In Vivo Delivery of Atoh1 Gene to Rat Cochlea Using a Dendrimer-Based Nanocarrier

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Gene therapy is a promising clinical solution to hearing loss. However suitable gene carriers for the auditory system are currently unavailable. Given the unique structure of the inner ear, the route of delivery and gene transfer efficiency are still not optimal at present. This study presented a non-viral delivery system of in vivo delivery of Atoh1 gene (a potentially therapeutic gene for hearing loss) to rat cochlea. We treated polyamidoamine (PAMAM) dendrimers by activating and modifying with Na-carboxymethyl-β-cyclodextrins (CM-β-CD) in sequence. A novel gene carrier (CM-β-CD modified activated PAMAM dendrimers, CMAP) was then constructed. CMAP nanoparticles could bind pRK5-Atoh1-EGFP plasmids to form vector-DNA complexes (dendriplexes) with a mean particle size of 132 ± 20 nm and zeta potential of 31 ± 3 mV. These dendriplexes were locally applied on the round window membrane and delivered to the inner ear by passive gradient permeation. Results showed that the Atoh1 gene was successfully transferred into the cells as indicated by the green fluorescence detected in the inner ear. A relatively selective gene transfer with high efficiency was achieved in the auditory hair cells but not much in other cell types in the cochlea. Auditory brainstem response was determined seven days after inoculation, indicating good tolerance. This approach may provide a novel tool for inner ear gene therapy and initiate the applications of biomaterials to treat auditory disorders.

KEYWORDS: Hearing Loss, Gene Delivery, Cochlea, PAMAM Dendrimers, Activation, Na-Carboxymethyl-β-Cyclodextrin Modification.

INTRODUCTION

Hearing loss is a major public health problem. For instance, about 36 million patients have varying degrees of hearing loss in the US (www.nidcd.nih.gov/) and 27.8 million patients have impaired hearing with communication difficulties in China (www.moh.gov.cn/). Deafness is mostly caused by acquired damage from noise or ototoxic drug use, leading to cellular and structural damage in the cochlea. The core component of the cochlea is Corti’s organ, the sensory epithelia, in which auditory hair cells are responsible for transducing sound vibrations to nerve impulses and the formation of hearing. Such damage usually originates in auditory hair cells, including inner hair cells (IHCs) and outer hair cells (OHCs), which cannot regenerate spontaneously in mammals. Further structural degradation of Corti’s organ after an initial trauma eventually leads to irreversible hearing loss.

Cochlear implantation is currently the major method used to treat deafness; however, hearing performance after implantation varies1,3 and this treatment is costly, thereby limiting its accessibility particularly in developing countries. Therefore, an ideal therapy should be developed to restore cochlear function with more accessibility and provide a way to regenerate damaged auditory hair cells3 or repair such cells at specific pathological stages. Gene therapy is one of the promising approaches for the treatment of hearing loss. Therapeutic genes that
encode proteins/peptides for inner ear gene therapy may be divided in two major groups: inner ear protectors and sensory cell regeneration factors. Genes that protect the inner ear include neurotrophic factors, otospiralin and apoptotic inhibitors.5–7 Studies have demonstrated the successful expression of these proteins in the cochlea as mediated by viral vectors and such protectors elicit varying degrees of protection on hair cells and neurons. Current studies on gene therapy to regenerate auditory epithelia are mainly based on atonal homolog 1 (Atoh1, Math1 in mice), a basic helix-loop-helix transcription factor. Atoh1 plays a critical role for hair cell differentiation during the development of the auditory system in mammals.8 Overexpression of Atoh1 gene in the cochlea may induce extra hair cell formation and restore hearing function.9–11 In our previous study, noise-damaged hearing function of adult guinea pigs was improved by introducing Atoh1-adenovirus in the cochlea, and this observation revealed a new function of Atoh1 as an important gene to promote repair/regeneration of stereocilia and maintain injured hair cells in adult mammal cochlea.12 Recently, a pharmaceutical industry conducted a pre-clinical study on the use of Atoh1 gene carried by adenovirus to treat deafness.13

Gene therapy for hearing loss requires manipulating a suitable target gene and an equally complex task of defining atraumatic and targeting delivery to the inner ear. The most commonly used delivery methods are based on viral vectors, such as adenovirus and adeno-associated virus.5,14 Viral vectors have high efficiency, but they lack cell specificity and are associated with numerous problems, including inflammation, mutation, limited carrying capacity and ethical issue.15 In contrast to viral vectors, non-viral vectors have no size limitation to a gene cassette, no immunogenic effect, and no risk of a viral vector-related disease or toxicity. Non-viral gene delivery that target inner ear cells include physical methods employing a physical force such as pressure or electric pulse, and chemical approaches using liposomes or polymers. Electroporation9 and gene gun,16 which have promising results in vitro, have not been proven effective in vivo. Liposomes can be easily constructed and applied in various cultured cells. Cationic liposomes have been used in vitro and in vivo to mediate transgene expression in the inner ear; however, these liposomes present some limitations such as the absence of relative tissue specificity and low transfection efficiency (approximately 3%) compared with viral vectors.17,18 Synthetic and naturally occurring cationic polymers, with linear or branched configuration, constitute another category of DNA carriers that have been widely used for gene delivery.19 However, only a few studies using cationic polymers have been performed in the cochlea. Hyperbranched polylsine NPs have been tested in vitro and in vivo, and the efficiency of transfection in primary cochlear cell culture is 8.7%.20 Polyethylenimine (PEI), one of the most actively studied polymer gene carriers, has been used to evaluate the possibility of conducting gene transfer to the cochlea in vivo. PEI/DNA complexes (polplexes) cannot spread to Corti’s organ and implement transfection after these substances are introduced with sustained delivery (osmotic pump infusion method).21 All of these polymer-based vehicles for DNA delivery lack selectivity to cochlear cells, present low efficiency, and exhibit significant cytotoxicity.

To date, ideal gene vectors for the auditory system are unavailable. With increasing knowledge on biological mechanisms of auditory hair cell regeneration/repair, gene delivery to the cochlea has become one of the major focuses of studies that manipulate the function of the inner ear.

Polyamidoamine (PAMAM) dendrimers are representative cationic polymers that have highly branched spherical shape and controllable structure. These dendrimers can mediate the delivery of single- and double-stranded, natural and synthetic DNA or RNA of any kind and size.22 Dendrimers form complexes with DNA via sequence-independent electrostatic interactions, thereby producing particles called dendriplexes. Dendrimers are considered “proton-sponges” that facilitate intracellular gene transfer via a proposed escape mechanism from lysosomes.23 Some groups have used dendrimers to transfer genetic materials to several types of cultured cells.24

Numerous derivatives of dendrimers have also been developed in recent years. Treatments involving activation by heating have been studied to enhance the transfection of PAMAM dendrimers.25 Activation is a random process that generates mixed populations of dendrimer molecules that differ slightly in molecular mass and structure. Carboxyl groups are formed at amido bond cleavage sites and the molecular mass of the dendrimers are reduced by 20–25%. This process can enhance transfection by two to three orders of magnitudes compared with that of unprocessed (intact) dendrimers in cultured eukaryotic cells.26 In addition, various dendrimer-derived modified polymers have been synthesized and evaluated in vitro.27–29 Cyclodextrins (CDs) and its derivatives have been used as pharmaceutical excipients for more than two decades.30 These biocompatible glucose oligomers have been studied extensively and utilized to enhance drug stability, solubility and bioavailability.31 Previous studies used CDs to modify PAMAM dendrimers and revealed a 200-fold enhancement in gene delivery to cultured CAT cells by improving surface distribution.32 In general, dendrimer-based gene transfer often provides higher efficiency than other non-viral methods, and can be used for various cell types.33 To the best of our knowledge, the use of cationic dendrimers in cochlear gene transfer has not been reported yet.

In this study, a novel CMAP (CM-β-CD modified activated PAMAM dendrimers) nanocarrier was constructed based on PAMAM dendrimers via activation and modification in sequence. Such nanocarrier was used to deliver Atoh1-EGFP genes to rat cochlea by permeation through
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an intact round window membrane (RWM). This study primarily aimed to evaluate the efficiency and safety of this nanocarrier in the auditory system in vivo. This study also aimed to determine whether or not these strategies could improve gene transfer to the cochlea particularly to auditory hair cells.

MATERIALS AND METHODS

Materials and Animals

Na-carboxymethyl-β-cyclodextrin (CM-β-CD, Mw = 1375 Da), EcoRI restriction endonuclease and Branched polyethylenimine (PEI, Mw = 25 kDa) were purchased from Sigma-Aldrich (St, Louis, MO, USA). PAMAM dendrimers (Generation 7.0, tetrafunctional ethylenediamine (EDA) cores) were purchased from Dendritech, Inc. (Midland, MI, USA). Ceftriaxone was purchased from Roche Ltd. (Basel, Switzerland). Terra-Cortril-P and Gelfoam® were purchased from Pfizer Ltd. (NY, USA). Ceftriaxone was purchased from Sigma-Aldrich (St, Louis, MO, USA). PAMAM amine (EDA) cores) were purchased from Dendritech, Inc. (Midland, MI, USA). Antibodies were purchased from Abcam, Inc. (Cambridge, UK).

Adult male Sprague Dawley rats (100–150 g) supplied by the medical experimental animal center of Chinese PLA General Hospital (Beijing, China) were used in this study. All animal procedures were approved and controlled by the local ethics committee and carried out according to the institutional animal care and use guidelines.

Methods

Construction of CMAP Dendriplexes

Preparation of pRK5-Atoh1-EGFP Plasmid. pRK5-Atoh1-EGFP plasmid was kind gift of Professor W. Q. Gao from Genentech, Inc. (South San Francisco, CA, USA), and amplified in Escherichia coli DH5α (Invitrogen, Grand Island, NY, USA). The plasmid contains CMV and SV40 promoters, Atoh1 and enhanced green fluorescent protein (EGFP) genes. For plasmid isolation, the Endotoxin removal (EDTA, 1 mM) buffer solution. After 30 min of incubation at RT, the samples were electrophoresed at 60 V for 2 h, and the migration of ethidium bromide (EB, 0.5 µg/ml) was prepared in a TAE (10 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA) buffer solution. After 30 min of incubation at RT, the samples were electrophoresed at 60 V for 2 h, and the migration of DNA bands were visualized using UV gel imaging system (Kodak, Rochester, New York, USA).

Characterization of CMAP Dendriplexes

The average size, size distribution and zeta potential of the dendriplexes were determined by dynamic light scattering (DLS) using ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd., Malvern, UK). Determinations were performed at 633 nm with a constant angle of 90° at 25 °C after samples were appropriately diluted in distilled water. The morphology of the dendriplexes was observed by transmission electron microscopy (TEM, FEI, Hillsboro, OR, USA) following negative staining with uranylacetate.

Gel Retardation Assay

The ability of CMAP NPs to condense plasmid DNA was examined by agarose gel electrophoresis. Dendriplexes were incubated in the present of sodium dodecyl sulfate (SDS, Beijing Chemical Reagent Co., Ltd.) at RT for 15 min. Following that, dendriplexes were incubated with EcoR I restriction endonuclease at 37 °C for 2 h. An agarose gel (1.0%, v/v) containing ethidium bromide (EB, 0.5 µg/ml) was prepared in a TAE (10 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA) buffer solution. After 30 min of incubation at RT, the samples were electrophoresed at 60 V for 2 h, and the migration of DNA bands were visualized using UV gel imaging system (Kodak, Rochester, New York, USA).

In Vivo Study of Gene Transfer Into Cochlea

Surgical Introduction to Cochlea

Adult male Sprague Dawley rats were used for the experiments of in vivo gene transfer. Surgery was carried out under the operating microscope (Carl Zeiss, Berlin, Germany). We chose the right ear for the inoculation of CMAP dendriplexes in seven animals. And each group treated by intact PAMAM dendrimers, activated PAMAM dendrimers, modified PAMAM dendrimers and PEI was performed in five animals. Draping materials and surgical instruments went through high-pressure/high-temperature sterilization process. The operating table and operating microscope were sterilized using ultraviolet light irradiation for 30 min. Animals were anesthetized with intraperitoneal injection of 10% chloral hydrate (4.5 ml/kg). Animals were in supine position, and the cervical body hair was shaved. The skin in the surgical area was disinfected with 75% ethanol. And then subcutaneous...
injection of 1% lidocaine was performed to reduce pain. A ventral neck incision was made and the muscles were dissociated until the ipsilateral otic bulla was exposed (Fig. 2, panel (A)–(B)). A window with a diameter of approximately 2 mm was opened and the round window niche could be found under the stapedial artery, in which RWM located. Gelfoam® (absorbable gelatin sponge, approximately 8 mm^3) soaked up different dendriplexes or PEI/DNA polyplexes (N/P: 6/1) was placed at the round window niche (Fig. 2, panel (C–D)). Then, a small piece of muscle was taken and used to cover the surface of the round window niche, which was fixed with a small amount of tissue glue. The incision was closed with sutures. Total operating time was approximately 30 min. During surgery, the animals’ eyes were protected by Terra-Cortril-P. Immediately after surgery, intramuscular injection of Ceftriaxone (0.5 g/kg) was performed and the injections were repeated daily for three days after surgery in order to prevent possible middle ear infection. Artificial perilymph (137 mM NaCl, 5 mM KCl, 21 mM MgCl, 1 mM NaHPO4, 11 mM NaHCO3 and 11 mM glucose) was inoculated in five animals using the same method and served as control.

**Histological Treatment and Microscopic Observation**

Animals were anesthetized and sacrificed seven days after surgery. Otic bullae were quickly removed and fixed with 4% paraformaldehyde (0.01 M in PBS) at RT for 2 h. To prepare the surface mount sample, the basilar membrane was peeled off (including the Corti’s organ and spiral ligament) under the stereomicroscope (Olympus, Shinjuku, Tokyo, Japan), and the tectorial membrane was removed. To prepare the cryosection sample, the cochlea was soaked in 10% EDTA (Beijing Chemical Reagent Co., Ltd.) at 4 °C for 1 week for decalcification. They were dehydrated with 15% and subsequently 30% sucrose solutions at RT for 2 h each and embedded overnight in the O.C.T compound at 4 °C. Whole cochlea 4 μm cryosections were made using CM1900 freezing microtome (Leica).

The samples were stained with 4′,6-diamidino-2-phenylindole (DAPI, 10 mg/ml, Abcam) in a dark room at RT for 10 min for the identification of nucleus. After washing with PBS, the specimens were soaked in 1 mM CuSO4 for 90 min in order to quench auto-fluorescence, and subsequently washed with PBS. Samples were mounted and observed by using a confocal laser scanning microscope (LSM 510, Carl Zeiss). The numbers of EGFP positive cells of 100 inner/outer hair cells in the apex, middle and basal turns of cochlea were hand counted respectively. The average transduction efficiencies in IHCs and OHCs were calculated severally.
**RT-PCR Analysis**

Total RNA was isolated from the experimental cochlea using TRizol® reagent (Invitrogen) according to the manufacturer’s instructions. A reverse transcription reaction was performed with 1 μg RNA using SuperScript™ RT-PCR System (Invitrogen). The PCR amplification reaction was performed after initial denaturation at 94 °C for 5 min, and for 40 cycles consisted of 94 °C 40 s, 60 °C 40 s, 72 °C 40 s, then final extension at 72 °C for 5 min. RT-PCR products were separated in 1% agarose gel in TAE buffer, and detected by UV gel imaging system. Contralateral ears were used as control. Primer sequences for Atoh1 were shown as below: F: 5'-GGTAAAAGAGTTGGGGGACC-3'; R: 5'-TGGACAGCTTCTTGTGGTGTTG-3'.

**Western Blot Analysis**

Extracts from experimental cochlea were separated by electrophoresis on 12% SDS-polyacrylamide gels. The proteins were transferred into a PVDF membrane at 100 V for 30 min. The membrane was pre-hybridized by gently shaking with 5% Bovine serum albumin (BSA, Invitrogen) in PBS for 1 h at RT and then incubated with anti-Atoh1 (1:1000) and anti-histone H3 (1:3000) antibodies (Abcam) overnight at 4 °C. After washing the membrane was incubated with the secondary antibody (1:2000 Abcam) at RT for 2 h. For detection, the washed membrane was covered with 2 ml BCIP/NBT liquid substrate (Sigma, Schnell-dorf). Bands were detected by exposure. Contralateral ears were used as control.

**Pilot Assay of Systemic Distribution**

To evaluate the systemic effect, the animals inoculated of CMAP dendriplexes were anesthetized and sacrificed seven days after surgery. The tissues of liver, spleen and brain were eviscerated and fixed with 4% paraformaldehyde at RT for 2 h, then dehydrated with 15% and 30% sucrose solutions at RT for 2 h in sequence. The specimens were embedded overnight in the O.C.T compound at 4 °C. 4 μm tissue sections were made and stained with DAPI. After washing with PBS, the sections were soaked in 1 mM CuSO4 solution for 90 min and subsequently washed with PBS. The sections were mounted and observed by using a confocal laser scanning microscope.

**Auditory Brainstem Response (ABR) Measurement**

To evaluate the adverse side effect to auditory system, ABR threshold tests were performed before and seven days after surgery using the TDT system (Tucker-Davis Technologies, Alachua, FL, USA). ABR thresholds were obtained from the ears treated by artificial perilymph (control group) in five animals, PEI polyplexes in five animals and CMAP dendriplexes in seven animals respectively. Animals were anesthetized as described above. The parietal scalp-electrode was the recording electrode; the electrode at the external ear was the reference electrode and the electrode at the tip of the nose was the ground electrode. The stimulus sound was “click,” the band-pass filter was 80–3000 Hz, and the number of superimposition was 1024. Scan time was 10 ms. Reaction potential was magnified 10,000 times, and the average number of superimposition was 1024. Starting from 90 dB SPL (Sound Pressure Level), the sound intensity was decreased in 10 dB steps until non-typical waveform was recorded. The corresponding value of the sound intensity was the ABR hearing threshold.

**Statistics Analysis**

Results are presented as mean values ± standard deviation (SD). Statistical analyses were performed using SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). For the studies of gene transfer efficiency in auditory hair cells, the EGFP positive percentages between IHCs and OHCs, and between intact, activated alone, modified alone and CMAP dendriplexes were analyzed using Student’s t-tests. Differences were considered to be statistically significant at p < 0.05.

**RESULTS AND DISCUSSION**

**Characterization of CMAP Dendriplexes**

The CMAP dendriplexes were characterized in detail. As presented in Figure 3, panel (A), the particle size distribution histogram indicates that the CMAP dendriplexes were monodispersed (PDI = 0.146) with a mean particle size of 132 ± 20 nm. As expected, these dendriplexes were cationic with a zeta potential of 31 ± 3 mV. Moreover, we studied the morphology of the CMAP/DNA dendriplexes by TEM. Figure 3, panel (B), shows the spherical NPs observed in the TEM image.

To evaluate the ability of CMAP to bind DNA, we performed a gel retardation assay and the results are shown in Figure 3, panel (C). CMAP dendriplexes were lagging when they were electrophoresed on an agarose gel, and these dendriplexes dissociated in the presence of anionic SDS detergents. The results indicated that CMAP NPs and plasmid DNA formed complexes reversibly via electrostatic interactions after they were mixed, and complexation was completed. After the mixture was incubated with EcoR I restriction endonucleases, the naked plasmid DNA was completely digested, but the DNA that formed a complex with CMAP remained intact, indicating that CMAP NPs could protect DNA against enzymatic degradation.

**In Vivo Transfection Study**

**Improved Gene Transfer Into Cochlea**

Figure 4, panel (A)–(F), shows the gene expression in cochlea seven days after different transgenic agents were introduced. In Figure 4, panel (G), the transfection efficiencies of CMAP detected in IHCs and OHCs were 47.57 ± 6.68% and 82.14 ± 9.67%, respectively, compared...
Figure 3. Characterization of CMAP dendriplexes. (A) Size distribution histogram of CMAP dendriplexes. (B) TEM image of dendriplexes. Scale bar = 1 μm. (C) Agarose gel electrophoresis analysis: DNA ladder (M), plasmid DNA (0.5 μg, lane 1); DNA incubated with EcoR I restriction endonuclease (lane 2); CMAP dendriplexes (lane 3); CMAP dendriplexes incubated with SDS (lane 4); CMAP dendriplexes incubated with EcoR I restriction endonuclease (lane 5); CMAP dendriplexes incubated with EcoR I restriction endonuclease after disruption by SDS (lane 6).

Figure 4. Gene expression in the cochlea seven days after introduction. Group of CMAP dendriplexes treatment (A)–(B): (A) Representative confocal fluorescence microscopy images of the basilar membrane in the surface mount samples. Scale bar = 50 μm for overall; 10 μm for IHC and OHC. (B) A typical image of gene expression in the whole cochlea in cryosection samples. Scale bar = 50 μm. (C) Schematic diagram of gene expression in the cochlea: (1) IHCs; (2) OHCs; (3) stria vascularis; (4) spiral ganglion cells; (5) mesothelial cells beneath Corti’s organ; (SV) scala vestibule; (SM) scala media; (ST) scala tympani. (D) Groups of intact/activated/modified PAMAM dendriplexes treatments. Scale bar = 50 μm. (E) Group of PEI polyplex treatment. Scale bar = 50 μm. (F) Control group (inoculation of artificial perilymph using the same method). Scale bar = 50 μm. (G) Comparison of transgenic efficiency among PEI (n = 5), intact PAMAM dendrimers (n = 5), activated only (n = 5), modified only (n = 5), and CMAP (n = 7, **p < 0.01). (H) Difference in transgenic efficiencies between IHCs and OHCs treated with CMAP dendriplexes (n = 7, **p < 0.01). (I) RT-PCR tests: CMAP dendriplex treatment (lane1); negative control (lane2). (J) Western blot analysis: CMAP dendriplex treatment (lane1); negative control (lane2).
with 0.29 ± 0.48% and 0.43 ± 0.79% of PEI, 1.14 ± 0.69% and 7.00 ± 2.38% of intact dendrimers, 1.71 ± 0.95% and 5.86 ± 2.41% of activated dendrimers, and 1.00 ± 1.16% and 7.43 ± 1.99% of modified dendrimers. In IHCs and OHCs, CMAP dendriplexes reached remarkably higher efficiencies than PEI polyplexes and unprocessed PAMAM dendriplexes, whereas either activation or modification was unable to enhance gene transfer separately. In addition, IHCs and OHCs significantly differed in terms of the efficiency of gene expression (Fig. 4, panel (H)). In the whole cochlea, faint EGFP fluorescence signals were also detected in the stria vascularis, spiral ganglion, and mesothelial cells beneath Corti’s organ (Fig. 4, panel (B)–(C)). No evident expression in the supporting cells was observed. As shown in Figure 4, panel (I)–(J), the gene transfer of Atoh1 was also validated by RT-PCR and western blot analyses. On the other hand, no apparent nuclear fragmentation was detected. Considering the potential biological effects of Atoh1 gene to auditory hair cells in two to three weeks after introduction, we did not perform a long-term observation.

PAMAM dendrimers used for gene transfer have been studied for more than two decades, and their activities have been investigated in many types of cell lines. In this study, intact PAMAM dendrimers presented a poor gene transfer performance in the auditory epithelia, whereas a remarkably higher efficiency of CMAP was achieved. This result indicated that transgenic performance of dendrimers in auditory system could be improved by combining activation and CM-β-CD modification. Activation involves the random removal of branches from a newly synthesized dendrimer molecule and produces a more flexible molecule that provides more efficient transfection and reduced cytotoxicity as well as a useful substance for a broader range of cell types. Commercialized transgenic reagents (Superfect® and Polyfect®) based on activated PAMAM dendrimers have been widely used in biomedical studies. Moreover, numerous surface groups of dendrimers can be derivatized with CDs simultaneously to modify certain properties such as steric stabilization. Amphoteric CDs do not form a complex with DNA by themselves, but these CDs can modulate the size and distribution of dendriplexes. In this mechanism, activation and modification possibly contribute to the enhancement of transgenic activities of CMAP NPs. Although our results showed that activation or modification could not separately improve gene transfer to the cochlea, the high transfection efficiency of CMAP dendriplexes suggested a synergistic, rather than additive, effect between activation and modification on the transfection efficiency of dendrimers in the inner ear. However, exact mechanisms involved in the relatively selective gene transfer observed in the hair cells remain unclear.

**Milder Impact on Hearing Function**

Auditory brainstem response (ABR) is a neurological test of the auditory brainstem that responses to acoustic stimuli. ABR was performed in this study to assess the side effects of CMAP dendriplexes introduced by topical administration and monitor hearing function.

The changes in the ABR threshold stimulated by surgically introducing different transgenic agents are illustrated in Figure 5. The average ABR threshold before introduction was 37.86 ± 4.88 dB SPL. On the seventh day after surgery, post-operative ABR threshold of animals in the CMAP dendriplex treatment group was 47.86 ± 3.93 dB SPL compared with 54.29 ± 4.50 dB SPL of PEI polyplex treatment group, and 42.86 ± 4.88 dB SPL of control animals. PEI group shows a significant increase in ABR threshold compared with the control group. The post-operative ABR threshold of CMAP group showed an evidently lower increase than that of PEI group, and this threshold did not differ from that of the control animals. Nonetheless, such threshold was slightly greater than the pre-operative threshold. At the same time, no significant increase in the post-operative ABR threshold was detected in the control animals.

PAMAM dendrimers are usually toxic to cells particularly when systemically injected in vivo. In general, the toxicity caused by dendrimers is attributed to destabilization of the cell membrane, thereby resulting in hemolysis. In contrast to cationic dendrimers with varying degrees of toxicity detected in multiple organs after systemic administration, CMAP dendriplexes were tolerated after these substances were topically administered in the cochlea. However, exact mechanisms remain unknown, but improvements on the cytotoxicity of dendrimers because of activation or CD modification have
already been proven. These processes that result in lower surface charge and shield several amine groups may reduce the potential cytotoxicity of CMAP NPs. In addition, a small number of auditory epithelia in Corti’s organ mean that fewer vectors are needed for transfection, thereby reducing the risk of triggering undesired toxic responses.

**Restricted Systemic Distribution**

A pilot study on systemic distribution was conducted. Brain, liver, and spleen tissues were harvested to characterize the expression of EGFP. No evident EGFP signals were detected in these main organs (Fig. 6).

The cochlear housing of the sensory epithelia prevents, or at least limits, unwanted systemic diffusion. The egress of transgenic agents from the cochlea that caused transduction in the brain, liver, or spleen was not detected because the vectors were not able to enter the systemic circulation and unable to transfet solid organs presumably because of the integrity of the blood-brain and blood-labyrinthine barriers.

**Potential Influence of Cochlear Construction and Microenvironment on Gene Transfer**

Synthetic gene delivery systems should be rationally designed to link the chemical structure of cationic complexation agents to the morphology and physicochemistry of respective nucleic acid complexes. Such properties are then linked to biological properties on a cellular and systemic level. We speculated that the advantages of CMAP nanocarriers, including high efficiency and remarkable safety, were more likely associated with unique physiological construction and microenvironment in the cochlea.

The physiological circulation of lymphatic fluids allows NPs to diffuse in areas that are distantly located from the site of inoculation. However, basilar membrane cells are arranged densely with a narrow intercellular space, thereby limiting the diffusion of NPs from the scala tympani (ST) to the scala media (SM) and the auditory epithelia in Corti’s organ. PEI polyplexes that have larger particle sizes (approximately 300 nm to 600 nm) cannot migrate into ST. For this reason, we speculated that the size of NPs may be related to their ability to diffuse in Corti’s organ; however, factors influencing the circulation and distribution of NPs in the cochlea have not been elucidated yet because few reports have only provided partial insights regarding this topic. The precise distribution and uptake of NPs in Corti’s organ should be investigated in future studies because such properties possibly accounts for the evident differences in gene transfer efficiency between IHCs and OHCs in our results.

**Atraumatic Delivery Route**

None of the experimental animals lost weight or died after surgery. At the time of sacrifice, all of the operated ears contained middle ear serous effusion without symptoms of local infection in the middle ear or inner ear. The gelatin sponge remained firmly in the bony groove against RWM in the test animals.

In addition to the development of vehicles for gene transfer to auditory system, the chosen route of delivery to the inner ear should be given careful consideration. For human applications, a delivery method that preserves hearing function and cochlear architecture should be developed. Several methods have already been tested. To ensure a safe delivery, transgenic agents are commonly applied topically on the cochlea via micro-osmotic pump infusion, cochleostomy, RWM inoculation and the application of Gelfoam soaked in vector-transgene complexes directly on RWM. With the exception of the last method, all of these methods involve breaching the cochlea and thus posing risks of hearing impairment from traumatic disruption of the cochlea. The Gelfoam approach provides a size-dependent passage of transgenic agents through an intact RWM, thereby administering a hypothesized “real” dosage of vectors at lower concentrations in the cochlea and a more sustained exposure time; these factors minimize potential toxicity as gene transfer in the cochlea becomes more possible. This CMAP-based approach was used effectively to transfer foreign genes to the cochlea, particularly in the auditory epithelia, thereby allowing preferential gene therapy in hair cells. This method may be used to rescue hair cells in animals with a targeted deletion of Atoh1, such as in Atoh1-self-terminating mouse model.

For this reason, we speculated that the
CONCLUSIONS
In this study, a novel CMAP nanocarrier based on PAMAM dendrimers was constructed and used to deliver Atoh1-EGFP genes to rat cochlea by topical administration in vivo. The results indicated that this non-viral delivery system was highly efficient and exhibited low toxicity to auditory epithelia. The strategies of constructing a CMAP nanocarrier, together with an atrumatic delivery route of permeation through intact RWM, provided a potential solution to transfect auditory system. With further studies, the present approach may be more useful to develop gene therapy for hearing loss.

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