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High Accumulation of Platinum-DNA Adducts in Strial Marginal Cells of the Cochlea Is an Early Event in Cisplatin but Not Carboplatin Ototoxicity

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ABSTRACT

Ototoxicity is a typical dose-limiting side effect of cancer chemotherapy with cisplatin but much less so with carboplatin. To elucidate the underlying molecular pathological mechanisms, we have measured the formation and persistence of drug-induced DNA adducts in the nuclei of inner ear cells of guinea pigs after short-term exposure to either cisplatin or carboplatin using immunofluorescence staining and quantitative image analysis. After application of carboplatin, all cells of the cochlea exhibited a similar burden of guanine-guanine intrastrand cross-links in DNA. In contrast, we observed a pronounced 3- to 5-fold accumulation of this cytotoxic adduct exclusively in the marginal cells of the stria vascularis between 8 and 48 h after treatment with cisplatin. In the kidney, the other critical target tissue of cisplatin toxicity, a similar high preferential formation of cytotoxic DNA adducts was measured in the tubular epithelial cells but not in other renal cell types. As for the ear, this excessive formation of DNA damage in a particular cell type was seen in animals treated with cisplatin but not those treated with carboplatin. Because cisplatin ototoxicity is often attributed to oxidative stress mediated by the generation of radical oxygen species (ROS), we have measured in parallel the levels of the lead DNA oxidation product 8-oxoguanine (8-oxoG) in cochlear cryosections. Compared with basal levels in untreated control cochleas, no additional formation of 8-oxoG was detectable up to 48 h after cisplatin treatment in the DNA of either inner-ear cell type. This suggests that the generation of ROS may be a secondary event in cisplatin ototoxicity.

Platinum derivatives are frequently used in cancer chemotherapy for patients with malignancies of, for example, the urogenital tract, the lung, the colon, the stomach, or the head and neck region. It is widely accepted that the antineoplastic efficiency of cisplatin [*cis*-diamminedichloroplatinum (II)] as well as carboplatin (diammine-cyclobutane-dicarboxylato-platinum) results from their interaction with the nuclear DNA of tumor cells (reviewed in Siddik, 2003). The drugs harboring two reactive groups initially induce monoadducts at nucleophilic sites (e.g., of guanine or adenine) and can lead subsequently to intra- and interstrand cross-links in the DNA. Once formed, these lesions can trigger apoptotic cascades (McKeage, 1995; Boulikas and Vougiouka, 2003) pre-

dominantly via the mitochondrial pathway (McDonald and Windebank, 2002; Lee et al., 2004). The vital role of DNA adducts in this process is confirmed by observations in human cells differing in their ability to repair drug-induced damage to their genome. Cells with impaired nucleotide excision repair functions are clearly more sensitive to cisplatin than their proficient counterparts (Furuta et al., 2002; Wu et al., 2003).

On the other hand, the frequently dose limiting side effects of cisplatin on normal cells and tissues (e.g., in the inner ear and the kidney) were mainly ascribed until now to pathways not primarily triggered by drug-mediated DNA damage. Most investigations attributed cisplatin ototoxicity and nephrotoxicity to free radical-induced cell damage, which is mediated by reactive oxygen species (ROS) (Husain et al., 2001; Minami et al., 2004).

Although there is no obvious reason for assuming completely different mechanisms of drug-induced cytotoxicity in

tumor cells and in normal tissues, little information regarding the formation and persistence of platinum-DNA adducts in the inner ear has been available until now. van Ruijven and coworkers (2005) used immunohistochemical methods to demonstrate the formation of DNA platination products in the cochlear cells of guinea pigs after long-term exposure to low-dose cisplatin (2 mg/kg b.wt. for 2 weeks). To investigate the role of drug-induced DNA damage for short-term ototoxicity after a high dose of cisplatin, we have employed monoclonal antibodies and a quantitative immunofluorescence assay to visualize and measure the major DNA platination product at the cell level of individual cells. We have analyzed the time courses of formation and persistence of DNA intrastrand cross-links in distinct cochlear cells of guinea pigs after a single injection of cisplatin or carboplatin.

Beside its ototoxic side effects, cisplatin also accumulates in the epithelial cells of renal proximal tubuli with consecutive tubular necrosis and interstitial nephritis (Dobyan et al., 1980; Uehara et al., 2005). Therefore, we have additionally studied the distribution of platinum-DNA adducts in the cortical and medullary portion of the kidney after application of cisplatin or carboplatin.

Because drug-induced oxidative stress is discussed as a major pathogenic factor for functional damage in the cochlea and hearing loss after cisplatin treatment, we have addressed this mechanism by measuring the levels of 8-oxoguanine, the lead DNA oxidation product formed after ROS exposure, in the nuclei of cochlear cells.

Materials and Methods

Animals, Drugs, and Experimental Design. Healthy pigmented guinea pigs (strain Dunkin Hartley; 250–400 g) with a positive acoustic pinna reflex were used in this study. After transportation, the animals were maintained in the central animal laboratory of the University of Essen for at least 1 week. They were housed under standard laboratory conditions and fed ad libitum. The study was performed according to the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals* and the laws of animal welfare in Germany. Twenty animals were divided into three experimental groups. The first group (vector control group, four animals) received an i.p. injection of 0.9% sodium chloride. The second group (10 animals) received an i.p. injection of 12.5 mg/kg b.wt. cisplatin (Platinex; Bristol-Myers Squibb Co., Stamford, CT), a standard dose to induce cisplatin ototoxicity in guinea pigs. The last group (six animals) received an i.p. injection of 80 mg/kg b.wt. carboplatin (Carboplat; Bristol-Meyers-Squibb). Before and 8, 24, or 48 h after injection, animals were sacrificed by decapitation under carbon dioxide anesthesia, and both auditory bullae and a part of the kidney were removed from each animal.

Cell Culture. Immortalized rat alveolar epithelial cells were seeded in Lab-Tek II two-chamber slides (Nalge Nunc, Naperville, IL). At near confluence, the medium was discarded, replaced by Hanks' balanced salt solution, and cells were exposed to various doses of H_2O_2 (0–50 μM). After incubation for 2 h at 37°C, slides were washed with PBS and stored until immunostaining at −80°C. Immunocytological assay (ICA) analysis of 8-oxoguanine (8-oxoG) was performed as described for tissue sections.

Preparation of Tissues. After fixation by intralabyrinthine perfusion of 95% ethanol/chloroform/glacial acetic acid [6:3:1 (v/v)] for 12 h, both cochleae from each animal were transferred to phosphate-buffered saline (PBS). Decalcification was performed in EDTA solution (10% in PBS) for 4 weeks. After washing, the specimens were placed in sucrose solution (5% in PBS for 12 h and 15% in PBS for 5 h) before freezing in liquid propane. Cryosections (10 μm) were

prepared from cochleae and from the kidney and placed onto specific adhesion slides (ImmunoSelect; Squarix Biotechnology, Marl, Germany) for optimal preservation of the delicate tissue structures during the immunostaining procedure. All slides were air-dried and stored at −80°C until further processing.

Immunocytological Assay (ICA) for Specific DNA Adducts. DNA lesions were immunostained in tissue sections using adduct-specific antibodies essentially as described previously (Seiler et al., 1993; Thomale et al., 1996) with some modifications. In brief, samples were fixed in methanol (−20°C; 12 h) and rehydrated in PBS, and the nuclear DNA was partly denatured by alkali treatment [70 mM NaOH, 140 mM NaCl, 40% methanol (v/v); 0°C, 5 min]. Cellular proteins were removed by a two-step proteolytic procedure: samples were first digested with pepsin (200 μg/ml in 20 mM HCl, 37°C, 10 min; Roche Diagnostics, Mannheim, Germany) and then with proteinase K (100 μg/ml in 20 mM Tris/HCl, 2 mM $CaCl_2$, pH 7.5, 37°C, 10 min; Merck, Darmstadt, Germany). After blocking with casein (1% in PBS; 25°C, 30 min) slides were stained either for Pt-[GG] intrastrand cross-links, the major DNA adduct of cisplatin and carboplatin, or for 8-oxoG, the lead adduct for DNA oxidation damage.

Pt-[GG]. Slides were incubated with the monoclonal antibody R-C18 (0.1 μg/ml in PBS/1% casein; 37°C, 2 h; Buschfort-Papewalis et al., 2002) which was established from immunized rats and characterized for its specific binding to Pt-(GG) intrastrand cross-links in genomic DNA (Liedert et al., 2006). After washing (0.05% Tween 20 in PBS; 25°C, 2 min), a three-step sandwich immunostaining was performed by subsequent incubation with: 1) FITC-labeled goat anti-(rat Ig) (Dianova; 6.5 μg/ml in PBS/1% Casein; 37°C, 45 min), 2) ALEXA FLUOR 488-labeled rabbit anti-(FITC) (5 μg/ml in PBS/1% casein; 37°C, 45 min; Invitrogen, Carlsbad, CA) and 3) ALEXA FLUOR 488 goat anti-(rabbit Ig) (5 μg/ml in PBS/1% casein; 37°C, 45 min; Invitrogen).

8-oxoG. Slides with cells or tissue sections were incubated with rabbit anti-(8-oxoG) antibody (1 μg of antibody/ml of PBS/1% BSA) for 16 h at 4°C. This antibody was characterized previously in various immunoassays (radioimmunoassay, enzyme-linked immunosorbent assay, ICA) for its specific binding to 8-oxoG and the sensitive detection of the DNA oxidation product in cultured cells and in lung tissue sections (Nehls et al., 1997; Seiler et al., 2001). After washing with PBS/BSA, adducts were visualized with goat anti-(rabbit Ig) $F(ab)_2$ fragments conjugated to tetramethylrhodamine B isothiocyanate (TRITC; 2 μg/ml PBS/1% BSA; 45 min, 37°C; Dianova, Hamburg, Germany).

Finally, the nuclear DNA of the cells was counterstained in both cases with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml in PBS; 25°C, 30 min; Serva, Heidelberg, Germany) and cells were covered with Vectashield densification compound (Vector Laboratories, Burlingame, CA).

Image Analysis. Visualization and measurement of antibody- and DNA-derived fluorescence was performed by a microscope-coupled digital image analysis system: fluorescence microscope Axioplan, lens Plan-Neofluar 40×/0.75; mercury lamp HBO 100 W; DAPI filter: excitation, 365 nm; emission, 397 nm; FITC/ALEXA 488 filter: excitation, 450 to 490 nm; emission, 515 to 565 nm; TRITC filter: excitation, 510 to 560 nm; emission, 590 nm (long pass filter) (Carl Zeiss GmbH, Jena, Germany); charge-coupled device camera C4880 (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany); ACAS 6.0 multichannel cytometry analysis system (Dunn, Asbach, Germany). The nuclear DNA of individual cells was localized and measured by the DAPI-derived signals, and the same pixels were scanned in parallel for antibody-derived ALEXA/FITC or TRITC fluorescence. Integrated signals from each nucleus were corrected for the corresponding DNA content and computed for at least 100 cells per time point.

Results

Cisplatin but Not Carboplatin Induced Excessive Accumulation of DNA Intrastrand Cross-Links in the Marginal Cells of the Stria Vascularis. As drug-induced modifications in the nuclear DNA are the primary molecular triggers for the onset of apoptotic processes in cells exposed to cisplatin or carboplatin, we have established a sensitive method to quantify such lesions in individual cell nuclei of the guinea pig inner ear. The major reaction product of both

Fig. 1. Visualization of DNA intrastrand cross-links in inner ear cells from guinea pigs treated with cisplatin. Cochlear cryosections from untreated control guinea pigs (left) or from animals 24 h after i.p. injection of cisplatin (12.5 mg/kg b.wt.; right) were immunostained for Pt-(GG) cross-links in DNA using the adduct-specific monoclonal antibody R-C18 and ALEXA488-labeled secondary antibodies [immunofluorescence (IF), green]. The nuclear DNA was counterstained with DAPI (blue). RM, Reissner's membrane; MC, marginal cells; IBC, intermediate and basal cells of the stria vascularis; NE, neurosensory epithelium with hair cells.

drugs with DNA is the intrastrand cross-link between neighboring guanine residues (Pt-[GG]), representing >75% of all adducts. We have used the monoclonal antibody R-C18, which recognizes this particular platination product with high specificity in genomic DNA to set up a quantitative ICA (Seiler et al., 1993; Liedert et al., 2006) for use with cryosections of the cochlea. In tissue samples from guinea pigs taken 24 h after a single i.p. injection of cisplatin (12.5 mg/kg b.wt.), the presence of Pt-(GG) adducts was detectable by antibody-derived green fluorescence in the nuclei of all investigated cell types (Fig. 1). The visualization already indicated a non-homogeneous distribution of the adduct staining among different cell types. The localization and the amount of DNA as well as the levels of Pt-DNA adducts in the nuclei of different cell types of the stria vascularis could be measured by quantitative image analysis. Adduct levels in individual cell nuclei were corrected for the corresponding DNA content and expressed as arbitrary fluorescence units (AFU). Due to the mild fixation and extensive proteolytic digestion, the neurosensory epithelium with the hair cells was less well preserved, which hampered the systematic evaluation of these cells. For the few specimens with an intact neurosensory epithelium and analyzable hair cells, results are reported as well.

Tissue sections from untreated control animals showed a regular pattern of DNA-DAPI staining in the nuclei of all different cell types of the cochlea with no or very low antibody-derived signals for Pt-DNA adducts. In the experimental groups treated with cisplatin, the quantification of Pt-(GG) in DNA at 8, 24, or 48 h revealed clear-cut adduct kinetics during this period and distinct intercellular differences (Figs. 1 and 2B). Due to ongoing DNA platination, the adduct levels increased between 8 and 24 h approximately 2-fold in all cell types; they dropped by 30 to 60% within the next 24 h by virtue of cellular DNA repair activity. For a given time point, the vast majority of the cochlear cells dis-
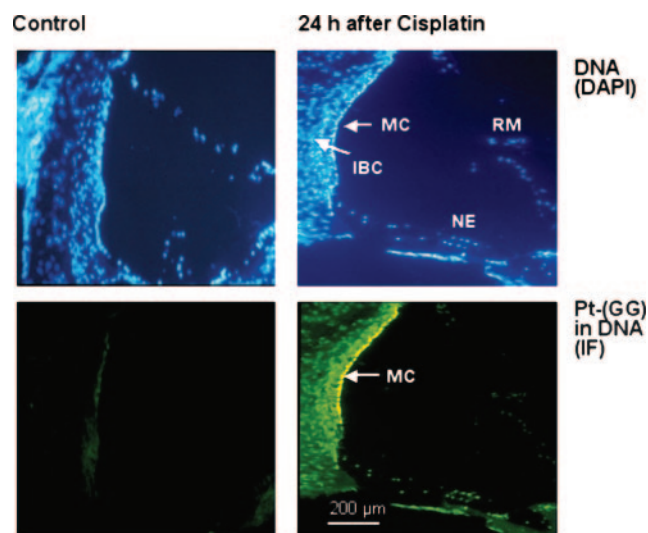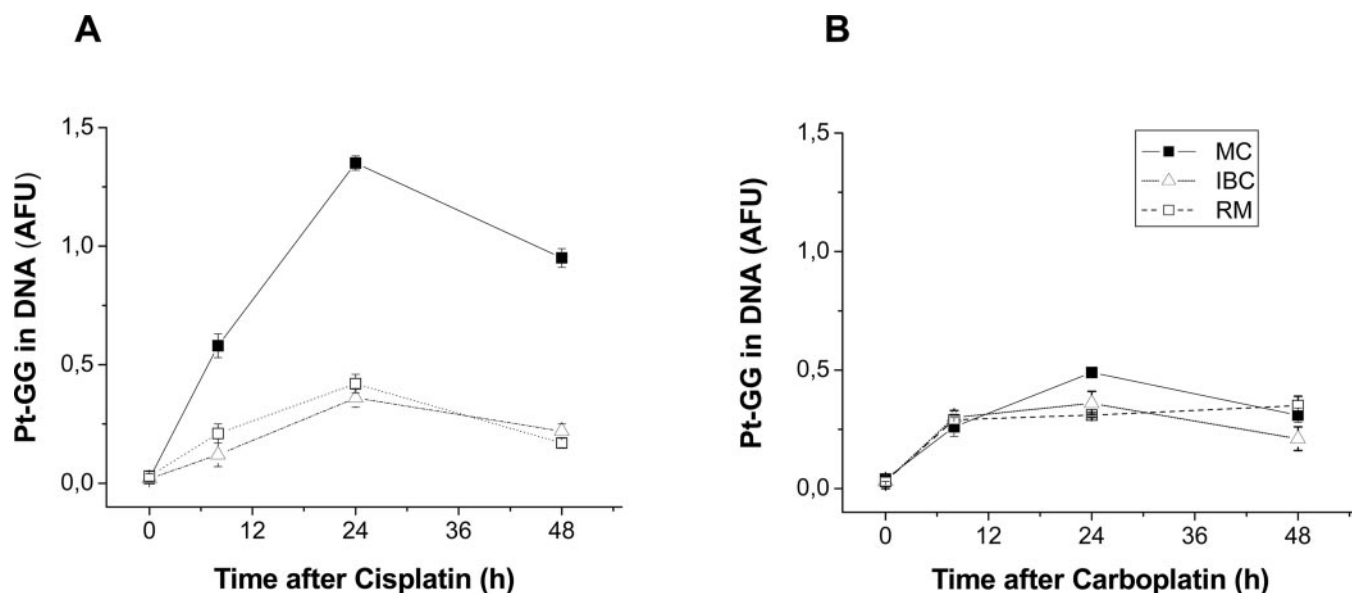

Fig. 2. Formation and repair of Pt-(GG) cross-links in the nuclear DNA of different inner ear cells of guinea pigs treated with cisplatin or carboplatin. Animals were i.p. injected with a single dose of cisplatin (12.5 mg/kg b.wt.) or carboplatin (80 mg/kg b.wt.) and sacrificed at different times after drug application. Cryosections were immunostained for specific DNA platination products (see Fig. 1), the adduct levels in individual cell nuclei were measured by quantitative image analysis, corrected for the actual DNA content and expressed as arbitrary fluorescence units (AFU). Data points represent mean values (bars, S.E.) of >100 evaluated nuclei per cell type. MC, marginal cells; IBC, intermediate and basal cells of the stria vascularis; RM, cells of Reissner's membrane.

played a rather uniform immunostaining without significant differences between specific cell types [e.g., the intermediate and basal cells of the stria vascularis or the cells of Reissner's membrane ($p > 0.05$, paired t test)]. It is noteworthy that the marginal cells of the stria vascularis consistently exhibited, at all time points along the cochlear duct, highly significant ($p = 0.0002$, paired t test) 3- to 5-fold higher burden of cisplatin adducts in their nuclear DNA compared with other cell types (Fig. 2A). In contrast, the immunostaining of single

Fig. 3. Immunostaining of Pt-(GG) cross-links in the nuclei of inner ear cells from guinea pigs 24 h after a single dose of cisplatin or carboplatin (see Fig. 2). Cryosections from the cochleae of exposed animals were stained as in Fig. 1. Increased nuclear immunostaining (IF) is seen in the marginal cells after administration of cisplatin but not of carboplatin.

Fig. 4. Accumulation of Pt-DNA adducts in kidney cells of guinea pigs 24 h after treatment with cisplatin or carboplatin (for details see Figs. 1 and 2). Strong IF is seen in the proximal tubule cells (TC) after administration of cisplatin but not of carboplatin.

well preserved outer hair cells in the neurosensory epithelium of all cochlear turns exhibited a low level of immunofluorescence similar to other cell types and displayed no specific accumulation of cisplatin-DNA adducts (Fig. 1). The number of analyzable cells in this population, however, was too small for statistical evaluation. Other cells of the cochlear neurosensory epithelium could not be clearly differentiated.

To prove whether the exceptionally high levels of DNA adducts in the marginal cells is a particular feature of cisplatin and might be held responsible for the pronounced ototoxicity of this drug, we have analyzed in parallel the adduct levels in inner ear cells from guinea pigs treated with carboplatin. Although cisplatin and carboplatin induce essentially the same set of reaction products in DNA (Kelland, 1993), use of the latter does not result in major ototoxic side effects during cancer chemotherapy in patients and in animal models.

A single application of carboplatin (80 mg/kg b.wt.) induced very similar levels of intrastrand cross-links in the intermediate and basal cells and the cells of Reissner's membrane compared with cisplatin treatment (Figs. 2B and 3). It is noteworthy that the levels of carboplatin-mediated DNA adducts in the marginal cells were comparatively low and did not differ significantly ($p > 0.05$, paired t test) from those of the other cell populations in the cochlea (Fig. 2B).

The DNA of Kidney Tubular Epithelial Cells Is Preferentially Adducted by Treatment with Cisplatin but Not with Carboplatin. Because cisplatin and carboplatin also differ in their nephrotoxic potential, we have compared the adduct formation in kidney cells of both experimental groups. At all investigated points in time, the accumulation of Pt-DNA-adducts in the renal cortex differed from that in tubulus medullary epithelium cells (Fig. 4 and Table 1). After application of cisplatin, the Pt-(GG) adduct concentrations of all cells of the glomeruli in the cortex as well as distal tubulus cells in the medullary portion did not differ significantly. The adduct levels in proximal tubulus cells, however, were 3- to 4-fold higher than in the nuclei of other kidney cells, and this difference was highly significant for measurements both 24 and 48 h after cisplatin ($p < 0.01$, paired t test; see Table 1).

In contrast, after application of carboplatin, no significant differences ($p > 0.05$, paired t test) were observed between the accumulations of DNA adducts in all the different cell populations of the glomeruli and the proximal and the distal tubulus cells (Table 1). Thus, these results support previous assumptions that the pronounced tissue-specific toxicity of cisplatin may be caused by the high formation of DNA lesions in distinct cell types of the affected organ.

No Increased Levels of 8-Oxoguanine in the DNA of Inner Ear Cells after Cisplatin Administration. Augmented oxidative stress provokes various types of DNA base damage; 8-oxoG is one of the lead adducts for ROS-induced DNA lesions (Barzilai and Yamamoto, 2004). Therefore, we
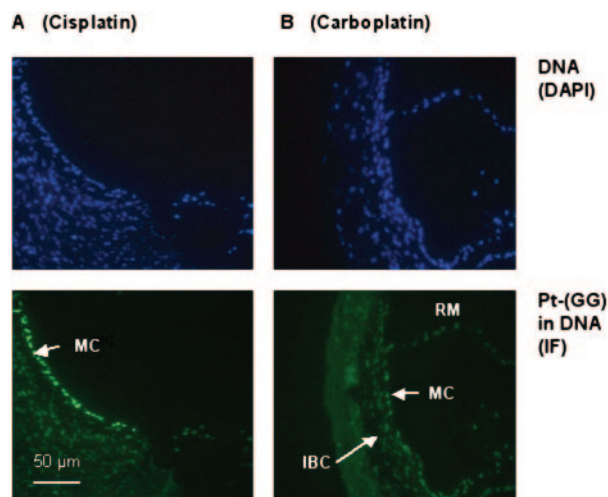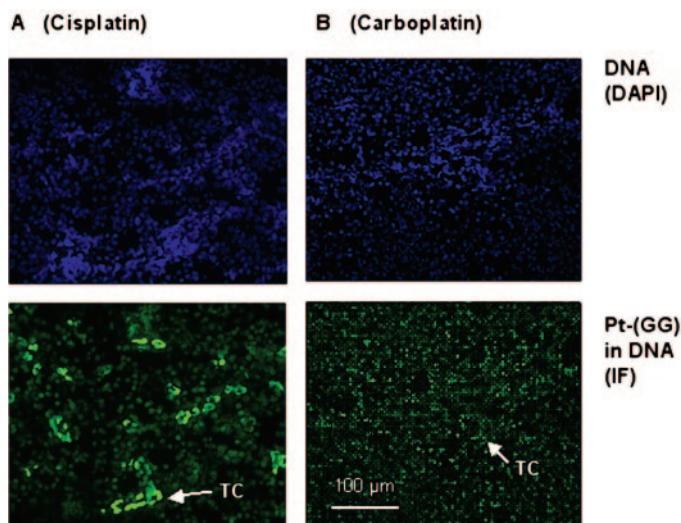
TABLE 1
Levels of Pt-(GG) adducts (AFU ± S.E.M.) in the nuclear DNA of the epithelium cells of proximal tubules (TC) and of cortex cells (CC) in kidneys from guinea pigs without treatment and at 24 or 48 h after application of cisplatin (12.5 mg/kg b.wt.) or carboplatin (80 mg/kg b.wt.)

Cell Type	Untreated Control	After Cisplatin		After Carboplatin	
		24 h	48 h	24 h	48 h
TC	0.02 ± 0.01	1.29 ± 0.07	0.84 ± 0.07	0.62 ± 0.04	0.49 ± 0.03
CC	0.01 ± 0.01	0.37 ± 0.02	0.22 ± 0.03	0.55 ± 0.02	0.41 ± 0.04

have determined the levels of this adduct in the nuclear DNA of cochlear histological sections using polyclonal 8-oxoG-specific antibodies for immunostaining (Fig. 5A). The ICA analysis revealed considerable antibody-derived fluorescence signals from all cochlear cells of control animals with only minor intercellular variation and no obvious differences from samples taken 8, 24, or 48 h after cisplatin administration. This observation was confirmed by quantitative evaluation with very similar AFU values in intermediate and basal cells as well as in cells of Reissner's membrane in untreated animals compared with the same cells after drug exposure ($p > 0.05$, paired t test; see Table 2). Although marginal cells displayed slightly (20–25%) higher levels of immunostaining compared with other cochlear cell types, both before and after cisplatin administration, this increase was not significant ($p > 0.05$, paired t test) and was independent of drug application.
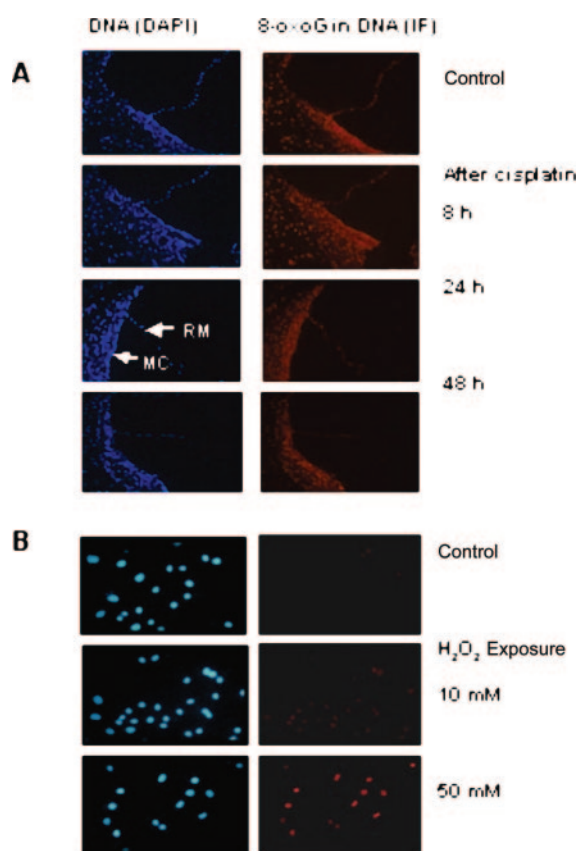

Fig. 5. Visualization of 8-oxoguanin in the nuclear DNA of cochlear cryosections and of cultured cells. Immunostaining was performed with adduct-specific antibodies and TRITC-labeled secondary antibodies (IF, red) and counterstained with DAPI (blue) as described under *Materials and Methods*. A, cryosections from cochleae of guinea pigs before and at 8, 24, or 48 h after application of cisplatin (12.5 mg /kg b.wt.). B, immunostaining of cultured rat lung epithelium cells without treatment or 2 h after exposure to H_2O_2 (10 or 50 mM).

To demonstrate the sensitivity of the ICA assay for ROS-induced DNA adducts and the low background binding of the antibody, we have immunostained cultivated rat lung epithelium cells without or 2 h after exposure to hydrogen peroxide (H_2O_2) (Fig. 5B). Antibody-derived TRITC signals were nearly absent in control cells but intermediate and high in the nuclei of cells exposed to 10 or 50 mM H_2O_2, respectively, These results confirmed earlier findings from cell culture experiments and from in vivo studies in lung tissues of rats exposed to ROS-inducing quartz particles (Nehls et al., 1997; Seiler et al., 2001).

Discussion

Different explanations for the pathophysiologic causes of cisplatin-induced ototoxicity have been discussed; most investigations ascribe cisplatin ototoxicity and nephrotoxicity to free radical-induced cell damage. However, direct evidence for ROS production could be detected only in cisplatin-exposed cochlear explants and in short time cultures (Dehne et al., 2001). Furthermore, several in vivo studies suggested the involvement of ROS in cisplatin ototoxicity using antioxidative strategies. These studies showed a reduction of cisplatin-induced inner ear toxicity by using drugs to bolster the innate oxidative stress defenses of the cochlea (Minami et al., 2004; Lynch et al., 2005).

It is generally accepted that the antineoplastic effect of cisplatin is mediated by the platination of nucleophilic centers in DNA bases, finally leading to intra- or interstrand cross-linking of the bases, to abnormal base pairing, or to DNA strand breakage. Assuming that the pathophysiological mechanisms of cisplatin-induced ototoxicity are the same as in tumor cell toxicity, we have analyzed the formation and persistence of drug-induced damage in the nuclear DNA of different cell populations in the inner ear of guinea pigs. After a single administration of cisplatin at a dose that leads to a pronounced hearing loss (Laurell and Engstrom, 1989; Hatzopoulos et al., 1999), a highly significant accumulation of drug-induced Pt-DNA adducts was observed in the marginal cells of the stria vascularis. Up to 48 h after treatment, the marginal cells exhibited a 3- to 5-fold higher adduct burden compared with directly neighboring cells of the stria vascularis as well as all other cell types of the cochlea. Single outer hair cells, which have been discussed as the main target of cisplatin-induced cell damage, exhibited the same comparatively low levels of Pt-(GG) adducts in all cochlear turns as, for example, basal and intermediate cells of the stria vascularis or the cells of Reissner's membrane. Our findings are in line with the platinum-195M labeling studies of Schweitzer et al. (1986, 1993), who reported a 2- to 3-fold increase in platinum uptake in the stria vascularis compared with the organ of Corti.

DNA intrastrand cross-links were readily detectable 8 h

TABLE 2
Levels of 8-oxoG (AFU ± S.E.M.) in the nuclear DNA of different cells in the inner ear of guinea pigs before and after treatment with cisplatin (12.5 mg/kg b.wt.)
Marginal cells (MC) or intermediate and basal cells (IBC) of the stria vascularis, cells of Reissner's membrane (RM).

Cell Type	Untreated Control	After Cisplatin		
		8 h	24 h	48 h
MC	0.15±0.02	0.15 ± 0.04	0.13 ± 0.03	0.12 ± 0.03
IBC	0.13 ± 0.03	0.13 ± 0.02	0.10 ± 0.02	0.11 ± 0.04
RM	0.13 ± 0.02	0.11 ± 0.04	0.12 ± 0.03	0.09 ± 0.02

after drug injection in all cell types of the inner ear and peaked at 24 h because of the rather slow pharmacokinetics and the delayed formation of secondary structures from the initial monoadducts (Siddik, 2003). The augmented adduct levels in the nuclei of marginal cells are not caused by their disability to repair such DNA lesions as all cells of the cochlea were capable in reducing the peak adduct burden by 30 to 60% within 24 h, most likely via the nucleotide excision repair pathway (Siddik, 2003; see Fig. 2).

When disregarding the impact of repair mechanisms, the level of DNA platination products is directly linked to the intracellular drug concentration, which in turn can be augmented by both excessive influx and decreased export. For the platinum drugs, such membrane transport mechanisms are not yet fully understood. We have shown that enhanced transgenic expression of the functional inhibition of the ABC membrane efflux transporter MRP2/cMOAT in various human cell lines influenced both the level of cisplatin-induced DNA adducts as well as the cellular drug sensitivity and the induction of apoptosis (Liedert et al., 2003; Materna et al., 2005). Similar observations were made for the membrane copper transporters CTR1 (import) and ATP7A or ATP7B (efflux) (Safaei and Howell, 2005). However, it remains unclear whether any of the four pump molecules is expressed in cells of the inner ear, and it is unlikely that their activities account for the excessive cisplatin-induced adduct formation in marginal cells because the transporters seem not to discriminate between different platinum-based drugs.

Because different cell types may not share the same relationship between drug-induced DNA damage and cytotoxicity, we have further substantiated the role of DNA adduct accumulation in drug-induced hearing loss by corresponding experiments with a second platinum-based anticancer drug, carboplatin, that produces the same types and pattern of DNA damage but has a far lower ototoxic potential than cisplatin. In clinical practice, the dosage of cisplatin and carboplatin is not directly comparable. In general, carboplatin is dosed approximately 4-fold higher than cisplatin (Bertolini et al., 2004). To exclude the possibility that the lack of ototoxicity is caused by an underdosage, we used 80 mg of carboplatin/kg b.wt., which is more than 6 times the customary cisplatin dose (12.5 mg/kg b.wt.). A single exposure to carboplatin led to comparable levels of DNA cross-links in all cochlear cells and a complete lack of the pronounced damage in marginal cells.

Until now, the main target for the ototoxic activity of platinum drugs was seen in different cell populations depending on the concentration of cisplatin (low dose of 2 mg/kg versus high doses up to 16 mg/kg) and the mode of application (long-term versus short-term). Low-dose, long-term exposure (1.5–2 mg/kg, daily) resulted in a loss of outer hair cells and shrinkage of the spiral ganglion cells with (Tange and Vuzevski, 1984) or without degeneration of strial marginal cells (van Ruijven et al., 2004).

Cochlear damage was observed predominantly in the stria vascularis after short-term cisplatin treatment with doses up to 16 mg/kg (Saito and Aran, 1994). Analyses by transmission electron microscopy also visualized strial edema, bulging, rupture, and compression of the marginal cells, with depletion of the organelles (Meech et al., 1998). Under similar conditions, hearing tests by auditory brainstem responses already showed, on the first day after cisplatin injection, a marked hearing loss and a decrease of endocochlear potentials as a result of damaged strial barrier (Laurell and Engstrom, 1989; Hatzopoulos et al., 1999). Together, these observations indicate that short- and long-term ototoxic side effects may differ according to the pharmacokinetics of cisplatin in inner ear fluids and tissues.

Our findings of an excessive accumulation of Pt-(GG) DNA adducts are in line with the activation of apoptotic mechanisms in strial marginal cells of mice 3 days after a local administration of cisplatin (Lee et al., 2004). Furthermore, apoptosis was detected in the stria vascularis and in the spiral ligament but not in the organ of Corti after injection of cisplatin (10 mg/kg) as opposed to carboplatin (50 mg/kg; Watanabe et al., 2002).

Based on these observations, we suggest a mechanism in which the excessive DNA platination in the marginal cells represents the earliest event in short-term cisplatin ototoxicity which then triggers their functional impairment and/or their potential apoptotic destruction. The derogation of marginal cells may lead to an impaired K^+ secretion into the endolymph as well as an impaired uptake of K^+ from the intrastrial space with subsequent dysfunction and loss of hair cells. Thus it can be hypothesized, that cisplatin but not carboplatin is (co-) transported from the intrastrial compartment by an active pump system into the cells, where it is trapped, accumulates, and subsequently reacts with DNA.

This view is supported by congruent findings in the kidney, where the accumulation of DNA adducts in the proximal tubulus cells was seen after administration of cisplatin but not of carboplatin. This corresponds to the clinically known concentration of cisplatin in the epithelial cells of the proximal tubuli with consecutive tubular necrosis and interstitial nephritis. The organic cation transporter OTC2, which is highly expressed in the kidney, has been found to play a significant role in cisplatin accumulation and in the tubular toxicity in rats (Yonezawa et al., 2005). Whether OTC2 or a similar membrane pump is expressed in marginal cells and discriminates between both platinum complexes remains to be proven.

The detection of basal oxidative DNA damage in all cell types of the inner ear independent of exposure to cisplatin is consistent with the incessant metabolic production of ROS (Evans and Halliwell, 1999). Despite efficient cellular repair mechanisms, this challenge leads to steady state levels of 8-oxoG lesions in the nuclear DNA of all cells. Analyses in human and in rodent tissues revealed molar ratios of approximately four 8-oxoG residues per 10^6 guanines in DNA, which translates into the presence of approximately 10.000 adducts per diploid cell (Seiler et al., 2001; Gedik and Collins, 2005). In this study, a single high-dose injection of cisplatin did not significantly increase the basal 8-oxoG levels in the DNA of marginal cells or other cochlear cells analyzed up to 48 h after exposure. Our results, however, do not completely rule out the concept of free radical-induced damage to other cell organelles (e.g., after long-term exposure to cisplatin).

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