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Research paper

Death pathways in noise-damaged outer hair cells

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Abstract

Using morphological criteria, death pathways in outer hair cells (OHCs) were determined in chinchilla organs of Corti that had been exposed to a high- or moderate-level octave band of noise (OBN) centered at either 0.5 or 4-kHz. The specimens were part of our large collection of plastic-embedded flat preparations of chinchilla cochleae. Three death pathways were identified: (1) oncotic – swollen, pale-staining cell with a swollen nucleus, (2) apoptotic – shrunken, dark-staining cell with a pyknotic nucleus and (3) a newly defined third pathway – no basolateral plasma membrane but cellular debris arranged in the shape of an intact OHC with a nucleus deficient in nucleoplasm. To minimize the secondary loss of OHCs from the entrance of endolymph into the organ of Corti, the specimens used for quantitative analysis of death pathways had the following characteristics: (1) the level to which they were exposed was less than or equal to 95 dB SPL, (2) the exposure duration was 6–216 h, (3) fixation for microscopic examination took place in vivo 1–2 h post-exposure and (4) there were no focal OHC lesions in the organs of Corti. Fifty-eight noise-exposed cochleae met these criteria. In these specimens, degenerating and missing OHCs were classified as to which death pathway the cells had followed or were following. Nine non-noise-exposed cochleae were also evaluated for OHC death pathways. The number of OHCs following the third death pathway was significantly greater in the noise-exposed cochleae than the non-noise-exposed cochleae for total exposure energies greater than those produced by 75 dB SPL for 216 h to a 0.5-kHz OBN and 57 dB SPL for 48 h to a 4-kHz OBN. In cochleae exposed to either octave band, OHCs dying by oncosis or apoptosis were uncommon.

Keywords: Cell-death pathways; Noise damage; Chinchilla; Histopathology

1. Introduction

The study of cell-death pathways in various tissues has been intensive since the 1970's. Two basic cell-death pathways have been identified that are common to most cell types in a number of species. These pathways are called necrosis (i.e., passive or unprogrammed cell death) and apoptosis (i.e., active or programmed cell death) (e.g., Clarke, 1990; Majno and Joris, 1996; Kitanaka and Kuchino, 1999). Although there are several ways in which to discriminate between death pathways, the morphological appearance of dying cells is considered to be the gold standard (Majno and Joris, 1995; Majno and Joris, 1996).

Cells that are swollen and ultimately rupture are usually said to be following the necrotic death pathway (e.g., Schweichel and Merker, 1973). However, Majno and Joris (1995) have stated that the death pathway that these swollen cells follow should be termed 'oncosis' (i.e., swollen) rather than 'necrosis' (i.e., dead). They further stated that the term 'necrosis' should be reserved for dead cells, regardless of which death pathway the cells followed. Morphologically, oncotic cells have pale-staining cytoplasm, chromatin clumped along the nuclear membrane and swollen organelles. When the mitochondria become grossly swollen, these changes are generally irreversible. An inflammatory response is evoked when the plasma membrane, nucleus and organelles within a cell rupture. At this point,

Abbreviations: IHC(s), inner hair cell(s); mEq/L, milliequivalents per liter; OBN, octave band of noise; OC(s), organ(s) of Corti; OHC(s), outer hair cell(s); pSPL, peak sound pressure level; SPL, sound pressure level; TUNEL, Td-mediated d-UDP-biotin nick-end-labeling

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the cell is termed 'necrotic' rather than 'oncotic'. Ultimately, residual debris from necrotic cells that followed the oncotic death pathway is phagocytized by mononuclear phagocytes, macrophages and monocytes (e.g., Majno and Joris, 1995; Jugdutt and Idikio, 2005).

Morphologically, cells dying by apoptosis are shrunken with dark cytoplasm and a pyknotic nucleus but an intact plasma membrane (e.g., Kerr et al., 1972). Coincident with the nuclear changes, the apoptotic cell loses its attachment to adjacent cells. The apoptotic cell develops surface blebs which then split off as round to oval membrane-bound structures called 'apoptotic bodies' that contain nuclear fragments and cell organelles. Apoptotic bodies are phagocytized and digested either by adjacent epithelial cells or professional phagocytes (i.e., macrophages derived from monocytes) or, in cases of endocrine and hollow organs, may be extruded into the lumen (e.g., Cummings et al., 1997). Because apoptotic bodies are rapidly phagocytized and their contents are not released into the surrounding tissue, cells dying by apoptosis evoke no inflammatory response (e.g., Cummings et al., 1997). In vivo, cells undergoing apoptosis complete the process in a few hours (e.g., Bursch et al., 1990; Majno and Joris, 1996; Cummings et al., 1997).

The histochemical technique called TUNEL (i.e., Td-mediated d-UDP-biotin nick-end-labeling) has been used extensively to identify apoptotic cells in normal and pathological tissues. TUNEL generally uses a fluorescent probe to label fragmented DNA, a hallmark of the apoptotic death pathway (e.g., Kressel and Groscurth, 1994; Ben-Sasson et al., 1995; Labat-Moleur et al., 1998). However, TUNEL is not absolutely specific for apoptotic cells. For example, in the small intestine, only 10% of the apoptotic cells are TUNEL-positive before they are shed into the lumen (Ben-Sasson et al., 1995). Nuclear fragments from necrotic or autolytic cells may also be TUNEL positive (e.g., Kressel and Groscurth, 1994; Ben-Sasson et al., 1995; Nishizaki et al., 1999). The above descriptions of oncotic, necrotic and apoptotic cells are based primarily on the light and transmission electron microscopic findings in normal and pathological liver, kidney and intestine.

In recent years, death pathways have been studied in outer hair cells (OHCs) of the organ of Corti (OC) following noise damage (e.g., Hu et al., 2000; Hu et al., 2002a; Hu et al., 2002b; Hu et al., 2006; Nicotera et al., 2003; Yang et al., 2004). In these studies, the noise was a 1–4 h continuous exposure to a narrow band or an octave band of noise (OBN) at 110–120 dB sound pressure level (SPL) or a series of impulses (e.g., 75 pairs of impulses at 155 dB pSPL at 1/s). In most of the cochleae analyzed in these studies, the hair-cell plasma membranes were not visible. Cell-death pathways were generally deduced on the basis of the morphological appearance of hair-cell nuclei stained with propidium iodide or Hoechst 33342, sometimes combined with f-actin staining of the hair-cell cuticular plates. Damaged or dead OHCs were classified as: (a) necrotic if their nuclei were swollen and weakly fluorescent, (b) apoptotic if their nuclei were shrunken and intensely fluorescent or (c) missing if their nuclei were not present and there was no f-actin staining in the presumed location of the cuticular plate. Hu et al. (2000, 2002a,b, 2006) and Yang et al. (2004) classified many of the OHCs in and around the center of a noise-induced lesion as apoptotic although some necrotic OHCs were also identified. Only one study presented quantitative data on necrotic, apoptotic, and missing OHCs after an intense noise exposure (Yang et al., 2004).

The present study was undertaken to determine the spectrum of morphological appearances that dying and dead OHCs assume after either high- or moderate-level noise exposures. These histological appearances were used to determine the death pathway that the cells were presumed to be following. Quantitative data were then collected on the prevalence of different death pathways in noise-damaged OHCs after moderate noise exposures.

2. Materials and methods

2.1. Data set

To begin a systematic study of cell-death pathways in the OC, we examined 83 cochleae from our permanent collection of about 1400 plastic-embedded control and noise-exposed specimens. Many of the animals from which these cochleae were obtained had been prepared for microscopic examination prior to 1985 and institutional approval of animal-use protocols was not required. Since 1985, chinchillas were covered by an approved protocol (Bohne, PI). All animals were handled in accordance with NIH guidelines for the care and use of laboratory animals.

Nine cochleae were obtained from 1–2-year-old non-noise-exposed chinchillas. This age range was used because it matched the age range of our noise-exposed chinchillas and 1–2-year-old chinchillas do not have significant age-related hair-cell loss (Bohne et al., 1990).

Seventy-four noise-exposed cochleae were obtained from chinchillas that had been exposed for 1–216 h to a 0.5- or 4-kHz OBN at 57–108 dB SPL. These specimens were examined in order to determine the full range of morphological appearances for noise-exposed OHCs that were dying or dead (see below).

Specimens for quantification of death pathways were restricted to 58 cochleae (Table 1) with the following characteristics: (1) the level to which they were exposed was less than or equal to 95 dB SPL, (2) the exposure duration was 6–216 h, (3) in vivo fixation for morphological examination took place from 1–2 h post-exposure and (4) there were no OHC focal lesions in any of the specimens. By limiting the quantification portion of the study to specimens that were fixed shortly after exposure termination, the OC damage involved losses of OHCs almost exclusively. Death of inner hair cells (IHCs) and pillar cells was minimal in these specimens and recovery time was too short for degeneration of the spiral ganglion and Schwann cells to have been

Table 1
Experimental groups used for quantification of OHC death pathways

Group[a]	Exposure parameters	N	$\text{Log}_2(E)$[b]
Control	–	9	–
0.5-kHz OBN # 1	65 dB SPL, 48 h	2	7.77
# 2	65 dB SPL, 216 h	2	9.94
# 3	75 dB SPL, 48 h	2	11.09
# 4	75 dB SPL, 216 h	2	13.26
# 5	85 dB SPL, 48 h or	4	14.42
	95 dB SPL, 6 h	6	14.74
# 6	95 dB SPL, 18 h or	1	16.32
	95 dB SPL, 24 h	4	16.74
# 7	95 dB SPL, 36 h or	1	17.32
	95 dB SPL, 48 h	6	17.74
Total		30	
4-kHz OBN # 1	57 dB SPL, 48 h	2	5.11
# 2	57 dB SPL, 216 h or	4	7.28
	65 dB SPL, 48 h	2	7.77
# 3	65 dB SPL, 216 h or	2	9.94
	72 dB SPL, 48 h	2	10.10
# 4	72 dB SPL, 216 h or	3	12.27
	80 dB SPL, 48 h	3	12.76
# 5	86 dB SPL, 24 h	8	13.75
# 6	86 dB SPL, 48 h	2	14.75
Total		28	

[a] Grouped by approximately equivalent total energy.
[b] Doubling of total energy (E).

initiated. Hence, the data presented here concern death pathways in OHCs only.

A focal lesion has been defined previously as a contiguous region in the OC in which hair-cell loss is equal to or greater than 50% over a minimum distance of 0.03 mm or three IHCs (Bohne and Clark, 1982; Bohne et al., 1990). Because there is usually damage to the reticular lamina at OHC focal lesions, secondary loss of hair cells may occur adjacent to these lesions after the noise exposure has terminated (Bohne and Harding, 2000). By excluding cochleae with OHC focal lesions from the data set, we avoided analyzing cells that were injured secondarily, due to ion leaks through the damaged reticular lamina (Bohne and Harding, 2000; Ahmad et al., 2003). The secondary loss of hair cells is dominated by swollen cells with pale cytoplasm and nuclei (Bohne, 1976a), morphological changes that we now term 'oncotic'. Based on the selection criteria above, cochleae having a moderate number of oncotic OHCs were not included in the quantitative analysis.

In cochleae exposed to the 4-kHz OBN, OHC death pathways were assessed in the region 63–81% distance from the apex (i.e., 3–8-kHz region). This region was examined because it included the area maximally stimulated by the 4-kHz OBN plus a half octave above the band. In control cochleae and those exposed to the 0.5-kHz OBN, OHC death pathways were assessed in the regions 22–40% distance (i.e., 0.375–1-kHz region) and 63–81% distance from the apex (i.e., 3–8-kHz region). The 0.375–1-kHz region was examined because it included the area stimulated maximally by the 0.5-kHz OBN plus a half octave above the band. The 3–8-kHz region was examined because it encompassed the locations of the hair-cell losses which often occur in the basal turn following exposure to the 0.5-kHz OBN (e.g., Fried et al., 1976; Bohne, 1976b).

2.2. Cochlear processing

Under deep anesthesia, the chinchilla cochleae had been fixed in-vivo by perfusion of scala tympani with a 1% solution of osmium tetroxide in potassium dichromate buffer (i.e., Dalton's) with 1.65% calcium chloride. Use of this fixative allowed simultaneous morphological assessment of cell nuclei, organelles and plasma membranes. However, this fixative is not compatible with immunohistochemical techniques (e.g., TUNEL).

The cochleae with most of the cochlear bone intact were dehydrated, infiltrated with epoxy resin (i.e., Durcupan), and then embedded in the same plastic. After the plastic polymerized, the cochleae were dissected into flat preparations of the cochlear duct which were re-embedded in thin layers of plastic (Bohne, 1972; Bohne and Harding, 1993). Although quantitative morphometric data could easily be collected in these preparations, plastic-embedding is not compatible with fluorescent stains such as propidium iodide or Hoechst 33342. OC length in these cochleae had been measured, and missing and degenerating hair cells had been counted throughout the specimens.

In some cochleae, OC segments containing specific types of dying OHCs were sectioned at a radial angle using an ultramicrotome (LKB Model 2128). One-micrometer-thick sections were mounted with heat on glass slides, stained with a mixture of 1% methylene blue and 1% Azure II (Richardson et al., 1960), and examined by bright-field microscopy.

2.3. Quantitative microscopic examination

The control and noise-exposed cochleae were examined at a horizontal angle by phase-contrast microscopy at magnifications of 625× or 1250×. Missing, recently dead and dying OHCs were classified, using morphological criteria, as to which death pathway the cells had followed or were following. Dying and dead OHCs were found to have one of the following histopathological appearances: (1) swollen cell body with pale cytoplasm and a nucleus shifted toward the reticular lamina, (2) shrunken, dark cell body with a shrunken, dark nucleus and (3) cellular debris arranged in the typical shape of an OHC body but lacking an intact basolateral plasma membrane. The nucleus was located near the cell's basal pole and contained a reduced amount of chromatin that was clumped along the nuclear membrane. Often, the apical plasma membrane, cuticular plate and an ill-defined stereocilia bundle were present over the debris. Other OHCs were grossly swollen, had ruptured plasma membranes and enlarged, pale nuclei. Based on cell-death studies in other tissues, severely damaged OHCs

with the above appearances were judged to be dying by the following death pathways: (1) oncotic (Figs. 1 and 2), (2) apoptotic (Figs. 1 and 3) and (3) a death pathway that was morphologically distinct from the oncotic and apoptotic pathways and, hence, has been termed here as a 'third death pathway' (Figs. 1 and 4). Grossly swollen cells with ruptured plasma membranes had the typical appearance of necrosis (Fig. 5). When third death pathway OHCs were identifiable by the morphological criteria listed above, they already had disrupted plasma membranes and, thus, were dead or necrotic according to the definition of Majno and Joris (1995), Majno and Joris (1996).

Once an OHC died, the phalangeal processes that originally surrounded that cell enlarged to form a phalangeal scar. Based on their phase-contrast microscopic appearance, two types of phalangeal scars were distinguished in the reticular lamina (Bohne, 1976c). Scars with a very pale line of union between adjacent processes forming the scar have been termed 'immature' whereas scars with a dense line of union between the processes have been termed 'mature' (Figs. 1 and 6). With both types of scar, no hair-cell body remained beneath the reticular lamina although, in some cases, cellular debris was seen. Debris was found more often beneath immature scars compared to mature scars, suggesting that the hair cells replaced by immature scars had degenerated more recently than those replaced by mature scars.

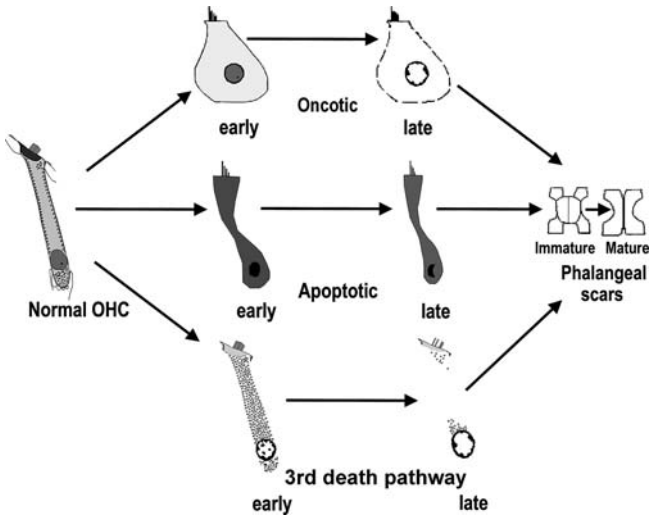

Fig. 1. Noise-damaged OHCs appear to die by one of three death pathways – oncotic, apoptotic or a newly defined third pathway. In a radial view, an oncotic OHC is swollen, pale and its nucleus is shifted towards the reticular lamina. When an oncotic cell enlarges further, its basolateral membrane ruptures and cell contents are spilled into the fluid spaces of the OC. An apoptotic OHC is shrunken, dark-stained and has a pyknotic nucleus. The plasma membrane of the apoptotic cell remains intact so the cell contents are not released into the fluid spaces of the OC. An OHC following the third death pathway lacks all or part of its basolateral membrane. Its apical membrane persists for awhile along with remnants of the stereocilia. Debris from the cell is arranged in a cylindrical shape, similar to the shape of an intact OHC. The cell's nucleus, generally deficient in nucleoplasm, first has a similar size and position to the nucleus in a normal OHC. At a later stage, the nucleus is enlarged and is nearly deficient in nucleoplasm. Cells with deficient plasma membranes (i.e., late oncotic. and early and late third death pathway) are properly termed 'necrotic' (i.e., dead). Once an OHC dies, the phalangeal processes surrounding the missing cell enlarge to form a phalangeal scar in the reticular lamina. In a horizontal view, an immature scar has a barely visible line of union between adjacent phalangeal processes forming the scar while a mature phalangeal scar has a dense line of union.

In the controls ($N = 9$) and the cochleae exposed to 0.5-kHz OBN ($N = 30$) or 4-kHz OBN ($N = 28$), the numbers of OHCs that were following the oncotic, apoptotic, or third death pathway and those that were necrotic were counted. Immature and mature phalangeal scars in which cellular debris was present in the Nuel spaces were classified as to which pathway led to the OHC's death. Scars that had no debris beneath the reticular lamina were classified as 'unknown' death pathway. The total number of OHCs that should have been present in the regions examined for each cochlea was estimated using equations that describe the relation between hair-cell density and total OC length for the different cochlear turns (Bohne et al., 1986). The counts of OHCs following the different death pathways divided by the estimated total OHCs gave the percentage of the total OHCs that were following the different death pathways.

2.4. Statistical analysis

OHC losses in the noise-exposed cochleae were analyzed relative to the total exposure energy (Pa^2 s). For each OBN, cochleae with similar total exposure energies were grouped together for subsequent analysis (Table 1). The Student's *t*-test was used to identify significant differences between the different groups and the control cochleae.

3. Results

3.1. Control cochleae

In the 0.375–1-kHz (i.e., apical) region, these cochleae were missing 0–2 IHCs (i.e., total of eight IHCs across nine specimens) with IHC loss averaging $0.2 \pm 0.2\%$. In the same region, 2–18 OHCs (i.e., total of 88 OHCs across 9 specimens) were missing; OHC loss averaged $0.7 \pm 0.4\%$. In the control cochleae and those exposed to the 0.5-kHz or 4-kHz OBN (see below), most missing IHCs had been replaced by mature phalangeal scars.

In these non-noise-exposed cochleae, most of the missing OHCs had been replaced by mature phalangeal scars and there was no cellular debris associated with the scars. Hence, the missing OHCs were classified as having died by an unknown death pathway. These cochleae contained a few OHCs that were following the third death pathway. Occasionally, OHCs were seen following the apoptotic death pathway. Relative to the total number of dead/dying OHCs, 65% were following the third death pathway, 1% was apoptotic, 0% was oncotic and 34% were unknown.

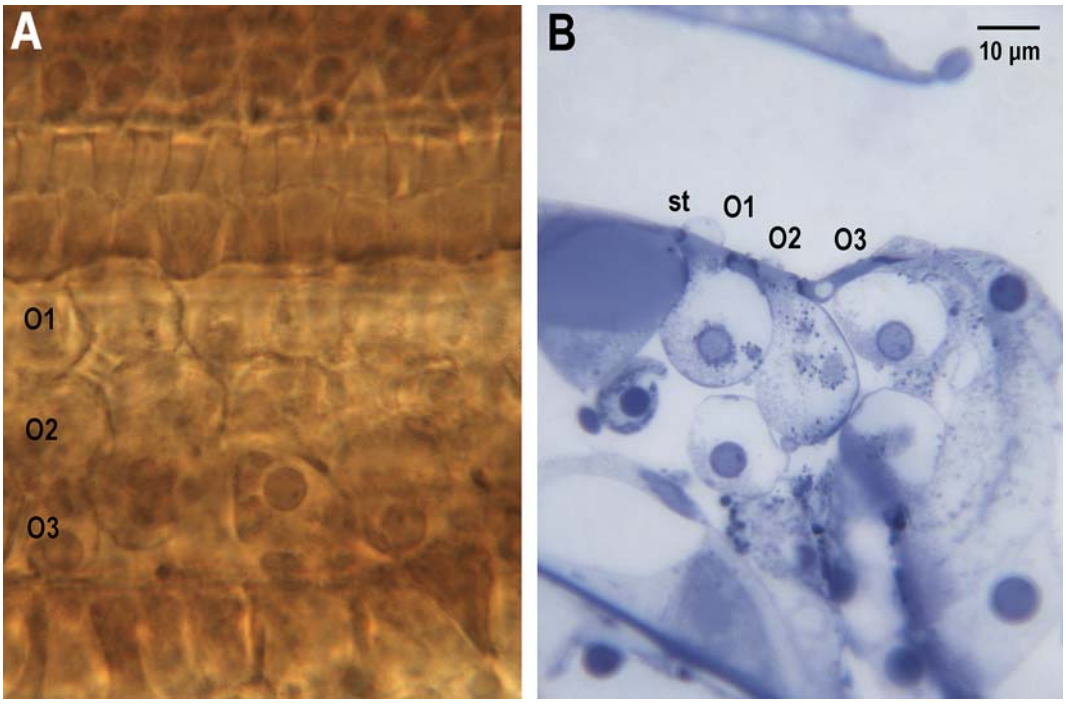

Fig. 2. Oncotic OHCs in the first (O1), second (O2) and third (O3) rows in the lower first turn of two cochleae that were exposed for 3.5 h to a 4-kHz OBN at 108 dB SPL and were fixed 1–2 h post-exposure. These specimens were not included in the quantitative analysis. (A) Flat preparation viewed by phase-contrast microscopy; (B) One-micrometer-thick, stained radial section. OHCs following the oncotic death pathway are swollen, have pale cytoplasm and pale-staining nuclei that are shifted toward the reticular lamina. The stereocilia (st) on these cells are often fused.

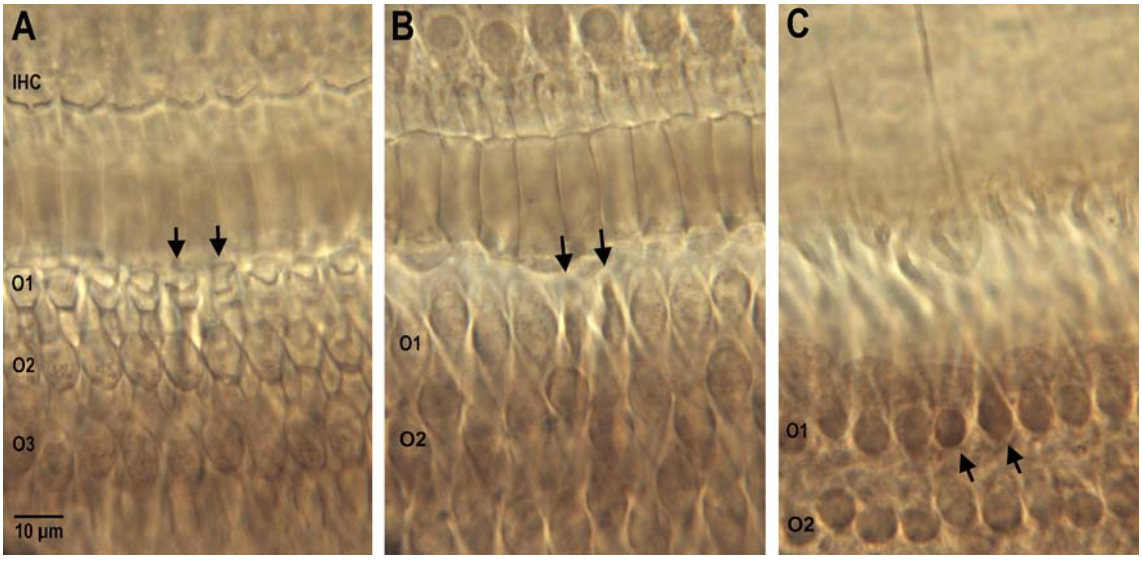

Fig. 3. Middle of the second turn in cochlea that was exposed for 6 h to a 0.5-kHz OBN at 95 dB SPL (Group # 5, Table 1) and was preserved 1 h post-exposure. At three different focal levels in the OC, two early apoptotic cells (arrows) are visible in the first row of outer hair cells (O1). (A) At the reticular lamina level, the cells are slightly darker and have abnormal stereocilia patterns; (B) At the OHC body level, the apoptotic cells are quite shrunken and dark compared to the neighboring OHCs; (C) At the OHC nuclear level, the apoptotic cell nuclei are darker but only slightly smaller than those in adjacent first row OHCs. IHC – inner hair cells; O2, O3 – outer hair cells in the second and third rows, respectively.

In the 3–8-kHz (i.e., basal) region, 0–4 IHCs (i.e., total of seven IHCs across nine specimens) were missing with IHC loss averaging $0.2 \pm 0.4\%$. In the same region, 3–14 OHCs (i.e., total of 60 OHCs across 9 specimens) were missing and OHC loss averaged $0.5 \pm 0.2\%$. Relative to the total number of dead/dying OHCs, 79% were following

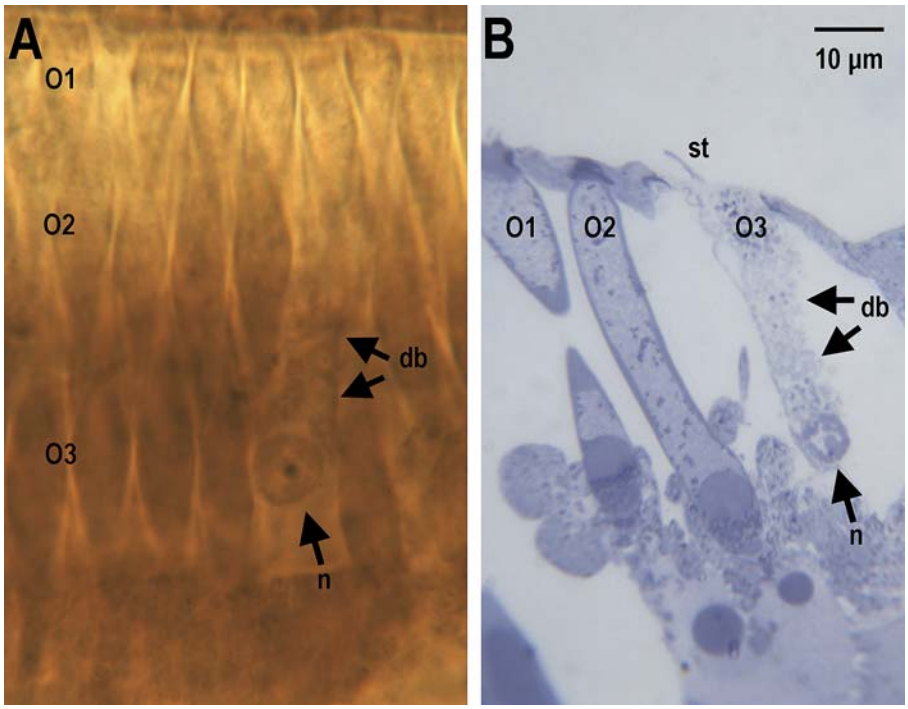

Fig. 4. Degenerating OHCs (arrows) following the third death pathway in the second turn of two cochleae that were exposed for 48 h to a 0.5-kHz OBN at 85 dB SPL (Group # 5, Table 1) and were fixed 1–2 h post-exposure. (A) Flat preparation viewed by phase-contrast microscopy. (B) One-micrometer-thick stained radial section. These degenerating OHCs in the second (A, O2) and third rows (B, O3) lack all or nearly all of their basolateral plasma membrane. Cellular debris (db, arrows) is arranged in a cylindrical fashion, similar to the shape of an intact OHC (B, O2). The nuclei (n, arrows) of the third death pathway OHCs are pale-stained and have chromatin clumped along the nuclear membrane. The apical membrane and stereocilia (st) persist longer than the basolateral membrane. Because these cells have deficient plasma membranes, they are already dead (i.e., necrotic). O1 – OHCs in the first row.

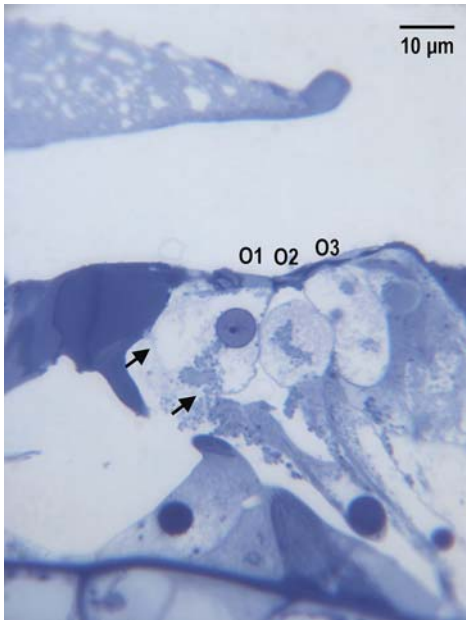

Fig. 5. One-micrometer-thick, stained radial section in the lower first cochlear turn of a cochlea that was exposed for 1 h to a 4-kHz OBN at 108 dB SPL and was fixed 1.5 h post-exposure. This cochlea was not included in the quantitative study. A first row OHC (O1) is necrotic (i.e., dead) as its basolateral plasma membrane is ruptured between the arrows. The cell contents have spilled into the Nuel space (by arrows). This cell died via the oncotic death pathway. OHCs in the second (O2) and third (O3) rows are oncotic as their basolateral membranes are still intact.

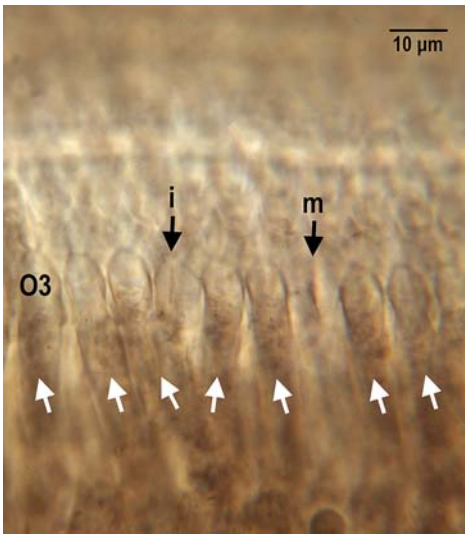

Fig. 6. Second turn of cochlea that was exposed for 24 h to a 0.5-kHz OBN at 95 dB SPL (Group # 6, Table 1) and was preserved 1.5 h post-exposure. Phase-contrast photomicrograph of the reticular lamina shows an immature (i) and a mature (m) phalangeal scar in the third row of OHCs (O3). Immature scars are distinguished from mature scars by the pale rather than dark line of junction between the processes forming the scar. The bodies of the third row OHCs that are present (white arrows) are out-of-focus; there are no OHC bodies beneath the scars.

the third death pathway, 3% were apoptotic, 0% was oncotic and 18% were unknown.

3.2. Cochleae exposed to 0.5-kHz OBN

The exposures for these cochleae ranged from 65 dB SPL for 48 h (Group # 1, Table 1) to 95 dB SPL for 48 h (Group # 7). Groups # 2 and 4 included cochleae that were exposed for 216 h. In the apical region, the cochleae exposed to the 0.5-kHz OBN were missing 0–8 IHCs (i.e., total of 74 IHCs across 30 specimens), with IHC loss averaging $0.5 \pm 0.5\%$. Fig. 7A shows, as a function of the logarithm to the base two (i.e., doublings) of total exposure energy, the percentages of missing OHCs in the apical region of the OC that were following the third, apoptotic and unknown death pathways. Relative to the total number of dead/dying OHCs ($N = 2852$ across 30 specimens), 78% were following the third death pathway, 1% was apoptotic, 1% was oncotic and 20% were unknown. Because of the very low percentage of oncotic OHCs in the noise-exposed cochleae in the quantitative portion of the study, these data were not plotted in Fig. 7. OHC loss by the third death pathway exceeded that by the apoptotic and unknown death pathways for all total energies. Apical OHC loss by the third death pathway was significantly greater (i.e., $p < 0.005$–0.001) in the 0.5-kHz OBN-exposed cochleae than in control cochleae for exposures with total energies greater than 2^{13} Pa^2 s (i.e., Group # 4, Table 1).

In the basal region, all cochleae exposed to the 0.5-kHz OBN were missing 0–10 IHCs (i.e., total of 43 IHCs across 30 specimens), with IHC loss averaging $0.5 \pm 0.6\%$. Fig. 7B shows, as a function of total exposure energy, the percentages of missing OHCs in the basal region that were following the third, apoptotic and unknown death pathways for the 0.5-kHz OBN-exposed cochleae. Total OHC loss was minimal but loss by the third death pathway exceeded that by the apoptotic and unknown death pathways for all total energies. Relative to the total number of dead/dying OHCs ($N = 187$ across 30 specimens), 73% were following the third death pathway, 6% were apoptotic, 1% was oncotic and 20% were unknown. These losses were not significantly different from the losses in the basal region of controls.

3.3. Cochleae exposed to 4-kHz OBN

In the basal region, all cochleae exposed to the 4-kHz OBN were missing 0–22 IHCs (i.e., total of 89 IHCs across 28 specimens, including 37 in seven IHC focal lesions), with IHC loss averaging $1.1 \pm 2.0\%$. The exposures for these cochleae ranged from 57 dB SPL for 48 h (Group # 1, Table 1) to 86 dB SPL for 48 h (Group # 6). Groups # 2, 3, and 4 included cochleae that were exposed for 216 h. Fig. 7C shows, as a function of total exposure energy, the percentages of missing OHCs in the basal region that were following the third, apoptotic and unknown death pathways. OHC loss by the third death pathway exceeded that by the apoptotic and unknown death pathways for all total energies. Relative to the total number of dead/dying OHCs ($N = 820$ across 28

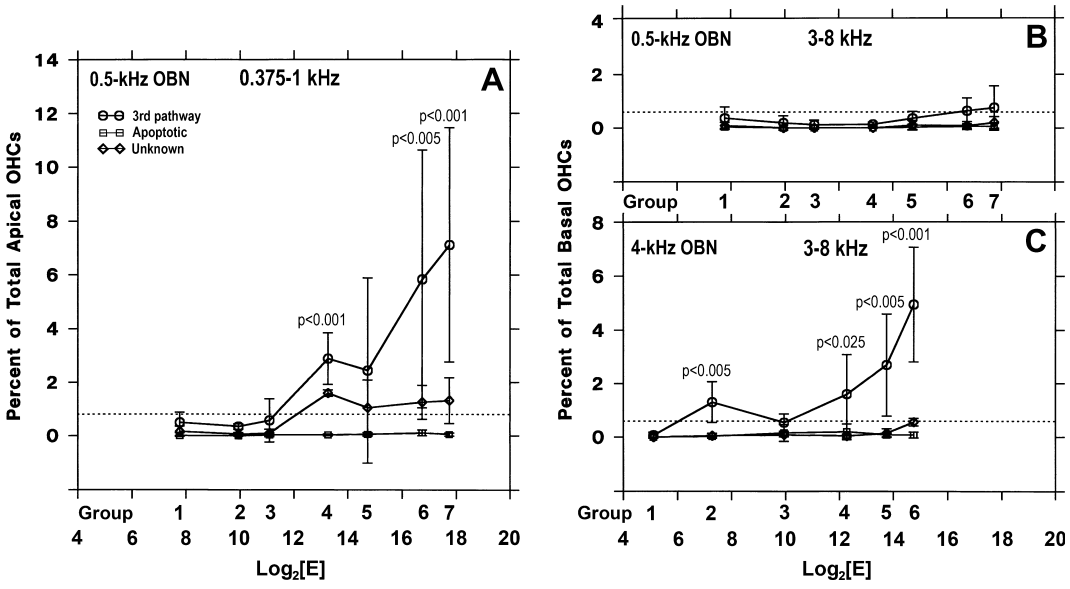

Fig. 7. The mean percentage (±SD) of OHCs following the third (circles), apoptotic (squares) or unknown (diamonds) death pathways as a function of exposure group and total exposure energy (E): (A) 0.375–1-kHz region in cochleae exposed to the 0.5-kHz OBN ($N = 30$), (B) 3–8-kHz region in cochleae exposed to the 0.5-kHz OBN (i.e., same cochleae as in 'A') and (C) 3–8-kHz region in cochleae exposed to the 4-kHz OBN ($N = 28$). Dotted lines in 'A', 'B' and 'C' indicate mean OHC death by the third pathway plus one standard deviation in control cochleae ($N = 9$). For these moderate exposures, the number of OHCs following the third death pathway exceeded those dying by the apoptotic pathway and those that were unknown. Despite considerable inter-animal variability, the number of OHCs following the third death pathway increased significantly in the apex for 0.5-kHz OBN exposures (A) with total energies greater than 2^{13} Pa^2 s (Group # 4) and in the base for 4-kHz OBN exposures (C) with total energies greater than 2^{10} Pa^2 s (Group # 3).

specimens), 89% were following the third death pathway, 5% were apoptotic, 1% was oncotic and 5% were unknown. OHC loss by the third death pathway was significantly greater (i.e., $p < 0.025$–0.001) in the 4-kHz-exposed cochleae than in control cochleae for total energies greater than 2^{10} Pa^2 s (i.e., Group # 3). The difference in OHC death by the third pathway between Group # 2 cochleae and controls was also significant ($p < 0.005$).

4. Discussion

The most significant finding in this study was that after moderate exposures to a 0.5- or a 4-kHz OBN, most of the dying OHCs were following a non-apoptotic, non-oncotic death pathway. This pathway, termed here the third death pathway, was morphologically distinct from the other two pathways. It is possible that some missing OHCs in our cochleae were apoptotic before they disappeared but they were classified as dying by an 'unknown' death pathway. However, even if all missing OHCs that were classified as unknown death pathway had been apoptotic before they disappeared, the number of apoptotic OHCs would still be considerably less than third death pathway OHCs. By not including cochleae with focal OHC lesions in the quantitative analysis, this study focused on OHCs that were damaged directly by noise rather than secondarily by such processes as ion leaks through the damaged reticular lamina.

4.1. Is the third death pathway a variant of oncosis?

Oncotic OHCs are swollen with pale cytoplasm and pale nuclei (Figs. 1 and 2). As these cells continue to swell, organelles rupture and disappear. When the plasma membrane ruptures, the cell is necrotic (Fig. 5). It is very unlikely that the organelles remaining in a necrotic OHC would reassemble into a cylindrical shape and the nucleus shift to the cell's original basal pole, thus causing an oncotic OHC to take on the appearance of a third death pathway OHC (Figs. 1 and 4).

4.2. Is the third death pathway a variant of apoptosis?

We believe that the third death pathway is not a variant of apoptosis for two reasons. First, the morphology of third death pathway OHCs (Figs. 1 and 4) is quite distinct from that of cells dying by apoptosis (Figs. 1 and 3) with respect to the condition of the plasma membrane, the appearance of the nucleus and the distribution of debris in the OC. Second, in many of the cochleae examined in this study, a few OHCs with the typical appearance of apoptosis were seen in the same cochlear regions as OHCs following the third death pathway. It seems highly unlikely that two morphologically distinct variants of apoptosis would appear in the same region of the cochlea simultaneously.

4.3. How do the present findings compare to findings in previous studies of cell death in the noise-damaged cochlea?

Previous studies of noise-induced OHC death reported that many of the cells in the center of the damaged zone were apoptotic; a few were necrotic (Hu et al., 2000, 2002a,b, 2006; Nicotera et al., 2003; Yang et al., 2004; Han et al., 2006). On the other hand, our results indicate that apoptotic OHCs were uncommon in cochleae fixed 1–2 h after a moderate noise exposure; third death pathway OHCs were much more common (Fig. 7).

We believe that there are plausible explanations for the discrepancies between the previous studies and the current study with respect to cell death pathways in the noise-damaged cochlea. First, in the studies by Hu and colleagues, OHC death pathways were determined primarily on the basis of nuclear shape, size and staining characteristics with propidium iodide or Hoechst 33342. The plasma membranes of the dying cells and cellular debris were not visible. OHCs following the third death pathway have swollen nuclei and little stainable nucleoplasm (Figs. 1 and 4). Thus, in Hu and colleagues' studies, some OHCs following the third death pathway may have been mistakenly identified as necrotic OHCs; other third death pathway OHCs may have been invisible. Second, to analyze death pathways in OHCs, fixed cochleae were dissected in buffer (i.e., so-called 'wet dissections') by Hu and colleagues and Han et al. (2006). Artifacts such as tissue distortion and fragmentation during the preparation of wet dissections may have resulted in the disappearance or redistribution of cellular debris in the OC. The loss of cellular debris from wet dissections of the OC would make it more difficult to identify OHCs following the third death pathway. Finally, Hu and colleagues identified cell types in flat preparations of the OC on the basis of nuclear position. In the noise-damaged OC, hair-cell and supporting-cell nuclei are often displaced from their usual positions (e.g., Covell, 1963; Beagley, 1965; Harding et al., 1992; Ahmad et al., 2003). Thus, determining cell type solely on the basis of nuclear position can be problematic in the severely noise-damaged OC.

In our laboratory, we have always prepared cochleae for microscopic examination by fixing them in vivo, embedding the entire specimen in plastic and then dissecting the specimen after plastic polymerization. Debris from degenerating hair cells likely remained very near where it was generated because the OC and basilar membrane were intact throughout fixation, dehydration, plastic infiltration and cochlear dissection. Being able to visualize cellular debris and determine its distribution in the damaged OC is important when attempting to identify death pathways in hair cells using morphological criteria. We have seen OHCs with morphological appearances like that shown in Fig. 4 since the middle 1970's. However, the prevalence of OHCs having this appearance was not realized until we began to use moderate noise exposures that were borderline for producing permanent threshold shifts, and we

began to evaluate hair-cell loss in noise-damaged cochleae on the basis of cell-death pathways.

4.4. Is the third death pathway unique to the cochlea?

The cochlea has certain features that make it unique in the body. First, there is a +80 to +100 mV potential in the endolymphatic space (e.g., Wangemann and Schacht, 1996) and thus, a 150–170 mV potential drop across the apical plasma membrane of OHCs. Second, OHCs are held in the reticular lamina by tight junctions between their apices and the phalangeal processes from the surrounding outer pillars and/or Deiters' cells. The reticular lamina forms the boundary between the endolymphatic space and the fluid spaces within the OC (e.g., Bohne, 1976c; Slepecky, 1996). The apical plasma membranes of the OHCs are bathed in endolymph (i.e., 157 mEq/L K^+ and 1 mEq/L Na^+) while their basolateral plasma membranes are bathed in a perilymph-like fluid (i.e., 148 mEq/L Na^+ and 4 mEq/L K^+) (e.g., Wangemann and Schacht, 1996).

Endolymph is similar in ionic composition to intracellular fluid. Thus, exposure of the intracellular compartment to endolymph via a damaged apical membrane should not cause additional injury to an OHC. However, once an OHC degenerates, the basolateral membranes of nearby non-damaged OHCs are exposed to an increased concentration of potassium and a reduced concentration of sodium than is found in perilymph. Perhaps alteration of the external environment of the basolateral membrane of OHCs leads to their disruption.

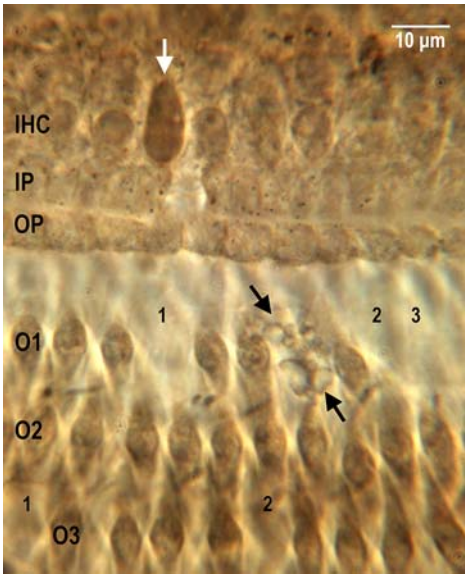

Fig. 8. Second turn of gerbil cochlea that was exposed for 48 h to a 0.5-kHz OBN at 110 dB SPL and was fixed 10 min post-exposure. Five OHCs [i.e., three 1st row (O1, 1, 2, 3) and two 3rd row (O3, 1, 2)] are missing. Cellular debris (between black arrows) is arranged in a cylindrical pattern where one 1st row OHC is degenerating. One inner pillar (IP) is missing near center. One darkly stained, shrunken IHC (white arrow) appears to be dying by the apoptotic pathway. O2 – Second row OHCs; OP – outer pillar heads.

4.5. Is the third death pathway unique to chinchilla OHCs?

Our permanent collection of plastic-embedded flat preparations includes the cochleae from several noise-exposed gerbils that were fixed less than one h post-exposure. Gerbils that had been exposed for 48 or 120 h to a 0.5-kHz OBN at 110 dB SPL sustained moderate losses of OHCs at about 15–20% distance from the apex. Inspection of these specimens revealed a moderate amount of cellular debris dispersed in the fluid spaces of the OC. In some instances, the debris was arranged in a cylindrical fashion beneath the reticular lamina (Fig. 8). Thus, our very preliminary data suggest that the third death pathway may also occur in gerbil OHCs.

4.6. What is the underlying mechanism of the third death pathway?

The underlying process(es) that lead to OHC loss by the third death pathway are unknown. However, under the transmission electron microscope, the apical membranes of OHCs in cochleae with moderate temporary threshold shifts appear damaged, as if they were turning over more rapidly than usual (Schmitt et al., 2004). Perhaps OHCs that cannot maintain the repair of their apical plasma membranes die by the third death pathway. Also, we do not yet know if the third death pathway is an 'active' or a 'passive' process. Clearly, further work must be done on cell-death pathways in the noise-damaged cochlea. Determining death pathways and the mechanisms of cell death are important for developing pharmacological treatments to prevent or minimize noise-induced OHC death.

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