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Nephrotoxicity of platinum complexes is related to basolateral organic cation transport

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Nephrotoxicity of platinum complexes is related to basolateral organic cation transport.

Background. Cisplatin and its analogs oxaliplatin and carboplatin are widely used antitumor drugs. Nephrotoxicity is a common and relevant adverse effect that occurs especially in cisplatin therapy. Cellular and molecular mechanisms of cisplatin-induced nephrotoxicity are not completely understood. The nephrotoxicity of platinum complexes was evaluated by a new in vitro system that utilizes the high Trans Epithelial Electrical Resistance (TEER) of the C7 clone of the MDCK (Madin-Darby canine kidney) cells. By means of this assay system we addressed the question whether the side of application of renal epithelia influences platinum complex toxicity.

Methods. C7 cells were grown in membrane filter cups, and the apical or basolateral membranes were exposed to 100-μmol/L cis-, oxali-, or carboplatin. TEER and caspase-3 activity were determined. Cimetidine was used as an inhibitor of organic cation transporters (OCTs). C7 cell lysates were analyzed for OCT-1 and -2 by Western blot analysis.

Results. TEER dropped by 89.5 ± 9.3% (mean ± SEM; $N = 6$) within 24 hours after addition of cisplatin to the basolateral side of C7 cells, while caspase activity increased up to 840.6 ± 17.4% (mean ± SEM; $N = 6$) compared to control cells. Exposure of the apical membrane to cisplatin reduced TEER by only 13.4 ± 8.7% (mean ± SEM; $N = 6$), and increased caspase-3 activity up to 213.9 ± 7.6% (mean ± SEM; $N = 6$). Oxaliplatin and carboplatin reduced TEER to a lesser extent than cisplatin. Oxaliplatin lowered TEER stronger than carboplatin. In general, basolateral application led to higher caspase activities and lower TEERs. The OCT-inhibitor cimetidine inhibited the TEER decrease induced by platinum complexes. Immunoblotting confirmed the presence of OCT-2 in C7 cells.

Conclusion. Toxic effects of platinum complexes on renal epithelia depend on the platinum complex used and the site of application. We conclude that cell polarity and basolateral transport mechanisms are essential in nephrotoxicity of platinum drugs.

Key words: nephrotoxicity, apoptosis, caspase-3, cell polarity, cisplatin analogs.

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Cisplatin and its analogs carboplatin and oxaliplatin are widely used antitumor drugs. Among others, platinum complexes are effective against cancer of the lung, testis, ovary, cervix, endometrium, oropharynx, bladder, colon, and rectum [1–4]. It is generally accepted that platinum complexes exert their main antitumor properties through binding to DNA, and several specific adducts have been identified [5–7].

Next to other adverse effects like nausea and diarrhea, nephrotoxicity is a major disadvantage that occurs especially in cisplatin therapy. Despite the dose dependency of the antineoplastic effect, nephrotoxic risks preclude the use of higher doses to maximize the therapeutic effect. A current study with high-dose cisplatin treatment in solid tumors showed that 42% of the treated patients suffered from nephrotoxic injury [8]. Several mechanisms have been implemented in dose-dependent and cumulative cisplatin-induced nephrotoxicity. Besides DNA damage, oxidative stress, inhibition of protein synthesis, mitochondrial dysfunction, and involvement of receptors of the tumor necrosis factor (TNF) family, apoptosis of the renal epithelial cells is of critical importance [9–12].

For a long time nephrotoxic injury was thought to be due to necrosis, until apoptosis was identified as an alternate mechanism of nephrotoxic cell death. Apoptosis enables cells to kill themselves under physiologic conditions, but it is also the specific mechanism through which cells with damaged genetic contents (i.e., cancer cells during cisplatin therapy) are removed [13]. Caspases, a class of cysteine proteases that are key regulators of the apoptotic pathway, have come into the focus of cisplatin-induced nephrotoxic injury [14]. In LLC-PK_1 cells, a cell line obtained from proximal tubules of pigs, cisplatin leads to activation of initiator caspases-2, -8, and -9, and consecutively to activation of the executioner caspase-3 [15]. The hallmark of apoptosis is the cleavage of nuclear chromatin by DNases that can be activated by caspase-3 [16].

Although cisplatin-induced nephrotoxicity has been subject of extensive research, the knowledge about the

influence of cell polarity and transport mechanisms is limited. There are no universally accepted platinum transport proteins identified to date, and the mechanism by which cisplatin enters cells is not fully understood [17]. Endo et al [18] found a carrier-mediated uptake of cisplatin by epithelial cells derived from proximal tubules of the opossum (OK cell line). Accumulation of cisplatin in the cells was more effective from the basolateral than from the apical cell culture medium. Okuda et al [19] investigated the role of basolateral membrane transport in cisplatin-induced toxicity in LLC-PK_1 cells, but they did not distinguish between apoptosis and necrosis because only lactate dehydrogenase (LDH) release into the culture medium was measured.

We have established a new model for the investigation of platinum complex-induced toxicity in renal epithelia by utilizing the C7 clone of the MDCK (Madin-Darby canine kidney) cells [20]. The C7 cells build a tight monolayer, which is reflected by their high Trans Epithelial Electrical Resistance (TEER) of up to 20,000 $\Omega \cdot cm^2$. By means of this assay system we addressed the question whether site of application has an effect on platinum complex–induced cell death. Here we report that platinum complex toxicity strongly depends on the site of application (i.e., basolateral application violates monolayers more than apical application). Cisplatin, oxaliplatin, and carboplatin induce loss of epithelial monolayer integrity by apoptosis via activation of caspase-3 to different extents.

METHODS

Cell culture and reagents

The phenotypic and genotypic properties of MDCK-C7 cells have been described in detail previously [20]. In short, the C7 clone resembles principal cells of the renal collecting duct epithelium with high transepithelial electrical resistance. C7 cells were grown in flasks under standard conditions (37°C, 5% CO_2) in minimal essential medium (MEM) with Earle's salts, nonessential amino acids, glutamic acid, and 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). For TEER measurements, 400,000 MDCK-C7 cells were seeded in a filter cup (growth area 4.2 cm^2, 1×10^6 pores/cm^2, 0.4 μm pore diameter; Becton Dickinson Labware, Franklin Lakes, NJ, USA) (Fig. 1). The polygonal flat C7 cells grow firmly attached to the microfilter membrane and form an epithelial monolayer. To prevent bacterial growth during repeated TEER measurements penicillin (100 U/mL) and streptomycin (1 μg/mL) were present in the media. Medium exchange and transepithelial electrical resistance measurement started three days after seeding MDCK-C7 cells. TEER was measured at least once per day in six-well dishes. TEER values were corrected for background resistance. After another two to four days, platinum complexes were added to the upper or lower medium compartment.

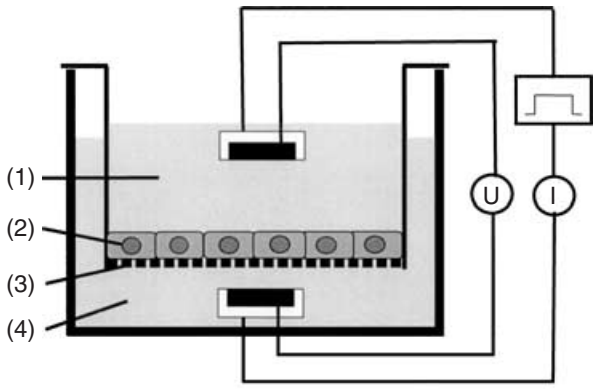

Fig. 1. Experimental setup. C7-MDCK cells (2) were seeded in microfilter membrane cups (3) mounted in wells to form a tight and polarized monolayer that divides the cell culture medium in an upper (1) and lower (4) medium compartment. The basolateral side of the cells is freely accessible for platinum drugs by adding them to the lower medium. Substances have contact to the apical membrane after adding them to the upper medium.

Application of platinum complexes

Cisplatin [cis-Platinum(II) diamine dichloride], oxaliplatin [SP-4–2-(1R-trans)]-1,2-cyclohexanediamine-N,N′)[ethanedioata(2-)-O,O′-platinum], and carboplatin [cis-diamine(1,1-cyclobutanedicarboxylato)-platinum] were purchased as crystalline powders (Sigma Aldrich, Taufkirchen, Germany) and dissolved in phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} [(mmol/L): 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4; 1 $MgCl_2$, 1 $CaCl_2$; pH 7.4 at 37°C]. Controls were performed by adding solvent to both medium compartments because there was no difference between the addition of solvent to the upper or lower medium.

Application of organic cation transporter inhibitor

The organic cation transporter (OCT) inhibitor cimetidine [21] (Sigma Aldrich) was dissolved in PBS with Ca^{2+} and Mg^{2+} (see application of platinum complexes; 107 mmol/L stock solution). Cimetidine was added 1 hour before addition of oxaliplatin (100 μmol/L) or carboplatin (100 μmol/L) and 6 hours before addition of cisplatin (10 μmol/L) to the basolateral medium.

Measurement of transepithelial electrical resistance

For transepithelial electrical resistance measurements, published in detail previously [22], commercially available STX-2 chopstick electrodes (WPI; Sarasota, FL, USA) were used. The concentric electrodes deliver current pulses (ΔI) and detect changes in transepithelial voltage (ΔV). The electrical resistance can be derived

continuously and measured with an ohmmeter. The measurement of the transepithelial electrical resistance provides a continuous assay system. Because measurements are performed under sterile conditions, they can be repeated in the same culture dish as often as desired at appropriate time intervals. Platinum complexes were added after C7 monolayers developed a resistance of ≥ 8 k$\Omega \cdot cm^2$. A resistance ≥ 1 k$\Omega \cdot cm^2$ already implicates a tight MDCK-C7 monolayer. Decreases in transepithelial resistance reflect changes in monolayer integrity. Resistances were set to 100% at the time of platinum complex addition.

Determination of caspase-3 activity and protein contents

The caspase assay was based on the cleavage of the 7-amino-4-coumarin (AMC) derived substrate Z-DEVD-AMC by caspase-3 and closely related proteases. Caspase-3 activity was measured with slight modifications of the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR, USA). Cell culture media were centrifuged at 1500 rpm for 5 minutes, and the cell pellet was added to the corresponding filter or well. Filter cups were placed in empty wells. Lysis buffer (200 µL) was added to each filter before cells were subjected to a short freeze-thaw cycle of 10 minutes at −80°C. Cells were scraped off and centrifuged (6000 rpm, 5 min, 4°C). The supernatants were used to determine protein contents and enzymatic activity of the samples. The Pierce Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used for quantification of total protein at a wavelength of 546 nm. Supernatant (60 µL) was mixed with 60 µL of 2× substrate in microplates for fluorescence readings. Specificity of substrate cleavage was tested by addition of the Ac-DEVD-CHO-inhibitor to the reaction mixture. The reaction mixture was incubated for 30 minutes at room temperature. AMC was determined using a Fluoroscan II fluorometer (Labsystems, Helsinki, Finland) with an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Based on the standard curve of AMC and the results of protein quantification, caspase activity was calculated as µmol/L AMC per mg protein. For clarity, the mean caspase activity of control samples at the time of platinum complex application was set to 100%. The results are therefore expressed as a ratio of the mean caspase activity of samples 48 hours after solvent or platinum complex application, and the mean caspase activity of controls shortly before addition.

Western blot analysis of C7 cell lysate

C7 cells were harvested by trypsination and heated (95°C, 5 min) in reducing sample buffer [(mmol/L): 100 Tris-HCl, 200 DTT; pH 6.8 with 4% SDS, 10% sucrose, 0.015% bromphenolblue]. Protein content was determined by the amido-black method [23]. Polyacrylamide gels 7.5% were loaded with 20 µg protein per lane and run at 50 V in buffer [(mmol/L): 25 Tris, 192 glycine, 3.5 SDS] in an electrophoretic chamber (Bio-Rad, München, Germany).

Gels were blotted on a polyvinylidene fluoride (PVDF) membrane at 80 mA for 1.5 hours. The blots were blocked with 5% skim milk in PBS without Ca^{2+} and Mg^{2+} (see application of platinum complexes) at 4°C overnight, incubated with primary antibody (1:5000 Anti OCT1/2, Cat. nos. OCT11-A and OCT21-A, respectively; Alpha-diagnostic, San Antonio, TX, USA) for 1 hour at 37°C, and secondary antibody (1:5000 antirabbit IgG, peroxidase conjugate no. A0545; Sigma Aldrich) for 1.5 hours at room temperature. After incubation, blots were washed in PBS with 0.05% Tween and exposed to x-ray film (AGFA, Leverkusen, Germany) using the enhanced luminescence kit (West Femto; Pierce).

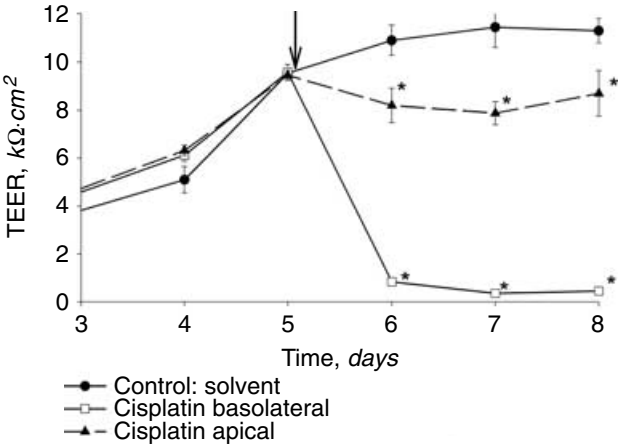

Fig. 2. Changes in transepithelial electrical resistance after addition of cisplatin. Transepithelial electrical resistance of MDCK C7 cell monolayers was measured in 24-hour time intervals before and after addition of 100 µmol/L cisplatin or solvent to the apical or basolateral medium. Cisplatin or solvent was added to the culture medium at day 5 (arrow) after seeding the C7 cells. Results are mean ± SEM, $N = 6$ each plot, $^*P < 0.05$.

Statistical analysis

Data of experiments are given as mean value ± standard error of the mean (SEM). Statistical significance was tested with the *U* test (Mann-Whitney-Wilcoxon), a reliable test for small numbers with unknown statistical distribution. α was set to 0.05 as threshhold for significance.

RESULTS

Transepithelial electrical resistance measurements

Platinum complexes were added when TEER was higher than 8 k$\Omega \cdot cm^2$. The mean transepithelial resistance was 9.5 ± 0.3 k$\Omega \cdot cm^2$ when cisplatin was added (Fig. 2). Apical application of cisplatin led to a decrease in transepithelial resistance from 9.5 ± 0.2 k$\Omega \cdot cm^2$ to

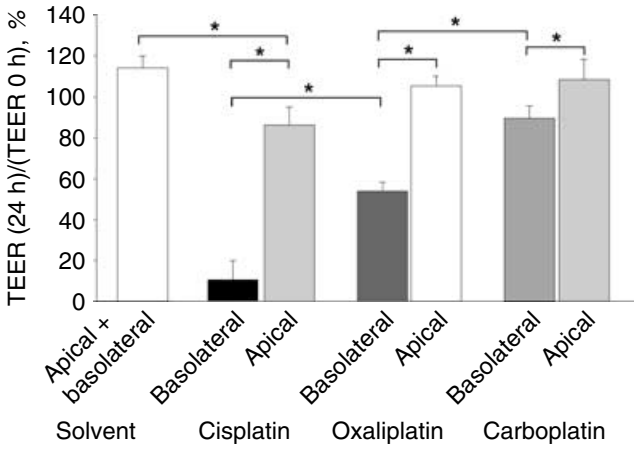

Fig. 3. Transepithelial electrical resistance 24 hours after addition of platinum complexes. Cis-, oxali-, and carboplatin (100 µmol/L final concentration) were added either to the apical or basolateral medium compartment. Solvent was added to the upper and lower medium for controls. The resistance of every monolayer at the time of platinum complex application was set to 100%. Results are mean ± SEM, $N = 6$ each bar, $^*P < 0.05$.

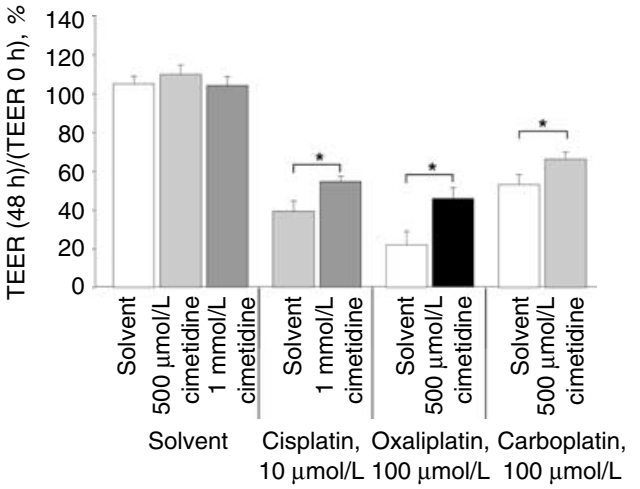

Fig. 4. Application of the organic cation transporter inhibitor cimetidine. Cisplatin (10 µmol/L), oxaliplatin (100 µmol/L), and carboplatin (100 µmol/L) were added to the basolateral medium in combination with cimetidine (500 µmol/L or 1 mmol/L) or solvent.

8.2 ± 0.7 kΩ·cm^2 within 24 hours. Addition of cisplatin to the basolateral medium induced a complete breakdown of transepithelial resistance from 9.6 ± 0.3 kΩ·cm^2 to 0.8 ± 0.1 kΩ·cm^2 within the same time. Addition of solvent (PBS with Ca^{2+} and Mg^{2+}) for controls to either medium compartment did not affect electrical resistance (Fig. 2). TEER of controls increased by 14.4 ± 5.7% within 24 hours (Fig. 3). There was no significant difference between controls and the application of oxaliplatin (105.6 ± 4.4%) or carboplatin (108.6 ± 9.8%) to the apical medium, whereas application of cisplatin lowered the resistance significantly to 86.6 ± 8.7% of the initial resistance. Basolateral application of cisplatin (10.5 ± 9.3%) induced a complete TEER breakdown, and oxaliplatin (54.2 ± 4.3%) reduced TEER significantly to a higher extent than carboplatin (89.6 ± 6.1%).

The OCT inhibitor cimetidine inhibited the TEER decrease induced by platinum complexes (Fig. 4). Twenty-four hours after basolateral addition of oxaliplatin the difference between cimetidine and controls was already statistically significant (data not shown). Cimetidine alone (500 µmol/L and 1 mmol/L) had no effect on TEER.

Caspase-3 activity

Addition of solvent to the apical and basolateral medium (Fig. 1) did not increase caspase-3 activity significantly within 24 hours (108 ± 6.4%) (Fig. 5). Addition of cisplatin (840.6 ± 17.4%), oxaliplatin (241.8 ± 13.4%), or carboplatin (138.5 ± 11.5%) to the basolateral medium led to a stronger enhancement of caspase activity than addition to the apical medium. Cisplatin in the apical medium raised caspase activity (213.9 ± 7.6%),

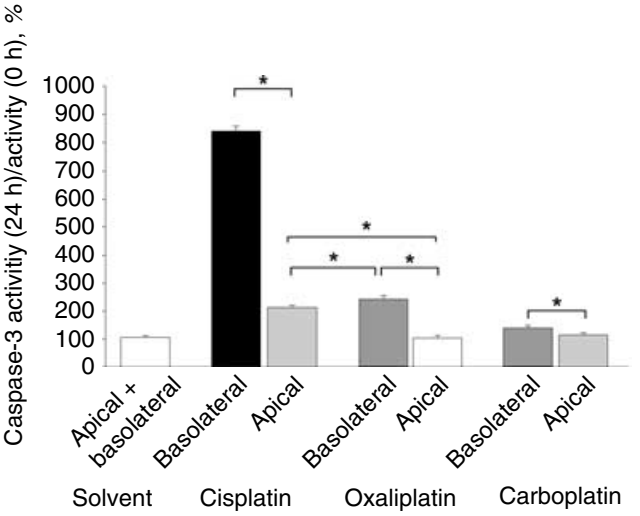

Fig. 5. Caspase-3 activity 24 hours after addition of platinum complexes. When electrical resistance of MDCK-C7 cells on filter membranes was higher than 8 kΩ·cm^2, cis-, oxali-, and carboplatin (100 µmol/L final concentration) were added either to the apical or basolateral medium compartment. Solvent was added to the upper and lower medium for controls. Caspase-3 activity of controls before platinum complex application was set to 100%. Results are mean ± SEM, $N = 6$ each bar, $^*P < 0.05$.

whereas application of oxaliplatin (104.2 ± 9.7%) or carboplatin (115.4 ± 8.0%) in the apical medium had no significant effect.

Western blot analysis of C7 cell lysate

Immunoblotting of C7 cell lysates confirmed the presence of more OCT-2 than OCT-1 (Fig. 7). The strongest band in the Western blot for OCT-2 had a molecular weight of about 65 kD, which is in accordance with the

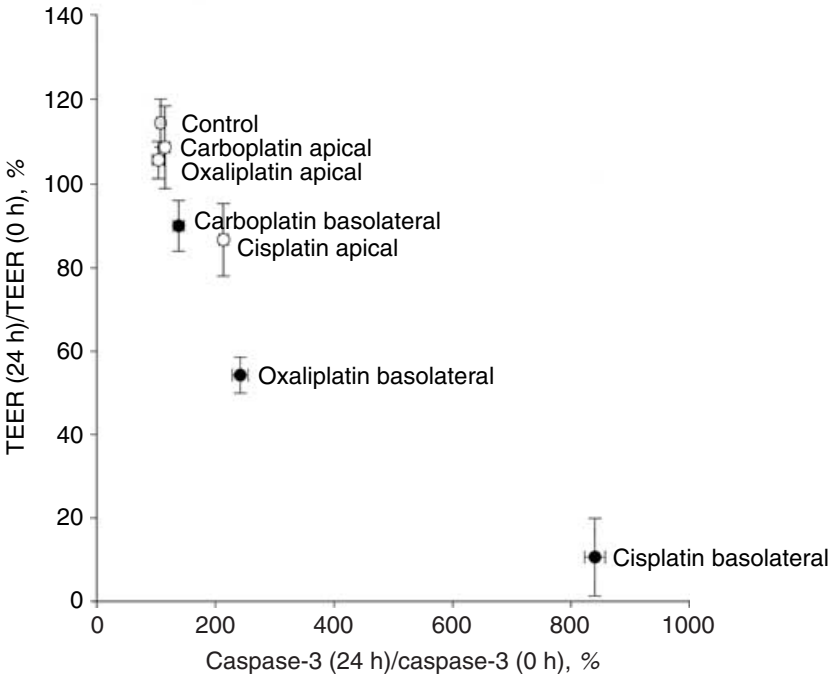

Fig. 6. Synopsis of caspase-3 activity and transepithelial electrical resistance (see Figs. 3 and 4). Cells were grown in filter cups and platin complexes (100 μmol/L final concentration) were added to the upper or lower medium compartment.

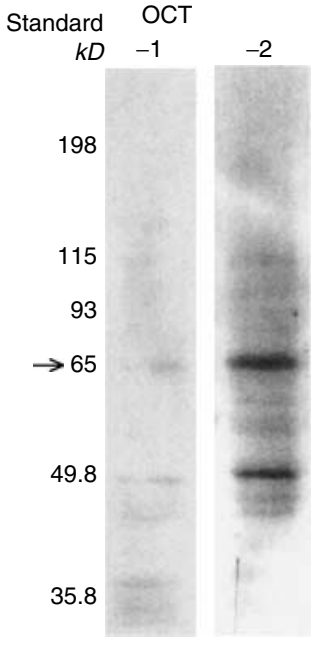

Fig. 7. Western blot analysis of C7 cell lysate. OCT-2 but not OCT-1 was detected in the lysate of MDCK-C7 cells by using polyclonal antibodies and a protocol described in the **Methods** section.

calculated molecular weights of organic cation transporters in rats, mice, or humans [24, 25].

DISCUSSION

Basic mechanisms of platinum complex toxicity in polarized renal cells were investigated in the present study. Here we report that the cisplatin analogs oxaliplatin and carboplatin induce activation of caspase-3 in renal epithelia. So far, the activation of caspase-3 by carboplatin and oxaliplatin has only been associated with their antineoplastic effect [6, 26]. Furthermore, we can show that cisplatin, oxaliplatin, and carboplatin induce caspase-3 activation to different extents, and that this activation depends on the site of application in a renal epithelium.

Studies in renal and nonrenal cell models revealed that caspase-3 is activated by a variety of stimuli, including receptor-mediated activation of caspase-8 [27, 28]. Members of the tumor necrosis factor receptor (TNFR) family are especially directly involved in caspase activation and subsequent cisplatin-induced renal failure [29–31]. Additionally, MDCK cells express TNFR1 and TNFR2 [32].

Altogether, high caspase-3 activities are correlated with low TEERs in our model system (Fig. 6). TEER is a useful addition to biochemical parameters because it contains valuable information about monolayer integrity. It reflects functional changes in epithelial permeability. Hence, platinum complex–induced apoptosis is accompanied by an increased permeability of the epithelial monolayer and vice versa.

The difference between apical and basolateral platinum complex application is essentially based on the negligible paracellular shunt of our model system. Paracellular diffusion of platinum complexes from the apical to the basolateral side would consequently lead to basolateral uptake of cisplatin, and thus, result in TEER breakdown and increased caspase activities. It is likely that a basolaterally located transporter is responsible for

the different effects between apical and basolateral application of platinum complexes. Because the reactive species of cisplatin are positively charged, few candidate transporters have to be considered. OCTs, for example, are polyspecific, potential-driven transporters that are involved in the basolateral entry step in organic cation secretion [25]. They transport substrates like metabolic products or the model cation tetraethylammonium (TEA) from the basolateral side into the cytosol before apical secretion via a proton-cation exchanger [21, 33].

We suppose that organic cation transporters (OCTs) are involved in the transport of platinum drugs into the cytosol, and thus are responsible for differences between apical and basolateral application of platinum complexes. The nephrotoxic effect of cisplatin is preferentially located in the S3 segment of the proximal tubulus [14]. Interestingly, high expression levels of OCTs can be found in this part of the nephron [34]. rOCT1 has been detected in the basolateral membrane of S1 and S2 segments of renal proximal tubules, whereas rOCT2 is predominantly located in the basolateral membranes of S2 and S3 segments [35]. Although the renal collecting duct is not the primary location of renal side effects in cisplatin therapy, tight epithelia derived from this segment are a versatile model for studying differentially sorted transporters in apical and basolateral membranes.

The organic cation transporter OCT-2 has been identified in the basolateral, but not in the apical membrane of MDCK-cells [17, 35, 36]. MDCK cells consist of at least two subclones [20], and OCT-2 is located in the C7 clone used in the present study. For OCT-2 in MDCK cells, an IC_{50} of 6.3 μmol/L for cimetidine (5 μmol/L [^{14}C]TEA) has been found [36]. Accordingly, the OCT inhibitor cimetidine delayed TEER breakdown after addition of platinum complexes to the basolateral medium. Concentrations (100 μmol/L) of platinum complexes are commonly used for in vitro studies [15, 37]. In vivo, local concentrations of platinum drugs are too different to be determined due to reabsorption and secretion processes. In a pharmacokinetic study of cisplatin, the peak plasma levels varied from 0.2 up to 14 μmol/L [38]. Erdlenbruch et al [38] found concentrations higher than 300 μmol/L for unbound platinum in urine after chemotherapy. In rat models, plasma concentrations of 55 μmol/L and renal tissue concentrations of 57 μmol/L of unmodified cisplatin have been measured after bolus injection [39]. In patients with normal renal function receiving 130 mg/m^2 oxaliplatin as a 2-hour infusion without hydration, the maximum total plasma concentration of oxaliplatin was 6.5 μmol/L with 42% unbound platinum complex [40]. In similar studies peak plasma concentrations higher than 12 μmol/L were observed [41]. A broad, mean terminal half-life ($t_{1/2}\beta$) of oxaliplatin ranging from 30 up to 240 hours has been determined [41, 42]. There are no reports about plasma levels in the current high-dose oxaliplatin treatment trials so far. High-dose therapeutic regimens (i.e., 500 mg/m^2 over 1 hour for 3 consecutive days) have been established for carboplatin. The platinum complex concentration in patients was up to 65 μmol/L in plasma and 61 μmol/L in ultrafiltrated plasma [43].

The observed differences between the platinum complexes in our in vitro system match their clinical properties. Carboplatin is a very attractive drug compared with cisplatin because of its very low nephrotoxicity [44].

CONCLUSION

The differences between basolateral and apical susceptibility of renal epithelia together with the different pharmacokinetic properties of platinum drugs are crucial determinants of nephrotoxic adverse effects. Additionally, further investigation of polarization and protein sorting of renal epithelia could contribute to a better understanding of the basic mechanisms of platinum complex–induced nephrotoxic cell injury.

Taken together, our in vitro model provides a system that is enabled to continuously study the effects of drugs in sufficiently separated media, and nearly eliminates problems of diffusion between medium compartments. The high TEER of the C7 clone is a sensitive and easy to measure parameter. It is therefore a valuable tool for studying basic mechanisms of platinum complex–induced toxicity in polarized epithelia.

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