Overexpression of *Bcl-2* Prevents Neomycin-Induced Hair Cell Death and Caspase-9 Activation in the Adult Mouse Utricle *In Vitro*

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ABSTRACT: **Mechanosensory hair cells of the inner ear are especially sensitive to death induced by exposure to aminoglycoside antibiotics. This aminoglycoside-induced hair cell death involves activation of an intrinsic program of cellular suicide. Aminoglycoside-induced hair cell death can be prevented by broad-spectrum inhibition of caspases, a family of proteases that mediate apoptotic and programmed cell death in a wide variety of systems. More specifically, aminoglycoside-induced hair cell death requires activation of caspase-9. Caspase-9 activation requires release of mitochondrial cytochrome c into the cytoplasm, indicating that aminoglycoside-induced hair cell death is mediated by the mitochondrial (or "intrinsic") cell death pathway. The Bcl-2 family of pro-apoptotic and anti-apoptotic proteins are important upstream regulators of the mitochondrial apoptotic pathway. Bcl-2 is an anti-apoptotic protein that localizes to the mitochondria and promotes cell survival by preventing cytochrome c release. Here we have utilized transgenic mice that overexpress *Bcl-2* to examine the role of Bcl-2 in neomycin-induced hair cell death. Overexpression of *Bcl-2* significantly increased hair cell survival following neomycin exposure in organotypic cultures of the adult mouse utricle. Furthermore, *Bcl-2* overexpression prevented neomycin-induced activation of caspase-9 in hair cells. These results suggest that the expression level of Bcl-2 has important effects on the pathway(s) important for the regulation of aminoglycoside-induced hair cell death.** © 2004 Wiley Periodicals, Inc. J Neurobiol 60: 89–100, 2004

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INTRODUCTION

Auditory and vestibular hair cells die in response to a number of stimuli, including aging, noise trauma, and exposure to ototoxic drugs. Hair cell death resulting from exposure to aminoglycoside antibiotics has been shown by both morphological and molecular techniques to demonstrate properties associated with programmed cell death or apoptosis (Kerr et al., 1972; Li et al., 1995; Lang and Liu, 1997; Vago et al., 1998; Forge and Li, 2000; Cunningham et al., 2002; Matsui et al., 2002, 2003). Most importantly, aminoglycoside-induced hair cell death can be inhibited using broad-spectrum inhibition of the caspase family of cell death-associated proteases (Forge and Li, 2000; Williams and Holder, 2000; Cheng et al., 2003; Cunningham et al., 2002; Matsui et al., 2002, 2003). In addition, hair cells that are protected by caspase inhibition remain functional (Matsui et al., 2003). Neomycin-induced hair cell death is dependent upon the activation of caspase-9 (Cunningham et al., 2002). Caspase-9 activation requires a signal from the mitochondria in the form of release of mitochondrial cytochrome c. The presence of cytochrome c in the cytoplasm results in formation of a multiprotein complex consisting of cytochrome c, apaf-1, dATP, and procaspase-9 (Li et al., 1997; Zhou et al., 1999; Yuan and Yankner, 2000). The assembly of this complex results in activation of caspase-9 and cell death.

The most important proteins mediating cell death vs. survival decisions at the level of the mitochondria are the members of the Bcl-2 family of pro- and anti-apoptotic molecules. Bcl-2 family members act upstream of caspase activation and serve as checkpoints in the regulation of apoptosis. The Bcl-2 family is comprised of proteins that either promote (e.g., Bax and Bid) or inhibit (e.g., Bcl-2 and Bcl-X_L) apoptosis. These proteins form homodimers and heterodimers, and the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members within a cell is important in determining whether the cell lives or dies in response to a damaging stimulus (Oltvai et al., 1993). Bcl-2 is localized primarily to the outer mitochondrial membrane (Hockenbery et al., 1990; Krajewski et al., 1993) where it functions to inhibit the mitochondrial permeability transition (MPT) that serves as a mechanism for cytochrome c release (Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997; Shimizu et al., 1998; Newmeyer et al., 2000). Overexpression of *Bcl-2* blocks cytochrome c release (Kluck et al., 1997; Yang et al., 1997). In contrast, the pro-apoptotic Bcl-2 family member Bax promotes cytochrome c release (Jurgensmeier et al., 1998).

Bcl-2 overexpression can prevent apoptosis in a large number of systems. Overexpression of *Bcl-2* can prevent neuronal cell death both during normal development and during experimental ischemia (Martinou et al., 1994). In the auditory system, overexpression of *Bcl-2* in transgenic mice prevents afferent deprivation-induced cell death of neurons in the anteroventral cochlear nucleus (Mostafapour et al., 2002). Here we have utilized *Bcl-2* overexpressing transgenic mice to examine the role of Bcl-2 aminoglycoside-induced hair cell death.

METHODS

Animals

Our experiments were conducted independently in two laboratories, and data from each laboratory are presented. Both laboratories used transgenic mice (line NSE 73a) that overexpress the human *Bcl-2* gene under the control of the neuron-specific enolase (NSE) promoter (Martinou et al., 1994). The mice used at the University of Washington were the generous gift of Dr. D.-F. Chen (Harvard Medical School, Boston). These mice were housed in the University of Washington Animal Care Facility. The *Bcl-2* overexpressing mice used at the Central Institute for the Deaf (CID) were generously provided by Dr. Eugene Johnson, Jr. (Washington University) and were bred in the CID animal care facility. All experimental protocols were approved by the appropriate Institutional Animal Care and Use Committee (University of Washington or Washington University/CID). In order to reduce the possibility of effects of other genes co-segregating with the *Bcl-2* transgene, we backcrossed the *Bcl-2* overexpressing transgenic mice to C57Bl6 for a minimum of five generations prior to the onset of our studies. Because NSE73a females are infertile (Martinou et al., 1994), transgenic male mice were bred to wild-type C57Bl/6 female mice. The resulting litters consisted of approximately 50% transgenic and 50% wild-type animals. The genotype of each mouse was determined by tail-clip DNA analysis using polymerase chain reaction (PCR).

Mouse Utricle Organ Cultures

Transgenic and wild-type animals were killed by overdose of pentobarbital and then decapitated. Utricles were dissected using sterile technique and were cultured free-floating (1–6 utricles per well) in 24-well tissue culture plates. Utricles were cultured whole without removing otoconia. Culture medium consisted of basal medium Eagle supplemented with Earle's balanced salt solution (BME and EBSS, 2:1 v/v) and 5% fetal bovine serum (FBS) (all medium components from Gibco Invitrogen, Carlsbad, CA). Neomycin sulfate (Pharma-Tek, Huntington, NY) was prepared as a 100-mM stock solution in sterile water and added directly to culture wells at final concentrations of

either 1 or 2 mM. No neomycin was added to control cultures. Utricles were incubated for 12–24 h at 37°C in a 5% CO_2/95% air environment (Forma Scientific, Marietta, OH).

All cultures for sectioned material were performed at CID, where utricles were cultured in medium 199/10% FBS at 37°C for 24 h, followed by another 24 h in control medium or medium supplemented with 1 mM neomycin. Specimens were then fixed, embedded in epoxy resin, and sectioned at 3 μm. Serial sections were collected and stained with toluidine blue.

Immunochemistry

Whole-mount utricles were double-labeled for calmodulin and calbindin as previously described (Cunningham et al., 2002). Calmodulin labels both hair cell types in the mammalian utricle, the Type I hair cells, which are found primarily in the striolar region, and the smaller Type II hair cells of the extrastriolar region (Ogata and Slepecky, 1998; Ogata et al., 1999). Calbindin labels only the Type I hair cells of the mouse utricle (Dechesne et al., 1988). Utricles were fixed in 4% paraformaldehyde in phosphate buffer. Otoconia were removed from fixed utricles by a stream of phosphate-buffered saline (PBS) applied via a syringe. Utricles were then incubated in blocking solution (2% bovine serum albumin/0.4% normal goat serum/0.4% normal horse serum/0.4% Triton X-100 in PBS) for 3 h at room temperature (RT). Endogenous peroxidases were quenched using 1% H_2O_2 in PBS for 30 min at RT. Utricles were double-labeled using a monoclonal antibody against calmodulin (Sigma, St. Louis, MO) and a polyclonal antibody against calbindin (Chemicon, Temecula, CA). Utricles were incubated overnight at 4°C in both primary antibodies diluted in blocking solution (calmodulin, 1:200; calbindin, 1:250). Utricles were washed in PBS/0.1% Triton X-100 and incubated for 2 h at RT in secondary antibodies diluted in blocking solution as follows: biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA); Alexa 594 conjugated goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR). Utricles were then washed and incubated for 2 h in avidin-biotin-horseradish peroxidase complex (Vector). Tissues were rinsed in 0.05 M Tris buffer (pH 7.4) and placed in diaminobenzidine (DAB, 0.375 mg/mL in 0.05 M Tris + 0.1% H_2O_2) for 5–12 min at RT. Utricles were whole-mounted in Vectashield (Vector) and coverslipped.

For human Bcl-2 immunochemistry, utricles were double-labeled as above using the monoclonal anti-calmodulin antibody (above) and a hamster monoclonal antibody directed against human Bcl-2 (PharMingen, San Diego, CA). This antibody has been shown by both western blotting and immunochemistry to be monospecific for the human Bcl-2 protein; the antibody does not recognize mouse Bcl-2 (Hockenbery et al., 1990; McDonnell et al., 1990). Primary antibody was diluted 1:200 in blocking solution. The secondary antibodies were Alexa 594 conjugated goat anti-hamster IgG and Alexa 488 conjugated goat anti-mouse IgG (both 1:500; Molecular Probes).

Hair Cell Counts

For hair cell counts in whole-mount preparations, utricles double-labeled with calmodulin and calbindin were examined on a Leitz Aristoplan upright microscope using a 40× objective. Hair cell density was measured using a 10 × 10 eyepiece reticule. At the magnification used, each square of the reticule was 30 μm on each side. Calbindin-positive hair cells in the striolar region were counted in each of four 900 μm^2 areas using a Texas Red filter set. Calmodulin-positive hair cells in the extrastriolar region were counted in four 900μm^2 areas under Nomarski differential interference optics. The eight 900 μm^2 areas for examination were selected to be completely inside the sensory epithelium and to include only striolar or only extrastriolar hair cells. The four regions of the striola were selected to be spaced approximately evenly across the striola, and the four extrastriolar regions were selected arbitrarily from the calmodulin-positive/calbindin-negative region. A labeled cell was counted if at least half of the cell body was contained within the selected square. Hair cells were examined at multiple focal planes in order to ensure that damaged hair cells below the surface of the epithelium would be counted. The four striolar and four extrastriolar hair cell counts were each averaged to produce one striolar and one extrastriolar hair cell density for each utricle examined.

For counts of hair cells in plastic sections, serial semithin (3 μm) sections were cut through the utricle and mounted onto untreated slides. In each utricle, one section was chosen randomly from every 10 serial sections (30 μm intervals) through the entire organ. This procedure resulted in the analysis of 8—12 sections per utricle. A total of four utricles from wild-type mice and seven utricles from *Bcl-2* overexpressing mice were examined. The length of the utricular sensory epithelium was measured for each section using light microscopy (100× objective) and Neuroleucida imaging software (MicroBrightField, Williston, VT). Hair cell numbers were expressed as a function of sensory epithelium linear density (number of hair cells/100 μm sensory epithelium). Hair cells were distinguished easily from other cell types in the utricular macula. A cell was counted as a hair cell if it had an identifiable cuticular plate and a cell body that did not contact the basal lamina. Type I and Type II hair cells, which normally are identified by the morphology of the innervation (Wersäll, 1956; Jørgensen and Christensen, 1989), could not be distinguished since the neural endings contacting the hair cells retract and degenerate *in vitro* (Oesterle and Rubel, 1993). All hair cell counts were conducted blinded with respect to mouse genotype.

In Situ Substrate Detection of Activated Caspase-9

Caspase-9 activity was examined using the fluorescent caspase-9 substrate, fam-LEHD-fmk (Intergen, Purchase,

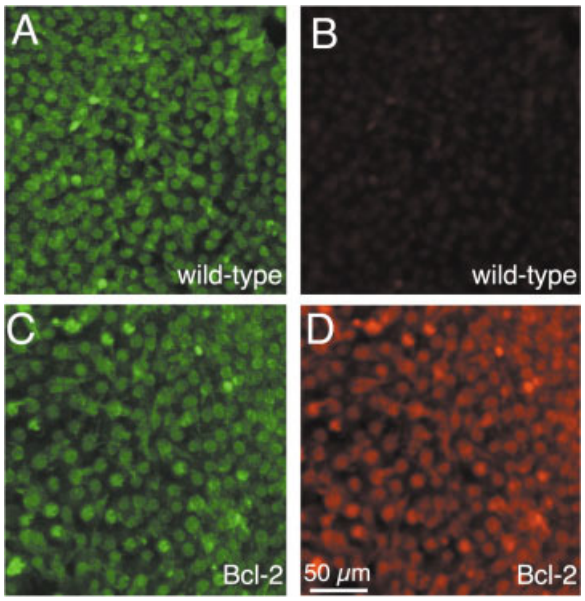

Figure 1 Immunochemistry for human Bcl-2. Immunochemistry was performed on utricles from wild-type (A, B) and *Bcl-2* overexpressing (C, D) mice. Utricles were double-labeled using an antibody against calmodulin (A, C) and an antibody that recognizes human Bcl-2 but does not cross-react with mouse Bcl-2 (B, D). Anti-calmodulin labels all of the hair cells in both wild-type (A) and *Bcl-2* overexpressing (C) utricles. Human Bcl-2 immunoreactivity is present in the hair cells of the transgenic (*Bcl-2* overexpressing) utricle (D) and absent from the wild-type utricle (B). Scale bar = 50 μm.

NY) as previously described (Cunningham et al., 2002). Utricles were cultured in the presence or absence of 1 mM neomycin for 12 h. The 12-h timepoint was selected for detection of activated caspase-9 in order to facilitate examination of hair cells that were lost by 24 h in 1 mM neomycin (Cunningham et al., 2002). Fam-LEHD-fmk was added directly to the culture medium (final concentration = 5 μM) for the final hour in culture. After 1 h in substrate, utricles were washed three times for 15 min each at 37°C in the wash buffer supplied by the manufacturer. Utricles were then fixed overnight at 4°C in the fixative supplied by the manufacturer. Following fixation and otoconia removal, utricles were incubated for 2 h at RT in rhodamine phalloidin (1:50 in PBS, Molecular Probes) to label hair cell stereocilia. Utricles were then rinsed with PBS, mounted, and coverslipped. Whole-mounted utricles were viewed on a confocal laser scanning microscope (MRC-1024, BioRad, Hercules, CA) using LaserSharp version 2.1 software (BioRad). In order to ensure that labeled cells were hair cells and not supporting cells, z-series optical sections were limited to a z-axis depth of 5 μm or less from the lumenal surface (apex of hair cells) and were collected to include the phalloidin-labeled stereocilia. Data were processed digitally using NIH Image and Adobe Photoshop (Adobe, Mountain View, CA).

Statistical Analyses

Data from hair cell counts were subjected to either an unpaired two sample *t* test assuming unequal variances using Excel 98 (Microsoft Corporation, Redmond, WA), or analysis of variance using StatView (SAS, Cary, NC).

RESULTS

Human Bcl-2 Is Expressed in the Hair Cells of Utricles from *Bcl-2* Overexpressing Transgenic Mice

The transgenic mice used in this study express human *Bcl-2* under the control of the neuron-specific enolase

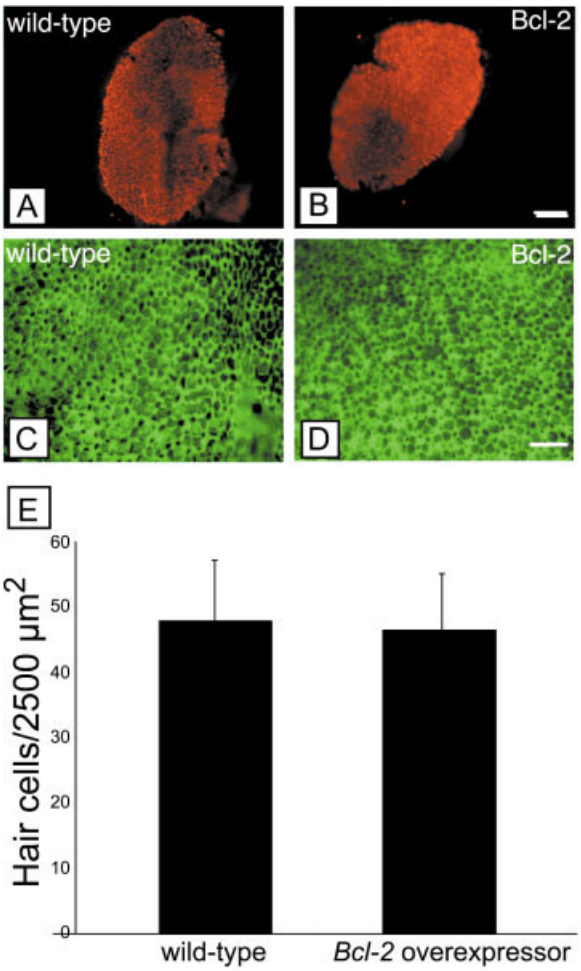

Figure 2 Hair cell densities in wild-type and *Bcl-2* overexpressing utricles. Utricles from wild-type (A, C) and *Bcl-2* overexpressing (B, D) mice were labeled using anti-Myosin VIIa (red, A, B) and phalloidin (green, C, D). Hair cells were counted in two 2,500 μm^2 areas in each utricle (E). Hair cell densities were essentially equal in wild-type and *Bcl-2* overexpressing utricles. Scale bars = 100 μm in A, B and 20 μm in C, D. Bars in E represent mean hair cell densities ± S.D. for n = 3 wild-type utricles and n = 3 transgenic utricles.

(NSE) promoter (Martinou et al., 1994). In order to confirm that the *Bcl-2* overexpressing transgenic mice express human Bcl-2 in utricular hair cells, immunohistochemistry was performed using an antibody that recognizes human Bcl-2 and does not cross-react with mouse Bcl-2 (Hockenbery et al., 1990; McDonnell et al., 1990). Figure 1 shows mouse utricles that were double-labeled for the hair cell marker calmodulin (green) and human Bcl-2 (red). Hair cells from wild-type mice express calmodulin [Fig. 1(A)] but do not express human Bcl-2 [Fig. 1(B)]. Hair cells from transgenic *Bcl-2* overexpressing mice express both calmodulin [Fig. 1(C)] and human Bcl-2 [Fig. 1(D)]. Z-series optical sectioning using a confocal microscope confirmed that all of the hair cells in the utricles of Bcl-2 overexpressing mice were immunoreactive for human Bcl-2, and no immunoreactivity for human Bcl-2 was detected in the support cell layer. These results are in agreement with the expression pattern of NSE, which is expressed in mature murine vestibular hair cells (Dechesne et al., 1985). These data confirm that the transgenic *Bcl-2* overexpressing mice express the human *Bcl-2* transgene in the hair cells of the utricle.

Untreated Wild-Type and *Bcl-2* Overexpressing Utricles Contain Similar Numbers of Hair Cells

In order to determine whether utricles from wild-type and *Bcl-2* overexpressing mice were similar in appearance and hair cell numbers, untreated (no neomycin) utricles were fixed and double-labeled using phalloidin and anti-myosin VIIa. Results are shown in Figure 2. Wild-type [Fig. 2(A,C)] and *Bcl-2* overexpressing [Fig. 2(B,D)] utricles were similar in size and shape, and the labeling patterns with both myosin VIIa [Fig. 2(A,B)] and phalloidin [Fig. 2(C,D)] were indistinguishable between the two genotypes. Hair cell counts of double-labeled tissue [Fig. 2(E)] confirmed that utricles from wild-type and *Bcl-2* overexpressing mice contain identical numbers of hair cells.

Bcl-2 Overexpression Promotes Hair Cell Survival following Neomycin Exposure

In order to examine the role of Bcl-2 in neomycin-induced hair cell death, utricles from *Bcl-2* overexpressing transgenic mice and wild-type control animals were cultured for 24 h in the presence or absence of 1 mM neomycin. Utricles were double-labeled for calmodulin and calbindin and examined in whole mount. Figure 3 shows a representative example of calbindin labeling of the Type I hair cells in the striolar region of wild-type and transgenic (*Bcl-2* overexpressing) mouse utricles. In the absence of neomycin, utricles from both wild-type [Fig. 3(A)] and transgenic [Fig. 3(C)] mice demonstrate normal-looking striolar regions with typical "sickle" shapes and normal-appearing numbers of Type I hair cells. However, after 24 h in the presence of 1 mM neomycin, the utricles of wild-type mice [Fig. 3(B)] contain substantially fewer surviving Type I striolar hair cells than utricles from *Bcl-2* overexpressing mice [Fig. 3(D)]. These results suggest that *Bcl-2* overexpression inhibits neomycin-induced hair cell death in the transgenic mice.

In order to quantify the differences in hair cell survival, hair cells were counted in whole-mount preparations of utricles from both wild-type and *Bcl-2* overexpressing mice cultured in the presence or absence of neomycin. Figure 4 shows the dose-response relationships between hair cell density and neomycin concentration for both wild-type and *Bcl-2* overexpressing mice. Hair cell densities (hair cells/900 μm^2) are shown as means $\pm$ S.D. for each group. These data are shown separately for the extrastriolar and striolar regions of the utricle, since the hair cells of the striolar region have been shown to be more susceptible to neomycin-induced death than those of the extrastriolar region (Cunningham et al., 2002). For both regions, the hair cell densities are essentially the same between wild-type and *Bcl-2* overexpressing utricles cultured in the absence of neomycin (control). For both regions and both genotypes, hair cell survival decreases as neomycin concentration increases. However, *Bcl-2* overexpression provides significant protection against neomycin-induced hair cell death in both the striolar and extrastriolar regions (ANOVA, $p < 0.01$ for extrastriola; $p = 0.01$ for striola).

Neomycin-exposed utricles from wild-type and transgenic mice were further examined in sectioned tissue. The utricles were fixed, embedded in plastic, and serial sections were collected. Representative sections are shown in Figure 5. Neomycin-exposed utricles from wild-type mice contain few surviving hair cells and numerous apoptotic bodies [Fig. 5(A)]. In contrast, neomycin-exposed utricles from *Bcl-2* overexpressing mice contain many surviving hair cells, often with intact stereocilia bundles [Fig. 5(B)].

Quantification of hair cell survival was independently performed in sectioned tissue. For this analysis, utricles from both wild-type and *Bcl-2* overexpressing mice were cultured for 24 h in the presence of 1 mM neomycin. Hair cells were counted in serially sectioned, plastic-embedded tissue. Hair cell counts are expressed as mean ($\pm$ S.E.M.) number of hair cells/

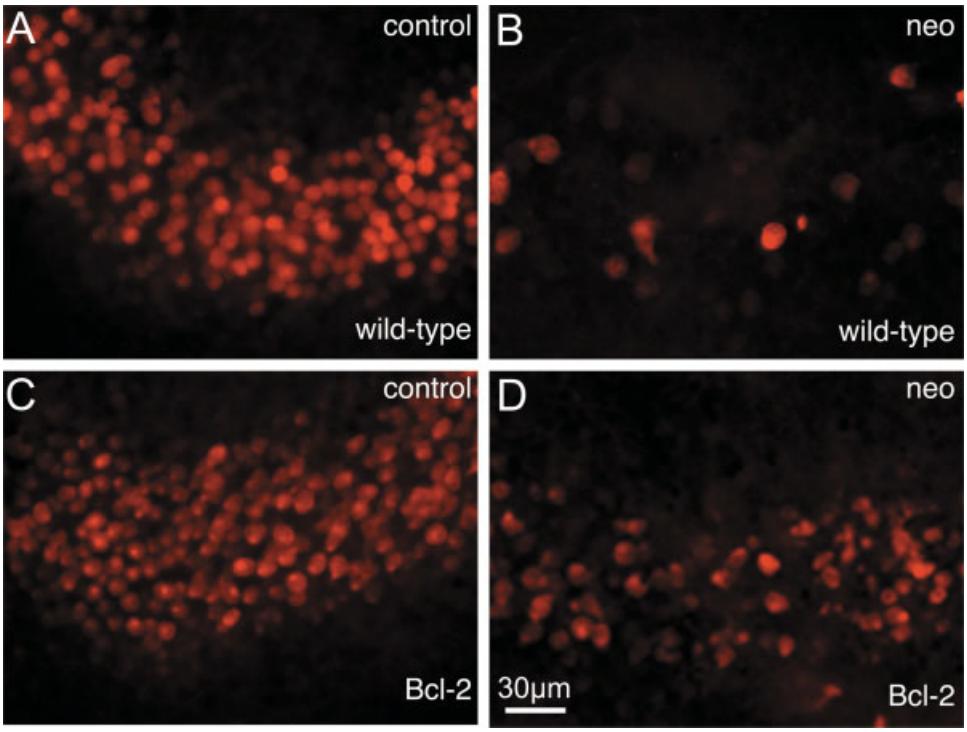

Figure 3 *Bcl-2* overexpression promotes hair cell survival following neomycin treatment. Utricles from wild-type (A, B) and *Bcl-2* overexpressing (C, D) mice were cultured for 24 h in control media (A, C) or in media containing 1 mM neomycin (neo) (B, D). Representative whole mounts are shown of fixed utricles that were immunolabeled using an antibody directed against calbindin, which labels the Type I hair cells in the striolar region of the mouse utricle. In control cultures, the striolar regions of both wild-type (A) and *Bcl-2* overexpressing (C) utricles appear normal and contain a high density of Type I hair cells. Following 24 h in 1 mM neomycin, utricles from wild-type mice contain very few surviving hair cells in the striolar region (B). A representative utricle from a *Bcl-2* overexpressing mouse (D) shows increased hair cell survival in the striolar region relative to wild-type mice. Scale bar = 30 μm. Note: The size of the scale bar is representative of the size of the squares in the eyepiece reticule (30 μm per side) used to quantify hair cell densities (see Methods).

100 μm sensory epithelium. As shown in Figure 5(C), this counting technique also revealed that hair cell survival following neomycin exposure is significantly higher in *Bcl-2* overexpressing utricles than in wild-type utricles ($p < 0.01$). Thus, separate counts of hair cells from whole-mounted and sectioned utricles independently confirmed that *Bcl-2* overexpression provides significant protection against neomycin-induced hair cell death.

Since the data obtained thus far were for cultures in which utricles were fixed immediately after a 24-h exposure to neomycin, these data do not address whether neomycin-exposed hair cells of *Bcl-2* overexpressing utricles would go on to die if examined later. Therefore, we examined whether *Bcl-2* overexpression could continue to inhibit neomycin-induced hair cell death in longer-term cultures. Utricles from wild-type and *Bcl-2* overexpressing mice were cultured for 24 h in 1 mM neomycin. Utricles were then washed and incubated for another 24 h in neomycin-free culture medium prior to fixation. Utricles were double-labeled for calmodulin and calbindin, and hair cells were counted in whole-mounted tissue. Results are shown in Table 1. *Bcl-2* overexpression provides significant protection against neomycin-induced hair cell death in these longer-term cultures in both sensory regions ($p < 0.01$ for both striolar and extrastriolar regions). Comparison of the 24-h and 48-h treatment groups reveals that for both wild-type and *Bcl-2* overexpressing utricles exposed to neomycin, there is considerable loss of hair cells between 24 and 48 h *in vitro*. Neomycin-exposed wild-type utricles showed decreases in hair cell density of 66% in the striola and 38.5% in the extrastriolar region over this period. In the same time period under the same conditions, *Bcl-2* overexpressing utricles exposed to neomycin showed somewhat smaller decreases of 40.6% and 30.4% in the striolar and extrastriolar regions,

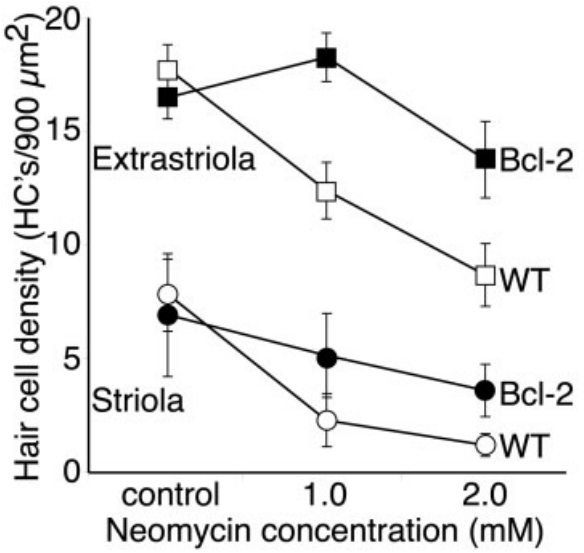

Figure 4 Relationship between neomycin concentration and hair cell density. Utricles from wild-type (open symbols) and *Bcl-2* overexpressing (filled symbols) mice were cultured for 24 h in control media or in media containing 1 mM or 2 mM neomycin. Utricles were double-labeled for calmodulin and calbindin immunoreactivity. For each utricle, hair cells were counted in each of four 900 μm^2 regions in the extrastriolar region and four 900 μm^2 regions in the striolar region. Hair cell counts from each region were averaged to give a mean striolar hair cell density and mean extrastriolar hair cell density for each utricle examined. In control cultures, utricles from wild-type and *Bcl-2* overexpressing mice contained approximately equal numbers of hair cells. For both the striolar and extrastriolar regions, hair cell density decreased as neomycin concentration increased (ANOVA, $p < 0.01$). For both regions, overexpression of *Bcl-2* resulted in a significant increase in hair cell survival following neomycin treatment (ANOVA, $p < 0.01$ for extrastriola; $p = 0.01$ for striola). Shown are mean hair cell densities ± S.D. for $n = 10$ utricles per group.

respectively. This hair cell loss occurs despite the fact that there is no neomycin present in the cultures during the final 24 h *in vitro*. Two-way analysis of variance (ANOVA) revealed significant main effects of both genotype and hours (24 vs. 48) for both striolar and extrastriolar hair cells (ANOVA, $p< 0.01$ for all four analyses). However, no significant interaction effect (genotype * hours) was present (ANOVA, $p = 0.62$ striola; $p = 0.49$ extrastriola).

Bcl-2 Overexpression Prevents Activation of Caspase-9 in Hair Cells

We have previously shown that activation of caspase-9 is necessary for neomycin-induced hair cell death in the cultured mouse utricle (Cunningham et al., 2002). Bcl-2 acts upstream of caspase-9 activation by inhibiting cytochrome c release (Kluck et al., 1997; Yang et al., 1997; Chao and Korsmeyer, 1998). Therefore, we examined whether overexpression of *Bcl-2* prevents activation of caspase-9 in neomycin-exposed hair cells. Utricles from wild-type and *Bcl-2* overexpressing mice were cultured for 12 h in 1 mM neomycin. Caspase-9 activation was assayed using the fluorescent caspase-9 substrate fam-LEHD-fmk (see Methods; also see Cunningham et al., 2002). After the culture period and incubation in the caspase-9 substrate, utricles were fixed, and stereocilia were labeled using rhodamine phalloidin. Representative results are shown in Figure 6. Utricles from both wild-type [Fig. 6(A)] and *Bcl-2* overexpressing [Fig. 6(C)] mice show very little activation of caspase-9 when cultured in neomycin-free medium. In the presence of neomycin, utricles from wild-type mice show robust activation of caspase-9 in hair cells [Fig. 6(B)]. However, utricles from *Bcl-2* overexpressing mice show very little activation of caspase-9 when cultured in the presence of 1 mM neomycin for 12 h [Fig. 6(D)].

The caspase-9 substrate data were quantified by counting the total number of hair cells in each utricle that demonstrated activation of caspase-9 (Fig. 7). Hair cells expressing activated caspase-9 were counted in wild-type ($n = 4$ control; 7 neomycin-treated) and *Bcl-2* overexpressing ($n = 6$ control; 7 neomycin-treated) utricles after 12 h in culture. In control media, both wild-type and *Bcl-2* overexpressing utricles contained fewer than five hair cells demonstrating activated caspase-9. When cultured in the presence of neomycin, wild-type utricles contained 57.6 ± 11.1 (mean ± S.D.) hair cells with activated caspase-9. In contrast, *Bcl-2* overexpressing utricles cultured in neomycin contained only 8.14 ± 4.78 activated caspase-9-positive hair cells. This difference in caspase-9 activation between wild-type and *Bcl-2* overexpressing utricles was highly significant ($p < 0.001$). These data indicate that *Bcl-2* overexpression prevents activation of caspase-9 in hair cells exposed to neomycin.

DISCUSSION

The purpose of this study was to examine the hypothesis that Bcl-2 plays a role in modulating aminoglycoside-induced hair cell death. The study utilized transgenic mice that overexpress human *Bcl-2* under the control of the neuron-specific enolase promoter (Martinou et al., 1994). Immunochemistry using an

antibody that recognizes human Bcl-2 (but not mouse Bcl-2) confirmed that the *Bcl-2* transgene is expressed in hair cells of the utricles of *Bcl-2* overexpressing transgenic mice. Hair cell counts of double-labeled utricles demonstrated that untreated wild-type and *Bcl-2* overexpressing utricles contain the same number of hair cells.

Bcl-2 Overexpression Prevents Neomycin-Induced Hair Cell Death

Our primary finding is that overexpression of *Bcl-2* provides significant protection against aminoglycoside-induced hair cell death. This finding was obtained independently in two different laboratories using markedly different methods of quantifying hair cell numbers. Using hair cell counts obtained from whole-mount preparations of utricles exposed to 1 mM neomycin, overexpression of *Bcl-2* improved hair cell survival by 45% in the striolar region and 69% in the extrastriolar region. Remarkably similar results were obtained independently using similar culture conditions (24 h in 1 mM neomycin) but a very different method of counting hair cells. Using hair cell counts from serially sectioned tissue, *Bcl-2* overexpression resulted in 45% more surviving hair cells relative to utricles from wild-type animals. Thus, our results are in agreement with many studies that have shown that Bcl-2 promotes cell survival in a large number of systems and in response to a variety of death-inducing stimuli (Sentman et al., 1991; Strasser et al., 1991; Martinou et al., 1994; Putcha et al., 1999; Mostafapour et al., 2002).

Bcl-2 overexpression continued to promote hair cell survival in longer-term cultures. We cultured utricles from *Bcl-2* overexpressing and wild-type mice in 1 mM neomycin for 24 h and then cultured them for an additional 24 h in neomycin-free media. Utricles from the *Bcl-2* overexpressing mice contained significantly more hair cells in both the striolar and extrastriolar regions after the 48-h culture period ($p < 0.01$ for both striolar and extrastriolar regions). However, neomycin-exposed utricles from both wild-type and *Bcl-2* overexpressing mice continued to lose hair cells during this additional 24-h period. Both wild-type and *Bcl-2*

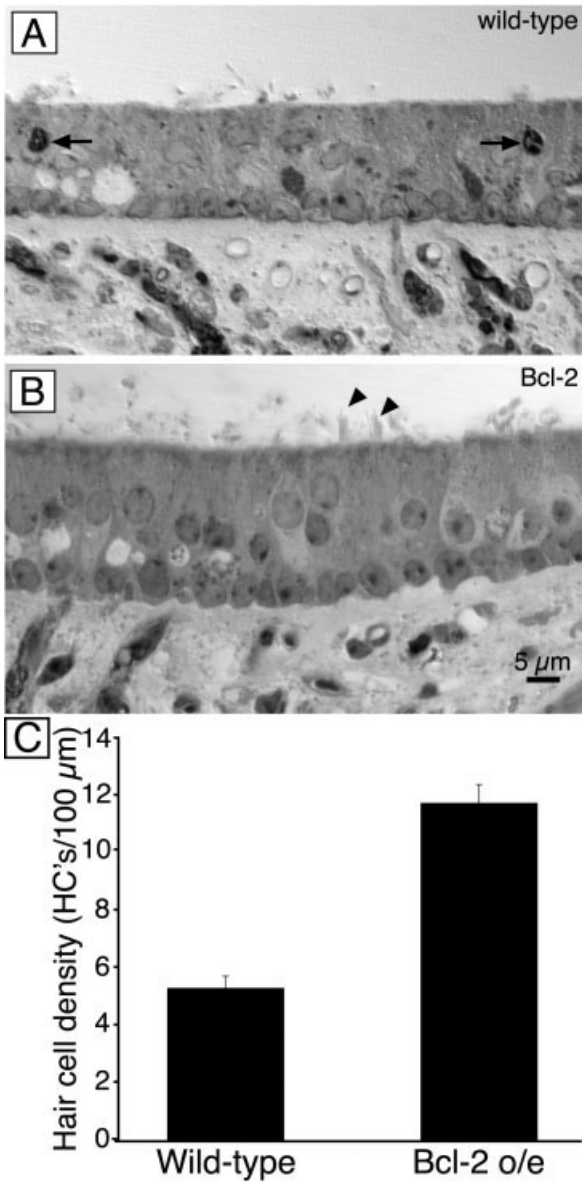

Figure 5 Sections of neomycin-exposed utricles. Shown are representative toluidine blue-stained sections from wild-type (A) and *Bcl-2* overexpressing (B) utricles that were cultured for 24 h in 1 mM neomycin. Wild-type utricles (A) contain few surviving hair cells and multiple apoptotic bodies (arrows) following neomycin exposure. In contrast, neomycin-exposed utricles from *Bcl-2* overexpressing mice (B) show many more surviving hair cells, some of which contain visible stereocilia bundles (arrowheads). C: Hair cell densities obtained from sections of neomycin-treated utricles. Utricles from wild-type and *Bcl-2* overexpressing mice were cultured for 24 h in 1 mM neomycin. Utricles were then fixed, sectioned at 3 μm, and stained with toluidine blue. Using a light microscope at 100×, surviving hair cells were counted, and the length of each sensory epithelium was measured. Hair cell density is reported as number of hair cells/100 μm sensory epithelium; 8–12 sections per organ and 4–7 organs per experimental condition were examined. Following neomycin exposure, utricles from *Bcl-2* overexpressing mice contained significantly more surviving hair cells than did utricles from wild-type mice ($p < 0.01$). Scale bar in B = 5 μm.

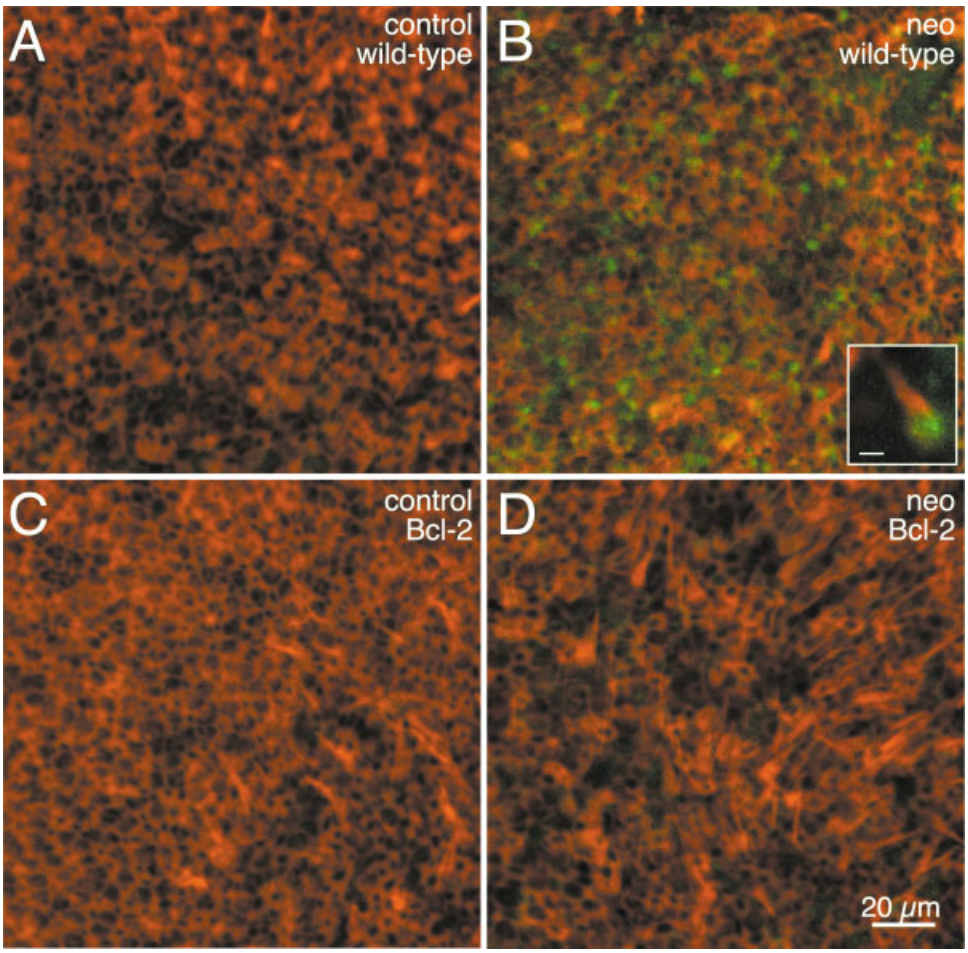

Figure 6 Overexpression of *Bcl-2* prevents activation of caspase-9 in hair cells exposed to neomycin. Utricles from wild-type (A, B) and *Bcl-2* overexpressing (C, D) mice were cultured for 12 h in control media (A, C) or media containing 1 mM neomycin (B, D). During the final hour in culture, the green fluorescent caspase-9 substrate fam-LEHD-fmk was added to the culture media. Utricles were then fixed and stereocilia were labeled using rhodamine phalloidin (red). In control cultures (no neomycin), utricles from wild-type (A) and *Bcl-2* overexpressing (C) mice contained little or no detectable caspase-9 activation. When cultured in the presence of neomycin, utricles from wild-type mice showed robust activation of caspase-9 in hair cells (B). However, utricles from *Bcl-2* overexpressing mice showed very little activation of caspase-9 in response to neomycin exposure (D). Inset in B shows a higher magnification image of a hair cell that is positive for activated caspase-9 in the cell body (green) and has a stereocilia bundle labeled red with phalloidin. Scale bar in inset = 5 μm. Scale bar in D = 20 μm.

overexpressing utricles showed greater decreases in hair cell density following neomycin exposure than did utricles cultured in control medium (no neomycin) throughout the 48-h culture period. Taken together, these data suggest that *Bcl-2* overexpression improves hair cell survival in the 48-h cultures relative to that seen in wild-type utricles. However, hair cell death does continue to occur in the *Bcl-2* overexpressing utricles in the 24 h following the removal of neomycin. The absence of an interaction effect (genotype * hours) for the final 24 h in culture indicates that the rate of hair cell loss during this period is approximately equal for wild-type and *Bcl-2* overexpressing utricles. Thus, while utricles from *Bcl-2* overexpressing mice contain significantly more hair cells after 48 h in culture than do utricles from wild-type mice, the protective effect of Bcl-2 occurs primarily during the first 24 h in culture. During the second 24 h in culture, the protective effect of Bcl-2 is overwhelmed in some hair cells, resulting in death of those cells.

Table 1 Hair cell densities from utricles cultured 24 or 48 h[1]

Genotype Region	Control (no Neo) 24 h	Control (no Neo) 48 h	24 h (+ Neo)	48 h (+Neo 24 h/−Neo 24 h)
WT extrastriola	17.75 ± 1.06 ($n = 5$)	16.07 ± 0.86 ($n = 5$)	12.39 ± 1.24 ($n = 6$)	7.63 ± 1.55 ($n = 5$)
Bcl-2 o/e extrastriola	16.48 ± 1.94 ($n = 6$)	15.78 ± 1.78 ($n = 4$)	18.25 ± 1.08 ($n = 6$)	12.71 ± 0.78* ($n = 5$)
WT striola	7.53 ± 1.54 ($n = 6$)	5.04 ± 0.85 ($n = 5$)	2.29 ± 1.15 ($n = 6$)	0.78 ± 0.43 ($n = 5$)
Bcl-2 o/e striola	6.925 ± 2.70 ($n = 6$)	5.50 ± 0.84 ($n = 4$)	5.15 ± 1.86 ($n = 6$)	3.06 ± 0.74* ($n = 5$)

[1] Hair cell densities (mean ± S.D.) for both the extrastriolar and striolar regions of wild-type (WT) and *Bcl-2* overexpressing (Bcl-2 o/e) mouse utricles.

*For both the extrastriolar and striolar regions, *Bcl-2* overexpression provided significant protection (relative to WT) against neomycin (Neo)-induced hair cell death in the 48-h cultures (two-way ANOVA, $p < 0.01$ for both striolar and extrastriolar regions).

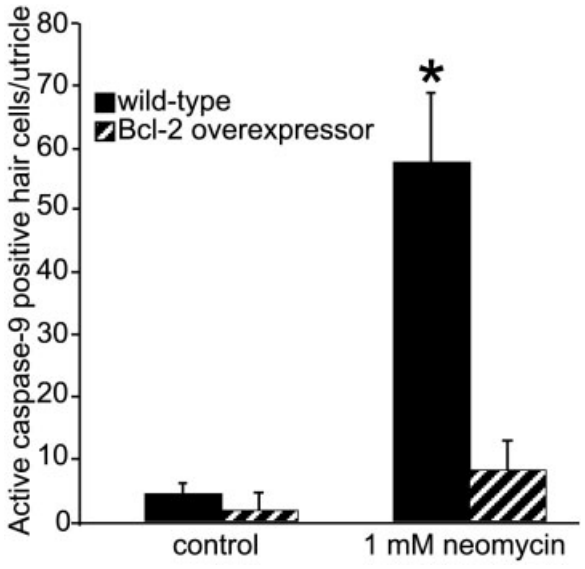

Figure 7 Quantification of hair cells expressing activated caspase-9. Hair cells demonstrating activation of caspase-9 (using the fluorescent substrate fam-LEHD-fmk) were counted in control and neomycin-treated utricles from wild-type and *Bcl-2* overexpressing mice. Each bar represents the number (mean ± S.D.) of active caspase-9-positive hair cells per utricle after 12 h in culture. Note: Hair cells expressing activated caspase-9 were counted at only one timepoint (12 h in culture either with or without neomycin). Therefore, these counts represent a "snapshot" of caspase-9 activation and likely underestimate the total number of hair cells in which caspase-9 was activated. Wild-type: $n = 4$ control and 7 neomycin-treated; *Bcl-2* overexpressing: $n = 6$ control and 7 neomycin-treated. *Statistically significant ($p < 0.001$) difference between wild-type and *Bcl-2* overexpressing utricles in the number of caspase-9 positive hair cells after neomycin exposure.

Bcl-2 Overexpression Prevents Activation of Caspase-9 in Hair Cells

We had previously shown that activation of caspase-9 is required for neomycin-induced hair cell death (Cunningham et al., 2002). Here we show that *Bcl-2* overexpression prevents activation of caspase-9 in hair cells exposed to neomycin. This finding is consistent with the known mechanism of Bcl-2 function. At the mitochondrial membrane, Bcl-2 functions to prevent release of mitochondrial cytochrome c into the cytoplasm (Kluck et al., 1997; Yang et al., 1997; Chao and Korsmeyer, 1998). By inhibiting the release of cytochrome c, Bcl-2 prevents the activation of caspase-9 (Li et al., 1997).

Model for Mitochondrially-Mediated Hair Cell Death

Our findings are consistent with a model in which aminoglycoside-induced hair cell death is mediated by mitochondria. This model is also supported by several reports showing that reactive oxygen species (ROS) are produced in hair cells exposed to aminoglycosides (Clerici et al., 1996; Hirose et al., 1997, 1999; Sha and Schacht, 1999; Takumida and Anniko, 2001), and that free radical scavengers can protect hair cells against aminoglycoside-induced death (Garetz et al., 1994; Song and Schacht, 1996; Sinswat et al., 2000; Sha et al., 2001; Wang et al., 2003). If sufficient ROS are produced, the cell's intrinsic antioxidant mechanisms are overwhelmed (Sha et al., 2001), and reactive oxygen species promote the release of mitochondrial cytochrome c into the cytoplasm (Atlante et al., 2000). The release of cytochrome c into the cytoplasm results in activation of

caspase-9 and initiation of apoptotic death. Once activated, caspase-9 activates downstream caspase-3 in hair cells (Cunningham et al., 2002). Activated caspsase-3 participates in the destruction of the cell by cleaving many proteins necessary for cell survival, including Bcl-2, inhibitors of deoxyribonucleases, and cytoskeletal proteins (Lazebnik et al., 1994; Cheng et al., 1997; Kothakota et al., 1997; Kirsch et al., 1999). It appears likely that overexpression of *Bcl-2* inhibits the release of cytochrome c in hair cells, thereby inhibiting the activation of this mitochondrial apoptotic pathway.

In summary, we have shown that overexpression of *Bcl-2* inhibits aminoglycoside-induced hair cell death in the mouse utricle *in vitro*. Furthermore, overexpression of *Bcl-2* is sufficient to prevent the activation of caspase-9 that is required for neomycin-induced hair cell death. Along with data from other researchers, our data are consistent with a model in which aminoglycoside-induced hair cell death is mediated by mitochondria. Expansion of our understanding of the cellular biology of hair cell death is an important step in identifying potential targets for therapeutic intervention.

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