Cisplatin-Induced Renal Cell Apoptosis: Caspase 3-Dependent and -Independent Pathways

BRIAN S. CUMMINGS and RICK G. SCHNELLMANN

Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, South Carolina

Received January 15, 2002; accepted March 1, 2002

This article is available online at http://jpet.aspetjournals.org

ABSTRACT

The chemotherapeutic cisplatin causes renal dysfunction and renal proximal tubular cell (RPTC) apoptosis. The goal of these studies was to examine the role of p53, caspase 3, 8, and 9, and mitochondria in the signaling of cisplatin-induced apoptosis. Cisplatin (50 μM) produced time-dependent apoptosis in RPTCs, causing cell shrinkage, a 50-fold increase in caspase 3 activity, a 4-fold increase in phosphatidylserine externalization, and 5- and 15-fold increases in chromatin condensation and DNA hypoploidy, respectively. Mitochondrial membrane potential and ATP levels did not change at any time during cisplatin exposure. Caspase 8 and 9 activities also did not increase during treatment. Cisplatin increased nuclear p53 expression 4 h after treatment, preceding both caspase 3 activation and chromatin condensation.

Treatment with the p53 inhibitor α-(2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-p-tolyethanone (PFT) before cisplatin exposure inhibited p53 nuclear expression at 4, 8, and 12 h and inhibited phosphatidylserine externalization and caspase 3 activation at 12 h. Neither DEVD-fmk nor ZVAD-fmk inhibited cisplatin-induced p53 nuclear expression. Both DEVD-fmk and ZVAD-fmk completely inhibited caspase 3 activity but, like PFT, partially inhibited cisplatin-induced chromatin condensation, annexin V labeling, and DNA hypoploidcy after 24 h. These data demonstrate that at least 50% of cisplatin-induced apoptosis in RPTC is mediated by p53 and that p53 activates caspase 3 independently of either caspase 9 or 8 or mitochondrial dysfunction. Furthermore, 50% of cisplatin-induced RPTC apoptosis is independent of p53 and caspases 3, 8, and 9.

cis-Diaminedichloroplatinum (cisplatin) is a common chemotherapeutic agent used in the treatment of solid tumors (Lieberthal et al., 1996; Lau, 1999). One major drawback of cisplatin is its propensity to cause nephrotoxicity (Blachley and Hill, 1981). Cisplatin-induced renal proximal tubule cellular (RPTC) damage can develop after one dose and may result in either acute renal failure and/or renal electrolyte wasting (Safirstein et al., 1984; Lau, 1999). It is thought that the high amount of RPTC death after cisplatin treatment is a key factor in the development of acute renal failure.

Cisplatin-induced RPTC death was originally thought to be the result of oncosis, a type of cell death that is ATP-independent and characterized by cell and organelle swelling and lysis (Chopra et al., 1982). However, histological examination of cisplatin-treated kidney tissue demonstrated pathology characteristic of both apoptosis and oncosis (Schumer et al., 1992). Apoptosis is a controlled type of cell death that is energy-dependent and characterized by cell shrinkage, chromatin condensation, membrane budding, phosphatidylserine externalization, and activation of a family of cysteine proteases called caspases (Salvesen and Dixit, 1997; Cummings et al., 2000b). Caspase activation is thought to be a key step in the genesis of apoptosis, and numerous stimuli activate caspases, including those that activate plasma membrane death receptors (caspase 8) and cause mitochondrial dysfunction (caspase 9). Caspases are either initiators or executioners. Initiator caspases include caspases 8 and 9, and activation of these caspases results in activation of downstream or executioner caspases such as caspases 3 and 7 (Salvesen and Dixit, 1997). Executioner caspases are responsible for many of the biochemical characteristics of apoptosis, including cleavage and activation of poly(ADP-ribose) polymerase and of the inhibitor of caspase activator domain protein, which leads to DNA fragmentation. Studies have demonstrated that cisplatin induces both renal cell apoptosis and oncosis, depending on the concentration used (Chopra et al., 1982; Schumer et al., 1992; Lieberthal et al., 1996; Lau, 1999; Zhan et al., 1999).

Although studies have revealed that cisplatin induces renal cell apoptosis, the mechanism is not well understood. Lieberthal et al. (1996) showed that the morphological char-
acteristics of apoptosis were present in cisplatin-treated mouse renal proximal tubule cells, but the caspases involved were not studied. Lau (1999) demonstrated that caspase 3, but not caspase 1, was activated in LLC-PK1 cells treated with cisplatin, but the initiators of caspase 3 activation were not determined. Other studies in renal cell lines undergoing apoptosis induced by cisplatin demonstrated that caspase 3 can be activated by caspase 9, which is activated by the release of cytochrome c from the mitochondria (Schumer et al., 1992; Zhan et al., 1999). Studies in nonrenal cell models revealed that caspase 3 is activated by a variety of stimuli, including receptor-mediated activation of caspase 8 (Sun et al., 1999), caspase 9 activation (Schuler et al., 2000), alterations in the expression of the apoptotic proteins Bax and Bcl-2 (Sawada et al., 2000), and reactive oxygen species (Ye et al., 1999).

In addition to caspase 3 being activated by events centered at mitochondria, caspase 3 may be activated by p53-mediated activation of caspase 8 and 9 (Bennet, 1999; Ye et al., 1999). p53 is a tumor suppressor protein that increases in expression and translocates to the nucleus in cells undergoing apoptosis (Bennet, 1999; Komarov et al., 2000). It is activated in response to DNA damage, alterations of the cell cycle, and hypoxia (Ye et al., 1999). A role for p53 in cisplatin-induced renal cell apoptosis has been suggested (Gonzales et al., 2000), but to date, no studies have correlated p53 to activation of caspase 3, 8, and 9 during renal cell apoptosis. Such data would greatly aid in understanding the mechanism of renal cell death during cisplatin treatment. Furthermore, elucidation of how cisplatin treatment of renal cells causes apoptosis would increase our knowledge of the mechanisms of acute renal failure induced by other chemotherapeutic agents (Moos and Fitzpatrick, 1998; Komarov et al., 2000).

We determined the role of p53, mitochondria, and caspase 3, 8, and 9 in the signaling of cisplatin-induced renal cell apoptosis. Data from this study demonstrate that 50% of cisplatin-induced RPTC apoptosis is mediated by p53 and that p53 activates caspase 3 independently of either caspase 9 or 8 or mitochondria dysfunction. Furthermore, and just as importantly, work presented herein demonstrates that 50% of cisplatin-induced renal cell apoptosis is mediated by additional mechanisms independent of p53 and caspases 3, 8, and 9.

### Experimental Procedures

#### Materials

Female New Zealand White rabbits (1.5–2.0 kg) were purchased from Myrtle’s Rabbitry (Thompson Station, TN). L-Ascorbic acid-2-phosphate (magnesium salt) was obtained from Wako Bioproducts (Richmond, VA). The antibody to p53 and α-2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-p-tolyethanone (PPT) were purchased from Calbiochem (La Jolla, CA). DEVD-afc, IETD-afc, and LEHD-afc were purchased from Biovision, Inc. (Palo Alto, CA). The caspase 3 inhibitor DEVD-fmk, the general pan caspase inhibitor ZVAD-fmk, and annexin-FITC were obtained from R & D Systems (San Diego, CA). Hyperfilm ECL was purchased from Amersham Biosciences (Cleveland, OH), and cisplatin, propidium iodide, and all other chemicals and materials were obtained from Sigma-Aldrich (St. Louis, MO).

#### Isolation of Proximal Tubules and Culture Conditions

Rabbit renal proximal tubules were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved conditions as described previously (Nowak and Schnellmann, 1995, 1996). The cell culture medium was a 1:1 mixture of Dulbeco’s modified Eagle’s medium/Ham’s F-12 (without d-glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM l-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 mM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added to fresh culture medium immediately before daily media change. In general, confluent RPTCs were treated with inhibitors or diluent control (typically DMSO at <0.1% [v/v]) for 30 min before treatment with cisplatin. Aliquots of RPTCs were used for various assays as detailed below.

#### Measurement of Annexin V and Propidium Iodide Staining

Annexin and PI staining were determined using confocal microscopy and flow cytometry as described previously with modifications (Schutte et al., 1998; Goldberg et al., 1999; Meijeriman et al., 1999). Briefly, media were removed, RPTCs were washed twice with phosphate-buffered saline (PBS), and incubated in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2, pH 7.4) containing annexin V-FITC (25 μg/ml) and PI (25 μg/ml) for 10 min. Cells were washed three times in binding buffer and prepared for either flow cytometry or confocal laser scanning microscopy. For flow cytometry RPTCs were released from the monolayers using a rubber policeman and staining was quantified using a FAC-SCalibur flow cytometer (BD Biosciences, San Jose, CA). For each measurement 10,000 events were counted. Microscopy was performed using a confocal laser scanning microscope (model 410; Carl Zeiss Inc., Thornwood, NY). RPTCs were fixed for 10 min in 3.75% formaldehyde, washed three times with PBS, mounting media was added, and cover slips applied.

#### Determination of Caspase Activities

Caspase 3, 8, and 9 activities were determined using the fluorometric substrates DEVD-afc (caspase 3 substrate), IETD-afc (caspase 8 substrate), and LEHD-afc (caspase 9 substrate) following the protocols of the Caspase Activity Assay kit from BioVision, Inc. At 2, 4, 8, 12, and 24 h both attached and detached cells were isolated by scraping the dish with a rubber policeman and centrifugation at 400 for 10 min. The supernatant was removed, and the pellet was suspended in 100 μl of lysis buffer (BioVision, Inc.) and incubated at 4°C for 10 min followed by centrifugation at 12,000g for 10 min. Aliquots (50 μl) of the supernatant were removed and placed in a 96-well microplate containing reaction buffer (BioVision, Inc.). Substrate was added, and the microplate was incubated at 37°C for 30 min. Activity was monitored as the linear cleavage and release of the afc side chain and compared with a linear standard curve generated on the same microplate.

#### Immunocytochemistry and Assessment of Chromatin Condensation

RPTCs were exposed to either the solvent control or cisplatin for 4, 8, 12, and 24 h, fixed for 20 min using 10% buffered formalin/4% formaldehyde, and washed with PBS. Samples were permeabilized, washed, and nonspecific binding blocked by incubation of RPTCs in PBS/8% bovine serum albumin for 30 min. After washing RPTCs were incubated at 4°C overnight with either the primary antibody against p53 (10 μg/ml) or an IgG control, washed three times, and incubated with a secondary antibody conjugated to FITC and propidium iodide (25 μg/ml) for 2 h. Samples were washed three times, covered with mounting media, and coverslips applied. Visualization of staining was done using a confocal laser scanning microscope (model 410; Carl Zeiss, Inc.). Apoptotic nuclei were those nuclei that exhibited chromatin condensation at the periphery of the nucleus. Chromatin condensation and p53 staining were evaluated using a double blind protocol.

#### Measurement of DNA Hypoploidy, Cell Cycle, and Detachment

Cell cycle analysis and DNA hypoploidy were assessed using methods described previously (Cummings et al., 2000a). Briefly, RPTCs were washed twice with sample buffer [PBS plus glucose (1 g/l)], dislodged using Cellstripper (Mediatech, Herndon, VA), centrifuged at 400g for 10 min, and suspended in sample buffer. Cells were fixed in ice-cold ethanol (70% [v/v]) and stained with propidium iodide.
(50 μg/ml) in sample buffer containing RNase A (100 U/ml) for 30 min at room temperature with gentle shaking. Samples were analyzed within 24 h by flow cytometry with a FACScalibur flow cytometer (BD Biosciences). The amount of cell detachment was assessed using flow cytometry and determined by counting the number of cells in an equal volume of media for 1 min and using a hemacytometer. Both of these methods gave similar results.

**Measurement of Mitochondrial Function.** Mitochondrial function and cellular energetics were assessed by measurement of mitochondrial membrane potential and ATP and GTP levels. Mitochondrial membrane potential was assessed using the fluorometric dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyanobiphenyl (JC-1) (Molecular Probes, Eugene, OR) as described by Chen and Smiley (1992) with modifications. Briefly, treated RPTCs were exposed to JC-1 (20 μg/ml) in media for 10 min, washed three times in PBS, harvested by scraping, and JC-1 fluorescence determined at 530 nm/590 nm (excitation/emission). ATP and GTP levels were analyzed by reverse phase-high-performance liquid chromatography as described previously (Groves and Schnellmann, 1996).

**Protein Determination.** Protein determination was determined using the bicinchoninic acid assay method as described by Sigma-Aldrich.

**Statistical Analysis.** RPTCs isolated from one rabbit represented one experiment (n = 1). The appropriate analysis of variance was performed for each data set using SigmaStat statistical software (SPSS, Inc., Chicago, IL). Individual means were compared using Fisher’s protected least significant difference test with P ≤ 0.05 considered indicative of a statistically significant difference between mean values.

### Results

**Concentration Dependence of Cisplatin-Induced RPTC Apoptosis.** Treatment of RPTC with cisplatin resulted in concentration-dependent apoptosis as assessed by the externalization of phosphatidylserine using annexin V-FITC staining and flow cytometry (Fig. 1). The earliest detectable increase in annexin V-FITC staining above control was detected at 12 h of exposure to 50 and 100 μM cisplatin. Cisplatin at concentrations less than or equal to 100 μM induced significant increases in annexin V-FITC staining without causing increases in PI staining. Higher concentrations of cisplatin (200 and 400 μM) resulted in a decrease in annexin V staining alone and an increase in cells staining positive for PI alone and annexin V and PI. Confocal microscopy demonstrated that annexin V was localized to the outer leaflet of the plasma membrane in cells exhibiting annexin V-FITC staining alone (data not shown), confirming that increases in annexin V-FITC staining were the result of binding to externalized phosphatidylserine and not a result of internalization of the annexin V. Cisplatin-induced apoptosis was further confirmed by caspase 3 activation, chromatin condensation, and DNA hypoploidy (see below). Based on these data 50 μM cisplatin was used for the rest of the experiments.

**Effect of Cisplatin on Caspase Activity, ATP, GTP, and Mitochondrial Membrane Potential.** To investigate the mechanism of cisplatin-induced renal cell apoptosis phosphatidylserine externalization was correlated to the activation of caspases (Fig. 2, A and B). No significant increases in the activities of caspase 3, 8, or 9 were detected at either 2 or 4 h and at no time did the activity of any caspase increase in control RPTC. In contrast, cisplatin treatment of RPTC for 8 h increased caspase 3 activity 5.4-fold. Cisplatin-induced increases in caspase 3 activity were time-dependent because activity increased 12.4- and 54.7-fold after 12 and 24 h, respectively. In contrast to caspase 3, neither caspase 8 nor 9 activity increased at any time during cisplatin treatment, suggesting that these initiator caspases are not responsible for the activation of caspase 3. The increase in caspase 3 activity at 8 h preceded increases in annexin V-FITC staining in RPTCs at 12 h (Fig. 2B). No increase in annexin V-FITC staining was detected in RPTCs at either 4 or 8 h (data not shown).

Because cisplatin has been reported to inhibit mitochondrial function and overall cellular energetics (Brady et al., 1990; Kruidering et al., 1997), the effect of cisplatin treatment on ATP and GTP contents and on mitochondrial membrane potential were determined (Fig. 2, C and D; Table 1). ATP levels also were assessed to test the hypothesis that GTP decreases during cisplatin-induced apoptosis. This hypothesis is supported by recent studies that report a decrease in cellular GTP levels during anticancer agent- and ischemic-induced apoptosis (Vitale et al., 1997; Dagher and Potlikin, 2000). ATP and GTP levels in control RPTCs did not change significantly at any time throughout the experiment (12 h). ATP levels during the 12-h cisplatin exposure period did not significantly change. In contrast to ATP, cisplatin treatment resulted in a small increase in GTP levels after 12 h of exposure. Cisplatin treatment did not decrease mitochondrial membrane potential at any time during exposure as determined by JC-1 fluorescence (Table 1). These data suggest that cisplatin-induced caspase 3 activation is not mediated by decreases in cellular energetics or mitochondrial membrane potential. These results also indicate that cisplatin-induced caspase 3 activation is independent of caspase 8 or 9 activity and precedes annexin V labeling.

**Role of p53 in Cisplatin-Induced RPTC Apoptosis.** Because a role for p53 in apoptosis has been identified (Benet, 1999; Ye et al., 1999; Komarov et al., 2000; Sawada et al.,
we examined p53 during cisplatin exposure. Immunocytochemistry using confocal laser scanning microscopy demonstrated that cisplatin treatment of RPTCs increased both cytosolic and nuclear p53 levels above controls as early as 4 h after treatment (Figs. 3 and 4). The level of nuclear p53 expression continued to increase at both 8 and 12 h after treatment but decreased after 24 h. The increase in nuclear p53 staining in cisplatin-treated RPTCs preceded any increase in chromatin condensation and annexin V labeling by at least 4 h (Figs. 2B, 3E compared with 3I, and 4, A and B). Cisplatin also slightly increased p53 levels in the cytosolic fraction after 4 h, but the level was far below that seen in the nuclear fraction and did not change throughout the time course of the experiment (data not shown). Thus, cisplatin treatment of RPTCs results in the induction of p53 expression and the appearance of p53 in the nucleus before increases in either chromatin condensation, caspase 3 activation, or annexin V labeling. To investigate the hypothesis that p53 mediates caspase 3 activation in cisplatin-treated RPTCs, the effects of the p53 inhibitor PFT (Komarov et al., 2000), the caspase 3 inhibitor DEVD-fmk, and the general pan caspase inhibitor ZVAD-fmk were determined. Treatment of RPTCs with PFT before cisplatin exposure decreased nuclear p53 staining at 4, 8, and 12 h compared with cisplatin alone (Figs. 4 and 5). In contrast, treatment of RPTCs with either DEVD-fmk or ZVAD-fmk before cisplatin treatment did not alter p53 localization or expression compared with RPTCs treated with cisplatin alone (Fig. 5, A–C, versus 5, I–P). p53 expression and localization were similar among all treatment groups after 24 h. These temporal experiments also measured the effect of PFT
on caspase 3 activities at both 12 and 24 h. Cisplatin treatment of RPTCs increased caspase 3 activities at both 12 and 24 h (Fig. 6A). Treatment of RPTCs with PFT before cisplatin treatment totally inhibited caspase 3 activity at 24 h. Higher concentrations of PFT (30 μM) did not result in increased inhibition of caspase 3 (data not shown). Treatment of RPTCs with DEVD-fmk or ZVAD-fmk before cisplatin treatment totally inhibited caspase 3 activity at both 12 and 24 h. These data suggest that p53 functions upstream of caspase 3 and mediates its activation during cisplatin-induced RPTC apoptosis.

To determine whether inhibition of p53 and caspase 3 protected RPTCs from cisplatin-induced apoptosis, the ability of PFT, DEVD-fmk, and ZVAD-fmk to inhibit phosphatidylserine externalization, DNA hypoploidy, and chromatin condensation was analyzed (Figs. 4B, 6B, and 7). Treatment of RPTCs with PFT, DEVD-fmk, and ZVAD-fmk before cisplatin exposure reduced phosphatidylserine externalization to control levels at 12 h (Fig. 6B). In contrast, PFT had no affect on cisplatin-induced RPTC phosphatidylserine externalization at 24 h. Interestingly, DEVD-fmk and ZVAD-fmk only inhibited phosphatidylserine externalization approximately 40%, even though caspase 3 was totally inhibited by these compounds at 24 h (Fig. 6A). Cisplatin treatment of

Fig. 3. Effect of cisplatin on p53 expression. Confluent monolayers of RPTCs were treated with cisplatin for 4, 8, 12, and 24 h and fixed, and p53 and PI staining were analyzed by immunocytochemistry using confocal laser scanning microscopy. A–D, p53 staining in control cells. E and F, p53 staining in cisplatin-treated cells. I–L, PI staining for E–H. Data are representative of at least three separate experiments.

Fig. 4. Quantification of the effect of cisplatin on p53 expression and chromatin condensation. A, quantification of cisplatin-induced increases in RPTC p53 expression in the presence and absence of inhibitors. Confluent RPTC monolayers were treated with PFT (10 μM), DEVD-fmk (50 μM), ZVAD-fmk (50 μM), or solvent control [DMSO at <0.1% (v/v)] before cisplatin (50 μM) treatment. p53 staining was determined by immunocytochemistry using confocal laser scanning microscopy. Each individual nuclei was scored for the presence of p53 staining. Legend is exactly as given for B. B, quantification of cisplatin-induced increases on RPTC chromatin condensation in the presence and absence of inhibitors. Data are represented as the mean ± S.E.M. of at least three separate experiments. Means with different subscripts are significantly different from one another (P < 0.05).
RPTCs resulted in a significant increase in chromatin condensation as early as 8 h after treatment (Figs. 3 and 4B). Treatment of cells with either PFT or DEVD-fmk and ZVAD-fmk before cisplatin treatment significantly decreased chromatin condensation compared with RPTCs treated with cisplatin alone. Similar to the results seen with phosphatidylserine externalization, inhibition of p53 or caspases only partially inhibited chromatin condensation at 24 h. Cisplatin treatment of RPTCs resulted in a significant increase in the sub-G0/G1 peak, increasing DNA hypoploidy from 2 ± 1% in control cells to 42 ± 1% in cisplatin-treated cells (Fig. 7, A and B). PFT, DEVD-fmk, and ZVAD-fmk treatment of RPTCs before cisplatin exposure all resulted in approximately a 40% decrease DNA hypoploidy compared with RPTCs treated with cisplatin alone (Fig. 7C). Very little DNA hypoploidy was detected in RPTCs exposed to cisplatin for 12 h, supporting previous observations that this event occurs relatively late in apoptosis. These results suggest that approximately 50% of cisplatin-induced apoptosis measured in RPTCs at 24 h is independent of caspase 3, 8, and 9 and p53.

To further study the effect of p53 and caspase inhibitors on cisplatin-induced RPTC apoptosis we determined cell cycle distribution of RPTCs in the presence and absence of cisplatin. Control confluent monolayers of RPTCs were primarily composed of cells in the G1/G0 (60%) and G2/M (16%) phases, with the rest being either in S phase (varied from 5 to 10%).
or present to the left of the G\textsubscript{2}/G\textsubscript{0} peak (<10% with no definite peak) (Figs. 7, A and B, and 8A). Treatment of RPTCs with cisplatin for 24 h resulted in a reduction of cells in the G\textsubscript{2}/G\textsubscript{0} and G\textsubscript{2}/M phases (Fig. 8). No significant alterations in cell cycle were seen in RPTCs treated for 12 h (data not shown). The loss of cells from the G\textsubscript{2}/G\textsubscript{0} and G\textsubscript{2}/M phases was accompanied by the appearance of a sub-G\textsubscript{2}/G\textsubscript{1} peak (Fig. 7C). Pretreatment of RPTCs with either PFT or caspase inhibitors did not alter the loss cells from either the G\textsubscript{2}/G\textsubscript{1} or G\textsubscript{2}/M peaks. However, pretreatment of RPTCs with PFT or caspase inhibitors did decrease the amount of cells that detached from the plate compared with cells treated with cisplatin alone (Fig. 8C). Thus, although PFT, DEVD-fmk, or ZVAD-fmk do not protect a specific cell population from apoptosis they do decrease the overall amount of cell death and detachment. Therefore, inhibition of p53 and caspases inhibits cisplatin-induced apoptosis as determined by annexin V-FITC staining, chromatin condensation, caspase activation, DNA hypoploidy, and cell detachment. However, inhibition of p53 and caspases does not fully inhibit cisplatin-induced apoptosis, suggesting that a significant amount (50%) of apoptosis in these cells progresses independently of p53 or caspase 3, 8, and 9.

**Discussion**

Renal cell death is a consequence of cisplatin treatment during chemotherapy and is one of the major factors limiting its use (Blachley and Hill, 1981). Pathological examination of kidneys exposed to cisplatin in vivo demonstrates pathology to the S1 and S2 segments of the proximal tubule characteristic of both apoptosis and oncosis (Chopra et al., 1982; Safirstein et al., 1984; Schumer et al., 1992). This is similar to results with cisplatin in vitro where apoptosis or oncosis is dependent on the concentration and length of exposure (Lieberthal et al., 1996; Lau, 1999). In vitro, caspase 3 is
activated during cisplatin-induced renal cell apoptosis (Lau, 1999), but the mechanisms responsible for caspase 3 activation are not fully understood. The goal of this article was to determine the role of p53, mitochondria, and caspase 8 and 9 in cisplatin-induced activation of caspase 3 and renal cell apoptosis.

Cisplatin-induced apoptosis was concentration- and time-dependent with concentrations of 100 μM or less inducing apoptosis exclusively, and those higher than 200 μM inducing oncosis. Apoptosis was confirmed by cell morphology, annexin V labeling, caspase 3 activation, chromatin condensation, and DNA hypoploidy. Thus, similar to previously reported studies using mouse RPTCs or renal cell lines (Lieberthal et al., 1996; Lau, 1999), primary cultures of rabbit RPTCs treated with low concentrations of cisplatin undergo apoptosis.

Cisplatin treatment of RPTCs induced caspase 3 activation with initial increases observed at 8 h and a 54-fold increase observed at 24 h. Caspase 3 activity was not detected at either 2 or 4 h. The increase in caspase 3 activity at 8 h preceded the first detectable increases in phosphatidyserine externalization by at least 4 h. In contrast to caspase 3, caspase 8 or 9 activity did not increase during the 24-h exposure period. Furthermore, pretreatment with the general pan caspase inhibitor ZVAD-fmk resulted in an equal amount of protection against apoptosis as RPTCs treated with the caspase 3 inhibitor DEVD-fmk. These results suggest that cisplatin-induced RPTC apoptosis is caspase 8- and 9-independent and, in part, caspase 3-dependent (see below).

In contrast to oncosis, apoptosis is an ATP-dependent process (Levin et al., 1999). We addressed the role of the mitochondria in cisplatin-induced apoptosis by measuring ATP and GTP concentrations and mitochondrial membrane potential. Neither ATP, GTP, nor mitochondrial membrane potential decreased during cisplatin treatment. The maintenance of ATP throughout cisplatin treatment agrees with studies demonstrating that neither cellular ATP nor energetics decreased during cisplatin-induced U937 cellular apoptosis or

---

**Fig. 8.** Effect of cisplatin on RPTC cell cycle and detachment. Confluent RPTC monolayers were treated either with PFT (10 μM), DEVD-fmk (50 μM), ZVAD-fmk (50 μM), or solvent control (DMSO at <0.1% [v/v]) before treatment with cisplatin (Cis, 50 μM). After 24 h the media were removed for cell counting, and RPTCs on the monolayers were isolated, fixed in ethanol, and stained with propidium iodide for cell cycle analysis using flow cytometry. A, effect of PFT, DEVD-fmk, and ZVAD-fmk on cisplatin-induced changes in the amount of cells in the G1/G0 phase of the cell cycle. B, effect of PFT, DEVD-fmk, and ZVAD-fmk on cisplatin-induced changes in the amount of cells in the G2/M phase of the cell cycle. C, effect of PFT, DEVD-fmk, and ZVAD-fmk on cisplatin-induced detachment of RPTCs. Data are represented as the mean ± S.E.M. of at least three separate experiments. Means with different subscripts are significantly different from one another (P < 0.05).
apoptosis induced by H₂O₂ in bovine endothelial cells (Lelli et al., 1998; Liang and Ulliyatt, 1998). Our data differ from studies demonstrating that GTP decreases during apoptosis induced by the anticancer agent tiazofurin or ischemia (Vitale et al., 1997; Dagher and Plotkin, 2000). Reasons for this discrepancy could be differences in the stimuli of apoptosis. Tiazofurin induces apoptosis by inhibition of inosine 5'-monophosphate dehydrogenase, whereas ischemia induces cell death by multiple mechanisms, including mitochondrial dysfunction (Vitale et al., 1997; Dagher and Plotkin, 2000). In contrast, cisplatin-induced apoptosis is believed to be the result of DNA damage (Wetzel and Berberich, 2001). The mechanisms resulting in increased GTP levels after 12 h of cisplatin treatment are unknown. However, this increase is small and occurs late in the initiation of apoptosis. Thus, data presented in this study support the conclusion that cisplatin activates caspase 3 by a mechanism independent of mitochondrial dysfunction.

p53 is a tumor suppressor molecule that increases in content and translocates to the nucleus in response to DNA damage (Bennet, 1999). Treatment of RPTCs with cisplatin resulted in increased p53 content and nuclear localization as early as 4 h after treatment. The nuclear localization of p53 at 4 h preceded increases in caspase 3 activity and chromatin condensation by at least 4 h. Experiments using the p53 inhibitor PFT revealed that PFT decreased nuclear p53 accumulation, and prevented caspase 3 activation after 12 h of cisplatin treatment. In contrast, neither DEVD-fmk nor ZVAD-fmk had any effect on p53 translocation. These results demonstrate that p53 is needed, in part, for cisplatin-induced apoptosis and that p53 nuclear localization precedes increases in caspase 3 activity.

The use of the caspase inhibitors and the PFT inhibitor revealed a specific sequence of events that occurs during cisplatin-induced RPTC apoptosis (Fig. 9). The sequence of events is as follows: p53 nuclear localization, increased caspase 3 activity and chromatin condensation, and annexin V and DNA hypoploidy. In many ways these results are predictable from previous reports in the literature (Lieberthal et al., 1996; Lau, 1999; Zhan et al., 1999; Schuler et al., 2000). However, these events occurred in the absence of increases in caspase 8 or 9 activity or mitochondrial dysfunction. Furthermore, complete inhibition of caspase 3 with DEVD-fmk and caspases in general with ZVAD-fmk did not completely inhibit annexin V labeling, DNA hypoploidy, and chromatin condensation. Consequently, it seems there is a second caspase-independent cell death pathway that induces many of the characteristics of apoptosis. Regardless of the mechanisms involved, data from this study demonstrate that only about 50% of cisplatin-induced RPTC apoptosis is dependent on caspase activation. It will be interesting to determine whether these same results are seen in other cell types, especially fibroblasts and tumor cells.

Pretreatment of RPTCs with the caspase inhibitors completely inhibited phosphatidyserine externalization in cisplatin-treated RPTCs at 12 h but only partially inhibited phosphatidyserine externalization at 24 h. Reasons for this are not known but phosphatidyserine externalization can occur independently and dependently of caspases (Vanags et al., 1996; Kagan et al., 2000). Phosphatidyserine externalization is controlled by the enzymes scramblase and translocase, and caspases do not cleave these two enzymes (Vanags et al., 1996; Kagan et al., 2000). However, caspases may indirectly control their activity by cleaving cytoskeleton proteins attached to the phospholipids, including focal adhesion kinases and the actin-capping protein α-adducin (van De Water et al., 1999, 2000). Data in this article indicate that cisplatin-induced phosphatidyserine externalization is initially mediated by caspases. However, as the length of cisplatin exposure increases other factors independent of caspases control phosphatidyserine externalization. These factors include increases in intracellular Ca²⁺, which has been shown to alter scramblase and translocase activity (Vanags et al., 1996; Kagan et al., 2000).

Similar to results with phosphatidyserine externalization, caspase inhibitors were unable to fully inhibit cisplatin-induced DNA hypoploidy, chromatin condensation, and cellular detachment after 24 h. The inability of caspase inhibitors to fully inhibit DNA hypoploidy and chromatin condensation may be the result of cisplatin directly causing DNA crosslinking, leading to DNA damage irrespective of caspase activation of poly(ADP-ribose) polymerase and other nucleases (Damia et al., 2001; Wetzel and Berberich, 2001).

Pretreatment of RPTCs with PFT fully inhibited cisplatin-induced p53 nuclear translocation, caspase 3 activation, and phosphatidyserine externalization after 12 h. In contrast, at 24 h PFT only afforded a partial protection against cisplatin-induced apoptosis. Readdition of PFT 12 h after the initial cisplatin treatment, or increasing the concentration of PFT to 30 µM did not result in decreased caspase 3 activity at 24 h (data not shown). The effect of PFT on p53 nuclear translocation in other cells is reversible, and PFT is ineffective if added after the initial stimuli of injury (Komarov et al., 2000). Thus, the ability of PFT to inhibit cisplatin induced increases in caspase 3 activity and annexin V binding at 12 h...
but only partially inhibitory after 24 h could be a result of the reversibility of this compound.

Although data in this study firmly place activation of p53 upstream of caspase 3 it is doubtful that p53 is directly activating caspase 3. Other studies have demonstrated that p53 activates caspase 3 by a variety of mechanisms, including the activation of the proapoptotic proteins Bid and Bax and stimulation of cytochrome c release, all of which increase caspase 3, 8, and 9 activities in numerous cell types in response to chemical-induced apoptosis (Schuler et al., 2000; Chen et al., 2001). The data in this article do not demonstrate a role for caspase 8 or 9 as intermediaries between p53 and caspase 3. However, data in this article do not exclude roles for cytochrome c and Bax. Finally, caspase 3 may be activated by caspase 12, which itself is activated by the release of endoplasmic reticulum Ca²⁺, independently of caspase 8 or 9 (Nakagawa et al., 2000). A role for caspase 12 cannot be ruled out by the use of the inhibitor ZVAD-fmk because the specificity of this compound for caspase 12 is not known.

In conclusion, we have demonstrated the novel observation that cisplatin-induced renal cell apoptosis is mediated by p53 activation of caspase 3 independently of either caspase 8 or 9. This signaling pathway has a major role in the initial RPT-apoptosis, accounting for at least 50% of apoptosis. As cell death progresses a parallel and distinct mechanism results in an apoptotic-like cell death that has similar morphological and biochemical characteristics to apoptosis but is not inhibited by inhibitors of either p53 or caspase 3, 8, and 9.

Acknowledgments
We acknowledge Drs. Grazyna Nowak and Paul A. Nony for help with confocal microscopy, flow cytometry, and the caspase assays. We also thank Dr. Mike Aeol for performing the ATP and GTP assays.

References


