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Mechanisms of rapid sensory hair-cell death following co-administration of gentamicin and ethacrynic acid

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ABSTRACT

Concurrent administration of a high dose of gentamicin (GM; 125 mg/kg IM) and ethacrynic acid (EA; 40 mg/kg IV) results in rapid destruction of virtually all cochlear hair cells; however, the cell death signaling pathways underlying this rapid form of hair-cell degeneration are unclear. To elucidate the mechanisms underlying GM/EA-mediated cell death, several key cell death markers were assessed in the chinchilla cochlea during the early stages of degeneration. In the middle and basal turns of the cochlea, massive hair-cell loss including destruction of the stereocilia and cuticular plate occurred 12 h after GM/EA treatment. Condensation and fragmentation of outer hair-cell nuclei, morphological features of apoptosis, were first observed 5–6 h post-treatment in the basal turn of the cochlea. Metabolic function, reflected by succinate dehydrogenase histochemistry and mitochondrial staining, decreased significantly in the basal turn 4 h following GM/EA treatment; these early changes were accompanied by the release of cytochrome *c* from the mitochondria into the cytosol and intense expression of initiator caspase-9 and effector caspase-3. GM/EA failed to induce expression of extrinsic initiator caspase-8. These results suggest that the rapid loss of hair cells following GM/EA treatment involves cell death pathways mediated by mitochondrial dysfunction leading to the release of cytochrome *c*, activation of initiator caspase-9 and effector caspase-3.

1. Introduction

Ototoxic drugs, such as the aminoglycoside antibiotics, platinum-based anticancer agents and loop inhibiting diuretics are frequently used to selectively damage specific cell types within the inner ear in order to assess the physiological consequences of a particular type of lesion or to investigate the effects of acute or chronic cochlear pathology. Aminoglycoside antibiotics such as gentamicin (GM) preferentially damage the hair cells. Outer hair cells (OHC) are more vulnerable than inner hair cells (IHC) (Dallos and Harris, 1978; Schmiedt et al., 1980) and hair-cell damage begins in the base of the cochlea and spreads towards the apex as the dose or duration of treatment increases (Forge and Schacht, 2000). The base-to-apex damage gradient has been linked to intrinsic differences in the level of antioxidant enzymes (Sha et al., 2001) and also differences in aminoglycoside uptake (Dai and Steyger, 2008; Dai et al., 2006). The uptake of GM into hair cells begins in the base and spreads to the apex. Uptake is first seen in OHC, initially in the third row, after two days of treatment and then in IHC after 8 days of treatment. Despite the early entry of the drug, functional hearing impairment and hair-cell loss are only observed 10–14 days after the start of daily treatments (Hiel et al., 1993). Aminoglycosides enter hair cells near the apical pole and accumulate in lysosomes and mitochondria until a cytotoxic level is reached (Ding et al., 1995a; Hashino et al., 1997; Hiel et al., 1993). In cases where the dose is non-lethal, clearance of GM from hair cells is extremely slow, extending out to a year.

The antibacterial effects of aminoglycosides result from their binding to the 16s RNA of the 30 S ribosomal subunit and the formation of inert 70 S ribosome; this inhibits protein synthesis in bacteria and also the mitochondria of mammalian cells (Mehta and Champney, 2002; Michel-Briand, 1978).

The ototoxic effects of aminoglycoside antibiotics can be greatly enhanced by co-administration with loop inhibiting diuretics such as ethacrynic acid (EA) (Brummett et al., 1975; McFadden et al., 2002; Nourski et al., 2004; Prazma et al., 1974; Tran Ba Huy et al., 1983). Combined treatments of aminoglycoside antibiotics and (EA) have been used to create animal models with massive IHC and OHC loss. Such models have been used to study the effects of electrical stimulation on surviving spiral ganglion neurons, the time course of neural degeneration in the absence of trophic

Abbreviations: GM, gentamicin; EA, ethacrynic acid; EP, endolymphatic potential; Cis, cisplatin; HC, Hensen cells; HBSS, Hank's balanced salt solution; IHC, inner hair cell; MAPK, mitogen-activated protein kinase; OHC, outer hair cell; PC, pillar cell; PI, propidium iodide; SDH, succinate dehydrogenase; XIAP, X-linked inhibitor of apoptosis

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support from hair cells or hair-cell regeneration induced by viral-mediated gene transfer (Izumikawa et al., 2005; Matsushima et al., 1991; McFadden et al., 2004). The massive hair-cell loss that occurs shortly after GM/EA treatment occurs because EA accelerates the influx of GM from the stria vascularis into endolymph followed by entry through the apical surface of the hair cell into the cytoplasm (Ding et al., 1995a, 2003; Hiel et al., 1992; Tran Ba Huy et al., 1983).

The biological events that contribute to the rapid destruction of hair cells following GM/EA treatment are largely unknown. Some in vitro studies with chronic kanamycin treatment suggest that hair-cell death may occur by necrosis (Jiang et al., 2006). On the other hand, hair-cell death from aminoglycoside antibiotics applied in vivo and in vitro is thought to occur by apoptosis (Nakagawa et al., 1998; Ylikoski et al., 2002). Aminoglycoside induced cell death leads to the loss of mitochondrial membrane potential and activation of caspase-3; activation of caspase-3 was blocked by X-linked inhibitor of apoptosis (XIAP) (Dehne et al., 2002; Tabuchi et al., 2007). Aminoglycosides also upregulate Harakiri, a proapoptotic factor, and the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (MAPK) (Kalinec et al., 2005). EA depletes glutathione S-transferase, a cellular antioxidant, and increases the production of reactive oxygen species (Babson et al., 1994; Khadir et al., 1999; Wang et al., 2007). The biological mechanisms that lead to the rapid and massive loss of IHC and OHC following GM/EA treatment are poorly understood, but previous studies suggest that cell death is likely mediated by caspases, a family of aspartate-specific cysteine proteases (Ding et al., 2007). Caspase-8 mediated cell death is initiated through the extrinsic pathway involving cell death receptors on cell membrane while caspase-9 mediated cell death is triggered by intrinsic cell death pathways involving the mitochondria. We recently reported that rapid hair-cell death resulting from co-administration of EA and cisplatin was initiated by caspase-8 (Ding et al., 2007); however, it is unclear if GM/EA induced cell death is mediated in the same manner. To address this question, we treated chinchillas with a dose of GM/EA that destroys all of the hair cells within 24 h and then used several anatomical and biological markers to determine it cell loss was initiated by caspase-8 and/or caspase-9. In contrast to hair-cell death mediated by EA and cisplatin, our results show for the first time that GM/EA-mediated cell death is initiated through the caspase-9 pathway.

2. Methods

2.1. Subjects

Thirty normal chinchillas, 1–3 years of age and weighing between 560 and 670 g were used in this study. Twenty-four animals were treated with concurrent administration of GM (gentamicin sulfate, Sigma, 125 mg/kg, i.m.) and EA (Sodium Edecrin®, Merck, 40 mg/kg, i.v., right jugular vein) as described previously (Ding et al., 2003; McFadden et al., 2004). As noted in our previous studies, co-administration of this dose of GM and EA abolishes cochlear function 15–30 min post-treatment and 100% of the hair cells are missing 24 h post-treatment (Ding et al., 2003; McFadden et al., 2004). The 48 cochleas from the GM/EA treated animals were evaluated with various staining techniques as indicated in Table 1. After the GM/EA injections, animals were sacrificed at 4, 5, 6, 12, or 24 h. The remaining six chinchillas (12 cochleas) were used as controls for various staining techniques. The numbers of ears evaluated by each labeling technique before and at various times following GM–EA treatment are shown in Table 1.

2.2. Histology

Chinchillas were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.), decapitated and their cochleas were quickly removed and prepared for histological analysis using several different staining techniques described below. Silver nitrate was used to label the stereocilia on the hair cells as previously described (McFadden et al., 2004). After the cochlea was removed, the oval window was opened, a hole was made at the apex and a 0.5% solution of silver nitrate in distilled water was perfused through the round window three times to exchange the fluid in scala tympani. Afterwards, the cochlea was perfused with distilled water followed by 10% formalin (pH 7.2) and then immersed in fixative for 24 h. The organ of Corti was dissected out, trimmed and mounted in glycerin on glass slides as a flat surface preparation. Slides were exposed to sunlight for approximately 1 h to enhance the brownish-black staining of the stereocilia. Specimens were examined under a light microscope at 400× (Zeiss Axioskop) and photographed (Olympus DP10 or Nikon Coolpix). Images were transferred to a PC computer, processed with Adobe Photoshop (version 5.5).

Some specimens were labeled with TRITC-conjugated phalloidin or FITC-conjugated phalloidin, which preferentially labels filamentous actin in hair-cell stereocilia, as previously described (Ding et al., 2002a; Zhang et al., 2003). Cochleas were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. Afterwards the organ of Corti was microdissected out, rinsed in PBS and immersed into 0.25% Triton X-100 for 5 min and then placed in TRITC-conjugated (Sigma P1951, 1:200) or FITC-conjugated phalloidin (Sigma 77415, 1:200) in PBS for 30 min. After rinsing with PBS, the organ of Corti was mounted on slides in 50% glycerol with 100 mg/ml of 1,4-diazabicyclo [2.2.2] octane anti-fade compound (Sigma, D2522, DABCO) and coverslipped. Samples were examined under a confocal microscope (BioRad MRC1024) using appropriate filters for FITC (excitation: 494 nm, emission: 518 nm) and TRITC (excitation: 543 nm, emission: 571 nm) fluorescence.

Propidium iodide (PI) (excitation 530 nm, emission 615 nm), which binds to DNA and RNA, was used to label the nuclei in some cochleas. Specimens were fixed, dissected, permeabilized as described above and then immersed in 10 U/ml of RNAse for 30 min to eliminate background labeling of cytoplasmic RNA (Benbow et al., 2000). Specimens were rinsed in PBS and stained with PI (10 μg/ml for 10 min) to selectively label DNA. Samples were subsequently stained with FITC-labeled phalloidin (Sigma, P5282) for 30 min to label the stereocilia bundles and hair-cell cuticular plates, mounted on glass slides and examined under a confocal microscope (BioRad MRC1024) as described above.

Table 1
Number of samples used for each labeling technique.

Label #1 Label #2	Silver nitrate	PI FITC phalloidin	SDH	MitoTracker TRITC phalloidin	Cytochrome *c* TRITC phalloidin	Caspase 9 PI	Caspase 8 PI	Caspase 3 PI
Normal	1	1	1	2	1	2	2	2
4 h post			3	3	3			
5 h post			3			3	3	3
6 h post	2	3	3			3	3	3
12 h post	2	3	3			3	3	3
24 h post	2							