研究成果の刊行に関する一覧表（雑誌）

発表者名	論文タイトル名	発表誌名	巻・号	ページ	出版年
Hiraoka K., Yamamoto S., Otsuru S., Nakai S., **Tamai K.**, Morishita R., Ogihara T., Kaneda Y.	Enhanced tumor-specific long-term immunity of hemagglutinating [correction of hemaggluttinating] virus of Japan-mediated dendritic cell-tumor fused cell vaccination by coadministration with CpG oligodeoxynucleotides.	J Immunol.	173(7)	4297-4307	2004
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Takeda S., Shiosaki K., **Kaneda Y.**, Nakasatomi T., Yoshizaki H., Someya K., Koono Y., Eda Y., Kino Y., Yamamoto N., and Honda M.	Hemagglutinating virus of Japan protein is efficient for induction of CD4+ T-cell response by a hepatitis B core particle-based HIV vaccine.	Clinical Immunology	112	92-105	2004
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No influence of tumor growth by intramuscular injection of hepatocyte growth factor plasmid DNA: safety evaluation of therapeutic angiogenesis gene therapy in mice

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Abstract

Recently, a novel therapeutic treatment for ischemic diseases using angiogenic growth factors to augment collateral artery development has been proposed. As intramuscular injection of naked human hepatocyte growth factor (HGF) plasmid DNA induced therapeutic angiogenesis in several animal test subjects, we have started a clinical trial to treat peripheral arterial disease. However, one might assume that over-expression of angiogenic growth factors could enhance tumor growth. To resolve this issue, we examined the over-expression of HGF in tumor bearing mice. Tumors on their backs were prepared with an intradermal inoculation of A431, human epidermoid cancer cells expressing c-Met. These mice were intramuscularly injected with human HGF plasmid or control plasmid into the femoral muscle. Human HGF concentration was increased only in the femoral muscle, but not in blood. Although recombinant HGF stimulated the growth of A431 cells in vitro, temporally and locally HGF elevation in hindlimb had no effect on tumor growth in mice.

Keywords: Angiogenesis; Safety; Cancer; Peripheral vascular disease; Gene therapy

Critical limb ischemia is estimated to develop in 500–1000 individuals per million per year [1]. In a large proportion of these patients, the anatomical extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Most importantly, there is no optimal medical therapy for critical limb ischemia. Therefore, novel therapeutic modalities are needed to treat these patients. Recently, the efficacy of therapeutic angiogenesis using gene transfer of angiogenic growth factors such as VEGF (vascular endothelial growth factor) has been tested in human patients with critical limb ischemia and myocardial ischemia [2–4]. Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia or myocardial infarction. In addition to VEGF, hepatocyte growth factor (HGF) originally cloned as hepatocyte mitogen [5] is also of particular interest, as HGF exclusively stimulated the growth of endothelial cells without replication of vascular smooth muscle cells (VSMC) [6–8]. Indeed, others and we have previously reported that HGF is a potent angiogenic growth factor that is useful for effective therapeutic angiogenesis in several animal tests [9,10]. Based upon the preclinical study, we have started human clinical trials of gene

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therapy to treat peripheral arterial disease using naked plasmid DNA encoding the HGF gene by intramuscular injection into ischemic legs. Although human clinical trials using angiogenic growth factors including HGF do not exhibit severe adverse effects at the present time, one might assume that the potential risks such as the promotion of tumor growth might exist. As HGF is well known as a pleiotropic effector of cells expressing the c-Met tyrosine kinase receptor [11–13], HGF produced by mesenchymal cells may act predominantly on cells of epithelial origin in an endocrine and/or paracrine fashion [14,15]. HGF especially regulates cell growth, cell motility, and morphogenesis of various types of cells [5]. HGF-Met signaling clearly plays a role in tumor development and progression, as c-Met was originally isolated as the product of human oncogene, trp-met, encodes an altered Met protein possessing constitutive, ligand-independent tyrosine kinase activity, and transforming ability [16,17]. The oncogenic capability of HGF-Met signaling is likely due to the inappropriate use of mitogenic and angiogenic signals. HGF-Met signaling also induces the invasiveness and metastatic potential [18–20]. To answer this safety question, we examined the effects of local over-expression of HGF by intramuscularly injection of plasmid DNA on tumor growth. The present study documented no influence of HGF plasmid DNA gene therapy on tumor growth despite the increase in local HGF concentration in mice.

Materials and methods

Construction of plasmids. To produce a HGF expression vector, human HGF cDNA (2.2 kb) was inserted into pVAX1 (3.0 kb) (Invitrogen, Carlsbad, CA, USA) at the *Bam*HI and *Not*I sites. pVAX1 not containing HGF cDNA was used as a control. pCMV-luciferase-GL3 (7.4 kb) was constructed by cloning the luciferase gene from the pGL3-Promoter Vector (Promega, Madison, WI, USA) into pcDNA3 (5.4 kb) (Invitrogen, USA) at the *Hin*dIII and *Bam*HI sites. Plasmids were purified with the Qiagen plasmid isolation kit (Hilden, Germany).

Human recombinant HGF. Human recombinant HGF was purified from a culture medium of Chinese hamster ovary cells or C-127 cells transfected with an expression plasmid containing human HGF cDNA [9,21].

Cell scattering and proliferation assay. A431 cells were cultured in DMEM supplemented with 10% FBS. Then the cells were plated in 6-well tissue culture dishes at 1×10^5 cells/well in 3.0 ml DMEM with 1% FBS and cultured for 48 h in the absence or presence of various concentrations of HGF [21]. For proliferation assay, A431 cells were cultured in DMEM supplemented with 10% FBS. Then the cells were plated in 96-well tissue culture dishes at 3×10^3 cells/well in 200 μl DMEM with 1% FBS and cultured for 24 h. After washing with DMEM, the cells were cultured for 48 h in DMEM with 1% FBS in the absence or presence of various concentrations of HGF [21]. The number of cells was counted with MTS colorimetric assay system (Promega, USA).

In vivo gene transfer using direct intramuscular injection approach. All procedures were approved by the Osaka University Committee on Animal Research. Six-week-old male BALB/cA nu/nu mice bearing tumor on their back were prepared by an intradermal inoculation of 5×10^6 A431 cells in 0.1 ml PBS [21,22]. A431 human epidermoid carcinoma cells express c-Met/HGF receptor but scarcely produce HGF. A431 cells produce IL-1, one of the HGF-inducing soluble factors [21,23]. One week later, the mice received an intramuscular injection of 200 μg human HGF plasmid DNA in 0.1 ml saline on hindlimb [24,25]. After 4 weeks observation, mice were killed under anesthesia and examined for body weight, tumor weight, tumor invasion, central necrosis, and metastasis. HGF concentrations in blood and tumor tissues were measured by enzyme-immunoassay (EIA).

In vivo gene transfer into the tumor tissues. A431 cells were intradermally implanted into nude mice same as above and one week later, the direct injection of 200 μg human HGF plasmid DNA or empty vector was injected into tumor tissues. After 4 weeks observation, mice were killed under anesthesia and examined for tumor properties.

Measurement of HGF concentration in hindlimb and other tissues. To document the successful transfection of HGF vector into the hind limb, we examined the production of human immunoreactive HGF [8,26]. Before and at 1, 4, 7, and 14 days after transfection, the hindlimb of the mouse transfected with HGF vector, opposite leg, back skin, liver, and blood serum were removed and stored at −70 °C until use. On the day of extraction, the tissue was thawed at 4 °C, weighed, and homogenized by polytron in assay solution. Each specimen was centrifuged at 20,000*g* for 30 min at 4 °C, to remove the lysate. The concentration of HGF in the hindlimb was determined by EIA using anti-human HGF antibody [8,26]. In brief, rabbit anti-rat or anti-human HGF IgG was coated on 96-well plates (Corning, NY) at 4 °C for 15 h. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), the conditioned medium was added to each well and the preparation was incubated for 2 h at 25 °C. The wells were washed three times with PBS containing 0.025% Tween 20 (PBS–Tween), biotinylated rabbit anti-human HGF IgG was added, and the preparation was incubated for 2 h at 25 °C. After washing with PBS–Tween, the wells were incubated with horseradish peroxidase-conjugated streptavidin–biotin complex in PBS–Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/ml *o*-phenylenediamine, 100 mM sodium phosphate, 50 mM citric acid, and 0.015% H_2O_2. The enzyme reaction was stopped by adding 1 M H_2SO_4 and absorbance at 490 nm was measured. The antibody against human HGF reacts with only human HGF and not with rat HGF [27]. Mouse endogenous immunoreactive HGF was also measured by EIA using anti-mouse HGF antibody, as the antibody against mouse HGF reacts with only mouse HGF, and not with human HGF [26]. The lower detection limit of this method was 0.1 ng/ml.

Assay for luciferase activity. The mice transfected with luciferase gene were killed under anesthesia at 24 h post-injection. The tissues (tumor, left hindlimb, and right hindlimb) were harvested and placed individually in flat-bottomed 1.5 ml Eppendorf microfuge tubes. Luciferase activity assay was performed as described previously [28]. Luciferase levels were normalized by determining the protein concentrations of the tissue extracts [28]. Luciferase units were expressed as relative light units (RLU) per microgram of tissue protein.

Statistical analysis. All statistical analyses were done with the Stat View, release 4.11 (Abacus Concepts, CA, USA). The results were compared with Student's *t* test. All data are expressed as means ± SD. The differences were considered statistically significant at a P value of <0.05.

Results

As co-expression of c-Met/HGF receptor and HGF molecules in the same cell, which generates an autocrine stimulatory loop, is reported to be oncogenic [18,29], we initially identified the in vitro effects of HGF on the growth cells expressing c-Met. We chose A431 human epidermoid carcinoma cells, since A431 cells express

c-Met, but rarely produce HGF. Initially, we examined the effects of HGF on tumor scattering. As shown in Fig. 1, addition of rHGF induced the scattering of A431 cells in a dose-dependent manner. In addition to scattering, a significant increase in the number of A431 cells was observed under rHGF stimulation in a dose-dependent manner (Fig. 2, $P < 0.01$).

Using these A431 cells, we next examined the effects of local over-expression of HGF on tumor growth in mice harboring A431 tumors. Initially, we measured tissue HGF concentration in tumor induced by A431 cells implantation. As reported previously, A431 cells themselves rarely produced HGF, but secreted the HGF inducers for fibroblasts such as interleukin 1 [21,23]. Indeed, as shown in Fig. 3A, human HGF concentration both in A431 cells and the medium of A431 cells in vitro could not be detected. Similarly, neither could mouse HGF concentration be detected in either the A431 cells or the medium of A431 cells in vitro. In contrast, human HGF concentration was readily detected in tumor tissues in vivo. Tumors induced by A431 cells most likely secrete human HGF locally. In addition, mouse endogenous HGF concentration was also readily detectable by tumor harboring. As A431 cells secreted inducers for HGF such as interleukin 1 [21,23], endogenous HGF levels would be induced. In this

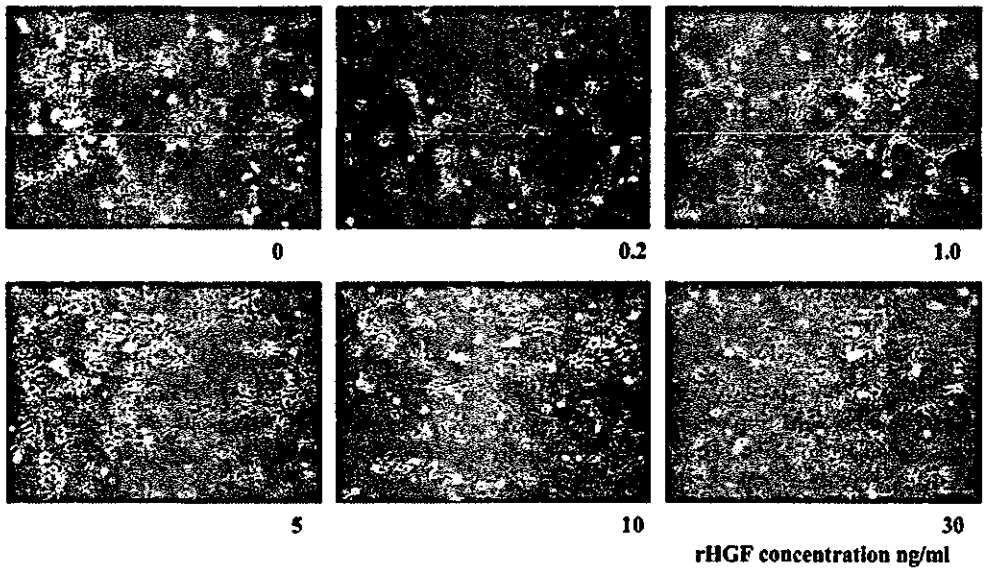

Fig. 1. Effects of human recombinant HGF protein (rHGF) on cell scattering. A431 cells were plated at a density of 1×10^5 cells/well on 6-well plates and cultured for 48 h in various concentrations of rHGF.

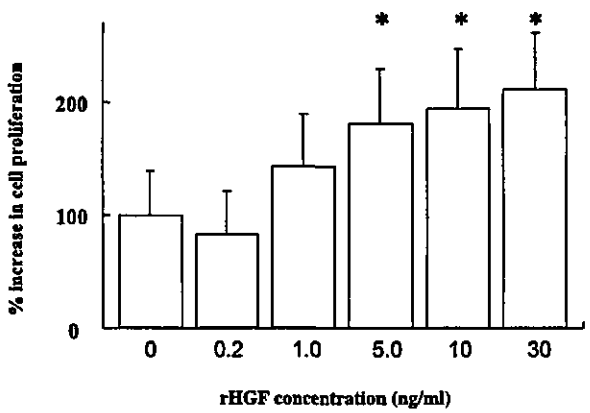

Fig. 2. Effects of rHGF on cell proliferation. A431 cells were plated at a density of 3×10^3 cells/well on 96-well plates and cultured for 48 h in various concentrations of rHGF for every 16 wells. Cell numbers of each well were measured using MTS assay.

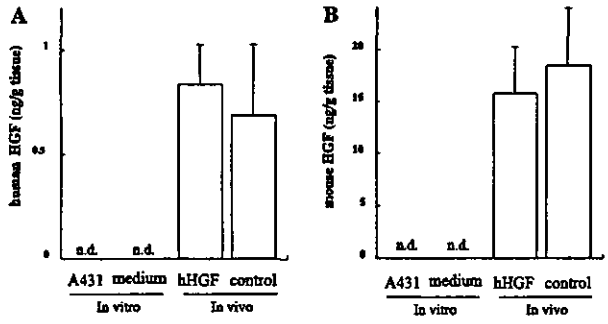

Fig. 3. HGF concentration in vitro and in vivo. (A) Human HGF concentration in A431 cells and medium in vitro and tumor tissues in vivo. The mice received intradermal injections of A431 cells (5×10^6 cells) on their back on day 0, intramuscularly injections of human HGF plasmid (hHGF, $n = 3$) or empty vector (pVAX1, $n = 3$) on hindlimb on day 7. Their tumors were resected on day 28 and HGF concentration was measured by EIA. (B) Mouse HGF concentration in same objects.

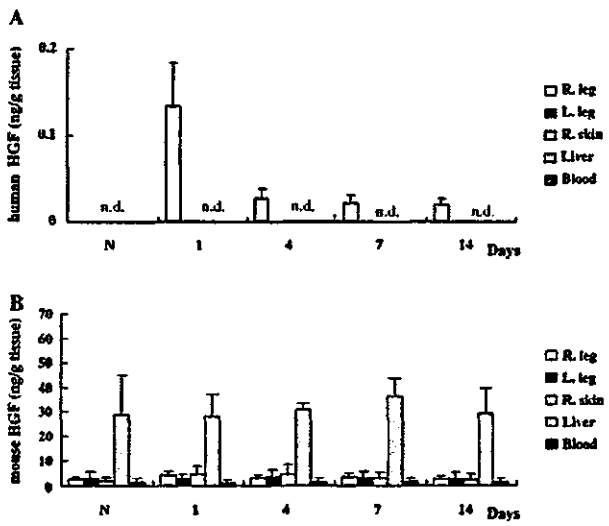

Fig. 4. HGF concentration in various tissues. (A) Change of human HGF concentration in right femoral muscle (R. leg), left femoral muscle (L. leg), right back skin (R. skin), liver, and blood of BALB/cA nu/nu mice before (N), and 1, 4, 7, and 14 days after intramuscularly injection of 200 μg naked human HGF plasmid on right hindlimb $n = 4$. (B) Change of mouse HGF concentration in the same tissues of BALB/cA nu/nu mice before and after intramuscular injection of 200 μg naked human HGF plasmid on right hindlimb $n = 4$.

model, naked plasmid DNA was intramuscularly injected into hindlimb. As shown in Fig. 4A, human HGF concentrations were only detected in the right hindlimb directly transfected with human HGF plasmid. Other organs such as contra-lateral left hindlimb, skin, and liver did not exhibit a detectable HGF level. In addition, neither could serum HGF concentration be detected. In contrast, mouse endogenous HGF concentration could be detected in all tissues. However, HGF level in the liver was extremely high as compared to other organs. Local transfection of HGF plasmid DNA did not affect mouse endogenous HGF level (Fig. 4B). Interestingly, the human and mouse HGF levels in the tumors were also not affected by intramuscular injection of HGF plasmid DNA (Figs. 3A and B).

Based upon the increase in local HGF concentration, we examined the effects of over-expression of HGF in hindlimb on tumor growth. Transfection of human HGF plasmid DNA into peripheral arterial disease mouse model at same dosage resulted in a significant increase in blood flow and the capillary density (data not shown). However, as expected, there was no significant difference in tumor size, weight, invasion, and central necrosis between the mice intramuscularly transfected with human HGF plasmid and control vector (Fig. 5). In addition, no significant difference in tumor size, weight, invasion, and central necrosis could be detected between the mice intramuscularly transfected with control vector and the untreated mice (Fig. 5). Metastasis was not detected in all mice. None of the mice transfected with HGF gene died during the observation period.

Since mice were bearing tumors on their right back, we examined the site effects of direct injection of HGF plasmid DNA. There was no significant difference in tumor properties between the mice intramuscularly injected with HGF plasmid on the right hindlimb and the left hindlimb (data not shown). We next examined whether intratumor injection of HGF plasmid DNA accumulates tumor invasion and growth. There was no significant difference in tumor size between the mice injected with empty vector into tumor tissues and the untransfected mice. In addition, no significant difference in tumor size could be detected between the mice injected with HGF plasmid DNA and empty vector

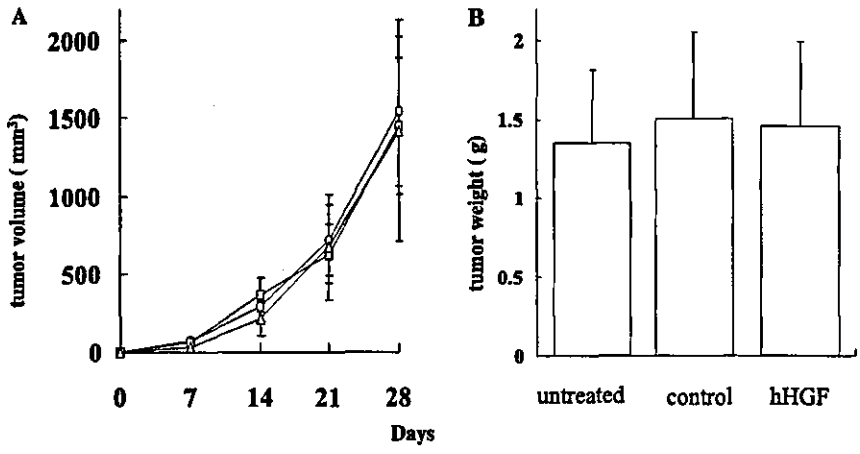

Fig. 5. Growth of tumor cells in vivo. (A) Effect of intramuscular injection of naked human HGF plasmid on the growth of tumor cells in vivo. Mice received intradermal injections of A431 cells (5×10^6 cells) on their right back on day 0 and intramuscular injections of human HGF plasmid (hHGF; ○, $n = 9$) or empty vector (pVAX1; △, $n = 9$) on left hindlimb on day 7 or not (N; □, $n = 9$). Tumor volume on their back was calculated as $1/2 \times \text{length} \times \text{width}^2$ (length > width). (B) Tumor weight on day 28. Tumors were dissected on day 28 and measured by weight.

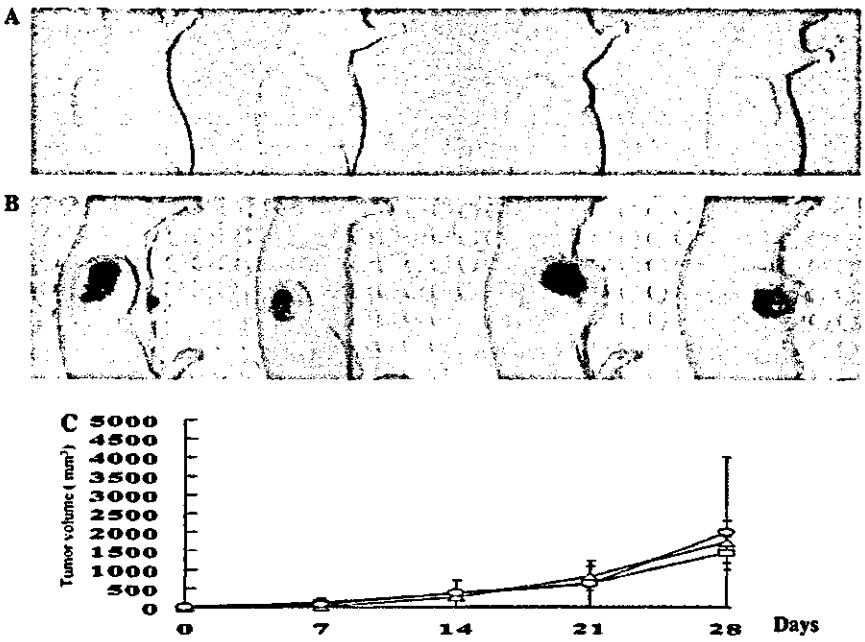

Fig. 6. Effect of direct injection of naked human HGF into the tumor on the growth of tumor. Mice were intradermally injected with A431 cells (5×10^6 cells) on their right back on day 0. Direct injection of human HGF plasmid (○, $n = 4$) or empty vector (△, $n = 4$) into tumor tissues was performed on day 7, whereas control mice were not transfected (□, $n = 9$). (A) Mice transfected with human HGF plasmid DNA (○, $n = 4$). (B) Mice transfected with empty control vector (△, $n = 4$). (C) Effect of direct injection of naked human HGF into the tumor on the growth of tumor.

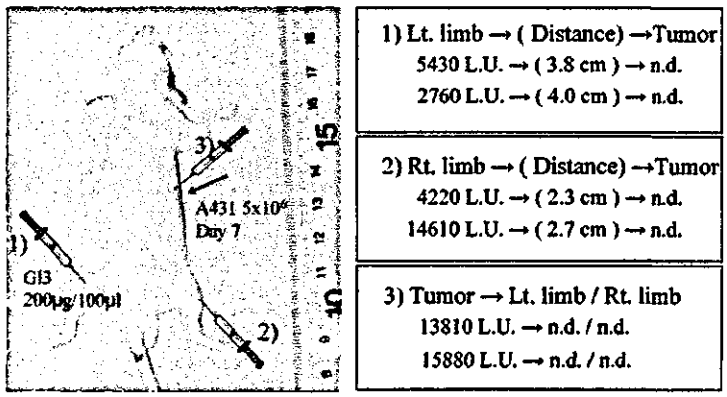

Fig. 7. Measurement of luciferase activity after injection of plasmid DNA: (1) Intramuscular injection into left hindlimb, (2) intramuscular injection into right hindlimb, and (3) injection into tumor. n.d., not detected.

(Fig. 6). Distant metastasis could not be detected in all mice. Finally, we tested the possibility that plasmid DNA should occur within the tumor by leakage of plasmid DNA from the hindlimb to the circulating system. As shown in Fig. 7, our present study denied this possibility. Intramuscular injection of pCMV-luciferase-GL3 plasmid DNA into the hindlimb resulted in the detection of luciferase activity only in hindlimb, while no luciferase activity could be detected in the tumor. In contrast, even with an injection into the tumor, luciferase activity was only detected in the tumor and not the hindlimb. These results demonstrate that the transfection of the injection of plasmid DNA results in the limited expression within the injection site.

Discussion

As the feasibility of therapeutic angiogenesis using angiogenic growth factors such as VEGF, FGF, and HGF has been reported in experimental models [9], in vivo transfer of these genes into the muscle is currently being tested as gene therapy for untreatable peripheral arterial disease. In this study, we evaluated the most

important safety issue regarding the use of plasmid vector encoding angiogenic growth factors in the patients who have tumors. The two important questions are: (1) the promotion of tumor by the intramuscular injection of naked plasmid DNA and (2) the expression of transgene in tumors by the leakage of plasmid DNA from the injected sites. In this study, we chose HGF as a model of safety evaluation, since the previous reports demonstrated the oncogenic capability of HGF-Met signaling, the invasiveness, and metastatic potential [18–22]. Indeed, we confirmed the stimulatory effects of human rHGF on scattering and proliferation using A431 cells in vitro. Although HGF transgenic mice develop a remarkably broad array of histologically distinct tumors of both mesenchymal and epithelial origin [30], in this study, no change was detected in tumor growth in mice intramuscularly transfected with HGF plasmid DNA into the hindlimb of tumor bearing mice. In addition, no evidence of metastasis and death could be detected throughout the observation period. Although human and mouse HGF concentrations were significantly increased in tumor tissues in vivo than in A431cells in vitro, there was no significant difference in local HGF concentration in tumor tissue between the mice intramuscularly injected with human HGF plasmid and control vector, while local human HGF was overexpressed in the hindlimbs. In fact, human HGF concentration was significantly elevated only in femoral muscle where human HGF plasmid was injected, but not elevated in other tissues and blood. In addition, despite an extremely high concentration of HGF in tumor tissues on mice, there was no significant difference in circulating HGF concentration between the tumor bearing mice and no treatment mice. How about the comparability of the present study with human clinical trial? In this study, we employed 200 μg human HGF plasmid DNA by the intramuscular injection into the hindlimb, while the body weights of the mice were pretty small about 30 g/body. Even at this dose, we confirmed that transfection of HGF plasmid DNA is enough to induce blood flow and the number of capillary density. That amount per body weight is equal to about 400–600 mg in a human case. In a human clinical trial, a total of 4 or 8 mg of human HGF plasmid DNA was used in trial. Even in extremely large amounts of human HGF plasmid, no promotion of the tumor was detected. Regarding the expression of plasmid DNA within the tumor from the leakage of plasmid DNA from the hindlimb to the circulating system, our present study denies this possibility. Intramuscular injection of plasmid DNA resulted in the detection of luciferase activity only in the hindlimb, while no luciferase activity could be detected in the tumor. These results denied the potential side effects of therapeutic angiogenesis using plasmid DNA in cancer patients with peripheral arterial disease.

However, there are still some elements of this series of safety evaluations to limit the conclusions. In this study, we used the mice bearing the tumors on their backs, but in clinical situation there are many different types of tumors that arise from variable tissues. In addition, other angiogenic growth factors such as VEGF may not be same as HGF, since serum concentration of VEGF, but not HGF, was increased after gene transfer into the human legs using naked plasmid DNA [31]. Nevertheless, the present study clearly demonstrated that local over-expression of HGF did not induce tumor promotion. HGF gene therapy is safe for mice tumor and further studies have to be done in the case of human diseases. The present information is important to consider the stimulation of new vessel formation by HGF as a new therapeutic option in angiogenesis-dependent conditions such as wound healing, inflammatory diseases, ischemic heart disease, myocardial infarction, and peripheral arterial disease.

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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 (*lac*Z 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*lacZ* (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*lacZ*, 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*lacZ* 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