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Apoptotic outer hair cell death in the cochleae of aging Fischer 344/NHsd rats

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Abstract

Apoptotic cell death has been implicated in cochlear degeneration during aging. To better understand the impact and the biological process of outer hair cell (OHC) apoptosis, we investigated the contribution of apoptotic cell death to the formation of the OHC lesions, and observed the temporal patterns of the occurrence of apoptotic events associated with the mitochondrial pathway in Fischer 344/NHsd rats, with ages ranging from 20–27 months. The results showed that the ratio of apoptotic to necrotic OHCs was 8:1. During the process of cell degeneration, the onset of Bax expression, cytochrome c release, and nuclear DNA fragmentation preceded the onset of nuclear condensation. In contrast, the activation of caspases-3 and -9, as well as the degradation of F-actin, took place after the onset of nuclear condensation. The results of this study suggest that the initiation of nuclear degradation is a caspase-3-independent process. Moreover, the study revealed that OHCs with Bax expression or cytochrome c release could enter either the apoptotic or necrotic pathway, suggesting the presence of a regulatory mechanism that guides degenerating OHCs to die via either the apoptotic or necrotic pathway.

Keywords

Aging; apoptosis; necrosis; outer hair cell; Fischer 344/NHsd rat

1. Introduction

Age-related hearing loss (ARHL) is the most common form of acquired hearing loss in the adult population. As our society matures, there are more people living into their 60s, 70s, 80s, and beyond. Within the elderly segment of our society, hearing loss is second to arthritis as a handicapping condition. Certainly, ARHL in humans is a reflection of both the genetics of the individual and the history of exposure to noise, drugs, and diseases. While there are data on the prevalence and pathology associated with ARHL, the mechanisms underlying ARHL are not well understood.

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Apoptotic cell death has been implicated in cochlear degeneration from aging. Apoptosis is an energy-dependent process, characterized by a set of unique morphological and biological features (Lallemend et al., 2005; Nicotera et al., 2003; Van De Water et al., 2004). Usami et al. (1997) reported the appearance of nuclear DNA fragmentation, a phenotype of apoptosis, in the cochlea of senescence accelerated mice. Zheng et al. (1998) used a semi-quantitative method to correlate the number of cochlear cells having DNA fragmentation with the functional changes in distortion-product otoacoustic emissions (DPOAE) in aged gerbils and found that a greater DPOAE decrement was associated with greater apoptotic activity in the aged cochleae. These studies clearly implicated apoptotic cell death in the cochlear pathogenesis and suggest the role of cochlear apoptosis in the generation of ARHL.

Apoptosis is a tightly controlled cell death process, requiring the participation of a cascade of signal transductions and the interaction of multiple apoptotic molecules. Apoptosis involves two essential signal pathways, the membrane and the mitochondrial pathways. Using a luminescent assay, Nevado et al. (2006) documented an increase in caspase-9 activity in aged cochleae. In contrast, caspase-8 activity was not significantly affected by age, suggesting the involvement of the mitochondrial pathway in the apoptotic process of aged cochleae. Consistent with the notion of mitochondrial involvement, Riva et al. (2005) reported the release of cytochrome c in aging inner ears. In addition, activation of caspase-3, a downstream event of caspase-9 activation, also has been observed in aged cochleae (Alam et al., 2001; Nevado et al., 2006). So far, the involvement of multiple apoptotic events in aging cochleae has been well established. However, the temporal sequence and the spatial distribution, as well as the interplay, of these apoptotic events in hair cells are still not known.

Under stress, cells can die through several death pathways. For example, studies have shown that exposure to intense noise causes hair cells to die through apoptosis or necrosis (Hu et al., 2000). A third cell death pathway, featured morphologically by nuclear condensation with maintenance of cell shape, but loss of the plasma membrane, also has been observed in noise-traumatized cochleae (Bohne et al., 2007). In the aging cochlea, although apoptotic OHC death has been identified, its prevalence and the ratio of the apoptotic to necrotic contribution to pathogenesis of OHC lesions in aging cochleae remain to be determined.

The current study was designed to expand our current understanding of age-related cochlear degeneration. Three specific aims were: (1) to determine the contribution of apoptotic and necrotic cell death to OHC lesions; (2) to describe the temporal patterns of the apoptotic events related to the mitochondrial pathway of apoptosis, including Bax expression, cytochrome c translocation, and caspase-9 activation, as well as downstream events, including activation of caspase-3, fragmentation of nuclear DNA, and degradation of F-actin; and (3) to learn how these apoptotic events are interrelated.

2. Materials and Methods

2.1. Animals

A total of 45 Fischer 344/NHsd rats, obtained from Harlan Laboratories, were used in the study. The animals were assigned to one of two groups: the young rat group, ages 3–5 months ($n = 19$) or the aging rat group, ages 20–27 months ($n = 26$). The cochleae from these animals were further assigned to defined groups for physiological and pathological examinations except for one accidentally-damaged young cochlea during dissection. All procedures involving use and care of the animals were reviewed and approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

2.2. Measurement of auditory brainstem response (ABR) thresholds

ABR thresholds were measured in 13 of the 19 young rats and 15 of the 26 aging rats. The animals were anesthetized through the inhalation of Isoflurane (3–4% at the flow rate of 1.5L/min for induction and 0.8L/min for maintenance). ABR thresholds were measured with needle electrodes placed subdermally over the vertex (the positive electrode), the ipsilateral mastoid (the negative electrode), and the shoulder blade (the ground electrode). The ABR was elicited with tone bursts generated digitally (TDT, SigGen) and played out through a D/A converter (TDT, RP2.1, 100 kHz sampling rate) in a signal averaging system. Tone bursts were presented with a high frequency speaker (model: AS-TH400A) at 5, 10, 20, and 40 kHz (0.5 ms rise/fall time, no plateau, alternating phase) at the rate of 21 per second. The stimulus levels were varied in 5 dB descending steps, and the ABR thresholds were defined as the lowest intensity that elicited a response.

The output of the electrodes was led to the head stage of a differential amplifier (TDT, RA4LI), followed by an amplifier (TDT, RA16PA), and then routed by a fiber optic cable to a real-time processor (TDT, RA16BA, 24 kHz sampling rate) that is part of an auditory-evoked response averaging system controlled by TDT software (BioSig). The input signal was amplified (~50,000×), filtered (100–3000 Hz), processed with an artifact reject at 80% of the full scale, averaged (250 sweeps), and then stored and displayed on a computer.

2.3. Quantification of dying and missing OHCs

2.3.1. Propidium iodide (PI) staining—PI staining was performed to visualize the cell nuclei. In most specimens, the PI staining was used as a counterstaining for another histochemical or immunohistological assay. For these specimens, PI staining was performed in fixed tissues after the completion of the first staining (see the following sections). Then, the specimens were incubated in the PI solution (5 µg/ml in PBS) for 10 min.

To determine the viability of OHCs, PI staining was used *in vitro* in 10 cochleae (5 young and 5 aging cochleae). PI is a membrane-impermeable dye. When performed *in vitro*, this probe is excluded from the cells that have normal integrity of the plasma membrane. For *in vitro* staining, the cochleae were stained before fixation. After sacrifice of the animals, the cochleae were collected. Then, the PI staining solution (about 50 µl at the concentration of 5 µg/ml in 10 mM PBS) was gently perfused into the cochleae through the round window, and the excessive solution was allowed to efflux through the oval window. Then, the cochleae were incubated in the same staining solution for 10 min at room temperature. After the PI staining, the cochleae were fixed with 10% buffered formalin.

2.3.2. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)—

A TUNEL assay was used to detect nuclear DNA fragmentation. The procedure has been previously described (Nicotera et al., 2003). Briefly, the cochleae were fixed with 10% buffered formalin and dissected. The organs of Corti were transferred to ice-cold 70% (v/v) ethanol and stored overnight at –20° C. The tissues were washed and then incubated with DNA-labeling solution (Molecular Probes, Inc.) containing 10 µl of reaction buffer, 0.75 µl of TdT enzyme, 8.0 µL of BrdUTP, and 31.25 µL of dH₂O for 16 hrs at room temperature. The tissues were stained with Alexa Fluor 488 dye-labeled anti-BrdU antibody (5 µL of antibody plus 95 µL of washing buffer) at room temperature for 1 hr. After the labeling, the tissues were further stained with PI.

2.3.3. F-actin staining—F-actin, an important structural protein of OHCs, was stained to illustrate the cuticular plates for quantification of missing OHCs and for assessment of protein degradation of OHCs during apoptosis. The procedure for the F-actin staining has been previously described (Hu et al., 2002). Briefly, upon completion of cochlear dissection, the

organs of Corti were transferred to the freshly-prepared staining solution, containing FITC-phalloidin (1:250, Sigma), 0.25% Triton-X-100, and 1% bovine serum albumin in PBS and were incubated at room temperature for 30 min.

2.3.4. Criteria for identification of apoptotic, necrotic, and missing OHCs—The criteria for determination of apoptotic, necrotic, and missing OHCs have been previously described (Nicotera et al., 2003; Yang et al., 2004).

Apoptotic OHCs: The OHCs showing condensed nuclei were counted as apoptotic cells. Nuclear condensation was determined when the nucleus became smaller in size, with the increase of PI fluorescence, than were the nuclei of neighboring viable cells. We also used the TUNEL assay as a complementary method to confirm the nature of apoptosis. All OHCs exhibiting nuclear condensation had a strong TUNEL fluorescence.

Necrotic OHCs: The primary criterion for determination of necrotic OHCs was nuclear swelling illustrated by PI staining. The morphologies of nuclear swelling were characterized by enlarged sizes, malformed shapes, and loss of normal texture of the nuclei. When the PI staining was performed *in vitro*, necrotic nuclei had a strong PI fluorescence, indicating the presence of membrane leakage.

Missing OHCs: The missing OHCs were recognized in the areas of the cuticular plates that lost F-actin labeling. In certain apoptotic cells, particularly those at the later stage of the death process, the F-actin fluorescence in the cuticular plates was significantly decreased, but the condensed nuclei were still present. To prevent double counting of the same cell as both missing and apoptotic, examinations of the cuticular plates and the nuclei were always carried out simultaneously. Priority was given to the presence of a condensed nucleus and that cell would be scored as apoptotic, regardless of the status of F-actin.

2.4. Assessment of apoptotic events

2.4.1. Caspase staining—Caspases-3 and -9 were detected using two fluorescent probes, FAM-DEVD-FMK and FAM-LEHD-FMK, respectively (Intergen Company, Purchase, NY). The basic labeling procedures for detecting the two caspases were similar and have been described previously (Nicotera et al., 2003). Briefly, the animal was anaesthetized with a mixture of xylazine (6 mg/kg, i.m.) and ketamine (50 mg/kg, i.m.). Either the left or right cochlea was surgically exposed using a conventional ventral approach. The cochlea was perfused through the round window with approximately 30 μ l of a freshly-prepared staining solution containing one of two caspase probes (diluted according to the manufacturer's instructions) and incubated for 1 hr. The cochlea was then perfused with the manufacturer-provided buffer, followed by the manufacturer-provided fixative. The animal was sacrificed and the cochlea was harvested. The cochlea was then dissected in 10mM PBS.

2.4.2. Bax staining—The animals were sacrificed and the cochleae were quickly removed from the skull and fixed with 10% buffered formalin for 4 hrs. Then, the cochleae were dissected in 10 mM PBS and the organs of Corti were harvested. The tissues were permeabilized in a Triton X-100 solution containing 0.2% Triton X-100 and 0.5% bovine serum albumin in PBS (pH 7.4) for 1 hr at room temperature. Then, the tissues were incubated with a rat monoclonal anti-Bax antibody (1:200, Abcam Inc, Cambridge, MA) in a blocking solution (Blocker Casein, Pierce) overnight at 4° C. After rinsing with PBS, the tissues were stained with the second antibody, FITC-labeled rabbit polyclonal against Rat IgG H&L (1:100, Abcam Inc.) in 10 mM PBS, for 1 hr at room temperature. After another rinse in PBS, the specimens were further stained with the PI solution for 10 min. To assess non-specific staining, a few

pieces of specimens were stained with the second antibody only (omitting the primary antibody).

2.4.3. Detection of cytochrome c—The animals were sacrificed and the cochleae were quickly removed from the skull and fixed with 10% buffered formalin for 4 hrs. The procedure for cytochrome c staining has been previously described (Nicotera et al., 2003). Briefly, after dissection, the organs of Corti were incubated with a blocking solution (Blocker™ Casein in PBS, Pierce Chemical Company) for 60 min at room temperature. The specimens were permeabilized with 0.2% Triton X-100 in PBS for 30 mins and then incubated with a primary antibody (anti-cytochrome c, 6H2.B4; BD PharMingen, San Diego, CA) at a concentration of 2.5 µg/ml in the blocking solution overnight at 4° C. The cochlear tissues were then incubated with a second antibody (Cy2-conjugated goat anti-mouse IgG, 1: 200, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hr. The specimens were doubly stained with PI for 10 min. To assess non-specific labeling, several pieces of specimens were incubated only with the second antibody (without incubation with the primary antibody).

2.5. Experimental procedures

Animals first received the ABR test. Then, they were assigned to defined groups for pathological assessments. The animals were either anesthetized for *in vivo* staining (caspase staining) or sacrificed for immunolabeling after fixation or for PI staining either before or after fixation. After the staining, the organs of Corti were mounted on slides (whole mount) for microscopic examination. First, the organs of Corti were thoroughly examined with a fluorescence microscope. The numbers of apoptotic, necrotic, and missing OHCs were quantified along the length of the organ of Corti. These quantitative data were used to construct the cochleograms. Then, the cochlear regions showing active OHC death were further examined and photographed with confocal microscopy. Use of confocal microscopy to inspect the OHC pathology reduced the ambiguity in the distinction between OHC nuclei and supporting cell nuclei. The numbers of the cochleae used for each test are presented in Table 1.

2.6. Statistical analyses

A two-way ANOVA (Age × Frequency) was used to analyze the differences between the mean ABR thresholds of the two aging groups (young and aging) across the four frequencies of the acoustic stimuli (5, 10, 20, and 40 kHz). Age and Frequency were analyzed as between-subjects variables. If a significant main effect occurred for Age or Frequency, post hoc testing with a Tukey test was performed to delineate the nature of the differences. A *t*-test was used for comparison of the numbers of apoptotic and necrotic OHCs in the aging cochleae, and a one-way ANOVA was used for comparison of the difference in the numbers of OHCs showing different phenotypes of apoptosis. For all statistical analyses, the significance value was set at $p < .05$.

3. Results

The average hearing thresholds of ABR at the frequencies of 5, 10, 20, and 40 kHz for the two age groups (young and aging) are presented in Fig. 1A. There are significant threshold shifts ($p < .05$) at all tested frequencies between the young and the aging group.

3.1. Progression of OHC degeneration in aging Fischer 344/NHsd rats

Aging degeneration involves both OHCs and inner hair cells. In the present study, the focus was placed on OHC pathology because OHCs were the primary site of sensory cell degeneration.

Morphological examination of the cochleae from young control rats revealed only a few missing OHCs ($0.44\% \pm 0.73\%$ mean $\pm$ SD) scattered over the length of the organs of Corti. In contrast, the aging cochleae exhibited evident OHC pathologies, including apoptotic and necrotic changes, as well as signs of missing OHC. To assess the progression pattern of cochlear pathology, we divided the aging cochleae into two groups based on the animals' age: the 20- to 23-month group and the 24- to 27-month group. Fig. 1B shows the average percentages of degenerated OHCs (the sum of the missing OHCs and the OHCs with condensed or swollen nuclei) in the two aging groups and the young control group. Note that a large number of missing or degenerated OHCs are present in the apical and basal ends of the aging cochlea, whereas the middle part of the aging cochlea has a smaller number of missing or degenerated OHCs. The figure shows that the growth of OHC lesions accelerated as rats aged. From 20–23 months to 24–27 months, the basal lesion grows significantly faster than does the apical lesion (an increase of 32.2% in the basal part vs. an increase of 12.8% in the apical part of the cochleae, $p < .05$).

3.2. Ratio of apoptosis and necrosis in aging cochleae

The presence of the two cell death pathways, apoptosis and necrosis, in aging cochleae was identified using nuclear morphology and was confirmed by biological assays. Figs. 2A and 2B show two regions of the organs of Corti in two aging cochleae stained with PI *in vitro*. The arrows in 2A and 2B point to the OHC nuclei with an increased PI fluorescence, an indication of cell membrane disruption. Note that the nuclei of these dying cells appear condensed, suggesting that these cells were dying by apoptosis. Necrotic OHCs were also identified in the organs of Corti of the aging cochleae. The arrowheads in Fig. 2B point to the nuclei displaying typical nuclear morphologies of necrosis, i.e., the increase of the PI fluorescence (indicative of membrane deterioration) and enlargement of the nuclear size.

To determine the contributions of each mode of cell death to the pathogenesis of OHC lesions of aging cochleae, the numbers of OHCs showing signs of apoptosis or necrosis were counted from 19 aging cochleae (20–27 months). Fig. 3 shows the comparison of the average numbers of apoptotic and necrotic OHCs per cochlea. The number of apoptotic OHCs is significantly greater than that of necrotic cells ($p < .001$), with a ratio of 8:1.

3.3. Early events of OHC degeneration

Using the nuclear morphology as a temporal index, the apoptotic events were divided into the early and the late events of OHC degeneration. The onset of the early events was prior to the appearance of nuclear shrinkage.

3.3.1. Expression of Bax protein in OHCs of aging cochleae—Examination of Bax immunoreactivity in young cochleae (< 5 months) showed only a weak Bax fluorescence in the organ of Corti, except for a few scattered OHCs, where increased Bax fluorescence was present. Fig. 4A shows a typical image of Bax staining in a young cochlea (4 months).

There was a marked increase of Bax immunoreactivity in the aging cochleae, but the level of the increase varied across individual OHCs. Some cells exhibited strong Bax fluorescence, whereas others lacked Bax fluorescence. Double labeling of OHCs with PI revealed that Bax immunoreactivity was present in the OHCs with normal nuclear morphology, as well as in the OHCs with swollen nuclei. Fig. 4B shows an area of the organ of Corti from an aging cochlea (20 months). Arrows point to the OHCs with increased Bax fluorescence. These cells exhibit normal nuclear morphology. The double arrow indicates a Bax-positive OHC showing nuclear swelling. Fig. 4C shows an area of another cochlea in which nuclear condensation is visible. Note that the OHCs displaying clear nuclear condensation lack Bax fluorescence (marked by

the arrowheads). These results suggest that Bax expression is an early event of OHC degeneration.

The average of the numbers of OHCs showing strong Bax immunolabeling per cochlea was 645 ± 98 (mean $\pm$ SD), approximately 15% of the total OHC population. These cells were distributed in the entire length of the organ of Corti, but were more prominent in the basal end of the organ of Corti (Fig. 4D).

3.3.2. Release of cytochrome c—Microscopic examination of immunolabeled cytochrome c revealed no detectable cytochrome c immunoreactivity in the normal OHCs of young cochleae, except for a few cells that occasionally exhibited the cytochrome c fluorescence. Fig. 5A shows the cytochrome c staining of a young cochlea (5 months). Note that there is no cytochrome c fluorescence in any of the OHCs, except for one that shows nuclear malformation and a slight increase in cytochrome c fluorescence (marked by the arrow).

In the aging cochleae, there was a marked increase in the number of OHCs showing cytochrome c labeling. In general, cytochrome c fluorescence was present in OHCs having normal nuclei or having the nuclei with increased PI fluorescence, but without marked shrinkage (arrows in Figs. 5B and 5C). Cytochrome c fluorescence was also present in OHCs having swollen nuclei (arrows in Fig. 5D). In contrast, it disappeared in OHCs showing nuclear condensation (marked by the arrowhead in Fig. 5C). The average number of OHCs exhibiting cytochrome c immunoreactivity per cochlea was 278 ± 78 (mean $\pm$ SD), accounting for approximately 6.5% of the total OHC population. These positively-stained cells were present in all cochlear turns, but more prominent in the basal and apical parts of the cochlea (Fig. 5F).

3.3.3. Nuclear DNA degradation—A TUNEL assay was used to detect the presence of nuclear DNA fragmentation. All OHC nuclei having clear nuclear condensation showed TUNEL fluorescence (see the green fluorescence marked by the arrows in Figs. 6A and 6B). Certain OHCs, primarily in the basal end of the cochlea, with a relatively normal appearance of the nuclei, also exhibited strong TUNEL fluorescence (Fig. 6B, marked by the arrow within the white frame; also see the inset in Fig. 6B for a higher magnification view). These cells are likely to be at the early stages of OHC degeneration. The necrotic cells also exhibited TUNEL fluorescence, but with less intensity (data not shown).

3.4. Late events of OHC degeneration

3.4.1. Activation of caspases-3 and -9 in the aging cochlea—Caspases are a family of cysteine proteases responsible for the initiation and execution of the apoptotic process. In the young cochleae, there was no detectable activity of the two examined caspases (data not shown).

In the aging cochleae, the OHCs having normal nuclear morphology lacked caspase activity. The OHCs showing slight nuclear condensation also lacked caspase activity. Detectable caspase activity appeared only in the OHCs exhibiting marked nuclear condensation. The basic patterns of caspase-3 and caspase-9 distribution in the organ of Corti were similar. Fig. 7A illustrates a typical image of caspase-3 staining in an aging cochlea. The arrows indicate strong caspase-3 activity (the green fluorescence) around two condensed OHC nuclei. The neighboring OHCs with normal nuclear morphology lack caspase-3 activity. Fig. 7B shows an example of caspase-9 staining. Note that the activity of caspase-9 is distributed in the OHCs with condensed nuclei.

The number of OHCs showing caspase fluorescence was 3 to 7 per cochlea, significantly lower than the numbers of the Bax-positive and cytochrome c-positive OHCs (ANOVA, $p < .01$, Fig.

8). Like the Bax and cytochrome c distribution, caspase-positive cells were more evident in the basal end of the cochlea.

3.4.2. Cleavage of F-actin during the process of apoptosis—F-actin is an important structural protein in the OHC, distributed primarily in the stereocilia and the cuticular plate of the OHC. F-actin was examined to assess the progress of structural protein degradation during apoptosis. In the young cochleae, the F-actin distribution in the OHC population was homogeneous. All OHCs exhibited a strong F-actin fluorescence in their cuticular plates (data not shown).

In aging cochleae, F-actin fluorescence was strong in the OHCs with normal nuclear morphology or with slight nuclear condensation. Reduction of the fluorescence intensity of F-actin was observed in the OHCs with more advanced nuclear shrinkage. However, the correlation between the extent of F-actin reduction and the degree of nuclear condensation was not strong.

Fig. 9 shows examples of the relationship between the levels of F-actin reduction and the level of nuclear condensation. The double arrows in Fig. 9A point to the OHCs showing slight nuclear condensation. These cells maintain normal F-actin labeling (the double arrows in 9B). The arrows in 9B and 9D point to the cuticular plates with partial reduction of F-actin staining. The corresponding changes in the nuclei of these cells are presented in Figs. 9A and 9C (arrows). Note that F-actin reduction appears after the occurrence of marked nuclear condensation. In addition to the partial reduction of F-actin staining, a complete loss of F-actin staining was seen in some apoptotic OHCs (marked by the arrowheads in Figs. 9D and 9F), suggesting that complete loss of F-actin staining precedes the complete degradation of apoptotic nuclei. In the site of the organ of Corti where the OHCs had completed degraded, phalangeal scars were commonly seen (marked by the asterisks in Figs. 9D and 9F).

4. Discussion

4.1. Sequence of apoptotic events

It has been well established that apoptosis is a tightly controlled death process. During this process, a cascade of apoptotic events is initiated and cellular structures are disassembled. To better understand the process of age-related OHC apoptosis in Fischer 344/NHsd rats, we investigated the temporal sequence of the manifestation of multiple apoptotic events in aging cochleae. Ideally, the methodology for this study would be a dynamic observation of apoptotic activity in living cochlear tissues. However, such an approach is technically unfeasible due to the fact that the mature OHCs are unable to survive in a culture condition. Therefore, in the current study, we used an alternative strategy. A common temporal index, the nuclear morphology, was used as a temporal marker for estimation of the temporal sequence of apoptotic events. Selection of the nuclear morphology as the temporal marker was based on the following considerations: (1) nuclear condensation is an essential event of the apoptotic process (Rogalinska, 2002); and (2) nuclear condensation progresses in a step-by-step fashion that can be assessed by monitoring the changes in the nuclear morphology.

The current study revealed that the onset of three apoptotic events, Bax expression, cytochrome c release, and nuclear DNA fragmentation, preceded nuclear condensation. In contrast, the onset of three other apoptotic events, activation of caspase-9 and caspase-3, as well as F-actin degradation, was evident after the occurrence of marked nuclear shrinkage.

Both Bax and cytochrome c are pro-apoptotic proteins. Overexpression of Bax protein in mammalian cells has been found to result in the induction of apoptosis through the release of cytochrome c from mitochondria (Jurgensmeier et al., 1998; Sawa et al., 2000). Although the

precise mechanism of the Bax-mediated release of cytochrome c is not clear, the consequence of this action is well documented, which includes activation of caspase-9 and then caspase-3 (Gorman et al., 2000; Jordan et al., 2003).

In the current study, strong Bax and cytochrome c immunoreactivity were observed in OHCs displaying normal or slightly condensed nuclei. When marked nuclear shrinkage occurs, the immunolabeling of cytochrome c and Bax disappeared. These results indicate that Bax overexpression and cytochrome c translocation take place in the early stage of OHC degeneration.

The apoptotic events that take place after the occurrence of nuclear condensation include activation of caspases-9 and -3 and degradation of F-actin. Conceptually, caspase-9 activation precedes the activation of caspase-3. However, we did not find a temporal gap between the two events, possible due to the rapidness of the signal relay from caspase-9 to caspase-3. Like caspase activation, degradation of F-actin is observed in apoptotic cells with marked nuclear condensation, whereas it is not evident in the OHCs with early nuclear condensation. The onset of F-actin degradation appeared to be concurrent with that of caspase activation, suggesting that F-actin cleavage in aging OHCs is mediated by caspase-3. This finding is consistent with our previous observation that inhibition of caspase-3 activity delayed the F-actin degradation in the noise-traumatized cochleae (Hu et al., 2002).

4.2. The onset of the nuclear events of apoptosis preceded the onset of caspase activation

In the current study, we found strong fluorescence of caspases-9 and -3 in the OHCs showing marked nuclear shrinkage. However, no detectable caspase fluorescence was found in the OHCs having mild nuclear condensation (an increase of PI staining intensity and a slight reduction of the nuclear size). Although we could not rule out the possibility of the occurrence of a low level activation of caspase, the current results showed that massive activation of the caspases occurs only at the later phase of nuclear condensation. This result suggests that caspase-3 activation may not be the essential initiator of the nuclear event of apoptosis.

Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of apoptosis. There is strong evidence from studies of non-cochlear tissues that the nuclear event of apoptosis is associated with the activity of caspase-activated DNase (CAD). Normally, CAD exists as an inactive complex (ICAD). The activation requires the involvement of caspase-3, which cleaves ICAD to release CAD. Studies have shown that inactivation of the caspase-3 gene delays cell death and largely abolishes chromatin condensation and DNA fragmentation in many cell types (Woo et al., 1998; Zheng et al., 1998). Similarly, inactivation of CAD prevents the advance of chromatin condensation (Liu et al., 1998). These studies clearly demonstrate the role of caspase-3 in nuclear degradation.

The results of our study, however, which showed that massive caspase-3 activation lags behind the onset of nuclear condensation, appear to be inconsistent with these earlier studies. The current result suggests that, in the aging process of cochlear hair cells, caspase-3 activation is indispensable for the initiation of nuclear condensation.

At least two alternative molecular pathways have been linked to the initiation of the nuclear event of apoptosis. The first pathway still involves the caspase family, but with other members of the family. For example, Ruchaud et al. (2002) reported the involvement of caspase-6 in chromatin condensation and nuclear fragmentation through cleaving lamins, intra-nuclear proteins that maintain the shape of the nucleus and mediate the interactions between chromatin and the nuclear membrane. The second pathway is caspase-independent. Susin et al. (2000) reported a caspase-independent pathway involving apoptosis-inducing factor (AIF). These previous studies suggest that nuclear condensation during apoptosis can be initiated by multiple

factors. Because caspase-3 activation is not completely disassociated with nuclear condensation in aging hair cells, but rather acts in a delayed fashion, we suspect that the initiation of nuclear condensation is a caspase-3-independent process, whereas the progression of the nuclear condensation is associated with caspase-3 activity. It is possible that the AIF-dependent and the caspase-3-dependent pathways are activated in a sequential fashion, with the AIF-induced nuclear apoptosis preceding that induced by CAD. This pattern of apoptosis has been previously reported (Susin et al., 2000). An understanding of the molecular mechanisms responsible for nuclear degradation of aging apoptotic OHCs warrants future investigation.

4.3. Apoptosis vs. necrosis in the generation of the OHC lesions in aging cochleae

The numbers of degenerated OHCs (active apoptotic and necrotic OHCs, as well as missing OHCs) were quantified for the cochleae of Fisher 344/NHsd rats between the ages of 20 to 27 months. Our results revealed that, while both apoptotic and necrotic OHCs are present in aging cochleae, the number of apoptotic OHCs is significantly greater than that of necrotic OHCs, with a ratio of 8:1. The study provides strong evidence that the mode of action of age-related pathogenesis of OHC lesions in Fischer 344/NHsd rats proceeds primarily through apoptosis, with necrosis being a lesser contributor. This result is consistent with earlier studies showing TUNEL labeling of apoptotic cells in the SAM mouse (Usami et al., 1997). The result also complement the research of Nevado et al. (2006), who found an increase of caspase-3 activity in biochemical studies of the cochlea of Sprague-Dawley rats. Bohne et al. (2007) reported a third form of cell death in noise-traumatized cochlea, one in which a cell dies and leaves no plasma membrane, only cellular debris shaped in the form of OHC following acoustic trauma. In the current study, we were unable to confirm the presence of such pathology in the aging cochlea due to the limitations of the light microscopy, which was unable to reveal the fine structures of the cells.

One interesting finding of the current study is that cytochrome c translocation and Bax expression manifest not only in the OHCs displaying nuclear condensation, but also in the OHCs with swollen nuclei. This observation suggests that the OHCs with the early apoptotic activity can end up dying by either apoptotic or necrotic process. The occurrence of Bax expression and cytochrome c release in cells having necrotic nuclei has been observed in other pathological conditions (Nicotera et al., 2003; Shiraishi et al., 2001). Several possible regulatory mechanisms responsible for directing a cell to die by apoptosis or necrosis have been proposed. The regulatory factors include the energetic status and/or the level of oxidative stress of dying cells (Galan et al., 2001; Gumprich et al., 2000; Komatsu et al., 2000; Lieberthal et al., 1998; Shiraishi et al., 2001). It is possible that all the degenerated OHCs are initially set to die through the process of apoptosis. However, during apoptosis, possibly at the initial phase, certain cells are diverted to necrosis due to the deterioration of energy production or to a high level of oxidative stress. An understanding of the precise molecular mechanisms that are responsible for controlling cell death regulation in aging hair cells requires further research.

4.4. Discrepancy between the numbers of Bax-positive and cytochrome c-positive cells and the numbers of caspase-positive cells and cells with condensed nuclei

Another interesting finding of the current study is that the numbers of Bax-positive and cytochrome c-positive hair cells are significantly greater than those of caspase-positive cells and cells having condensed nuclei. Clearly, the hair cells showing caspase activity and nuclear condensation are in the later phases of apoptosis. However, the status of Bax-positive and cytochrome c positive cells is complicated. As discussed earlier, certain OHCs with Bax expression and cytochrome c translocation could enter the necrotic pathway. Given the finding that, in the aging cochleae, the ratio of apoptotic to necrotic cells is 8:1, we think that the shift of the cell death pathway alone can only account for a small portion of the difference. The

second possibility is that the early phase of OHC degeneration progresses more slowly than does the late phase of apoptosis. The accelerated apoptotic execution in the late phase of apoptosis quickly eliminated caspase-positive cells and cells having nuclear condensation. Consequently, these cells were less likely to be seen in the pathological observation.

The number of Bax-positive cells is greatest among the examined parameters in the aging cochleae. Certainly, the expression of Bax cannot be a sign of imminent cell death for all the positive cells. An increase in Bax expression without a commitment to apoptosis has been observed in the aging process of brain tissue (Thees et al., 2005). Here we offer two additional explanations for the large number of Bax-positive cells in the aging cochleae. First, the pro-apoptosis function of Bax is regulated by certain anti-apoptotic proteins. Evidence has emerged that the role of Bax in the initiation of apoptosis is related to the level of Bcl2 expression (Cory et al., 2005; Perianayagam et al., 2003; Van Der Vliet et al., 1997). Therefore, the ratio of Bax/Bcl2, rather than Bax alone, is a more important indicator of apoptosis. Second, in addition to the pro-apoptotic role, recent studies have shown that Bax plays a non-apoptotic role in the maintenance of normal cell functions. For example, a study has shown the Bax action on mitochondrial channel activity and on synaptic transmission (Jonas et al., 2005). Bax also has been shown to participate in the regulation of Ca^{2+} homeostasis (Aiba-Masago et al., 2002), as well as in mitochondrial morphogenesis (Karbowski et al., 2006). Therefore, in certain hair cells, expression of Bax may be related to functional changes in aging hair cells, rather than to the generation of apoptosis. An understanding of the precise role of Bax and cytochrome c in the apoptosis of aging cochlea requires future research.

Abbreviations

ARHL, Age-related hearing loss; OHC, Outer hair cell; PI, Propidium iodide; PBS, Phosphate Buffered Saline; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

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Figure 1.

A. Comparison of the thresholds of ABRs between the two age groups. The aging group has a significant elevation of the thresholds at all tested frequencies (re: the young group, $p < .05$). Fig. 1B. Comparison of the average percentage of the degenerated OHCs among the 3-month, 20- to 23-month, and 24- to 27-month groups. Note that the basal end of the cochleae exhibits a greater expansion of the OHC lesion with the increase in age. N = the number of cochleae.

Figure 2. The nuclear morphology of aging OHCs in a whole mount specimen stained with PI *in vitro*. The arrows in 2A and 2B point to the shrunken OHC nuclei with an increased PI fluorescence. The arrowheads in Fig. 2B indicate the OHCs having swollen nuclei. Bar: 10 μ l.

Figure 3.

Comparison of the average number of apoptotic and necrotic OHCs in aging cochleae. The average number of apoptotic OHCs is significantly greater than that of the necrotic cells ($p < .001$), with a ratio of 8:1. Bar: Standard deviation.

Figure 4.

Bax immunostaining in a young cochlea (4A) and two aging cochleae (4B and 4C). There is no detectable Bax fluorescence in the young organ of Corti. In contrast, the aging cochleae show increased Bax immunoreactivity (the green fluorescence) in OHCs. Bax fluorescence is present in the OHCs exhibiting normal nuclear morphology (arrows in 4B and 4C), but absent in the OHCs displaying nuclear condensation (arrowheads in 4B). The double arrow in 4B points to a Bax-positive OHC with a swollen nucleus. Fig. 4D: Percentage distribution of the total Bax-positive hair cells from the base (left) to the apex (right) of the organ of Corti. Bar: 10 μ l.

Figure 5.

Cytochrome c labeling in the basal turns of young (5A) and aging (5B, 5C, and 5D) cochleae. The arrow in 5A points to a malformed nucleus with a slight increase of cytochrome c fluorescence. There is no detectable cytochrome c fluorescence in the remaining OHCs of the young cochlea. In the aging organs of Corti, an increased immunolabeling (the green fluorescence) is present in certain OHCs (marked by the arrows in B, C, and D). The cytochrome c positive cells have either normal nuclear morphology (arrows in 5B), mild nuclear condensation (arrows in 5C), or nuclear swelling (arrows in 5D). In contrast, the OHC having marked nuclear condensation lacks cytochrome c labeling (the arrowhead in 5C). Fig.

5D: Percentage distribution of the total cytochrome c-positive hair cells from the base (left) to the apex (right) of the organ of Corti. Bar: 10 μ l.

Figure 6. Typical TUNEL in an aging cochlea. Condensed and fragmented nuclei exhibit a strong TUNEL fluorescence (yellow-green, marked by arrows). The arrow in the white frame of 6B points to a TUNEL-positive OHC with normal nuclear morphology (also see the inset for a high-magnification view). Bar: 10 μ l.

Figure 7. Typical examples of the caspase-3 (7A) and caspase-9 (7B) staining in aging cochleae. The arrows point to the OHCs displaying a strong caspase fluorescence (the green fluorescence). Note that these cells exhibit marked nuclear condensation. Bar: 10 μ l.

Figure 8.

Comparison of the numbers of positive OHCs showing Bax expression, cytochrome c release, nuclear shrinkage, or caspase activation.

Figure 9.

The reduction of F-actin fluorescence in the cuticular plates of apoptotic OHCs. The left column images (9A, 9C, and 9E) illustrate PI-stained OHC nuclei. The right column images (9B, 9D, and 9F) show the corresponding change in the F-actin staining illustrated in 9A, 9C, and 9E, respectively. The double arrows in 9A and 9B point to OHCs having condensed nuclei, but retaining F-actin fluorescence in the cuticular plates. The arrows in 9A and 9B, as well as in 9C and 9D, show apoptotic OHCs with reduced F-actin staining in the cuticular plates. The arrowheads in 9C and 9D, as well as in 9E and 9F, point to apoptotic OHCs showing the complete loss of F-actin staining in the cuticular plates. The asterisks in 9D and 9F point to the sites of missing cells where phalangeal scars have been formed. Bars: 10 μ l.

Table 1

The number of the cochleae used for each test

Assay	Young Rats	Aging Rats
PI in vitro	5	5
TUNEL + PI	4	5
F-actin + PI	10	20
Cyto C + PI	5	6
Casp-3 + PI	4	5
Casp-9 + PI	4	5
Bax	5	6