Original Article

Adenoviral–mediated Hath1–EGFP gene transfer into guinea pig cochlea through intact round window membrane

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Abstract Objective To study expression of adenoviral–mediated Hath1–EGFP gene in the guinea pig cochlea after transfer through intact round window membrane (RWM), and to assess its effects on hearing.

Methods Twenty adult guinea pigs were used, of which: 12 were surgically inoculated with Ad–Hath1–EGFP in the bony groove of round window niche, and 8 with artificial perilymph. Auditory brainstem response (ABR) thresholds were determined in all animals before and 5 days after surgery. On post-surgery day 5 and day 14, animals were sacrificed and whole mounts of cochlea and frozen sections were examined.

Results ABR tests showed no significant change of hearing after the surgery. Strong fluorescence staining in the cochleae was seen in Ad–Hath1–EGFP groups. The highest levels of gene expression were seen in the post-surgery day 5 group with little decrease on post-surgery day 14. The contralateral cochlea and those in the control groups were free of fluorescence staining.

Conclusion The transgenic Hath1–EGFP can be effectively delivered into the inner ear through intact RWM, in an atraumatic manner.

Key words Gene transfer; round window membrane; Adenovirus; guinea pig; Hath1

Introduction

As neurosensory cells in the mammalian auditory system—cochlear hair cells can transduce sound stimulation into electrical signals which are conveyed to CNS through acoustic nerve to maintain normal hearing. However, many factors including excessive sound stimulation, ageing, ototoxic drugs and autoimmune diseases can induce irreversible damages of cochlear hair cells, resulting in permanent sensorineural hearing loss. Previous studies have demonstrated that although spontaneous replacement responses can be found in birds and lower vertebrates after hair cells idamage, regeneration of hair cells and recovery of hearing function are yet to be found in the mature mammalian cochlea. In recent years research reports concerning application of exogenous genes with induction and differentiation-promoting functions in the regeneration of adult mammalian inner ear hair cells have challenged the conventional idea that adult mammalian inner ear hair cells cannot regenerate. The delicate cochleas structure, poses a difficult challenge effective yet safe transfer of target genes into the cochlea without cochlear
structure damage. The aim of this in vivo study is to investigate the possibility of gene delivery through the intact round window membrane using an adenoviral vector system without cochlea structure damage in a guinea pig model. The results from this study show significant levels of Hath1-EGFP gene expression in the cochlea, and may serve as a foundation for further study on inner ear gene therapy.

1 Materials and methods

1.1 Material

Recombination adenovirus with Hath1 and enhanced green fluorescent protein vector (Ad–Hath1–EGFP\textsuperscript{3,4}) were provided by Prof. Gao (Genentech USA).

1.2 Animals

Randomly bred Hartley–strain guinea pigs weighing 250–300 g were used in this study. Twenty guinea pigs were randomly divided into a control (n = 8) and an experiment group (n=12). The left ear served as the test ear.

1.3 Auditory brainstem response (ABR)

Under general anesthesia, a needle electrode was placed at the vertex as the recording electrode, and another two needle electrodes into both ears as the reference. The ground electrode was placed subcutaneously at the nose. Stimuli were Clicks (0.1 ms), 1024 responses were superimposed bandpass–filtered (80 ~ 3000 Hz), and then averaged on an Intelligent Hearing System Smart EP2.22 system (IHS USA) which was also used for analyzing the recordings. ABR thresholds were determined via visual identification of reproducible responses with descending click intensities at 10 dB steps. ABR threshold were tested before and five days after operative procedure for each animal.

1.4 Surgical procedures

Under general anesthesia, the tympanic bulla was opened, and the round window niche exposed. A approximately 5ul Ad–Hath1–EGFP (experiment group) or artificial perilymph (control group) was injected into the round window niche. To prevent immediate backflow of the viral suspension, the cannula was left in place for 5 min following the inoculation. The tympanic bulla then was closed with a muscle flap and the incision closed with sutures.

1.5 Cochlear whole mount preparation

Animals were sacrificed 5 days or 14 days after surgery (3 animals from the experiment group, and 2 animals from the control group). Following decapitation under deep anesthesia, the temporal bones were quickly opened and the stapes removed. The round window membrane and cochlear tips were perforated using a fine needle. The cochlea was gently perfused with 4% paraformaldehyde fixative in 10mM phosphate buffered saline (PBS), pH 7.4, through the cochlea tip. The entire temporal bones was immersed in the fixative for 2 hours and rinsed in 10 mM PBS twice. The whole mount was then examined under a fluorescence microscope.

1.6 Frozen section preparation

Three animals from the experiment group and 2 from the control group were sacrificed 5 days or 14 days after surgery by decapitation under deep anesthesia. Both temporal bones were removed and the fluid spaces of the inner ear perfused with 4% paraformaldehyde in phosphate buffer saline (PBS). The specimens were decalcified for 3 days in 10% EDTA, cryo–protected in 30% sucrose for 24h and transferred into cryo–molds filled with OCT embedding compound. The molds were vacuumed at low pressure for 10 min, to remove trapped air. The specimen was kept in OCT at 4°C for 24 h to for adequate infiltration into the inner ear. It was appropriately aligned and, snap–frozen in liquid nitrogen for 0.5 min before sectioning. Sections (8 μm thick) were cut on a Leica Cryomicrotome 2800. A approximately 200 serial sections were collected from each ear onto electrostatic glass slides, which were examined under a fluorescence microscope.

1.7 Immunocytochemistry and Histological Analysis

The cochlea whole mount was stained with Probedium Iodide (PI Sigma), a DNA specific labeling agent, and then examined using a Radiance2100 con–focal fluorescence microscope (Bio–Rad company) located at the Academy of Military Medical Sciences.
1.8 Statistical analysis The SPSS statistical software package was used. All values are given as means ± SD. Comparisons were made using the student’s t-test.

2 Results

2.1 ABR threshold There was no significant difference in ABR thresholds between the inoculated and contralateral ears, or between the experiment and control groups at 5 days post surgery (P > 0.05); ABR thresholds at 5 days post surgery were not significantly different from those before the surgery for both the inoculated and the contralateral ears (P > 0.05).

2.2 Gross examination of the ear At 5 and 14 days post-surgery, the middle ear cavity was found clean with no signs of inflammation or hemorrhage in either the middle ear or inner ear. The round window membrane was also clean, free of hyperaemia. Small amount of exudation was visible in the round window niche in animals examined at 5 days post-surgery, but not in those examined at 14 days post-surgery.

2.3 Hath1 expression in cochlea Under the fluorescence microscope, bright green fluorescent light (representative of gene expression) was seen in cochleae with inoculation. Green fluorescent was visible throughout the cochlea, from the basal turn to apical turn (Fig. 2a–d). The high levels of fluorescent light were seen 5 days after the surgery, and showed little decrease in intensity in animals examined on day 14. The Hath1 expression was focused in the supporting cell area involving the inner sulcus cell (IS) and spiral ganglion cell (SGC). It was also seen in epithelial cells in the scala tympani and vestibule (Fig. 2e). Hath1 expression was not detected in the non-inoculated ear or ears in the control group (Fig. 2f). PI after staining revealed punctiform absence of outer hair cells in inoculated ears (Fig. 2g), but not in inner and outer hair cells of the control group.

3 Discussion

Cochlear gene transfer studies in animal models have utilized mainly two methods: direct injection through the round window membrane (RWM) or intracochlear infusion through a cochleostomy. However, structural damage from surgical trauma and inflammatory reaction and hearing loss associated with these methods lead us to search for a less invasive delivery method. Another concern is injury to adjacent important structures caused by surgical maneuvers, which has restricted its application⁵,⁶. An alternative technique in gene transfer is through the cerebrospinal fluid and the induction is achieved via the cochlear aqueduct to reach both inner ears. However, animal studies show that expression of target genes in the cochlea, limited by the number of receptors, is mainly distributed in basal turns bilaterally. Expression is also seen in the bottom part of the brain, raising the question of a potential risk to the brain. These have restricted clinical application of such techniques⁷.

Based on the understanding of anatomical characteristics of the cochlea and biological features of the RWM as a semipermeable membrane with considerable permeability⁸, we introduced a new and minimal invasive gene delivery technique, which involves delivery of Ad–Hath1–EGFP into the round window niche and its penetration of the RWM into the cochlea. The advantages of this technique include minimal disturbances to cochlear structural integrity and no risk of lymphatic leakage or liquid extravasation from RWM⁹,¹⁰. The surgical approach in this technique is simple and allows pres-
ervation of normal physiological functions of the cochlea. Examination of the middle ear cavity at 5 and 14 days post-surgery in this study showed no signs of inflammatory reaction or hemorrhage in either the middle ear or inner ear. There was no significant difference in ABR threshold before and after the surgery or between the experiment and control groups (P > 0.05), suggesting no influence on hearing by the surgical maneuvers. Punctiform loss of outer hair cells seen only in inoculated ears may be indicative of injury to adjacent hair cells from viral biotoxicity, which seems to have little effects on auditory function.

The carrier employed in this study, i.e. AD virus,
has a high transfection efficiency (possibly 100%), capable of infecting both dividing and nondividing cells. It is easy to culture and purify and can be prepared at high titres. Its unconformity with host cell genome makes it a relative safe carrier, with a bright future of application. Our data show that the target gene was widely expressed in the cochlea at 5 days after the surgery, involving the supporting cells and spiral ganglion cells. There was a gradual decrease in the intensity of fluorescent light from the basilar turn to the top turn, suggesting that the target gene carried by AD virus was disperse throughout the whole cochlear area via inner ear lymph fluid, with relatively high expression in the region adjacent to injection compared to other areas. The difference in target gene expression in the cochlea may be due to relatively slow circulation metabolism of inner ear lymph fluid or the inability of the defective AD virus to cross the labyrinth membrane between the inner and outer spatium perilymphaticum. In addition, the tropism of the virus towards the tissue cell is determined by the virus receptors in the tissues. Virus adhesion towards tissue cells usually takes several to dozens of minutes, which is influenced not only by the virus adhesion protein, the electrification condition and cellular structure of the specific cell receptor, but also by various environmental factors including humidity, presence of ions, pH value and route of viral invasion.

The minimal change in fluorescent light intensity between day 5 and day 14 indicates gene expression stability. However, abundant early and late protein production following virus injection of virus can cause host immune reaction and subsequent clearance, which can limit expression period. By combining recombinated AD virus and immune depressant, some researchers have been able to prolong the expression period. Further investigation is needed to achieve long-term expression of exogenous gene carried by AD virus.

Safe drug administration via appropriate route is especially important in treating inner ear diseases. The technique used in the present study is easy, atraumatic, and effective in delivering transgenes through intact RWM. Compared with other more invasive gene delivery methods, this technique represents a safer and more clinically viable route of cochlear gene delivery. The technique at its present form needs further improvements. Auditory thresholds need to be systematically tested at more time points and at different frequencies. Spatial and temporal characteristics of gene expression in the inner ear with hearing damage induced by drug or noise need to be studied and gene transduction efficiency need to be quantified.

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