Caspase Inhibitors, but not c-Jun NH2-Terminal Kinase Inhibitor Treatment, Prevent Cisplatin-Induced Hearing Loss

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ABSTRACT

Cisplatin (CDDP) is a highly effective chemotherapeutic agent but with significant ototoxic side effects. Apoptosis is an important mechanism of cochlear hair cell loss following exposure to an ototoxic level of CDDP. This study examines intracellular pathways involved in hair cell death induced by CDDP exposure in vivo to develop effective therapeutic strategies to protect the auditory receptor from CDDP-initiated hearing loss. Guinea pigs were treated with systemic administration of CDDP. Cochlear hair cells from CDDP-treated animals exhibited classic apoptotic alterations in their morphology. Several important signaling events that regulate the death of CDDP-injured cochlear hair cells were identified. CDDP treatment induced the activation and redistribution of cytosolic Bax and the release of cytochrome c from injured mitochondria. Activation of caspase-9 and caspase-3, but not caspase-8, was detected after treatment with CDDP, and the cleavage of fodrin by activated caspase-3 was observed within damaged hair cells. Intracochlear perfusions with caspase-3 inhibitor (z-DEVd-fmk) and caspase-9 inhibitor (z-LEHD-fmk) prevent hearing loss and loss of sensory cells, but caspase-8 inhibitor (z-IETD-fmk) and cathepsin B inhibitor (z-Fa-fmk) do not. Although the stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK) signaling pathway is activated in response to CDDP toxicity, intracochlear perfusion of JNK inhibitor, D-JNKI-1, did not protect against CDDP ototoxicity but instead potentiated the ototoxic effects of CDDP. The results of the present study show that blocking a critical step in apoptosis may be a useful strategy to prevent harmful side effects of CDDP ototoxicity in patients having to undergo chemotherapy.

INTRODUCTION

Cisplatin (cis-diamine-dichloroplatinum II; CDDP) is a highly effective and widely used anticancer agent (1). The risk of ototoxic and nephrotoxic side effects commonly hinders the use of higher doses that could maximize its antineoplastic effects (2). CDDP has been shown to induce auditory sensory cell apoptosis in vitro (3, 4) and in vivo (5, 6). Devarajan et al. (7) recently have reported CDDP-induced apoptosis in an immortalized cochlear cell line. In this model, CDDP toxicity was associated with an increase in caspase-8 activity, followed by truncation of Bid, translocation of Bax, release of cytochrome c, and activation of caspase-9. This suggests that death receptor and mitochondrial pathways are involved in CDDP-induced apoptosis of hair cells. However, results obtained in vitro may not be directly applicable in vivo.

The present study explores the mechanisms of CDDP-induced ototoxicity in vivo and develops an efficacious strategy for protection against CDDP-induced hearing loss. In addition to death receptor and mitochondrial pathways, we also examine the c-Jun NH2-terminal kinase (JNK) signaling pathway (8) on CDDP-induced apoptosis in cochlear sensory cells.

MATERIALS AND METHODS

Animals. The care and use of animals followed the animal welfare guidelines of the Institut National de la Santé et de la Recherche Medicale and was approved by the French Ministère de l’Agriculture et de la Forêt. Adult females, pigmented guinea pigs (250 to 300 g; Charles River Laboratories, Wilmington, MA) were used in this study.

Preparation of Otoprotective Molecules for Perfusion. The specific inhibitors of caspase-3 (z-DEVd-fmk), caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), and cathepsin (z-Fa-fmk; Calbiochem, La Jolla, CA) were 83-mmol/L, 85-mmol/L, 81-mmol/L, and 72-mmol/L stock solution, respectively, in 100% DMSO (Merck, Whitehouse Station, NJ), and D-JNKI-1 was a 1-mmol/L stock solution in 0.1 mol/L PBS at pH 7.2.

Before perfusion, stock solutions of the putative otoprotective compounds were diluted in artificial perilymph to final concentrations of 100 μmol/L for caspases and cathepsin inhibitor and 10 μmol/L for D-JNKI-1. Before surgery, the osmotic minipump (1 μL/h, over 7 days; Alza Corp., Palo Alto, CA) was filled under sterile conditions with 200 μL of artificial perilymph or artificial perilymph containing one of the putative otoprotective agents.

Minipump Implantation and Electrophysiologic Recordings. Experiments were designed to record compound action potential (CAP) in awake animals from a plug fixed on the head of the animal during minipump implantation. Briefly, the animals were anesthetized with an intraperitoneal injection of a 6% solution of sodium pentobarbital (Sanofi, New York, NY; dose, 0.3 mL/kg). Each bulla was opened under sterile conditions, and a recording electrode was positioned on the round window membrane. A hole 0.25 mm in diameter was gently drilled in the base of the right cochlea for installation of the perfusion catheter.

CAPs of the auditory nerve were elicited by tone bursts of alternating phase (1-ms increase/decrease; 8-ms duration) and applied to the ear at a rate of 10/s from 0 to 100 dB sound pressure level in 5-dB steps in a free field via a JBL 075 earphone (JBL, Northridge, CA). Cochlear responses were amplified (gain, 2000), averaged (128 samples), and stored on a Pentium PC computer operating at 100 MHz (Dell Dimension; Dell, Austin, TX). CAP recordings were measured peak to peak between the negative depression N1 and the subsequent positive wave P1. The threshold of the CAP was defined as the intensity in decibel sound pressure level needed to elicit a measurable response (3, 4).

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay. ApopTag in situ apoptosis detection kit (Intergen, Purchase, NY) was used. The incorporated digoxigenin nucleotides were immunostained with a FITC-conjugated antidigoxigenin antibody. The tissues were counterstained with propidium iodide (PI). FITC and PI fluorescent signals were observed with an MRC 1024 laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Hoechst Staining. Cell nuclei morphology was assessed by Hoechst 33342 dye (Sigma Chemical Co., St. Louis, MO). Deparaffinized cochlear sections were stained with a 1:100 dilution of rhodamine-conjugated phalloidin (R-145; Molecular Probes, Eugene, OR) for 1 hour at room temperature. The sections then were counterstained with Hoechst 33342 (0.002%, w/v) at room temperature for 10 minutes and examined on a Leica DMRB microscope (blocks of filters N2.1 and A4; Leica Microsystems, Inc., Wetzlar, Germany).

Immunocytochemistry. Cytochrome c was detected in cryostat sections (10 μm) with mouse monoclonal anti–cytochrome c antibody (1:200; Pharmingen, San Jose, CA) and FITC-conjugated secondary antibody (1:200 dilution; goat antimouse IgG antibody B13113C; BioSys, Ann Arbor, MI). Bax was detected with rabbit polyclonal antibody to the NH2-terminus of Bax (1:750; Upstate Biotechnology, Lake Placid, NY) that recognizes the activated form of Bax and an Alexa 488–labeled secondary antibody (1:1000 dilution; goat antirabbit IgG antibody; Molecular Probes). Immunostained
specimens were counterstained with PI. The subcellular distribution of Bax was confirmed by double labeling with two primary antibodies [i.e., a rabbit polyclonal antibody to the NH2-terminus of Bax and a mouse monoclonal antibody to mitochondrial heat shock protein (HSP) 70, the specific mitochondrial resident protein of the hsp70 family; clone, JG1; 1:500; Affinity BioReagents, Golden, CO]. Secondary antibodies were Alexa 488–labeled goat antirabbit antibody and Alexa 568–labeled goat antimouse antibody (1:500; Molecular Probes).

Activated caspase-3 and cleavage of fodrin were detected using a Tyramide Signal Amplification kit (TSA Fluorescence System kit; NEN Life Science Products, Boston, MA). Cryostat sections were incubated with two primary antibodies [i.e., a monoclonal antibody against calbindin (1:600 dilution; Sigma) and a rabbit polyclonal antibody against active caspase-3 (1:6000; BioVision Incorporated, Mountain View, CA)] or a rabbit polyclonal antibody against cleaved α-fodrin (Asp185). This latter antibody recognizes fodrin fragments that are cleaved exclusively by caspases (1:400; Cell Signaling, San Diego, CA). Secondary antibodies were biotinylated antirabbit antibody (1:300; P.A.R.I.S., Compiègne, France) and Cy3-conjugated goat antimouse IgG antibody (1:500; Jackson ImmunoResearch Labs, West Grove, PA). Fluorescent tags were visualized with a confocal microscope (Bio-Rad). In control specimens without primary antibodies, neither FITC nor Cy3 fluorescent tags were observed.

**In vivo Substrate Detection of Activated Caspases.** Caspase activity was examined in vivo using the fluorescent substrates fam-LETD-fmk (caspase-8 substrate), fam-LEHD-fmk (caspase-9 substrate), and fam-DEVD-fmk (caspase-3 substrate) obtained from Intergen. Fluorescence substrate (2.5 μM), fam-LETD-fmk (caspase-8 substrate), fam-LEHD-fmk (caspase-9 substrate), and fam-DEVD-fmk (caspase-3 substrate) obtained from Intergen. Fluorescence substrate (2.5 μM) was perfused directly into the cochleae of untreated and CDDP-treated animals. The method of perfusion used has been described previously (9). Briefly, after the cochlea was exposed ventrally, a 1-hour perfusion of the scala vestibuli. After the perfusion of the substrate, wash buffer was perfused for 1 hour. Cochleae then were fixed with a final perfusion of fixative for 45 minutes. The cochleae were removed and double labeled with rhodamine-conjugated phalloidin.

**Western Blot Analyses.** Tissue proteins were homogenized in sample buffer (10) separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated overnight at 4°C with anti-JNK polyclonal antibody (1:1000; Upstate Biotechnology), followed by incubation with peroxidase-conjugated secondary antibody (Roche Applied Science, Indianapolis, IN). Protein-antibody complexes were revealed with the Western Lightning Chemiluminescence Reagent (PerkinElmer Inc., Wellesley, MA). Blots were incubated overnight at 4°C with anti-JNK polyclonal antibody (1:500; Jackson ImmunoResearch Labs, West Grove, PA). Fluorescent tags were visualized with a confocal microscope (Bio-Rad). In control specimens without primary antibodies, neither FITC nor Cy3 fluorescent tags were observed.

**Transmission Electron Microscopy Assessment of Hair Cell Integrity.** The transmission electron microscopy procedure has been described previously (11). Briefly, animals were decapitated during deep anesthesia, and their cochlear wall and allowed to flow out of the cochlea through a hole in the scala vestibuli. After the perfusion of the substrate, wash buffer was perfused for 1 hour. Cochleae then were fixed with a final perfusion of fixative for 45 minutes. The cochleae were removed and double labeled with rhodamine-conjugated phalloidin.

**RESULTS**

This study examined the mechanisms by which CDDP damage induces apoptosis of affected hair cells and evaluated the otoprotective effects of five protective molecules on preservation of structural integrity and function in CDDP-treated guinea pig cochleae.

**Functional and Morphologic Assessment of Hair Cell Integrity.** In the present study, we used two types of CDDP administration [i.e., a single high dose of CDDP (10 mg/kg, intraperitoneally) and a multiple dose of CDDP (2 mg/kg/d, intraperitoneally, for 5 days)] that correspond to the high therapeutic doses of CDDP used to manage human cancers (12). When compared with multiple doses of CDDP, a single high-dose administration resulted in a similar pattern of hearing loss that occurred more rapidly (i.e., 6 versus 3 days, respectively) but remained stable for at least 6 days (Fig. 1A). To reduce the animal mortality that results from single high-dose administration of CDDP, quantification of cell death of hair cells and long-term protection were studied after multiple-dose CDDP injections. Even with this precaution, 6 animals within the group of 42 animals treated with multiple doses of CDDP died during the course of treatment. Nine animals within this multiple-dose group did not show a clear pattern of hearing loss. Because of this interindividual variability with respect to CDDP-induced hearing loss, we ensured that CDDP otopotoxicity had occurred by recording CAP audiograms in each CDDP animal from the nonperfused contralateral cochlea. Only animals with a hearing loss of ≥30 dB at 20 kHz by day 6 in this contralateral ear were included in this study (i.e., 27 of 42 animals).

In the contralateral ears, 3 days after the beginning of CDDP treatment (day 3), only the highest frequencies tested (i.e., 16 to 26 kHz) showed measurable hearing losses. During the continuing course of the CDDP treatment (i.e., between days 3 and 5), hearing losses continued to develop, progressing to the middle (i.e., 6 to 16 kHz) and then the lower (<6 kHz) frequencies. One day after the end of treatment (day 6), significant hearing losses were observed across all of the frequencies, with higher frequencies being most affected (Fig. 1A).

Comparison of surface preparations of untreated cochleae with cochleae subjected to systemic CDDP treatment (Fig. 1B) revealed that many of the outer hair cells (OHCs) were missing from the CDDP-exposed cochleae. Counting of remaining hair cells was performed on surface preparations of the organ of Corti stained with a rhodamine-phalloidin conjugate. These counts showed a large loss of OHCs from the basal turns of the CDDP-treated, nonperfused contralateral cochleae (n = 4), with a typical gradation of base to apex pattern of hair cell loss and a typical gradient of OHC loss that progressed from the first to the third row of OHCs (Fig. 1A, inset). A few areas of inner hair cell (IHC) loss were observed located in the most basal portion of CDDP-treated cochleae (Fig. 1A, inset).

**DNA Fragmentation and Nuclear Morphology.** To determine the nature of cell death, we performed terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) on untreated (n = 2) and CDDP-treated (n = 6) cochleae. TUNEL-positive cells were not observed in any of the cochleae from untreated control animals (Fig. 1C). In contrast, the basal part of cochleae from CDDP-treated animals showed TUNEL-positive labeling of a variety of cell types within the organ of Corti, especially OHCs and occasionally IHCs and support cells (Fig. 1C).

Cell nuclei within sensory cells of cochleae from untreated control animals stained uniformly with Hoechst dye (Fig. 1D). After CDDP treatment, characteristic pathologic changes in the nuclear staining were observed in the basal and middle turns of cochleae (i.e., the appearance of karyopyknosis in the nuclei of some OHCs and IHCs; Fig. 1D).

**Bax Redistribution.** We examined the activation and subcellular localization of Bax by using an antibody directed to the NH2-terminus of Bax. Cochleae from untreated control animals (n = 2) exhibited a diffuse, pattern of light immunostaining barely above a background level, indicating an absence of Bax activation (Fig. 2A). Two to 4 hours after a single high-dose injection of CDDP (n = 3), the pattern...
of Bax immunostaining of the OHCs of the basal and middle cochlear turns changed to a dense, punctate pattern of immunostaining consistent with the activation and redistribution of Bax from the cytosol to the mitochondria of affected sensory cells (Fig. 2A). Double labeling of cells showed that activation of Bax colocalized with mt HSP 70, the specific mitochondrial resident protein of the hsp70 family, in treated animals but not in control untreated cochleae (Fig. 2C).

release of cytochrome c. Subcellular localization of cytochrome c was examined by immunostaining. In the cochlea of untreated, control animals (n = 2), immunolabeling with an anti-cytochrome c antibody showed an unequal, punctate distribution of reaction product within the cytoplasm (Fig. 3A). This pattern of immunostaining is consistent with mitochondrial localization of cytochrome c. Six hours after a single, high-dose injection of CDDP (n = 3), the pattern of hair cell and support cell cytoplasm immunostaining for cytochrome c changed from punctate to a diffuse and weak pattern of immunostaining with reaction product uniformly distributed throughout the cytoplasm of the affected cells (Fig. 3B). The pattern of immunostaining in the CDDP specimens was no longer consistent with mitochondrial localization of cytochrome c in the auditory hair cells and the organ of Corti support cells.

specific caspases. Activation of caspases was examined using fluorescent substrate detection tests in untreated control cochleae and cochleae observed at 6 and 12 hours after a single high-dose injection of CDDP. In untreated control cochleae (n = 6; Fig. 4A), few hair cells contained the green fluorescent labeling that indicated cleaved caspase-8, -9, and -3 substrates. A slight but not significant increase (P > 0.05) of caspase-8, -9, and -3-positive auditory hair cells was noted at 6 hours after CDDP treatment (Fig. 4B; n = 9). Similarly, a slight but not significant increase (P > 0.05) of caspase-8-positive cells was noted at 12 hours (Fig. 4A and B; n = 3). In contrast, high-dose CDDP-exposed cochlea at 12 hours after treatment showed a robust and significant increase in the number of caspase-9-positive OHCs (Fig. 4A and B; P < 0.001; n = 3), whereas the proportion of caspase-9-positive IHCs remained approximately the same as measured at 6 hours. Finally, a significantly increased percentage of caspase-3-positive IHCs and OHCs was evident at 12 hours after high-dose CDDP treatment (Fig. 4A and B; P < 0.01; n = 3).

Caspase-3 activation also was determined by immunolabeling using an antibody specific for activated caspase-3. Activated caspase-3 was never detected in the cochlear cells from untreated control animals (Fig. 4C; n = 3). By contrast, marked caspase-3 activation was observed in some OHCs and a few IHCs of the basal and middle turns (Fig. 4C; n = 3) of CDDP-treated animals at 12 hours after high-dose CDDP.

Cleavage of fodrin by Caspases. Using an antibody specific for the M15, 000 NH2-terminal fragment of cleaved fodrin, we looked for the presence of cleaved fodrin in high-dose CDDP-exposed cochlear cells. Cleaved fodrin was never detected in the cochlear cells from untreated control animals (Fig. 4D; n = 2). In contrast, there was marked immunostaining for cleaved fodrin in the region of the cuticular plates of some OHCs and a few IHCs located in the basal and middle cochlear turns of CDDP-treated animals observed between 6 and 48 hours after injection of a single high dose of CDDP (Fig. 4D; n = 6).
JNK Signaling Pathway. To identify involvement of the JNK signal pathway in CDDP-induced apoptosis of cochlear hair cells in vivo, activation of JNK was measured by immunolabeling of Western blot analyses obtained from untreated cochleae (n = 4) and single high-dose (10 mg/kg) CDDP-treated cochleae removed at 3, 6, 12, 24, and 48 hours after CDDP treatment (n = 4 per time point). High-dose CDDP treatment induced strong phosphorylation of JNK (p-JNK) first detected at 3 hours after treatment, which persisted through 48 hours, with a peak level of p-JNK protein reached at 6 hours after injection. In contrast, there was no change in the expression levels of total JNK protein observed during the 48-hour course of CDDP treatment (Fig. 5A). The level of p-JNK in CDDP-treated cochleae compared with...
untreated cochlea (Fig. 5A) reached a peak at 6 hours after high-dose CDDP exposure. Similar results were obtained for phospho-c-Jun (p-c-Jun) and expression levels of c-Jun proteins. A single high dose of CDDP induced distinct bands of p-c-Jun labeling that increased in intensity during the 3- to 48-hour period following CDDP treatment. No change in the expression level of c-Jun protein was observed during the course of CDDP treatment (Fig. 5B). The level of p-c-Jun in CDDP-treated cochlea compared with untreated cochlea (Fig. 5B) reached a peak of expression at 12 hours after high-dose CDDP.

Casparase-3 Inhibitor II and Casparase-9 Inhibitor I, but not Cathespin B Inhibitor I and Casparase-8 Inhibitor III, Prevent Hearing Loss and Loss of Sensory Cells. Substrate detection and immunolabeling results indicate that procaspase-9 and procaspase-3, but not procaspase-8, were activated in the cochlear hair cells of CDDP-treated animals. To further investigate involvement of caspase-8 in CDDP-induced apoptosis of cochlear cells, we perfused a caspase-8 inhibitor III (z-IETD-fmk) into the scala tympani (n = 5). There was no protective effect on CDDP-induced hearing loss in animals treated with 100 μmol/L of z-IETD-fmk (Fig. 6A). The roles of caspase-9 and caspase-3 were investigated by perfusing the caspase-9 inhibitor I (z-LEHD-fmk) and caspase-3 inhibitor II (z-DEVD-fmk) into the scala tympani of CDDP-treated animals. Either 100 μmol/L of z-LEHD-fmk (n = 5; Fig. 6A) or 100 μmol/L of z-DEVD-fmk (n = 6; Fig. 6A) inhibitors prevented most of the CDDP-initiated hearing loss. Although 100 μmol/L of z-DEVD-fmk prevented CDDP-induced hearing loss, it prevented neither the subcellular redistribution of Bax (Fig. 2B; n = 2) nor the mitochondrial release of cytochrome c into the cells’ cytoplasm (Fig. 3C; n = 2). However, it did significantly suppress the occurrence of TUNEL-positive hair cells within 48 hours after the onset of CDDP treatment (Fig. 6B; P < 0.001; n = 3). Quantitative evaluation performed at the end of CDDP treatment (day 6) of the z-DEVD-fmk–perfused cochlea revealed that there were only a few OHCs lost from the basal turn and that there was no apparent damage to the middle or apical turns of these z-DEVD-fmk/CDDP-treated cochlea (Fig. 6C; n = 3). The otoprotective effect of z-DEVD-fmk against CDDP-induced ototoxicity was confirmed by ultrastructural analysis of the organ of Corti (Fig. 6D), in which no degeneration-related cellular abnormalities were observed in CDDP-treated cochlea (n = 2) protected by z-DEVD-fmk.

Because z-DEVD-fmk also might inhibit cathepsins, we performed additional experiments using the cathepsin inhibitor (z-FA-fmk). No protective effect against CDDP ototoxicity was observed with perfusion of 100 μmol/L z-FA-fmk (Fig. 6A; n = 5), suggesting that the protective effect of z-DEVD-fmk was caused by inhibition of caspase activation rather than an effect on cathepsins.

A Peptide Inhibitor of JNK (D-JNKI-1) Potentiated the Otoxic Action of CDDP. JNK and c-Jun activation increased over time following exposure to high-dose CDDP treatment (Fig. 5A and B). Thus, we blocked the JNK pathway by using D-JNKI-1, a cell-permeable peptide that competitively blocks the ability of JNK to phosphorylate its targets. Intracochlear perfusion of 10 μmol/L D-JNKI-1 solution did not rescue the cochlea from CDDP-initiated ototoxic damage (n = 6); rather, it potentiated CDDP-initiated hearing loss between 10 and 26 kHz (Fig. 5C, left). Statistical analysis revealed that the potentiation of CDDP-induced hearing loss by D-JNKI-1 was significant at the frequencies of 20 and 26 kHz (P < 0.05) when compared with CDDP-treated animals. Morphologic evaluation of the organ of Corti indicated that the combination of D-JNKI-1 with...
CDDP treatment caused greater damage than CDDP treatment alone (Fig. 5C, right; n = 3). The basal turns of the D-JNKI-1–perfused, CDDP-treated cochleae also lost a few IHCs. Perfusion with D-JNKI-1 prevented neither the subcellular redistribution of Bax (Fig. 2B; n = 2) nor the release of cytochrome c from the mitochondria (Fig. 3D; n = 2) that occurs following CDDP treatment. Ultrastructural examination showed that D-JNKI-1–perfused cochleae (n = 2) displayed extensive damage and degeneration of the hair cells and more extensive damage to other cochlear-related structures (Fig. 5D). The most frequent pathology observed within the somas of OHCs was large cytoplasmic vacuoles and chromatin condensations within their nuclei, and a few OHCs showed degenerative changes indicative of cellular necrosis.

**DISCUSSION**

The pattern of hair cell loss correlated with the electrophysiologic determination of a high-frequency hearing loss. CDDP-induced apoptosis of OHCs, IHCs, and nonsensory cells of the organ of Corti as shown by TUNEL labeling, Hoechst staining, and ultrastructural analysis correlates well with the findings of recent reports (5, 13, 14). Identification of intracellular death signaling pathways activated in CDDP-stressed hair cells may offer the possibility of developing a strategy for otoprotection against CDDP-induced hearing loss.

**Death Receptor Pathway.** One major signaling pathway that leads to the apoptosis of damaged cells is the Fas/APO-1–dependent (cell death receptor) pathway. Activated Fas/APO-1 receptors interact causing the activation of receptor-associated procaspase-8, which can activate downstream effector caspases (15). In contrast to the in vitro study showing a transient activation of caspase-8 in an immortalized cell line (7), the present study did not observe a significant increase in caspase-8 activation in CDDP-treated guinea pig cochleae. It is worth noting that this immortalized auditory cell line represents OHC precursors and not adult-like OHCs (7). Thus, the different patterns of caspase-8 activation observed in vitro (7) and in our in vivo study might be caused by the differences in these two experimental models. Additional functional data reported in our study reveal that local scala tympani perfusion of z-IETD-fmk, a caspase-8 inhibitor, was ineffective in preventing either CDDP-induced hair cell death or hearing loss. This result suggests that in vivo CDDP-induced apoptosis of cochlear cells is caspase-8 independent.

**Mitochondrial Pathway.** Another cell death pathway that participates in apoptosis of stress-damaged hair cells is the mitochondria pathway (16). Mitochondria are a target of reactive oxygen species and a source for the generation of additional reactive oxygen species. In situ-generated reactive oxygen species can cause cytochrome c release in primary cultures of cerebellar granule neurons (17). Cyto-
CDDP-treated cochleae (n = 3) and z-DEVD-perfused, CDDP-treated cochleae (n = 3). The cytocochleogram derived from z-DEVD-fmk-perfused, CDDP-treated cochleae shows only minimal OHC loss and no IHC loss. D, transmission electron micrographs from the basal turn of a z-DEVD-fmk-perfused cochlea from a CDDP-treated animal. The IHC (I) and the three OHCs (O₁, O₂, and O₃) are well preserved, and their stereocilia (arrows) have a normal appearance. O₁, Deiters’ cells; O₂, outer pillar cell; O₃, inner pillar cell; ph, phalangeal cell; b, border cell; bar, 5 μm.

Fig. 6. A, audiograms from perfused cochleae and contralateral, nonperfused cochleae in CDDP-treated animals. Hearing losses are similar between the z-IETD-perfused, z-FA-perfused cochleae, and contralateral, nonperfused cochlea. In contrast, only high-frequency hearing losses (i.e., between 20 and 26 kHz) are seen in the z-DEVD-perfused and z-LEHD ears; lower frequency hearing losses are totally prevented. B, counting of TUNEL-positive hair cells in a 1-mm length of surface preparation of the organ of Corti from control untreated (n = 3) and 2 days after treatment in CDDP-treated (n = 3) cochleae and z-DEVD-perfused, CDDP-treated cochleae (n = 3). Note the significant increase of TUNEL-positive hair cells in the CDDP-exposed cochlea and the minimal number in the z-DEVD-fmk-perfused, CDDP-treated cochlea (***, P < 0.001). C, a cytocochleogram from z-DEVD-fmk-perfused cochlea from a CDDP-treated animal. The IHC (I) and the three OHCs (O₁, O₂, and O₃) are well preserved, and their stereocilia (arrows) have a normal appearance. O₁, Deiters’ cells; O₂, outer pillar cell; O₃, inner pillar cell; ph, phalangeal cell; b, border cell; bar, 5 μm.

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Consistent with other studies (3, 7, 18, 19), we show that an upstream initiator caspase (i.e., caspase-9) and a downstream effector caspase (i.e., caspase-3) were activated in the CDDP-damaged OHCs and some IHCs located in the basal turn of the cochlea. The intracochlear perfusion of caspase-3 inhibitor (z-DEVD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) dramatically reduced the ototoxic effects of CDDP, as evidenced by a lack of DNA fragmentation with almost no apoptotic cell death of hair cells or other cell types within the cochlea and almost no loss of hearing in the CDDP-treated animals. z-DEVD-fmk is a potent, cell-permeable, and irreversible inhibitor of caspase-3. We cannot exclude that other members of the caspase-3 subfamily are not involved in CDDP otoxicity because it is known that caspase-2 and -7 also are inhibited by z-DEVD-fmk (20). Mandic et al. (21) reported on the possible involvement of calcium-dependent protease calpains in CDDP toxicity in a human melanoma cell line, suggesting a role for calpains in CDDP otoxicity. Although experiments yet have to be done in vivo, the lack of a protective effect of calpain inhibitors on auditory hair cell and neurons in culture argues against this hypothesis (4). The present results suggest that CDDP otoxicity is mediated through a molecular pathway that involves the mitochondria and the sequential activation of initiator and effector caspsases.

JNK Signaling Pathway. The JNK signaling transduction pathway that phosphorylates the NH₂-terminal region of c-Jun (22, 23) is involved in the control of apoptosis in certain models of oxidative stress damage. A protective role for c-Jun NH₂-terminal phosphorylation against DNA damage-induced apoptosis is supported by the results of recent studies (24, 25). These studies have shown that the JNK signal cascade is activated by CDDP-induced DNA damage and is necessary for the repair of DNA-CDDP adducts and for cell viability. Therefore, to identify the involvement of this signal pathway in CDDP-induced apoptosis of cochlear cells in vivo, levels of activated JNK were measured. Our results show that CDDP treatment induced a significant increase in activated JNK and that inhibition of this signal pathway by scala tympani perfusion of d-JNKI-1, a cell-permeable peptide that blocks JNK-mediated activation of c-Jun (26), prevented neither the CDDP-initiated activation and subcellular redistribution of Bax nor the mitochondrial release of cytochrome c. Contrarily, inhibition of this signal cascade by d-JNKI-1 increased the sensitivity of cochlear hair cells to damage by CDDP. This result suggests that the JNK pathway is not involved in the CDDP-induced hair cell death but may have a role in DNA repair and maintenance of CDDP-damaged sensory cells.

Clinical Implications. As yet, there is no reliable test to predict which of those patients treated with CDDP will develop clinically significant otoxicity. It now is possible to introduce chemoprotectors, antiapoptotic agents, and other compounds across the round window membrane of the cochlea in humans (27, 28). If successful, the application of an otoprotective strategy for patients who begin to show signs of ototoxic side effects should greatly enhance the quality of life for patients who have to undergo high-dose CDDP chemotherapy.
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