

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% H₂O₂ 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 \pm 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/*pclacZ* 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 μ g 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. 2A](#)). The expression of human HGF could even be detected in rats 12 days after transfection with *hHGF* ([Fig. 2A](#)). Interestingly, an increase of rat HGF was also observed in CSF from the KM + HGF group ([Fig. 2B](#)), 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. 2D](#), 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. 4A](#). 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. 4C](#)). 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. 4B](#) 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. 4E-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. 4I). In contrast, inner and outer HCs in the KM + HGF group were well preserved as well as in the control rat (Fig. 4J).

Evaluation of hearing function

According to the experimental time course in Fig. 5A, 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. 5B). 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. 5A. As seen in Fig. 5C, 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 virus (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 fusogenic envelope derived from inactivated HVJ

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

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

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

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

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

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Table 1**Luciferase activity at 24 h after transfection of *HGF* gene via the cisterna magna**

	RLU/mg tissue
Cerebral cortex	151.2 ± 224.7 (n=4)
Medulla	225.3 ± 88.2 (n=4)
Cerebellum	112.3 ± 61.4 (n=4)
Cochlea	146.6 ± 44.7 (n=8)

After intrathecal injection of the luciferase gene via the cisterna magna, luciferase activity was measured from tissues dissected from the cerebral cortex, medulla, cerebellum, and cochlear. Transgene expression was not detected in other organs including the liver, lung, and spleen.

Fig. 1

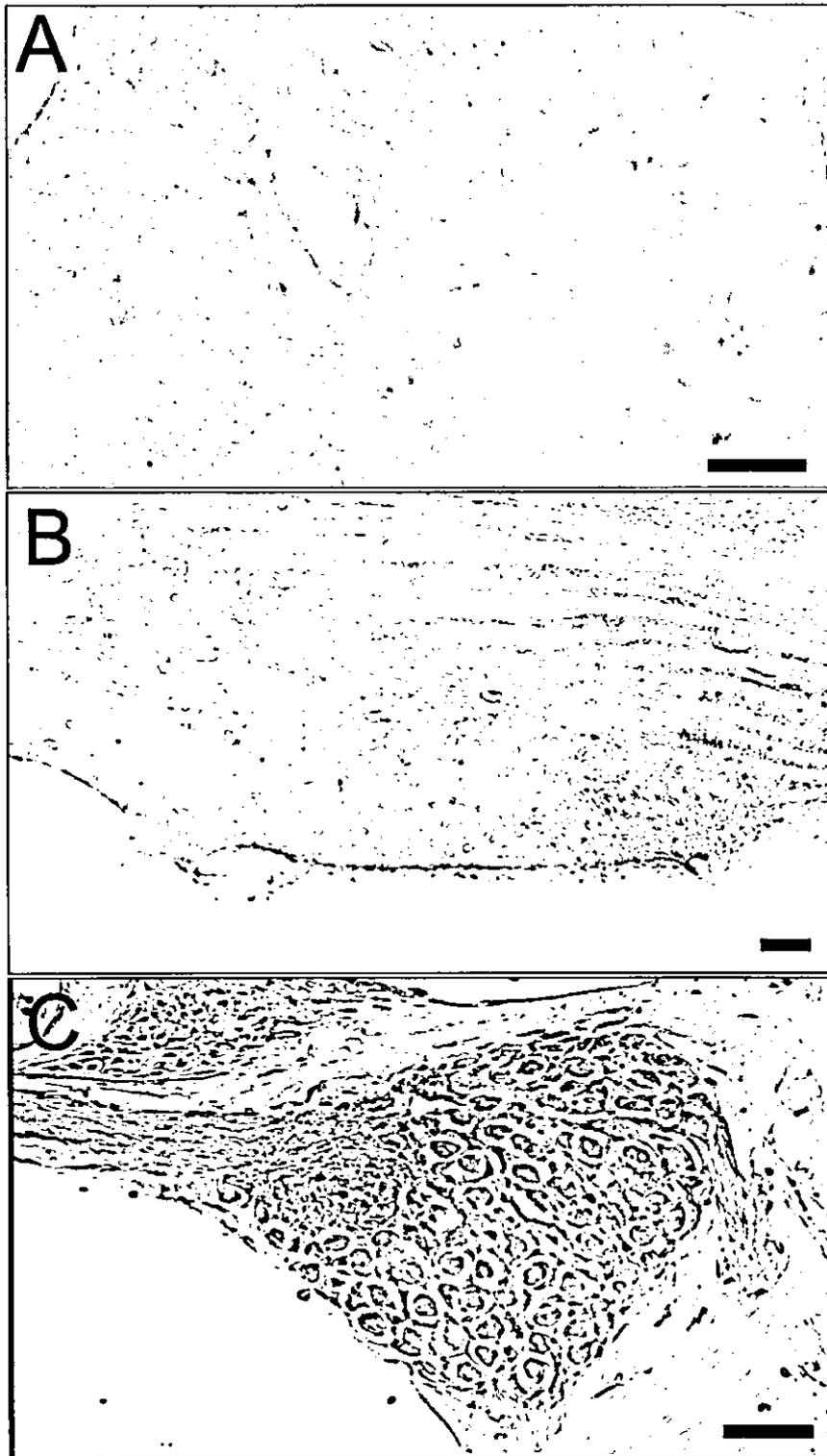


Figure 1. Localization of *lacZ* expression in the brain and cochlea. *lacZ* expression in the medulla (A), cochlear nucleus (B), and SGCs (C) of normal rats was detected by X-gal staining on day 7 after intrathecal injection of HVJ-E containing the *E. coli* β -galactosidase gene *lacZ*. Scale bar: 100 μ m.

Fig. 2

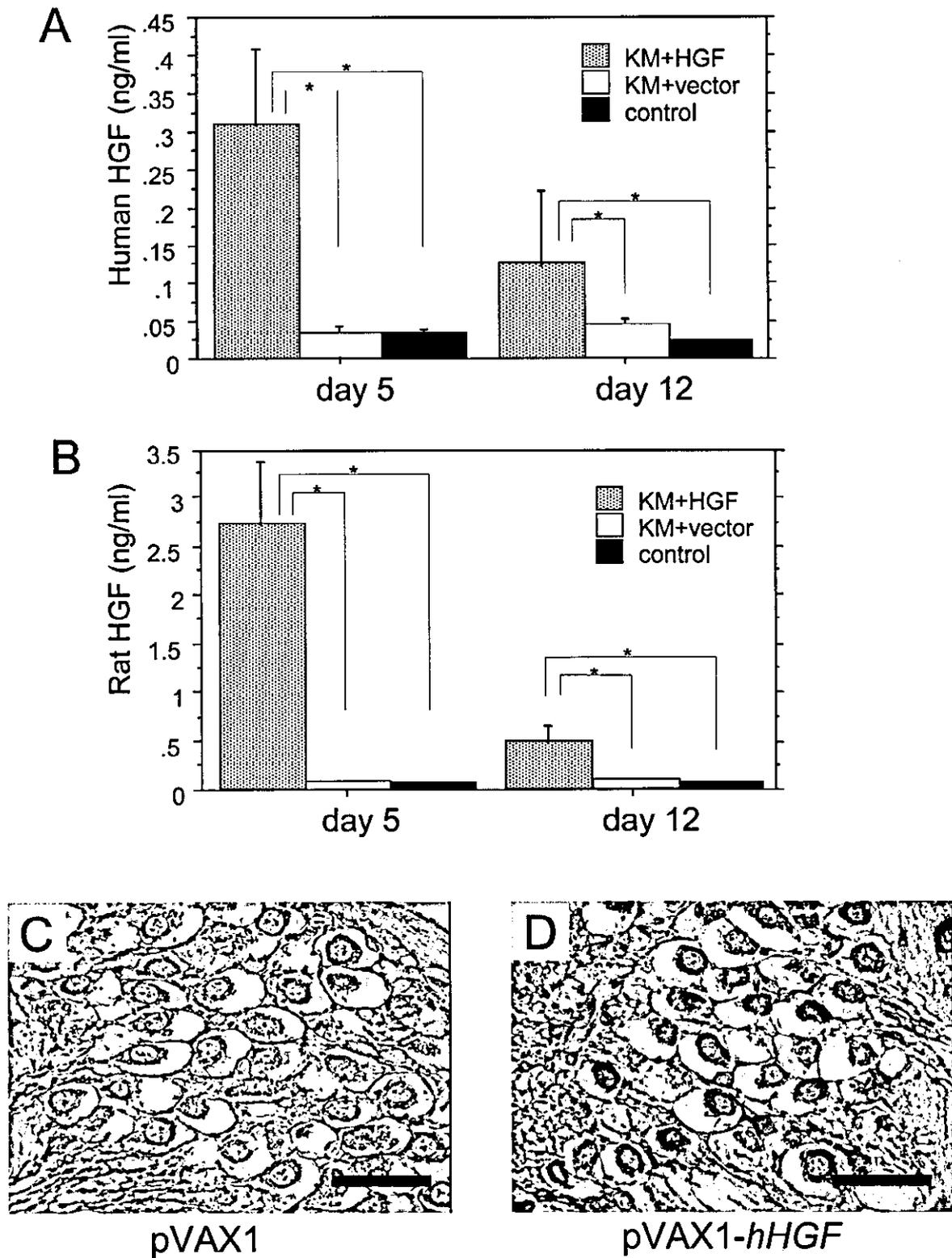


Figure 2. Expression levels of exogenous and endogenous HGF in CSF and SGCs. Exogenous human HGF (A) and endogenous rat HGF (B) in CSF from the KM + HGF, KM + vector, and control groups were measured on days 5 and 12 after transfection with the human HGF transgene ($n=4$ for each). SGCs from the mid-turn of cochleae treated with KM + vector (C) or KM + HGF (D) were immunostained with anti-human HGF antibody. $*P < 0.01$. Scale bar: 50 μm ; $n=4$ for each group.

Fig. 3

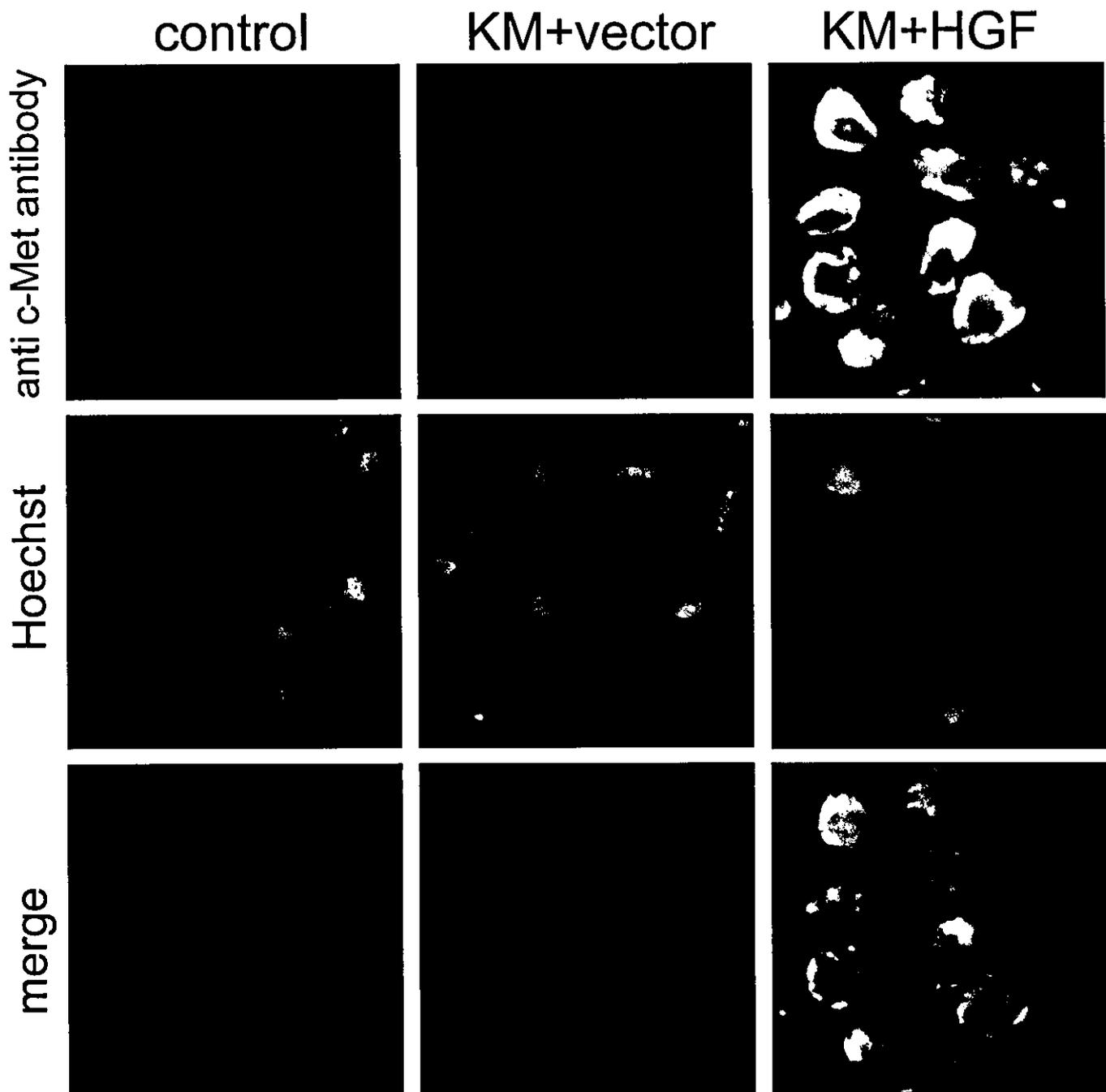


Figure 3. Enhancement of c-Met expression in SGCs by *HGF* gene transfer. Immunohistochemistry of SGCs from the intact rats, KM + vector group, and KM + HGF group was performed. Samples were stained with anti-c-Met antibody (upper) and counterstained with Hoechst 33342 (middle). Merged images are also presented (bottom).

Fig. 4

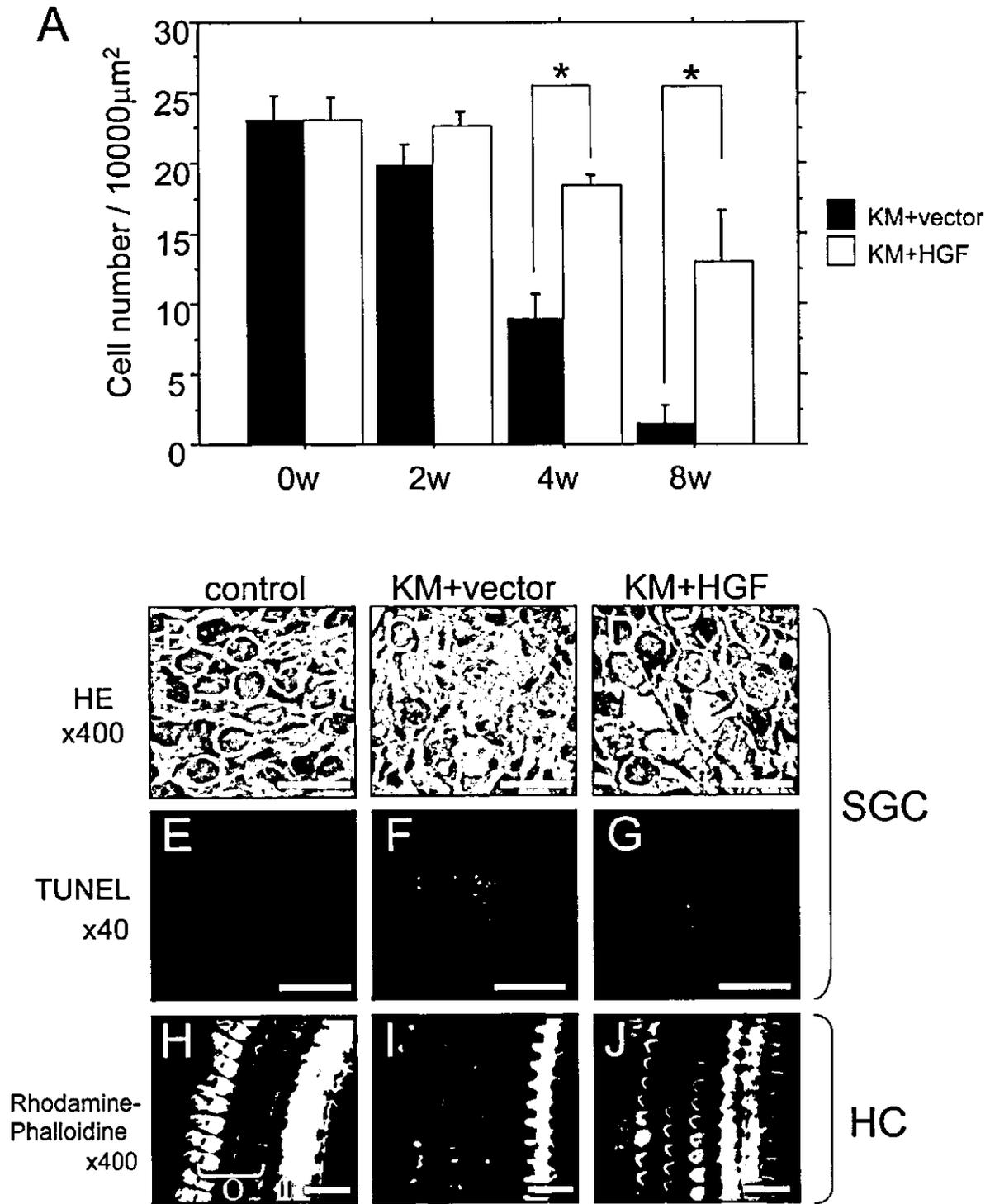


Figure 4. Protective effect of the HGF transgene on SGC and HC treated with kanamycin. Numbers of hematoxylin-positive cells of SGC of the rats treated with KM + vector or KM + HGF are counted at various time points (*A*; $n=6$ for each group). Mid-modiolar 10 μ m cryosections from rats without treatment (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 week 4. TUNEL staining of the contiguous sections of SGCs from the same rats as described above is shown in *E*, *F*, and *G*. Fluorescent image of HC of the rats in the control, KM + vector group, and KM + HGF group is shown in *H*, *I*, and *J*. O: outer hair cell; I: inner hair cell. Scale bar: 50 μ m in *B-D* and *H-J*, 500 μ m in *E-G*. * $P < 0.01$.

Fig. 5

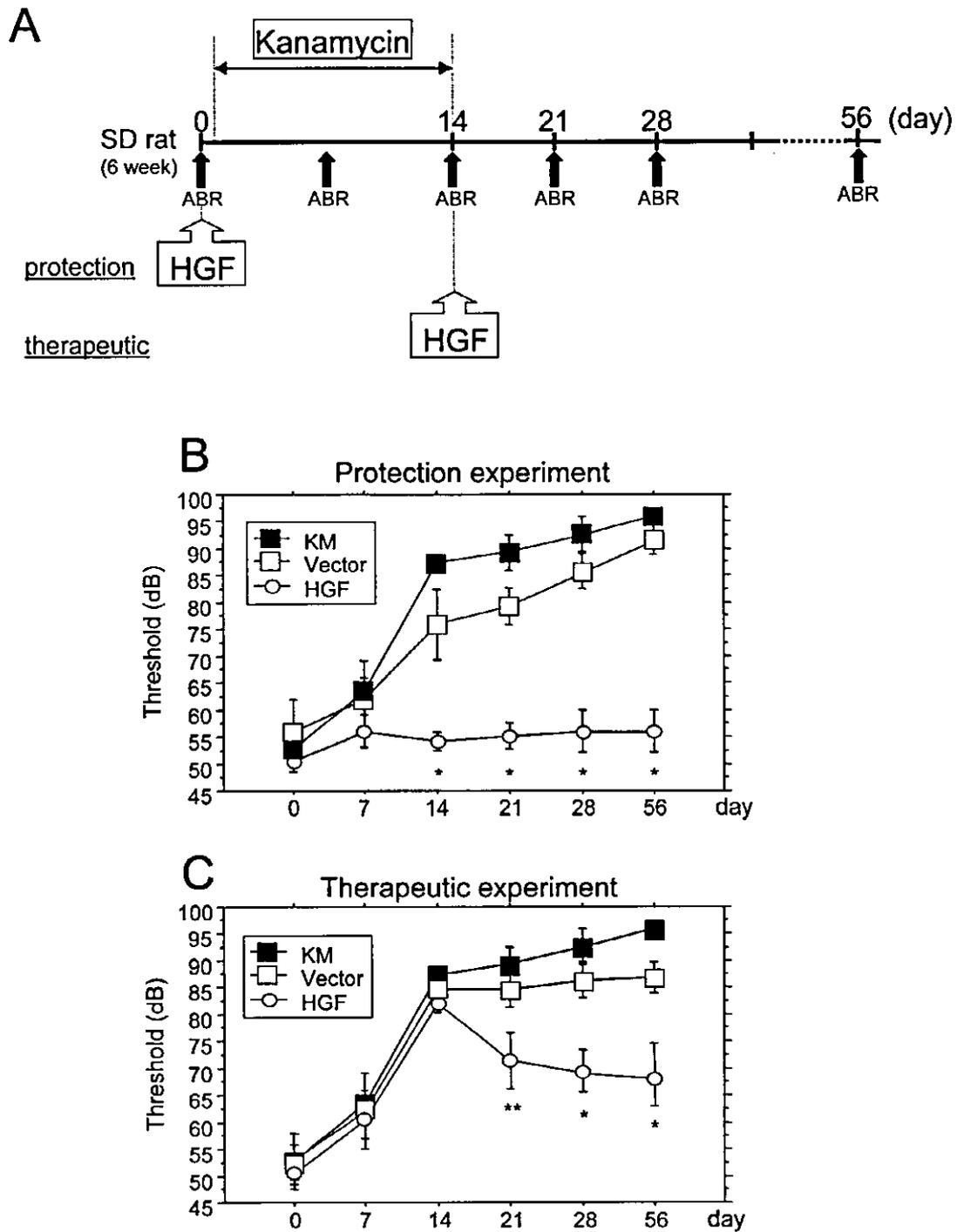


Figure 5. Hearing function of rats treated with KM, KM + vector, or KM + HGF was evaluated by the auditory threshold using ABR. Time course of the experiment was illustrated in *A*. In the protection experiment (*B*), rats treated with the HVJ-E containing control vector (vector) or HVJ-E containing the human *HGF* gene (HGF) immediately before 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 the 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. Means \pm SD of each value are indicated. ■: KM group; □: KM + vector group; ○: KM + HGF group. * $P < 0.01$; ** $P < 0.05$.

New Vector Innovation for Drug Delivery: Development of Fusigenic Non-Viral Particles

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Abstract: Efficient and minimally invasive drug delivery systems have been developed to treat intractable human diseases. One approach has been the development of chimeric vector systems combining at least two different vector systems. Based on this concept, chimeric drug delivery systems that combine viral and non-viral features have been developed. Fusigenic non-viral particles have been constructed by conferring viral fusion proteins onto non-viral vectors. HVJ (hemagglutinating virus of Japan; Sendai virus)-liposomes were constructed by the combination of DNA-loaded liposomes with a fusigenic envelope derived from HVJ (hemagglutinating virus of Japan, Sendai virus). Reconstituted HVJ-liposomes were also developed by the insertion of isolated fusion proteins of HVJ into DNA-loaded liposomes. Recently, the technology has been developed to incorporate macromolecules directly into inactivated HVJ particles without liposomes. The resulting HVJ envelope vector introduced plasmid DNA, efficiently and rapidly into both cultured cells *in vitro* and organs *in vivo*. Furthermore, proteins, synthetic oligonucleotides and drugs have also been effectively introduced into cells using the HVJ envelope vector. The HVJ envelope vector will be a promising tool for both *ex vivo* and *in vivo* gene therapy experiments.

Key Words: chimeric vector, non-viral vector, HVJ, cell fusion, HVJ-liposomes, HVJ envelope vector, gene therapy.

I. INTRODUCTION

Gene therapy appears to be promising for treating intractable human diseases [1], but further development of effective gene transfer vector systems is key to the advancement of human gene therapy [2]. Efficient and minimally invasive vector systems appear to be most appropriate for both gene therapy and drug delivery. Numerous viral and non-viral (synthetic) methods for gene transfer have been developed [3-6], and in general, viral methods are more efficient than non-viral methods for the delivery of genes to cells. However, viral vectors are not available for drug delivery. Moreover, the safety of viral vectors is of concern due to the concomitant introduction of genetic elements from parent viruses, leaky expression of viral genes, immunogenicity, and changes in the host genome structure, whereas non-viral vectors are less toxic and less immunogenic [5, 6]. From these perspectives, much attentions have been paid on the development of non-viral vector systems. Nevertheless, most non-viral methods are less efficient for transfer of macromolecules, particularly *in vivo*. One approach to deal with these issues is the chimeric combination of viral and non-viral vectors.

Various modifications have been made to enhance the efficiency of gene delivery by non-viral vectors. Although liposomes have been used to target and introduce macromolecules into cells, gene transfer efficiency was low and varied during the early days of liposome development. The synthesis of cationic lipids produced a revolutionary improvement in gene transfer efficiency in 1987 [7]. Felgner

et al. also developed a new model of liposome/DNA complex called a "lipoplex". Until then, DNA had been incorporated into liposomes, but, with lipoplex, an electrostatic complex was made between negatively charged DNA and positively charged cationic liposomes. Numerous cationic lipids have been synthesized to further improve transfection efficiency and to reduce cytotoxicity of lipoplex [6]. Nevertheless, in lipoplex-mediated transfection DNA is still taken up into cells by phagocytosis or endocytosis, not by fusion.

To solve the problem of degradation of the molecules before reaching the cytoplasm, fusion-mediated delivery systems have been developed. A fusigenic viral liposome with a fusigenic envelope derived from hemagglutinating virus of Japan (HVJ; Sendai virus) was constructed [8, 9]. HVJ has been shown to fuse with cell membrane at neutral pH, and HN and F-fusion proteins of the virus, contributes to the cell fusion [10]. For fusion-mediated gene transfer, DNA-loaded liposomes were fused with UV-inactivated HVJ to form the fusigenic viral-liposome, HVJ-liposome, which is 400 to 500 nm in diameter. This fusion-mediated delivery resulted in the advantageous protection of the molecules in the endosomes and lysosomes from degradation [11].

A similar approach has been performed to enhance gene transfer efficiency of receptor-mediated gene delivery system by combining fusion peptide derived from influenza virus hemagglutinin [12]. A tissue-specific gene delivery system has been developed by binding tissue-specific molecules to a poly-L-lysine/DNA complex. Binding asialoglycoprotein and transferrin to a poly-L-lysine/DNA complex successfully targets DNA to hepatocytes and cancer cells, respectively [13, 14]. However, the limitation of this system is the degradation of the DNA in the lysosomes. To

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avoid such degradation, a fusion-mediated gene delivery system has been investigated. Influenza virus is known to fuse with cell membranes at acidic pH, and hemagglutinin (HA) protein on the viral envelope is known to be involved in the fusion activity. It has also been elucidated that an N-terminal peptide of influenza HA subunit HA-2 can fuse with cell membranes. The transferrin/poly-L-lysine/DNA complex bound with the HA-2 peptide has been shown to increase gene transfer efficiency in cultured cancer cells more than 1,000 fold compared with that in the absence of the peptide [12].

A more direct and practical approach is the conversion of a fusigenic virion to a non-viral gene delivery particle. Numerous viruses such as influenza, VSV and HVJ are known to induce cell fusion. We have recently succeeded in developing an HVJ envelope vector system [15]. In this review article, we will also explain the new vector system

II. DEVELOPMENT OF HVJ-LIPOSOMES

One approach to improve the vector systems involves the insertion of fusion proteins into liposomes to enhance gene delivery [8, 9]. HVJ, also known as Sendai virus, is able to fuse with cell membranes and also with liposomes [10]. DNA-loaded liposomes are fused with UV-inactivated HVJ to form HVJ-liposomes (Fig. 1). The resulting vesicle, the HVJ-liposome, consists fusion proteins on the envelope and DNA on the inside. The resulting HVJ-liposome is

approximately 400 to 500 nm in diameter. These liposomes are able to encapsulate DNA smaller than 100 kb, with a DNA trapping efficiency of approximately 20%. RNA, oligodeoxynucleotides (ODN), proteins, and drugs can also be enclosed and delivered to cells. It has been suggested that the advantage of fusion-mediated introduction of macromolecules may be the protection of macromolecules from degradation in the endosome and lysosome before reaching the cytoplasm. Nakamura *et al.* have clearly demonstrated this hypothesis using FRET (fluorescence resonance energy transfer) in the introduction of antisense oligonucleotides into cell nucleus [11].

HVJ-liposomes have been shown to be useful for *in vivo* gene transfer. When HVJ-liposomes containing the LacZ gene were injected directly into one lobe of a rat liver, approximately 70% of cells expressed LacZ gene activity, and no pathological hepatic changes were observed [16]. In this experiment, gene transfer to rat liver cells was not inhibited by repeated injections. After repeated injections, the anti-HVJ antibody generated was not sufficient to neutralize HVJ-liposomes. Cytotoxic T cells recognizing HVJ were not detected in rats transfected repeatedly with HVJ-liposomes. Thus, one advantage of HVJ-liposomes would be allowance of repeated administration [16].

To improve gene transfer efficiency, lipid components of liposomes have been investigated. Subsequently, new anionic liposomes called HVJ-AVE liposomes; i.e., HVJ-

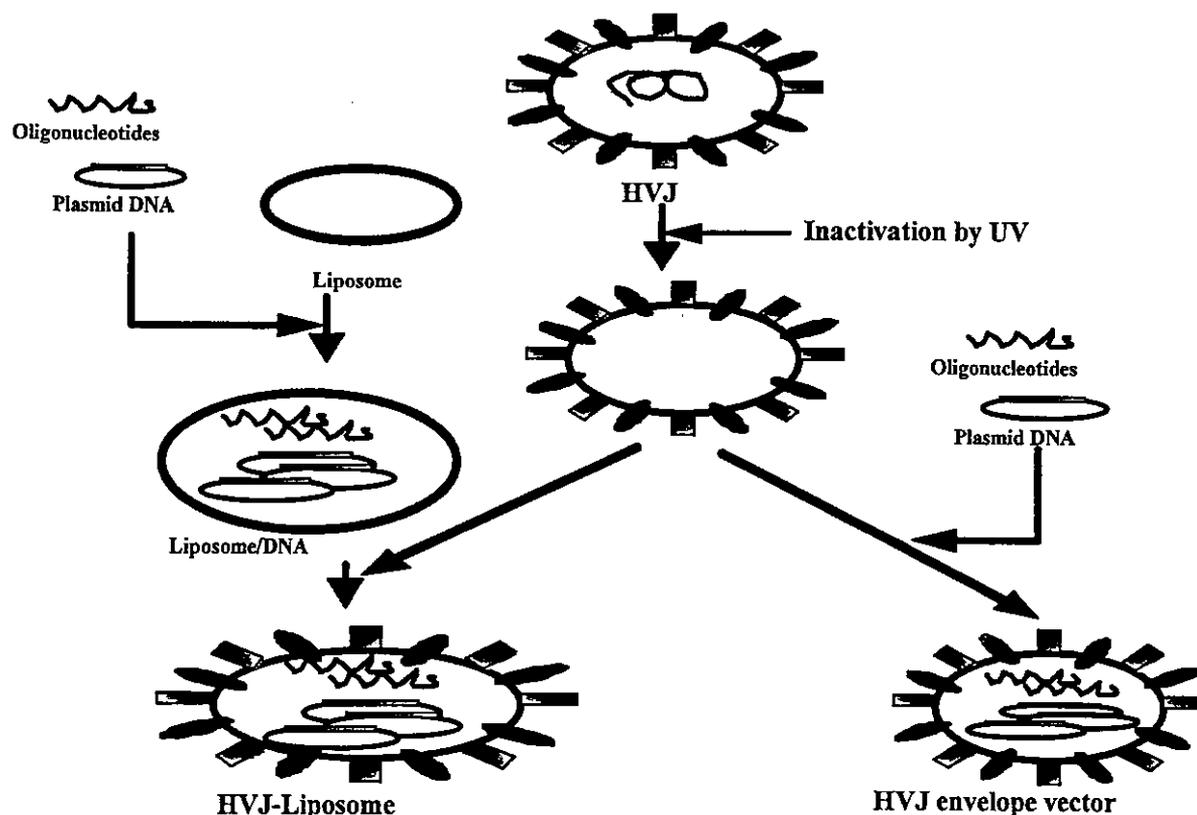


Fig. (1). Development of HVJ-liposomes and HVJ envelope vector. Either plasmid DNA or synthetic oligonucleotides are able to be incorporated into both vectors and efficiently delivered to cells by the fusion activity of HVJ. However, since viral genome is completely inactivated and degraded, viral proteins are never produced in the cells.

artificial viral envelope liposomes have been developed. The lipid components of AVE liposomes have been to be very similar to the HIV envelope and mimic the red blood cell membrane [17]. HVJ-AVE liposomes have yielded gene expression in liver and muscle 5 to 10 times higher than that observed with conventional HVJ-liposomes [18]. Additional improvements have been made through the construction of cationic-type HVJ-liposomes using cationic lipids. Of the cationic lipids, positively charged DC-cholesterol (DC) [19] has been the most efficient for gene transfer. For luciferase expression, HVJ-cationic DC liposomes were 100 times more efficient than conventional HVJ-anionic liposomes [17]. HVJ-cationic DC liposomes have been shown to be more appropriate for gene transfer to cancer cells. In gene transfer to intraperitoneally disseminated colon cancers, HVJ-cationic liposomes introduced either luciferase DNA or FITC-ODN predominantly to tumor nodules in the mouse abdomen.

III. RECONSTITUTED FUSION PARTICLES

To promote fusion-mediated gene delivery, reconstituted particles containing fusion proteins of HVJ have been developed [20-22]. It has been possible to construct HVJ-liposomes using inactivated whole HVJ virion and isolated fusion proteins can be used instead of the whole virion. HVJ virion was completely lysed with detergent, and the lysates were mixed with DNA solution. In some cases, several lipids were added to the mixture. By removing the detergent using dialysis or a column procedure, reconstituted HVJ particles containing DNA were constructed. Instead of the whole virion of HVJ, fusion proteins (F and HN) isolated from the virion were mixed with the lipids/DNA mixture in the presence or absence of detergent. Since F protein is recognized by the asialoglycoprotein receptor on hepatocytes, reconstituted HVJ particles containing only F protein have been constructed to specifically target hepatocytes *in vivo* [22]. In another approach, fusion proteins, F and HN, have been purified from the HVJ virion and liposomes containing F and HN were constructed by the detergent-lysis-dialysis method [23, 24]. The resulting fusion liposomes were fused with DNA-loaded liposomes to form reconstituted HVJ-liposomes [24]. These reconstituted fusion liposomes were as effective as conventional HVJ-liposomes with the fully intact HVJ virion in terms of delivery of both FITC-ODN and the luciferase gene to cultured cells. LacZ gene was also transferred directly to mouse skeletal muscle *in vivo* using these reconstituted fusion particles.

IV. DEVELOPMENT OF HVJ ENVELOPE VECTOR SYSTEM

The disadvantage of HVJ-liposome is the complicated procedure to isolate and produce both inactivated HVJ and DNA-loaded liposomes. Another limitation is that the fusion activity of the HVJ-liposomes decreases to approximately 2% of native HVJ because of the reduction of density of fusion proteins on the surface of HVJ-liposomes. To solve these problems, the HVJ envelope vector system has been developed as illustrated in Fig. (1) [15]. HVJ is completely inactivated by either UV-irradiation or β -propiolactone treatment. Exogenous plasmid DNA is incorporated into the

inactivated HVJ by treatment with mild detergent and centrifugation. By this procedure, approximately 15 - 20% of added DNA is able to be incorporated into the inactivated HVJ envelope. Electronmicroscopy confirms that DNA is incorporated into all of the particles of inactivated HVJ. The largest DNA tested was a 14 kb plasmid DNA, with a resultant trapping efficiency of approximately 18%. Without centrifugation, the DNA trapping efficiency is reduced to approximately 3% - 5%. Without detergent treatment, no DNA becomes incorporated into the viral particle. Synthetic oligonucleotides, proteins and peptides can be incorporated into the HVJ envelope by a similar strategy.

The HVJ envelope vector differs from the reconstituted HVJ particles that are prepared by reassembling lipids and fusion proteins after solubilization of the virus particle. In the preparation of the HVJ envelope vector, plasmid DNA is incorporated into inactivated HVJ particles by treatment with mild detergent without destruction of the virion and without the dialysis, purification or addition of lipids or proteins which are used for the preparation of reconstituted HVJ particles [20-22]. Therefore, the composition of the HVJ envelope vector is very similar to that of native HVJ.

For *in vitro* transfection, the HVJ envelope vector containing luciferase expression plasmid was mixed with protamine sulfate, and this mixture was added to cultured cells. Protamine sulfate was absolutely necessary for *in vitro* gene transfer with the HVJ envelope vector to augment attachment of the HVJ envelope vector to the cell surface by providing a cationic charge. The HVJ envelope vector was useful for gene transfer to various cell lines, and a short incubation period (i.e., a 10-min incubation) was sufficient for high expression of the target gene. When the HVJ envelope vector containing the GFP expression plasmid was added to BHK-21 cells, GFP expression was approximately 80%, as determined by flow cytometry. Under such conditions, little cell damage was observed. Fluorescence isothiocyanate-labeled oligodeoxynucleotides (FITC-ODN), proteins such as IgG, bovine serum albumin and human insulin were also transferred to cultured cells at an efficiency of more than 95%. The HVJ envelope vector is much more efficient in gene transfer to primary culture cells, such as rat neuronal cells, human aortic endothelial cells, mouse dendritic cells and rat cardiac myocytes, than other lipofection reagents. Additionally, cells in a suspension are also appropriate targets for HVJ envelope vector. Thus, the HVJ envelope vector should be useful for *ex vivo* gene therapies. Another advantage of the present HVJ envelope vector system is its utility for *in vivo* application. The HVJ-envelope vector is more effective than HVJ-liposomes for *in vivo* gene transfer. LacZ or luciferase gene transfer to lung, liver, uterus, eye, skin, muscle, and brain of animals such as mouse, rat, rabbit and monkey are achieved by direct injection of the HVJ envelope vector. FITC-ODN were also efficiently delivered to rat lung, cartilage of monkey joints and tumor masses. Among the organs we have tested, the HVJ envelope vector is more effective than HVJ-liposomes for gene transfer to liver, uterus, brain, eye, and lung with similar levels of expression detected in muscle and skin. This predominance of the HVJ envelope vector over HVJ-liposomes may be due to the stronger fusion activity of the HVJ envelope vector in comparison to HVJ-liposomes.