Hearing Research 240 (2008) 52–56

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Research paper

Response of the flat cochlear epithelium to forced expression of *Atoh1*

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ARTICLE INFO

Article history:
Received 22 October 2007
Received in revised form 5 February 2008
Accepted 17 February 2008
Available online 7 March 2008

Keywords:
Cochlea
Deafness
Aminoglycosides
Neomycin
Adenovirus
Guinea pig
Atoh1
Gene transfer

ABSTRACT

Following hair cell elimination in severely traumatized cochleae, differentiated supporting cells are often replaced by a simple epithelium with cuboidal or flat appearance. *Atoh1* (previously *Math1*) is a basic helix–loop–helix transcription factor critical to hair cell differentiation during mammalian embryogenesis. Forced expression of *Atoh1* in the differentiated supporting cell population can induce transdifferentiation leading to hair cell regeneration. Here, we examined the outcome of adenovirus mediated over-expression of *Atoh1* in the non-sensory cells of the flat epithelium. We determined that seven days after unilateral elimination of hair cells with neomycin, differentiated supporting cells are absent, replaced by a flat epithelium. Nerve processes were also missing from the auditory epithelium, with the exception of infrequent looping nerve processes above the habenula perforata. We then inoculated an adenovirus vector with *Atoh1* insert into the scala media of the deafened cochlea. The inoculation resulted in upregulation of *Atoh1* in the flat epithelium. However, two months after the inoculation, *Atoh1*-treated ears did not exhibit clear signs of hair cell regeneration. Combined with previous data on induction of supporting cell to hair cell transdifferentiation by forced expression of *Atoh1*, these results suggest that the presence of differentiated supporting cells in the organ of Corti is necessary for transdifferentiation to occur.

1. Introduction

The cochlear sensory epithelium contains two types of differentiated epithelial cells: hair cells and supporting cells. When hair cells degenerate, supporting cells expand and replace them to maintain a confluent layer of cells lining the scala media and separating endolymph from perilymph. In some cases, supporting cells in lesioned ears remain differentiated and the organ of Corti maintains its tall appearance despite the lack of hair cells. However, in many cases the supporting cells that remain after hair cell loss do not maintain their differentiated state. As a result, the area of the organ of Corti becomes a flat or cuboidal simple epithelium with no patterned organization (Forge et al., 1998; Kim and Raphael, 2007). The condition of supporting cells in deaf ears will dictate the choice of therapy, once therapies such as hair cell regeneration or stem cell implantation become a reality.

The flat epithelium has been described after several types of trauma. For instance, ears that receive cochlear implants often exhibit a flat epithelium in both human and animal models (Nadol et al., 1994). A variety of etiologies may lead to degeneration of the auditory epithelium to the flat state, including severe presbycusis (Bhatt et al., 2001), extremely severe ototoxic injury (Coco et al., 2007; Forge et al., 1998; Kim and Raphael, 2007) or hereditary cochlear pathologies (Webster, 1992). In many cases, the loss of hair cells does not initially involve supporting cell degeneration, but over time the non-sensory auditory epithelium is replaced by a flat epithelium. Because of the prevalence of this pathology in humans, the flat epithelium constitutes the substrate for potential future therapy in many clinical cases. It is therefore important to characterize the flat epithelium and determine how it responds to therapeutic manipulations.

In the present study, we have used the neomycin model to eliminate hair cells and induce transformation of supporting cells into the flat epithelium state. We tested the ability of the flat epithelium to be transduced with an adenovirus and whether forced expression of a developmental gene, *Atoh1*, in the flat epithelium can induce transdifferentiation of these cells into new hair cells. *Atoh1* is the mouse homolog of the *Drosophila* gene *atonal*, a basic helix–loop–helix transcription factor that acts as a 'pro-hair cell gene' (Jones et al., 2006). Forced expression of *Atoh1* in deaf ears with differentiated supporting cells can induce transdifferentiation of these supporting cells to new hair cells (Izumikawa et al., 2005; Shou et al., 2003).

Abbreviations: Green fluorescent protein (GFP); Scanning electron microscopy (SEM); Phosphate buffered solution (PBS); Figure (Fig)

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doi:10.1016/j.heares.2008.02.007

We found that the adenovirus-mediated expression of a reporter gene in the flat epithelium was robust. However, forced expression of *Atoh1* did not induce noticeable changes in the morphology of the flat epithelium. The results point to the importance of designing ways to prevent supporting cell degeneration and indicate that once the auditory epithelium is flat, therapies other than *Atoh1* over-expression should be considered.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the University of Michigan Institutional Committee on Care and Use of Animals (UCUCA) and performed using accepted veterinary standards. We used 72 young adult guinea pigs (Elm Hills Breeding Laboratory). At the beginning of the experiments, animals weighed 250–400 g and displayed normal Preyer's reflex. All animals were deafened unilaterally with neomycin (see below) and received one of the following treatments: Ad.*Atoh1* ($n = 33$), Ad.*Atoh1-GFP* ($n = 21$), Ad.*GFP* ($n = 3$), Ad.empty (adenovirus with no gene insert) ($n = 9$), 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) ($n = 2$) and deafening alone ($n = 4$).

2.2. Deafening and inoculation surgery

All animals were deafened unilaterally (left ear), with a single bolus injection of 60 μl of 10% neomycin (Pharma-Tek, Huntington, NY) diluted in sterilized water. Neomycin was selected at this concentration because it leads not only to complete elimination of all hair cells in turns 1–3 of the guinea pig cochlea, but also to a drastic change in the morphology of supporting cells. The animals were anesthetized by the combination with Rompun (intramuscularly, xylazine, 10 mg/kg, Bayer, Shawnee Mission, KS, USA) and Ketalar (intramuscularly, ketamine HCl, 40 mg/kg, Parke Davis, Morris Plains, NJ, USA). We injected 1% lidocaine hydrochloride (subcutaneously, 0.5 ml) for local anesthesia in the postauricular region. The animals were placed at a prone position on a heated pad. An incision was made along the left postauricular region. The temporal bone was exposed and then opened by scalpel drilling and forceps to gain a view of the entire round window membrane. Using the bent tip of a 30 gauge needle and a 100 μl Hamilton syringe, 60 μl of 10% neomycin was injected into the scala tympani through the round window membrane, over 1 min. After the injection, the opening in the temporal bone was closed with carboxylate cement (Duleron) and the skin was sutured in two layers.

The vectors or artificial endolymph were inoculated into the scala media of left ear seven days after the deafening surgery, using the procedure described previously (Ishimoto et al., 2002) except that inoculation was into the second turn of the cochlea. Animals were sacrificed and prepared for morphological analysis (immunocytochemistry, plastic sections or SEM) six days after deafening, or two months after viral vector inoculation. In addition, a group of animals ($N = 4$) was used for immunocytochemical detection of *Atoh1* gene expression at a time point seven days after viral vector inoculation.

2.3. Adenoviral vectors

We used advanced generation replication-deficient recombinant adenoviral vectors with E1, E3 and partial E4 regions deleted (Brough et al., 1997). The vectors were Ad.*Atoh1*, Ad.*Atoh1-GFP*, Ad.*GFP* and Ad.empty. All vectors were provided by GenVec Inc. (Gaithersburg, MD, USA). The *Atoh1* gene insert was driven by the human cytomegalovirus promoter and the *GFP* gene was driven by the chicken beta-actin promoter. We used undiluted vectors at a concentration of 1×10^{12} particles purified virus per ml. The viral suspensions were stored at −80 °C until thawed for use.

2.4. Immunocytochemistry

Animals were deeply anesthetized with xylazine and ketamine as above, decapitated, and the temporal bones were removed. The inner ears were perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 h. Further dissection was performed to remove the stria vascularis, Reissner's membrane and the tectorial membrane. Then the tissue was permeabilized with 0.3% Triton-×-100 in PBS for 10 min. Non-specific binding of secondary antibody was blocked with 5% normal goat serum in PBS for 30 min. Immunocytochemistry was performed using primary antibodies: a mouse monoclonal anti-neurofilament 200 kDa antibody (Sigma, St. Louis, MO, diluted 1:200) or mouse monoclonal anti-Atoh1 (Hybridoma Core, University of Iowa), followed by a secondary antibody, a goat monoclonal anti-mouse conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA) for 30 min. To double stain for actin, we used FITC-conjugated phalloidin (Molecular Probes, Junction City, OR, diluted 1:200). The specimens were further dissected to separate individual cochlear turns and mounted on glass slides using CrystalMount (Biomeda, Foster City, CA). The samples were examined and photographed using a Leica DMRB epifluorescence microscope (Leica, Eaton, PA) with a Cooled SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

2.5. Scanning electron microscopy

Animals were deeply anesthetized (as described above) and perfused transcardially with 0.15 M cacodylate buffer, followed by 2% glutaraldehyde in the same buffer. Cochleae were removed and the otic capsule opened to continue fixation for 2 h. The tissues were postfixed using the osmium thiocarbohydrazide method (Osborne and Comis, 1991). The specimens were dehydrated with ethanol and dried by the critical point method with CO_2 in a SamDri-790 (Tousimis, Rockville, MD). The samples were fixed to stubs with silver paste and photographed digitally using a Philips XL30 Field Emission Gun scanning electron microscope (FEI, Hillsboro, OR).

2.6. Plastic sections

Animals were anesthetized and decapitated and the temporal bones removed and placed in 4% paraformaldehyde in PBS for 2 h. The otic capsule was dissected away and the modiolus along with the organ of Corti, were decalcified for a 2–3 days in 3% EDTA with 0.25% glutaraldehyde. Once tissues appeared soft, specimens were postfixed with 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol and embedded in Embed 812 epoxy resin. Sections were obtained with glass knives and photographed using a Leica DMRB microscope.

3. Results

Morphological analysis reported here is based on observations in the first three turns of the cochlea. Little variation was seen among individuals. In normal ears that were not deafened, the combined staining with phalloidin and neurofilament shows presence of hair cells and nerves extending in the direction of the hair cells (Fig. 1a). In contrast, in animals sacrificed at six days after the deafening procedure, whole-mounts of the organ of Corti stained