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Establishment of a model of cochlear lesions in rats to study potential gene therapy for sensorineural hearing loss

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ABSTRACT

Introduction: Sensorineural hearing loss seriously influences a patient's daily life, and no effective treatments exist to date. Gene therapy is a potential treatment for regenerating hair cells to restore hearing.

Methods: In this study, we established a cochlear lesions model to study hair cell regeneration by co-administration of kanamycin and furosemide. After the injections, we assessed the survival of outer hair cells (OHC), inner hair cells (IHC), supporting cells (SC), spiral ganglion neurons (SGN) and peripheral axons. Moreover, we used two viral vectors to detect the transgene distribution.

Results: Our results showed at 12 h post-treatment, numerous OHC were missing in the basal turn. At 24 h post-treatment, all OHCs in basal half of the cochlea were lost, and by 48 h, OHC loss had spread to the apical coil. Four days after the injections, all OHCs were absent. At 1 mo post-treatment, the organ of Corti had collapsed. In contrast, most of the SC remained 4 d after the injections. The loss of SGN and peripheral axons was consistent with this time course post-treatment. The results of transgene distribution suggested the correlative gene can be transferred into the organ of Corti using adenoviruses (AdV) vectors and lentiviruses (LV) vectors in our cochlear lesion model.

Comparison with Existing Method(s): We assessed the details of HC death at more time point and chosen the time point for gene transfer in this model.

Conclusions: We conclude that this cochlear lesion model would be suitable for the study of hair cell regeneration.

1. Introduction

Sensorineural hearing loss is a common condition that seriously influences a patient's daily life. It is caused by damage to the sensory hair cells (HC) in the organ of Corti after exposure to noise, ototoxic drugs, and aging, and thus so far, no effective treatments exist. In humans and other mammals, the loss of the sensory HC and the subsequent inability to regenerate them result in permanent hearing loss. However, studies of the avian inner ear showed that nonsensory supporting cells (SC) could give rise to new sensory HC via mitosis and direct transdifferentiation [1]. Furthermore, previous studies proved that prospectively identified post-mitotic, postnatal SCs can proliferate and subsequently transdifferentiate into HC in neonatal mice [2]. After overexpressing the Atoh1 gene, nonsensory cells could successful regenerate new HC in mature guinea pigs [3]. These results indicate that postnatal mammalian SC is potential targets for therapeutic manipulation. From these studies, we also concluded that the signaling pathways that are required for the regeneration of hair cells are repressed in adult mammals. Therefore, gene transfer is a powerful technique to deliver potentially therapeutic genes into these target cells to study the regeneration of hair cells [4]. This process comprises transferring correlative genes into either HC or SC with the intent of regenerating new HC or inducing the transdifferentiation of SC into HC to restore hearing. These investigations required a stable and repeatable animal model of cochlear lesions that was characterized by the absence of all OHC, while most SCs remained.

Recently, aminoglycosides have been used in research because they are the major causative factors of acquired hearing loss,

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balance dysfunction and the eradication of hair cells; thus, they can be utilized to create animal models of cochlear implantation for the study of hair cell regeneration [5,6]. Several ototoxic deafening protocols use aminoglycosides, including intracochlear infusions of aminoglycosides [7], repeated injections of aminoglycosides to achieve HC damage [8], and co-administration of aminoglycosides and a loop diuretic [9]. Among these protocols, the combination of a single dose of aminoglycosides closely followed by a single dose of a loop diuretic induces rapid hair cell loss in humans and experimental animals. This protocol has been developed for guinea pigs and mice [9,10]. In contrast to guinea pigs and mice, rats have recently been considered an ideal animal model for investigating hair cell regeneration because they present several advantages, including the size of their otic capsules, lower costs, and resilience after complicated surgery, and convenience for surgical interventions. Moreover, the rat genome has been sequenced and presents important anatomical and physiological similarities to that of humans [11]. However, thus far, no details exist on the investigation of co-administrating kanamycin and furosemide in rats.

In this study, we present an experimental rat model in which hair cell loss has been induced through a combination of a single dose of the aminoglycoside kanamycin, followed by a single dose of the loop diuretic furosemide. We also tested the transgene distribution of two viral vectors. The results show cochlear lesion model would be suitable for the study of hair cell regeneration.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (8 weeks old, 250–300 g) were anesthetized using a 1-ml/kg intramuscular injection of an anesthetic mixture composed of 500 mg ketamine, 20 mg xylazine, and 10 mg acepromazine in 8.5 ml water and were then sacrificed by exsanguination. All animal studies were conducted under the National Institutes of Health guidelines and were approved by the Committee on Animal Research, the Fourth Military Medical University, China. The animal center of the Fourth Military Medical University provided the Sprague-Dawley rats used in the experiments.

2.2. Drug administration

HC death was experimentally induced in rats by administering single doses of kanamycin (500 mg/kg, dissolved in phosphate-buffered saline [PBS], intramuscular), followed immediately by a single dose of furosemide (200 mg/kg, jugular vein). Animals were killed 1, 2, 4, 7, or 30 days after the injections.

2.3. Production of viral vectors

A replication-deficient adenoviral vector (human AdV, serotype 5) was used to encode green fluorescent protein (GFP), driven by the cytomegalovirus (CMV) promoter. This virus was designated Ad5.CMV-GFP (1.8×10^{12}) plaque-forming units [PFU]/ml), and the E1 and E3 regions were deleted. Viral suspensions in 3% sucrose were stored at −80 °C until thawed for use.

The LV vectors were produced as described previously. Briefly, the transfer plasmids pHR-CMV/CAG/EF-1a/PGK-GFP-WHV was co-transfected with pMD.G and pCMVDR8.91 into 293 T cells. The supernatant was collected 2 or 3 d following transfection and was concentrated by two rounds of ultracentrifugation at 141,000 × *g* for 1.5 h. The final pellet was dissolved in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS). The number of transducing units (TUs) for the LV-CMV-GFP vector was determined by infecting 100,000 293 T cells using a serial dilution of the vector. The dilution resulting in 30% GFP-positive cells was used to calculate the number of TUs per ml.

2.4. Surgical procedures for the scala media inoculation

Deafened rats were anesthetized as described above. The animals were placed in a supine position on a thermoregulated heated pad. Their bullae were exposed through a dorsal postauricular approach, and a diamond drill was used to open them. After identifying the stapedial artery, 3 μl of viral vector suspension was injected into the basal turn of the scala media by penetrating the stria vascularis over 5 min. To prevent the immediate backflow of the viral suspension, the cannula was left in place for 10 min. After the injections, the cochleostomy was covered with connective tissue [12]. The incision was closed in two layers. We inoculated the contralateral ear with 3 μl of artificial endolymph (NaCl 1 mM, KCl 126 mM, $KHCO_3$ 25 mM, $MgCl_2$ 0.025 mM, $CaCl_2$ 0.025 mM and K_2HPO_4 1.4 mM) as the control group by repeating the same procedure. The animals were sacrificed 4 d after vector delivery.

2.5. Tissue preparation

Animals were sacrificed after anesthesia at 12 h, 24 h, 48 h, 4 d, 7 d, and 28–30 d. For experiments involving the immunofluorescence of whole-mount preparations, the temporal bones were isolated. After removing the bulla, the stapes was lifted from the oval window, and small openings were made in the apical and basilar turns. Cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) was perfused directly into the cochlea through the openings for 6 h. For samples that would undergo cryosectioning, animals were euthanized by intracardiac perfusion with 4% PBS-buffered paraformaldehyde after anesthesia. Following fixation, the cochleae were decalcified in 10% EDTA (pH 7.4) for 24–48 h at 4 °C, sequentially infiltrated with 30% sucrose overnight followed by O.C.T. compound, frozen rapidly with dry ice, and cut into 10-μm sections with a cryostat.

2.6. Tissue labeling

To label the filamentous actin that is abundantly expressed in stereocilia HC, phalloidin conjugated to a fluorophore or Rhodamine (diluted 1:200 or 1:500 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the whole-mount preparations at a concentration of 5 μl. Although phalloidin labeling is a fast and easy means to identify HC loss, it is not definitive because the absence of cilia does not always accurately reflect the loss of underlying HC bodies. Therefore, a mouse monoclonal anti-parvalbumin antibody (diluted 1:1000 in PBS; Abcam, Hong Kong, China) was used to label HC bodies in our study. To label IHC, we use an anti-calbindin antibody (diluted 1:200 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) [13]. Monoclonal anti-acetylated tubulin (diluted 1:200 in PBS; Abcam, Hong Kong, China) was used to label type I and type II spiral ganglion neurons (SGN) and the peripheral axons [14].

To immunofluorescently label whole-mount preparations, the tissues were treated with 0.3% Triton X-100 for 30 min in PBS to make the membranes more permeable to antibodies. To prevent nonspecific binding of the primary antibody, tissues were then incubated for 1 h in a blocking solution consisting of 10% normal serum, 0.03% saponin, and 0.1% Triton X-100 in PBS [15]. Primary antibody incubations were performed for 2 d at 4 °C in PBS containing 3% serum, 2 mg/ml bovine serum albumin, and 0.1% Triton. Fluorescent-labeled secondary antibodies (Alexa 488 or 594, Molecular Probes, Invitrogen) were used at a dilution of 1:300