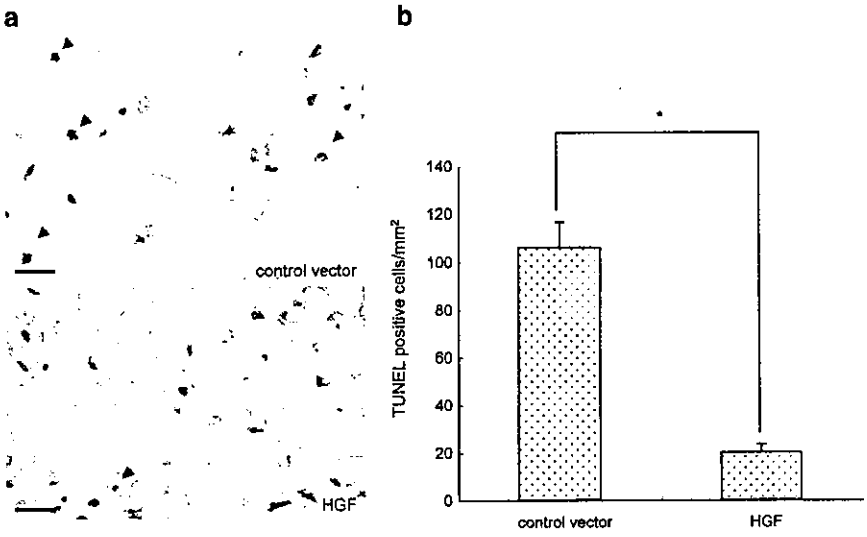

Figure 3. a, TUNEL staining of coronal sections of cerebral cortex in boundary of infarct area after 1 day of focal cerebral ischemia. Bar=100 μm. b, Number of TUNEL-positive cells. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.01$ vs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

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.[17,29] Recently, HGF has been the center of interest in neuroprotective substances, because HGF works as a survival factor for embryonic motor neurons.[30] Moreover, sensory and sympathetic neurons and their precursors respond to HGF with increased differentiation, survival, and axonal outgrowth.[30] 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.[19] In the present

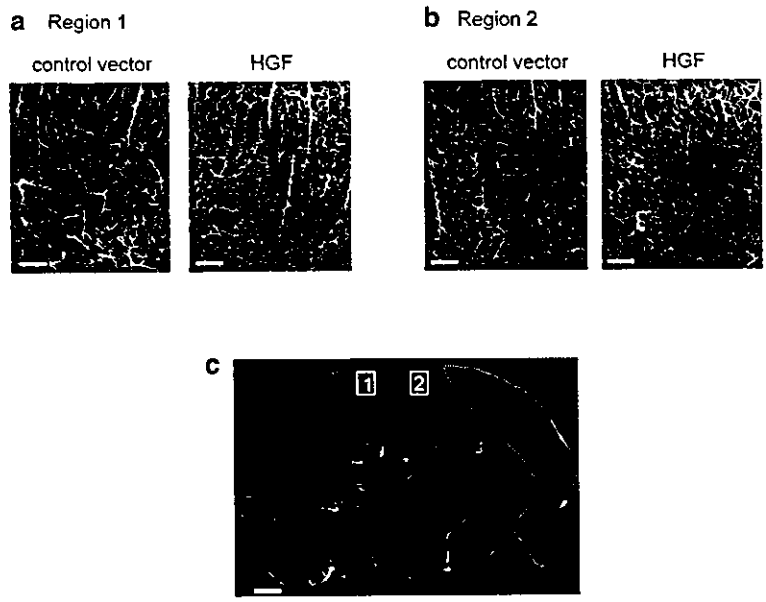

Figure 4. Vascular patterns in cerebral cortex at 24 hours after MCA occlusion. Region of interest was set at 2 points on surface of cerebral cortex (c). Rats transfected with human HGF gene showed a significant increase in capillary density in both intact (a) and infarcted (b) hemispheres. Dotted line in c represents infarcted region. a and b, bar=100 μm; c, bar=1 mm. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector.

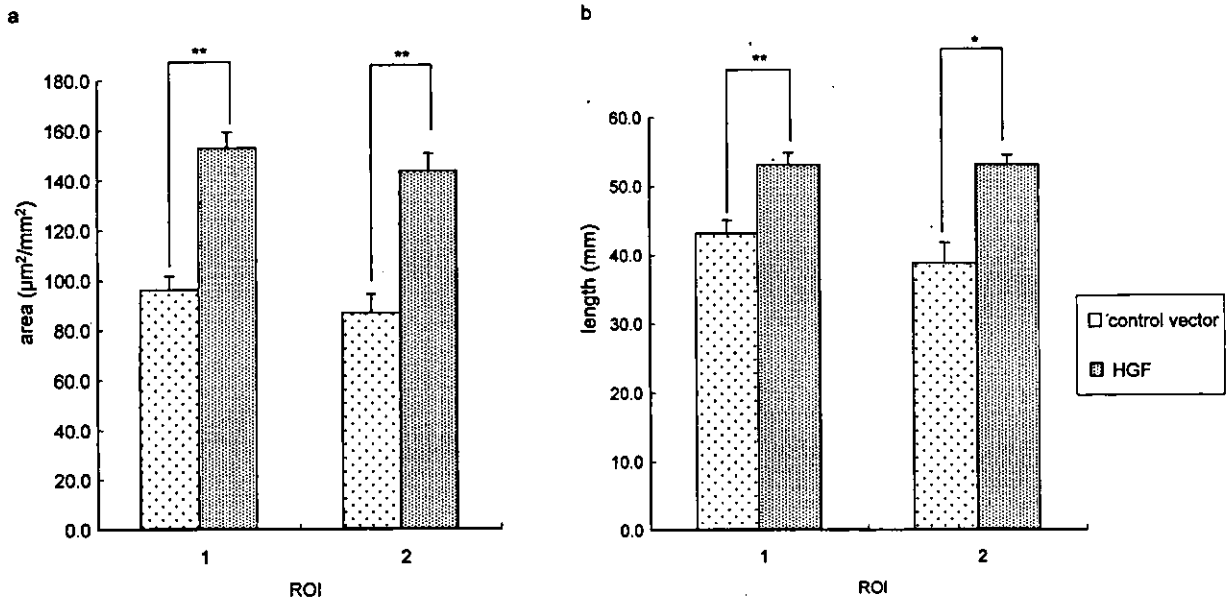

Figure 5. Capillary density as assessed by quantitative analysis at 24 hours after MCA occlusion. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. Number of ROI is same as in Figure 4. *$P<0.05$, **$P<0.01$ vs control vector (n=7 in each group).

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.[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

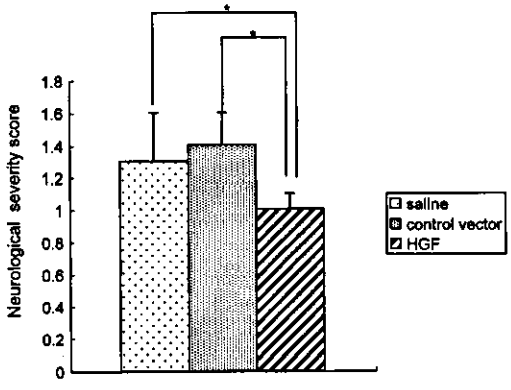

Figure 6. Neurological severity score at 24 hours after MCA occlusion. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.05$ (n=7, each group).

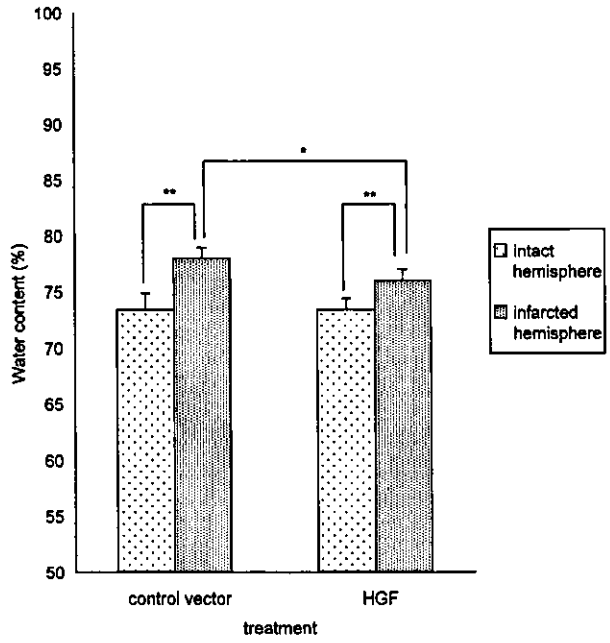

Figure 7. Water content after 24 hours of cerebral ischemia. Water content (WC) was calculated as WC (%)=(wet wt−dry wt)/(wet wt)×100. *$P<0.05$ (n=7 in each group).

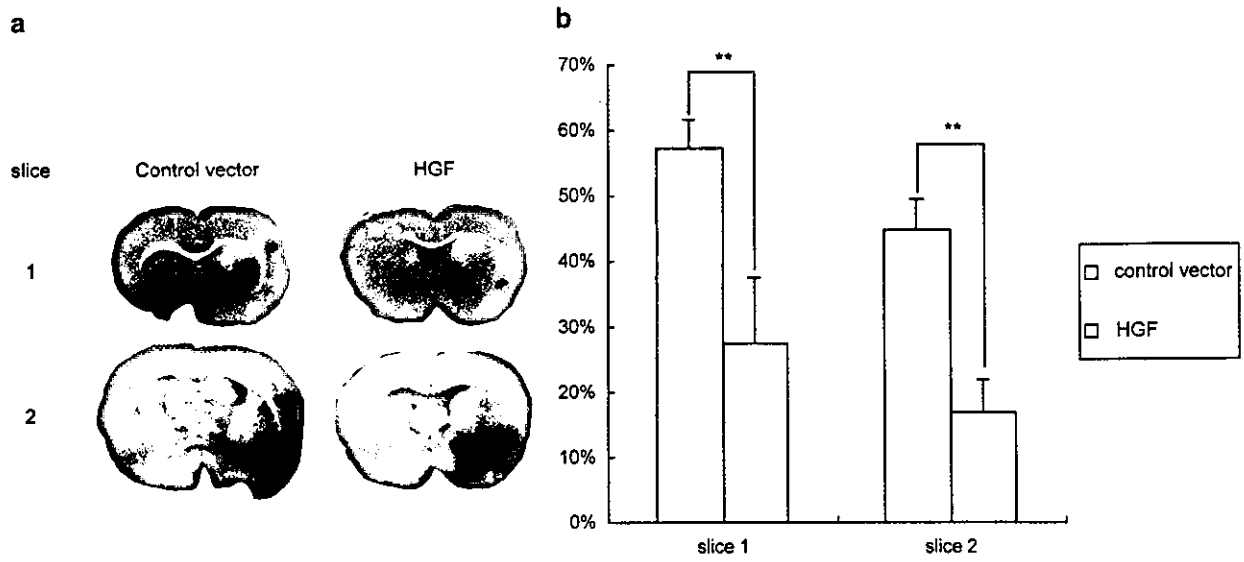

Figure 8. a, Coronal sections at 9 hours after MCA occlusion. Evans blue dye was injected at 6 hours after occlusion. To identify infarcted area, slice 1 was stained with TTC. Blue area indicates leakage of Evans blue dye. b, Percent area of Evans blue dye. Degree of leakage (%) was calculated as leakage (%)=[LT−(RT−RB)]/LT×100. RB indicates area stained blue. $^{**}P<0.01$ vs control vector (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.[15–17,30,32,33] The mechanism of the neuroprotective effects of HGF is inhibition of apoptosis via the MAP kinase pathway[32] and the phosphatidylinositol-3 kinase/Akt pathway.[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[34] and rat transient focal cerebral ischemia model,[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,[10] early postischemic (1 hour) administration of recombinant VEGF165 to ischemic rats significantly increased BBB leakage, hemorrhage, and ischemic lesions.[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.[27,28,35] Indeed, human gene therapy in patients with limb ischemia using VEGF also demonstrated lower-limb edema as a side effect,[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.[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.

Acknowledgments

This work was partially supported by a Grant-in-Aid from the Organization for Pharmaceutical Safety and Research, a Grant-in-Aid from the Ministry of Public Health and Welfare, a Grant-in-Aid from Japan Promotion of Science, and the Ministry of Education, Culture, Sports, Science, and Technology, the Japanese Government.

References

1. Kalaria RN, Bhatti SU, Lust WD, et al. The amyloid precursor protein in ischemic brain injury and chronic hypoperfusion. *Ann N Y Acad Sci.* 1993;695:190–193.
2. Kurumatani T, Kudo T, Ikura Y, et al. White matter changes in the gerbil brain under chronic cerebral hypoperfusion. *Stroke.* 1998;29:1058–1062.
3. Krupinski J, Kaluza J, Kumar P, et al. Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke.* 1994;25:1794–1798.
4. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest.* 1994; 93:662–670.
5. Pearlman JD, Hibberd MG, Chuang ML, et al. Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nat Med.* 1995;1:1085–1089.
6. Baumgartner I, Pieczek A, Manor O, et al. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation.* 1998; 97:1114–1123.
7. Rosengart TK, Lee LY, Patel SR, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation.* 1999;100:468–474.
8. Sugimori H, Speller H, Finklestein SP. Intravenous basic fibroblast growth factor produces a persistent reduction in infarct volume following permanent focal ischemia in rats. *Neurosci Lett.* 2001;300:13–16.
9. Tsuzuki N, Miyazawa T, Matsumoto K, et al. Hepatocyte growth factor reduces infarct volume after transient focal cerebral ischemia in rats. *Acta Neurochir Suppl.* 2000;76:311–316.
10. Hayashi T, Abe K, Itoyama Y. Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. *J Cereb Blood Flow Metab.* 1998;18:887–895.
11. Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.* 1982;239:57–69.
12. Ferrer I, Krupinski J, Goutan E, et al. Brain-derived neurotrophic factor reduces cortical cell death by ischemia after middle cerebral artery occlusion in the rat. *Acta Neuropathol (Berl).* 2001;101:229–238.
13. Schabitz WR, Sommer C, Zoder W, et al. Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. *Stroke.* 2000; 31:2212–2217.
14. Zarnegar R, Michalopoulos GK. The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J Cell Biol.* 1995;129: 1177–1180.
15. Sun W, Funakoshi H, Nakamura T. Localization and functional role of hepatocyte growth factor (HGF) and its receptor c-met in the rat developing cerebral cortex. *Brain Res Mol Brain Res.* 2002;103:36–48.
16. Zhang L, Himi T, Morita I, et al. Hepatocyte growth factor protects cultured rat cerebellar granule neurons from apoptosis via the phosphatidylinositol-3 kinase/Akt pathway. *J Neurosci Res.* 2000;59: 489–496.
17. Hayashi K, Morishita R, Nakagami H, et al. Gene therapy for preventing neuronal death using hepatocyte growth factor: in vivo gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. *Gene Ther.* 2001;8:1167–1173.
18. Kaneda Y, Nakajima T, Nishikawa T, et al. Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther.* 2002;6:219.
19. Shimamura M, Morishita R, Endoh M, et al. HVJ-envelope vector for gene transfer into central nervous system. *Biochem Biophys Res Commun.* 2003;300:464–471.
20. Koike H, Morishita R, Iguchi S, et al. Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene. *FASEB J.* 2003;17: 779–781.
21. Belayev L, Alonso OF, Busto R, et al. Middle cerebral artery occlusion in the rat by intraluminal suture: neurological and pathological evaluation of an improved model. *Stroke.* 1996;27:1616–1622; discussion 1623.
22. Bederson JB, Pitts LH, Germano SM, et al. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke.* 1986;17:1304–1308.
23. Petullo D, Masonic K, Lincoln C, et al. Model development and behavioral assessment of focal cerebral ischemia in rats. *Life Sci.* 1999; 64:1099–1108.
24. Matsuo Y, Mihara S, Ninomiya M, et al. Protective effect of endothelin type A receptor antagonist on brain edema and injury after transient middle cerebral artery occlusion in rats. *Stroke.* 2001;32:2143–2148.
25. Katayama S, Shionoya H, Ohtake S. A new method for extraction of extravasated dye in the skin and the influence of fasting stress on passive cutaneous anaphylaxis in guinea pigs and rats. *Microbiol Immunol.* 1978; 22:89–101.
26. Cavaglia M, Dombrowski SM, Drazba J, et al. Regional variation in brain capillary density and vascular response to ischemia. *Brain Res.* 2001;910: 81–93.
27. Zhang ZG, Zhang L, Jiang Q, et al. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest.* 2000;106:829–838.
28. Zhang Z, Chopp M. Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia. *Trends Cardiovasc Med.* 2002;12:62–66.
29. Kiprianova I, Freiman TM, Desiderato S, et al. Brain-derived neurotrophic factor prevents neuronal death and glial activation after global ischemia in the rat. *J Neurosci Res.* 1999;56:21–27.
30. Maina F, Klein R. Hepatocyte growth factor, a versatile signal for developing neurons. *Nat Neurosci.* 1999;2:213–217.
31. Tomita N, Morishita R, Taniyama Y, et al. Angiogenic property of hepatocyte growth factor is dependent on upregulation of essential transcription factor for angiogenesis, ets-1. *Circulation.* 2003;107: 1411–1417.
32. Hamanoue M, Takemoto N, Matsumoto K, et al. Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. *J Neurosci Res.* 1996;43:554–564.
33. Hossain MA, Russell JC, Gomez R, et al. Neuroprotection by scatter factor/hepatocyte growth factor and FGF-1 in cerebellar granule neurons is phosphatidylinositol 3-kinase/akt-dependent and MAPK/CREB-independent. *J Neurochem.* 2002;81:365–378.
34. Yoshimura S, Morishita R, Hayashi K, et al. Gene transfer of hepatocyte growth factor to subarachnoid space in cerebral hypoperfusion model. *Hypertension.* 2002;39:1028–1034.
35. van Bruggen N, Thibodeaux H, Palmer JT, et al. VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J Clin Invest.* 1999;104:1613–1620.
36. Baumgartner I, Rauh G, Pieczek A, et al. Lower-extremity edema associated with gene transfer of naked DNA encoding vascular endothelial growth factor. *Ann Intern Med.* 2000;132:880–884.
37. Morishita R, Aoki M, Hashiya N, et al. Hepatocyte growth factor (HGF) angiogenic gene therapy: promises for cardiovascular diseases. *Gene Ther Reg.* 2002;1:343–359.

Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene[1]

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SPECIFIC AIM

The current therapeutic angiogenesis strategy to treat ischemic disease using angiogenic growth factors has been limited to use of a single gene. To enhance the angiogenic activity, we examined the angiogenic activity of cotransfection of genes of an angiogenic growth factor, hepatocyte growth factor (HGF), and a vasodilator substance, prostacyclin synthase.

PRINCIPAL FINDINGS

1. Angiogenesis induced by intramuscular (i.m.) injection of HGF or prostacyclin synthase plasmid in mouse model

Accompanied by a significant increase in human immunoreactive HGF in the hind limb transfected with human HGF vector, injection of human HGF vector into the ischemic hind limb resulted in a significant increase in blood flow from 2 wk after transfection as assessed by laser Doppler imaging (**Fig. 1**, $P<0.01$). Moreover, transfection of human HGF vector significantly increased capillary density in the mouse ischemic hind limb around the injection site compared with the hind limb transfected with control vector at 4 wk after transfection ($P<0.01$). These results clearly demonstrated that transfection of human HGF vector into the ischemic hind limb induced therapeutic angiogenesis, which could be applied for the treatment of peripheral arterial disease. In contrast, no human HGF could be detected in muscles transfected with control vector at 4 days after transfection ($P<0.01$). Similarly, human 6 keto PGF1α could be detected in the ischemic hind limb transfected with human prostacyclin synthase, but not control vector ($P<0.01$). Human HGF protein was increased by cotransfection of the HGF and prostacyclin synthase genes. Injection of human prostacyclin synthase vector resulted in a weak but significant increase in capillary density at 4 wk after transfection. The increase in blood flow induced by the prostacyclin synthase gene was similar to that induced by the HGF gene at 2 wk after transfection (Fig. 1, $P<0.01$). Cotransfection of the HGF and prostacyclin synthase genes demonstrated a further increase in blood flow compared with single gene transfection of HGF or prostacyclin synthase alone at 2 wk after transfection (Fig. 1, $P<0.01$). Similarly, capillary density was most increased in mice transfected with the HGF and prostacyclin synthase genes compared with the HGF vector ($P<0.01$).

2. Angiogenesis induced by i.m. injection of HGF or prostacyclin synthase plasmid in rabbit model

Similar to the mouse model, human immunoreactive HGF was observed in rabbits transfected with human HGF vector, but not control vector at 4 days after transfection (HGF: 2.1±0.4 ng/g tissue vs. control; not detected; $P<0.01$). Injection (i.m.) of HGF plasmid into the ischemic limb on day 10 after surgery produced significant augmentation of collateral vessel development as assessed by angiography on day 30 in the ischemia model, as shown in **Fig. 2** ($P<0.01$). Serial angiograms revealed progressive linear extension of collateral arteries from the origin stem artery to the distal point of the reconstituted parent vessel in HGF-treated animals. Consistent with induction of angiogenesis, a significant increase in blood flow as assessed by a Doppler flow wire under basal conditions was observed in rats transfected with HGF plasmid compared with rats transfected with control vector ($P<0.01$). A significant increase in the blood pressure ratio of ischemic limb to normal limb was also observed in rabbits transfected with HGF plasmid compared with control vector ($P<0.01$). Cotransfection of the HGF and prostacyclin synthase genes demonstrated a further increase

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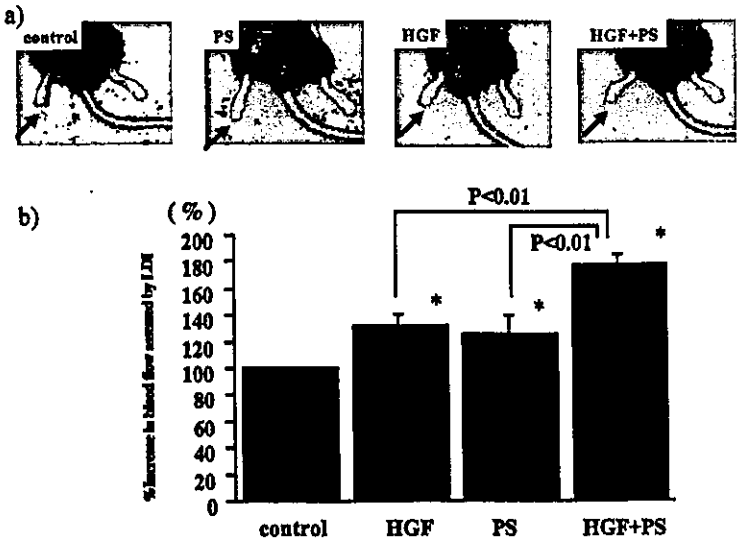

Figure 1. Effect of i.m. injection of human HGF plasmid or prostacyclin synthase plasmid in an ischemic limb model. *a)* Typical image of blood flow analyzed by laser Doppler imager at 2 wk after transfection. Injection (i.m.) of plasmid was performed in the right ischemic hind limb. Panels show color-coded images representing blood flow distribution. Low or no perfusion is displayed as dark blue; the highest perfusion is displayed as white. *b)* Quantitative analysis of blood flow in right hind limb. Control = muscle from ischemic hind limb transfected with control vector, HGF = muscle from ischemic hind limb transfected with human HGF vector, PS = muscle from ischemic hind limb transfected with prostacyclin synthase vector, HGF + PS = muscle from ischemic hind limb transfected with human HGF vector and prostacyclin synthase vector. Each group contains 7–8 animals. $^{**}P < 0.01$ vs. control.

in angiographic scores compared with single gene transfection of the HGF or prostacyclin synthase gene alone (Fig. 2, $P<0.01$). Similarly, capillary density was most increased in rabbits transfected with the HGF and prostacyclin synthase genes compared with HGF vector ($P<0.01$).

3. Attenuation of decreased in neuronal velocity by i.m. injection of HGF and prostacyclin synthase plasmids in mouse diabetic ischemic hind limb model

Using diabetes ischemic hind limb animal models, we have investigated the hypothesis that experimental diabetic ischemic neuropathy can be reversed by coadministration of HGF and prostacyclin synthase genes. To answer this question, we used a new diabetic neuropathy model produced by creating hind limb ischemia in diabetic mice. Different from a previous report using a simple diabetes model, the diabetic ischemia model was characterized by severe impairment of nerve conduction velocity. In these ischemic mice with streptozotocin-induced diabetes, consistent with a profound reduction in the number of vessels, severe peripheral neuropathy developed in parallel, characterized by significant slowing of motor and sensory nerve conduction velocity, compared with nondiabetic control animals ($P<0.01$). In contrast, 4 wk after i.m. gene transfer of the HGF and prostacyclin synthase genes, vascularity and blood flow in the ischemic hind limb of treated animals were similar to those of nondiabetic control mice. In addition, constitutive overexpression of the HGF and prostacyclin synthase genes resulted in restoration of large and small fiber peripheral nerve function. Improvement of nerve function was not accompanied by a change in blood glucose level or body weight, since transfection of the HGF and prostacyclin synthase genes did not alter these parameters (data not shown). These findings suggest the feasibility of a novel treatment strategy for peripheral neuropathy.

CONCLUSION AND SIGNIFICANCE

A novel therapeutic strategy using angiogenic growth factors to expedite and/or augment collateral artery

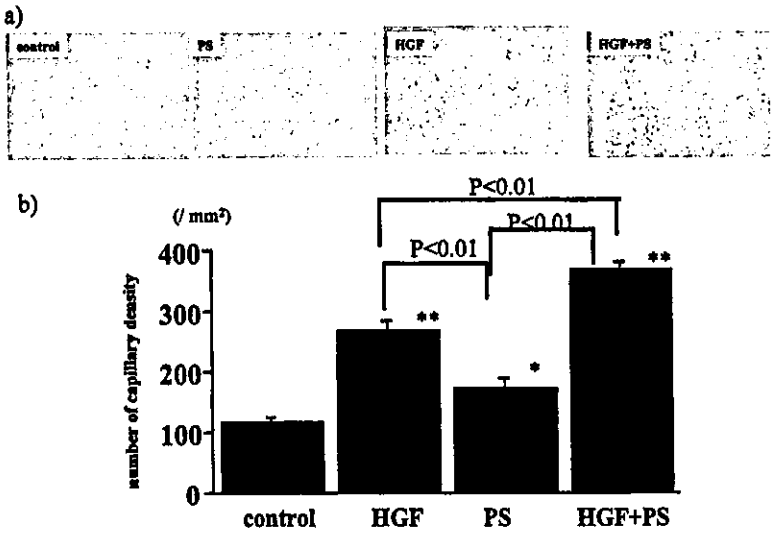

Figure 2. Effect of transfection of human HGF plasmid or prostacyclin synthase plasmid on capillary density. *a)* Representative cross sections (200×). *b)* Effect of transfection of human HGF vector on number of vessels. Control = muscle from ischemic hind limb transfected with control vector, HGF = muscle from ischemic hind limb transfected with human HGF vector, PS = muscle from ischemic hind limb transfected with prostacyclin synthase vector, HGF + PS = muscle from ischemic hind limb transfected with human HGF vector and prostacyclin synthase vector. Each group contains 7–8 animals. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

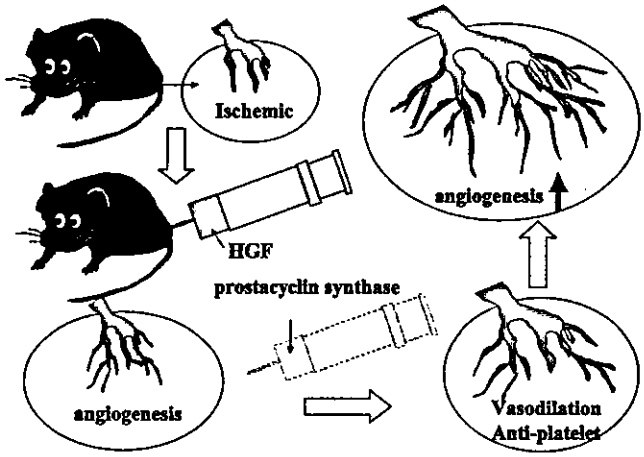

Figure 3. Schema of therapeutic angiogenesis with cotransfection of HGF and prostacyclin synthase genes.

development has recently entered the realm of treatment of ischemic diseases. Although the clinical utility of gene therapy using VEGF (VEGF165, VEGF121, VEGF-2), HGF, FGF, and HIF-1 has been demonstrated, the current clinical trials and animal experiments are limited to use of a single angiogenic growth factor to stimulate angiogenesis. Here, we introduce a new strategy, therapeutic angiogenesis using cotransfection of the HGF and prostacyclin synthase genes, as gene therapy for treatment of patients with critical limb ischemia. The reason we chose prostacyclin synthase was to consider the utility of vasodilator agents such as prostaglandins and phosphodiesterase type III inhibitors to treat human patients with PAD. A combination of angiogenesis induced by HGF and vasodilation of newly generated blood vessels induced by prostacyclin would enhance blood flow recovery and maintain new vessel formation (see **Fig. 3**). As expected, blood flow and capillary density in mice cotransfected with the HGF and prostacyclin synthase genes was much greater than that with single transfection of "naked" plasmid DNA.

As diabetic patients often present with advanced coronary and peripheral artery disease, in developing human gene therapy it is important to know whether compensatory mechanisms for vascular ischemia are affected in this condition. Accordingly, we and others have reported a delay of angiogenesis in a diabetic state. Nevertheless, our previous studies demonstrated the utility of HGF gene transfer to stimulate neovascularization of ischemic tissues in a diabetes model. Peripheral neuropathy is common and ultimately accounts for significant morbidity in diabetes. However, there are currently no therapeutic options for patients with diabetic neuropathy. Earlier work using animal models of hind limb ischemia also documented favorable effects of VEGF gene transfer on ischemic peripheral neuropathy. Numerous reports have shown direct effects of reduced blood flow or ischemia on nerve conduction velocity and integrity of the peripheral nervous system. Diabetic neuropathy has been causally related to microangiopathy and endoneurial ischemia. It is intriguing to note that the neurological and neurophysiological findings in a prospective study of patients undergoing $phVEGF_{165}$ gene transfer for critical limb ischemia showed clinical improvement in electrophysiological measurements in diabetic patients. Although the model used in this study was more severe compared with the previous work, cotransfection of the HGF and PGIS genes was able to improve the electrophysiological findings. As HGF has been reported to have direct effects on nerve cells, the results of these experiments do not exclude the possible contribution of direct effects of HGF on nerve integrity.

What is the clinical relevance of the second-generation therapeutic angiogenesis strategy presented in this study? First, although a single i.m. injection of HGF plasmid was sufficient to prevent necrosis, enhancement of therapeutic angiogenesis by increasing the dose or injection time or transfection efficiency using viral and nonviral vector systems would be important. Indeed, the clinical efficacy using the VEGF gene did not reach 100%. To treat a wider range of patients such as those with critical limb ischemia with diabetes or on hemodialysis, it is necessary to achieve higher efficiency. However, when increasing the dose or injection time, cost and toxicity issues might be a problem. Alternatively, using highly efficient vector systems may cause side effect issues and toxicity due to the vector itself. Indeed, adenoviral vectors often used clinically cause deleterious side effects. Stimulation of collateral formation induced by HGF plasmid DNA with prostacyclin is relatively safe, since prostacyclin is widely used to treat patients for a long time, without severe side effects. Second, it is important to achieve therapeutic effects at an earlier time point such as 2 wk after transfection. Using single gene transfection, therapeutic efficacy appears from 3 or 4 wk after transfection, since the increase in blood vessels induced by the expression of growth factors from a transgene requires a long period. Thus, to consider the clinical setting, this second generation of therapeutic angiogenesis may be useful to treat PAD patients. Alternatively, pharmaceutical drugs such as oral prostacyclin analogs are widely used to treat PAD patients, and the combination of gene therapy using a single gene with oral drugs is more likely in the clinical setting. A recent report using VEGF121 is attractive. The authors reported that the vasodilator response to nitroglycerine was significantly restored in patients transfected with the VEGF121 gene. Thus, it is likely to stimulate collateral formation after gene therapy combined with oral vasodilator drugs in clinical practice. These experimental findings are thus encouraging for the treatment of diabetic neuropathy and PAD, although these areas clearly require further investigation and monitoring with regard to safety issues.

Here we have demonstrated that cotransfection of the prostacyclin synthase and HGF genes was more effective than single gene transfection to stimulate angiogenesis and improve diabetic neuropathy in a mouse hind limb ischemia model. These data provide important information for the clinical application of therapeutic angiogenesis to treat peripheral arterial disease. FJ

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Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats

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ABSTRACT

Hearing impairment, which is the most prevalent sensory deficit of human beings, needs a breakthrough in therapeutic technologies. One technology is the usage of a vector system to reach the inner ear, and another is by a therapeutic molecule. Here we developed a novel gene therapy strategy by combining hepatocyte growth factor (HGF) with hemagglutinating virus of Japan envelope (HVJ-E) vector. When HVJ-E containing human HGF gene was injected intrathecally into the cerebrospinal fluid via cisterna magna of rats, the vector reached the inner ear region, and human HGF gene expression was detected in the spiral ganglion cells (SGCs) of the inner ear. Expression of endogenous rat HGF and its receptor, c-Met, was also induced in SGCs by human HGF. Kanamycin treatment results in hearing impairment by inducing degeneration of hair cells (HCs) and apoptosis of SGCs in rats. By HGF gene transfer before kanamycin treatment, both loss of HCs and apoptosis of SGCs were prevented. Furthermore, hearing function, evaluated by auditory brainstem response, was maintained at a normal level. When HGF gene transfer was performed 2 wk after kanamycin treatment, hearing impairment was significantly recovered. These results indicate a novel and effective therapeutic strategy against sensorineural hearing impairment.

Key words: hair cell • spiral ganglion • cochlear implants • gene therapy

Hearing impairment is the most prevalent sensory deficit of human beings, affecting more than 1 in 10 individuals. Hearing impairment can be caused by a variety of factors: infection, noise, aging, and ototoxic substances such as aminoglycosides and cisplatin (1). These substances exhibit deleterious effects on the hair cell (HC) in the organ of Corti and the spiral ganglion cell (SGC; refs 2, 3). HCs are the mechanosensory epithelial cells that convert auditory stimuli to electric signals, and SGCs are the primary afferent auditory neurons located in the Rosenthal's canal within the modiolus of the cochleas. HCs and SGCs play a direct role in mediating auditory stimuli to the central auditory neurons. The loss of HC prevents the transduction of acoustic signals, leading to a retrograde neuronal degeneration of SGC, in which a

loss of SGC exacerbates hearing dysfunction (3). In general, HC and SGC in mammals are believed not to have the capacity for postembryonic cellular mitosis to generate new HC and SGC. Therefore, the loss of either HC or SGC results in intractable hearing impairment.

For the treatment of the severely deaf, cochlear implants have been implanted in the cochleas of many patients with severe/profound deafness and have been shown to provide effective results. The electrode of the cochlear implant is inserted into the fluid space of the inner ear, bypassing damaged or absent HCs, thus delivering an electrical stimulus directly to SGCs. This device provides significant improvement of the quality of sound perception. However, the effectiveness of cochlear prosthesis depends on the quality and amount of the remaining auditory nerve, and the state of their degeneration severely diminishes the hearing benefits obtained from the prosthesis. Previous studies have shown a clear relationship between the total number of viable auditory neurons available for stimulation and the performance of subjects receiving cochlear implants (4). These data have indicated that stable and satisfactory results of the implant depend on the development of a therapeutic strategy for preserving or regenerating the auditory neurons, mainly SGCs. Recent studies have revealed that some neurotrophic factors, such as neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF), improve the survival of inner ear auditory neurons, including SGCs (5–11).

Hepatocyte growth factor (HGF), which was originally identified as a potent motogen for hepatocytes and cloned in 1989 (12, 13), has since been shown to be a pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activities toward a variety of cells including neural cells. Both HGF and c-Met, a HGF receptor with a membrane-spanning tyrosine kinase, are expressed in various regions of the brain (14, 15) and peripheral neurons (16, 17). More importantly, functional coupling between HGF and c-Met plays important roles in the development and maintenance of neural system (18–20). Previous studies have revealed that *HGF* gene transfer could prevent postischemic delayed neuronal death in the hippocampus (21–24). Altogether, these studies suggest that HGF functions as a neurotrophic factor to maintain the physiological structure and function of the nervous system. HGF, however, has not been used for the treatment of hearing impairment to date. The expression of HGF and c-Met was detected in the epithelial cells, neural cells, and mesenchymal cells in the embryonic cochlea of rats by immunohistochemical analysis using an antibody specific for each molecule (unpublished data). Moreover, we found that the expression of c-Met in SGC was enhanced when rats were treated with kanamycin (25). We therefore hypothesized that HGF may play an important role in the regulation of the hearing function. Hence, in this study, we investigated the effect of HGF on HCs, SGCs, and hearing function before and after aminoglycoside insult to provide a novel strategy for prevention as well as improvement of hearing impairment. The animal model of deafness induced by aminoglycosides is the most commonly used experimental model, whereby the damage of HCs is followed by the loss of SGCs (26, 27). Because the loss of HC induced by aminoglycosides is dose dependent, animal models with various extents of hearing impairment can be established (26, 27). As described above, in most cases of hearing impairment, the cause is directly or indirectly related to the degeneration or the death of HCs and SGCs (4). Hence, kanamycin was used in this investigation to mimic the most likely clinical circumstances of hearing impairment.

In the present study, to achieve a minimally invasive therapeutic approach for gene transfer to the inner ear, we attempted to transfer a novel nonviral vector, hemagglutinating virus of Japan envelope (HVJ-E) vector (28), containing marker genes (*lacZ* or luciferase gene), into the

cerebrospinal fluid (CSF) via the cisterna magna. The HVJ-E vector is effective for gene transfer to neurons both in vitro and in vivo. When the HVJ-E vector containing a reporter gene was transfected to cultured neuronal cells or delivered intrathecally to CSF, gene expression was detected in various areas of the CNS (29).

In this study, using this new delivery system, we examined the neuroprotective effect of HGF on the survival of SGCs in a hearing-impaired animal model and investigated the new possibility of gene therapy for the treatment of hearing impairment.

MATERIALS AND METHODS

Plasmid DNA

pc*lac*Z (9.2 kb) was constructed by inserting a *Hin*dIII-*Bam*HI fragment of pSV-β-galactosidase (Promega, Madison, WI) into pcDNA3 (5.4 kb; Invitrogen, San Diego, CA) at the *Hin*dIII and *Bam*HI sites. pCMV-luciferase-GL3 (pcLuc-GL3: 7.4 kb) was constructed by cloning the luciferase gene from the pGL3-basic Vector (Promega) into pcDNA3 (Invitrogen). pVAX1-*hHGF* (5.2 kb) was constructed by inserting the human HGF gene (*hHGF*) into pVAX1 (3.0kb) (Invitrogen) at the *Bam*HI and *Not*I sites. Plasmids were purified with the QIAGEN plasmid isolation kit (Qiagen, Hilden, Germany).

HVJ-E vector

The HVJ-E vector was constructed by incorporating plasmid DNA into inactivated HVJ particles as described previously (28). In short, ultraviolet (UV)-inactivated HVJ (Z strain), 4.5×10^{10} particles, was mixed with 300 μg of plasmid DNA and 0.3% Triton-X 100. The suspension was then washed with balanced salt solution (BSS: 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH7.6) and centrifuged, and the pellet was resuspended with a final volume of 100 μl BSS for intrathecal infusion. HVJ-E containing pc*lac*Z, pcLuc-GL3, pVAX1-hHGF, pcDNA3, or pVAX1 plasmid DNA was used in the present study. The incorporation rate of DNA into HVJ-E was ~15% (28). Total amount administered was 4.5×10^{10} particles of HVJ-E containing 45 μg plasmid DNA per rat.

Experimental animals and treatment groups

Sprague-Dawley male rats (6 wk of age; 190-210 g) exhibiting a normal Preyer's reflex were obtained from Charles River Japan (Atsugi, Japan). All procedures were conducted in accordance with the guidelines of the Animal Committee of Osaka University. Animals of all groups were hearing impaired bilaterally by aminoglycoside intoxication as previously reported (1–3). Kanamycin sulfate (Meiji Seika, Tokyo, Japan) was administered daily by subcutaneous injection (400 mg/kg/day) for 14 consecutive days. In the protection experiment, rats were injected intrathecally with either HVJ-E containing pVAX1-*hHGF* (KM + HGF group) or control plasmid (pVAX1) (KM + vector group) on the first day of kanamycin injection. Rats in the KM group did not receive an intrathecal inoculation but only a kanamycin injection. In the therapeutic experiment, all animals were first hearing impaired with kanamycin for 14 days and hearing impairment was confirmed by auditory brainstem response (ABR), and on day 14 (14th day of

kanamycin injection), they were injected intrathecally with HVJ-E containing pVAX1-*hHGF* (KM + HGF group) or pVAX1 (KM + vector group).

In vivo gene transfer to the subarachnoid space

In this study, we employed infusion of an HVJ-E suspension into the cisterna magna of rats for in vivo gene transfer into the CNS and the inner ear. After induction of anesthesia with ketamine (Sankyo) and xylazine (Bayer Ltd.), the head of each animal was fixed in the prone position, and the atlanto-occipital membrane was exposed through an occipitocerebral midline incision for infusion into the subarachnoid space. A stainless steel cannula (27 gauge; Becton-Dickinson) was then introduced into the cisterna magna (subarachnoid space). After withdrawal of the CSF (100 μl) for confirmation of the cannula position and to avoid increased intracerebral pressure, HVJ-E (100 μl) containing a reporter gene (pcLuc-GL3, pc*lacZ*), *hHGF* (pVAX1-*hHGF*), or a control vector (pVAX1, pcDNA3) was infused at a rate of 50 μl/min. Subsequently, the animals were placed in a "head down" position for 30 min. All rats showed no signs of weight loss (data not shown) or abnormal behaviors (e.g., shivering, rotation, paralysis, or immobility) after the administration.

***lacZ* expression assay**

On day 7 after intrathecal injection of HVJ-E containing pc*lacZ*, which encodes *E. coli* β-galactosidase (β-gal), the rats were killed and the organs harvested from the rats were prepared for X-gal staining as described previously (21).

Assay for luciferase activity

Rats transfected with the luciferase gene were killed under anesthesia 24 h after transfection. Organs (brain, lung, spleen, liver, and cochlea) were harvested and placed individually in 50 ml FALCON tubes. Luciferase activity was measured using a luciferase assay kit (Promega), as described previously (30). Luciferase levels were normalized by determining the protein concentrations of the tissue extracts (31). Luciferase units were expressed as relative light units (RLU) per milligram of tissue protein.

Enzyme-linked immunosorbent assay for human HGF in CSF

CSF (100 μl) from the rats, isolated 5 and 14 days after the injection of HVJ-E containing *hHGF*, was used for the experiments. The concentration of human HGF and rat HGF in the CSF was determined by enzyme-linked immunosorbent assay (ELISA) using anti-human and anti-rat HGF antibody, respectively, according to the instructions of the manufacturer (Institute of Immunology, Tokyo, Japan). The antibody against human HGF is specific only for human HGF and not rat HGF, whereas the antibody against rat HGF reacted only with rat HGF but not human HGF.

Histological and immunocytochemical analyses

The rats were deeply anesthetized by intraperitoneal injection of pentobarbital (250 mg/kg) and perfused transcardially with phosphate buffered saline (PBS), pH 7.4. Then the rats were killed by decapitation, and the cochleas were collected. For the immunostaining of SGCs, the cochlea was

removed from the remaining temporal bone and immersed in 70% ethanol overnight at 4°C for fixation. After complete fixation, cochleas were decalcified in 3% EDTA for a week at 4°C. Immunostaining procedures were as described previously (32). In short, tissues were dehydrated by being dipped into a series of ethanol and xylene, embedded in paraffin, and cut into 6 μm sections. Sections were then rehydrated, and nonspecific peroxidase activity was blocked by incubating with 0.3% H_2O_2 for 30 min. Sections were incubated overnight at 4°C with either rabbit anti-human HGF IgG [prepared by Ueki et al. (33)] or anti-mouse c-Met antigen (SP260: Santa Cruz). After being washed in PBS, sections for human HGF immunostaining were incubated with anti-rabbit biotinylated secondary antibody, followed by peroxidase-conjugated avidin (Vector Laboratories). Subsequently, the ABC visualization kit (Vector Laboratories) was used according to the instructions of the manufacturer, and sections were counterstained with hematoxylin. Sections for c-Met immunostaining were incubated with anti-rabbit fluorescent antibody (Alexa Fluor 546: Molecular Probes) followed by counterstaining with Hoechst 33342 (Molecular Probes). Images were examined under a light microscope (Eclipse TE300: Nikon, Tokyo, Japan) equipped with a digital image processing system (Hamamatsu Photonics, Shizuoka, Japan).

For the observation of HCs, the cochlea was dissected from the rat transcardially fixed with 4% paraformaldehyde (PFA), and the lateral wall, stria vascularis, tectorial membrane, and Reissner's membrane of the cochlea were removed, and the organ of Corti was separated from the modiolus in half-turn segments. Then, the tissue was permeabilized with 0.3% Triton X-100 in PBS for 10 min and incubated in rhodamine-phalloidin (R-415: Molecular Probes Inc.) diluted 1:100 in PBS for 30 min. After a PBS rinse, the organ of Corti was mounted on a glass slide and examined using a confocal microscope (Radiance 2000: Bio-Rad).

For quantitative SGC analysis, the cochleas from the KM + HGF group and KM group in the protection experiment and the therapeutic experiment were removed after cardiac perfusion with 4% PFA, overnight fixation, and decalcification. Cochleas were then placed in 20% sucrose in PBS overnight at 4°C and rapidly frozen in Tissue-Tek (Miles, Inc., Elkhart, IN) on dry ice. Cryosections (10 μm) were cut on a Leica CM3050S cryostat (Leica Microsystems), mounted on Superfrost slides (Fisher Scientific, Houston, TX), and stained with hematoxylin.

TUNEL staining was performed according to the manual of the manufacturer (ApopTag® Plus fluorescein in situ apoptosis detection kit, Intergen). The cryosections from the KM + vector group, KM + HGF group, and control rats on week 4 were stained for detection of apoptosis.

Evaluation of auditory function

To evaluate auditory function, we performed ABR. The ABRs were measured 1 day before the first day of kanamycin administration to determine the baseline responses and were again recorded 7, 14, 21, 28, and 56 days from the first day of the kanamycin treatment. Before each test of auditory function, the animals were anesthetized with an intramuscular injection of a ketamine (50 mg/kg) xylazine (10 mg/kg) solution. Needle electrodes were placed subcutaneously at the ipsilateral right pinna (reference electrode), the contralateral pinna (ground electrode), and the vertex (active electrode). All recordings were performed in a sound-proof room with a Neuropack IV (MEM-4104) system (Nihon-Kohden, Tokyo, Japan). The electric potentials were evoked by single-wave 100 μs click sounds (10/ s), and these monaural stimuli were delivered to the right ear by a loudspeaker. Responses were digitally filtered (bandpass: 50–3000 Hz), amplified, and

averaged (500 responses). The intensity of the stimulus was varied in 2 dB stepwise increments to determine the threshold. The threshold is defined as the lowest intensity level at which responses could still be recorded in two consecutive trials to confirm response reproducibility.

Statistical analysis

All values are means ± SD. ANOVA with Bonferroni/Dunn's post hoc test was used to determine the significance of differences in multiple comparisons. Differences with P values <0.05 were considered significant.

RESULTS

In vivo transfection of β-gal and luciferase gene into CSF using HVJ-E system

The first aim of this study was to determine whether intrathecal administration of the HVJ-E vector would result in efficient transduction to HC, SGC, and CNS in vivo. The brain and cochlea from the rats injected intrathecally with HVJ-E/pc*lac*Z were prepared for X-gal staining. Figure 1 shows that β-gal expressing cells were present in the SGCs, cerebral cortex, and medulla.

Luciferase expression was also examined in the brain, cochlea, lung, spleen, and liver of the rats injected intrathecally with HVJ-E/pcLuc-GL3. One day after injection, strong transgene expression was observed in the cochleas and various areas of the brain (Table 1). The highest luciferase activity was observed in the medulla, which is the area of the brain nearest to the injection point. Luciferase activity in the cochlea was found to be comparable to that in the brain. In contrast, on day 1 and day 5 after transfection, luciferase activity was not detected in the lung, spleen, or liver of the luciferase-injected rats, nor in any of the tested organs from the control-injected rats (data not shown). We further optimized the amount of HVJ-E for the highest gene expression in the brain and cochlea by intrathecal administration of 3×10^{10}, 4.5×10^{10}, and 6×10^{10} particles of HVJ-E containing 20, 30, and 40 μg luciferase gene, respectively. When 4.5×10^{10} particles of HVJ-E were administered, the highest activity was observed in the brain and cochlea as compared with other cases. Therefore, we adopted the dose of 4.5×10^{10} particles of HVJ-E for the administration of HGF gene. Assays were repeated at least twice, and reproducible results were obtained.

In vivo transfection of *HGF* gene into the subarachnoid space

HGF is a secretory protein, which is known to function in an autocrine/paracrine manner on epithelial cells (34) and also on the nervous system (20, 35). To determine the successful transfer of human *HGF* gene into the SGCs and CNS, we first measured the protein level of HGF in the CSF by ELISA (n=4, each group). As expected, human HGF protein was not detected in the CSF of the control rats using an antibody specific for human (but not rat) HGF (33). However, 5 days after transfection, human HGF was readily detected in the CSF of the rats transfected with *hHGF* (Fig. 2*A*). The expression of human HGF could even be detected in rats 12 days after transfection with *hHGF* (Fig. 2*A*). Interestingly, an increase of rat HGF was also observed in CSF from the KM + HGF group (Fig. 2*B*), although human *HGF* gene but not rat *HGF* gene was administered. The concentration of human HGF in CSF may not be elevated to sufficient pharmacological levels (mean value: 0.31 ng/ml on day 5). On the other hand, endogenous rat HGF concentration was ~10

times that of the human HGF (mean value: 2.74 ng/ml on day 5). The concentration of human HGF was also examined, but it was not detected in the serum of the rats administered with *hHGF* or control plasmid (data not shown). These results indicated that *hHGF* was successfully transfected, resulting in the secretion of human HGF protein into the CSF and that exogenous human HGF enhanced secretion of endogenous rat HGF. Assays were repeated at least twice, and reproducible results were obtained.

To determine whether the human *HGF* transgene was expressed in SGCs, we immunocytochemically examined the exogenous HGF expression in the SGCs obtained from rats inoculated with *hHGF* and compared the findings with the result from the control group. As shown in Fig. 2*D*, human HGF was markedly observed in the cytoplasm of SGCs and the percentage of human HGF positive cells was >70%, thus indicating that the *HGF* transgene was introduced not only into the CNS but also into SGCs. The high concentration of human HGF in CSF may also suggest secretion from the transfected cells in the brain.

We next examined the expression of c-Met, which is the tyrosine kinase receptor of HGF, on SGCs. A previous study conducted by us showed that kanamycin treatment alone could enhance the c-Met expression in SGCs (25). Consistent with the previous data we have obtained, we demonstrated an increase of c-Met expression in SGCs of the KM + vector group. In the KM + HGF group, the expression of c-Met was greatly enhanced (Fig. 3), suggesting that the increased c-Met and HGF expression affects the survival of SGCs synergistically. c-Met expression in SGC was measured semiquantitatively by evaluating the spot intensity of the Western blotting image using NIH Image software. The ratio of the c-Met expression was 1:3.8:12.5 (control: KM + vector: KM + HGF).

The effect of HGF on HC and SGC damaged by kanamycin treatment

We examined whether HGF can rescue the loss of the HC and SGC induced by kanamycin insult. The number of SGCs was assessed on mid-modiolar sections stained with hematoxylin. The cochleas receiving only the HVJ-E containing control vector showed a similar number of cells compared with cochleas before receiving kanamycin treatment (data not shown). These results indicated that damage to the SGCs was not induced by intrathecal injection of HVJ-E. In the KM + vector group, on the 14th day of the 2-wk long kanamycin treatment, there was a trend, albeit not a significant one, of reduction in the number of SGCs, as shown in Fig. 4*A*. Subsequent observation showed a significant reduction of SGCs in the KM + vector group 4 and 8 wk after the first day of kanamycin administration. On the other hand, in the KM + HGF group, the cochleas showed significantly more surviving SGCs on weeks 4 and 8 as compared with the KM + vector group. On week 8, the surviving cell count in the KM + HGF group was ~6 times higher than that in the KM + vector group (13.3±3.2 cells/10,000 μm^2 vs. 2.2±1.8 cells/10,000 μm^2, $P<0.05$). These results revealed that HGF gene transfer has a protective effect on SGC survival. Light microscopic examination demonstrated that there were many cells showing vacuolated cytoplasm and nuclei containing clumped chromatin in the KM + vector group (Fig. 4*C*). In the KM + HGF group, however, there were considerably less cells showing such appearances and most cells had a similar appearance as the control (Fig. 4*B* and *D*). To determine whether the loss of SGCs was the consequence of apoptotic cell death, we performed TUNEL staining of SGCs. TUNEL staining showed lower numbers of positive cells in the KM + HGF group as compared with the KM + vector group and control rats (Fig. 4*E-G*). These results suggested that SGCs undergo apoptosis in

response to kanamycin treatment and that *HGF* gene transfer by intrathecal HVJ-E inoculation was protective against kanamycin insult.

The number of HCs was assessed on fluorescence images of rhodamine phalloidin-stained whole-mount preparations of the organ of Corti. Severe loss of the outer HCs and partial loss of the inner HCs were observed in the KM group, and they were replaced by scars (Fig. 4*I*). In contrast, inner and outer HCs in the KM + HGF group were well preserved as well as in the control rat (Fig. 4*J*).

Evaluation of hearing function

According to the experimental time course in Fig. 5*A*, we evaluated the protective effect of *HGF* gene transfer on hearing function by analyzing the changes of hearing function using ABR. The KM group showed a significant threshold shift (Fig. 5*B*). The mean shift was 32.6 dB on day 14, and they continued to show significant threshold shift (35-40 dB) by day 56. On the other hand, the KM + HGF group showed nearly normal ABR threshold. Interestingly, the KM + vector group also showed significant reduction of the shift on days 14 and 21, although an increase of the shift was later observed. Statistically significant differences were found between the KM + HGF group and the KM group and between the KM + HGF group and the KM + vector group on days 14, 21, 28, and 56.

Toward examining the potential for human gene therapy, we transferred *HGF* gene into rats after hearing impairment was observed, as described in Fig. 5*A*. As seen in Fig. 5*C*, transfection of *hHGF* into the subarachnoid space of the hearing-impaired rats significantly reduced the threshold shift in ABR, compared with rats transfected with the control vector. We also measured the number of SGCs in mid-modiolar sections of the cochleas from rats transfected with *hHGF* and control vector after kanamycin treatment. As expected, transfection of *hHGF* resulted in a significant reduction of the number of SGC loss (7.2±1.2 cells/10,000 μm^2 on week 8). These results suggested that SGC protection induced by HGF gene transfer, either before or after kanamycin insult, improved hearing function.

DISCUSSION

In this study, we demonstrated that intrathecal injection of HVJ-E containing *hHGF* into CSF effectively prevented the loss of HC and SGC by inhibition of apoptosis. Consequently, hearing impairment was prevented when the *HGF* gene was administered shortly before kanamycin treatment, and hearing function was recovered even after induction of hearing impairment by kanamycin. These data indicated the high potential of *HGF* gene therapy using the HVJ-E vector for both the prevention and treatment of hearing impairment. The success of this gene therapy is due to two novel issues. One is the novel nonviral vector system, and another is the therapeutic molecule with multiple functions.

Several vectors, including adenoviral vector and adeno-associated virius (AAV), have been used for gene delivery to the inner ear (5, 6, 9, 10, 36–42). In this study, we employed the HVJ-E vector system as a delivery method to the inner ear. This vector is the so-called second generation of the HVJ vector, since we have previously developed a chimeric vector system, the HVJ-liposome, that combines DNA-loaded liposomes with a fusigenic envelope derived from inactivated HVJ

(43–45). The liposome in which plasmid DNA is packaged is fused with UV-inactivated HVJ to form the HVJ-liposome. This vector has been available for gene transfer to various organs, and thus the CNS was an appropriate target for gene delivery. However, this system is of limited use because the fusion activity of the HVJ-liposome is greatly decreased compared with that of native HVJ as a result of the reduced density of fusion proteins (HN and F) on HVJ-liposomes. Another problem is that vector production is somehow complicated by the presence of two distinct vesicles, HVJ particles and liposomes. To address these problems, we have developed a simple method to convert inactivated HVJ into a gene transfer vector by introducing plasmid DNA directly into inactivated HVJ particles, by treatment with mild detergent and centrifugation in the presence of plasmid DNA (28). Therefore, the amount of fusion proteins in the HVJ-E vector particles is the same as that in the native HVJ. Our previous studies demonstrated the successful delivery of DNA to cultured cells and animal tissues such as the liver, skin, uterus, lung, eye, tumor tissues (28), and brain (29).

There are essentially four surgical approaches certified for gene transfer to the inner ear: *1*) direct injection into the cochlea with cochleostomy, *2*) administration through the round window membrane with injection via the membrane or with permeation by placing a piece of vector-soaked gelform on the intact membrane, *3*) inoculation into the inner ear through the posterior semicircular canal with canalostomy, and *4*) inoculation into the endolymphatic sac (36–39). Various viral vectors such as adenovirus vector, herpes virus vector, and AAV vector have been directly injected into the inner ear using either one of the four approaches described above. However, each approach has its own advantages and disadvantages in terms of invasiveness and efficacy. In this experiment, we injected the HVJ-E vector intrathecally into the CSF to avoid invasion to the inner ear by direct injection to the cochlea. Using this approach, we verified transgene expression in SGCs by enzyme activity and immunostaining, and no significant damage was observed in either brain or ear tissues. This fact suggests that the HVJ-E vector itself reached the SGCs of the inner ear after the administration into the CSF. Several possible pathways from the CSF to the inner ear have been indicated. Lalwani et al. (40) reported that, after unilateral cochlear infusion of the AAV vector containing the GFP gene, reporter gene expression was detected not only in the inoculated cochlear but also in the contralateral (uninoculated) cochlea and the brain. They speculated three potential routes of this spread: *1*) via the temporal bone marrow space, *2*) via the bloodstream, and *3*) via the cochlear aqueduct, which connects the CSF space to the scala tympani of the basal turn of the cochlea (41). Moreover, Stöver et al. (42) reported functional communication between the CSF and the perilymphatic space of the cochlea. Here, we reported the first therapeutic application of intrathecal approach via cisterna magna for cochlear gene transfer. Since we did not observe any luciferase activity in distant organs when the vector was injected intrathecally, the most likely pathway to reach the inner ear region from CSF is considered to be via the cochlear aqueduct. If the vector had been disseminated systemically via the bloodstream, transgene expression should have been detected in distant organs such as the spleen and lung, as luciferase activity was detected primarily in the spleen after intravenous injection (28). Although safety issues regarding the dissemination of gene transfer vectors beyond the targeted cochlea will need to be addressed before its application to human gene therapy, this approach may be advantageous, especially for bilateral cochlear gene therapy.

Neurotrophic factors such as NGF, BDNF, GDNF, and NT-3 have been used as therapeutic molecules for the auditory systems and shown to improve the survival of auditory neurons (5–11). HGF, however, has not been used for this purpose to date. HGF was first identified as a potent

mitogen for mature hepatocytes and was cloned in 1989 (12, 13). In addition to its hepatotrophic effect, HGF was revealed to exhibit neurotrophic activity in the hippocampus, cerebral cortex, sensory neurons, and motor neurons (14, 20). Recently, Hayashi et al. (21) reported that *HGF* gene transfer to the subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. Sun and Nakamura et al. (46) reported that introduction of the *HGF* gene into neurons of ALS-model mice attenuates motor-neuronal degeneration and increases the lifespan of these mice. We demonstrate here that human HGF was detected in both CSF and SGCs and that it induced the expression of rat endogenous HGF. Moreover, the induction of HGF increased expression of the HGF receptor c-Met in SGCs to augment signal transduction of HGF. HGF is also known to have anti-apoptotic activity by increasing the ratio of bcl-2 to bax through the PI3K/Akt pathway (47) and to have the capacity to induce angiogenesis and increase blood flow (48–51). These functions of HGF can be enhanced by a positive feedback mechanism. Recent studies have shown that the feedback mechanism involved in HGF signaling is mediated by an essential transcription factor, ets. Aoki et al. (48) demonstrated that HGF up-regulates ets activity and ets-1 protein. Not only rat HGF, but also exogenously expressed human HGF, stimulates endogenous HGF expression through the induction of ets activity (49). A RAS-RAF-MEK-ERK signaling pathway is involved in the activation of ets-1 transcription by HGF (52). When ets-1 expression was inhibited by the transfection of antisense ets-1 oligodeoxynucleotides, HGF expression was markedly decreased (49, 50). In this study, the biological effects of HGF appeared to be up-regulated multifold by such a positive feedback mechanism, although the level of human HGF in CSF was much lower than rat HGF after stimulation by human HGF. *HGF* gene therapy for the auditory system may have several advantages over the previous gene therapy strategies using neurotrophins, but further comparative experiments using other neurotrophins are needed. Although the precise measurement of cochlear blood flow would be difficult, further study of vascular function in the cochlea after HGF gene transfer will provide novel information regarding cochlear function. Moreover, there exists another possibility in which HGF could cause the regeneration of HC or SGC as implied in this study, and we are now investigating the regenerative effect of HGF on inner ear cells. Combined therapy of cochlear implant and HGF gene therapy, i.e., administering the *HGF* gene during the operation of cochlear implant, would be also effective.

Hearing impairment was associated with the loss of HC and SGC, and the prevention of their loss was achieved by the protective effect of HGF against apoptotic cell death. HGF expression was also effective for the recovery of hearing function, after previous impairment by kanamycin treatment. Thus, *HGF* gene therapy is a potent candidate for the treatment of sensorineural hearing impairment. This research provides a new insight and approach for clinical treatment for hearing impairment by combining the *HGF* gene with the HVJ-E vector delivery system.

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REFERENCES

1. Mills, J. H., and Going, J. A. (1982) Review of environmental factors affecting hearing. *Environ. Health Perspect.* **44,** 119–127

2. Spoendlin, H. (1975) Retrograde degeneration of the cochlear nerve. *Acta Otolaryngol.* **79,** 266–275

3. Bichler, E., Spoendlin, H., and Rauchegger, H. (1983) Degeneration of cochlear neurons after amikacin intoxication in the rat. *Arch. Otorhinolaryngol.* **237,** 201–208

4. Incesulu, A., and Nadol, J. B., Jr. (1998) Correlation of acoustic threshold measures and spiral ganglion cell survival in severe to profound sensorineural hearing loss: implications for cochlear implantation. *Ann. Otol. Rhinol. Laryngol.* **107,** 906–911

5. Bowers, W. J., Chen, X., Guo, H., Frisina, D. R., Federoff, H. J., and Frisina, R. D. (2002) Neurotrophin-3 transduction attenuates Cisplatin spiral ganglion neuron ototoxicity in the cochlea. *Mol. Ther.* **6,** 12–18

6. Chen, X., Frisina, R. D., Bowers, W. J., Frisina, D. R., and Federoff, H. J. (2001) HSV amplicon-mediated neurotrophin-3 expression protects murine spiral ganglion neurons from cisplatin-induced damage. *Mol. Ther.* **3,** 958–963

7. Ernfors, P., Duan, M. L., ElShamy, W. M., and Canlon, B. (1996) Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3. *Nat. Med.* **2,** 463–467

8. Gabaizadeh, R., Staecker, H., Liu, W., Kopke, R., Malgrange, B., Lefebvre, P. P., and Van de Water, T. R. (1997) Protection of both auditory hair cells and auditory neurons from cisplatin induced damage. *Acta Otolaryngol.* **117,** 232–238

9. Gao, W. Q. (1998) Therapeutic potential of neurotrophins for treatment of hearing loss. *Mol. Neurobiol.* **17,** 17–31

10. Staecker, H., Kopke, R., Malgrange, B., Lefebvre, P., and Van de Water, T. R. (1996) NT-3 and/or BDNF therapy prevents loss of auditory neurons following loss of hair cells. *Neuroreport* **7,** 889–894

11. Yagi, M., Kanzaki, S., Kawamoto, K., Shin, B., Shah, P. P., Magal, E., Sheng, J., and Raphael, Y. (2000) Spiral ganglion neurons are protected from degeneration by GDNF gene therapy. *J. Assoc. Res. Otolaryngol.* **1,** 315–325

12. Nakamura, T., Nawa, K., and Ichihara, A. (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* **122,** 1450–1459

13. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342,** 440–443

14. Honda, S., Kagoshima, M., Wanaka, A., Tohyama, M., Matsumoto, K., and Nakamura, T. (1995) Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor. *Brain Res. Mol. Brain Res.* **32,** 197–210

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

16. Hashimoto, N., Yamanaka, H., Fukuoka, T., Dai, Y., Obata, K., Mashimo, T., and Noguchi, K. (2001) Expression of HGF and cMet in the peripheral nervous system of adult rats following sciatic nerve injury. *Neuroreport* **12,** 1403–1407

17. Hashimoto, N., Yamanaka, H., Fukuoka, T., Obata, K., Mashimo, T., and Noguchi, K. (2001) Expression of hepatocyte growth factor in primary sensory neurons of adult rats. *Brain Res. Mol. Brain Res.* **97,** 83–88

18. Jung, W., Castren, E., Odenthal, M., Vande Woude, G. F., Ishii, T., Dienes, H. P., Lindholm, D., and Schirmacher, P. (1994) Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. *J. Cell Biol.* **126,** 485–494

19. Maina, F., Hilton, M. C., Ponzetto, C., Davies, A. M., and Klein, R. (1997) Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. *Genes Dev.* **11,** 3341–3350

20. Maina, F., and Klein, R. (1999) Hepatocyte growth factor, a versatile signal for developing neurons. *Nat. Neurosci.* **2,** 213–217

21. Hayashi, K., Morishita, R., Nakagami, H., Yoshimura, S., Hara, A., Matsumoto, K., Nakamura, T., Ogihara, T., Kaneda, Y., and Sakai, N. (2001) Gene therapy for preventing neuronal death using hepatocyte growth factor: in vivo gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. *Gene Ther.* **8,** 1167–1173

22. Miyazawa, T., Matsumoto, K., Ohmichi, H., Katoh, H., Yamashima, T., and Nakamura, T. (1998) Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: a novel neurotrophic factor. *J. Cereb. Blood Flow Metab.* **18,** 345–348

23. Yamada, K., Moriguchi, A., Morishita, R., Aoki, M., Nakamura, Y., Mikami, H., Oshima, T., Ninomiya, M., Kaneda, Y., Higaki, J., et al. (1996) Efficient oligonucleotide delivery using the HVJ-liposome method in the central nervous system. *Am. J. Physiol.* **271,** R1212–R1220

24. Yoshimura, S., Morishita, R., Hayashi, K., Kokuzawa, J., Aoki, M., Matsumoto, K., Nakamura, T., Ogihara, T., Sakai, N., and Kaneda, Y. (2002) Gene transfer of hepatocyte growth factor to subarachnoid space in cerebral hypoperfusion model. *Hypertension* **39,** 1028–1034

25. Doi, K., Hasagawa, T., Fuse, Y., Uno, Y., Fujii, K., and Kubo, T. (1999) Change of auditory system in animal model of cochlear implant. *Otol. Jpn* **9,** 85–90

26. Wu, W. J., Sha, S. H., McLaren, J. D., Kawamoto, K., Raphael, Y., and Schacht, J. (2001) Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. *Hear. Res.* **158,** 165–178

27. Forge, A., and Schacht, J. (2000) Aminoglycoside antibiotics. *Audiol. Neurootol.* **5,** 3–22

28. Kaneda, Y., Nakajima, T., Nishikawa, T., Yamamoto, S., Ikegami, H., Suzuki, N., Nakamura, H., Morishita, R., and Kotani, H. (2002) Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol. Ther.* **6,** 219

29. Shimamura, M., Morishita, R., Endoh, M., Oshima, K., Aoki, M., Waguri, S., Uchiyama, Y., and Kaneda, Y. (2003) HVJ-envelope vector for gene transfer into central nervous system. *Biochem. Biophys. Res. Commun.* **300,** 464–471

30. Tomita, N., Morishita, R., Higaki, J., Tomita, S., Aoki, M., Kaneda, Y., and Ogihara, T. (1996) Sustained transgene expression by transfection of renin gene into liver of neonates. *Biochem. Biophys. Res. Commun.* **224,** 43–49

31. Tsujie, M., Isaka, Y., Nakamura, H., Imai, E., and Hori, M. (2001) Electroporation-mediated gene transfer that targets glomeruli. *J. Am. Soc. Nephrol.* **12,** 949–954

32. Sun, W., Funakoshi, H., and Nakamura, T. (1999) Differential expression of hepatocyte growth factor and its receptor, c-Met in the rat retina during development. *Brain Res.* **851,** 46–53

33. Ueki, T., Kaneda, Y., Tsutsui, H., Nakanishi, K., Sawa, Y., Morishita, R., Matsumoto, K., Nakamura, T., Takahashi, H., Okamoto, E., et al. (1999) Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat. Med.* **5,** 226–230

34. Tsao, M. S., Zhu, H., Giaid, A., Viallet, J., Nakamura, T., and Park, M. (1993) Hepatocyte growth factor/scatter factor is an autocrine factor for human normal bronchial epithelial and lung carcinoma cells.PG - 571-9. *Cell Growth Differ.* **4,** 571–579

35. Yang, X. M., Toma, J. G., Bamji, S. X., Belliveau, D. J., Kohn, J., Park, M., and Miller, F. D. (1998) Autocrine hepatocyte growth factor provides a local mechanism for promoting axonal growth. *J. Neurosci.* **18,** 8369–8381

36. Kawamoto, K., Oh, S. H., Kanzaki, S., Brown, N., and Raphael, Y. (2001) The functional and structural outcome of inner ear gene transfer via the vestibular and cochlear fluids in mice. *Mol. Ther.* **4,** 575–585

37. Stöver, T., Yagi, M., and Raphael, Y. (1999) Cochlear gene transfer: round window versus cochleostomy inoculation. *Hear. Res.* **136,** 124–130

38. Jero, J., Mhatre, A. N., Tseng, C. J., Stern, R. E., Coling, D. E., Goldstein, J. A., Hong, K., Zheng, W. W., Hoque, A. T., and Lalwani, A. K. (2001) Cochlear gene delivery through an intact round window membrane in mouse. *Hum. Gene Ther.* **12,** 539–548