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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*lac*Z were prepared for X-gal staining. Figure 1 shows that β-gal expressing cells were present in the SGCs, cerebral cortex, and medulla.

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

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

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

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

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

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

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

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

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

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

Evaluation of hearing function

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

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

DISCUSSION

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

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

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

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

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

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

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

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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

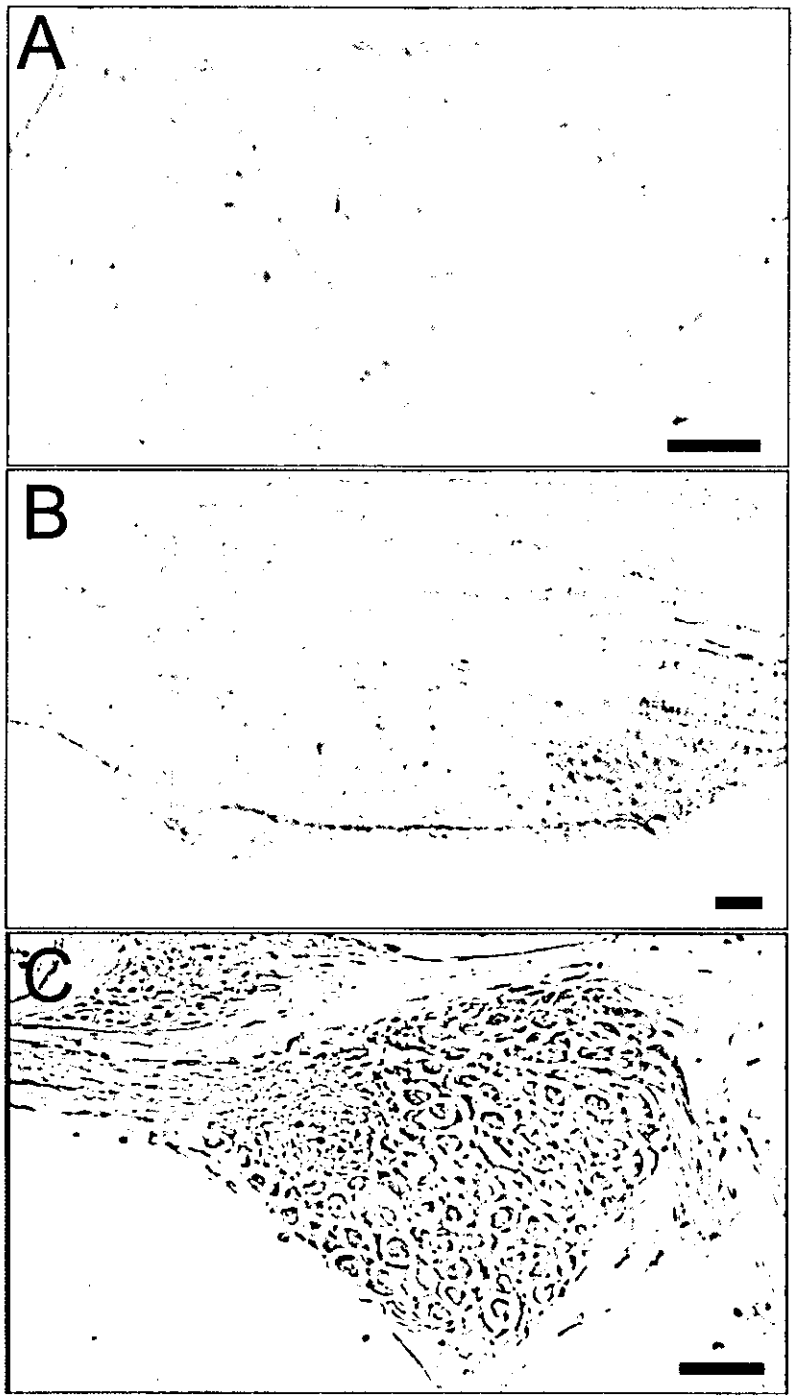

Figure 1. Localization of *lacZ* expression in the brain and cochlea. *lacZ* expression in the medulla (*A*), cochlear nucleus (*B*), and SGCs (*C*) of normal rats was detected by X-gal staining on day 7 after intrathecal injection of HVJ-E containing the *E. coli* -galactosidase gene *lacZ*. Scale bar: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

Fig. 2

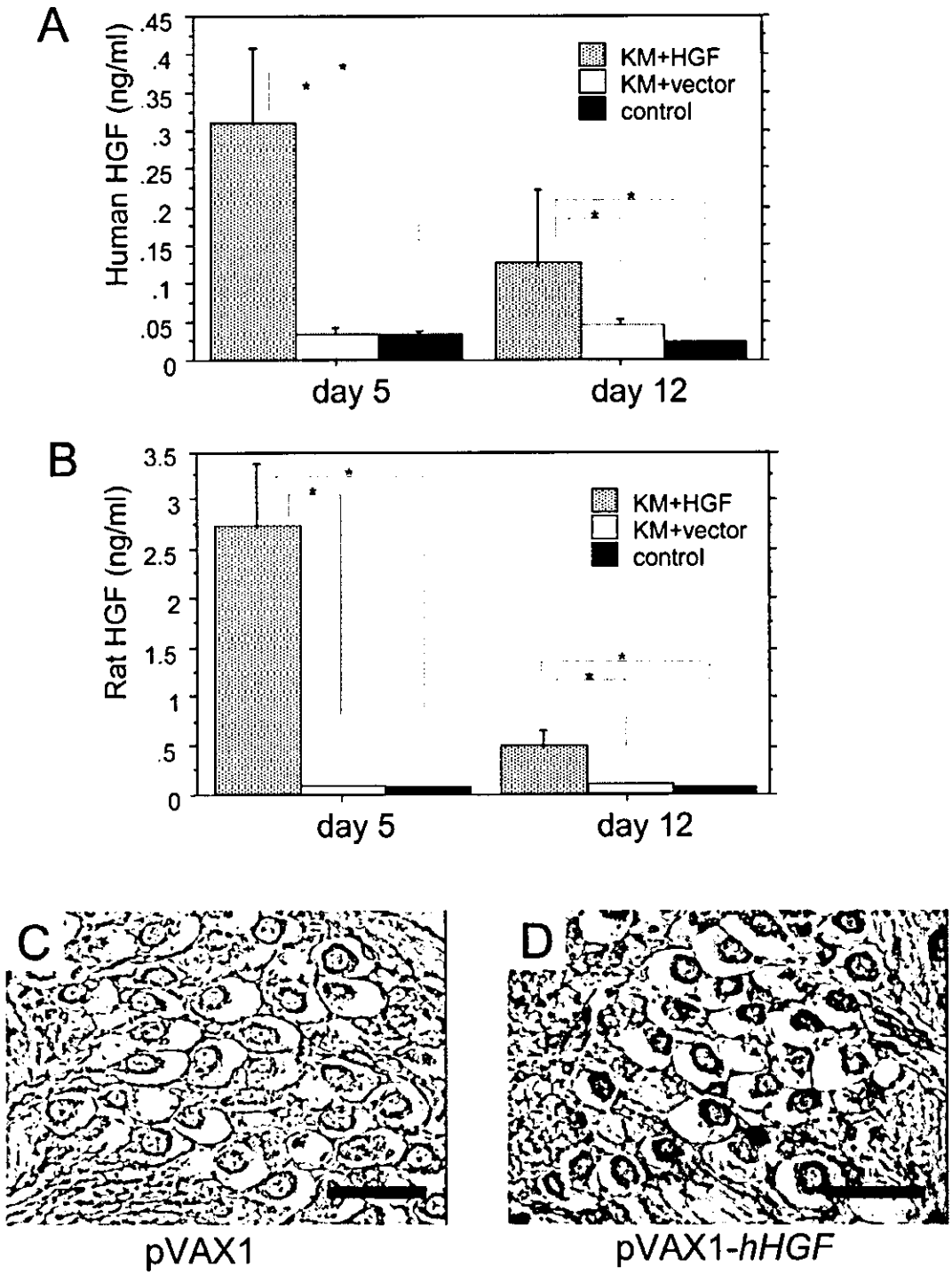

Figure 2. Expression levels of exogenous and endogenous HGF in CSF and SGCs. Exogenous human HGF (*A*) and endogenous rat HGF (*B*) in CSF from the KM + HGF, KM + vector, and control groups were measured on days 5 and 12 after transfection with the human HGF transgene (n=4 for each). SGCs from the mid-turn 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

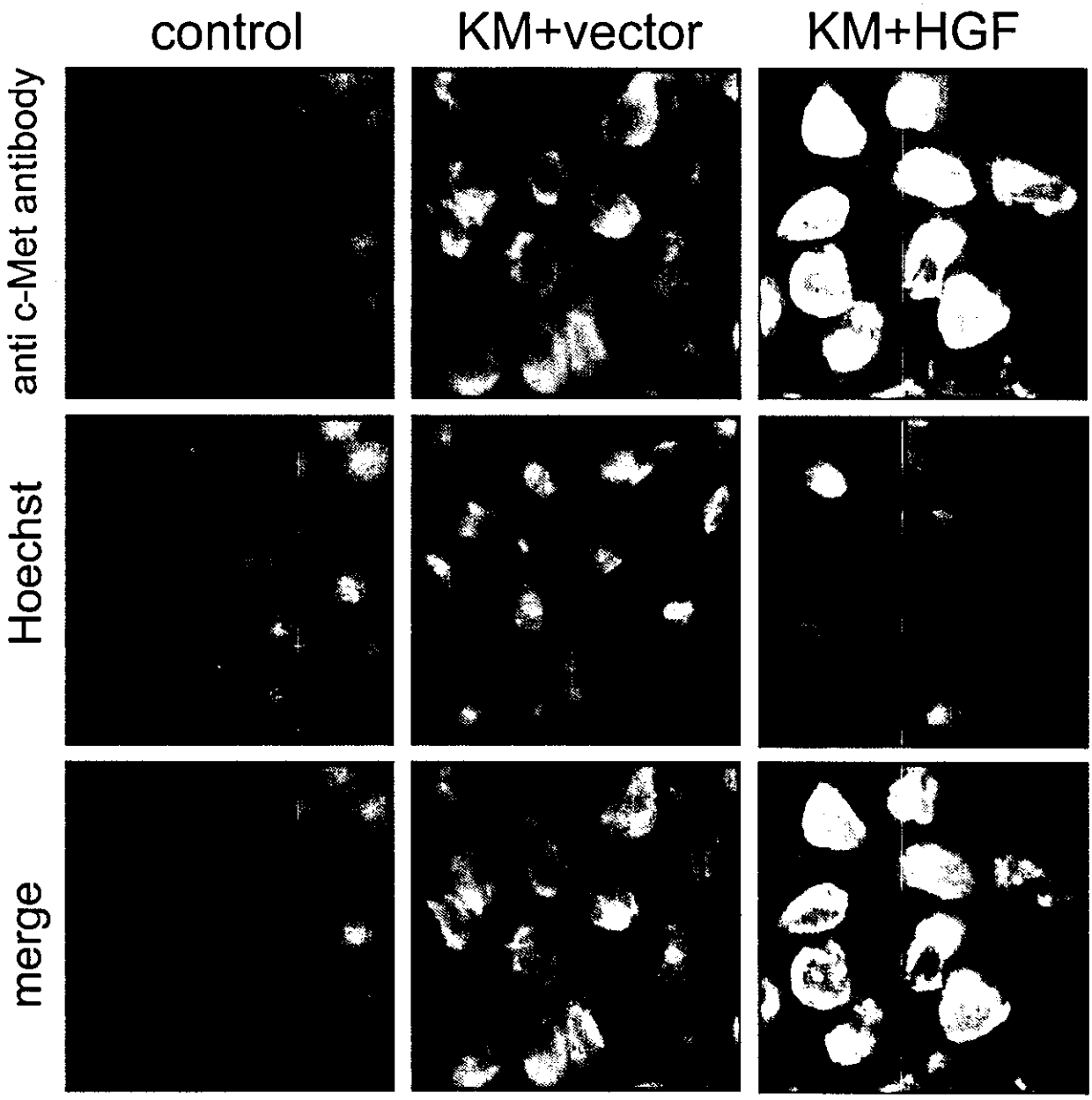

Figure 3. Enhancement of c-Met expression in SGCs by *HGF* gene transfer. Immunohistochemistry of SGCs from the intact rats, KM + vector group, and KM + HGF group was performed. Samples were stained with anti-c-Met antibody (upper) and counterstained with Hoechst 33342 (middle). Merged images are also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

Fig. 4

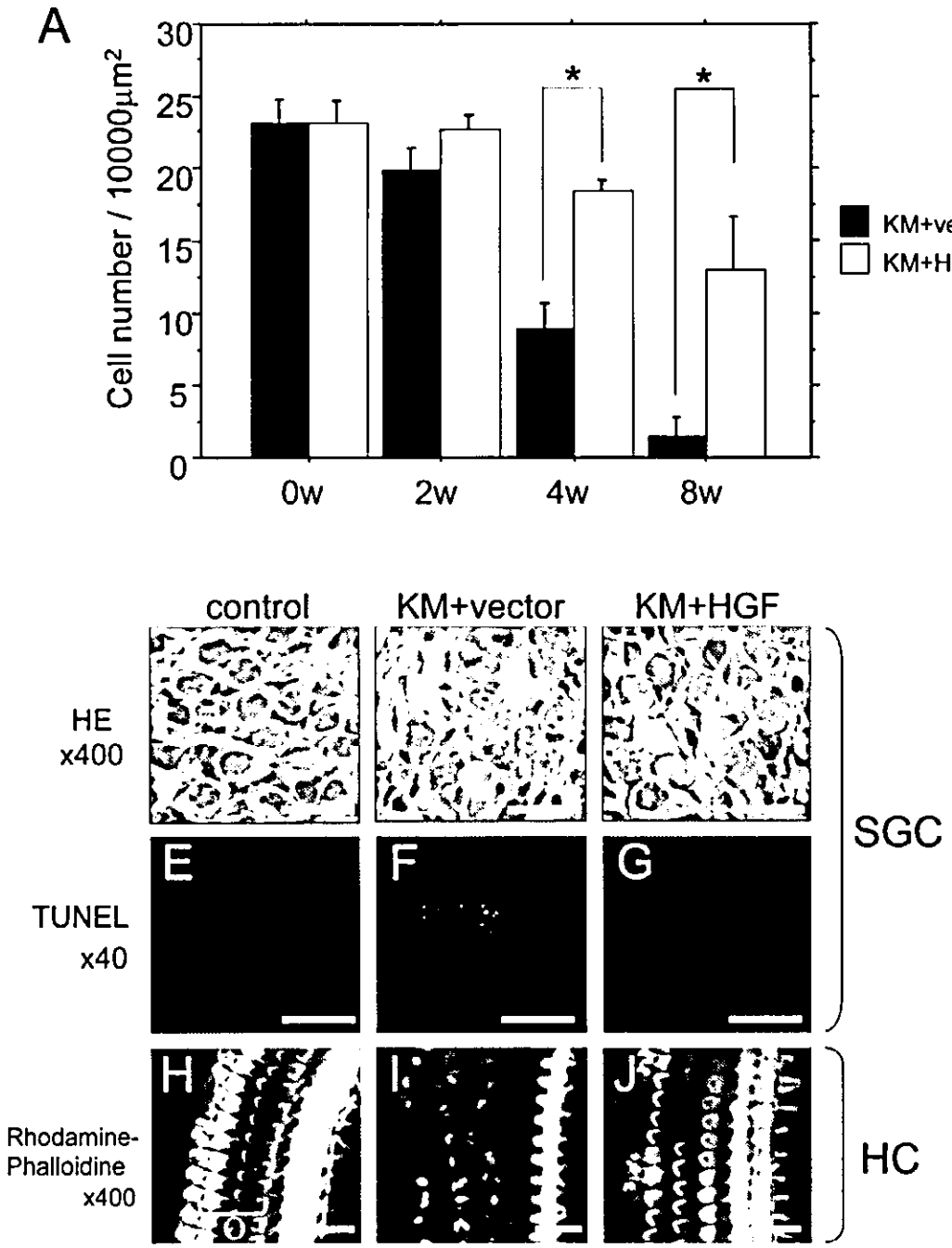

Figure 4. Protective effect of the HGF transgene on SGC and HC treated with kanamycin. Numbers of hematoxylin-positive cells of SGC of the rats treated with KM + vector or KM + HGF are counted at various time points (***A***; n=6 for each group). Mid-modiolar 10 μm cryosections from rats without treatment (control; ***B***), treated with kanamycin and HVJ-E containing control vector (***C***), or HVJ-E containing the human HGF gene (***D***) were stained with hematoxylin on 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 μm in ***B-D*** and ***H-J***, 500 μm in ***E-G***. *$P < 0.01$.