ORIGINAL ARTICLE

Cochlear protection against cisplatin by viral transfection of X-linked inhibitor of apoptosis protein across round window membrane

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We have previously demonstrated that both age-related and noise-induced hearing loss are reduced in transgenic mice that ubiquitously overexpress X-linked inhibitor of apoptosis protein (XIAP). In view of the therapeutic implications of these findings, we have developed a minimally invasive surgical method to deliver adenoid-associated virus (AAV) across the round window membrane (RWM) of the cochlea, enabling efficient gene transfer to hair cells and sensory neurons in this enclosed structure. This RWM approach was used in the present study to evaluate the effectiveness of AAV-mediated XIAP overexpression in protecting against cisplatin-induced ototoxicity. Two weeks following surgery, AAV-derived XIAP was detected in the majority of inner and outer hair cells, resulting in a threefold elevation of this antiapoptotic protein in the cochlea. The protection of AAV-mediated XIAP overexpression was evaluated in animals treated with cisplatin at a dose of 4 mg kg⁻¹ per day for 4–7 consecutive days. The XIAP overexpression was found to attenuate cisplatin-induced hearing loss by ~22 dB. This was accompanied by a reduction of the loss of vulnerable hair cells and sensory neurons in the cochlea by 13%.

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INTRODUCTION

Platinum-based chemotherapeutics including cisplatin and carboplatin are commonly used in the treatment of many forms of cancer, particularly those that occur in childhood.¹ In combination with a high standard of supportive care, these agents have markedly increased the survival rates of children with cancers in developed countries.² However, the therapeutic benefits of platinum-based chemotherapeutics are compromised by the high rates of hearing loss (>60%) associated with the use of these drugs in pediatric patients.^{3,4}

Hearing loss induced by cisplatin is largely caused by the death of sensory cells, especially outer hair cells (OHC) in the cochlea, although the loss of spiral ganglion neurons (SGN) and injury of lateral wall structures such as the stria vascularis and spiral ligament may also be contributing factors.^{5–8} At concentrations achieved by chemotherapeutic doses of cisplatin, cell death occurs mainly through the induction of apoptosis that is mediated by two mechanisms.⁹ The first is directly related to the antineoplastic effects of cisplatin that inhibit mitosis.² This is initiated by the covalent binding of cisplatin to guanine bases in DNA, causing genotoxic stress that triggers p53-mediated activation of the intrinsic (mitochondrial) apoptotic pathway.^{2,10} The second mechanism is attributed to free radical-induced apoptosis.^{2,9–16} This is partially linked with the first mechanism because DNA damage impairs the synthesis of antioxidant enzymes, resulting in elevated levels of injurious free radicals.⁹

Many studies have demonstrated that preventing apoptosis is effective in alleviating cisplatin-induced cochlear damage and

hearing loss.^{17–23} Previous studies from our group have shown that overexpression of X-linked inhibitor of apoptosis protein (XIAP) delays age-related hearing loss²⁴ and attenuates noise-induced hearing loss.²⁵ Other laboratories have also shown that adeno-associated virus (AAV)-mediated XIAP overexpression decreases cisplatin-induced hearing loss.^{20,21}

Some advantages of using AAV-mediated XIAP cochlear gene therapy to prevent cisplatin ototoxicity are long-lasting protection produced by sustained elevation of this antiapoptotic protein, and the gene transfer that is restricted to the cochlea reducing the risk of impacting other organs. However, previous studies that have demonstrated AAV-mediated XIAP overexpression protects against cisplatin ototoxicity did not provide data about the transfection effectiveness and details regarding the degree to which sensory hair cell loss was attenuated.^{20,21} Moreover, the AAV-XIAP vector used in these studies was delivered by cochleostomy, a traumatic surgical approach that might cause hearing loss.

We have recently established a minimally invasive method for AAV-mediated gene transfer in the cochlea via the round window membrane (RWM).²⁶ In combination with a newly developed AAV vector in which gene transfer effectiveness is increased by the point mutation of a surface tyrosine residue (Y733F),^{27–29} we achieved a 40–50% transfection rate of OHC, a sensory cell population not previously reported to be transfected by AAV as well as a transfection rate of over 80% for inner hair cells (IHCs).²⁶ One basis of this progress, we have re-evaluated the effectiveness of AAV-mediated XIAP overexpression against cisplatin ototoxicity.

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AAV-XIAP was delivered to the cochlea using our RWM approach followed by confirmation of AAV-mediated XIAP overexpression and quantification of the degrees to which cisplatin-induced hair cells (HC) and SGN losses were reduced.

RESULTS

In the first step of our experiment, we counted the number of HCs that were transfected by the AAV-XIAP vector delivered using our RWM approach. This was done by immunohistochemical detection of the 6Myc tag on XIAP encoded by the AAV-XIAP vector. In agreement with our previous findings,²⁶ a high degree of transfection was achieved with >90% of IHCs and ~50% of OHCs in the basal turn displaying 6Myc immunoreactivity (Figure 1). Although the transfection rate declined toward the apex of the cochleae, a large portion of hair cells were still transfected even in the third turn (>50% for IHCs and ~30% for OHCs).

In the second step, we examined the protective effects of AAV-XIAP against cisplatin-induced ototoxicity in 11 animals. For each animal, our RWM approach was performed on both ears for transfection. One served as the control ear and did not receive the AAV-XIAP vector but only sham surgery, whereas the other served as the experimental ear and received the AAV-XIAP vector. Auditory brain stem responses (ABRs) were measured and compared in seven animals before and 10 days after surgery for the control and experimental ears at 1, 2, 4, 8, 16 and 32 kHz to generate threshold–frequency curves. ABR thresholds did not show any significant differences between the control and experimental ears, as well as before and after the surgery (Figure 2a). These findings support the safety of our RWM approach to achieve gene transfer in the cochlea. Following the second measurement of ABRs, all experimental animals received cisplatin (4 mg kg⁻¹, once daily for 4–7 days). ABRs were measured again 7 days after the cessation of the cisplatin. A larger threshold shift was detected at all frequencies in the control relative to the experimental ears following cisplatin administration (Figure 2b). Cisplatin-induced hearing loss was seen across the

whole tested frequency range with the largest shifts occurring in the three middle frequencies (4, 8 and 16 kHz). The average hearing threshold over 4–16 kHz was 32.85 ± 1.44 dB sound pressure level (SPL) in the experimental ears that received AAV-XIAP. This was significantly lower than the value of 54.76 ± 4.87 dB SPL in the control ears (paired *t*-test, *df* = 6, *t* = 4.396, *P* = 0.0046).

To further quantify XIAP transfection effectiveness and the impact of cisplatin on the expression of this protein, western blotting was performed to detect the levels of both endogenous XIAP (en-XIAP) and 6Myc-XIAP derived from the AAV-XIAP vector β -actin as internal reference. Cochlear proteins were extracted from three types of samples or animals for this purpose. (1) Four cochleae were collected from four guinea pigs receiving binaural 6Myc-XIAP transfection but no cisplatin treatment, one ear from each of those animals was used for western blotting and other ears were for HC transfection rate and SGN count (see method section). (2) Eight cochleae were collected from four animals that received 6Myc-XIAP transfection surgery in one ear and then treated with cisplatin systematically. (3) Still another four animals that were used in other experiment. Those animals received 6Myc-XIAP transfection in one ear, but no cisplatin treatment. The sample size was four for every group and every treatment.

In the no-cisplatin-treated samples, levels of en-XIAP were the same in control (sham surgery) and 6Myc-XIAP-transfected ear (comparison between the two ears within group 3, data not shown), suggesting that the 6Myc-XIAP did not impact the expression of en-XIAP. The levels of 6Myc-XIAP were ~2.6 fold as high as the en-XIAP (comparison between the two XIAPs within the ears of group 1, Figure 3, 6Myc-XIAP1: 0.456 ± 0.056 , en-XIAP1: 0.170 ± 0.012 , *t*-test, *df* = 6, *t* = 4.997, *P* = 0.002) in those animals. In the group 2 treated with cisplatin, levels of en-XIAP were increased significantly as compared with that in group 1 without cisplatin. As the increase was the same in both the control ears (receiving sham surgery) and experimental ears (receiving AAV-6Myc-XIAP), the value was pooled as 0.342 ± 0.052 (en-XIAP2, Figure 3). Compared with the en-XIAP1 in the ears of no cisplatin control animals, the increase was 100% (0.170 ± 0.012 versus 0.342 ± 0.052 , *t*-test, *df* = 6, *t* = 3.21, *P* = 0.019). However, the level of 6Myc-XIAP in the cisplatin-treated animals (6Myc-XIAP2) was not significant different from 6Myc-XIAP1 in saline-treated control animals (Figure 3). Although en-XIAP levels were increased by cisplatin administration, 6Myc-XIAP still contributed more to the total pool of XIAP (6Myc-XIAP > en-XIAP2) in the 6Myc-XIAP transfected ears.

The protective effects of 6Myc-XIAP were further demonstrated morphologically by the quantification of hair cell loss. Cisplatin administration resulted in a significant loss of OHCs that was reduced by delivery of the AAV-XIAP vector. Figure 4 shows the representative images of surface preparation from no-cisplatin blank control (a), no-myc-XIAP control ear treated with cisplatin (b) and the experimental ear treated with cisplatin (c, transfected with 6Myc-XIAP). Figure 5 shows the loss of OHCs in the control and experimental ears of seven animals treated with cisplatin. Cisplatin mainly caused OHC loss at the basal end of the cochlea: no significant OHC loss was found above the middle portion of the cochlea (below 10 kHz, Figure 5a). Moreover, OHC loss was significantly less in the ears treated with the AAV vector. Cell counts revealed that average OHC cell loss in the AAV-transfected ears (899.9 ± 170 /cochlea, mean $\pm$ s.d.) was significant less than that of the no-transfected control ears (1040.7 ± 171 /cochlea, paired *t*-test, *df* = 6, *t* = 16.227, *P* < 0.001, Figure 5b).

XIAP overexpression also protected against the loss of SGNs produced by cisplatin. In the Rosenthal's canal of a cochlea from the control ear of a saline-treated animal, SGNs are tightly packed and fill the entire region of the canal (Figure 6a). However, in the cochlea from the control ear of an animal treated with cisplatin there seems to be fewer SGNs and a distinct margin of cell loss that surrounds the canal (Figure 6b). Consistent with the induction

Figure 1. Representative confocal images of AAV-mediated XIAP transfection in the cochlea. Transfection was assessed by immunohistochemical detection of the 6Myc tag on AAV-derived XIAP.

Figure 2. The ABR audiogram. (a) control recording and 10 days after the surgery. (b) 7 days after the last day of cisplatin treatment. Bar: SEM. $N = 7$ in each group. Pre-S/Post-S: pre and post surgery.

Figure 3. Quantification of both en-XIAP and 6Myc-XIAP by western blotting. 6Myc-XIAP, transfected, en-XIAP: endogenous; 1: from animals treated with saline, 2: from animals treated with cisplatin. $N = 4$ in each group. * $P < 0.05$.

Figure 4. Images of BM surface preparation stained with Rhodamine phalloidin. All samples were from basal turn. (a) Blank normal control (without cisplatin). (b) and (c): cisplatin-treated (b: ear with sham surgery, c: ear transfected with 6Myc-XIAP).

of programmed cell death by cisplatin, condensed SGNs that have an apoptotic-like morphology are clearly visible (arrow heads Figure 6b). By contrast, in the experimental ear of an animal treated with cisplatin, fewer apoptotic cells are observed and there appears to be less cell loss (Figure 6c).

To quantify these changes, intact SGNs were counted in cochlear sections from the control and experimental ears of animals treated with either saline or cisplatin. A one-way ANOVA was performed against the factor of cisplatin treatment over three levels (ctrl, cisplatin, cisplatin+6Myc-XIAP) and showed a significant difference across groups ($F_{2,20} = 18.925$, $P < 0.001$) for the SGN counts of the first turn. Here, relative to the average SGN counts in no-cisplatin-treated animals (85.88 ± 5.36), there were significantly fewer SGNs in cochlea from the control ears (without 6Myc-XIAP transfection) of cisplatin-treated animals (68.28 ± 5.95 ; $t = 6.093$, $P < 0.001$, *post hoc* comparison, Holm–Sidak method, indicated by asterisks in Figure 7a). The loss of SGNs produced by cisplatin was attenuated in the cochlea from the experimental ears that received the AAV-XIAP vector (78.68 ± 4.19). The difference between the ears in cisplatin-treated animals with and without 6Myc-XIAP transfection was also significant ($t = 3.783$, $P = 0.001$, *post hoc* test Holm–Sidak method). These trends were the same in both the first and second turn of the cochlea (Figure 7b).

DISCUSSION

In the present study, XIAP overexpression was achieved using an AAV vector in which a surface tyrosine residue (Y733F) had been mutated to improve transfection effectiveness.³⁰ We have previously shown that a high degree of OHC transfection in guinea pig cochlea is achieved using this vector at high titers (close to 10^9 ; ref. 14).²⁶ AAV-XIAP was delivered by diffusion across the RWM rather than by violating the otic capsule with a cochleostomy that may cause trauma and hearing loss. In preventing ototoxicity, the development of a non-traumatic approach is critical for clinical applications in which the patient population has near-normal hearing function. This is especially the case in cisplatin-induced hearing loss because immunosuppression resulting from chemotherapy increases the risk of labyrinthitis.

As the RWM is not permeable to AAV, a digestive procedure using collagenase was used to gently increase permeability of this membrane to AAV. In agreement with our previous reports,²⁶ we achieved a high degree of gene transfer for both OHCs and IHCs using this new trans-RWM protocol. Furthermore, this method for cochlear gene therapy did not cause hearing loss (Figure 1). These findings indicate that our RWM approach is an effective and safe

Figure 5. Comparison for OHC loss between ears treated with sham surgery (Ctrl) and that received 6Myc-XIAP. Left: cytochleogram for % OHC loss as the function of distance and frequency. Right: averaged HC loss for each cochlea. *** $P < 0.001$. $N = 7$ in each group.

Figure 6. Representative images of SGNs in the cross view of Rosenthal's canal. (a): blank normal control shows the tightly packed SGNs with close contact to the wall of the canal. (b and c): from cisplatin-treated animal (b: sham surgery control, c: ear with 6Myc-XIAP transfection). Both b and c show a loss of tight SGN packing and dying SGNs (white arrow heads) and a margin region without SGNs along the inner surface of the canal.

Figure 7. Comparison of SGN counts across groups of blank normal control (Ctrl) and those animals treated with cisplatin, one ear with 6Myc-XIAP transfection (Cis-Myc-XIAP) and the other with sham surgery (Cis alone). The numbers are the averaged SGNs in each sectional view crossing Rosenthal's canal. $N = 7$ in each group. *** $P < 0.001$. $N = 7$ in each group.

method for cochlear gene therapy that may be readily adapted in the clinic.

The 6Myc tag on XIAP encoded by the AAV vector enabled us to compare the degree of XIAP overexpression obtained with respect to endogenous levels of this antiapoptotic protein. AAV-XIAP delivered by our RWM approach produced about a threefold elevation of total XIAP levels in the cochlea. Levels of endogenous XIAP were not reduced by this increase in 6Myc-XIAP produced by the AAV-XIAP vector (Figure 3). We have previously noted that the expression of endogenous XIAP is unaltered in transgenic mice

that ubiquitously overexpress 6Myc-XIAP.²⁴ Furthermore, overexpression of 6Myc-XIAP did not attenuate the increase in endogenous XIAP levels produced by cisplatin (Figure 3). These findings further support the safety of AAV-mediated XIAP overexpression by demonstrating that regulation of the endogenous gene was unaltered.

Previously, the protection effect of AAV-mediated XIAP overexpression against cisplatin was tested conceptually with positive outcome.^{20,21} In those earlier reports, however, the AAV-mediated XIAP transfection to the hair cells (especially the OHCs) in the

organ of Corti was not demonstrated and quantified. To our knowledge, successful AAV-mediated gene transfection to OHCs was not reported prior to our previous study in which virus with tyrosine mutation in high titer was used.²⁶

In agreement with this clear evidence of AAV-mediated XIAP gene transfer to sensory hair cells in the cochlea, these vulnerable cells were protected both morphologically and functionally against cisplatin-induced death. Relative to control ears in guinea pigs treated with cisplatin, the experimental ears that received the AAV-XIAP vector displayed a 20 dB improvement in ABRs averaged over 4–16 kHz. This was accompanied by a reduction in the loss of OHCs in the cochlea of experimental relative to control ears (Figure 5). In addition to OHCs, XIAP overexpression also increased the survival of SGNs (Figure 7).

AAV-mediated XIAP overexpression also reduced cisplatin-induced hearing loss at the lowest frequency (1 kHz) tested (Figure 1). However, hair cell loss was limited to the basal half of the cochlea associated with higher frequency hearing. This suggests OHCs in the apical half may have been functionally damaged or in the process of dying although appearing physically intact at the time of our histological assessments. Indeed, the degree of functional hearing protection over the entire frequency range examined appears larger than the magnitude of cellular protection achieved. This further suggests that some remaining hair cells in the control cochlea of cisplatin-treated animals may have been functionally compromised and in the process of undergoing cell death that was not detectable by our morphological measurements of viability. Extended observations at longer time periods after the cessation of cisplatin treatment may enable us to resolve this discrepancy.

The mechanisms responsible for SGN death after cisplatin are not entirely clear. In the present study, the damaging effects of cisplatin on SGNs were assessed as a reduction in SGN numbers and morphological features consistent with apoptosis (condensed nuclei and organelles, Figure 6). SGN death may have resulted from direct cellular damage by cisplatin or may have been secondary to the loss of sensory hair cells that injures synapses between IHCs and SGNs.³¹ However, the latter mechanism is unlikely because the degenerative death of SGNs usually takes a relatively long time (months to years) to develop after sensory hair cell loss or damage to the synapses between SGNs and IHCs,^{32,33} whereas SGN death detected in the present study occurred within days after cisplatin treatment.

In conclusion, we have found that delivery of AAV-XIAP via the RWM safely and robustly increases XIAP expression in sensory hair cells of the cochlea protecting them from the injurious effects of cisplatin. SGN that are vulnerable to damage by cisplatin were also protected by this RWM approach for AAV-mediated XIAP overexpression in the cochlea. Preservation of these sensory hair cell and neuronal populations was accompanied by a marked reduction in cisplatin-induced hearing loss. AAV-mediated XIAP overexpression did not alter the endogenous expression of XIAP in the cochlea further supporting the safety of this approach. These findings suggest that XIAP overexpression achieved by delivery of an AAV-XIAP vector via the RWM may be a clinically tractable strategy to prevent cisplatin-induced hearing loss.

MATERIALS AND METHODS

Subjects and procedures

In total 18 young (2–3-month-old) adult albino guinea pigs were used in these experiments (Charles River Co., Senneville, QC, Canada). All passed otologic evaluation for normality of external and middle ears, and baseline test of auditory brainstem responses (ABR) for hearing sensitivity.

Out of the 18 animals, 4 received binaural transfection of AAV-XIAP and were used for measuring AAV-XIAP transfection in HC, for the control counts of SGNs and western blotting for both en-XIAP and 6Myc-XIAP. Two weeks after the transfection surgery, the four animals were killed and one

cochlea from each animal was used for making a cochleogram to measure the transfection rate, and the other ear for western blotting to quantify the levels of both en- and 6Myc-XIAP without cisplatin.

The remaining 14 animals were used for the transfection-induced protection experiments. Transfection surgery was carried out in each animal binaurally: with one ear randomly selected for receiving AAV-6-myc-XIAP and the other ear for saline of equal volume. Another ABR test was carried out 10 days after the transfection surgery to ensure that there was no significant hearing owing to the surgery. This was followed by the administration of cisplatin. One week after final injection of cisplatin, 11 animals had survived. The final ABR was then performed followed by morphologic observations (in 7 out of 11 animals) or western blotting (in 4 out of 11 animals) for measuring the level of both 6Myc- and en-XIAPs after cisplatin treatment.

All experimental procedures were approved by the ethics committees for laboratory animals at Dalhousie University in Canada. They also conformed to the NIH guidelines for laboratory animals.

ABR recording

Guinea pigs were anesthetized with a mixture of ketamine and xylazine (40 mg kg⁻¹ and 10 mg kg⁻¹ intraperitoneally, respectively) for ABR recording. The body temperature of the animals was maintained at ~38 °C using a thermostatic heating pad during the recording. The ABR tests were performed in a sound-attenuating audiometric chamber. All stimuli were generated and all responses were recorded and processed using Tucker–Davis Technologies hardware and software (TDT System III, Alachua, FL, USA). Tone bursts at 1, 2, 4, 8, 16 and 32 kHz were generated digitally with duration of 10 ms and rise-fall time of 1 ms. The acoustic stimuli were presented at a rate of 21.1 per second and delivered using an electrostatic transducer (EC1, TDT) via a plastic tube, which was inserted into the animal's external ear canal. This closed field stimulation ensured separate evaluations of hearing in each ear. The evoked responses were collected using three subdermal electrodes positioned at the vertex and both mastoid regions. The evoked potentials were filtered with a bandpass filter between 100 and 3000 Hz, and averaged 1000 times. The hearing sensitivity at each frequency was evaluated in a downward sequence of 5-dB steps from 90 dB SPL and was determined as the lowest SPL to which a visible, repeatable wave III could be identified.

Surgery for gene transfection

The subjects were anesthetized similarly to the ABR-testing protocol. The surgery was performed under sterile condition and the body temperature was maintained at 38 °C. The animal's head was fixed with a stereotaxic restraint, and the hair covering the post-auricular area was cut. After local analgesia with lidocaine, a retro-auricular incision was made to expose the mastoid. A hole of 2–3 mm in diameter was drilled into the mastoid to expose the round window niche and the bony wall of the cochlea immediately inferior to the round window niche. A micropump (Micro4 from WPI, FL, USA) was used to drive a micro-syringe to apply the following solution. A solution of type II collagenase (C6885, Sigma-Aldrich, Oakville, ON, Canada) at a concentration of 30 mg ml⁻¹ was freshly prepared in Ringer's solution just before the surgery. The solution of 10 µl was slowly pumped into the round window niche over a 10 min period, after which residual solution inside the round window niche and in the bulla was suctioned out. A piece of Gelfoam (5–10 mm³) was inserted gently to make contact with the RWM. Next, 10 µl of viral vector was applied to the RWM via the Gelfoam before closing the incision. The XIAP vector was rAAV8-mut733 with surface tyrosine mutation as described previously.³⁰ The viral vector was provided by the Retinal Gene Therapy Group, University of Florida, USA at the titers of 2.38 × 10¹³. The XIAP vector was tagged with 6-myc tag for identification. In the ear with sham surgery, the AAV vector was replaced by the same volume of saline.

Cisplatin application

Cis-Diamineplatinum (II) dichloride was obtained from Sigma-Aldrich (Cat#479306) and was freshly prepared with saline into concentration of 0.5 mg ml⁻¹. The guinea pigs were given cisplatin intraperitoneally at a daily dose of 4 mg kg⁻¹. Starting at the third day of cisplatin administration, the hearing status of the animals was monitored by measuring ABR in the control ear. The cisplatin application was stopped if a larger than 20 dB threshold shift was seen in the average across 4, 8 and 16 kHz, or 7 days of consecutive application of cisplatin was reached.

Morphology and immunostaining, and western blotting

Under deep anesthesia with an overdose of pentobarbital (100 mg kg⁻¹), the animal was decapitated and the temporal bone was rapidly removed and the cochleae extracted. To prepare the cochlea for fluorescence detection of hair cells, the apex of the cochlea and the round window were punctured and then the cochlea was perfused through the apical hole with fixative (phosphate-buffered saline containing 2.5% paraformaldehyde) that exited through the round window. The cochlea was then immersed in fixative overnight at 4°C. The basilar membrane and the organs of Corti were dissected under microscope. The basilar membrane samples for determining transfection rate were first treated with 0.25% triton X-100 for 1 h, and then blocked by 10% goat serum for 1 h, followed by primary antibody against 6-myc (Polyclonal from rabbit, Ab9106, Abcam, Cambridge, MA, USA), at a concentration of 1:300 overnight at 4°C. After washing three times in phosphate-buffered saline, the samples were treated with the second antibody (goat anti rabbit, 70243-Cy2 from Jackson ImmunoResearch Laboratories Inc., Bar Harbor, ME, USA) at a dilution of 1:200 for 2 h at 4°C. To evaluate the hair cell loss, the basilar membrane was stained with Rhodamine phalloidin (Invitrogen R-415) at 1:200 in 1 × phosphate-buffered saline for 2 h at room temperature after triton X-100 treatment. Then the samples were washed again three times in the phosphate-buffered saline in a dark room.

After staining procedures, samples were mounted in glycerin on glass slides and examined by fluorescent microscopy (Axioplan2, Zeiss, Toronto, ON, Canada) or confocal microscopy (Laser Scanning, ZEISS LSM510 META). Only cells that clearly displayed fluorescence were counted as positive. The transfection and HC loss cochleograms were then established by counting the 6-myc fluorescence-positive cells or missing HCs with the microscope focusing on the level of HCs in the organ of Corti. The cytochleogram was established as the spatial-percentage count of missing/transfected hair cells along the cochlear duct.

To quantify SGN loss, a protocol similar to that described in our previous reports^{25,34} was used. In brief, after the basilar membrane was dissected, the modiolus of the cochleae was decalcified with 5% EDTA for 24 h and further fixed in 1% osmium acid for 1 h at room temperature. The samples were then embedded in Epon after dehydration in grade ethanol dilution from 20–100%. Semi-thin cross-sections of 1.5–2 mm were made, transferred to a glass slide and stained with 1% Toluidine blue for 1 min, and then examined under a light microscope. The number of SGN cell bodies was counted in Rosenthal's canal at two sides of the modiolus in each turn. At each location, 10 sections were taken to cover a distance over 0.2 mm and the SGN cell body counts were averaged across the 10 sections.

Western blotting was performed according routine protocols. As much soft tissue as possible was harvested from each cochlea, which was used as one independent sample. Each cochlea was homogenized in radio-immunoprecipitation buffer (1% Triton X-100, 1% sodium dodecyl sulfate, 8.77% NaCl, 2.42 Tris-HCl base and 5% deoxycholic acid, pH 8) and then centrifuged at 14 000 g for 10 min at 4°C. Supernatants were transferred to a new tube for measuring protein concentrations. Twenty micrograms of protein from each sample was used to run gel electrophoresis. The blots were probed with a primary antibody against an epitope common to both endo- and exo-XIAPs (1:1500, XIAP Ab mouse, BD Biosciences 610762, Mississauga, ON, Canada), equivalent protein loading was confirmed using an antibody against β -actin (1:20 000; Sigma-Aldrich A5441). XIAP was visualized using an anti-mouse IgG horseradish peroxidase-linked antibody (1:10 000; Vector Laboratories (Burlingame, CA, USA), PI-2000) and an ECL Plus Kit (GE Health Care, Milwaukee, WI, USA). The immunoreactive bands were read with a Storm 840 gel analysis system. The β -actin band was used as internal reference for the level of both en-XIAP and myc-XIAP.

Statistics

For simplicity, the hearing threshold changes were evaluated by using the frequency averaged threshold across 4, 8 and 16 kHz. The HC loss was presented as a spatial-percentage HC count. Paired *t*-tests were used to evaluate potential differences in the ABR threshold shifts and the total HC loss between ears with XIAP and no-XIAP control. One-way ANOVA was performed to evaluate the difference of SGNs counts across three groups, followed by *post hoc* tests. Unpaired *t*-tests were used to evaluate the potential changes in both myc-XIAP and en-XIAP between normal and cisplatin-treated animals.

All data are presented as Mean $\pm$ s.e.m., unless otherwise marked.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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