Munehisa Shimamura, Naoyuki Sato, Satoshi Waguri, Yasuo Uchiyama, Takuya Hayashi, Hidehiro Iida, Toshikazu Nakamura, Toshio Ogihara, Yasufumi Kaneda, Ryuichi Morishita

Abstract—There is no specific treatment to improve the functional recovery in the chronic stage of ischemic stroke. To provide the new therapeutic options, we examined the effect of overexpression of hepatocyte growth factor (HGF) in the chronic stage of cerebral infarction by transferring the HGF gene into the brain using hemagglutinating virus of Japan envelope vector. Sixty rats were exposed to permanent middle cerebral artery occlusion (day 1). Based on the sensorimotor deficits at day 7, the rats were divided equally into control vector or HGF-treated rats. At day 56, rats transfected with the HGF gene showed a significant recovery of learning and memory in Morris water maze tests (control vector 50 ± 4 s; HGF 33 ± 5 s; P < 0.05) and passive avoidance task (control vector 132.4 ± 37.5 s; HGF 214.8 ± 26.5 s; P < 0.05). Although the total volume of cerebral infarction was not related to the outcome, immunohistochemical analysis for Cdc42 and synaptophysin in the peri-infarct region revealed that HGF enhanced the neurite extension and increased synapses. Immunohistochemistry for glial fibriary acidic protein revealed that the formation of glial scar was also prevented by HGF gene treatment. Additionally, the number of the arteries was increased in the HGF group at day 56. These data demonstrated that HGF has a pivotal role for the functional recovery after cerebral infarction through neurotogenesis, improved microcirculation, and the prevention of gliosis. Our results also provide evidence for the feasibility of gene therapy in the chronic stage of cerebral infarction. (Hypertension. 2006;47:1-10.)

Key Words: cerebral ischemia ■ genes ■ microcirculation ■ rats

Gene Transfer of Hepatocyte Growth Factor Gene Improves Learning and Memory in the Chronic Stage of Cerebral Infarction

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Middle cerebral artery occlusion (MCAo) is one of the most common causes of focal stroke in humans and causes severe sensorimotor deficits and cognitive dysfunction. The ischemic changes closely resemble those produced in a MCAo model in rats, which causes infarction mainly in the dorsolateral and lateral portions of the neocortex and the entire caudoputamen. Several growth factors are upregulated immediately after MCAo, such as fibroblast growth factor (FGF), brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), and thought to protect neurons or promote angiogenesis after MCAo. In fact, the extension of infarction is prevented by administration of growth factors or gene transfer of growth factors before or immediately after MCAo. However, the therapeutic time window of such treatment is too short for clinical use, because they focused on preventing the extension of neuronal death in the penumbra in the acute stage.

Recently, HGF and c-Met/HGF have been reported to be upregulated mainly in the peri-infarct region as long as 28 days after permanent MCAo and up to 14 days in FGF or VEGF. HGF is a well-known potent pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activity in a variety of cells. Both HGF and the c-Met/HGF receptor of membranes spanning tyrosine kinase are expressed in various regions of the brain. HGF is also involved in the development and maintenance of cortical neurons during differentiation, motogenesis, neuritogenesis, and neuronal survival during the development of the rat cerebral cortex. Interestingly, HGF promotes proliferation and neuronal differentiation of neural stem cells from mouse embryos. In vivo, it has also been demonstrated that HGF promotes angiogenesis in cerebral ischemia in rodents without disrupting the blood–brain barrier. From these viewpoints, we speculated that HGF might play a pivotal role in the functional recovery in the chronic stage of ischemic insult, and its overproduction could improve the cognitive dysfunction. To clarify this speculation, we transferred the human HGF gene into the brain 7 days after MCAo, using the hemagglutinating virus of Japan (HVJ)-
envelope vector,\textsuperscript{10} and examined behavioral tests, MRI, and histological changes. Here, we demonstrated that gene therapy delayed for as long as 7 days improved outcome from ischemic stroke, and HGF is an important growth factor for the recovery of cognitive function in the chronic stage of MCAo through reconstitution of the neuronal network.

\section*{Methods}

\subsection*{Preparation of HVJ-Envelope Vector}

HVJ-envelope vector was prepared as described previously.\textsuperscript{24,25} Briefly, virus suspension (15,000 hemagglutinating units) was inactivated by UV irradiation (99 mJ/cm\textsuperscript{2}) and mixed with plasmid DNA (400 \(\mu\)g) and 0.3\% Triton-X. After centrifugation, it was washed with 1 mL of balanced salt solution (10 mmol/L Tris-Cl (pH 7.5), 137 mmol/L NaCl, and 5.4 mmol/L KCl) to remove the detergent and unincorporated DNA. After centrifugation, the envelope vector was suspended in 100 mL of PBS. The vector was stored at 4°C until use.

\subsection*{Construction of Plasmids}

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.\textsuperscript{26} This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered constitutive. The control vector had the same structure as the expression vector plasmid, including the promoter but not containing HGF cDNA. Plasmids were purified with a QIAGEN plasmid isolation kit (Qiagen).

\subsection*{Surgical Procedure}

Male Wistar rats (270 to 300 g; Charles River Japan, Atsugi, Japan) were used in this study. To generate a permanent MCAo model, the right middle cerebral artery (MCA) was occluded by placement of poly-l-lysine-coated 4-0 nylon around the origin of the MCA, as described previously.\textsuperscript{2} In vivo gene transfer was performed by intracisternal injection as described previously.\textsuperscript{25} Briefly, rats were anesthetized with ketamine (Sankyo) and xylazine (Bayer Ltd). Torso twisting, rats were held by the tail on a flat surface. The degree of left forelimb was evaluated by the degree of left forelimb flexion. For forelimb, we used the following categories (maximum score is 4). For forelimb, we used the following categories (maximum score is 4). For forelimb, we used the following categories (maximum score is 4). For forelimb, we used the following categories (maximum score is 4). For forelimb, we used the following categories (maximum score is 4). For forelimb, we used the following categories (maximum score is 4).

\subsection*{Protocol for Treatment and Behavioral Tests}

Ten rats were only anesthetized (sham operation), and 60 rats were subjected to MCAo (day 1). Based on the neuromuscular function and body weight evaluated on day 7, the rats were divided equally into control vector-treated (n = 23) and HGF-treated (n = 23) groups. Rats showing no palsy on day 7 or that died before day 7 were excluded from the present study (n = 14). On day 55, neuromuscular function and locomotor activity were evaluated in the surviving rats (n = 20 for control vector-treated and n = 22 for HGF-treated rats).

\subsection*{Sensorimotor Deficit and Locomotor Activity}

Although there are various batteries for testing sensorimotor deficit, we used a simple protocol\textsuperscript{27} to evaluate sensorimotor deficit, which used the following categories (maximum score is 4). For forelimb flexion, rats were held by the tail on a flat surface. For torso twisting, rats were held by the tail on a flat surface. The degree of body rotation was checked. For lateral push, rats were pushed either left or right. Rats with right MCA occlusion showed weak or no resistance against a left push. For hind limb placement, one hind limb was removed from the surface. Spontaneous locomotor activity was also measured via the open field test for 30 minutes using an automated activity box (Muromachi Kikai).

\subsection*{MWM Task}

A cylindrical tank 1.5 m in diameter was filled with water (25°C), and a transparent platform 15 cm in diameter was placed at a fixed position in the center of 1 of the 4 quadrants (O’Hara & Co, Ltd). In the hidden platform test, the platform was set below the water level, and it was not seen by the rats. The platform was fixed at 1 quadrant, and the starting point was changed in each trial. A previous study showed a difference in the latency of reaching the platform until day 6 of the session between rats exposed to MCAo 12 to 14 weeks before and control rats, if the tests were performed twice a day.\textsuperscript{28} Based on the results, we carried out the tests twice a day for 6 days. If the rat could not reach the platform, the latency was set at 60 s. In the visible platform test, a flag was placed on the platform, which could be seen by the rats. The tests were carried out twice a day for 6 days. In this trial, the platform and the starting point were changed in each trial. Throughout the tests, the path of swimming was captured by a charge-coupled device video camera and analyzed by National Institute of Health image.

\subsection*{Passive Avoidance Task}

A step-through type of passive avoidance task was used in the present study. The apparatus (Medical Agent) consisted of an illuminated chamber and a dark one. To habituate the rats, they were placed in the illuminated chamber, and the door was opened so that they could enter the dark one. Rats have a habit of entering the dark chamber, because they prefer darkness. In an acquisition trial, the rats were placed in the illuminated chamber and exposed to a 0.6-mA foot shock when they entered the dark chamber. Each trial was continued until the rat learned not to enter the dark chamber for 300 seconds. In retention trials, the rats were placed in the illuminated room 3 days after the acquisition trial. We evaluated the latency (maximum: 300 s) of their staying in the illuminated room.

\subsection*{Immunohistochemical Study}

For histopathologic analysis, other rats (control vector-treated [n = 4] or HGF-treated [n = 4]) rats in each experiment) were treated the same as described above and euthanized on day 11, 14, or 56, followed by transcardial perfusion fixation with normal saline followed by 4% paraformaldehyde. The brain was removed, postfixed, cryoprotected, and cut on a cryostat at 12 \(\mu\)m. After blocking, sections were incubated in 3% normal goat serum and anti-MAP2 (1:100; mouse monoclonal; Sigma-Aldrich, St Louis, MO), GFAP (1:1000; mouse monoclonal; Chemicon, Temecula, CA), and Cdc42 (1:100; mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-mouse goat fluorescent antibody (1:1000 for MAP2 and GFAP, 1:250 for Cdc42, Alexa Flour 546, Molecular Probes). For immunostaining of human HGF or synaptophysin, sections were treated with 2\% H\textsubscript{2}O\textsubscript{2} to block endogenous peroxidase and then incubated with an antibody against human HGF-\(\beta\) (H714; 1:250; rabbit polyclonal; Immuno-Biological Laboratories, Gunma, Japan) or synaptophysin (1:500; mouse monoclonal; Chemicon, Temecula, CA) at 4°C O/N. They were incubated with streptavidin-horseradish peroxidase (Vectorstain Elite ABC; Vector Laboratories, Burlingame, CA), and the biotin-streptavidin-peroxidase complex was detected with diaminobenzidine (human HGF) or tetramethylbenzidine (synaptophysin) peroxidase substrate solution (Vector Laboratories). Negative control sections from each animal received identical preparations for immunohistochemical staining, except that primary antibodies were omitted.

\subsection*{Quantitative Histological Analysis}

To quantify the immunoreactivity for GFAP and synaptophysin, the acquired image was imported into Adobe Photoshop (version 7.0, Adobe System). The color image was converted into a grayscale image. This was imported into Mac SCOPE (version 2.5, Mitani Corporation). The region of interest was set at the peri-infarct region.
in the cerebral neocortex. The peri-infarct region is defined as the area surrounding the lesion, which morphologically differs from the surrounding normal tissue (Figure 1b, part A).29,30 The number of pixels for which the signal was $/H11022$/25 was counted. Immunoreactivity was calculated by the equation: % Area = (Number of high signal pixels)/(Total number of pixels). To quantify the cerebral edema, we calculated the percentage of measured infarct area in the corrected infarct area at 0.7 mm from bregma. The corrected infarct area was calculated as \[LT-(RT-RI)\], where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarct area.10 The infarct region is edematous when the percentage is $/H11022$/100%. The infarct brain is atrophic if the percentage is $/H11021$/100%.

Alkaline Phosphatase Staining

For alkaline phosphatase (ALP) staining, sections were washed in Tris-HCl and incubated for 30 minutes in substrate solution (a mixture of naphtol AS-BI phosphate [$/H9268$-Aldrich] and fast red violet LB salt [$/H9268$-Aldrich]). Five consecutive sections in each rat were observed, and acquired images were imported into Adobe PhotoShop. The color image was converted into a grayscale image. Then, the ROI was set as the region in the peri-infarct region. The area or length of vessels was analyzed with an Angiogenesis Image Analyzer (version 1.0, Kurabo).

Statistical Analysis

All of the values are expressed as mean±SEM. ANOVA was used to determine the significance of differences in multiple comparisons. $P<0.05$ was considered significant.

Results

Transfer of HGF Gene Improves Learning and Memory After Cerebral Infarction

To test for successful gene transfer via the subarachnoid space, the concentration of human HGF in CSF was measured by ELISA at 4, 7, 14, and 21 days after gene transfer. As expected, human HGF could be detected in the CSF of rats transfected with human HGF vector 4 at 7 days after gene transfer, whereas human HGF protein could not be detected in control rats (Figure 1a). Human HGF protein was detected in the pia mater in the

Figure 1. (a) Concentrations of human HGF in cerebrospinal fluid at 4, 7, 14, and 21 days after gene transfer (11, 14, 21, and 28 days after middle cerebral artery occlusion). Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *P<0.01 vs Control. (b, part A) HE staining at 4 days after gene transfer (11 days after middle cerebral artery occlusion). I, infarct region; PI, peri-infarct region; N, normal region. Bar=100 μm. (B through D) Representative images of immunohistochemical staining for human HGF. (B) Peri-infarct region in rats transfected with control vector (rectangle area in A). Bar=50 μm. (C) Contralateral intact region in rats transfected with HGF vector. Bar=50 μm. (D) Peri-infarct region in rats transfected with HGF vector. Arrowhead showed immunopositive cells for human HGF. Bar=50 μm.

Figure 2. Magnetic resonance images of brain. (a) Volume of infarction in all rats calculated in T2-weighted images. Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22). (b) Typical T2-weighted image of coronal section of rat brain. The images were divided into 3 groups: types A, B, and C (described in text). Most rats showed type A, and fewer showed type B or type C.
normal region (Figure 1b, part C), as well as in the pia mater and parenchyma in the infarct and peri-infarct region 4 days after gene transfer using immunohistochemistry (Figure 1b, part D). Although HE staining at 4 days after gene transfer showed that the infarct brain is atrophic in this timing, as reported previously,31 there was no significant difference between rats transfected with the human HGF gene and control vector (control vector 87.1±8.1%, HGF 81.0±4.3%; P value not significant).

To confirm the severity of cerebral infarction, all of the rats were examined by T2-weighted MRI on day 96. Although the total volume of infarction calculated in T2-weighted images was not different between rats transfected with the human HGF gene and control vector (Figure 2a), the pattern of cerebral infarction was divided into 3 groups: (1) type A, high-intensity area seen in the dorsolateral and lateral portions of neocortex and the entire caudoputamen; (2) type B, high-intensity area seen in the dorsolateral and lateral portions of neocortex and in part of the caudoputamen; and (3) type C, high-intensity area seen in part of the lateral neocortex and caudoputamen (Figure 2b). In type C, most part of lateral neocortex was intact.

In the hidden platform test of MWM, which examined spatial learning and memory, the latency in rats transfected with control vector was markedly longer as compared with sham-operated rats, and the latency in rats transfected with HGF vector was significantly shorter than that of rats transfected with control vector (Figure 3a). There were no differences both in swimming speed and visible platform test, which excluded the possible influences of visual loss, sensorimotor deficit, and motivation on the results,32 between rats transfected with control and HGF vector (data not shown). Thus, spatial learning and memory partly, but significantly, recovered in rats transfected with HGF vector. In the passive avoidance task, which was used to measure associated learning and memory,32 the retention of memory was longer in rats transfected with the HGF vector (Figure 3b), which demonstrated a trend toward significance (P=0.053). Sensorimotor deficit and locomotor activity were also tested, because they have some influence on tests of cognitive function.32 Sensorimotor deficit had spontaneously recovered to some extent by day 55 in both groups, and there was no difference between the

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**Figure 3.** Learning and memory in the chronic stage of cerebral infarction. (a) Hidden platform test in MWM test in all rats. Although rats subjected to middle cerebral artery occlusion hardly reached the hidden platform as compared with sham-operated rats, rats transfected HGF vector could reach faster than that of control vector. (b) Retention trial in passive avoidance task in all rats. The latency of rats staying in the illuminated chamber was calculated. (c) Sensorimotor deficit and (d) spontaneous locomotor activity in all rats. There is no sensorimotor deficit in sham-operation rats in “c” (shown as “n.d.”). Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22); Sham, sham-operated rats (n=10); *P<0.05, **P<0.01 vs Sham, #P<0.05 vs Control.
2 groups (Figure 3c). Locomotor activity of rats subjected to MCAo was increased as compared with sham-operated rats, as described before, but there was no difference in rats transfected with control and HGF vector (Figure 3d).

To exclude the influence of the pattern of cerebral infarction on the cognitive function, we additionally focused on type A rats. The volume of cerebral infarction in type A rats was not different between rats transfected with human HGF gene and control vector (Figure 4a). Even type A rats transfected with HGF vector showed the improvement in the learning and memory in MWM test (Figure 4b). Also, rats transfected HGF vector showed the significantly longer retention of memory in the passive avoidance task (Figure 4c). Type A rats showed no significant difference in sensorimotor deficit and locomotor activity (data not shown).

These data supported the results that in the MWM and passive avoidance task were not influenced by the sensorimotor and locomotor activity and the volume and pattern of cerebral infarction. Overall, these data suggest that rats transfected with HGF vector maintain their memory longer as compared with those transfected with control vector.

HGF Enhances Neuritogenesis and Synaptogenesis

To examine whether HGF induced neuritogenesis and/or synaptogenesis, we focused on Cdc42, which belongs to the Rho family of GTPases and has positive effects on neuronal process extension, and synaptophysin, which is used as presynaptic markers and synaptogenesis. According to previous reports that the neuronal process extension occurred until 14 days after focal cerebral ischemia and synaptogenesis in the chronic stage of the insult, we measured the immunopositive cells against Cdc42 at day 14 and synaptophysin at day 56. Although the number of Cdc42-positive neurons was the same in the contralateral neocortex in both groups, the peri-infarct region in the neocortex of rats transfected with the HGF vector showed a significant increase in the number of Cdc42-immunoreactive cells (Figure 5a and 5b). Also, the immunoreactivity of synaptophysin was significantly increased at day 56 in rats transfected with the HGF gene, especially in the peri-infarct region (Figure 5c and 5d).

HGF Prevents Glial Scar Formation

Then, we investigated whether HGF had influences on astrocytes, because the neuron–glia interaction is also impor-
The immunoreactivity of GFAP was increased on days 14 and 56 in the peri-infarct region in both groups, and the immunoreactivity on day 14 was significantly higher in rats transfected with HGF vector (Figure 6). In contrast, the fewer immunopositive cells against GFAP could be detected in rats transfected with the HGF vector on day 56 as compared with the control vector (Figure 6).

Because some viral vectors, such as adenoviral vector, cause diffuse encephalomalacia and substantial leukoencephalopathy,38 we also performed hematoxylin/eosin staining to examine the inflammation. As expected, there was no inflammatory lymphocyte infiltration in HGF and control vector–transfected rats compared with sham-operated rats (data not shown).

**Discussion**

Disruption of blood flow to the brain initiates a cascade of events that produces neuronal death and leads to neurological dysfunction. From this viewpoint, we and others have reported that pretreatment with neurotrophic factors, such as FGF and HGF, has beneficial effects to prevent brain injury. However, considering their clinical application, pretreatment with neurotrophic factors might not be feasible. Unfortunately, few reports have revealed beneficial effects of treatment after infarction. To develop new therapeutic strategies to treat brain infarction, in this study, we examined the effects of overexpression of HGF after infarction, because HGF has unique actions in the central nervous system, as (1) a survival

**Figure 5.** (a) Representative images of immunohistochemical staining for Cdc42 on day 14 in rats transfected with control and HGF vector. The number of cells immunoreactive for Cdc42 was significantly increased in the pyramidal neurons in the peri-infarct region (#) of rats transfected with HGF vector, I, infarct region. (b) Quantitative analysis for Cdc42-immunoreactive cells in peri-infarct region (#). (c) Typical images of immunohistochemical staining for synaptophysin on day 56 (●). (d) Quantitative analysis for the immunoreactivity of synaptophysin. In the peri-infarct region, the immunoreactivity was significantly increased in rats treated with HGF gene. Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *P<0.05, **P<0.01 vs Control. Bar=100 μm. PI, peri-infarct region in neocortex; C, contralateral region in neocortex.
factor for embryonic motor neurons; (2) a stimulatory factor for the differentiation, survival, and axonal outgrowth of sensory and sympathetic neurons; (3) a neurotrophic factor; and (4) a potent angiogenic growth factor. The present study demonstrated that overexpression of HGF resulted in significant improvement of the results in MWM and the passive avoidance task on day 56, without any difference in infarct size and pattern. This study demonstrated that treatment with HGF postinfarction improved learning and memory.

Interestingly, the overexpression of HGF did not act on the disability of sensorimotor function and locomotor activity. The discrepancy of the recovery between the sensorimotor and cognitive functions has also been reported recently. The authors reported that the functional recovery was observed not in the cognitive function but in the sensorimotor deficits when MHP36 stem cells were grafted into the cerebral parenchyma, whereas only spatial learning was improved in rats with intraventricular grafts. Although the reason why the discrepancy was caused was unclear in the present study, we speculate that the functional recovery might be dependent on the kind of growth factor or the route of administration because of the different mechanisms in recovery from sensorimotor and cognitive deficits. Because the improvement of the sensorimotor deficits is also important, additional study is necessary to achieve the improvement of sensorimotor deficits.

The region where the significant histological difference was observed was the peri-infarct region, which was the border region between the frontal and parietal cortex. Because the neocortex was also an important site for learning and memory, we speculate that the functional recovery enhanced by HGF is dependent on that region in the present study. In fact, both the immunohistochemical analysis for Cdc42, synaptophysin, and GFAP and the ALP staining revealed significant differences in that region. Cdc42 belongs to the Ras superfamily of small GTPases and is expressed in hippocampus, cerebellum, thalamus, and neocortex in the rats. In general, Rac and Cdc42 have positive effects on process extension, whereas Rho has a positive effect on process retraction. HGF activated Cdc42, concomitant with the formation of filopodia and lamellipodia, in epithelial cells, although it was not still demonstrated in neurons. Considering that the immunoreactivity for Cdc42 in pyramidal neurons, which possess a high density of cholinergic terminals, was enhanced at day 14, the reconstitution of the neural network through neurite extension, so-called “neuritogenesis,” might be in progress at the early stage of HGF gene treatment. Also, the immunoreactivity of the presynaptic marker synaptophysin was increased at day 56 in rats treated with the HGF gene, which implies that the neuritogenesis resulted in the formation of new synapses. These results suggested that HGF enhanced neuritogenesis and synaptogenesis, which might contribute to the recovery of cognitive dysfunction.

The association of neurogenesis is also the center of interest, because HGF is involved in the development and maintenance of cortical neurons during differentiation and motogenesis in the neocortex. In general, adult neurogenesis in the neocortex is still controversial. It is also unclear whether adult neurogenesis occurs in the neocortex in rats after focal cerebral ischemia, because Jiang et al. showed the existence of neurogenesis, but Zhang et al. failed to detect neuronal nuclei and 5-bromodeoxyuridine double-labeling cells in the neocortex. In the present study, the fact that the volume of infarction was not decreased by transfection of the HGF gene and the density of matured neurons assessed by immunohistochemistry for MAP2 was not different (data not shown) implied that neurogenesis was not related to the functional recovery.
Another possible mechanism is that exogenously added HGF would transiently activate astrocytes and induce the production of other neurotrophic factors, resulting in the promotion of neuritogenesis. In fact, immunoreactivity for GFAP was increased on day 14 but decreased on day 56 to the contrary. Similar results were also observed in the recent report showing the effectiveness of forced arm use and brain-derived neurotrophic factor in MCAo. A recent study showed that the activated astrocytes possess qualities that will promote neuronal survival and regeneration, and they do not, by themselves, produce inhibitory extracellular matrix, whereas reactivated astrocytes stimulated by cytokines, including interleukin 1β, interferon γ, tumor necrosis factor α, and transforming growth factors, contribute to the glial scar formation, which inhibit neuronal survival or regeneration. It was also demonstrated that exogenous HGF regulated c-Met expression in cultured astrocytes and might induce other neurotrophic factors from activated astrocytes. Thus, it is likely that the effect of HGF was direct action and/or indirect action via neuron–glia interactions on neuritogenesis. This study also revealed an increase in microvessels only in the peri-infarct region but not in normal regions. Although the relationship between the improved microcirculation and behavior is still unclear, a recent report demonstrated that restoration of perfusion by collateral growth and new capillaries in the ischemic border zone around a cortical infarct supported long-term functional recovery in rats. Additionally, others reported that some patients who received tissue plasminogen activator therapy with no immediate clinical improvement in spite of early recanalization showed delayed clinical improvement. From the viewpoints, it is likely that the improvement of microcirculation is an important factor for the functional recovery. Although additional study is necessary, the improvement of microcirculation by HGF might be an alternative mechanism to improve learning and memory.

The influence of HGF on cerebral edema is another important issue. In general, the peak of cerebral edema is 3 days, and a significant decrease is 7 days after permanent MCAo in rats. Afterward, the infarct brain becomes atrophic. In the present study, the infarct region was atrophic in rats transfected with the HGF gene, as well as the control vector, and there was no significant difference in the volume. Thus, HGF gene transfer did not exacerbate the cerebral edema. Considering that VEGF
exacerbated cerebral edema. HGF might be safer than VEGF. Additional study is necessary to compare the effectiveness of HGF to other growth factors.

The amount of HGF produced by this method (0.1 to 0.4 ng/mL) is relatively low because of the limited transfected cells in the surface brain and ischemic region, as compared with that of previous reports showing the effectiveness of recombinant human HGF protein for the cerebral ischemia. Nevertheless, this low concentration might be enough to have the beneficial effects, because HGF elicited surviving neurotrophic effect at 0.5 to 1 ng/mL in primary cultured hippocampal neurons and enhanced neurite extension at 0.1 to 100 nM (0.1 to 100 ng/mL) in neocortical explant. Indeed, several previous articles demonstrated that the similar amount of HGF produced by gene transfer showed the neuroprotective and/or angiogenic property in several experimental rodent models. Because the higher concentration of HGF is more effective for survival and neurite extension in in vitro study, several improvements, such as modification of the HVJ-envelope vector and HGF plasmid, are required to achieve better outcome.

Perspectives

Overall, the present study is the first to demonstrate that HGF gene therapy delayed for as long as 7 days improved the outcome from ischemic stroke through the reconstitution of the neuronal network and improvement in the microcirculation. In clinical use, the present study might be attractive to support the application of HGF for the treatment of the patients in the chronic stage of brain infarction. Although most of the previous reports demonstrated the effectiveness of growth factors before the insult or within several hours of the onset by the inhibition of apoptosis and extension of the ischemic lesion, it is difficult to administer them in time in most patients. Additionally, some patients improve their cognitive dysfunction spontaneously within several days after cerebral infarction. Also, the intracisternal injection is too difficult in the acute stage of cerebral infarction, because it is possible that the brain edema is worsened by intracisternal injection itself. In contrast, the present study is more closed to the real clinical situation for the treatment of the patients with chronic brain stroke. Although additional study is necessary to determine whether other growth factors are effective or not in the chronic stage, gene therapy using HGF may provide new therapeutic options for treatment after cerebral ischemia.

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