Cisplatin is a chemotherapeutic drug whose cytotoxicity is key to its therapeutic and side effects. Nephrotoxicity, mainly due to renal tubular injury, poses its most important therapeutic limitation. Tubular necrosis is derived from epithelial cell death by apoptosis and necrosis in the proximal and distal tubuli. The mode of cell death has been related to drug concentration, with necrosis occurring with high concentrations and apoptosis with lower concentrations. To fully understand the toxic effects of cisplatin to potentially improve its pharmaco-toxicological profile, it is necessary to unravel the cellular events and signaling pathways implicated in the appearance of both modes of cell death. We used cultured human lymphoma and renal tubule cells to investigate the biochemical and phenotypic characteristics of the death mode induced by increasing concentrations of cisplatin. Our results indicate that pronecrotic concentrations of cisplatin early activate the apoptotic machinery, which is in turn directly blocked by cisplatin at the level of effector caspases. Aborted apoptosis induces a death phenotype lacking some typical characteristics of this process, which more closely resembles necrosis. Furthermore, unidentified Bcl-2- and mitochondria-independent pathways are induced by pronecrotic and not by proapoptotic concentrations of cisplatin. Cisplatin-induced cell necrosis is the result of an aborted apoptosis at the level of effector caspases. Yet, Bcl-2-independent effects lead to cell death, which may pose potential targets for pharmacological intervention aimed at reducing cisplatin nephrotoxicity.

Key Words: cisplatin; apoptosis; necrosis; caspase inhibition; Bcl-2; cell cycle.
proximal tubule might be more easily subject to necrotic concentrations of cisplatin than the distal tubule, which might explain why necrosis is mainly found in the former.

The mode of cell death is not a trivial issue for anticancer drug utility. On the contrary, it is in the core of the therapeutic and side effects. On the one hand, apoptosis and necrosis exert different effects on the surrounding tissue and the whole organism. The main reason is that necrosis activates an inflammatory and immune response, whereas apoptosis does not (Doonan and Cotter, 2008; Mills et al., 1999). The influence of inflammation on the antitumor effect is uncertain, whereas it is mostly detrimental when considering its role in side effects. On the other hand, the underlying mechanisms and signaling pathways leading to cell death by apoptosis and necrosis may differ substantially or share some common elements, depending on the stimulus and the scenario (Golstein and Kroemer, 2007; Harwood et al., 2005; Vandenabeele et al., 2010). Until recently, necrosis was considered a passive mode of cell death. However, it is now understood that, in some instances, it results from the activation of an extant cellular programme still present in many cell types, which is overcome and normally inhibited by the evolved apoptotic machinery (Golstein and Kroemer, 2007; Vandenabeele et al., 2010). Furthermore, the mode of cell death caused by cytotoxic agents may be switched from one type to another by experimental or pathophysiological determinants, such as oxygen availability or the presence of exogenous or endogenous modulators of cell death pathways (Melino et al., 2000; Nicotera and Melino, 2004; Troyano et al., 2003; Zhang et al., 2008b). In fact, apoptosis is highly dependent on ATP availability (Chiarugi, 2005; Nicotera et al., 1998), which in turn strongly relies on a correct mitochondrial function and respiration (Ankarcrona et al., 1995; Hatefi, 1993).

Knowledge of the mechanisms and determinants leading to one form of cell death or another induced by anticancer platinated drugs may unravel new targets for the improvement of their pharmaco-toxicological profile, specifically by ameliorating their nephrotoxicity. On these grounds, the aim of this work was to gain further insight into the necrotic mechanisms underlying cisplatin’s cytotoxicity, namely to study the mechanisms through which high concentrations of cisplatin cause cell necrosis and not apoptosis, as lower concentrations do. We show that necrotic concentrations of the drug activate the apoptotic program, which is aborted at the level of effector caspases resulting in a necrotic-like death phenotype.

**MATERIALS AND METHODS**

**Reagents, cells, and protocols.** Where not otherwise indicated, reagents were purchased from Sigma (Madrid, Spain). A human proximal tubule cell line (HK2; from the ATCC collection; LGC Standards, Barcelona, Spain) and human lymphoma Jurkat T cells were used for the study. Jurkat cell Bcl-2 stable transfectants, and their controls carrying the empty vector (pcDNA3.0) have been already described (Lopez-Hernandez et al., 2004). HK2 and Jurkat cells were treated for 4, 8, and 18 h with cisplatin (0–1000µM). Jurkat cells, and Bcl-2 and pcDNA3.0 stable transfectants, were treated for 18 h with cisplatin (0–1000µM). In another set of experiments, Jurkat cells were treated for 3 h with cisplatin (0–1000µM) in the presence or absence of 0.25 µg/ml anti-Fas antibody (clone CH11, Millipore, Madrid, Spain) or 100µM cycloheximide. During the treatments, cells were photographed under live-cell, light microscope (Axiovert 200M, Zeiss; and Nikon Eclipse TS100, Barcelona, Spain) for visual inspection. For some visual experiments, HK2 cells were transfected with pEGFP with the aid of JetPEI (Polyplus Transfection, New York, NY), treated with cisplatin, and photographed under fluorescence microscopy (Axiovert 200M, Zeiss). In some experiments, cells were fixed in ice-cold 70% ethanol overnight for cell cycle analysis. In others, protein extracts were obtained in extraction buffer A (25mM PIPES [pH 7], 25mM KCl, 5mM EGTA, 0.5% NP-40, and a mixture of protease inhibitors consisting of 1µM phenylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) or extraction buffer B (120mM KCl, 10mM NaCl, 1mM KH2PO4, 20mM 4-[(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid [HEPES]-Tris [pH 7.1], 2mM succinate, 100 µg/ml digitonin, and a mixture of protease inhibitors consisting of 1µM phenylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin). Protein content was determined with a commercial kit (Bio-Rad, Madrid, Spain) based on the Lowry method, according to the manufacturer’s instructions. Extracts obtained with buffer A were used for caspase activity assays, Western blot studies, and DNA fragmentation measurement. Extracts obtained with buffer B were used for analyzing mitochondrial release of cytochrome c by Western blot.

**MTT assay.** Viable cell number was determined by incubating cell cultures with 0.5 µg/ml 3-[4,5-di methylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) for 4 h. Then, 10% SDS in 0.1M HCl was added 1:1 (vol/vol) and left overnight at 37°C. Finally, absorbance was measured at 570 nm.

**Caspase activity.** Twenty micrograms of protein from cell extracts were incubated with 100 µl reaction buffer (50mM HEPES, pH 7.4; 100mM NaCl; 1mM EDTA; 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; 10% sucrose; and 5mM DTT) containing 100 µM of the fluorogenic caspase substrate rhodamine 110, bis-(N-CBZ-t-aspartyl-t-glutamyl-t-valyl-t-aspartic acid amide (Z-DEVD)2Rh110; Anaspec, Fremont, CA). Incubation was performed at 37°C for 30 min in a Fluoroskan Ascent FL fluorometer (Thermo, Rockford, IL), and fluorescence intensity was measured every 3 min at 538 nm, upon excitation at 485 nm. Caspase activity was calculated by determining the slope of the linear relationship between fluorescence intensity and time.

**In vitro inhibition of DEVDase activity.** Jurkat cells were treated for 3 h with vehicle (control) or a mix of 0.25 µg/ml anti-Fas antibody (Upstate-Millipore, Madrid, Spain) plus 100µM cycloheximide. Cell extracts were obtained in extraction buffer A (see above) and named “nonactivated” and “activated,” respectively. Extracts were then incubated in vitro with increasing concentrations of cisplatin (0–500µM) for 15 min at 37°C. Immediately after that, DEVase activity was measured in an aliquot from each sample.

**Cellular ATP content.** Cellular ATP content was determined with the commercial Bioluminescent Somatic Cell Assay kit (Sigma-Aldrich) according to the manufacturer’s instructions.

**Western blot.** Thirty micrograms from each cell extract were separated by acrylamide electrophoresis. Proteins were transferred to an Immobilon-P Transfer Membrane (Millipore) and incubated with antibodies against cleaved caspases 3 and 9 (Cell Signaling, Danvers, MA), cleaved Bid (tBid, Cell Signaling), cytochrome c (BD Pharmingen, Madrid, Spain) or Erk-1 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescent detection (Immobilon Western Chemiluminescent HRP Substrate kit, Millipore) with photographic films (Kodak, Madrid, Spain).

**DNA fragmentation.** DNA fragmentation was determined by ELISA with the commercial kit Cell Death Detection Plus (Roche Diagnostics, Barcelona,
Spain) according to the manufacturer’s instructions. Ten micrograms of protein were used from each cell extract.

**Cell viability by propidium iodide exclusion.** Cells were incubated for 15 min with 50 μg/ml propidium iodide (PI, Sigma Aldrich) in the dark. Immediately, cells were analyzed by flow cytometry for PI fluorescence (FLH-2) in a FACScalibur cytometer (BD Pharmingen).

**Phosphatidylserine externalization.** Cells were harvested by centrifugation and double stained with reaction buffer (10mM HEPES, pH 7; 140mM NaCl; 2.5mM CaCl2) containing 5 μl annexin V-APC (BD Pharmigen) and 50 μg/ml PI (Sigma Aldrich) for 15 min at room temperature. Cell population analysis for annexin V (FLH-4) and PI (FLH-2) was performed by flow cytometry in a FACScalibur cytometer (BD Pharmingen).

**Cell cycle analysis.** Thirty minutes before harvest, 10μM 5-bromo-2-deoxyuridine (BrdU, Sigma Aldrich) was added to the cell culture. Subsequently, cells were fixed in ice-cold 70% ethanol overnight and stained with 0.01 mg/ml PI (Sigma Aldrich) and 0.01 mg/ml anti-BrdU-FITC antibody (Abcam, Cambridge, United Kingdom). Then, 0.01 mg/ml PI was added to cell suspensions and 1 hour later they were analyzed by flow cytometry (FACScalibur, BD Pharmingen) with the CellQuest software. Singlet discrimination was done by means of an FL2-A versus FL2-W representation.

**Statistical analysis.** Data are expressed as the average ± SD of the indicated number of experiments (n). Statistical analysis was performed with the SPSS 17 software (SPSS Inc., Chicago, IL) using the one-way ANOVA analysis followed by the post hoc Scheffe’s test. p < 0.05 was considered statistically significant.

**RESULTS**

**Cell Death Phenotype Induced by Increasing Concentrations of Cisplatin**

HK2 and Jurkat cell death caused by cisplatin was dependent on the concentration of the drug (Fig. 1A). Both cell lines showed a similar concentration-effect relationship, as did other renal and cancer cell lines of different species tested in our laboratory (data not shown). For clarity’s sake, we included in the figures images from HK2 cells, in which the cell morphology is better appreciated. For all cell lines, it is true that concentrations of cisplatin up to 30μM caused mainly a mode of cell death with clear morphological and biochemical characteristics of apoptosis, whereas the commonest phenotype observed with concentrations equal or higher than 300μM had clearly lost many of these characteristics and more closely resembled those of necrosis. With 100μM cisplatin, both apoptotic and necrotic looking cells could be observed to coexist. As Supplementary material, we included 20-h movies of HK2 cultures treated with different concentrations of cisplatin (or DMSO as control), where the evolution of cell death can be seen with detail. Figure 1B shows the death phenotype of HK2 cells treated with 30 and 1000μM cisplatin for 18 h, where typical dismantling into apoptotic bodies is seen with 30 and not with 1000μM. This was also observed with Jurkat cells (data not shown).

Figure 1C shows light microscopy photographs of HK2 cells treated with 0, 30, 100, and 300μM cisplatin for 8 and 18 h. After 4 h of treatment, no morphological alterations were obvious with any of the concentrations (data not shown; see live-cell microscopy in Supplementary materials). After 8 h, cells treated with low concentrations of cisplatin started showing evidence of an apoptotic process, such as membrane blebbing and shrinkage, which lead to a complete cell dismantling into apoptotic bodies by 18 h. Along with the increment in cisplatin concentration, morphological features of apoptosis became less frequent and a necrotic-like phenotype took over. With high concentrations (≥300μM), cells died almost unanimously to necrosis. Cell swelling occurred as early as 8 h after the beginning of exposure. In both extremes of the concentration range, apoptosis and necrosis seemed to be the only mode of cell death. However, with intermediate ones, coexistence of both phenotypes was detected. This is most evident with the 100μM concentration which seemed to pose an inflection point.

**Biochemical Characteristics of Different Death Phenotypes Induced by Cisplatin**

In agreement with the phenotype, both HK2 and Jurkat cells displayed typical biochemical patterns of apoptosis and necrosis after 18 h of treatment with proapoptotic (≤100μM) and pronecrotic (>100μM) concentrations of cisplatin, respectively. Indeed, in both cell lines, DEVDase (caspase) activity (Fig. 2A) and DNA fragmentation (Fig. 2B) showed patterns associated to these two death phenotypes in a manner dependent on the concentration of cisplatin. Jurkat cells showed a perfect correlation between cisplatin concentration, mode of cell death and cytochrome c release, caspase 3 activation, and bid activation (Fig. 2D). Caspase 3 activation behaved in the same way in HK2 cells, although all concentrations of cisplatin-induced cytochrome c release from mitochondria (even pronecrotic ones; Fig. 2D). The increase in DEVDase activity produced by proapoptotic concentrations of cisplatin in HK2 cells was completely prevented by a cocktail of inhibitors of apoptotic proteases (caspases, cathepsins, and calpains; Fig. 2E). This cocktail also inhibited the DNA fragmentation induced proapoptotic concentrations of cisplatin, but it was incapable of preventing the DNA fragmentation induced by pronecrotic ones, including 100μM (Fig. 2F). Furthermore, after only 8 h of treatment with 30 and 300μM cisplatin, Jurkat cells showed some DNA fragmentation in the absence of DEVDase activity (Figs. 3B and 3C).

Interestingly, at early time points, high (necrotic) concentrations of the drug gave rise to apoptotic signs of cell death between 4 and 8 h after the onset of the treatment. These signs included cytochrome c release, caspase 3 activation, caspase (DEVDase) activity, and internucleosomal DNA fragmentation (Figs. 3A–C), despite being incapable of culminating the process into hard apoptotic endpoints and into a typical apoptotic morphology. These results indicate that pronecrotic
concentrations of cisplatin activate the apoptotic machinery, which notwithstanding cannot progress or is aborted before it completes the apoptotic program.

**Energetic Status: Cellular ATP Reserve**

Data on the cellular content of ATP showed that after 4 h of treatment, the cell’s ATP pool was not modified in HK2 cells. The ATP pool in Jurkat cells was modified only with exposure to 1000 μM cisplatin, as no differences were observed at all the other concentrations that were tested. After 8 h, the ATP pool in HK2 cells was still intact with all cisplatin concentrations (Fig. 3D). However, a dramatic fall in the cell’s ATP reserve was observed in Jurkat cells with high concentrations of cisplatin (≥100μM). After 18 h, there was a concentration-dependent fall in ATP reserve in both cell lines, which was more pronounced in Jurkat cells. Disconnection between ATP level and evidence of apoptotic signs was evident for pronecrotic concentrations of cisplatin but especially for the concentration of 100μM (Fig. 2C). In both cell lines, the 100μM concentration caused an ATP fall very similar to that

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**FIG. 1.** Antiproliferative effect of cisplatin and morphological phenotypes induced by cisplatin. (A) MTT-based proliferation/viability, concentration-effect profile of Jurkat and HK2 cells treated for 24 h with cisplatin (0–1000μM). Data represent average ± SD of n = 3. * p < 0.05 with respect to 0μM cisplatin. (B) Representative photographs (n = 3) of HK2 cells transiently transfected with EGFP, treated for 20 h with 0 (control), 30 or 1000μM cisplatin, and photographed under fluorescence microscopy (magnification: 400×). Cells treated with 30μM cisplatin show a typical apoptotic phenotype, whereas those treated with 1000μM lack morphological evidence of apoptosis. (C) Representative light microscopy photographs (n = 4) of HK2 cells treated with 0 (control), 30, 100, and 300μM cisplatin for 8 and 18 h. White arrows: apoptotic cells; Black arrows: necrotic cells. Twenty hour time course photographic movies of cells treated with 0 through 1000μM cisplatin are provided as Supplementary material.
caused by 300 and 1000 μM, despite activating several apoptotic mediators and producing the appearance of different apoptotic characteristics. It is known that apoptosis is an ATP requiring process, especially in the initiation phase (Chiarugi, 2005). Concentrations of cisplatin (100 and 300 μM) that depleted ATP by 8 h in Jurkat cells induced clear apoptotic

FIG. 2. Biochemical characterization of cell death induced by cisplatin. HK2 and Jurkat cells were treated for 18 h with 0–1000 μM cisplatin. (A) DEVDase (caspase) activity measured in cell extracts. (B) Internucleosomal DNA fragmentation. (C) Cellular ATP content. (D) Representative images of Western blot analysis of cytochrome c release, activated caspase 3, full-length PARP (upper band) and a proteolytic fragment (lower band), Bid proteolytic activation (tBid), and Erk-1 (as a control). (E) DEVDase (caspase) activity in cell extracts from HK2 cells treated as before, in the presence or absence of a cocktail of caspase (25 μM Z-VAD-fmk, Calbiochem), cathepsin (10 μM CA-074, Sigma Aldrich), and calpain (10 μM E64D, Sigma Aldrich) inhibitors (PI). (F) Internucleosomal DNA fragmentation of cells treated as in E. Data represent the average ± SD of n = 3–4 independent experiments. AU, arbitrary units. *, p < 0.05 with respect to the same concentration of cisplatin in the control (-) group.
signs such as DEVDase activity and DNA fragmentation (Figs. 3B–D). Altogether, these results indicate that the ATP requirement for apoptotic events is limited to the first few hours of incubation with cisplatin (about the first 4 h). In both cell lines, ATP is maintained high in such a temporal frame, suggesting that other factors different from ATP depletion are responsible for the necrotic phenotype observed with high concentrations of cisplatin.

**FIG. 3.** Early biochemical parameters of HK2 and Jurkat cells treated with cisplatin. Both HK2 and Jurkat cells were treated with 0–1000 μM cisplatin for 4 and 8 h and, at the end, cytochrome c release, caspase 3 activation, and Erk-1 (as a control) were determined by Western blot (A); DEVDase (caspase) activity was measured in cell extracts (B); Internucleosomal DNA fragmentation was determined (C); and Cell ATP content was quantified (D). Data represent the average ± SD of n = 3 independent experiments. AU, arbitrary units. * and **, p < 0.05 with respect to 0 μM cisplatin in its group.
Stable overexpression of the antiapoptotic protein Bcl-2 protected Jurkat cells from the apoptotic cell death induced by low concentrations of cisplatin but not from the nonapoptotic (necrotic) death caused by high concentrations (≥100μM) of this drug. However, Bcl-2 did not inhibit the cell cycle arrest induced by cisplatin. As depicted in Figure 4A, the number of metabolically active cells remaining after treatment for 18 h with 10μM cisplatin was almost identical to the number of cells at the beginning of the experiment (18 h before t = 0). The number of dead cells (i.e., propidium iodide positive; PI+) after treatment with 10μM cisplatin was statistically indistinguishable from that in the control condition (Fig. 4B). Furthermore, as shown in Figures 4C and 4D, 10μM cisplatin induced a marked cell cycle arrest in early S phase, which almost fully explains its antiproliferative effect. Overexpression of Bcl-2 did not modify the number of living cells after treatment with 10μM cisplatin, indicating that Bcl-2 has no effect on cell cycle arrest. This was corroborated by the results shown in Figures 4C and 4D on the distribution of cells along the cell cycle phases.

The 30μM concentration induced net cellular death because the number of metabolically active cells (Fig. 4A) after 18 h of treatment was lower than at the beginning of the experiment (t = 0). Indeed, the number of PI positive cells escalated to 60%, which accounted for most of the antiproliferative effect (Fig. 4B). Remaining cells were stagnated in S phase (Figs. 4C and 4D). Overexpression of Bcl-2 prevented cell death but not cell cycle arrest. As such, the number of Bcl-2 overexpressing cells treated with 30μM cisplatin was the same as at the beginning of the experiment (t = 0). For concentrations of 100μM and over, further net death was induced, which could not be protected by Bcl-2. Only with 100μM was Bcl-2 capable of exerting modest protection, reinforcing the idea that this is a transitory concentration between apoptosis and necrosis. The cell death caused by necrotic concentrations of cisplatin was not only independent of Bcl-2 but also apparently from mitochondrial damage. As depicted in Figure 4F, Bcl-2 overexpression prevented cytochrome c release induced by all concentrations of cisplatin, despite being incapable of inhibiting necrotic cell death. DNA fragmentation appears to require mitochondrial injury because Bcl-2 prevented its appearance with apoptotic and necrotic stimulation (Fig. 4E).

Our results also show that proapoptotic concentrations of cisplatin did not cause cell death in nonproliferating cells, whereas pronecrotic ones acted identically on proliferating and nonproliferating cells. Cessation of proliferation was induced by serum deprivation for 3 days. Figure 5A shows the proliferating profiles during 4 days of HK2 cells in medium containing 10% serum and 0% serum. To discard the effect of serum deprivation per se on cisplatin’s effect, we also implemented a third group of HK2 cultured for 3 days in 10% serum-containing medium and then for another 24 h (the period of treatment with cisplatin) in serum-deprived medium. As depicted in Figure 5A, the black arrow in Figure 5A indicates the moment when, in another set of experiments, cisplatin treatment was initiated. The effect of cisplatin on these three groups of cells is shown in Figure 5B. It can be appreciated that cisplatin did not cause cell death in nonproliferating, serum-deprived cells, which was not due to serum deprival but to the quiescent status, as demonstrated by the effect of 30μM cisplatin in serum-deprived cells previously maintained in growing conditions with normal (10% serum) medium for 3 days. Hundred micromolar cisplatin, as well as higher concentrations, exerted similar effects in all three groups, independently of the proliferation status. Precisely, 100μM cisplatin caused about a 50% loss in the number of metabolically active cells (compared with the untreated condition), in all groups.

In order to study whether pronecrotic concentrations of cisplatin interfere with the execution of apoptosis, which might explain the absence of a typical apoptotic phenotype, we decided to incubate Jurkat cells with an extrinsic and an intrinsic inducer of apoptosis (anti-Fas antibody and cycloheximide, respectively), in the presence of increasing concentrations of cisplatin (0 through 1000μM). We used this system because both anti-Fas and cycloheximide induced clear signs of apoptosis after only 3 h of treatment in Jurkat cells. In this time frame, cisplatin did not cause detectable biochemical or morphological signs of apoptosis, per se. However, as shown in Figures 6 and 7, cisplatin interfered with the correct execution of some aspects of the apoptotic program induced by Fas stimulation or treatment with cycloheximide, resulting in an altered phenotype. Precisely, the increase in DEVDase (caspase) activity induced by anti-Fas or cycloheximide was inhibited in a concentration-dependent manner by cisplatin (Fig. 6A). The inhibition of caspase activity did not seem to result from interference with the activation of effector caspases because neither cytochrome c release from mitochondria nor initiator caspase 9 or executor caspase 3 proteolytic activation were affected by increasing concentrations of cisplatin, in Jurkat cells (Fig. 6B). Only the highest concentration used (1000μM) had some uncertain effects, whereas lower concentrations were ineffective. However, the inhibition in caspase activity was the result of direct action of cisplatin on active caspases because incubation of cell extracts from cells previously activated for apoptosis (bearing high caspase activity) with increasing concentrations of cisplatin (0–1000μM) inhibited DEVDase activity in a concentration-dependent manner (Fig. 6C). Higher concentrations of cisplatin were needed in vitro (with respect to in vivo treatments) in order to achieve the same level of caspase inhibition. This was
FIG. 4. Effect of Bcl-2 on cisplatin-induced cell death types. Jurkat cells stably overexpressing the antiapoptotic protein Bcl-2, and control cells stably transfected with the empty vector (pcDNA) were treated for 18 h with 0–1000 μM cisplatin. (A) The effect of cisplatin on cell proliferation/viability was studied by the MTT method. (B) The percentage of viable cells was also determined by PI exclusion. (C and D) The distribution of cells among the cell cycle phases was determined by staining DNA with PI and measuring BrdU incorporation simultaneously, through the use of flow cytometry. (E) DNA fragmentation. (F) Representative images of Western blot showing cytochrome c release and Erk-1 (as a control) in cells treated as indicated above but for 8 h instead of 18 h. As positive controls in some types of experiments, cells were also treated with anti-Fas antibody (α-Fas) or 100 μM cycloheximide (Chx). CISP, cisplatin; data represent the average ± SD of n = 3–4 independent experiments. AU, arbitrary units. • and •, p < 0.05 with respect to 0 μM cisplatin in the same group. †, p < 0.05 with respect to the same concentration of cisplatin in the control pcDNA group.
probably due to the concentration of cisplatin inside the cells, resulting in higher intracellular concentrations, as described previously (Ghezzi et al., 2004).

Clearly defined endpoints for apoptosis induced by anti-Fas or cycloheximide, such as phosphatidylserine externalization and internucleosomal DNA fragmentation, were not modified by cisplatin, at any of the concentrations used (Figs. 7A and 7B). Both events are largely independent from executor caspases, as demonstrated by our present results (Figs. 2F and 6) and elsewhere (Lopez-Hernandez et al., 2003). However, cell dismantling into apoptotic bodies, a process dependent on executor caspases (Lopez-Hernandez et al., 2003, 2004), was clearly handicapped in Jurkat cells treated with anti-Fas or cycloheximide and coincubated with pronecrotic concentrations of cisplatin but not in cells coincubated with proapoptotic concentrations of cisplatin (Fig. 7C). Although some cells splitting into apoptotic bodies could still be observed in cells coincubated with 300μM cisplatin, these apoptotic bodies are fewer in number and larger in size (white arrows); in addition, many cells showed a different phenotype characterized by compacted intracellular material and shrunken nuclei in one corpse (black arrows). This indicates that pronecrotic concentrations of cisplatin interfere with the process of cell dismantling into apoptotic bodies.

**DISCUSSION AND CONCLUSIONS**

The cytotoxicity of the anticancer drug cisplatin is at crossroads of its therapeutic action and its side effects. As indicated above, both necrosis and apoptosis have been detected in tubular cells after treatment with cisplatin (Ferrer et al., 2003; Shibuya et al., 2003). Thus, unraveling the mechanisms leading to the appearance of both death phenotypes has great clinical interest. A first question to answer is whether apoptosis and necrosis are two facets or epiphenomenons of the same process, which bear a common axis susceptible of turning into a therapeutic target; or, on the contrary, apoptosis and necrosis induced by cisplatin are completely different processes that should be considered separately. Furthermore, as opposed to apoptosis, necrosis leads to the activation of an inflammatory response. Inflammation exerts an uncertain role in the antitumor effect of cisplatin, which might be beneficial under certain circumstances (Proskuryakov and Gabai, 2010). However, inflammation (Faubel et al., 2007; Pabla and Dong, 2008; Pan et al., 2009; Ramesh and Reeves, 2002; Zhang et al., 2007) and the innate immune response (Zhang et al., 2008a) amplify and mediate the associated renal damage. Our results show that, after 18 h, pronecrotic concentrations of cisplatin cause a death phenotype lacking apoptotic features. However, our results also show that at earlier time points (4 and 8 h of treatment), pronecrotic concentrations activate typical apoptotic pathways that, notwithstanding, are incapable of giving rise to an apoptotic mode of demise characterized by typical apoptotic end points, such as cell shrinkage and cell dismantling into membrane bound, tightly sealed apoptotic bodies.

In our model, pronecrotic concentrations of cisplatin interfere with the correct execution of apoptosis mainly downstream of mitochondria. This interference is due, at least partially, to the inhibition of the catalytic activity of executor caspases (responsible for the DEVDase activity), which leads to a skewed cell dismantling into apoptotic bodies. In fact, apoptotic body release, that is, individualization of membrane blebs, is an executor caspase-dependent process (Lopez-Hernandez et al., 2003, 2004). This may explain in part why cells treated with pronecrotic concentrations of cisplatin do not show the typical apoptotic phenotype, although they evidence some signs of apoptosis activation in early phases. According to these results, it might be hypothesized that blocking early proapoptotic signaling
FIG. 6. Effect of cisplatin on Fas- and CHX-induced caspase activity and caspase activation. Jurkat cells were treated for 3 h with vehicle, as control (-), 0.25 μg/ml anti-Fas antibody (α-Fas) or 100μM cycloheximide (CHX), in the presence of 0–1000μM cisplatin. At the end of the treatment, DEVDase (caspase) activity was measured in cell extracts (A), and cytochrome c release, proteolytic caspase 9 and caspase 3 activation, and Erk-1 (as a control) were determined by Western blot (B). p12, p15, p17, and p35 refer to the size of the detected proteins (in kDa). (C) Cell extracts from Jurkat cells treated with vehicle (nonactivated extract) or a mix of anti-Fas antibody plus CHX (preactivated extract) for 3 h were incubated in vitro with 0–5000μM cisplatin for 15 min at 37°C, and immediately, DEVDase activity was measured. Data represent the average ± SD of n = 3 independent experiments. AU, arbitrary units. *, p < 0.05 with respect to 0μM cisplatin in the same group. **, p < 0.05 with respect to the same concentration of cisplatin in the control group.
FIG. 7. Effect of cisplatin on Fas- and CHX-induced apoptotic end points. Jurkat cells treated for 3 h with vehicle, as control (-), 0.25 μg/ml anti-Fas antibody (α-Fas) or 100 μM cycloheximide (CHX), in the presence of 0–1000 μM, cisplatin were analyzed for phosphatidylserine (PS) externalization (A) and internucleosomal DNA fragmentation (B). There morphological phenotype was also visually inspected and photographed under light microscopy (400×; C). AU, arbitrary units PI; Data represent the average ± SD of n = 3 independent experiments. *, p < 0.05 with respect to the same concentration of cisplatin in the control group. White arrows: apoptotic cells; Black arrows: necrotic cells.
would prevent the lethal effect of both proapoptotic and pronecrotic concentrations of the drug. However, Bcl-2 overexpression inhibits the death of cells treated with proapoptotic but not with pronecrotic concentrations of the drug. This suggests that, in addition to interfering with the apoptotic machinery, pronecrotic concentrations of cisplatin activate other signaling pathways or cause additional cellular injury resulting in cell death, independently of the apoptotic programme and also independent of mitochondrial injury. Accordingly, these new targets must be identified for the design of new strategies aimed at better controlling cisplatin’s side effects. Interestingly, although a profound ATP depletion was observed after treatment with pronecrotic concentrations of cisplatin, this does not seem to be the cause of the aborted apoptosis because ATP-requiring apoptotic events are detected in early time points.

Jurkat cells stimulated for apoptosis with an activator of the extrinsic (anti-Fas antibody) or the intrinsic pathway (cycloheximide) undergo a rapid, effective, and massive rupture into apoptotic bodies. In the presence of pronecrotic concentrations of cisplatin, cell dismantling is partially or completely aborted in most cells, which acquire a death phenotype closely resembling that of Jurkat cells primed to apoptosis, in which apoptotic Na\(^+\) load is prevented (Bortner and Cidlowski, 2003). Experimental inhibition of Na\(^+\) load blocks cell shrinkage and cell dismantling into apoptotic bodies but, interestingly, it has no effect on the appearance of other characteristics of apoptosis, such as caspase activation, chromatin condensation, and DNA and nuclear fragmentation. This, to a certain extent, resembles the effect of pronecrotic concentrations of cisplatin observed in our cells, in which some caspase activation and DNA fragmentation are observed in the absence of cell shrinkage, membrane blebbing, and formation of apoptotic bodies. As such, channels and transporters for electrolytes involved in cell volume regulation and apoptotic volume decrease (Heimlich et al., 2004; Yu and Choi, 2000) might be targets of pronecrotic concentrations of cisplatin, which merits further investigation.

Our experiments also show that the cytotoxic effect of cisplatin is independent of its cytostatic action because apoptotic cell death can be inhibited (e.g., by Bcl-2 overexpression) without affecting cell cycle arrest. Because tumor cell proliferation rate is, in general terms, much higher than tubule cell proliferation (due to epithelium turnover or repair), this might be exploited for reducing cisplatin’s nephrotoxicity conserving its cytostatic effect. Furthermore, in agreement with a previous report, the apoptosis induced by cisplatin is dependent on the cell cycle. Indeed, cell death by apoptosis is significantly inhibited in nonproliferating cells (Fig. 5; Price et al., 2004). However, our study demonstrates that pronecrotic concentrations of cisplatin cause cell death identically in proliferating and nonproliferating cells (Fig. 5).

In conclusion, our results indicate that concentrations of cisplatin inducing a necrotic-like cell death mode are capable of activating the apoptotic machinery. However, they interfere with the execution phase by inhibiting effector caspases. In principle then, renal inhibition of apoptosis at the initial steps would help to control both the apoptotic and the necrotic epithelial death. Yet, pronecrotic concentrations of the drug also cause other cellular effects independent of the apoptotic machinery, which lead to cell death. The open question is how to inhibit necrotic cell death by both preventing the abortion of apoptosis and the onset of apoptotic programme-independent pathways or deadly injuries. Considering typical cisplatin dosage patterns, it is probable that tubule cells are only transiently subject to pronecrotic concentration of the drug. But even so, transient exposure to pronecrotic concentrations has been shown to commit cells to a necrotic mode of cell death (Lieberthal et al., 1996; Shino et al., 2003; Yano et al., 2007). As such, further investigation on the mechanisms leading to cellular necrosis might substantially help to improve the pharmaco-toxicological profile of platinum-based chemotherapeutic drugs.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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CISPLATIN-INDUCED NECROTIC CELL DEATH


