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**Dursun, Belda, Zhbin He, Hilary Somerset, Dong-Jin Oh, Sarah Faubel, and Charles L. Edelstein.** Caspases and calpain are independent mediators of cisplatin-induced endothelial cell necrosis. *Am J Physiol Renal Physiol* 291: F578–F587, 2006. First published April 18, 2006; doi:10.1152/ajprenal.00455.2005.—The role of caspases and calpain in cisplatin-induced endothelial cell death is unknown. Thus we investigated whether caspases and calpain are mediators of cisplatin-induced apoptosis and necrosis in endothelial cells. Cultured pancreatic microvascular endothelial (MS1) cells were exposed to 10 and 50 μM cisplatin. Apoptosis or necrosis was determined by Hoechst 33342 and propidium iodide (PI) nuclear staining. Cells treated with 10 μM cisplatin had normal ATP levels, increased caspase-3-like activity, excluded PI and demonstrated morphological characteristics of apoptosis at 24 h. Cells treated with 50 μM cisplatin had severe ATP depletion, increased caspase-3-like activity, and displayed extensive PI staining indicative of necrosis at 24 h. There was a dose-dependent increase in caspase-2-like activity and Smac/DIABLO protein. Calpain activity increased significantly with 50 μM, but not 10 μM cisplatin at 24 h. With 50 μM cisplatin, ATP levels were significantly reduced starting at 18 h, caspase-2- and caspase-3-like activities were significantly increased starting at 18 h, and LDH release started at 8 h with maximum increase at 18–24 h. Calpain activity was not increased before 24 h. The increase in LDH release and the nuclear PI staining with 50 μM cisplatin at 24 h was reduced by either the pancaspase inhibitor, Q-VD-OPH, or the calpain inhibitor, PD-150606. Calpain inhibitor had no effect on caspase-3-like activity. In conclusion, in cisplatin-treated endothelial cells, caspases, the major mediators of apoptosis, can also cause necrosis. A calpain inhibitor protects against necrosis without affecting caspase-3-like activity suggesting that calpain-mediated necrosis is independent of caspase-3.

**CISPLATIN IS ONE OF THE MOST important chemotherapeutic agents in the treatment of solid tumors; especially testicular, ovarian, head, neck, and lung cancers.** (21) Nephrotoxicity is the major side effect of cisplatin which limits its efficacy as a potent chemotherapeutic agent and also makes it one of the most widely studied nephrotoxins. Proximal tubular epithelial cells are thought to be the primary target of cisplatin-induced acute renal failure (ARF) (6, 25, 29, 31). Whether cisplatin causes apoptosis or necrosis to endothelial cells and the mechanisms whereby this injury occurs is not known.

Micropuncture studies in rats indicate that the decrease in renal plasma flow by cisplatin precedes the decrease in glomerular filtration rate (GFR) (37, 45), raising the possibility that endothelial injury comes before tubular injury. Thus cisplatin may impair the circulation in the vasa recta leading to damage to the adjacent S3 segments of the proximal tubule. In a rat model of cisplatin nephrotoxicity, damage to glomerular capillaries including endothelial cells has been described (27). Also, vascular toxicity presenting as thrombotic microangiopathy (19, 23), myocardial infarction (22), cerebrovascular events (8), Raynaud’s phenomenon (43), and complete occlusion of femoral artery (30) has been reported in patients receiving cisplatin. Cisplatin either alone or in combination with bleomycin also results in ARF with characteristics of hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (23, 44). Endothelial cell injury and dysfunction are thought to play an important role in the pathogenesis of ischemic acute tubular necrosis (34). Thus the present study in endothelial cells has relevance in understanding the effect of cisplatin in ARF.

Caspases and calpain are intracellular cysteine proteases (36, 42). Caspases participate in two distinct signaling pathways: 1) promotion of apoptotic cell death (caspase-3) and 2) activation of proinflammatory cytokines (caspase-1) (26, 41). Calpain is the major calcium-dependent cytosolic cysteine protease which is involved predominantly in intracellular signaling, cytoskeletal stability, necrosis, and apoptosis (42). The effect of caspases and calpain in cisplatin-induced endothelial cell death is unknown.

Given this background, the aim of the present study was to determine whether caspase and calpain are mediators of cisplatin-induced death and to investigate the pathways and morphology of cisplatin-induced death in microvascular endothelial cells.

**METHODS**

**Cell culture and reagents.** MS1 (MILE SVEN 1) mouse endothelial islet cells purchased from American Type Culture Collection (ATCC Catalog No. CRL-2279, Manassas, VA) were used in the experiments. The line retains the properties of endothelial cells including uptake of acetylated LDL and expression of both factor VIII-related antigen and the VEGF receptor (1, 2, 18). The expression of VEGFR-1 and VEGFR-2 in MS1 microvascular endothelial cells (data not shown) was confirmed by Western blot analysis.

Cells were grown in DMEM supplemented with 5% heat-inactivated fetal calf serum, 1% penicillin streptomycin, 4 mM l-glutamine, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate. Cells were kept in a humidified incubator gassed with 5% CO2-95% air at 37°C and the media were renewed two to three times weekly. The cells were maintained in six-well culture plates and subcultures were obtained by trypsinization. Experiments were performed when the plates became at least 80% confluent.

Cisplatin was purchased from Aldrich. Q-Val-Asp(nomonethylated)-Oph [QVD-OPH] was purchased from MP Biochemicals (Au-
rora, OH). PD-150606, a selective nonpeptide calpain inhibitor, was obtained from Calbiochem (Catalog No. 513022). DMSO was obtained from Aldrich. The vital nuclear dye bisbenzamide (Hoechst 33342) and propidium iodide (PI) were obtained from Molecular Probes (Eugene, OR).

**Cisplatin treatment.** After they reached confluence (at least 80%), MS1 cells were replaced with fresh DMEM including serum, glucose, and sodium bicarbonate, and then incubated with different concentrations of cisplatin for the indicated period of time. Cisplatin was freshly prepared and dissolved in DMSO (0.1%; final concentration) at the time of the experiment. Control MS1 cells were treated with the vehicle (0.1% DMSO). Optimum exposure time and the appropriate concentration of cisplatin were determined from samples taken at different time points and from different concentrations of the drug (data not shown); finally, treatments with 10 and 50 μM concentrations of cisplatin for 24 h were determined to be used for the rest of experiments to reflect the two aspects of cell death (apoptosis and necrosis).

**Caspase and calpain inhibition.** To determine the role of caspases and calpain in cisplatin-induced endothelial cell death, cells were incubated with 50 μM pancaspase inhibitor, QVD-OPH, or 50 μM calpain inhibitor, PD-150606, in two different concentrations of cisplatin (10 or 50 μM) for 24 h.

**ATP content.** Cell ATP content was determined by the luciferin-luciferase method on cell extracts (9). Control and treated monolayers were washed with 2 ml of Dulbecco phosphate-buffered saline (DPBS) and harvested in 1 ml of buffer A (0.220 M mannitol, 0.070 M sucrose, 0.5 mM EGTA, 2 mM HEPES, 0.1% fatty acid free BSA), scraped with a rubber policeman, transferred to an Eppendorf tube, and incubated on ice for 30 min. Extracts were centrifuged at 750 g. ATP determination kit (A-22066) from Molecular Probes was used in the bioluminescence assay for the quantitative determination of ATP with recombinant firefly lucerase and its substrate α-luciferin according to manufacturer instructions. ATP values were expressed as percent (%) of controls. The average of three ATP values was expressed as a single ATP value.

**LDH release.** Leakage of lactate dehydrogenase (LDH) was measured as an index of lethal membrane injury (necrosis), as described previously (3, 14). Briefly, at the end of the incubation of cells with cisplatin, the incubation medium was collected and the remaining pellet was dissolved in 1.5% Triton X-100 to the original volume. Percent LDH release was calculated by determining the ratio of LDH in the medium compared with that in the lysed MS1 cell pellet plus the medium.

**Caspase activities.** The substrates used in fluorescence or colorimetric assays preferentially detect active members of a given caspase group rather than a specific caspase. Therefore, the term “caspase-1-, -2-, -3-like activity” is used. Caspase-1, -2, and -3-like activity were determined by using fluorescent substrates as previously described (39). The cell pellets were mixed with a lysis buffer containing 25 mM Na HEPES, 2 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM PMSF, and 1 μM pepstatin A. The lysate was then vortexed and immediately frozen in liquid nitrogen and stored at −80°C. Lysate protein was measured by the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as standards. The calibration curve was performed with samples containing 50 μM Tris base, 150 mM NaCl, 2 mM EDTA, 1% Na deoxycholate, 1% NP-40, 0.1% SDS, 50 mM NaF, and 200 mM Na2VO4, and 0.1% β-mercaptoethanol, pH 7.2, plus protease inhibitors 1 mM mol/l AEBSF, 15 μmol/l pepstatin A, 14 μmol/l E-64, 40 μmol/l bestatin, 22 μmol/l leupeptin, and 0.8 μmol/l aprotonin. The homogenates were centrifuged (10,000 g at 4°C for 10 min) to remove unbroken cells and debris. Supernatants were mixed with sample buffer containing 50 μmol/l Tris base (pH 6.8), 0.5% glycerol, 0.01% bromophenol blue, and 0.75% SDS and heated at 95°C for 5 min. Equal amounts of protein (50 μg/lane) were fractionated by Tris-glycine-SDS-13% polyacrylamide gel electrophoresis (PAGE). The electrophoretically separated proteins were then transferred to an Immobilon P membrane (Millipore, Bedford, MA) by wet electroblotting for 60 min. The membrane was blocked with 5% nonfat dry milk in TBST [50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, and 0.1% Tween 20] buffer at pH 7.5 for 1 h at room temperature. Immunoblot analysis for caspase-3 was performed with the following primary antibody: a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1–277 representing the full-length precursor form of caspase-3 of human origin (1:200; catalog number sc-7148; Santa Cruz Biotechnology, Santa Cruz, CA). This antibody reacts with the precursor of caspase-3 (also designated as CCP 32, 32 kDa) as well as the act form (20 kDa). Purified recombinant caspase-3 (Upstate Group, Lake Placid, NY) was used as a positive control. Immunoblot analysis for caspase-2 was performed with the following primary antibody: a rabbit polyclonal antibody raised against a pep-
tide mapping at the NH2 terminus of caspase-2 of human origin (1:200; catalog number sc-623; Santa Cruz Biotechnology). Immunoblot analysis for Smac was performed with the following primary antibody: Smac (V-17) an affinity-purified goat polyclonal antibody against a peptide mapping near the NH2 terminus of mature Smac of human origin (1:200; catalog number sc-12683; Santa Cruz Biotechnology). The membranes were incubated overnight at 4°C with primary antibodies, washed in TBST buffer, and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 1:5,000 dilution in TBST buffer for 1 h at room temperature. Subsequent detection was carried out by enhanced chemiluminescence (ECL; Amersham), according to the manufacturer’s instructions. Prestained protein marker (Bio-Rad) was used for molecular mass determination.

**Immunofluorescence studies.** Nuclear morphology of the MS1 cells was examined to identify the mechanism of cell death. Nuclear morphology with characteristic features of apoptosis or necrosis remains the most reliable determinant for distinguishing the type of cell death in cell culture (26, 31, 32). The adherent MS1 cells were processed. The cells were stained with the DNA-specific dyes Hoechst 33342 and PI. Fluorescent microscopy was used to distinguish between apoptosis and necrosis, according to the previously described methods (31). Briefly, cells were washed in PBS and then stained with Hoechst 33342 (final concentration 5 μM) and PI (final concentration 0.5 μM) for 10 min at 37°C. Cells were washed again in PBS and then fixed with 3% paraformaldehyde. Fields of cells were photographed (magnification ×400), by using appropriate filters to examine Hoechst 33342 and PI fluorescence staining. Apoptotic cells were quantitated in at least randomly selected 200 cells stained with Hoechst 33342 by fluorescence microscopy using the appropriate filter. Nuclear morphology was assessed with the cell membrane-permeant supravital DNA dye Hoechst 33342 (excitation wavelength 348 nm; emission 479 nm). Hoechst 33342, unlike PI, enters and stains the nucleus of both viable cells and cells that have died by apoptosis or necrosis. Apoptosis was determined on the basis of condensed, pyknotic, and fragmented nuclei, as well as by increased fluorescent intensity of nuclei stained with Hoechst 33342. Plasma membrane integrity was assessed using the cell membrane-impermeant DNA dye PI (excitation wavelength 535 nm; emission 617 nm). Necrosis was determined on the basis of positive PI staining in red color, indicative of loss of membrane integrity.

**Statistical analysis.** Experiments were performed in triplicate and repeated at least three times. Data were analyzed for significance using ANOVA. Multiple means were compared using the Student-Newman-Keuls test. Values are expressed as means ± SE. A P value of <0.05 was considered significant.

**RESULTS**

**ATP levels.** Figure 1 shows the effect of varying concentrations of cisplatin with and without a pancaspase inhibitor, Q-VD-OPH, on cellular ATP content (% of controls) of microvascular endothelial cells. No reduction in cellular ATP content was observed with 10 μM cisplatin. However, when the dose of cisplatin was increased to 50 μM for 24 h, the ATP level of MS1 cells decreased remarkably, by 85% (P < 0.001 vs. controls and 10 μM cisplatin). Addition of the pancaspase inhibitor Q-VD-OPH did not cause a significant change in ATP levels of the cells exposed to different doses of cisplatin. These data demonstrate that ATP levels are decreased by 50 μM, but not 10 μM cisplatin, suggesting that the cells treated with the higher dose are more likely to undergo necrosis than apoptosis, because apoptosis, unlike necrosis, is an energy-requiring process.

**LDH release.** As depicted in Fig. 2, LDH release doubled with 10 μM cisplatin and increased sixfold with 50 μM cisplatin. Addition of the pancaspase inhibitor, QVD-OPH, significantly decreased LDH release (%) in cisplatin-treated cells, almost to the levels of controls, indicating complete protection. To determine whether calpain is a mediator of cisplatin-induced endothelial injury, LDH release was also measured in the presence of calpain inhibitor, PD-150606. Cotreatment with calpain inhibitor, PD-150606, decreased LDH release partially (almost 50%) in the higher concentration of cisplatin treatment only.

These results indicate that both the pancaspase inhibitor, Q-VD-OPH, or the calpain inhibitor, PD-150606, protect against cisplatin-induced LDH release in MS1 microvascular endothelial cells.

**Caspase-3 assays.** A dose-dependent increase in caspase-3-like activity was observed with the cisplatin treatment of MS1 cells (Fig. 3). There was a significant reduction in caspase-3-like activity of cisplatin-treated cells in combination with the pancaspase inhibitor, QVD-OPH.

As shown in Fig. 3, MS1 cells treated with 50 μM cisplatin displayed increased caspase-3-like activity. To further confirm this finding, immunoblot analysis for caspase-3 protein was performed. Caspases exist in cell cytosol as inactive pro form and require cleavage to become active. Immunoblots of cytosolic extracts were performed to detect the pro and active forms of caspase-3. As shown in Fig. 4, the active form of caspase-3 (~20 kDa) was increased significantly in 50 μM cisplatin. However, the intensity of the band reduced markedly with the addition of a pancaspase inhibitor, Q-VD-OPH, further demonstrating the role of caspase-3 in cisplatin-induced MS1 cell necrosis.

**Caspase-1-like activity.** Q-VD-OPH is a pancaspase inhibitor that inhibits the proinflammatory caspase-1, in addition to caspase-3 (4). To determine whether caspase-1 was also involved in the protection provided by Q-VD-OPH, caspase-1-like activity was measured. Caspase-1-like activity (nmol·min⁻¹·mg⁻¹) was 391.2 ± 20.64 in control cells, 374.6 ± 13.0 in 10 μM cisplatin-treated, and 334 ± 38.8 in 50 μM cisplatin-treated (P = not
significant). The lack of effect of cisplatin on caspase-1-like activity suggests that caspase-1 is not a mediator of cisplatin-induced microvascular endothelial injury, in vitro.

Caspase-2 and Smac/DIABLO assays. Caspase-2 is essential for permeabilization of mitochondrion and release of cytochrome c (28). Smac/DIABLO is a recently identified mitochondrial protein which activates caspase-9 and -3 by antagonizing inhibitor of apoptosis proteins (IAPs) (10). To determine whether the mitochondrial pathway of caspase-mediated injury was involved in cisplatin-induced endothelial cell death, caspase-2 and Smac/DIABLO were examined. A dose-dependent increase in caspase-2-like activity was detected with the cisplatin treatment of MS1 cells (Fig. 5). The dose-dependent increase in caspase-2-like activity was also confirmed by immunoblot analysis for caspase-2 protein (Fig. 6).

Immunoblotting for Smac/DIABLO protein demonstrated a dose-dependent increase in intensity in MS1 cells treated with the two different concentrations of cisplatin, with the highest intensity in the presence of 50 μM cisplatin (Fig. 7). The caspase inhibitor had no effect on Smac/DIABLO protein expression. The same samples were reprobed for actin to control for protein loading.

Calpain activity. Cells treated with 10 μM cisplatin had no increase in calpain activity (Fig. 8). However, calpain activity increased markedly in MS1 cells treated with 50 μM cisplatin. Calpain activity was also measured in the presence of pan-caspase inhibitor, Q-VD-OPH; Q-VD-OPH had no effect on calpain activity. Thus cotreatment with the pancaspase inhibitor, Q-VD-OPH, did not cause a significant reduction in calpain activities of cisplatin-treated cells, also confirming the specificity of Q-VD-OPH for caspases as opposed to calpain. Cotreatment with the calpain inhibitor PD-150606 completely inhibited calpain activity.

Interaction between caspases and calpain. To test whether there was an interaction between calpain- and caspase-mediated death pathways in cisplatin-treated endothelial cells, caspase-3-like activity was measured in the presence of

Fig. 2. Pancaspase inhibitor, Q-VD-OPH, completely protects against cisplatin-induced injury, whereas the calpain inhibitor, PD-150606, partially protects against cisplatin-induced necrosis. LDH release (%) was used as an assay of cell membrane damage. MS1 cells were exposed to vehicle (C), 10 μM cisplatin (10 μM), and 50 μM cisplatin (50 μM) for 24 h. There was a dose-dependent increase in percent LDH levels of cisplatin-treated MS1 cells. LDH release (%) increased significantly with 10 and 50 μM cisplatin. Pancaspase inhibitor, Q-VD-OPH, reduced LDH release (%) significantly in both 10 and 50 μM cisplatin-treated groups. The calpain inhibitor, PD-150606, reduced LDH release (%) significantly in 50 μM cisplatin-treated group, but not in 10 μM cisplatin-treated group. #P < 0.001 vs. C and 50 μM. **P < 0.01 vs. 10 μM and 10 μM plus PD-150606. ***P = NS vs. 10 μM. ****P < 0.001 vs. C, 50 μM plus PD-150606, and 50 μM plus QVD-OPH. #P = NS vs. C. ¶P < 0.001 vs. C, n = 6 in each group.

Fig. 3. Caspase-3-like activity is increased in cisplatin-induced injury. MS1 cells were exposed to vehicle (C), 10 μM cisplatin (10 μM), and 50 μM cisplatin (50 μM) for 24 h. With 10 μM cisplatin, a slight but significant increase was noted in caspase-3-like activity, whereas with 50 μM cisplatin a large increase of caspase-3-like activity was noted. Pancaspase inhibitor, Q-VD-OPH, reduced caspase-3-like activity significantly in both 10 and 50 μM cisplatin-treated groups. *P < 0.05 vs. C. **P < 0.001 vs. 10 μM. ***P < 0.001 vs. C and 10 μM. ****P < 0.001 vs. 50 μM, n = 6 in each group.

Fig. 4. Immunoblotting of caspase-3. Active caspase-3 (20 kDa) was increased significantly in 50 μM cisplatin. The intensity of the band reduced markedly with the addition of a pancaspase inhibitor, Q-VD-OPH, further demonstrating the role of caspase-3 in cisplatin-induced MS1 cell necrosis. Representative immunoblot of 3 separate experiments is shown.

Fig. 5. Caspase-2-like activity is increased in cisplatin-induced injury. MS1 cells were exposed to vehicle (C), 10 μM cisplatin (10 μM), and 50 μM cisplatin (50 μM) for 24 h. With 10 μM cisplatin, a slight but significant increase was noted in caspase-2-like activity, whereas with 50 μM cisplatin a large increase of caspase-2-like activity was noted. Pancaspase inhibitor, Q-VD-OPH, reduced caspase-2-like activity significantly in both 10 and 50 μM cisplatin-treated groups. *P < 0.05 vs. C. **P < 0.001 vs. 10 μM. ***P < 0.001 vs. C, 10 μM, and 50 μM plus Q-VD-OPH, n = 6 in each group.
calpain inhibitor. The calpain inhibitor did not affect caspase-3-like activity in cisplatin-treated cells (Fig. 9).

**Morphological studies.** With the color combination, control MS1 cells excluded PI staining and stained mostly with Hoechst 33342 (in blue color), as expected (Fig. 10A). In contrast, cells treated with 50 μM cisplatin for 24 h displayed extensive PI staining (in red color) indicative of loss of membrane integrity and necrosis (Fig. 10C). Cells treated with lower concentrations (10 μM) of cisplatin for 24 h stained mostly with Hoechst 33342 (Fig. 10B), although some cells demonstrated a staining pattern in between. The nuclei of 10 μM cisplatin-treated cells had abnormal morphological features typical of apoptosis. Those nuclei were smaller in size compared with controls which is indicative of homogenous masses of chromatin condensation in globular or crescent-shaped figures. Nuclear fragmentation and apoptotic bodies were also noted. Apoptotic cells were quantitated in at least 200 randomly selected cells stained with Hoechst 33342 by fluorescent microscopy. Percentage of apoptotic cells at 24 h was highest in 10 μM cisplatin. Apoptotic nuclear changes (to the extent seen with 10 μM cisplatin) were not a feature of 50 μM cisplatin (Fig. 11).

Effects of pancaspase inhibitor, Q-VD-OPH, and calpain inhibitor, PD-150606, were tested on cisplatin-induced injury. MS1 cells were exposed to vehicle (C), 10 μM cisplatin (10 μM), and 50 μM cisplatin (50 μM) for 24 h. With 10 μM cisplatin, no increase was noted in calpain activity, whereas with 50 μM cisplatin a large increase of calpain activity was noted. Calpain activity was not affected by the pancaspase inhibitor, Q-VD-OPH, in 50 μM cisplatin-treated groups. Calpain activity was completely inhibited by the calpain inhibitor PD-150606. *P < 0.001 vs. C and 10 μM. **P = NS vs. 50 μM. ***P < 0.001 vs. 50 μM, n = 6 in each group.

Fig. 8. Calpain activity is increased in the highest concentration of cisplatin-induced injury. MS1 cells were exposed to vehicle (C), 10 μM cisplatin (10 μM), and 50 μM cisplatin (50 μM) for 24 h. With 10 μM cisplatin, no increase was noted in calpain activity, whereas with 50 μM cisplatin a large increase of calpain activity was noted. Calpain activity was not affected by the pancaspase inhibitor, Q-VD-OPH, in 50 μM cisplatin-treated groups. Calpain activity was completely inhibited by the calpain inhibitor PD-150606. *P < 0.001 vs. C and 10 μM. **P = NS vs. 50 μM. ***P < 0.001 vs. 50 μM, n = 6 in each group.

with 50 μM cisplatin only (Fig. 10C), indicating that necrosis during cisplatin incubation was suppressed by Q-VD-OPH. Cells cotreated with 50 μM cisplatin plus PD-150606 for 24 h (Fig. 10F) were stained less with PI compared with cells treated with 50 μM cisplatin only (Fig. 10C), indicating that necrosis during cisplatin incubation was partially suppressed by PD-150606. Necrotic cells stained with PI were quantitated in the presence of inhibitors in five randomly selected high-power fields by fluorescent microscopy using the appropriate filter showed the same pattern of protection (Fig. 12).

**Time course experiments with higher dose (50 μM) of cisplatin.** In separate experiments, MS1 cells were harvested to determine the time-related changes after administration of 50 μM cisplatin. No change was observed in ATP levels within
first 8 h of exposure. However, at 18 and 24 h, ATP levels were significantly reduced (Fig. 13A).

Both caspase-2- and -3-like activities were significantly increased at 18 and 24 h (Fig. 13, B and C). Calpain activity was significantly increased at 24 h, but not in earlier time points (Fig. 13D). A time-dependent relationship was observed between the percentage of LDH release and exposure to the higher dose of cisplatin (Fig. 13E). The percentage of LDH release remained stable within the first 8 h of 50 μM cisplatin treatment. However, LDH release (%) was significantly increased at 18 and 24 h. Morphological changes with PI uptake (necrosis) followed the same pattern. Apoptosis was examined at 4, 8, and 12 h. At these time points, less than 1% of cells were apoptotic. Cells were released from the plate with 50 μM cisplatin starting at 12 h of exposure but not before. The cells that were released from the plate during the time course did not show characteristics of apoptosis and stained with PI, indicating that they were undergoing necrosis (data not shown).

DISCUSSION

In vitro, proximal tubular cells exposed to low doses of cisplatin have a small change in ATP levels and demonstrate features of apoptosis, whereas higher doses of cisplatin result in severe ATP depletion and necrosis (31). In LLC-PK1 cells treated with cisplatin, caspases mediate cell death as determined by Trypan blue exclusion (25). However, other studies in cisplatin-treated LLC-PK1 cells demonstrate that caspase-3...
activity declined with doses of cisplatin that induced necrosis (29) and that caspase-3 is crucial for apoptosis but not necrosis (48). Both caspase-dependent and -independent pathways of cisplatin-induced apoptosis have been described (6). Calpain is a possible mediator of caspase-independent cell death. There have been few studies on the role of calpain in cisplatin-induced cell death. Cell death in two malignant glioma cell lines treated with cisplatin was not mediated by calpain (16). However, in cisplatin-treated human melanoma cells, calpain-mediated apoptosis was dependent on caspases (16, 33). The pathways of cisplatin-induced cell death, involving caspases and calpain, are controversial. Calpain has not previously been reported to be a mediator of cisplatin-induced necrosis.

Vascular toxicity presenting as thrombotic microangiopathy (19, 23), myocardial infarction (22), cerebrovascular events (8), and Raynaud’s phenomenon (43) has been reported in patients receiving cisplatin. Endothelial cells in culture provide an opportunity to examine the contribution of caspases and calpain to this vascular toxicity. The role of caspases and calpain in cisplatin-induced apoptotic and necrotic death in endothelial cells is not known.

Caspases are the major mediators of apoptosis. Few reports indicate that caspases mediate necrosis as well (13, 25). In large doses of cisplatin (50 μM) where ATP depletion, a significant increase of LDH release, and substantial PI staining were observed, we detected high levels of caspase-3-like activity. LDH release and caspase-3-like activity of cisplatin-treated cells decreased dramatically with the pan-caspase inhibitor, Q-VD-OPH, further demonstrating that caspase-3 plays a role in cisplatin-induced microvascular endothelial cell necrosis. The results of the morphological studies confirm a role of caspases in endothelial cell necrosis.

There is evidence that the proinflammatory caspase-1 mediates necrotic cell death (13, 40). However, in the present study caspase-1-like activity was not increased in cisplatin-treated microvascular endothelial cells. An increase in renal caspase-1 activity in response to cisplatin has been demonstrated in vivo (17), suggesting that the activation of caspase-1 in cisplatin-induced ARF in vivo requires factors which are absent in vitro.

A possible explanation for the increased activation of caspase-3 in necrotic injury and the protective effect of its inhibition in the present model could be made by necrosis being mediated by some of the same factors as apoptosis, suggesting an integration of necrotic and apoptotic death pathways (7, 20, 38). Therefore, apoptosis and necrosis may share common pathways. Thus we studied the pathways of caspase-mediated endothelial cell necrosis.

There are two pathways that result in activation of caspase-3: the death receptor pathway and the mitochondrial pathway. The death receptor pathway is initiated with the activation of cell death receptors (Fas and tumor necrosis factor-α) resulting by activation of procaspase-8, which in turn activates caspase-3. Recent findings indicate that caspase-2 is a critical initiator of the mitochondrial apoptosis pathway which is required for the permeabilization of the mitochondria and release of cytochrome c (28). Smac/DIABLO is a recently identified mitochondrial protein which activates caspase-9 and -3 by antagonizing IAPs (10, 24, 46). In our model, both caspase-2-like activity and Smac/DIABLO protein were found to be increased in cisplatin-treated endothelial cells, with the highest increases in the higher dose (50 μM) of cisplatin that resulted in necrosis. These data provide evidence for the participation of the mitochondrial pathway in cisplatin-induced endothelial cell necrosis.

Calpain is a calcium-dependent cytosolic cysteine protease which is involved predominantly in intracellular signaling, cytoskeletal stability, necrosis, and apoptosis (11, 14). Thus we investigated whether calpain is a mediator of cisplatin-induced necrosis. Unlike caspases, the role of calpain in cell death pathways is less clear. Calpain can cleave caspases-7, -8, and -9, resulting in their inactivation (5). On the other hand, calpain was also found to cleave procaspase-12 to generate an active caspase and to cleave the antiapoptotic Bcl-X_L molecule into a proapoptotic molecule (24, 35, 46). Thus calpain may act both as a negative and positive regulator of cell death pathways in different circumstances. We previously demonstrated that calpain plays a role in hypoxia-induced necrosis to rat renal...
proximal tubules (12, 14, 15). The role of calpain in cisplatin-induced injury remains largely unexplored. We detected a significant increase in calpain activity in the higher dose of cisplatin-treated endothelial cells, which was consistent with necrosis. Furthermore, the increase in LDH release with 50 \% \text{H}_9262 \text{M} \text{cisplatin} was inhibited by approximately half in the presence of calpain inhibitor, PD-150606, demonstrating that calpain is a mediator of cisplatin-induced necrosis. The results of the morphological studies in the presence of PD-150606 were also consistent with this observation. Thus these results demonstrate that calpain is a mediator of cisplatin-induced necrosis. The pathways involved in the cross-talk between the calpain and caspase proteolytic systems remain controversial. Calpain activation may be upstream (13) or downstream of caspas (47). Thus we investigated whether there was an interaction between calpain- and caspase-mediated pathways in cisplatin-induced endothelial cell death. The calpain inhibitor PD-150606 was found to have no effect on caspase-3-like activity in cisplatin-treated cells, suggesting a separate caspase-3-independent pathway of calpain-mediated cell necrosis. Similarly, calpain activity which was elevated significantly with 50 \% \text{H}_9262 \text{M} \text{cisplatin} was unaffected by the pancaspase inhibitor, Q-VD-OPH. Time course experiments with the higher dose of cisplatin (50 \% \text{H}_9262 \text{M} \text{cisplatin}) were performed (Fig. 13). Maximal alterations in ATP levels, caspase-2-, and -3-like activity corresponded with the maximum increase in LDH release at 18 and 24 h. Calpain activity increased at 24 h, the time of maximum LDH release. Morphological studies with PI uptake (necrosis) followed the same pattern as well. Partial protection by PD-150606 against cisplatin-induced endothelial cell necrosis could be explained by the observation that the increase in calpain activity took place at an even later stage compared with caspas during the exposure with 50 \% \text{H}_9262 \text{M} \text{cisplatin}. Alternatively, the small amount of LDH release not blocked by the calpain inhibitor, but blocked by the caspase inhibitor, may represent secondary necrosis due to nonspecific
increased plasma membrane permeability. The time course suggests that both calpain and caspases mediate cisplatin (50 μM)-induced cell necrosis because the increase in calpain and caspase activity corresponds with the increase in LDH release. However, with 50 μM cisplatin, there was increased apoptosis compared with controls (Fig. 11). Thus the data with 50 μM cisplatin may represent apoptosis during the first 18 h of incubation followed by secondary necrosis at 18 and 24 h.

In summary, the present study in cisplatin-treated endothelial cells demonstrates the following novel findings: 1) caspases, the major mediators of apoptosis, can cause necrosis associated with an increase in caspase-2-like activity and Smac/DIABLO protein expression; and 2) the calpain inhibitor protects against necrosis without affecting caspase-3-like activity, suggesting that calpain-mediated necrosis is independent of caspase-3. We believe that this model demonstrates endothelial injury as a new target of research for the underlying mechanism of cisplatin-induced ARF. Further in vivo studies are necessary to determine the respective influences of calpains and caspases in cisplatin-induced microvascular endothelial injury in ARF.

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