ment within OPF gels of cationized gelatin microspheres loaded with plasmid DNA prolongs the release and subsequent bioavailability of the DNA compared to both the release of DNA from non-entrapped cationized gelatin microspheres and the persistence of injected DNA solution. A direct correspondence is apparent between the degradation of the cationized gelatin microspheres of either formulation embedded within OPF and the release of plasmid DNA from these composites (Figs. 2 and 3). Complete degradation of cationized gelatin microspheres embedded within OPF apparently required more time than for microspheres not embedded within OPF. It is likely that the slower apparent degradation of cationized gelatin microspheres embedded within OPF was due to the retention of cationized gelatin degradation fragments complexed with plasmid DNA within the OPF network.

Interestingly, the release of DNA from OPF alone and from either composite formulation was not significantly different. This result indicates that the OPF network controlled the rate of plasmid DNA release once the DNA was liberated from the cationized gelatin microspheres embedded within it. Indeed, the mesh size of OPF 10 K hydrogels is 13.6 ± 0.3 nm [22], whereas the apparent molecular size of plasmid DNA is much larger, on the order of hundreds of nanometers [8,29], as is the apparent molecular size of plasmid DNA–cationized gelatin complexes (164 ± 21.3 nm) [8]. Enzymes, however, are smaller than the mesh size of the OPF and can readily enter the hydrogels. Indeed, the entry of enzymes such as collagenase into OPF is required to degrade the CGMS component of the composites. Thus, the release of plasmid DNA from within the OPF network is likely dominated by the degradation of the OPF itself, as the small mesh size of OPF in relation to the size of plasmid DNA and of plasmid DNA-cationized gelatin complexes likely presents a significant barrier to the release of plasmid DNA by simple diffusion. However, as radiolabeling of the OPF was not feasible for this study due to a lack of primary amines in OPF, the in vivo degradation kinetics of OPF could not be examined for comparison with the observed DNA release kinetics.

In the case of the non-embedded cationized gelatin microspheres, it was expected that the release of DNA would be in good accordance with the degradation of the microspheres, as has been observed in previous studies [9,10,13]. Based upon previous results, it was anticipated that approximately 40–50% of the initial plasmid DNA and of the initial cationized gelatin would be remaining by day 3 [9,10,13]. However, this was not the case in the present study, as less than 10% of the initial plasmid DNA was remaining at day 3 for either microsphere formulation. Further, the degradation of cationized gelatin microspheres and the apparent release of plasmid DNA from the microspheres were not in accord in the present study for either microsphere formulation.

The use of radiolabeled materials required the assumption that no unbound radioisotope was present in the samples initially and that the radiolabel remained bound to the DNA or cationized gelatin throughout the duration of the study. This assumption was valid based upon experience from previous studies involving the release of radioiodinated plasmid DNA from CGMS [9,10,13]. Further, as seen in Fig. 1, the results of the radioiodination control study indicate that the radiolabel was associated with the plasmid DNA, with little to no unbound radioisotope present after purification. With respect to the possible dissociation of radioiodine from the plasmid, the primary mechanism for dissociation, in general, would be damage to the DNA induced by the hydrolytic action of nucleases or by comparable conditions, such that the plasmid is cleaved into small fragments, some of which contain the radiolabel. Release of these small fragments would result in an apparent release of the plasmid DNA. In the event that a small amount of unassociated radioisotope survived the purification procedure and was present in the samples initially or that radiolabel dissociated from the DNA or cationized gelatin during the course of the study, an apparent burst release or degradation effect would be observed.

Further, in the event that the plasmid DNA was not fully incorporated into the cationized gelatin microspheres, it is possible that an apparent burst release of DNA from the microspheres would be observed, as in the present study. However, as the volume of DNA solution added to the freeze-dried CGMS for loading was lower than the equilibrium swelling volume for the CGMS, the solution was completely incorporated into the CGMS, as in previous studies [9,10,13]. It follows that any DNA that did not incorporate into the

CGMS with the solution was associated with the surfaces of the CGMS. Therefore, the DNA was fully associated with the CGMS following loading, be it within the microspheres or on the surface of the microspheres.

The results indicate that a high degree of plasmid DNA was not fully incorporated within the cationized gelatin microspheres. Any DNA not incorporated within the CGMS would be either associated with the surface of the microspheres or injected in solution with the microspheres and would be expected to persist at the site of injection in a manner as observed for the injection of DNA solution alone. Indeed, no significant difference was observed between the release of plasmid DNA from non-embedded cationized gelatin microspheres and the persistence of injected DNA solution through day 7, whereas large differences were observed between released DNA and injected DNA over this time period in previous studies [9,10,13]. However, as DNA continued to be released beyond the limit of the observed persistence of injected DNA solution, it follows that some plasmid DNA was indeed incorporated within the cationized gelatin microspheres and subsequently released in a controlled manner.

With respect to the composites of OPF and cationized gelatin microspheres, any effect of unincorporated plasmid DNA on the observed release would be expected to be minimal, as one would anticipate unincorporated DNA to be released from the OPF scaffold in the same manner as DNA that was incorporated into the microspheres, based upon previously discussed size considerations. Although it is possible that the salt content of serum could accelerate dissociation of plasmid DNA from the CGMS relative to what one might observe in the absence of serum, the large size of plasmid DNA relative to the mesh size of OPF would result in retention of the plasmid within the OPF network of composites. Indeed, the observed release of plasmid DNA occurred in a sustained fashion in good accordance with the degradation of the embedded microspheres. Further investigation is warranted to more fully elucidate the apparent limitations on DNA incorporation within the cationized gelatin microspheres faced in the present study. Taking the effects of potentially unincorporated plasmid DNA upon the observed release kinetics into consideration, especially in the case of non-embedded cationized gelatin microspheres, it follows that the actual increases in the availability of DNA provided by cationized gelatin microspheres and by composites of the microspheres with OPF may be greater than those observed in the present study.

Although a prolonged bioavailability of therapeutic plasmid DNA is important to the success of a controlled release gene therapy system, the structural integrity of the DNA following release is of great importance to the potential of the DNA to transfect cells and be expressed. As characterization of the structural integrity of plasmid DNA released in vivo was not feasible, the structural integrity of plasmid DNA released from materials in vitro was assessed through agarose gel electrophoresis. The samples were housed in PBS containing the enzyme collagenase 1A, an enzyme known to digest gelatin through recognition of the sequence –X–Gly–Pro–R–, where X is a neutral amino acid [30]. The presence of collagenase was necessary to degrade the CGMS and to simulate physiological conditions. The collagenase 1A was present in the PBS at a concentration that approximates physiologically relevant concentrations [31].

The electrophoresis results indicate that plasmid DNA converts predominantly to the open-circular conformation when released from both the OPF alone and from composites of OPF and CGMS. The structure of the released DNA, however, does not change appreciably from the open-circular conformation with time of release for any of the material formulations examined. Although some conversion of plasmid DNA from the super-coiled to the open-circular conformation was observed, the literature suggests that there is little difference between the efficiency in transfection or transformation protocols of super-coiled and open-circular plasmid DNA, whereas linear DNA is much less efficient [32,33]. Although the integrity of plasmid DNA following release from composites of OPF and 3 mM CGMS was not assessed in vitro, the results would not be expected to differ from those of composites of OPF and 6 mM CGMS, as the only difference between the two groups is the higher concentration of glutaraldehyde employed in the crosslinking of 6 mM CGMS. Thus, the in vitro results demonstrate that plasmid DNA released from OPF and composites of OPF

and CGMS maintains viable structural integrity over the course of 42 days of release.

In addition to releasing plasmid DNA in a controlled manner while maintaining the structural integrity of the released DNA, the success of a candidate system for controlled DNA release in vivo requires that the components of the system be cytocompatible. Although potentially toxic materials such as TEMED and glutaraldehyde were employed in the fabrication of the materials examined in the present study, the cytotoxicity of the chemicals associated with OPF crosslinking has been addressed in previous reports [34,35]. Indeed, OPF crosslinked by the present method has been employed in studies involving cell encapsulation [19,20]. Further, extensive measures were taken to remove and deactivate glutaraldehyde following crosslinking of the CGMS, including treatment with glycine solution and a total of six washes with water. Indeed, gelatin microspheres crosslinked with glutaraldehyde in this manner were recently explored for the encapsulation of chondrocytes within composites of gelatin microspheres and OPF [36], demonstrating the cytocompatibility of the composite system.

Despite the limitations potentially associated with loading of plasmid DNA within the cationized gelatin microspheres, the increased persistence of plasmid DNA at the site of introduction provided by the microspheres and by the composites of cationized gelatin microspheres and OPF relative to the injection of DNA solution is apparent. Additionally, the prolonged persistence of plasmid DNA when delivered through cationized gelatin microspheres embedded within OPF rather than through the microspheres alone was demonstrated. Although no difference in DNA release was observed between OPF and composites of OPF and cationized gelatin microspheres, the presence of the microspheres may serve two valuable functions. First, the microspheres may act to serve as a porogen within the OPF hydrogel, allowing for the formation upon degradation of void volume into which tissue may grow. Although the term of plasmid DNA release can be controlled through the use of cationized gelatin microspheres alone [9,10], the degradation rate of the microspheres alone is too rapid with respect to typical rates of tissue ingrowth to allow for the microspheres to act as viable tissue scaffolds. The combination of cationized gelatin microspheres with OPF, however, may provide an ideal tissue scaffold by both prolonging the period of the bioavailability of the cationized gelatin and therapeutic plasmid DNA and by creating a porous OPF network into which tissue may grow. Second, plasmid DNA has been shown to form polyionic complexes with cationized gelatin upon loading and been proposed to be released with cationized gelatin degradation fragments as a complex with net positive charge [7]. This released complex of plasmid DNA with cationized gelatin has been proposed to protect the DNA from degradation by nucleases and to facilitate cellular entry [8]. Indeed, the release of plasmid DNA from cationized gelatin has been shown to prolong the duration of gene expression relative to the injection of plasmid DNA in solution form [7,9,10,12]. Thus, composites of cationized gelatin microspheres and OPF show promise as a vehicle for the controlled, sustained delivery of plasmid DNA for enhanced and prolonged gene expression. Further study is warranted, however, to investigate the effect of the controlled release of plasmid DNA from the composites upon gene expression.

5. Conclusion

Composites of cationized gelatin microspheres and a novel hydrogel material, oligo(poly(ethylene glycol)fumarate) were fabricated and investigated toward prolonging the release of plasmid DNA in vivo relative to the constituent materials. The effectiveness of the composites in prolonging plasmid DNA bioavailability relative to both injected plasmid DNA solution and non-embedded cationized gelatin microspheres was demonstrated. Interestingly, the release of plasmid DNA from composites was not significantly different than release from OPF alone. The release of plasmid DNA from the composites appears to be controlled by the degradation of the OPF and of the embedded microspheres. Despite the lack of difference in DNA release observed from OPF and the composites, the formation of composites provides the potential for the formation of porous OPF scaffolds to facilitate tissue infiltration and for enhanced gene expression through the release of plasmid DNA complexed to cationized gelatin degradation fragments. Thus, composites of

OPF and cationized gelatin microspheres show promise for application in the long-term controlled release of plasmid DNA.

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Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats[1]

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SPECIFIC AIM

No satisfactory therapy for sensorineural hearing impairment is yet available because the auditory sensory epithelium (hair cell (HC)) and its associated neuron (spiral ganglion cell (SGC)) are hardly regenerated in mammalians. We developed a novel gene therapy strategy to prevent and ameliorate hearing impairment by administration of the hemagglutinating virus of Japan envelope (HVJ-E) vector containing hepatocyte growth factor (HGF) gene into the cerebrospinal fluid (CSF).

PRINCIPAL FINDINGS

1. Gene transfer into the inner ear region by intrathecal injection of HVJ-E vector

We developed an effective gene delivery system to the inner ear with minimum invasiveness. We used an HVJ-E vector system, a novel nonviral vector with fusion activity derived from hemagglutinating virus of Japan (HVJ) (Sendai virus). To examine the efficiency, distribution, and safety of the HVJ-E vector, we injected HVJ-E containing marker genes *lacZ* gene and luciferase gene intrathecally into the CSF of rats via the cisterna magna. No significant damage was observed in either the brain or ear tissues. β-gal expression was observed in the SGC and stria vascularis, as well as in the cerebral cortex, cerebellum, and medulla. Luciferase activity was detected in the cerebral cortex, medulla, and cochlea from rats injected with the HVJ-E containing luciferase gene but not in other organs such as the lung, spleen, or liver. These results showed that the HVJ-E vector reached the inner ear region after the intrathecal administration and transduced the gene into the tissues without significant damage.

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

We used HGF as a therapeutic molecule for hearing impairment. We administered HVJ-E containing the human *HGF* gene (*hHGF*) into the CSF and measured the protein level of HGF by ELISA. Human HGF protein was detected in the CSF of the rats transfected with *hHGF* even after 12 days of transfection (mean value 0.31 ng/mL on day 5). An increase of rat HGF was also observed in the CSF from the rats administered with *hHGF* (mean value 2.74 ng/mL on day 5). We immunocytochemically checked exogenous HGF expression in the SGCs obtained from rats inoculated with *hHGF* (pVAX1-*hHGF*) and compared the findings with the result from the control group using the control vector (pVAX1) alone. Human HGF was clearly observed in the cytoplasm of SGCs and the percentage of human HGF positive cells was >70%. We next examined the expression of c-Met, a tyrosine kinase receptor of HGF, on SGCs. In rats administered *hHGF*, the expression level of c-Met was greatly enhanced in SGC cytoplasm.

3. Protective and therapeutic effect of HGF on the inner ear damaged by kanamycin insult

We examined whether HGF can rescue the loss of the HC and SGC induced by kanamycin (KM) treatment. KM was used to mimic the clinical situation of hearing impairment, in which the HC is damaged and lost, leading to the degeneration of SGC due to the lack of neurotrophic substances and electric stimuli. Severe loss of the outer HC and partial loss of the inner HC were observed in the rats inoculated with KM and HVJ-E/pVAX1 (KM+vector group) (**Fig. 1*I***). However, inner and outer HCs in the rats administered with KM and HVJ-E/pVAX1-*hHGF* (KM+HGF group), as well as those in the control rat, were well preserved (Fig. 1*H, J*). The number of surviving SGCs was assessed. A significant reduction of SGCs was observed in the KM +

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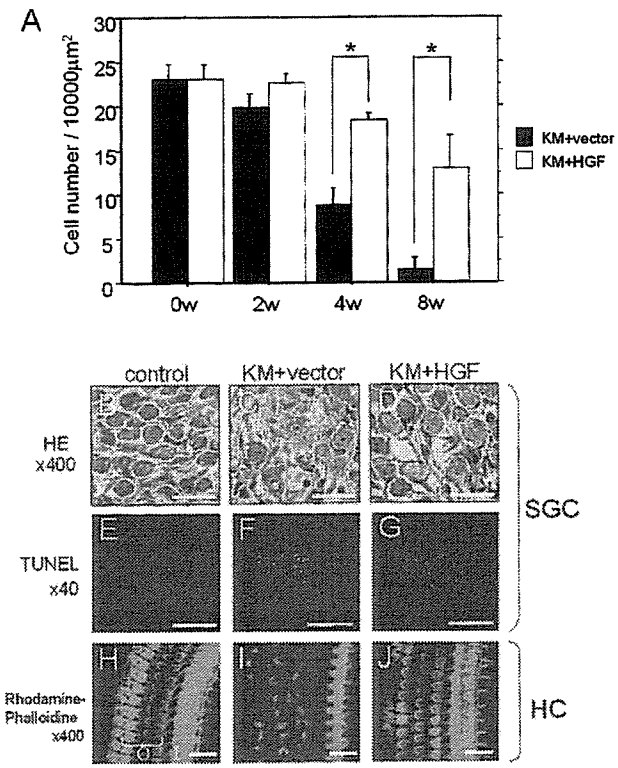

Figure 1. The protective effect of the HGF transgene on SGC and HC treated with kanamycin. Numbers of hematoxylin-positive cells of SGC of the rats treated with KM + vector or KM + HGF are counted at various time points (*A*) (n=6 for each group). Midmodiolar 10 μm cryosections from rats without treatment (control) (*B*), treated with kanamycin and HVJ-E containing control vector (*C*), or HVJ-E containing the human HGF gene (*D*) were stained with hematoxylin on wk 4. TUNEL staining of the contiguous sections of SGCs from the same rats as described above is shown in panels *E–G*. Fluorescent image of HC of the rats in the control, KM + vector, and KM + HGF groups is shown in panels *H–J*. O: outer hair cell, I: inner hair cell. Scale bar: *B–D, H–J)* 50 μm; *E–G)* 500 μm. $*P < 0.01$.

vector group 4 and 8 wk after KM administration (Fig. 1*A*). On the other hand, in the KM + HGF group, the cochlea showed significantly more surviving SGCs on wk 4 and 8 than the KM + vector group. On wk 8, the surviving cell count in the KM + HGF group was ~sixfold higher than those in the KM + vector group (13.3±3.2 cells/10000 μm^2 vs. 2.2±1.8 cells/10000 μm^2, $P<0.05$) (Fig. 1*A*). These results show that *HGF* gene transfer has a protective effect on HC and SGC survival. Light microscopic examination demonstrated there were many cells showing vacuolated cytoplasm and nuclei containing clumped chromatin in the KM + vector group (Fig. 1*C*). In the KM + HGF group, however, there were considerably fewer cells with such an appearance and most cells had an appearance similar to the control (Fig. 1*B, D*). TUNEL staining of SGC showed lower numbers of positive cells in the KM + HGF group than the KM + vector group and control rats (Fig. 1*E–G*). These results indicate that the death of HC and SGC in response to KM treatment could be inhibited by the intrathecal HVJ-E inoculation of the *HGF* gene. Hearing functions before and after KM treatment was also assessed by auditory brainstem response. Hearing impairment was prevented when the *HGF* gene was administered shortly before KM treatment (**Fig. 2*B***); even after induction of impairment by KM, hearing function could be recovered (Fig. 2*C*).

CONCLUSIONS AND SIGNIFICANCE

We demonstrated that intrathecal injection of HVJ-E containing *hHGF* into the CSF prevented the loss of HC and SGC by inhibition of apoptosis and showed a high therapeutic potential for both the prevention and treatment of hearing impairment. The success of this

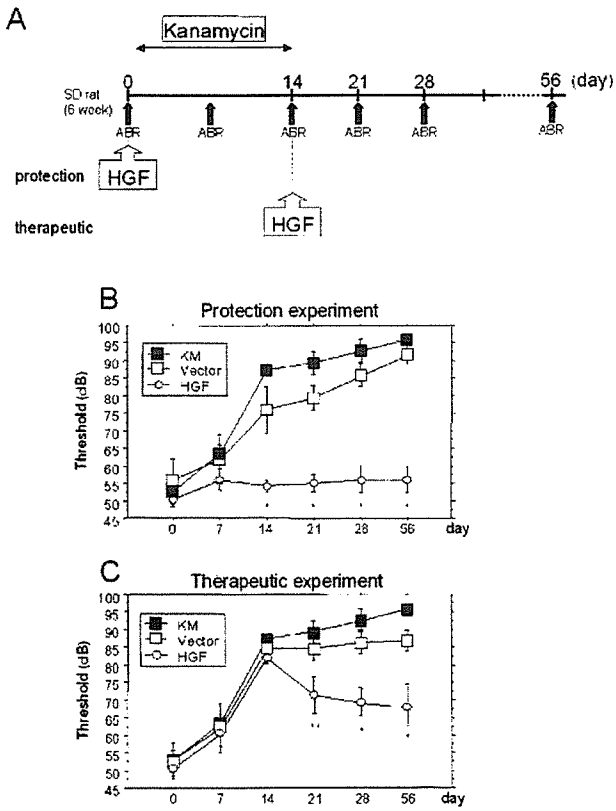

Figure 2. Hearing function of rats treated with KM, KM + vector, or KM + HGF was evaluated by auditory threshold using ABR. *A)* Time course of the experiment. In the protection experiment (*B*), rats treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) immediately prior to the kanamycin insult underwent evaluation of the auditory threshold on days 0, 7, 14, 21, 28, and 56. In the therapeutic experiment (*C*), rats were treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) 14 days after kanamycin insult and the auditory threshold was measured at each time point. KM means the auditory threshold of rats treated only with kanamycin. Six rats were used in each group. Mean and SD of each value are indicated.

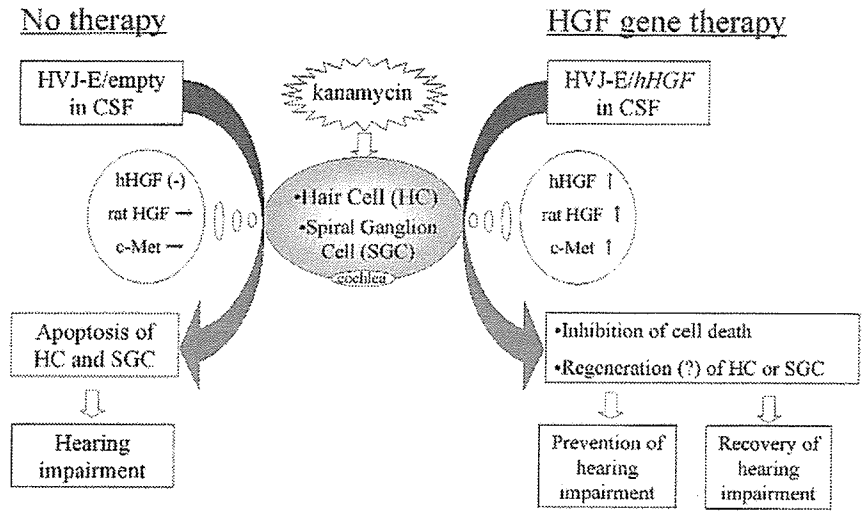

Figure 3. Schematic diagram.

gene therapy is due to two novel issues. One is the novel nonviral vector system; another, the therapeutic molecule with multiple functions.

We used the HVJ-E vector system as a delivery method to the inner ear. HVJ-E vector is constructed by treating HVJ with mild detergent and centrifugation in the presence of plasmid DNA. Our previous studies demonstrated the successful delivery of DNA using this method in vitro and in vivo. In this study, we injected the HVJ-E vector into the CSF to avoid invasion of the inner ear by direct injection to the cochlea. It is thought that HVJ-E most likely spread via the cochlear aqueduct, which connects the CSF to the perilymphatic space of the cochlea. Although safety issues regarding the dissemination of the vector beyond the targeted cochlea need to be addressed, this approach is advantageous, especially for bilateral cochlear gene therapy.

Several neurotrophic factors have been used as therapeutic molecules for the auditory systems. HGF, however, has not been used for this purpose so far. HGF was first identified as a potent mitogen for mature hepatocytes and proved to have multiple functions such as angiogenetic, anti-apoptotic, and neurotrophic activities. These functions of HGF can be enhanced by a positive feedback mechanism, mediated by an essential transcription factor, ETS. In this study, the biological effects of HGF appeared to be up-regulated multifold by such a feedback mechanism, although the level of human HGF in CSF was much lower than rat HGF after stimulation by human HGF. Therefore, *HGF* gene therapy for the auditory system is thought to have several advantages over the earlier gene therapy using neurotrophic factors. Further study of vascular function in the cochlea after HGF gene transfer will provide novel information regarding cochlear function. Moreover, another possibility exists: this study implies that HGF could cause the regeneration of HC or SGC.

Thus, *HGF* gene therapy is a potent candidate for treatment of auditory impairment. This research provides new insight and an approach for clinical treatment for hearing impairment by the combination of the *HGF* gene and the HVJ-E vector system. FJ

Novel Therapeutic Strategy to Treat Brain Ischemia

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

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

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

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

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

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

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

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

Methods

Preparation of HVJ-Envelope Vector

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

In Vivo Gene Transfer Into Subarachnoid Space in Normal Rats

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

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

Histological Examination

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

Evaluation of Effect of HGF Gene Transfer on Infarcted Area

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

Behavior Examination

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

Evaluation of Cerebral Edema After Permanent MCA Occlusion

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

Evaluation of Blood-Brain Barrier Permeability With Evans Blue Dye

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

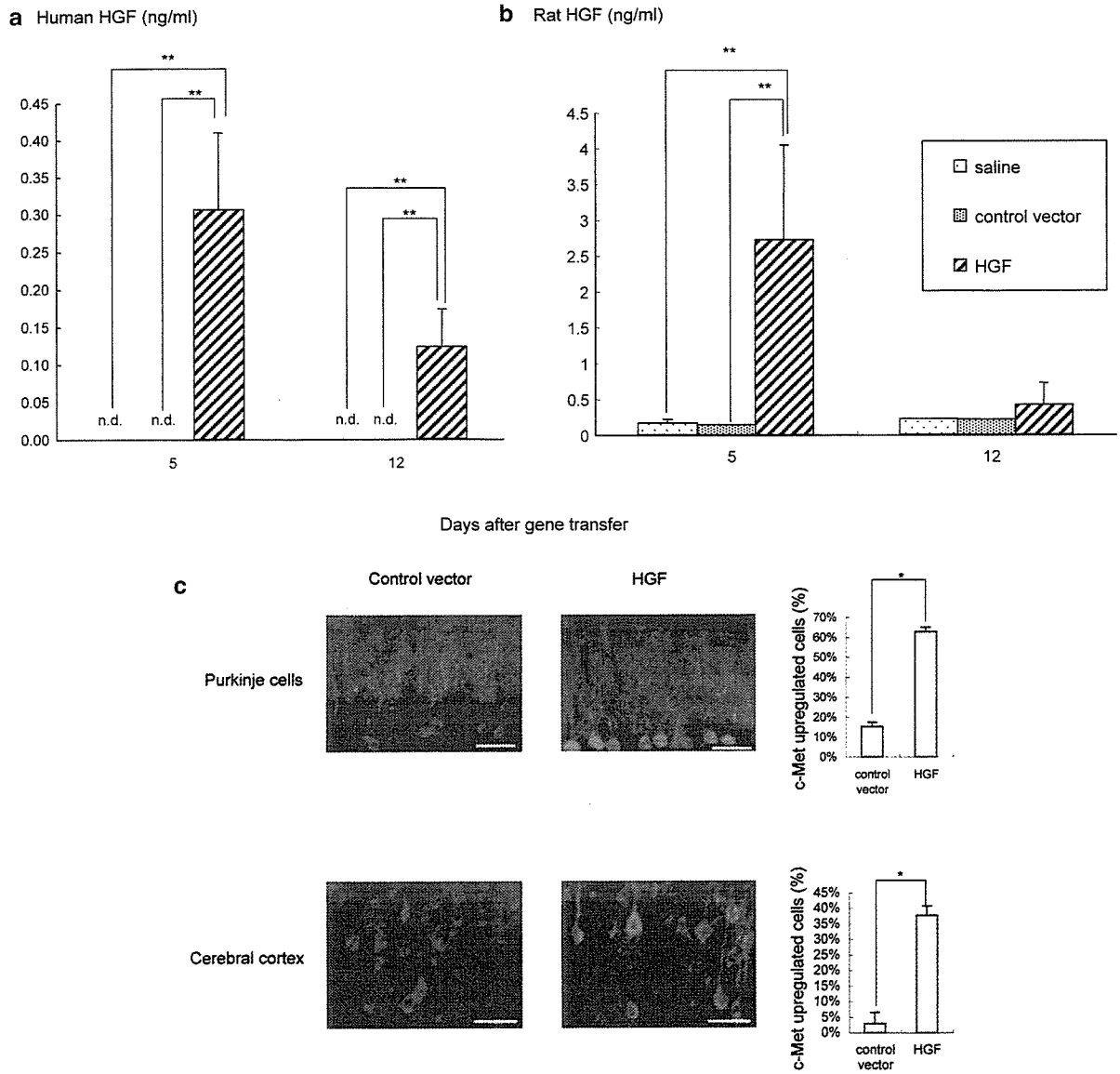

Figure 1. Concentrations of human (a) and rat HGF (b) in CSF at 5 and 12 days after gene transfer. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector; n.d., not detected; $^{**}P<0.01$. c, Immunohistochemical staining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

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

Evaluation of Capillary Density

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

Statistical Analysis

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

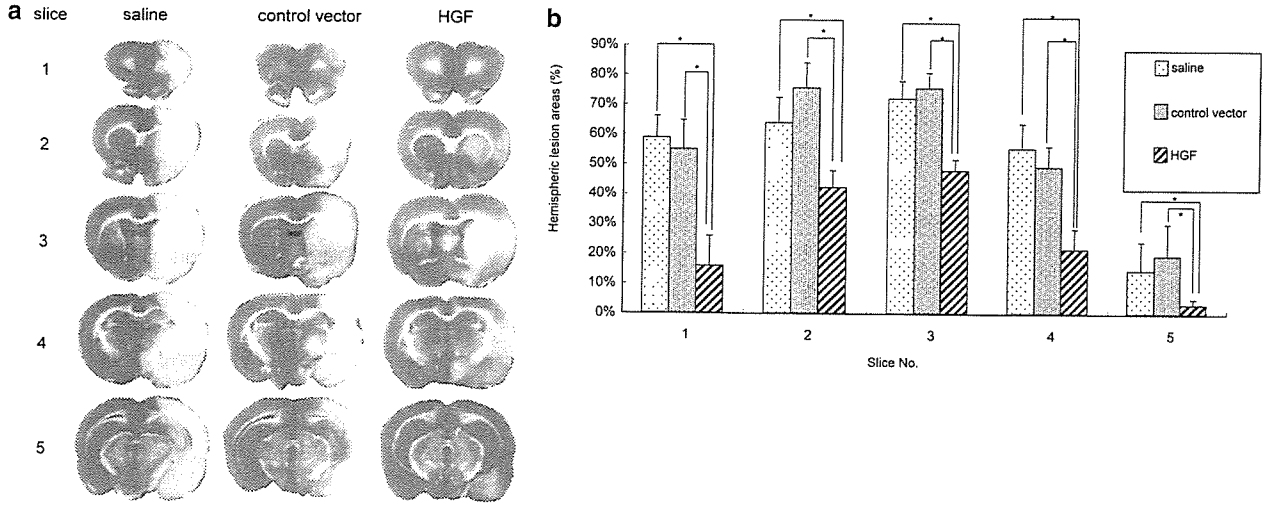

Figure 2. a, Reduction of infarcted area by HGF gene transfer: coronal sections stained with TTC at 24 hours after MCA occlusion. Red region shows intact area; white region shows infarcted area. b, Quantification of hemispheric lesion area 24 hours after ischemia. Corrected HLA was calculated as HLA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

Results

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

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

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

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

Inhibition of Destruction of BBB by Transfer of HGF Gene

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

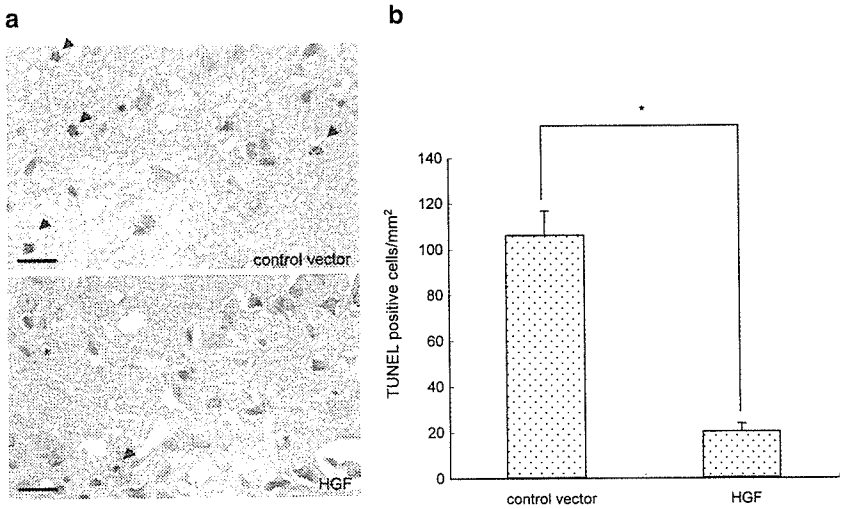

Figure 3. a, TUNEL staining of coronal sections of cerebral cortex in boundary of infarct area after 1 day of focal cerebral ischemia. Bar=100 μm. b, Number of TUNEL-positive cells. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.01$ vs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

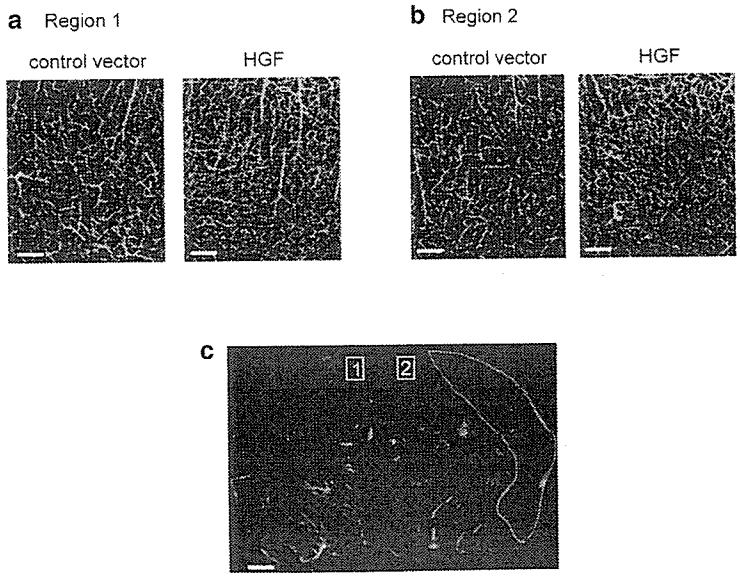

Figure 4. Vascular patterns in cerebral cortex at 24 hours after MCA occlusion. Region of interest was set at 2 points on surface of cerebral cortex (c). Rats transfected with human HGF gene showed a significant increase in capillary density in both intact (a) and infarcted (b) hemispheres. Dotted line 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.

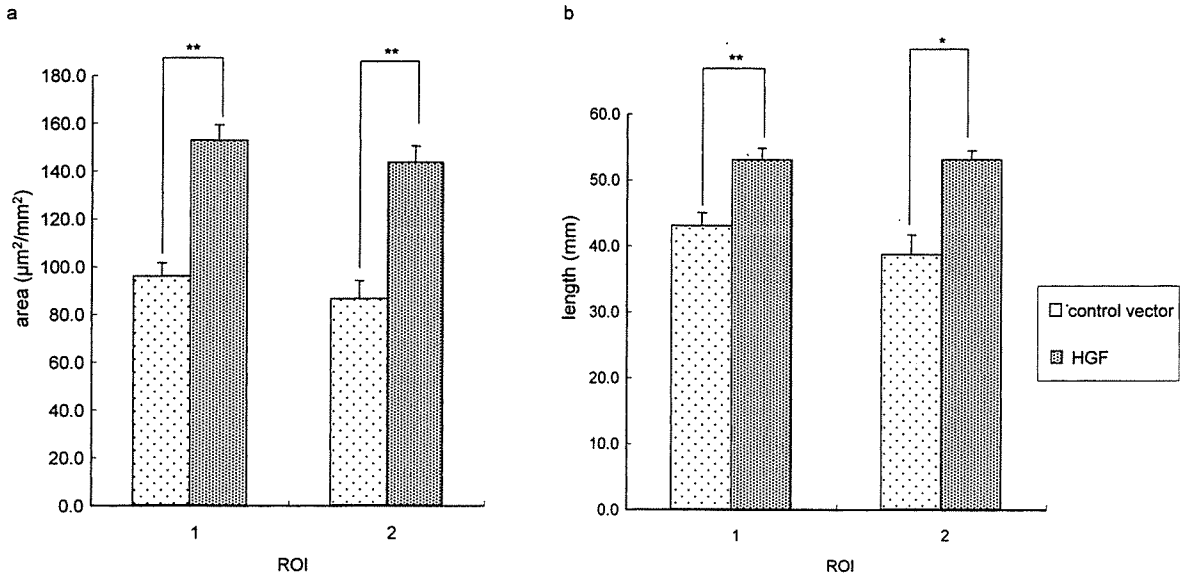

Figure 5. Capillary density as assessed by quantitative analysis at 24 hours after MCA occlusion. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. Number of ROI is same as in Figure 4. *$P<0.05$, **$P<0.01$ vs control vector (n=7 in each group).

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

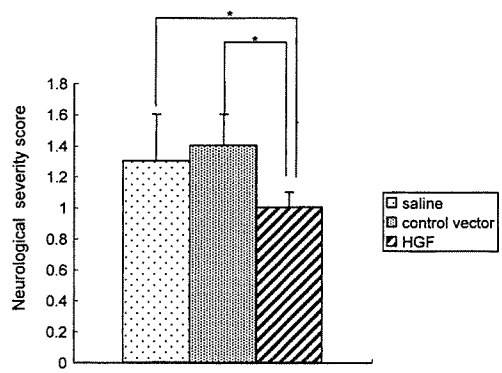

Figure 6. Neurological severity score at 24 hours after MCA occlusion. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.05$ (n=7, each group).

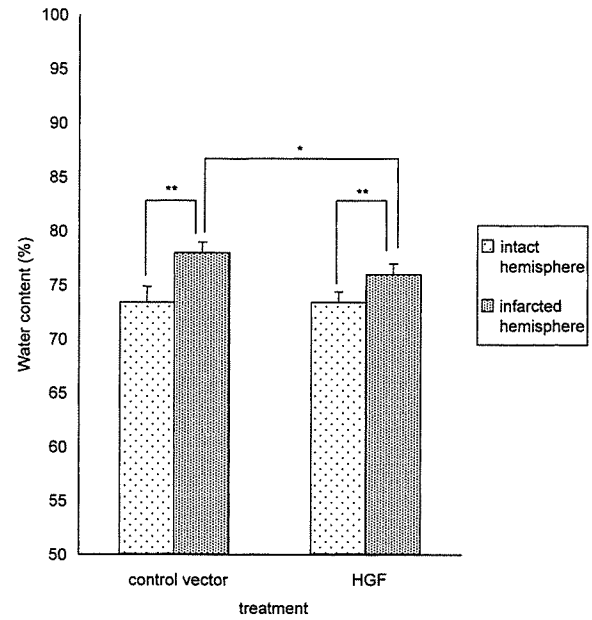

Figure 7. Water content after 24 hours of cerebral ischemia. Water content (WC) was calculated as WC (%)=(wet wt−dry wt)/(wet wt)×100. *$P<0.05$ (n=7 in each group).

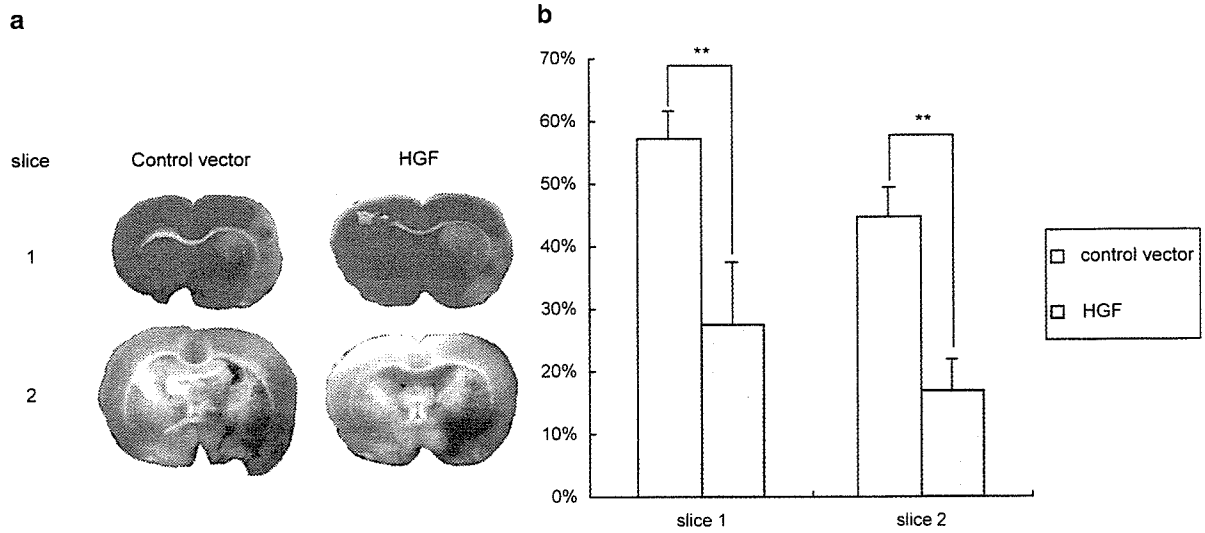

Figure 8. a, Coronal sections at 9 hours after MCA occlusion. Evans blue dye was injected at 6 hours after occlusion. To identify infarcted area, slice 1 was stained with TTC. Blue area indicates leakage of Evans blue dye. b, Percent area of Evans blue dye. Degree of leakage (%) was calculated as leakage (%)=[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.

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RESEARCH ARTICLE

Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin

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NK4, composed of the NH_2-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts as an HGF-antagonist and angiogenesis inhibitor. This study is an investigation to evaluate the feasibility of controlled release formulation of NK4 plasmid DNA in suppressing the tumor growth, and lung metastasis. Biodegradable cationized gelatin microspheres were prepared for the controlled release of an NK4 plasmid DNA. The cationized gelatin microspheres incorporating NK4 plasmid DNA could continuously release plasmid DNA over 28 days as a result of microspheres degradation following the subcutaneous injection. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA into the subcutaneous tissue significantly prolonged the survival time period of the mice bearing Lewis lung carcinoma tumor. Increases in the tumor volume and the number of lung metastatic nodules of NK4 plasmid DNA release group were suppressed to a significantly greater extent than that of solution-injected group (77.4 and 64.0%, respectively). The number of blood vessels and the apoptosis cells in the tumor tissue were significantly suppressed (80.4%) and increased (127.3%) against free NK4 plasmid DNA-injected group. Thus, the controlled release of NK4 plasmid DNA augmented angiogenesis suppression and apoptosis of tumor cells, which resulted in suppressed tumor growth. We conclude that this controlled release technology is promising to enhance the tumor suppression achieved by gene expression of NK4.
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Keywords: *controlled release; cationized gelatin microspheres; NK4; tumor metastasis*

Introduction

Hepatocyte growth factor (HGF) has been noted as the signal molecule that plays an important role in development, morphogenesis, and regeneration of living systems.[1–4] Recently, some therapeutic trials of angiogenesis induction,[5,6] chronic fibrotic diseases,[7–9] and tissue regeneration[10,11] by HGF have been performed experimentally and clinically to demonstrate the potential efficacy. On the other hand, for malignant tumors, HGF plays a definitive role in invasive, angiogenic, and metastatic behavior of cancer cells by way of the c-Met receptor.[12–16] Therefore, it is highly expected that inhibition of interaction between HGF and the c-Met receptor effectively suppresses the malignant activity of tumors. Based on this concept, Date *et al*[17] prepared an antagonist for HGF. The antagonist (NK4) is composed of the NH_2-terminal hairpin domain and the subsequent four-kringle domains of α-subunit of HGF. The NK4 binds to the c-Met/HGF receptor, but does not induce tyrosine phosphorylation of c-Met.[17] NK4 competitively inhibits some biological events driven by the HGF-Met receptor binding, including the invasion and metastasis of distinct types of tumor cells.[17,18] Moreover, NK4 has antiangiogenic activity and the antiangiogenic action is independent of its activity as an HGF antagonist.[19] The recombinant protein of NK4 has been used for tumor animal models to demonstrate the *in vivo* therapeutic feasibility and the blocking effect on HGF functions.[18–20] In addition, antitumor effects of stable expression of NK4 in cancer cells and recombinant adenovirus-mediated gene expression of NK4 have been reported.[21–25]

Based on recent advent of genomics, new genes have been discovered and will become therapeutically available for various diseases in the near future. In this connection, gene therapy is expected as a new and promising therapeutic choice.[26] Presently, several human clinical trials are being carried out to treat the cancer by utilizing the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses.[27] In spite of the high transfection efficiency, the trials are limited by the adverse effects of the virus itself, such as immunogenicity and toxicity or the possible mutagenesis of the cells transfected. Many types of cationized polymers[28] and cationized liposomes[29] have been explored and complexed with the plasmid DNA or antisense oligonucleotide for gene expression. This approach is to enable the plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size for enhanced efficiency of plasmid DNA transfection, which causes an increase in the gene expression. However, there are some problems to be solved, namely the transient and the low level of

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gene expression. One of the practically possible ways for better gene expression is to incorporate the plasmid DNA into an appropriate matrix for the controlled release over an extended time period. For the release matrix, we have taken advantage of a gelatin hydrogel prepared by the crosslinking of biodegradable polymer.[30] Since the polymer is positively charged, the plasmid DNA is likely to be immobilized in the hydrogel through the electrostatic interaction between the plasmid DNA and polymer molecule. The immobilized plasmid DNA could be released from the hydrogel if the hydrogel is degraded to generate the complex of water-soluble fragments of hydrogel polymer and plasmid DNA. This nonviral delivery system offers the advantages of biocompatibility of gelatin as a vector/carrier and the controllable biodegradability or achievement of local delivery and protection from rapid degradation of plasmid DNA by nucleases.

This study is the first report to demonstrate that the controlled release of NK4 plasmid DNA suppressed the tumor metastasis, in marked contrast to free plasmid DNA. The release mechanism driven by degradation of release carrier is quite different from that of plasmid DNA diffusion from the release carrier, which has been reported as the conventional release system of plasmid DNA.[31,32] We applied the cationized gelatin hydrogel to the controlled release of expression plasmid for human NK4, to evaluate the suppressive effects on tumor angiogenesis, growth, and metastasis in tumor-bearing mice. The results were compared to those with delivery of free plasmid to emphasize the efficacy of the release system in enhancing the biological activity of NK4.

Results

In vivo release profile of NK4 plasmid DNA from cationized gelatin microspheres

Figure 1 shows the time course of radioactivity remaining after the injection of cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA and ^{125}I-labeled free NK4 plasmid DNA or ^{125}I-labeled cationized gelatin microspheres. The remaining radioactivity decreased with injection time for every case. For cationized gelatin microspheres, the radioactivity gradually decreased over the time period of 28 days, whereas the radioactivity of free ^{125}I-labeled NK4 plasmid DNA injected more rapidly reduced, and disappeared from the injected site within 7 days. A good correlation in the time profile of *in vivo* retention was observed between the NK4 plasmid DNA incorporated and microspheres, demonstrating the controlled release of NK4 plasmid DNA as a result of microsphere degradation.

Prolonged survival and suppression of tumor growth and metastasis by cationized gelatin microspheres incorporating NK4 plasmid DNA

In the present study, we attempted to elucidate the antitumor effect of NK4 in metastatic murine tumor model. When Lewis lung carcinoma (LLC) cells were inoculated subcutaneously into mice, tumor cells formed tiny nodules 4 days after implantation, and metastatic nodules on the lung surface became visible 28 days after implantation.

Figure 2 shows the survival curve of tumor-bearing mice after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA or other agents. When the mice were injected with saline, empty cationized gelatin microspheres, and free NK4 plasmid DNA solution, all the mice died within 30 days. In contrast, the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of mice, wherein the

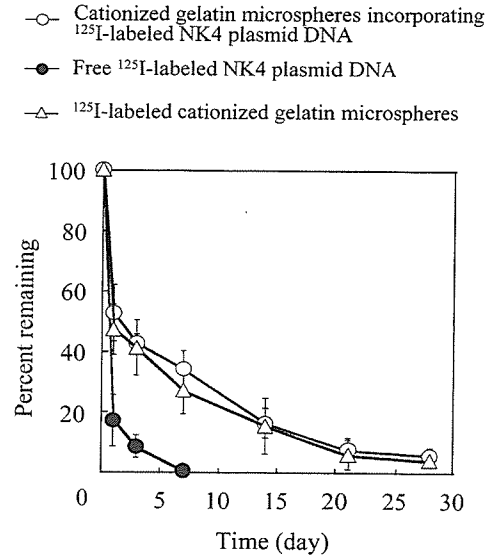

Figure 1 The time course of radioactivity of the remaining cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA and free ^{125}I-labeled NK4 plasmid DNA or ^{125}I-labeled cationized gelatin microspheres after the subcutaneous injection into the backs of tumor-bearing mice. The microspheres enabled NK4 plasmid DNA to remain in the injected site for a longer time period than in the solution form. The in vivo retention profile of NK4 plasmid DNA was in good accordance with that of microspheres as the release carrier.

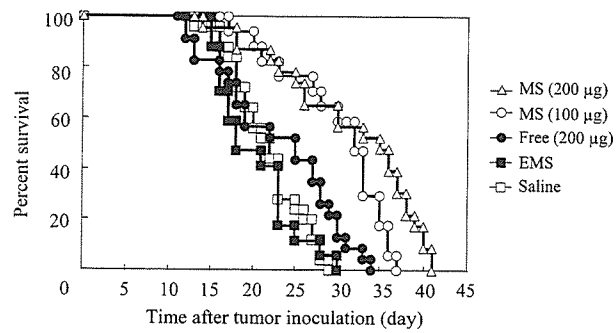

Figure 2 Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (MS (100 μg)) and 200 μg of NK4 plasmid DNA (MS (200 μg))*, 200 μg of free NK4 plasmid DNA (free (200 μg)), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the survival curve of saline-injected, control mice. The injection of microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of tumor-bearing mice, in contrast to that of free NK4 plasmid DNA.*

higher dose of NK4 plasmid DNA allowed longer survival of mice.

Figure 3a and b shows changes in primary tumor growth and the number of metastatic nodules after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA or other agents. The increase in the tumor volume was significantly suppressed by the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA, in marked contrast to free NK4 plasmid DNA. The tumor volume of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 68.3 and 77.4% of that of free NK4 plasmid DNA solution-injected mice, respectively. Lung metastasis was also suppressed by cationized gelatin microspheres incorporating NK4 plasmid DNA to a significantly greater extent than other agents. The number of lung metastasis node of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 62.3 and 64.0% of that of free NK4 plasmid DNA solution-injected mice, respectively. Injection of free NK4 plasmid DNA and empty cationized gelatin microspheres did not affect the number of lung metastasis, and the tumor volume and lung metastasis were similar to those of saline-injected, control mice.

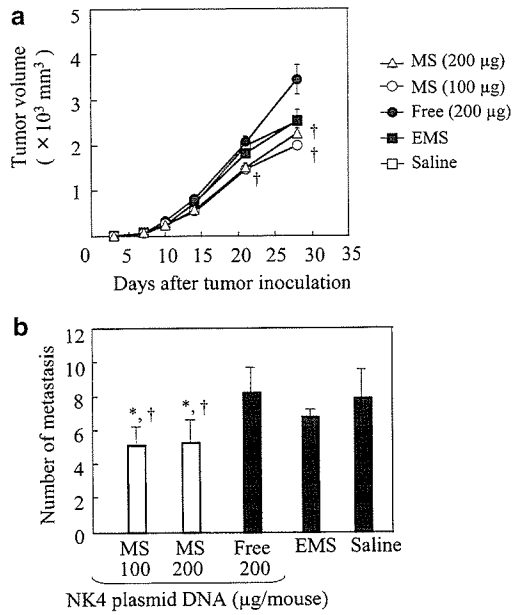

*Figure 3 In vivo tumor suppression effects of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA following the single injection into the subcutaneous tissue around the tumor mass. (a) Time course of tumor volume change: cationized gelatin microspheres incorporating 100 (MS (100 μg)) and 200 μg of NK4 plasmid DNA (MS (200 μg)), 200 μg of free NK4 plasmid DNA (free (200 μg)), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the tumor volume of saline-injected mice at the corresponding day. †, $P<0.05$: significant against the tumor volume of mice injected with 200 μg of free NK4 plasmid DNA. Irrespective of the NK4 plasmid DNA dose, the injection of microspheres incorporating NK4 plasmid DNA significantly suppressed the in vivo growth of tumor cells, in contrast to that of free NK4 plasmid DNA. (b) The nodule number of lung tumor metastasis 28 days after injection of cationized gelatin microspheres incorporating NK4 plasmid DNA (MS), free NK4 plasmid DNA (free), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the number of lung metastases of saline-injected mice. †, $P<0.05$: significant against the number of lung metastases of mice injected with 200 μg free NK4 plasmid DNA. The injection of microspheres incorporating both doses of NK4 plasmid DNA significantly decreased the nodule number of lung tumor metastasis, in contrast to that of free NK4 plasmid DNA.*

Angiogenesis and apoptosis in the tumor tissue

Figure 4a and b shows the immunohistochemical views and number of blood vessels of primary tumor tissue. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly decreased the number of blood vessels in the tumor tissue, and the size of tumor blood vessels was smaller compared to that of other agents. The number of blood vessels in the tumor tissue of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 82.1 and 80.4% of that of NK4 plasmid DNA solution-injected mice, respectively.

Since inhibition of tumor growth is associated with the increase in apoptosis of tumor cells, we analyzed change in apoptosis of tumor cells by TUNEL staining after injection of NK4 plasmid DNA (Figure 5a and b). The number of tumor cells undergoing apoptosis in the tumor tissue was significantly increased by the injection of cationized gelatin microspheres. In contrast, delivery of free NK4 plasmid DNA had no significant effect on apoptosis of tumor cells. The number of apoptosis cells in the tumor tissue of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 119.4 and 127.3% of that of free NK4 plasmid DNA solution-injected mice, respectively. Together with inhibition of tumor angiogenesis by delivery of NK4 plasmid DNA in cationized gelatin microspheres, these results suggest that inhibition of tumor growth might be caused by increased apoptosis of tumor cells associated with inhibition of tumor angiogenesis.

Figure 6a shows the time course of amount of NK4 protein expression in the primary tumor tissue, lung, and blood of tumor-bearing mice after injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA and 200 μg of free NK4 plasmid DNA. The NK4 concentration in the tumor and lung or blood increased with time up to day 7 to attain a maximum level, but thereafter decreased gradually. A low level of NK4 expression was detected in the lung tissue including metastatic nodules, whereas a high concentration of NK4 was observed in the blood. In contrast, no NK4 protein was detected at any sampling time and site for free NK4 plasmid DNA. A lacZ plasmid DNA was used to clarify the expression site. When cationized gelatin microspheres incorporating lacZ plasmid DNA were injected, gene expression was observed in the tumor mass around microspheres (Figure 6b).

Discussion

The gene delivery system is generally divided into two categories: viral and nonviral vectors. Although viral vectors such as retrovirus, adenovirus, and adeno-associated virus are potentially efficient, nonviral vectors have the advantages of less toxicity, less immunogenicity, and easier preparation. So far, several methods for delivering genes with nonviral carriers have been

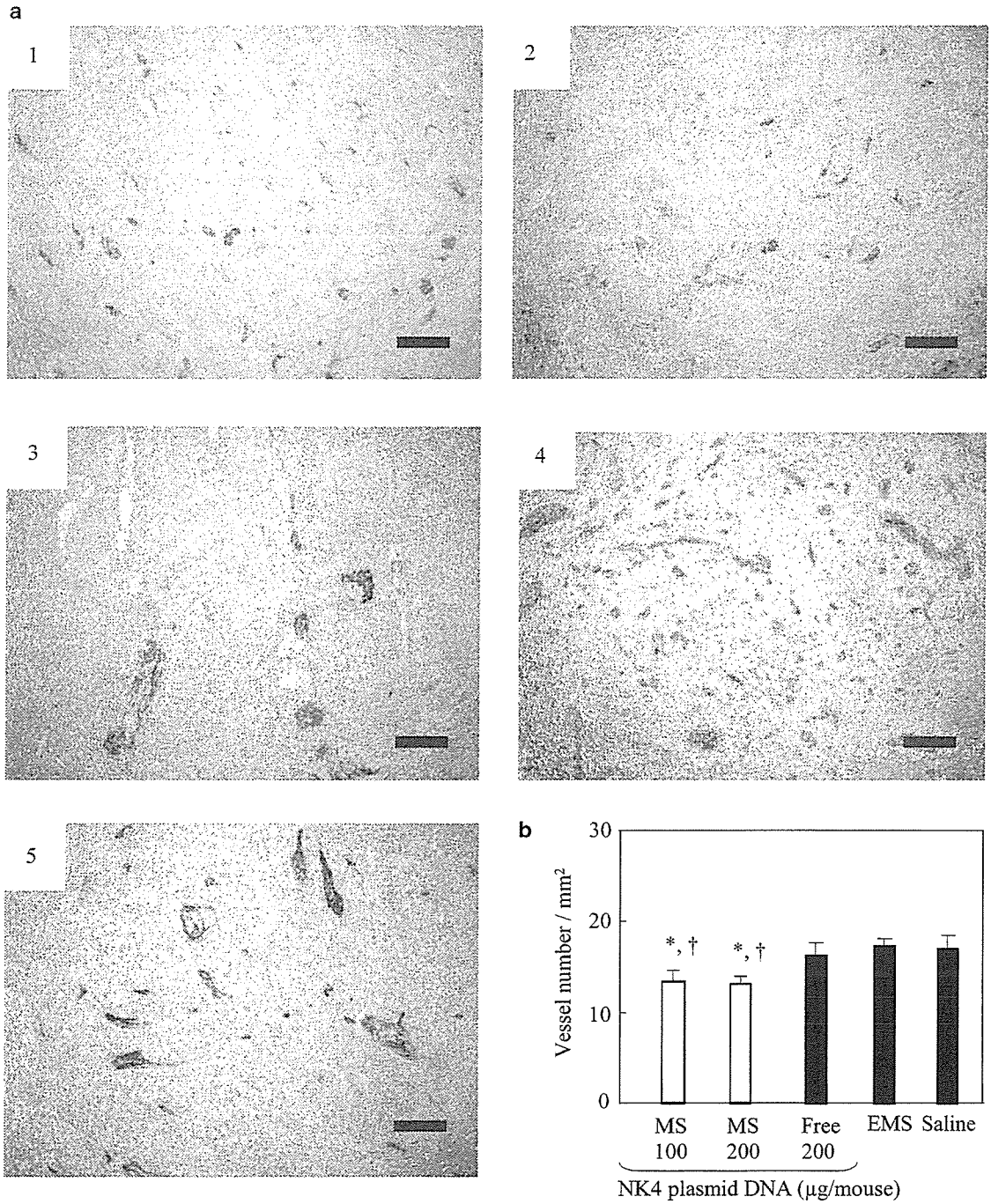

Figure 4 *(a) Immunohistochemical views of blood vessel formation of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (1) and 200 μg of NK4 plasmid DNA (2), 200 μg of free NK4 plasmid DNA (3), empty cationized gelatin microspheres (4), and saline (5) (magnification; ×100). The bar length is 200 μm. (b) The vessel number of tumor tissues 28 days after injection of cationized gelatin microspheres incorporating NK4 plasmid DNA (MS), free NK4 plasmid DNA (free), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the number of blood vessels formed of saline-injected mice. †, $P<0.05$: significant against the number of blood vessels formed of mice injected with 200 μg of free NK4 plasmid DNA. The injection of microspheres incorporating both doses of NK4 plasmid DNA significantly decreased the number of blood vessels formed around the tumor mass, in contrast to that of free NK4 plasmid DNA.*

developed, including naked plasmid DNA injection and complex formation with cationized polymers[28] or cationized liposomes.[29] However, there are several drawbacks with each nonviral vector, including a low efficiency of gene transfection compared with viral vectors and a transient gene expression. In this study, we introduce a system of prolonged gene expression based on the sustained release of plasmid DNA from cationized gelatin microspheres. The release mechanism driven by degradation of release carrier is quite different from that