayed as dark blue, whereas maximal perfusion was displayed as red. Good correlation of LDI with the number of blood vessels has been reported in several ischemia models. Our preliminary experiment also confirmed the previous observation. LDI was used to record perfusion of the brain surface, before, just after, and 7 and 14 days after occlusion. Through a midline scalp incision, a 12×12-mm bone window was made with an electric drill. Consecutive measurements were obtained over the same bone window. Color-coded images were recorded, and analyses were performed by calculating the average perfusion for total brain area of each rat. To account for variables, including ambient light and temperature, calculated perfusion was expressed as a ratio of postischemic to preischemic (untreated) brain.

**Histopathological Examination**

For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, after perfusion-fixing in 3% paraformaldehyde:20% sucrose solution for 1 day, 25-μm frozen sections in the coronal plane were taken at 100-μm intervals. Sections were stained with X-gal to identify infected neurons expressing β-galactosidase. For alkaline phosphatase (ALP) staining, 25-μm frozen sections under 5 mm from the surface in the cortex located at the midline in the bregma were taken at 100-μm intervals. At least 3 individual sections were analyzed. Omission of primary antibodies and staining with type- and class-matched irrelevant immunoglobulin served as a negative control for each antibody.

**ELISA for Human HGF and VEGF in Cerebrospinal Fluid**

Cerebrospinal fluid (CSF, 100 μL) from rats, both before and 7 and 14 days after occlusion of the bilateral carotid arteries, was used for the experiments. The concentration of human HGF in the CSF was determined by enzyme-immunoassay using anti-human HGF antibody (Institute of Immunology, Tokyo, Japan). The antibody against human HGF reacts with only human HGF and not with rat HGF. The concentration of rat and human VEGF was also measured by ELISA kit (R&D Systems) according to the manufacturer’s recommendations. Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells transfected with human HGF cDNA.

**Results**

**In Vivo Transfection of β-Galactosidase Gene Into Brain Using HVJ-Liposome Delivery System**

To develop an efficient method of gene transfer into the CNS, we initially directly infused HVJ-liposome complex containing β-galactosidase gene into the rat internal carotid artery. However, intra-arterial infusion into the carotid artery produced little expression of transgene in the brain and microvascular endothelial cells at 3 and 7 days after injection. Therefore, we injected HVJ-liposome complex into the lateral ventricle or the subarachnoid space. Injection of β-galactosidase gene using the HVJ-liposome method resulted in positive staining for β-galactosidase.
after injection (Figures 1A and 1B). Following injection of β-galactosidase gene into the lateral ventricle, β-galactosidase expression was mainly observed around the lateral ventricle and choroid plexus, consistent with our previous report. In contrast, β-galactosidase expression was observed in both sides of the brain following injection into the cisterna magna. As shown in Figures 1C and 1D, β-galactosidase–positive cells could be detected both in cerebral and cerebellar regions. In the treatment of cerebral hypoperfusion by angiogenesis in the clinical setting, it seems preferable to infuse into the subarachnoid space rather than inject into the lateral ventricle, using a stereotaxic frame.

**In Vivo Transfection of HGF or VEGF Gene Into the Subarachnoid Space**

To demonstrate the successful transfer of HGF or VEGF gene into the CNS, we first measured the protein expression of these molecules in the CSF by ELISA (n=4, each group). Expectedly, human HGF protein could not be detected in the CSF of control rats using specific anti-human antibodies before transfection, because anti-human HGF antibody could not react with rat HGF (Figures 2A and 2B). Seven days after transfection, human HGF was readily detected in the CSF of rats transfected with human HGF gene, but not before transfection (Figure 2A). Even at 14 days after transfection, human HGF could be detected in rats transfected with human HGF vector (P<0.01). Similarly, endogenous rat VEGF could be detected in the CSF of rats transfected with human VEGF gene throughout the experimental period, because the ELISA for VEGF could not distinguish rat and human VEGF. However, VEGF concentration in the CSF was significantly increased in rats transfected with human VEGF vector 7 days after transfection, and this continued up to 14 days after transfection (P<0.05, Figure 2).

**Angiogenesis on Brain Surface Induced by Transfection of Human HGF Gene**

Therefore, we analyzed the changes in cerebral blood flow (CBF) in untransfected rats before, immediately after, 7 days after, and 14 days after occlusion. Expectedly, CBF was markedly decreased immediately after occlusion of the bilateral carotid arteries and, thereafter, gradually increased time-dependently (Figures 3A and 3B). However, CBF was still significantly lower 7 and 14 days after occlusion compared with pretreatment (P<0.01, Figure 3B). In addition, CBF was also significantly lower 7 and 14 days after occlusion compared with sham-operated rats. Thus, we hypothesized that gene transfer of human HGF into the subarachnoid space might result in a beneficial effect on hypoperfusion observed in rats after occlusion of the bilateral carotid arteries.

Initially, we investigated whether transfection of angiogenic growth factor gene can be effective when performed before occlusion of the carotid arteries (30 minutes before ligation). Because the peak in the decrease of blood flow was at 1 week after ligation, we chose that point to test the effects of HGF or VEGF gene transfer on blood flow. Interestingly, transfection of HGF or VEGF gene significantly prevented the decrease in CBF induced by occlusion of the carotid arteries compared with rats transfected with control vector at

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**Figure 1.** Expression of β-galactosidase on brain surface at 1 week after transfection. A, View from bottom: (left) intra-arterial injection of HVJ-liposome (1 mL), (center) injection of HVJ-liposome (100 μL) into cisterna magna, (right) injection of HVJ-liposome (20 μL) into lateral ventricle; n=4 for each group. B, Representative cross-section of β-galactosidase staining in brain at 1 week after transfection: (top row, control) intra-arterial injection of HVJ-liposome (1 mL); (middle row, lateral ventricle) injection of HVJ-liposome (20 μL) into lateral ventricle; (bottom row, cisterna magna) injection of HVJ-liposome (100 μL) into cisterna magna; n=4 for each group. C, Expression of β-galactosidase on brain surface in the cortex region (left) and the cerebellar region (right). Left panel demonstrates the negative staining against β-galactosidase in the brain transfected with control vector, as negative control. D, Number of positive stained cells against β-galactosidase in each brain area; n=4 for each group.
1 week after transfection (Figure 3C, P<0.01). There was no significant difference in CBF between untransfected rats and rats transfected with control vector. In addition, we measured CBF in rats treated with recombinant HGF (200 μg), HGF gene (10 μg/mL), or a combination of recombinant HGF and HGF gene transfer. Each treatment was performed 10 minutes before occlusion of the carotid arteries. In rats treated with recombinant HGF alone, there was no significant increase in CBF compared with untreated rats at 1 week after transfection (Figures 3C and 3D). However, transfection of human HGF gene resulted in a significant increase in CBF 7 days after occlusion compared with rats transfected with control vector (P<0.05, Figure 3D). Furthermore, in rats treated with a combination of recombinant HGF and HGF gene transfer, CBF at 7 days was much greater than with HGF gene transfaction alone.

Looking toward human gene therapy, we transfected human HGF or VEGF genes immediately (5 minutes) after occlusion of the carotid arteries, because this is close to the clinical situation. Importantly, transfection of HGF gene into the subarachnoid space of ligated rats immediately after occlusion of the carotid arteries significantly improved the increase in CBF compared with rats transfected with control vector at 1 week after transfection (Figure 3E, P<0.01). Similarly, transfection of human VEGF gene also significantly attenuated the decrease in CBF induced by occlusion of the carotid arteries compared with control vector (P<0.05, Figure 3E). There was no significant difference in CBF between untreated rats and rats transfected with control vector. Finally, we measured the number of blood vessels in brain transfected with HGF after occlusion of the carotid arteries and without ligation. Each treatment was performed immediately after occlusion of the carotid arteries. Expectedly, transfection of human HGF vector into the brain of nonligated rats resulted in a significant increase in the number of vessels compared with control vector at 14 days after transfection (P<0.01, Figures 4A and 4B) and was accompanied by an increase in blood flow (Figures 4A and 4B). Similar to the experiments in nonligated brain, the number of vessels was significantly increased in the brain of ligated rats transfected with HGF gene (P<0.01). These results suggest that angiogenesis induced by HGF or VEGF gene transfer, either before or after occlusion of the arteries, may improve chronic cerebral hypoperfusion.

**Figure 2.** Human HGF (A) and VEGF (B) protein levels in rat cerebrospinal fluid determined by ELISA after transfection of human HGF or VEGF vector into cisterna magna. UT indicates untreated rats; 7d and 14d, 7 and 14 days after transfection. **P<0.01 versus UT; n=4 for each group.**

**Discussion**

The development of new blood vessels, or angiogenesis, begins with activation of endothelial cells in the parent vessel. Capillary growth is usually limited in adult tissues, but it can resume under hypoxic conditions and pathological conditions that are associated to a decreased oxygen supply, such as ischemia. Because inefficient vascular supply and the resultant reduction in tissue oxygen tension often lead to neovascularization to satisfy the needs of the tissues, to know the triggers of natural neovascularization is thought to be important. In the CNS, it is reported that VEGF gene is rapidly induced in the brain by ischemic insult. HGF gene is also expressed in the brain in the early phase of cerebral ischemia. Furthermore, recombinant VEGF is effective in reducing infarct volume when administered on the brain surface, and administration of recombinant HGF is also effective in reducing infarction size when continuously injected into the brain. How does administration of recombinant VEGF or HGF protect against cerebral infarction? To address this issue, we examined the angiogenic properties of these growth factors in the brain. It is well known that angiogenesis induced by ischemic stroke, particularly in the ischemic penumbra, correlates with longer survival in humans. Therefore, angiogenesis may play a pivotal role in the recovery of cerebral ischemia and in the prevention of stroke. The present study demonstrated that (1) gene transfer into the CNS was feasible by injection into the subarachnoid space by the HVJ-liposome method, (2) gene transfer of HGF or VEGF successfully caused angiogenesis in the brain, (3) transfection of HGF or VEGF gene was sufficient to increase CBF in the ischemic brain, suggesting effective treatment for chronic hypoperfusion of the brain caused by occlusion of the major vessels, and (4) therapeutic angiogenesis by VEGF or HGF was effective either before or after occlusion of the carotid arteries. Although blood flow as assessed by laser imaging may not be fully correlated with the deep circulation in brain, this report describes therapeutic angiogenesis to treat cerebrovascular disease using HGF gene transfer. Further studies are necessary to elucidate the mechanisms of the protective actions of HGF and VEGF. Interestingly, HGF has been reported to have the neurotrophic activity on neuron cell death. In contrast, early postischemic (1 hour) administration of recombinant VEGF165 to ischemic rats significantly increased blood-brain barrier leakage, hemorrhagic
Figure 3. A. Typical example of laser Doppler imaging (LDI) to evaluate cerebral blood flow after occlusion of carotid arteries. Low or no perfusion is displayed as dark blue, whereas the highest perfusion is displayed as white. B. Quantitative analysis of cerebral blood flow expressed as flux measured by LDI after occlusion of carotid arteries. pre indicates before occlusion; post, immediately after occlusion of carotid arteries; 7d, 7 days after occlusion; 14d, 14 days after occlusion. *P<0.05, **P<0.01 versus pre; n=6 for each group. C. Typical example of LDI to evaluate cerebral blood flow in rats transfected with HGF or VEGF gene before carotid artery occlusion at 1 week after transfection. D. Quantitative analysis of cerebral blood flow expressed as flux measured by LDI in rats transfected with HGF or VEGF gene before carotid artery occlusion at 1 week after transfection. Transfection was performed 10 minutes before occlusion of the carotid arteries. UT indicates untransfected rats; RC, recombinant HGF; GENE, HGF gene; GENE & RC, recombinant HGF and HGF gene; GENE (VEGF), VEGF gene. *P<0.05, **P<0.01 versus pre or UT; n=6 for each group. E. Quantitative analysis of cerebral blood flow expressed as flux measured by LDI in rats transfected with HGF or VEGF gene after occlusion of carotid arteries. Transfection was performed immediately after occlusion of the carotid arteries. pre indicates before occlusion of carotid arteries of untransfected rats; post, rats transfected with control gene immediately after occlusion of carotid arteries of untransfected rats; HGF, rats transfected with HGF gene immediately after occlusion of carotid arteries of untransfected rats; VEGF, rats transfected with VEGF gene after occlusion of carotid arteries. *P<0.05, **P<0.01 versus pre; #P<0.05 versus post; n=5 for each group.
Previously reported studies employed the infusion of recombinant protein continuously into the brain or subarachnoid space, the manipulation of which is rather harmful in clinical situations. This procedure resulted in rapid disappearance of recombinant angiogenic growth factors. To explore the clinical application, we also compared the angiogenic activity of recombinant protein with that of gene transfer in this study. Although administration of recombinant protein is sufficient to induce angiogenesis in peripheral vascular disease,\textsuperscript{7,8} our present study failed to demonstrate a significant increase in CBF by a single injection of recombinant protein. In contrast, transfection of HGF or VEGF gene was sufficient to increase CBF in the ischemic brain. Therefore, it is reasonable to apply gene transfer rather than recombinant therapy to achieve sustained expression and secretion of angiogenic growth factors in and on the surface of the ischemic brain.

On the other hand, we also elucidated a useful approach for gene transfer into the CNS for clinical gene therapy. As previously, we and others have reported high transfection efficiency of several gene transfer methods by administration into the lateral ventricle using a stereotaxic frame. The expression pattern of transgene observed in this study (mainly around the lateral ventricles and the choroid plexus) was consistent with our previous report.\textsuperscript{35} However, this approach seems to be far from the realm of clinical gene therapy. Fortunately, we found that transgene expression was observed on the brain surface following injection into the cisterna magna. Importantly, the previous report using the HVJ-liposome method documented that approximately 90% β-galactosidase-positive cells were stained with antineurofilament antibody, which are considered terminally differentiated neurons.\textsuperscript{37} In addition, Purkinje cells of cerebellar vermis, trigeminal nucleus, cochlear nucleus, ocularomotor nucleus, and other regions have been reported to be transfectable by the HVJ-liposome method. Together with the finding that HGF or VEGF can be secreted from cells because of the presence of signal peptides, secretion of HGF or VEGF into the subarachnoid space from transfected cells in the brain could result in beneficial effects for the injured brain. Indeed, HGF was still detectable in the CSF 14 days after transfection. Because the experimental condition may not be ideal for testing clinical usefulness, further studies are necessary in human gene therapy. Continuous development of systems related to the vector, promoter, or route of administration may help to provide human gene therapy for cerebrovascular disease in the future.

**Perspectives in Gene Therapy for Treatment of Cerebrovascular Disease**

Overall, the present study describes a novel therapeutic strategy using the angiogenic properties of HGF or VEGF, which might reduce the symptoms of chronic cerebral hypoperfusion. Recently, we have reported that transfection of HGF gene into the subarachnoid space prevented delayed neuronal death and was accompanied by a significant increase in HGF in the cerebrospinal fluid.\textsuperscript{27} Probably, HGF may be useful in the treatment of neurological clinical symptoms. Stimulation of new vessel formation by angiogenic growth factors is likely to create new therapeutic options in angiogenesis-dependent conditions such as stroke, moyamoya disease, and dementia. Recent clinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in peripheral and myocardial ischemia, as described above. From this viewpoint, the present report should stimulate additional investigations into gene therapy strategies including (1) how to overcome the presence of the blood-brain barrier, which prevents transgenes from reaching their cellular targets, (2) how to avoid deleterious effects in the brain, and (3) how to maintain brain function. In addition to these issues, it is time to take a hard look at practical issues that will determine the real clinical potential. These include (1) further innovations in gene transfer methods, (2) well-defined disease targets, (3) cell-specific targeting strategies, and (4) effective and safe delivery systems. As the development of gene therapy as it relates to the brain provides new information for the treatment of human cerebrovascular disease, further efforts to investigate the biology and pathophysiology of stroke, ische-
mic cerebrovascular disease, dementia, and atherosclerosis should be stimulated.

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