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Research paper

The fate of outer hair cells after acoustic or ototoxic insults

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Abstract

In epithelial sheets, clearance of dead cells may occur by one of several routes, including extrusion into the lumen, phagocytic clearance by invading lymphocytes, or phagocytosis by neighboring cells. The fate of dead cochlear outer hair cells is unclear. We investigated the fate of the "corpses" of dead outer hair cells in guinea pigs and mice following drug or noise exposure. We examined whole mounts and plastic sections of normal and lesioned organ of Corti for the presence of prestin, a protein unique to outer hair cells. Supporting cells, which are devoid of prestin in the normal ear, contained clumps of prestin in areas of hair cell loss. The data show that cochlear supporting cells surround the corpses and/or debris of degenerated outer hair cells, and suggest that outer hair cell remains are phagocytosed by supporting cells within the epithelium.

Keywords: Guinea pig; Mouse; Prestin; Overstimulation; Ototoxicity; Outer hair cell; Supporting cell

1. Introduction

The process of cell death during development and in mature tissues has been extensively studied. Cells can be eliminated either by their neighbors or by professional scavengers such as macrophages. In both cases, specific molecules mediate cell–cell signaling associated with clearance of cell corpses, sending "find-me", "eat-me" and "don't-eat-me" signals (Lauber et al., 2004). In sheets of epithelial cells, the highly regulated process of cell elimination is particularly important because of the need to maintain structural and functional integrity of the luminal surface (Rosenblatt et al., 2001).

The organ of Corti is the auditory sensory epithelium of mammals. It consists of a highly organized mosaic of hair cells and supporting cells. The strictly organized pattern of the organ of Corti makes it a useful model for studying the process of cell death and elimination. One type of hair cell in the mosaic is the outer hair cell (OHC). OHCs enhance the sensitivity and frequency selectivity of the organ of Corti mainly through the action of the membrane motor protein prestin (Belyantseva et al., 2000; Dallos and Fakler, 2002; Zheng et al., 2000). OHC death in mammals leads to a hearing deficiency that is permanent, because lost hair cells do not spontaneously regenerate (Daudet et al., 1998; Engstrom et al., 1970; Hawkins, 1973; Spoendlin, 1976; Wang and Li, 2000; Yamasoba et al., 2003). When hair cells die, the adjacent supporting cells quickly expand in a highly regulated manner and quickly close the gap that would be left by damaged hair cells (Forge, 1985; Leonova

Abbreviations: OHC, outer hair cell; IHC, inner hair cell; GFP, green fluorescent protein; qRT-PCR, quantitative real-time polymerase chain reaction; Ad, adenovirus; SPL, sound pressure level; PBS, phosphate buffered saline; DAB, 3,3′-diaminobenzidine tetrahydrochloride; EDTA, ethylenediaminetetraacetic acid

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and Raphael, 1997; Raphael and Altschuler, 1991a,b). Sites of hair cell degeneration where supporting cells seal the luminal surface are called phalangeal scars. The fate of the damaged or dead hair cells remains unknown. In exploring how the "corpses" of hair cells are removed from the epithelium, it is important to consider whether injured or damaged hair cells are ejected from the organ of Corti and removed by the immune system, or whether they remain within the organ of Corti where they are engulfed by neighboring supporting cells or invading phagocytic cells.

The aim of this study was to investigate the fate of OHC corpses following cochlear insults that lead to hair cell degeneration. We used ototoxic drugs or acoustic overstimulation to induce hair cell death. The level of *prestin* mRNA in the cochlea decreased after the insult. Prestin, an OHC protein that is normally absent from supporting cells (Belyantseva et al., 2000; Dallos and Fakler, 2002; Zheng et al., 2000), was used as a marker to identify intact OHCs and remnants of damaged OHCs. Using whole mounts and transverse sections of the organ of Corti, we found that sites of hair cell loss consisted of scarring supporting cells and prestin aggregates. Overall, the data suggest that supporting cells phagocytose injured or dead hair cells and/or their debris.

2. Experimental procedures

2.1. Animals and deafening

All animal experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan. We used young adult male guinea pigs (pigmented SPF animals from Elm Hill Breeding Labs, Chelmsford, MA) and 30–60-day old CD-1 mice of either sex (Charles River Breeding Labs, Wilmington, MA). In guinea pig experiments, hair cell lesions were induced by ototoxic insult or noise exposure. For ototoxic deafening, guinea pigs ($N = 11$) were given kanamycin (American Pharmaceutical Partners, Inc., Schaumburg, IL) and ethacrynic acid (Sodium Edecrin, Merck and Co., Inc., West Point, PA), as follows: a single dose of kanamycin (500 mg/kg, s.c.) was injected, followed 2 h later by ethacrynic acid (50 mg/kg, i.v.). Four of the 11 guinea pigs deafened with ototoxic drugs were inoculated with an adenoviral vector with a *GFP* gene insert (Ad.*GFP*) (GenVec, Gaithersburg, MD) 7 days after the deafening, to label supporting cells. These animals were sacrificed 4 days later and prepared for CFM analysis for prestin and GFP. For inducing acoustic lesions, guinea pigs ($N = 2$) were exposed for 4 h per day on two consecutive days to noise with a bandwidth of 1–20 kHz at an intensity of 120 dB SPL. Lesions in mice were produced by noise overstimulation. Eighteen mice ($N = 12$ for qRT-PCR and $N = 6$ for immunocytochemistry) were exposed to noise with a bandwidth of 1–20 kHz at an intensity of 120 dB SPL for 4 h. Animals with normal ears were used as controls ($N = 4$ guinea pigs, $N = 3$ mice for histocytochemistry and $N = 8$ mice for qRT-PCR).

2.2. Inoculation of guinea pig cochlea with Ad.GFP

To enhance visualization of scarring supporting cells, we expressed GFP in these cells. Deafening was performed with kanamycin and ethacrynic acid as described above. Seven days later, Ad.*GFP* was inoculated into the endolymph of the left cochlea. The inoculation was performed as previously described (Ishimoto et al., 2002) except that the vector solution was inoculated into the scala media of the apical turn. The adenovirus vector was replication-deficient recombinant adenoviruses with deleted E1, E3, and E4 regions (Brough et al., 1997). We used undiluted vectors at a concentration of 1×10^{12} total particles purified virus per milliliter.

2.3. Tissue dissection and fixation for histology

Animals deafened with ototoxic drugs were sacrificed 2, 4 or 9 days after the insult. Animals exposed to noise were sacrificed 6–7 days after the insult. Animals (mice or guinea pigs) were anesthetized with ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (AnaSed, Shenandoah, IA), decapitated, and their temporal bones removed. Inner ears were dissected out and perfused with 4% paraformaldehyde in PBS. Temporal bones were then incubated for 1 h at room temperature in the same fixative. Tissues were rinsed with PBS and the bone surrounding the organ of Corti was removed. Tissues were rinsed several times in PBS to remove debris. One cochlea of each animal was stained for fluorescence analysis and prepared as a whole mount of the organ of Corti. The other cochlea was stained with antibodies and 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO) and embedded in plastic for sectioning.

2.4. Staining for fluorescence analysis of prestin, F-actin, GFP and DNA

Tissues were permeabilized with 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO) for 1 h then rinsed thoroughly in PBS. To reduce non-specific antibody binding, tissues were incubated in a solution of 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) and 2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS for 2 h, or 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. Tissues were then rinsed in PBS and incubated with a prestin-specific rabbit antibody (a kind gift of Dr. Bechara Kachar, NIH-NIDCD) at a concentration of 0.0023 μg/ml, or with a goat anti-prestin antibody (1:100; Santa Cruz sc-22692). After a 10 min PBS rinse, tissues were incubated for 30 min with a secondary antibody (goat anti-rabbit or donkey anti-goat, both rhodamine-conjugated, Jackson