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Methodological paper

Comparison of activated caspase detection methods in the gentamicin-treated chick cochlea

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ABSTRACT

Aminoglycoside antibiotics induce caspase-dependent apoptotic death in cochlear hair cells. Apoptosis, a regulated form of cell death, can be induced by many stressors, which activate signaling pathways that result in the controlled dismantling of the affected cell. The caspase family of proteases is activated in the apoptotic signaling pathway and is responsible for cellular destruction. The initiator caspase-9 and the effector caspase-3 are both activated in chick cochlear hair cells following aminoglycoside exposure. We have analyzed caspase activation in the avian cochlea during gentamicin-induced hair cell death to compare two different methods of caspase detection: caspase antibodies and CaspaTag kits. Caspase antibodies bind to the cleaved activated form of caspase-9 or caspase-3 in specific locations in fixed tissue. CaspaTag is a fluorescent inhibitor that binds to a reactive cysteine residue on the large subunit of the caspase heterodimer in unfixed tissue.

To induce cochlear hair cell loss, 1–2 week-old chickens received a single injection of gentamicin (300 mg/kg). Chicks were sacrificed 24, 30, 42, 48, 72, or 96 h after injection. Cochleae were dissected and labeled for activated caspase-9 or caspase-3 using either caspase-directed antibodies or CaspaTag kits. Ears were co-labeled with either phalloidin or myosin VI to visualize hair cells and to determine the progression of cochlear damage. The timing of caspase activation was similar for both assays; however, caspase-9 and caspase-3 antibodies labeled only those cells currently undergoing apoptotic cell death. Conversely, CaspaTag-labeled all the cells that have undergone apoptotic cell death and ejection from the sensory epithelium, in addition to those that are currently in the cell death process. This makes CaspaTag ideal for showing an overall pattern or level of cell death over a period of time, while caspase antibodies provide a snapshot of cell death at a specific time point.

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1. Introduction

A single systemic injection of a high dose of gentamicin (an aminoglycoside antibiotic) can create a consistent pattern of hair cell death in the avian cochlea, with cell loss beginning in the basal (proximal) end of the basilar papilla and progressing apically (distally) with time (Bhave et al., 1995; Janas et al., 1995; Park et al., 1998; Roberson et al., 2000; Mangiardi et al., 2004; Duncan et al., 2006). Many studies have characterized changes in avian cochlear hair cells following treatment with gentamicin both in vivo and in vitro. Redistribution of F-actin labeling (Mangiardi et al., 2004) and changes in myosin VI and VIIa distribution and intensity (Duncan et al., 2006) have been reported to occur in chick cochlear hair cells prior to and during the process of ejection from the basilar papilla following gentamicin exposure. Additionally, recent morphological and biochemical evidence suggests that a significant amount of aminoglycoside-induced hair cell death occurs via apoptosis (Forge, 1985; Li et al., 1995; Nakagawa et al., 1997; Torchinsky et al., 1999; Forge and Li, 2000; Cunningham et al., 2002; Matsui et al., 2002, 2003, 2004; Cheng et al., 2003; Mangiardi et al., 2004; Duncan et al., 2006; Sugahara et al., 2006). Apoptosis

Abbreviations: TIAR, T-cell restricted intracellular antigen-related protein; AI, after injection; HHBSS, HEPES-buffered Hanks' balanced salt solution; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; ANOVA, analysis of variance; TUNEL, terminal uridine deoxynucleotidyl transferase nick end labeling; DNA, deoxyribonucleic acid; BWS, Belgian waterslager canary; DAPI, 4′6-diamidine-2-phenylindole diHCl

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appears to be the main mechanism of hair cell death in the avian basilar papilla following a single large dose of aminoglycosides (Matsui et al., 2002, 2003, 2004; Cheng et al., 2003; Mangiardi et al., 2004; Duncan et al., 2006). In contrast, hair cell death in mammalian vestibular and cochlear sensory epithelia appears to arise from both apoptotic and non-apoptotic pathways (Forge, 1985; Li et al., 1995; Nakagawa et al., 1997; Jiang et al., 2006; Bohne et al., 2007). The pathway taken seems to be related to the mechanism of cell death (noise vs. aminoglycosides) and the method of aminoglycoside administration (single large dose injection vs. multiple lower dose injections). In our studies of the gentamicin-treated chick cochlea, we have focused on the apoptotic pathways involving caspase activation, as this is the primary means of hair cell death in this sensory epithelium (Cheng et al., 2003; Matsui et al., 2003; Mangiardi et al., 2004).

Likened to cellular suicide, apoptosis is an actively driven process with distinct morphological and biochemical features. Cell shrinkage, nuclear and cytoplasmic condensation, chromatin fragmentation, and cysteine-aspartate protease (caspase) activation are all hallmarks of apoptotic cell death. It is the activation of caspases that ultimately results in apoptotic cellular degradation. Caspases can be divided into two groups: initiators (caspases-2, -8, -9, and -10) and effectors (caspases-3, -6, and -7). All cells contain caspases that are present constitutively but synthesized in an inactive precursor form. Once caspases are cleaved from an inactive pro-caspase to an activated caspase, it is the effector caspases that are responsible for cellular destruction.

Caspase-3 is the major effector protease involved in the apoptotic process and it can be activated by several initiator caspases found upstream in the signal cascade. Two of these upstream initiator caspases, caspase-8 and caspase-9, have been shown to be activated in the cochlea following sound exposure (Nicotera et al., 2003) and following aminoglycoside treatment in vivo and in vitro (Cunningham et al., 2002; Cheng et al., 2003). Involved in the extrinsic apoptotic pathway, caspase-8 is initiated after the binding and trimerization of death receptors on the cell membrane. Once activated, caspase-8 cleaves downstream pro-caspases by activation of bid and inducing mitochondrial cytochrome *c* release (Cryns and Yuan, 1998). Conversely, caspase-9 is involved in an intrinsic pathway associated with mitochondria-mediated activation and cytochrome *c* release into the cytosol (Cryns and Yuan, 1998; Robertson and Orrenius, 2002). Although caspase-8 and caspase-9 represent two distinct apoptotic signaling pathways, both have been shown to activate caspase-3 (Cheng et al., 2003; Nicotera et al., 2003). However, inhibition of caspase-9 prevented the activation of downstream caspase-3, whereas the inhibition of caspase-8 did not (Cunningham et al., 2002). Subsequently, much research has focused on the activation of caspase-9 and caspase-3 and their interactions with each other in promoting hair cell death.

Several morphological and biochemical markers of apoptosis have been identified in cochlear and vestibular hair cells following administration of aminoglycoside antibiotics both in vivo and in vitro. This includes the translocation of T-cell restricted intracellular antigen-related protein (TIAR) from the nucleus to the cytoplasm (Mangiardi et al., 2004), release of mitochondrial cytochrome *c* (Mangiardi et al., 2004; Matsui et al., 2004), nuclear condensation (Matsui et al., 2004) and activation of caspase-3 (Cunningham et al., 2002; Cheng et al., 2003; Mangiardi et al., 2004; Matsui et al., 2004), caspase-8, and caspase-9 (Cunningham et al., 2002; Cheng et al., 2003; Sugahara et al., 2006).

Two main methods have been employed in order to visualize caspase activation in cochlear and vestibular hair cells following aminoglycoside treatment or noise damage: antibodies raised against activated caspases (Cunningham et al., 2002; Mangiardi et al., 2004) and fluorogenic caspase substrates (Hu et al., 2002; Cunningham et al., 2002; Cheng et al., 2003; Matsui et al., 2004; Sugahara et al., 2006). The caspase antibodies bind to the large fragment of the activated caspase that results from the cleavage of the pro-caspase form. Fluorogenic caspase substrates consist of a short peptide sequence conjugated to a fluorescent probe (Cheng et al., 2003). These substrates act as inhibitors by binding to a reactive cysteine residue on the large subunit of the active caspase heterodimer. Both of these methods have been shown to reliably label caspase activation in hair cells; however, there has not been a study performed to directly compare these two caspase detection methods in the same tissue.

The purpose of this study is to compare both caspase-directed antibodies and fluorogenic caspase substrates (commercially available as CaspaTag in situ assay kits) as a measure of gentamicin-induced apoptotic cell death in the avian basilar papilla. To do this, we examined the timing of caspase activation following gentamicin treatment using both detection methods and then quantified the number of caspase-labeled cells at three different time points during the cellular death process. Results from this study indicate that there are important similarities, as well as significant differences, between the two detection methods in their capacity to label caspase activation in cochlear hair cells. Both caspase-directed antibodies and CaspaTag kits reliably label apoptotic cells expressing activated caspase-9 and caspase-3 in the avian cochlea. Additionally, the timing of caspase-9 and caspase-3 activation following gentamicin treatment is similar for both detection methods. However, antibodies against caspase-9 and caspase-3 tend to label only those cells that are currently in the process of apoptotic cell death and ejection from the sensory epithelium. Conversely, CaspaTag labels all the cells that have undergone apoptotic cell death and ejection from the sensory epithelium, in addition to those that are currently in the cell death process. This makes CaspaTag ideal for showing an overall pattern or level of cell death over a period of time, while caspase antibodies provide a snapshot of cell death at a specific time point.

2. Methods and materials

2.1. Animals

White Leghorn chickens (*Gallus domesticus*) were obtained at one week of age from specific pathogen-free avian supply (Charles River SPAFAS, Preston, CT). Upon arrival at the Children's Hospital animal care facility, chickens were housed in communal brooders with ad libitum access to food and water. All animal procedures were approved by the Children's Hospital Institutional Animal Care and Use Committee (IACUC).

2.2. Gentamicin injections

To create cochlear hair cell death, 11–16 day-old chicks were given a single, subcutaneous injection of gentamicin sulfate (300 mg/kg, Sigma, St. Louis, MO), with the time of injection designated as time "0 h" after injection (AI). Injections were performed either in the morning or early afternoon, so that the nephrotoxic effects of gentamicin could pass before the evening (Roberson et al., 2000; Duncan et al., 2006). An additional group of age-matched birds did not receive a gentamicin injection and served as unmanipulated experimental controls.

2.3. Sacrifice and cochlear dissection

To determine the time course of caspase activation following gentamicin treatment, birds were killed 24, 30, 42, 48, 72, or 96 h AI. At the time of sacrifice, animals were euthanized with an

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