ORIGINAL ARTICLE

Hyaluronic acid pretreatment for Sendai virus-mediated cochlear gene transfer

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Gene therapy with viral vectors is one of the most promising strategies for sensorineural hearing loss. However, safe and effective administration of the viral vector into cochlear tissue is difficult because of the anatomical isolation of the cochlea. We investigated the efficiency and safety of round window membrane (RWM) application of Sendai virus, one of the most promising non-genotoxic vectors, after pretreatment with hyaluronic acid (HA) on the RWM to promote efficient viral translocation into the cochlea. Sendai virus expressing the green fluorescent protein reporter gene was detected throughout cochlear tissues following application combined with HA pretreatment. Quantitative analysis revealed that maximum expression was reached 3 days after treatment. The efficiency of transgene expression was several 100-fold greater with HA pretreatment than that without. Furthermore, unlike the conventional intracochlear delivery methods, this approach did not cause hearing loss. These findings reveal the potential utility of gene therapy with Sendai virus and HA for treatment of sensorineural hearing loss.

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INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common human sensory deficit. Environmental and genetic factors contribute to SNHL resulting from degeneration of various cochlear tissues; the cochlear epithelium and the auditory nerve represent the most common cochlear tissues involved in SNHL. Mammalian cochlear hair cells (HCs) and spiral ganglion neurons (SGNs) lack the capacity for self-repair; therefore SNHL caused by cochlear tissue damage is irreversible.^{1–3} No effective treatment for SNHL is available, although some patients may benefit from hearing aids or cochlear implants. Recently, virus-mediated gene therapy has been proposed as a therapeutic strategy for SNHL. Viral vectors have proven to be much more effective than non-viral vectors for cochlear gene therapy.^{4–6} This technique has restored hearing function in several animal models, preventing SGN degeneration and even replacing lost HCs.^{7–10} In previous studies, adenovirus (AV),^{11,12} herpes simplex virus,¹³ lentivirus¹⁴ and adeno-associated virus (AAV)^{15,16} have been investigated for their suitability in cochlear gene therapy, but with limited success. Recent research has focused on Sendai virus (SeV), which has a number of characteristics desirable for human gene therapy: it is not genotoxic, it provides high transfection efficiency and it is not pathogenic in humans.^{17,18} SeV is a member of the Paramyxoviridae and is an enveloped virus with nonsegmented, negative-sense genomic RNA.¹⁹ Its genome is located exclusively in the cytoplasm of infected cells and never goes through a DNA phase, thus precluding integration of its genetic information into the host cell genome.²⁰ These properties make SeV an appropriate candidate for clinical use in cochlear gene therapy.

The cochlea is traditionally considered to be an ideal organ for local gene therapy because it is anatomically isolated from other organs, and the cochlear fluid streams internally. Therefore, local gene therapy in the cochlea would be less likely to produce side effects.²¹ An intracochlear approach, including cochleostomy and

injection through the round window membrane (RWM), has frequently been used for intracochlear delivery of therapeutic compounds and for virus-mediated gene therapy. Previous research has shown that SeV vectors injected via cochleostomy transfect a large variety of cells including cells of the stria vascularis, SGNs and sensory cells of the organ of Corti.²² However, this intracochlear approach presents a risk of perilymph leakage and hearing loss, which limits its potential for clinical application. In contrast, delivery of therapeutic compounds to the inner ear via absorption through the intact RWM is safe, easy to perform, atraumatic and ideal for clinical use. In fact, the intact RWM approach has been used to deliver drugs such as steroids and gentamicin into the cochlea for the treatment of idiopathic sudden SNHL and Meniere's disease.^{23,24} However, despite the advantages of the intact RWM approach, most viral vectors cannot readily penetrate the intact membrane.^{4,25–27} Therefore, it is necessary to develop a novel gene delivery system that efficiently delivers a meaningful concentration of viral vectors into the cochlea via the intact RWM. Toward this end, recent studies have demonstrated improved transfection efficiency of AV and recombinant AAV vectors after pretreatment of the RWM with facilitating agents such as collagenase²⁷ and hyaluronic acid (HA).²⁸

Here we investigated the SeV transfection efficiency using the intact RWM approach following HA pretreatment to facilitate gene transfer across the membrane. Green fluorescent protein (GFP) reporter expression was used to determine transfection efficiency and auditory function was monitored to assess the safety of the procedure. Our results revealed that the pre-application of HA facilitated safe and efficient cochlear transfection with SeV via the intact RWM. This treatment approach offers a significant advancement toward SeV-mediated gene therapy for SNHL.

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RESULTS

Auditory function

To evaluate the effects of SeV vector transfection with HA pre-application on hearing, auditory brainstem response (ABR; 4, 8, 16 and 32 kHz) was measured before and 3 days after surgery (Figure 1). Baseline thresholds before surgery were essentially equivalent among all groups. At 3 days after surgery, ABR thresholds in the HA alone (30 mg ml^{-1}) and the GFP-SeV/ ΔF alone groups largely overlapped with those of the saline-treated control group; there was no significant difference at any of the tested frequencies. In addition, compared with the saline-treated control group, there was no significant hearing loss after treatment with GFP-SeV/ ΔF in combination with HA (10 or 30 mg ml^{-1}) on the intact RWM at any of the tested frequencies. In contrast, in the group receiving injection of GFP-SeV/ ΔF through the RWM, significant threshold elevation occurred at 32 kHz compared with the remaining groups treated using an intact RWM approach. Furthermore, the cochleostomy approach using GFP-SeV/ ΔF also resulted in significant ABR threshold elevation at 16 and 32 kHz compared with all intact RWM approaches; however, there was no significant difference at any frequency among the groups that received injection through the RWM. These data suggest that HA and GFP-SeV/ ΔF , when placed on the RWM, do not cause hearing loss, and that the intact RWM approach appears to be safe.

Gene transfection

To assess whether HA applied onto the RWM could improve gene transfection, we used cochlear whole mounts to investigate GFP expression in the cochlear tissue after GFP-SeV/ ΔF treatment with or without HA (Figure 2a). In the GFP-SeV/ ΔF alone group, GFP expression was found only around the RWM; very few GFP-positive cells were observed in the cochlear epithelium.

Figure 1. GFP-SeV/ ΔF transfection of HA-pretreated intact RWM does not affect hearing function. ABR thresholds 3 days after surgery at each frequency did not differ among the five intact RWM groups. In contrast, an intracochlear approach resulted in significant threshold increase compared with intact RWM groups at high frequencies. Data represent mean values; error bars show s.e.m. (* $P < 0.05$, cochleostomy vs intact RWM groups; # $P < 0.05$, injection through the RWM vs intact RWM groups).

In contrast, in the HA-pretreated GFP-SeV/ ΔF groups (10 and 30 mg ml^{-1}), there was widespread GFP expression from the base to the middle turns of the cochlea as well as in HCs, supporting cells (SCs), cochlear nerve fibers (CNFs), fibrocytes within the osseous spiral lamina and the spiral ligaments. No obvious differences in GFP expression patterns were observed between the two HA concentrations (10 and 30 mg ml^{-1}). To verify the detailed SeV-mediated gene transfection patterns, we evaluated GFP expression in cross-sections. In cochleae pretreated with HA (30 mg ml^{-1}), we confirmed widespread GFP expression in the basilar membrane, outer hair cells (OHCs), SCs and CNFs (Figure 2b). In the SGNs of the cochlear base, an obvious increase in GFP expression was observed in the group pretreated with HA at 30 mg ml^{-1} compared with the group not pretreated with HA (Figure 2c).

Furthermore, to examine the transfection efficiency of GFP-SeV/ ΔF in HCs, we counted the GFP-positive OHCs and inner HCs (IHCs) according to frequency-specific regions in the HA-pretreated and HA-untreated groups (HA at 0, 10 or 30 mg ml^{-1}). In the 32 kHz region of the organ of Corti 3 days after surgery, many GFP-positive OHCs were observed in the HA-pretreated groups (Figure 3a). In contrast, very few GFP-positive HCs were observed in the HA-untreated group. HA pretreatment (10 and 30 mg ml^{-1}) yielded significant increases in transgene expression and transfection efficiencies in OHCs in the 32 kHz regions (Figure 3b). No significant difference was observed between HA concentrations (10 and 30 mg ml^{-1}). Transfection efficiencies were higher in OHCs than in IHCs. HA treatment did not produce a statistically significant difference in IHC transfection at any frequency (Figure 3c). In addition, the immunostaining signal for GFP showed a base-to-apex gradient pattern of distribution consistent with the GFP-SeV/ ΔF being delivered at the base of the cochlea where the RWM is located. To determine the maximum reach of GFP-SeV/ ΔF transfection from the RWM in HA-pretreated (HA 0, 10 or 30 mg ml^{-1}) and HA-untreated groups, we measured the distance from the RWM to the farthest GFP-positive cochlear cell, expressed as a percentage of the total cochlear length (Figure 3d). A significant increase in maximum reach of transgene expression from the RWM was observed in both HA-pretreatment groups (10 and 30 mg ml^{-1}) compared with that in the HA-untreated group; no significant difference was observed between HA concentrations (10 and 30 mg ml^{-1}). Therefore, these results indicated that HA pretreatment could promote transgene expression of the GFP-SeV/ ΔF vector placed on the RWM into the basal cochlea and increase the reach of transgene expression, but these effects did not occur in an HA concentration-dependent manner.

In addition, to investigate the extent of HC damage after SeV transfection, we performed a quantitative analysis of HC survival (Figures 3e and f). We did not detect an obvious decrease in HC survival among all groups, suggesting that transfection of SeV in combination with HA did not cause HC damage. This result is consistent with the functional assessment results following ABR examination.

Time course of GFP mRNA expression in the cochlea

The time course of GFP mRNA expression is critical to understanding the therapeutic time window of SeV vectors for SNHL. We assessed GFP mRNA expression before and after GFP-SeV/ ΔF treatment in the presence and absence of HA pretreatment (30 mg ml^{-1}) by quantitative reverse transcription (RT)-PCR at several time points (Figure 4). After GFP-SeV/ ΔF application to the untreated RWM, GFP mRNA expression increased to a peak at 3 days, and then rapidly decreased to nontreatment levels by 7 days. In HA-pretreated GFP-SeV/ ΔF -inoculated cochleae, GFP expression also peaked at 3 days after inoculation and was significantly increased by several 100-fold by the addition of HA.

Figure 2. HA increases GFP-SeV/ΔF transfection efficiency in cochleae. (a) In the GFP-SeV/ΔF alone group, GFP expression was found only around the RWM (white arrowheads); very few GFP-positive cells were observed in the cochlear epithelium. In the HA-pretreated GFP-SeV/ΔF group, widespread GFP expression was observed in the base to the middle turns of cochlea, and in SCs and HCs (yellow arrowheads), CNF (white arrows), fibrocytes within the OSL (pink arrows) and in the SL (pink arrowheads). (b) In cross-sections of cochleae pretreated with HA (30 mg mL⁻¹), widespread GFP expression was observed in the OHCs, SCs (yellow arrows), basilar membrane and the CNF. (c) An obvious increase in GFP expression was observed in the SGNs in the HA-treated group compared with those in the HA-untreated group. I, inner hair cell; O, outer hair cell; SL, spiral ligament; SCs, supporting cells; HCs, hair cells; OSL, osseous spiral lamina; CNF, cochlear nerve fibers. Scale bar, 50 μm.

These levels remained significantly high until day 14. The SeV/ΔF used in this study is nontransmissible because of the loss of the *F* gene, which mediates the attachment of virions into infected cells.²⁰ Therefore, once transfected in the cochlear tissues, SeV/ΔF cannot spread to neighboring cochlear cells. Nevertheless, HA-pretreated cochlear tissue transfected with GFP-SeV/ΔF demonstrated significantly elevated and sustained transgene expression compared with tissue from cochleae not pretreated with HA.

Furthermore, to confirm the safety of this approach, we performed quantitative RT-PCR analyses of the middle ear mucosa and the brain tissue after GFP-SeV/ΔF treatment in the presence and absence of HA pretreatment (30 mg mL⁻¹) at 3 h and 3 days (Figure 4). From these, GFP expression in the brain was not detected, suggesting that the GFP-SeV/ΔF applied on the RWM with or without HA was not transferred to the brain. However, in the middle ear mucosa at 3 days after treatment, we were able to

Figure 3. GFP-SeV/ΔF transfection efficiency is higher in the HA-pretreated groups. **(a)** Images were taken from the 32 kHz region of the organ of Corti 3 days after surgery. Transfected OHCs are indicated by white arrowheads. Transfected cochlear nerves are indicated by blue brackets. OHC regions are indicated by white brackets. OHCs, outer hair cells; IHCs, inner hair cells. Scale bar, 50 μm. **(b)** A significant increase in OHC transfection correlated with HA treatment in the 32 kHz region. Data represent mean values; error bars show s.e.m. (** $P < 0.01$). **(c)** HA treatment caused no statistically significant difference in IHC transfection efficiency at any frequency. Data represent mean values; error bars show s.e.m. **(d)** A significant increase in the maximum reach of transgene expression from the RWM was observed in the HA-treated groups. No significant difference was observed between HA concentrations (10 and 30 mg ml⁻¹). **(e, f)** The HC survival ratio is expressed as a percentage. No significant differences were observed in HC survival ratios between the groups. Data represent mean values; error bars show s.e.m. (* $P < 0.05$, ** $P < 0.01$); NS, not significant.

detect minor *GFP* expressions at a level largely lower than those detected in the cochlea. Moreover, HA did not affect the expression level in the middle ear mucosa. Because we applied GFP-SeV/ΔF on the RWM, which is located in the middle ear, we suggest that the preferred therapeutic reporter gene should be chosen carefully to avoid any damage to the middle ear from the treatment.

DISCUSSION

This study has demonstrated that HA-assisted, SeV-mediated gene therapy via the intact RWM enables efficient intracochlear transfection in a variety of cochlear cells including HCs, SCs, SGNs, CNFs, fibrocytes within the osseous spiral lamina and cells in the spiral ligament. In these cells, reporter expression increased to a maximum 3 days after treatment and was significantly increased by several 100-fold by pretreatment with HA. Levels remained high until day 14. This technique is easy to perform and carries minimal risk of hearing loss or biological side effects and is

therefore a promising novel method for human cochlear gene therapy.

The choice of an appropriate viral vector for gene transfer requires careful consideration. The patterns of virus-mediated transgene expression differ significantly between vectors. However, some vectors have evolved for efficient transfer into the host genome, and therefore, may provoke a strong immune response that could damage recipient cells and compromise cochlear function.^{29,30} Certain viral vectors such as AAV have the advantage of efficient transfection and long-term expression, which may be well suited for treating congenital genetic deafness.^{12,31} However, in some therapeutic applications for SNHL apart from congenital genetic deafness, long-term expression is not always essential to obtain therapeutic benefits. The effective therapeutic window may be relatively transient because inner ear tissues must be targeted shortly after injury, before they die.³² Therefore, viral-mediated cochlear gene therapy, when commenced at an appropriate time, may be more effective with a pulse of very efficient expression that does persist. In this study, we verified that SeV vectors enabled transient expression that

Figure 4. HA-pretreated cochlear tissue demonstrates higher and more sustained *GFP* expression after *GFP-SeV/ΔF* transfection. *GFP* expression increased in both HA-treated and -untreated transfected cells, with a peak at 3 days after surgery. Expression was significantly increased at 1, 3, 7 and 14 days after surgery with the addition of HA. In the brain, *GFP* mRNA expression was not detected. In the middle ear mucosa at 3 days after treatment, minor *GFP* mRNA expression was detected at a level well below that detected in the cochlea at the same time point. HA pretreatment did not affect the expression level in the middle ear mucosa. *GFP* mRNA expression levels were standardized to cochlear samples taken 3 h after *GFP-SeV/ΔF* application in the absence of HA. Data represent mean values; error bars show s.e.m. (**P* < 0.05).

peaked at 3 days (Figure 4). In addition, pretreatment with HA enabled significantly higher and sustained SeV-mediated transgene expression in cochleae for >2 weeks, which is sufficient to treat most types of SNHL, except for cases of congenital genetic deafness.²⁸ Our results will contribute to the more readily controlled, and therefore, likely to the more widely accepted use of SeV vectors plus HA for the clinical treatment of SNHL with fewer side effects. However, if transient expression is of limited clinical utility, the problem may still be resolved by repeated application when needed. However, further comprehensive experiments will be needed to address the chronic effects of gene transfection, if any, by SeV gene therapy.

An ideal route for cochlear gene delivery would allow simple, effective and targeted transfections without causing hearing loss. Perilymphatic or endolymphatic perfusion of viral vectors through a tiny hole drilled in the bony wall of the cochlea or via RWM injection is the conventional route for cochlear gene delivery. Although this allows for maximal transfection of the inner ear, it dramatically increases the risk of damage or infection. The potential for mechanical impairment and the complexity of the operation may limit its clinical utility. The RWM is the only membranous sealed window in the bony labyrinth. Diffusion via the intact RWM is a potential route for the delivery of biologically active molecules into the inner ear. This approach is safe, easy and atraumatic; therefore, it is an ideal method for clinical use. In fact, gentamicin has been delivered using this technique for the management of Meniere's disease with intractable vertigo.²⁴ In addition, transtympanic delivery of glucocorticoids has proven effective to treat cases of idiopathic sudden SNHL.²³ Furthermore, several studies have confirmed the feasibility of inner ear gene transfer mediated by viral vectors via the intact RWM in animal models.^{26,33} However, it has been difficult to deliver a sufficient quantity of these viral vectors into the inner ear through the intact RWM.^{4,25–27} This finding is consistent with our observation that *GFP* expression following SeV transfection was limited to cells around the RWM. In this study, very few *GFP*-positive cells were observed in the cochlear epithelium in the group treated with *GFP-SeV/Δ* alone. In addition, our ABR results suggest that our intact RWM approach did not cause hearing loss (Figure 1) and would not be expected to cause cochlear damage at 3 days after gene transfer (Figures 3e and f). However, the long-term effects

and safety with respect to cochlear functions after SeV gene transfection remain unknown. Therefore, in the future, additional experiments would be necessary to address the chronic effects on ABR thresholds as well as on distortion product otoacoustic emissions, which are used to quantify OHC function, because SeV gene transfection efficiency was higher in OHCs than in IHCs (Figures 3b and c). Furthermore, the use of 5-dB step measurements during ABR examination would be valuable for detailed functional analysis, whereas ABR experiments by 10-dB step measurements were performed in this study.

Permeability through the RWM can be impacted by several factors such as molecular size, configuration, concentration, liposolubility and electrical charge.^{34,35} With respect to molecular size, *in vivo* experiments have revealed that 1-μm microspheres can cross chinchilla RWMs, whereas 3-μm spheres cannot.³⁶ Thus, smaller molecules pass through the RWM, whereas larger molecules do not readily do so. In the current study, SeV, whose particle size is approximately Ø < 200 nm, could not pass through the RWM. The particle sizes of viral vectors tested by other groups for RWM-mediated gene therapy were Ø < 100 nm (retrovirus), Ø 70–90 nm (AV) and Ø 20 nm (AAV). Therefore, with respect to size only, these other viral vectors were expected to demonstrate an advantage regarding permeability through the RWM compared with the SeV vector. However, these other vectors were reported to have difficulty in passing through the RWM,^{4,25–27} suggesting that a smaller viral vector particle size is not sufficient for viral-mediated cochlear gene transfection by RWM. Thus, in comparison to other viral vectors, the mechanism of SeV transfection could potentially rely instead on polysialogangliosides, such as GD1a, GTMb and GQ1b, that are well-known natural receptors for SeV,³⁷ and whose expression has been demonstrated in the cochlea.³⁸ Therefore, the interaction of SeV applied on the HA-treated RWM with polysialogangliosides on the cochlear tissue surface suggests a possible mechanism for inducing the ensuing virus–inner ear cell fusion.

The RWM consists of three layers³⁹ (i) an outer epithelium facing the middle ear, (ii) a core of connective tissue and (iii) an inner ear epithelium bordering the inner ear. The outer epithelium consists of a single layer of light and dark cells containing dense cytoplasm and numerous microvilli. In this layer, cells are bounded by tight junctions with extensive interdigitations. The connective tissue

layer contains fibroblasts, collagen and elastic fibers, and blood and lymph vessels. The inner epithelium has loose junctions and a discontinuous basement membrane. Therefore, the major barrier to viral vector diffusion is likely to be the outer epithelial layer with tight junctions. It has been demonstrated that the efficiency of viral vector-mediated gene transfection is significantly increased if the RWM is pretreated or simultaneously treated with a facilitating agent such as histamine, HA or collagenase.^{27,28,40} These facilitating agents may act predominantly on the outer epithelial layer, which is the outermost part of the RWM. Among them, HA is a natural product frequently used in clinical settings. A concentrated form of HA is used in cosmetic and ophthalmic surgery and as a substrate for drug delivery. HA has also been used for cochlear implant surgery as a lubricant to decrease friction and facilitate safer and deeper insertion of electrodes into the scala tympani.⁴¹ More recently, HA has been used as a substrate for delivery of therapeutic molecules such as dexamethasone into the inner ear via the RWM.^{42,43} Therefore, the safety of HA has been studied extensively and is well established.

HA is a nontoxic, biodegradable compound. It is a viscous, high molecular weight, nonsulfated glycosaminoglycan polysaccharide that is present in many tissues throughout the body. For example, human and guinea pig perilymph contains HA in small amounts.⁴⁴ According to a previous report, the application of HA to the inner ear caused no detectable morphologic or functional changes at any level of the cochlea.^{43,45,46} The mechanism by which HA enhances intracochlear transfection may be based on several factors. The primary transfection mechanism via the RWM appears to be pinocytosis, a type of endocytosis.³⁵ HA tends to form large three-dimensional structures with a high density of negative charges, which attract osmotically active cations and water, creating a hydrated gel.^{44,46} These are readily transported by pinocytosis through the RWM; they then enter the perilymph through lymphatic and blood vessels.⁴⁷ Additional mechanisms may include increased osmotic pressure above the RWM,⁴⁶ modulated membrane permeability²⁸ or prolonged contact as a result of the high viscosity of HA, thereby allowing the passage of reagents through the RWM.⁴⁰ The acceleration of permeability with HA could be achieved with several mechanisms closely entangled. Because HA exhibits excellent biocompatibility and biodegradability, HA and its derivatives have been popularly used as temporal scaffolds for tissue engineering and drug delivery devices for therapeutic agents.^{48–51} With these considerations, prolonged contacts with RWM, as a result of the high viscosity of HA, might be a reasonable mechanism that allows the passage of the SeV vector through the RWM. This mechanism was consistent with our results that HA-assisted transfection efficiency was not only enhanced but also sustained for 2 weeks (Figure 4). Furthermore, regarding a cationization following HA interaction, it might be one other such possible mechanism. Recently, accumulating evidence indicates that the cationic SeV virosome consisting of the SeV protein and cationic lipid increased viral-mediated gene delivery.^{52,53} Until now, cationization effects for SeV vector itself by HA have not been reported. However, in experiments for SeV injection mixed with HA by cochleostomy, not but pretreatment, there was no effect of HA on transfection efficiency (our unpublished data), indicating that HA does not affect SeV but affects RWM. In addition, if delivered molecules or viral vectors themselves acquired the ability for accelerated RWM permeability, such as through HA-mediated cationization, then we would expect that upon HA pretreatment, the transfection efficiency in the middle ear would have also been higher with HA pretreatment to some extent. In our results, the SeV transfection efficiency in the middle ear was mostly the same between with or without HA pretreatment (Figure 4). On these results, we suggest that HA pretreatment might not lead to changes of molecules or viral vectors themselves but is more likely to have changed only the RWM permeability. We realize that this

does not exclude completely the possibility of cationization by HA because our treatments were limited only to the RWM. With regard to RWM, according to a previous report, the application of HA to the inner ear caused no detectable morphological or functional changes at any level of the cochlea. However, there is some possibility that HA cause imperceptible changes to RWM. Therefore, in the future, additional experiments would be necessary to observe RWM in the fine structure. Finally, we note that recently, HA has been demonstrated to be a ligand molecule of CD44 receptors.^{54,55} It has already been reported that CD44 receptors are expressed in the cochlear tissue.⁵⁶ Therefore, CD44 receptors might contribute to HA-assisted cochlear gene transfer. Further investigation is needed to define the precise mechanisms or pathways by which HA facilitates viral vector spread into the cochlea using the present technique. Furthermore, our data demonstrate that HA pretreatment could promote transgene expression of the GFP-SeV/ ΔF vector, although not in a concentration-dependent manner. Thus, lower concentrations of HA ($>10 \text{ mg ml}^{-1}$) would likely be sufficient to enhance SeV-mediated transfer into the cochlea. On the other hand, we noted that GFP expression in cochleae pretreated with HA was observed primarily in the basal turn and did not spread to the apical turn. Therefore, further improvements to increase transfection efficiency extending to the apical turn are needed to fine tune, the method for clinical applications.

Virus-mediated cochlear gene therapy for SNHL is progressing toward clinical use. In fact, the therapeutic use of gene therapy to restore SNHL is under investigation in many laboratories. In addition, human clinical trials have recently been approved for the use of AV transferred into SCs to treat auditory and balance dysfunction. For future strategies aiming to repair HCs or other inner ear cells using gene therapy, there is still a great need to develop better control over exogenous gene targeting and cell type-specific expression.³² The specificity of gene delivery using AV, AAV and retrovirus vectors can be modulated by changing promoters if a gene is required to be expressed in a specific cell type. However, to date, a number of inner ear gene delivery studies have used the hCMV promoter. Recently, it was reported that the use of a tissue-specific promoter such as the *GFAP* promoter provides a means to limit the expression of a gene to only SCs.⁵⁷ However, no such systems are applicable to SeV vectors because the viral genes are transcribed by viral RNA-dependent RNA polymerase. Therefore, a different system for controlling cell-specific gene expression is required when using SeV. Control of gene expression in SeV vectors has been previously demonstrated using several strategies; one of which is through the use of a single-chain fragment variable format antibody.⁵⁸ The single-chain fragment variable antibody usually retains the affinity and specificity of its parent antibody and has many advantages in therapeutic applications as it is a small molecule and has the potential to target drug delivery agents.^{59,60} Another means is by changing the position of the foreign gene⁶¹ because a gradient has been reported in reporter gene expression level that depended on position owing to the polarity of transcription. A third method is by incorporating the gene start signals recognized by the viral RNA polymerase.^{62,63} It has been shown that these signals within the SeV genome comprise distinct sequences that do not have equivalent transcriptional reinitiating efficiencies.⁶⁴ This suggests a potential for matching therapeutic gene expression levels to individual therapy programs when SeVs are used as vectors for human gene therapy. However, despite the potential of these approaches, for strategies that aim to repair the hair cells or other inner ear cells with SeV-based gene therapy, the current technological limitations of gene therapy such as the inability to target gene delivery to specific cell types and the lack of appropriate control over the level of gene expression must be addressed before moving these technologies toward the clinic.⁶⁵

SeV vectors have major advantages compared with other viral vectors, including low pathogenicity, powerful capacity for gene expression and a wide host range.⁶⁶ In addition, the cytoplasmic gene expression mediated by this vector is advantageous for applications, wherein chromosomal integration of exogenous genes may be undesirable. Such chromosomal integration of exogenous reprogramming genes limits their translation into clinical applications. These obstacles could be overcome with the use of SeV vectors. To date, in Japan, the feasibility of the clinical usage of SeV vectors has been examined in gene therapy for ischemia of the lower limbs.⁶⁷ No serious adverse events caused by gene transfer were detected in the patients over a long follow-up period. In addition, no infectious viral particles or representative elevation of proinflammatory cytokines were detected in any patient during the study.^{67,68} Thus, SeV vectors may prove extremely useful for gene therapy-mediated treatment of SNHL as well.

In the present study, we demonstrated that SeV vectors in combination with HA pretreatment do not cause hearing loss and that the intact RWM approach appears to be safe. Our findings highlight that HA-assisted, SeV-mediated gene therapy offers an easy and safe option for treatment of SNHL that provides very efficient transient gene expression targeted to various types of cochlear cells.

MATERIALS AND METHODS

Preparation of SeV vectors

SeV vectors were constructed and propagated as previously described.^{69–71} In this study, we used *F* gene-deleted SeV (SeV/ ΔF), which is nontransmissible because the *F* protein is critical for the fusion process.²⁰ A recombinant SeV/ ΔF was engineered to express the reporter gene *gfp* (GFP-SeV/ ΔF).⁷² Virus titers were determined and expressed as cell infectious units (CIU). Viral suspensions were preserved at -80°C and thawed on ice before use.

Animals and surgical procedures

Four-week-old female Hartley guinea pigs (Japan SLC, Inc., Shizuoka, Japan) with normal Preyer's reflex responses were used. All experimental procedures reported in this study were approved by the Institutional Animal Care and Use Committee of the National Defense Medical College, which adheres to EEC guidelines. All efforts were made to minimize the number of animals used.

Under general anesthesia with intraperitoneal injection of ketamine (50 mg kg^{-1}), medetomidine (1.0 mg kg^{-1}) and local analgesia with lidocaine, a retroauricular incision was made to expose the temporal bulla. A hole with a 2–3-mm diameter was drilled into the bony bulla to expose the RW niche. For the HA treatment arm, HA (hyaluronic acid sodium salt from rooster comb; Sigma H5388; Sigma, St Louis, MO, USA) was prepared in diluted sterile saline (10 and 30 mg ml^{-1}) just before the surgery. A piece of gelfoam (8 mm^3) immersed in HA was placed on the RW niche. Ten minutes later, the gelfoam and residual HA solution inside the RW niche were carefully removed by fine paper wicking, without perforating the membrane. Next, a piece of gelfoam soaked in $5\text{-}\mu\text{l}$ GFP-SeV/ ΔF (1×10^8 cell infectious units per ml) was placed on the RWM. The same method was used to apply GFP-SeV/ ΔF without HA pretreatment, HA pretreatment alone or saline alone as a sham surgery control. For the approach utilizing surgical injection through the RWM approach, the same method was used to expose the RW niche. Then, $5\text{-}\mu\text{l}$ GFP-SeV/ ΔF was injected gently through the RWM over 5 min using a 30-gauge needle with a 10- μl Hamilton syringe. In the cochleostomy group, the ventral side of the right bulla was opened and a hole was drilled in the lateral wall of the third turn. Through this, $5\text{-}\mu\text{l}$ GFP-SeV/ ΔF was injected into the scala tympani over 5 min. Finally, the hole in the bony bulla was sealed with muscle tissue and the skin was closed.

For this study, a total of 61 animals were divided into six groups: Group I, GFP-SeV/ ΔF alone ($n=20$); Group II, GFP-SeV/ ΔF pretreated with HA (10 mg ml^{-1}) ($n=6$); Group III, GFP-SeV/ ΔF pretreated with HA (30 mg ml^{-1}) ($n=20$); Group IV, saline alone in the right ear and HA (30 mg ml^{-1}) alone in the left ear ($n=5$); Group V, GFP-SeV/ ΔF injected into the perilymph via cochleostomy ($n=5$); and Group VI, GFP-SeV/ ΔF injected through the RWM ($n=5$).

Evaluation of hearing function (ABR)

ABR was measured to evaluate hearing function in each animal before and 3 days after surgery. Following anesthesia, stainless steel needle electrodes were placed subcutaneously at the vertex and on the ventrolateral aspect of the left and right ears for recording. Tone burst stimuli of 1 ms rise/fall time at frequencies of 4, 8, 16 and 32 kHz were generated. The amplitudes were specified by the sound generator and attenuated by a real-time processor and a programmable attenuator (RP2.1 and PA5; Tucker-Davis Technologies, Alachua, FL, USA). Stimuli were produced by a coupler-type speaker (ES1spc; Bio Research Center, Nagoya, Japan). ABR waveforms were recorded for 12.8 ms at a sampling rate of 4000 Hz using 50–5000 Hz band pass filter settings. Waveforms from 256 stimuli at a frequency of 9 Hz were averaged. ABR waveforms were recorded in 10 dB sound pressure level intervals down from the maximum amplitude until no waveform could be visualized. All values for the ABR thresholds in the text and figures are presented as means $\pm$ s.e.m.

Immunohistochemistry

Deeply anesthetized animals were decapitated, and both temporal bones were removed and immediately placed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C . Small openings were made in the round window, oval window and the apex of the cochlea in the same fixative. After immersion in the fixative overnight at 4°C , the temporal bones were decalcified in 0.1 M ethylenediaminetetraacetic acid (pH 7.4) containing 5% sucrose by stirring at 4°C for 3 days. For cochlear whole mounts ($n=4$ per group), the cochlea was microdissected into six pieces. Immunostaining was initiated by blocking the tissues for 1 h with 0.1% Triton X-100 in PBS supplemented with 5% goat serum. Fixed and permeabilized pieces were incubated overnight in an antibody diluent (DAKO, Carpinteria, CA, USA) with antibodies to GFP (Invitrogen, Carlsbad, CA, USA) or myosin VIIa (Proteus Biosciences, Ramona, CA, USA). Samples were washed three times for 20 min each with PBS. Primary antibodies were detected with secondary antibodies conjugated with Alexa 488 or 546 (Invitrogen). Some specimens were counterstained with F-actin (Invitrogen) and 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and viewed by means of confocal fluorescence microscopy (Nikon C2 system, Tokyo, Japan). For cross-sectioning ($n=2$ per group), fixed and decalcified temporal bones were immersed in 30% sucrose in PBS at 4°C for 12 h, incubated in optimal cutting temperature medium at room temperature for 1 h, and frozen in liquid nitrogen. The staining protocol was performed as described above. Specimens were viewed by epifluorescence microscopy (Axio Imager A1; Carl Zeiss Microimaging GmbH, Jena, Germany).

Transfection efficiency and hair cell counts

GFP-positive HCs identified with myosin VIIa antibodies were counted on each cochlear whole mount. ImageJ software (NIH, Bethesda, MD, USA) was used to measure the total length of the cochlear whole mounts, and a cochlear frequency map was computed to precisely localize IHCs based on frequency-specific regions. High-power images of frequency-specific regions according to the computed frequency map were assembled and analyzed. For the transfection efficiency of HCs, the number of GFP-positive HCs within a $100\text{-}\mu\text{m}$ area was counted in each of the four frequency-specific regions of the cochlea, and then a transfection cochleogram was established for the HCs. To determine the maximum reach of transgene expression, the distance from the farthest GFP-positive cochlear cell to the RWM was measured and expressed as a percentage of total cochlear length. For HC counts, the total numbers of IHCs and OHCs within a $100\text{-}\mu\text{m}$ area were counted in each of the four frequency-specific regions of the cochlea, and the HC survival rate was calculated.

Quantitative RT-PCR

Immediately after decapitation, the cochleae were dissected before surgery and at 3 h and 1, 3, 7, and 14 days after surgery and stored in RNeasy Lysis Buffer (Qiagen, Crawley, UK; $n=6$ per group). In the same way, samples of the brain tissue (8 mm^3), dissected from the cortex of the temporal lobe around the internal auditory meatus and the middle ear mucosa, including the tympanic membrane, were collected individually at 3 h and 3 days after surgery and then stored in RNeasy Lysis Buffer ($n=6$ per group). The total RNAs were extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Quantitative RT-PCR was performed on a Thermal Cycler Dice Real Time System using the One

Step SYBR PrimeScript PLUS RT-PCR Kit (RR096A; TaKaRa Bio, Shiga, Japan). Forward (F) and reverse (R) primer sequences were GFP-F 5'-CGC ACC ATC TTC-3' and GFP-R 5'-GGT CTT TGC TCA GGG CGG ACT-3'. PCR cycling conditions included 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Expression was determined using the $\Delta\Delta C_t$ method with glyceraldehyde 3-phosphate dehydrogenase as the internal control. All reactions were performed in duplicates. GFP mRNA expression was reported as *n*-fold glyceraldehyde 3-phosphate dehydrogenase mRNA. For the assessment of relative mRNA expression, levels were standardized to cochlear samples taken 3 h after GFP-SeV/ΔF application in the absence of HA.

Data analysis

Bonferroni's multiple comparison tests were performed to identify significant differences in ABR thresholds and transfection efficiencies. The two-tailed Mann-Whitney *U*-test was used to compare differences in mRNA expression. Data are presented in the figures as means $\pm$ s.e.m.; *P*-values < 0.05 were considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

TK and KM designed the experiments. TK, KM, KN, TF, MI, MH and AS performed research and analyzed the data. TK and KM co-wrote the manuscript. All authors discussed the results and commented on the manuscript.

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