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Age-related changes in the ratio of Mcl-1/Bax expression in the rat cochlea

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Abstract

Conclusion—Down-regulation of the ratio of Mcl-1/Bax expression may contribute to age-related sensory cell degeneration in the cochlea.

Objective—To better understand the involvement of Bcl-2 family members in the regulation of age-related sensory cell death, we examined the expression patterns of Bcl-2-associated protein X (Bax) and its suppressor, myeloid cell leukemia 1 (Mcl-1) in young and aging rat cochleae.

Methods—Young (2–3 months) and aging (27–28 months) Fischer rats were used. The auditory brainstem response (ABR) thresholds, elicited by tone bursts at 4, 8, 16, 32, and 40 kHz were measured. The expression patterns of Mcl-1 and Bax genes at both the mRNA and protein levels were examined using a real-time RT-PCR assay and Western blot. Distribution of Mcl-1 and Bax expression in the cochlear sensory epithelia was evaluated using immunohistology and nuclear staining.

Results—Aging cochleae exhibited a significant elevation of ABR thresholds. This change was accompanied by significant reduction in Mcl-1 expression at both the mRNA and the protein levels and in the ratio of expression levels of Mcl-1/Bax genes in the aging subjects. Importantly, the changes in Mcl-1 and Bax expression are spatially related to the sensory cells showing the sign of degeneration.

Keywords

Aging; hair cell; apoptosis; degeneration

Introduction

Age-related hearing loss is the most common cause of sensory hearing loss in the elderly population. Pathological analyses of aging cochleae have revealed significant sensory cell

degeneration, which involves both the apoptotic and necrotic pathways, with apoptosis being the major driving force for sensory cell degeneration [1, 2]. Anatomically, apoptotic cells appear more evident in the apical and basal end of the cochlea, corresponding to the site of the organ of Corti showing the greater extent of hair cell loss [2]. Molecular analyses have revealed a group of apoptosis-related genes possessing either pro- or anti-apoptotic properties [3]. Thus far, the molecular mechanisms regulating the aging process in the cochlea is not clear.

B-cell lymphoma 2 (Bcl-2) family is a large gene family that participates in the mitochondrial signaling pathway of apoptosis [4]. This family consists of both pro- and anti-apoptotic members. Bcl-2-associated protein X (Bax) is a pro-apoptotic member of the Bcl-2 family genes, and has been found to be expressed in aging cochleae [2]. Bax mediates apoptosis by inducing the release of cytochrome c from mitochondria, which in turn causes activation of caspases [5]. However, Bax is also expressed in young cochleae when cell degeneration is not evident [2]. This phenomenon suggests that the pro-apoptotic activity of Bax is suppressed in young cochleae. Thus far, the molecular mechanism responsible for controlling the Bax pro-apoptotic function in cochlear sensory cells is not clear. In studies with non-cochlear tissues, evidence has emerged that the pro-apoptotic activity of Bax is regulated by anti-apoptotic members of Bcl-2 family genes [6, 7]. A balanced expression of the pro- and anti-apoptosis genes is essential for the maintenance of normal cell physiology [8].

Multiple anti-apoptotic Bcl-2 genes are expressed in the rat cochlea [3]. Among these genes, myeloid cell leukemia 1 (Mcl-1) is highly expressed in the cochlear sensory epithelia [9]. Mcl-1 blocks the progression of apoptosis by binding and sequestering the pro-apoptotic Bcl-2 family proteins, Bak and Bax [10]. In noise-traumatized cochleae, upregulation of Mcl-1 has been reported [9], suggesting its involvement in stress-induced apoptosis. We therefore hypothesized that interaction of Mcl-1 and Bax is a contributing factor for regulation of age-related cochlear degeneration.

The current investigation was designed to examine the age-related expression changes in Mcl-1 and Bax. To this end, we examined the expression levels of these genes at both the mRNA and the protein levels. The study documents a significant decrease in the expression ratio of Mcl-1 to Bax in aging cochleae. Importantly, the study reveals a spatial correlation between the sensory cell degeneration and the expression change in these two genes. These results provide strong evidence for implication of these apoptosis-related genes in aging sensory cell degeneration.

Materials and methods

Subjects

Fischer rats (male and female) were used in this experiment. The animals were purchased from Animal Center of Weitong Lihua in Beijing and raised in Animal Center of Chinese PLA General Hospital. The animals were assigned into two groups: the young rat group with ages of 2-3 months (n=18, 200-250 g), and the aging rat group with the ages of 27-28

months (n=18, 300-350 g). The procedures involving use and care of the animals were reviewed and approved by Animal Center of Chinese PLA General Hospital.

Measurement of auditory brainstem response (ABR) thresholds

The auditory brainstem response (ABR) thresholds were recorded to assess the cochlear function. All ABR measurements were performed in a sound-proof booth. During testing, the animal was anesthetized by injection of ketamine (30 mg/kg) and acepromazine (0.1 mg/kg). The ABRs were elicited with tone bursts (4, 8, 16, 32 and 40 kHz) generated digitally (SigGen, TDT System III, USA). The responses were recorded through three stainless-steel needle electrodes, placed subdermally over the vertex (noninverting), behind the test ear (either the right or the left, inverting), and behind the shoulder blade (ground) of the animal. The stimulus levels were delivered in 5 dB descending steps from 100 to 20 dB SPL. Acoustic stimuli were calibrated using a sound level meter. The ABR threshold was defined as the lowest intensity that reliably elicited detectable responses.

Euthanasia and collection of cochlear tissues

The animals were anesthetized with injection of ketamine (30 mg/kg) and acepromazine (0.1 mg/kg) and then decapitated. The cochleae were quickly removed from the skull and were processed for either assessment of Mcl-1 and Bax expression patterns or inspection of sensory epithelium pathology. Table 1 shows the numbers of cochleae used for each experimental condition.

qRT-PCR

The transcriptional expression levels of Mcl-1 and Bax were examined using a qRT-PCR Assay kit from Applied Biosystems (TaqMan® Gene Expression Assays Kit, TaqMan® MGB probes, FAM™ dye-labeled, USA). Animals were decapitated. Either the right or the left cochlea was collected and dissected in an RNA stabilization reagent (RNAlater, Qiagen, USA). The cochlear tissues containing the sensory epithelia and the later wall were collected. Tissues from two cochleae were pooled to generate a sample. Total RNA was isolated using a TRIzol reagent (Invitrogen Inc., USA) according to the manufacturer's instruction. First strand cDNA was synthesized using a SuperScript™ III First –Strand Synthesis System for RT-PCR Kit (Invitrogen). qRT-PCR was performed using a Real Time PCR System (7900 HT, Applied Biosystems, USA). Upon completion of the PCR run, the Ct values were determined. Three biological replications were performed for each group. To quantify the relative expression levels of Mcl-1 and Bax transcripts, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from the same samples was processed and amplified with the two target genes.

Western blotting

The expression levels of Mcl-1 and Bax proteins were assessed using Western blotting. After sacrifice of the animal, both left and right cochleae were collected, and the tissues from the two cochleae containing the sensory epithelia and the lateral wall tissues were collected and pooled to generate one sample. The samples were homogenized at 4°C using a lysis buffer of 100 µl consisting of 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1% NP-40,

0.1% SDS and 1mM PMSF (Beijing Solarbio Science & Technology Co., Ltd, China). The homogenates were centrifuged at 12,000 g for 5 min at 4°C and the supernatants were collected. The protein concentration was measured using a Microplate Reader (DNM-9602A, Perlong Medical Equipment Co., Ltd, China).

Equal amounts of proteins (100 µg/each well) were loaded onto 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and separated by electrophoresis. Proteins were then electrophoretically transferred to a polyvinylidene fluoride membrane using an electrophoresis system (DYY-7C, Beijing luiyi Instrument Factory, China), according to the manufacturer's instructions. Identification of Mcl-1 and Bax proteins was achieved by using a mouse monoclonal antibody against Mcl-1 (1:100, sc-74437, Santa Cruz biotechnology Inc., USA) and a rat monoclonal Bax antibody (1:200, ab-3192, Abcam Inc., USA), respectively. The blots were first blocked with a blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween containing 10% non-fat milk) and then were probed with the primary antibody at 4°C overnight. After rinsing, the blots were incubated with a horseradish peroxidase-conjugated second antibody (Zhongshan Goldenbridge Biotechnology Co., Ltd, China). The stained membrane was visualized and imaged with a luminescent image analyzer ("AX-II", Guangdong Yuehua Medical Instrument Factory Co., Ltd, China). Three biological replications were performed for each group. β -actin was used as the reference level to quantify the relative expression levels of the Mcl-1 and Bax proteins.

Immunolabeling of Mcl-1 and Bax

The cochleae were gently perfused through the round window with cold 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS) at pH 7.4, and incubated in the same fixative overnight at 4°C. Following the fixation, the cochleae were dissected in 10 mM PBS. The bony wall and the spiral ligament were carefully removed, and the organ of Corti was separated from the modiolus and stored in PBS.

For Mcl-1 staining, the tissues were permeabilized with 0.2% Triton-X-100 in 10 mM PBS for 30 min and then were transferred into a blocking solution (Blocker™ Casein in PBS, Pierce Chemical Company, USA) for 1 h at room temperature. The specimens were incubated with a mouse monoclonal antibody against Mcl-1 (1:50, sc-74437, Santa Cruz Biotechnology Inc., USA) at 4°C overnight. After rinsing, the specimens were incubated with a secondary antibody (Alexa Fluor® 488 goat anti-mouse, Invitrogen Inc., 1: 100 in 10 mM PBS) for 2 h.

For Bax staining, the tissues were permeabilized in a Triton X-100 solution (0.2% Triton X-100 and 0.5% bovine serum albumin) in PBS (pH 7.4) for 1 h at room temperature. The specimens were incubated with a rat monoclonal anti-Bax antibody (1:200, Abcam Inc, USA) in a blocking solution (Blocker™ Casein, Pierce) at 4°C overnight. After rinsing, the tissues were stained with a secondary antibody (1:100, FITC-labeled rabbit polyclonal against rat IgG H&L, Abcam Inc.) in 10 mM PBS, for 1 h at room temperature.

To assess non-specific staining, a few pieces of the organs of Corti were stained with the second antibody only (without the primary antibody of Mcl-1 or Bax).

Assessment of sensory cell damage

For assessment of sensory cell damage, propidium iodide (PI), a DNA intercalating fluorescent probe, was used to visualize the morphological changes in nuclei. After Mcl-1 or Bax staining, the organs of Corti were incubated in the PI staining solution (5 µg/ml in 10 mmol/L PBS, Invitrogen Inc.) for 10 minutes at room temperature without light. Then, the specimens were rinsed in PBS and mounted on slides containing an antifade medium (ProLong™ Antifade kit, Invitrogen Inc.).

The criteria for defining the three types of hair cell pathology, apoptosis, necrosis, and missing, have been previously described [11]. The hair cells showing condensed nuclei, featuring a shrunken size with an increased PI fluorescence, were counted as apoptotic cells. The principal criterion for identification of necrotic hair cells was the presence of nuclear swelling illustrated by PI staining. The morphological characteristics of nuclear swelling included enlarged sizes, malformed shapes, and loss of normal texture of nuclear staining. Missing hair cells were recognized in the areas of the hair cells where nuclear staining was absent.

Data analyses

For ABR threshold analyses, a two-way ANOVA (age × frequency) was used to determine the differences between the mean ABR thresholds of the young and aging groups across the five tested frequencies (4, 8, 16, 32 and 40 kHz).

For the analysis of the mRNA expression changes in Mcl-1 and Bax in aging rats, a relative quantification method [12] was used. The expression levels of Mcl-1 and Bax gene were first normalized to the expression level of the reference gene (GAPDH) to generate the C_t for the young and the aging group. Then, the C_t was calculated with the formula: C_t of the aging group – C_t of the young group. A student's *t* test was used to evaluate the significance of the difference. The fold-change $2^{\Delta C_t}$ was considered biologically significant.

For Western blotting analyses, the staining intensities of Mcl-1 and Bax bands were quantified with image processing software (IMAGE-Pro Plus, Media Cybernetics Co., USA). The values were normalized to the values of β-actin derived from the same blot.

For the analyses of immunoreactivity of Mcl-1 and Bax staining as well as the pattern of sensory cell damage, the cochlear specimens were first examined with a microscope with epifluorescence illumination to identify sensory cell lesions. The lesions were further observed with a confocal microscope (Zeiss, LSM, 510). To define the distribution of Mcl-1 and Bax protein expressions along the organ of Corti, we identified the Mcl-1 and Bax positive-hair cells defined as visually detectable stronger immunolabeling in hair cells as compared with their neighboring hair cells. The percentages of Mcl-1 and Bax positive cells were quantified separately for the apical, the middle, and the basal portion of the organ of Corti in young and aging cochleae.

To quantify the levels of Mcl-1 and Bax immunoreactivity, the gray levels of Mcl-1 and Bax staining were measured with an image processing software (IMAGE-Pro Plus, Media Cybernetics Co.). The gray levels of the image pixels enclosed in the cytoplasm of sensory

cells were measured individually. To define the relation between the expression changes and the progression of sensory cell degeneration, we grossly categorized nuclear shrinkage into two levels: mild (75% of the normal size) and severe (25-50% of the normal size). The staining intensities of Mcl-1 and Bax in these categories of nuclear changes were compared. For each condition, at least 15 cells were measured for each cochlea.

For the statistical analyses, the significance value was set at $p < 0.05$.

Results

Aging rats exhibit a significant loss of hearing sensitivity

We first examined the functional status of the aging cochleae, so that the analysis of target gene expression can be placed in the context of the functional changes. To this end, we measured the ABR thresholds of the young and aging subjects. As compared with the ABR thresholds of the young rats, the aging rats exhibited a significant elevation in the ABR thresholds. Two-way ANOVA (age $\times$ frequency) with repeated measures revealed a significant age effect ($F = 734.4$, $df = 1$, $p < 0.001$) and a significant frequency effect ($F = 61.5$, $df = 4$, $p < 0.001$), with an average threshold difference of 34.4 ± 8.0 dB between the two age groups. The hearing loss was more prominent change in the high frequency range (32 and 40 kHz, post hoc testing with a Tukey test, $p < 0.001$) (Fig.1). This functional analysis indicates the presence of significant deterioration of cochlear function in aging rats.

Transcriptional changes in Mcl-1 and Bax in the aging cochlea

To evaluate the expression changes in Mcl-1 and Bax, we first examined their constitutive expression levels in the young cochlea. We found that both genes were expressed in the cochlear tissues.

In the aging cochlea, Mcl-1 expression was downregulated by 3.1 folds (Student's t test, $p = 0.009$) (Fig. 2A). The expression level of Bax, on the other hand, was slightly up-regulated (1.2 folds) (Fig. 2B). However, this change was not statistically significant (Student's t test, $p > 0.05$). To further define the changes in the transcriptional expression of Mcl-1 and Bax, we calculated the expression ratio of Mcl-1 to Bax, and compared the ratios between the young and the aging group. As shown in Fig. 2C, the ratio is significantly reduced in the aging cochleae (Student's t test, $p = 0.001$), suggesting a shift of expression balance of these two genes toward apoptosis promotion.

Expression changes in Mcl-1 and Bax proteins in the aging cochlea

Given the finding of transcriptional changes in the ratio of Mcl-1/Bax, we sought to determine whether the protein expression of these two genes is altered in the aging cochlea. To this end, Western blotting analyses were conducted using the cochlear tissues from young and aging cochleae.

For Mcl-1, Western blotting analysis revealed a strong band at 42 kDa and a faint band at 30 kDa, consistent with the reported molecular weights of the Mcl-1 and Mcl-1S proteins [9]. Noticeably, the band intensity of Mcl-1 from the aging tissues was weaker than that from the young cochlear tissues (Fig. 3A). The gray level analysis of the staining intensity confirmed

the visually detected difference (Student's *t* test, $p = 0.001$) (Fig. 3B), suggesting that the protein expression level of the Mcl-1 gene was downregulated in the aging cochleae.

For Bax, Western blot analysis revealed a band at 21 kDa, consistent with the molecular weight of Bax protein [8]. The staining intensity of the band appeared to be increased in aging cochlear samples as compared with the samples from young cochleae (Fig. 3C). However, this difference was not statistically significant (Student's *t* test, $p > 0.05$) (Fig. 3D).

We further calculated the ratio of the protein expression levels of Mcl-1 to Bax, and compared the ratios between the young and aging cochleae. We found that the ratio was reduced in the aging group as compared with that in the young group (Student's *t* test, $p = 0.001$) (Fig. 3E). This protein expression change is consistent with the transcriptional changes in these genes, further suggesting that the expression patterns of Mcl-1 and Bax are altered in the aging cochlea.

Expression distribution of Mcl-1 and Bax in cochlear sensory epithelium

The cochlea consists of heterogeneous cell populations. We therefore examined the spatial distribution of the Mcl-1 and Bax expression in the sensory epithelium, and determined the correlation between the expression changes and sensory cell degeneration using immunohistochemistry. For the control samples stained with the secondary antibody only (omitting the primary antibody of Mcl-1 or Bax), there was a weak background fluorescence, but the intensity was substantially lower than that observed in the samples stained with the primary antibodies (images not shown).

In the young cochlea, both Mcl-1 and Bax staining was observed in the organ of Corti and the immunoreactivity was stronger in the sensory cells, particularly outer hair cells. The Mcl-1 staining intensity (Fig. 4A) was, in general, stronger than that of Bax staining intensity (Fig. 4B). There is a slight difference in the staining intensity across individual hair cells.

In aging cochleae, the Mcl-1 immunoreactivity became more heterogeneous (Fig. 4C). Many hair cells showed a weak Mcl-1 immunoreactivity. However, certain cells exhibited an increased Mcl-1 fluorescence (indicted by arrows in Fig. 4C). These Mcl-1-positive cells were more evident in the apical and the basal sections of the organ of Corti. As compared with the corresponding sites of the young cochlea, the numbers of Mcl-1-positive cells in the apical and basal sections were increased in the aging cochleae (Fig. 5A, two-way ANOVA, $p < 0.01$).

The increase in Bax immunoreactivity was observed in the hair cells of the aging cochleae (Fig. 4D). Again, the sensory cells showing stronger Bax immunoreactivity (indicted by arrows in Fig. 4D) were more evident in the apical and basal ends of the cochleae, where sensory cell missing is more common than the middle section of the cochlea. As compared with the young cochleae, the aging ears exhibited a significant increase in the numbers of Bax-positive cells in the apical, middle and basal portions of the cochleae (Fig. 5B, two-way

ANOVA, $p > 0.001$). These observations indicate that the major changes in the expression levels of Mcl-1 and Bax protein occurs in the sensory cells.

Expression changes in Mcl-1 and Bax are correlated spatially with sensory cell degeneration

To determine whether the changes in Mcl-1 and Bax immunoreactivities were correlated with the changes in sensory cell pathology, we examined the staining patterns of these proteins in the sensory cells at different degenerative stages. Using the nuclear morphology as the index, we found both nuclear condensation and swelling. We defined the early nuclear condensation as the increase in PI fluorescence and slight reduction in the nuclear size (about 3/4 of the normal size) and the advanced nuclear condensation as moderate to severe reduction in the nuclear size (about 1/2 or 1/4 of the normal size). We found that all the sensory cells showing early nuclear condensation had an increased Mcl-1 and Bax immunoreactivities (Figs. 6A and 6B). The sensory cells displaying nuclear swelling also had strong Mcl-1 (Fig. 6C) and Bax (Fig. 6D) immunoreactivity. These sensory cells displaying nuclear swelling accounted for only a small portion (0.42%) of total degenerated hair cells (hair cells with condensed or swollen nuclei and missing hair cells). Mcl-1 and Bax staining was reduced or absent in the hair cells with advanced nuclear condensation (Figs. 6A and 6B).

Figs. 6E and 6F show the comparison of the mean gray level of Mcl-1 (Fig. 6E) and Bax (Fig. 6F) staining between the normal and the early stage of damaged hair cells in the aging cochleae. The hair cells with nuclear swollen and mild condensation in the aging cochleae show a significant difference in the gray level of Mcl-1 and Bax staining (Student's t test, $p < 0.001$). These observations suggest that Mcl-1 and Bax are involved in the early stage of sensory cell degeneration.

Discussion

The current study examined the expression changes in two apoptosis-related genes, Mcl-1 and Bax, in an aging Fischer rat model. We document a significant reduction in the expression ratios of Mcl-1/Bax at both the mRNA and the protein levels. Importantly, the expression changes in Mcl-1 and Bax are spatially correlated with the sensory cell degeneration. These results clearly link Mcl-1 and Bax genes to sensory cell degeneration in the aging cochlea.

Bax is an important pro-apoptotic player in apoptosis. Previous investigations have implicated this gene in sensory cell pathogenesis in the aging cochlea [2, 13]. Our current result showing an increase in Bax immunoreactivity in degenerative hair cells is consistent with these previous observations.

In the current investigation, we found a reduction in Mcl-1 expression at both the mRNA and the protein levels in the aging cochlea. This finding is consistent with previous observations showing the expression reduction in this gene in various pathological conditions such as growth factor deprivation [14], viral infection [15], exposure to chemotherapeutic agents [16], and UV irradiation [17]. The biological mechanism leading to

the expression reduction in Mcl-1 during aging degeneration is not clear, but is possibly related to both transcriptional and translational repression, because the reduction occurs at both the mRNA and the protein levels. Other factors, such as caspase-mediated cleavage, may also contribute to the reduction of Mcl-1 protein contents [18].

Here, we show that changes in Mcl-1 and Bax immunoreactivity are associated with the progression of sensory cell degeneration. Using the nuclear morphology as the index, we found that in hair cells with early signs of degeneration, Mcl-1 and Bax immunoreactivities were increased. In contrast, when sensory cells exhibited a moderate or severe nuclear condensation, stainings for Mcl-1 and Bax was reduced or absent. These results suggest that Mcl-1 and Bax participate in the early stages of sensory cell degeneration. It should be noted that the strong immunoreactivity of Bax and Mcl-1 appeared in not only the hair cells with condensed nuclei, but also the hair cells with swollen nuclei, suggesting that Mcl-1 and Bax are involved in both the apoptotic and necrotic pathways.

Interplay of Mcl-1 and Bax has shown to regulate apoptosis [19]. Mcl-1 can interact with a subset of the BH3-only pro-apoptotic Bcl-2 family proteins to suppress the formation of mitochondrial oligomeric Bax/Bak complexes [10]. In our current study, there is a threefold reduction in the ratio of Mcl-1/Bax expression in aging cochleae. This reduction may compromise the balance of the pro/anti-apoptosis action of these genes, causing the enhancement of apoptotic activities of the cells in aging cochleae.

In addition to the anti and pro-apoptotic roles, several studies have shown that Mcl-1 and Bax play certain non-apoptotic roles in the maintenance of normal cell function. For example, Mcl-1 expression increases in response to factors that regulate cell growth, differentiation, and activation [16]. Moreover, Bax participates in the regulation of Ca^{2+} homeostasis [20]. How cochlear Mcl-1 and Bax participate in these cellular activities is not clear, and warrant further investigations.

In summary, the present study reveals that the expression ratio of Mcl-1/Bax genes was significantly decreased in aging rat cochleae. This change is more evident in sensory cells and is related to sensory cell degeneration. These results implicated Mcl-1 and Bax in the regulatory mechanism of sensory cell degeneration. Modulation of the expression ratio of these two genes may serve a potential target for future therapeutic intervention for reduction of sensory cell degeneration in aging cochleae.

Acknowledgments

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Figure 1.

Comparison of the average ABR thresholds in the young and the aging rats. The aging rats exhibit a significant elevation in the thresholds at all tested frequencies. N=18 animals. Bars represent standard deviation. * $p < 0.001$.

Figure 2. Comparisons of the transcriptional expression levels of Mcl-1(A) and Bax (B), and the expression ratio of Mcl-1/Bax (C) in the young and the aging cochleae. Bars represent standard deviation. * $p < 0.01$.

Figure 3.

The protein expression levels of Mcl-1 and Bax assessed by Western blotting in the young and the aging cochleae. (A) An example of Western blot for the Mcl-1 protein examined in a young (3 months, 3m) and an aging (27 months, 27m) rat cochleae. (B) Comparison of the Mcl-1 expression levels in the young and the aging cochleae (N= 6 samples). (C) An example of Western blot for the Bax protein examined in a young (3 months) and an aging (27 months) rat cochleae. (D) Comparison of Bax expression levels between the young and the aging cochleae. N=6 samples. E: Comparison of the expression ratios of Mcl-1/Bax proteins between the young and the aging cochleae. Bars represent standard deviation. * $p < 0.01$.

Figure 4.

Typical examples of Mcl-1 and Bax immunostaining. (A and B) Mcl-1 (A) and Bax (B) immunoreactivity in the organs of Corti from the young rats. (C and D) Mcl-1 (C) and Bax (D) immunoreactivity in the aging organs of Corti. The immunoreactivity appears primarily in the cytoplasm of the sensory cells. Arrows mark the outer hair cells showing relatively strong immunoreactivity compared with neighboring cells. OHC1, OHC2 and OHC3 indicate the first, second and third rows of outer hair cells. The horizontal bar represents 15 μm.

Figure 5. Comparisons of the mean percentages of hair cells showing strong Mcl-1 (A) and Bax (B) immunoreactivity in the apical, the middle, and the basal portions of the organs of Corti between the young and the aging rats. Bars represent standard deviation. $*p < 0.01$.

Figure 6.

Spatial correlation between Mcl-1 and Bax immunoreactivity and sensory cell degeneration. The tissues were doubly staining with PI for illustration of nuclei. (A, - D) Mcl-1 (A and C) and Bax (B and D) immunoreactivity in the aging cochlea. The red color represents PI fluorescence and that green indicates either Mcl-1 or Bax immunostaining. Arrows in A and B point to the outer hair cells with relatively stronger Mcl-1 or Bax immunoreactivity. These cells have slightly condensed nuclei. The arrowheads in A and B point to the outer hair cells with moderate or severe nuclear condensation. These cells lack Bax or Mcl-1 immunoreactivity. The arrows in C and D indicate outer hair cells with swollen nuclei. These cells exhibit a stronger Bax or Mcl-1 immunoreactivity than those in the neighboring cells. Asterisks indicate the sites of missing hair cells. The horizontal bar represents 15 μ m. (E and F) Comparison of the mean gray level of Mcl-1(E) and Bax (F) staining between the normal and the early stage of damaged outer hair cells in the aging cochleae. Thin bars represent standard deviation. * $p < 0.01$.

Table 1

Number of cochleae used for each experimental condition.

Group	RT-PCR	Western Blotting	Immunolabeling and pathological assessment	
	Mcl1 and Bax	Mcl1 and Bax	Mcl1	Bax
Young	10	12	7	7
Aging	10	12	7	7