Protection of renal cells from cisplatin toxicity by cell cycle inhibitors

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Submitted 19 May 2003; accepted in final form 2 September 2003

Protection of renal cells from cisplatin toxicity by cell cycle inhibitors. Am J Physiol Renal Physiol 286: F378–F384, 2004. First published September 9, 2003; 10.1152/ajprenal.00192.2003.—The optimal use of cisplatin as a chemotherapeutic drug has been limited by its nephrotoxicity. Mice models have been used to study cisplatin-induced acute renal failure. After cisplatin administration, cells of the S3 segment in the renal proximal tubule are especially sensitive and undergo extensive necrosis in vivo. Similarly, cultured proximal tubule cells undergo apoptosis in vitro after cisplatin exposure. We have shown in vivo that kidney cells enter the cell cycle after cisplatin administration but that cell cycle-inhibitory proteins p21 and 14-3-3 are also upregulated. These proteins coordinate the cell cycle, and deletion of either of the genes resulted in increased nephrotoxicity in vivo or increased cell death in vitro after exposure to cisplatin. However, it was not known whether cell cycle inhibition before acute renal failure could protect from cisplatin-induced cell death, especially in cells with functional p21 and 14-3-3 genes. Using several cell cycle inhibitors, including a p21 adenovirus, and the drugs roscovitine and olomoucine, we have been able to completely protect a mouse kidney proximal tubule cell culture from cisplatin-induced apoptosis. The protection by p21 was independent of an effect on the cell cycle and was likely caused by selective inhibition of caspase-dependent and -independent cell death pathways in the cells.

Kidney failure; p21 cyclin kinase inhibitor; roscovitine

ONE OF THE MOST EFFECTIVE chemotherapeutic agents is the drug cisplatin, which is used in germ cell (e.g., testicular), head and neck, and cervical cancers (14, 28). Its usefulness is limited by its toxicity to noncancerous tissues, such as the proximal tubules of the kidney, which are especially sensitive (45). The mechanism underlying this selective toxicity in these cells is unclear, although it is likely that the anticancer activity is dependent on formation of DNA intrastrand cross-links (27). We have shown in vivo that cells in the kidney enter the cell cycle after cisplatin administration (32), and simultaneously genes for the p21 and 14-3-3ζ cell cycle inhibitors are upregulated (31, 33). Mice in which the p21 gene was deleted showed increased kidney cell cycle activity and increased cisplatin nephrotoxicity (32). Similarly, cultured cells having gene disruptions of p21 or 14-3-3ζ were more sensitive to cisplatin than were wild-type cells (31). These experiments showed that unregulated cell cycle activity in the kidney is a maladaptive reaction to acute renal failure. Although deletion of the p21 gene increased sensitivity to cisplatin, it is not clear that increased p21 would protect renal cells from injury. Here we report that cells cultured from mouse proximal tubules are completely protected from cisplatin toxicity by prior administration of an adenovirus vector directing the expression of p21.

Similarly, the cell cycle-inhibitory drug roscovitine is also effective in protecting kidney cells in vitro from cisplatin-induced apoptosis. We propose that increasing endogenous levels of p21 could be an effective therapy for cisplatin nephrotoxicity.

MATERIALS AND METHODS

Cell Culture

Mouse kidney proximal tubule (TKPTS) cells (10) were grown at 37°C in 5% CO2 in DMEM + Ham’s F-12 medium supplemented with 50 μM insulin and 7% fetal bovine serum (5). Cisplatin was added to cultures, where indicated, to a final concentration of 25 μM when cells were ~75% confluent, and the cells were grown for an additional 24 h. Adenovirus, either control expressing green fluorescent protein (GFP) or expressing human p21, was added to a final multiplicity of infection of 100. Roscovitine and olomoucine were dissolved in DMSO and added 4 h before cisplatin to final concentrations of 45 and 50 μM, respectively.

Except as noted, culture conditions followed the same schedule: cultured cells were maintained for 48 h after splitting before cisplatin was added as indicated, and cells were harvested 72 h after splitting. Adenovirus was added to the cells 24 h after splitting, and cell cycle inhibitors were added to the cells 44 h after splitting.

Cell Death Determination

Fluorescence-activated cell sorter analysis. Cells were harvested by trypsinization, pooled with the culture medium containing floating cells, and collected by centrifugation (10 min, 500 g). The cell pellets were resuspended in 0.3 ml of PBS containing 5 nM EDTA, and 0.7 ml of ethanol was added. Cells were incubated at 4°C for 16 h, collected by centrifugation (10 min, 2,000 rpm), and resuspended in 0.5 ml of PBS-EDTA. RNase A was added (50 μg, 10 mg/ml), and the suspension was incubated at 25°C for 30 min. Propidium iodide was added (450 μl, 100 μg/ml), and the samples were analyzed using a FACScalibur machine (Becton Dickinson). For each culture condition, ≥1 × 105 cells were analyzed. The percentage of cells in sub-G1/G0 (apoptotic fraction) (9), G1/G0, S, and G2/M phases was determined using a cell cycle analysis program (WinMDI 2.8).

Hoechst 33258 staining. Cells were grown on coverslips as described above. After times as indicated, cells were rinsed with PBS, fixed for 5 min with neutral-buffered formaldehyde, and stained with Hoechst 33258 (10 μg/ml in PBS) for 10 min. Under these conditions, Hoechst 33258 stains the nuclei of all cells blue when examined by fluorescence microscopy using a 4′,6-diamidino-2-phenylindole dihydrochloride filter. The nuclei of apoptotic cells display chromatid condensation and/or nuclear fragmentation, whereas normal cell nuclei are homogeneously stained. Apoptosis was assessed in ≥300 cells with the use of a fluorescent microscope with a 4′,6-diamidino-2-phenylindole dihydrochloride filter and a ×40 objective.

Light microscopy. Cells were photographed using Hoffman optics before they were harvested.
Western Blot Analysis

Proteins were isolated from 25-cm² culture flasks. Cell pellets were collected after trypsinization, and 0.5 ml of lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 1.5 mM MgCl₂, and 1 mM EDTA) was added. The samples were sonicated for 2 min, and cell debris was pelleted in an Eppendorf centrifuge at 4°C for 20 min. Protein concentration in the supernatant was determined using a Bio-Rad protein assay. Protein (25 µg) was electrophoresed using 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After they were blocked with milk, the membranes were incubated at 4°C for 16 h with primary antibody (Santa Cruz Biotechnology). Proteins bound to the antibody were visualized using enhanced chemiluminescence (Amersham).

Statistical Analysis

Probabilities were determined using Student’s t-test with two-tailed distribution.

RESULTS

Cisplatin-Induced Apoptosis in TKPTS Cells

Cells cultured for 72 h in the absence of cisplatin had a low level of apoptosis, 2.13 ± 0.83%, as determined by FACS analysis (Fig. 1A). Addition of 25 µM cisplatin for the last 24 h increased this level to 40.7 ± 5.2% \((P = 0.0023; \text{Fig. } 1E; \text{see Fig. } 4)\). Apoptosis was also confirmed by Hoechst nuclear staining (Fig. 2). Addition of adenovirus expressing GFP had no effect on the percentage of cells in apoptosis, with or without cisplatin (data not shown).

Effect of Cyclin-Dependent Kinase Inhibitors on the Cell Cycle

FACS analysis on untreated TKPTS cells (Fig. 1A) revealed that 43.5 ± 2.2% were in the G₁/G₀ phase of the cell cycle, 39.3 ± 3.6% were in the S phase, and 15.1 ± 4.3% were in the G₂ phase. Addition of p21 adenovirus (Fig. 1B) slightly decreased the percentage of cells in the G₁/G₀ phase to 33.3 ± 2.7%. Treatment with olomoucine (Fig. 1C) and roscovitine (Fig. 1D) resulted in a more significant reduction in the G₁/G₀ phase to 24.0 ± 1.5 and 16.2 ± 3.6%, respectively. The percentage of cells in the S phase was increased slightly by p21 adenovirus and olomoucine to 48.6 ± 1.7 and 48.0 ± 0.2%, respectively, and reduced slightly by roscovitine to 31.6 ± 2.3%. The percentage of cells in the G₂ phase was not affected by p21 adenovirus (17.7 ± 1.6%) but was increased by olomoucine and roscovitine to 24.5 ± 1.5 and 50.4 ± 0.9%, respectively. These values are summarized in Table 1. The addition of GFP-expressing adenovirus had no effect on the cell cycle (data not shown).

Effect of Cell Cycle Inhibitors on Apoptosis

Apoptosis was determined by FACS analysis (Fig. 1) of propidium iodide-stained cells (see MATERIALS AND METHODS).
and confirmed by fluorescence microscopy of Hoechst 33258-stained cells (Fig. 2). Light microscopy of cultured cells before they were harvested (Fig. 3) confirmed severe morphological changes induced by cisplatin, which were inhibited in the presence of p21 adenovirus or roscovitine. The addition of roscovitine did not affect the level of background apoptosis, which was 1.82 ± 0.74%; olomoucine slightly increased apoptosis to 3.6 ± 0.15%, whereas p21 adenovirus slightly lowered apoptosis to 0.38 ± 0.10%. Administration of cisplatin increased apoptosis to 40.7 ± 5.2% (Fig. 1E), which was not affected by olomoucine (Fig. 1G), with apoptosis observed in 36.3 ± 2.14% of the cells after cisplatin. However, addition of p21 adenovirus 24 h before cisplatin treatment or roscovitine 4 h before cisplatin reduced the level of apoptosis to background levels: 1.18 ± 0.3 (Fig. 1F) and 1.89 ± 0.49% (Fig. 1H), respectively (Fig. 4).

**Induction of p53 and p21 in TKPTS Cells**

We previously reported that the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21 were induced in kidney after cisplatin administration (6). The p53 protein was present at low levels, and p21 was undetectable in untreated TKPTS cells (Fig. 5, lane 1), and both were induced after cisplatin treatment (lane 2). Similarly, the induction of p21 from the p21 adenovirus can also be seen (lanes 3 and 4). The two p21 proteins are slightly different in molecular weight: the endogenous mouse gene product in the TKPTS cells was smaller than the adenovirus human p21 protein. The different levels of induction of p21 from the adenoviral vector (lanes 3 and 4) are likely caused by the time of exposure to adenovirus in the cell extract in lane 3 (48 h vs. 24 h in lane 4), because p21 expression in the adenovirus was controlled by a cytomegalovirus promoter, which should not be affected by cisplatin. A small induction of p53 and endogenous mouse p21 was noted in the presence of p21 adenovirus after the addition of cisplatin (lane 3), even though these cells were protected from apoptosis.

**Inhibition of Caspase-3 by p21 and Roscovitine**

The executioner caspase, caspase-3, is highly activated in TKPTS cells by 25 μM cisplatin. Activation was measured by two electrophoretic assays by direct measurement of the conversion from an inactive proenzyme (Fig. 6, bottom) or measurement of the cleavage of poly(ADP)ribose polymerase (PARP), a caspase-3 substrate, to an 89-kDa protein (Fig. 6, top). There was no activation of caspase-3 or cleavage of PARP in untreated cells or in cells treated with p21 adenovirus or roscovitine (lanes 1, 2, and 3, respectively). Treatment of cells with 25 μM cisplatin activated caspase-3, which resulted in cleaved PARP (lane 4). Pretreatment with p21 adenovirus (lane 5) or roscovitine (lane 6) before cisplatin almost totally prevented caspase-3 and PARP cleavage.

**DISCUSSION**

Cisplatin is an effective chemotherapeutic drug and is widely used for treatment of testicular, head and neck, and cervical cancers (14, 28). The generally accepted biological target of cisplatin is DNA, to which it binds and forms several types of adducts, primarily intrastrand purine cross-links (50). The dose of cisplatin that can be used is greatly limited by its

Table 1. Cell cycle analysis

<table>
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<th>Control</th>
<th>p21 Adenovirus</th>
<th>Olomoucine</th>
<th>Roscovitine</th>
</tr>
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<tbody>
<tr>
<td>G1/G0</td>
<td>43.5±2.2</td>
<td>33.3±2.7*</td>
<td>24.0±1.5*</td>
<td>16.2±3.6*</td>
</tr>
<tr>
<td>S</td>
<td>39.3±3.6</td>
<td>48.6±1.7*</td>
<td>48.0±0.2*</td>
<td>31.6±2.3*</td>
</tr>
<tr>
<td>G2/M</td>
<td>15.1±4.3</td>
<td>17.7±1.6</td>
<td>24.5±1.5*</td>
<td>50.4±0.9*</td>
</tr>
<tr>
<td>Sub-G1/G0</td>
<td>2.1±0.8</td>
<td>0.4±0.1*</td>
<td>3.6±0.2*</td>
<td>1.8±0.7*</td>
</tr>
</tbody>
</table>

Values are means ± SD. Data are from Fig. 1. Cells were untreated control, treated for 48 h with 100 multiplicity of infection p21 adenovirus (p21), treated for 28 h with 50 μM olomoucine, or treated for 28 h with 45 μM roscovitine. All cells were cultured for 3 days after splitting and harvested immediately after treatments. *Significantly different from control.
nephrotoxicity to noncancerous tissue (45), such as the S3 segment of the kidney proximal tubule, which is especially sensitive. Inasmuch as nonreplicating cells are relatively immune to toxicity from DNA-damaging agents, the mechanism for this selective toxicity in quiescent kidney tubule cells is not known, although it has recently been speculated that cisplatin is metabolized to a toxic compound(s) in these cells (54).

We have shown that, shortly after cisplatin administration, cells of the kidney enter the cell cycle but also induce cell cycle inhibitors: genes for the p21 cyclin-dependent kinase inhibitor and 14-3-3 (31, 33). The activities of p21 have been extensively characterized since its observation in a cyclin D coimmunoprecipitate (59). It had initially been shown to be induced by the p53 transactivating factor as a response to DNA damage (9), as a protein capable of interacting with and inhibiting cyclin-dependent kinases (16), and as a factor that could induce senescence in cultured cells (37). Although p21 had been originally associated with apoptosis (8), the gene was likely induced as a reaction to the high level of p53 that was used to mediate apoptosis in this system. We have found that cisplatin is more toxic in animals or cells in which the p21 or 14-3-3 gene is deleted, suggesting that cell cycle-inhibitory proteins are beneficial to the kidney in vivo and to cultured cells in vitro after cisplatin administration. However, although the lack of the p21 gene is deleterious, it has not been established whether expression of additional p21 in these cells before or after cisplatin administration would ameliorate toxicity. Several laboratories have reported that expression of p21 or other cell cycle-inhibitory proteins (p27, p16, and p18) by adenovirus vectors induces apoptosis in several cell lines (24, 46, 48, 55), although in other cell lines, p21-adenoviral vectors can provide...

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**Fig. 3.** Light microscopy of TKPTS cells before they were harvested. Cells were untreated (A), treated for 24 h with 25 μg/ml cisplatin (B), treated with 100 MOI p21 adenovirus for 48 h (C), treated with 100 MOI p21 adenovirus for 24 h and then for 24 h with 25 μg/ml cisplatin (D), treated with 45 μM roscovitine for 28 h (E), or treated with 45 μM roscovitine for 4 h and then for 24 h with 25 μg/ml cisplatin (F). All cells were cultured for 3 days after splitting.

**Fig. 4.** Induction of apoptosis in TKPTS cells by cisplatin. Data from Fig. 1 are displayed as a histogram. Cells were not exposed to cisplatin or treated for 24 h with 25 μg/ml cisplatin. Before cisplatin treatment, cells were untreated (none), treated for 24 h with 100 MOI p21 adenovirus (p21), treated with 50 μM olomoucine (Olo), or treated with 45 μM roscovitine (Rosco). All cells were cultured for 3 days after splitting. Error bars, SD. In a comparison of untreated with cisplatin-treated cultures, there were no significant differences between cultures pretreated with p21 adenovirus and those pretreated with roscovitine, but cisplatin significantly increased apoptosis without pretreatment (P = 0.0023) or with olomoucine pretreatment (P = 0.029).

**Fig. 5.** Induction of p53 and p21 protein in TKPTS cells. Total cellular proteins isolated from untreated cells (lane 1), cells treated for 24 h with 25 μg/ml cisplatin (lane 2), cells treated with 100 MOI p21 adenovirus for 24 h and then for 24 h with 25 μg/ml cisplatin (lane 3), and cells treated with 100 MOI p21 adenovirus for 24 h (lane 4) were analyzed by Western blotting. All cells were cultured for 3 days after splitting and were harvested immediately after treatments.
protection from apoptosis (15, 44, 60). Similarly, cell cycle-inhibitory drugs such as roscovitine and olomoucine, which are potent and selective inhibitors of cell cycle kinases Cdk2 and Cdc2, induce apoptosis in a variety of cell types (3, 7, 11, 19, 34–36, 49) but prevent apoptosis in others (1, 2, 12, 17, 18, 21–23, 29, 40, 41, 43, 56).

We investigated the effect of the addition of p21 adenovirus, roscovitine, and olomoucine on TKPTS cells using FACS analysis to determine the distribution of cells in various stages of the cell cycle (Table 1). Roscovitine and olomoucine had similar effects on the cell cycle, significantly increasing the percentage of cells in the G2/M phase while, at the same time, decreasing the percentage of cells in the G1/S phase. Morphologically, we did not observe substantial increases in the number of mitotic cells after treatment, so it is likely that the cell cycle inhibition is at the G2 checkpoint, rather than during mitosis. After treatment of the cells with p21 adenovirus, the vector-encoded human p21 protein could be identified (Fig. 5, lanes 3 and 4); it was slightly larger than the endogenous mouse TKPTS cell-encoded p21 induced by cisplatin treatment (Fig. 5, lane 2). However, the p21 adenovirus treatment did not significantly increase the percentage of cells at the G1 or G2 checkpoint (Table 1). Similarly, expression of human p21 protein had little effect on the generation time of the cells, increasing the doubling time slightly from 11.9 to 14.4 h. These data indicate that any effect of the induced p21 protein on these cells is unlikely to be the result of p21 as an inhibitor of the cell cycle.

Using an in vitro model of acute renal failure in which cisplatin is administered to a mouse proximal tubule cell culture, we have directly investigated the protective effect of cell cycle inhibitors as protectors of nephrotoxicity. An adenovirus vector delivering p21 to the cells completely protected them from cisplatin-induced cell death, even though the vector did not totally prevent a DNA lesion after cisplatin, as indicated by the small amounts of endogenous p53 and p21 induction. Similarly, roscovitine, a pharmacological inhibitor of cyclin-dependent kinases, protected from cisplatin-induced cell death. However, olomoucine, another cyclin-dependent kinase inhibitor with a spectrum of kinase inhibition similar to roscovitine, had virtually no protective effect. Roscovitine and olomoucine inhibited the cell cycle, as evidenced by a significant increase in the proportion of cells in the G2/M phase (Table 1), showing that cell cycle inhibition per se is not the sole mechanism of the protection. It is likely, therefore, that if the protection by p21 and roscovitine is by virtue of their inhibition of the same substrate or pathway, this inhibition need not cause cell cycle arrest, and olomoucine should not inhibit this substrate to the same extent. At 45 μM roscovitine, there is inhibition of cyclin kinases 1, 2, and 5 and of Erk1 and Erk2 (34), which is similar to the spectrum of inhibition caused by olomoucine. Although roscovitine inhibits the stress kinase JNK-1, the IC50 is 200 μM (30), which is several times higher than the concentration that was protective, and it does not inhibit p38 kinase (20). Similarly, p21 also inhibits Cdk1 and Cdk2 and stress kinases, including JNK-1 and p38, but does not inhibit Erk1 and Erk2 (51). It is unlikely, therefore, that inhibition of any one of these kinases is solely responsible for the protective effect observed with p21 and roscovitine.

The p21 protein has been shown to bind to several substrates, which, in the case of procaspase-3, -8, and -10, inhibits their activation (52, 60). Caspase activation has been associated with cytotoxicity-induced apoptosis in renal cells (4, 13, 25, 26, 38, 39, 42, 47, 53, 57, 58, 60), and we now report that pretreatment with p21 adenovirus or roscovitine almost completely inhibits activation of caspase-3 (Fig. 6). This inhibition should affect caspase-dependent apoptosis. However, we are not certain that this mechanism completely explains the protection from cisplatin apoptosis, because several reports indicate only partial protection against this injury using caspase inhibitors (4, 13, 38, 58, 61). Similarly, the use of caspase-inhibitory proteins, such as Bcl-2 (53, 61), the cowpox protein CrmA (53), XIAP (47), and the baculovirus protein p35 (25), also provided only partial protection from apoptosis. Studies have supported the possibility that caspase-independent mechanisms of apoptosis contribute to renal cell death after cisplatin treatment (4, 39), which could explain the only partial protection afforded by caspase inhibitors. This would indicate that p21 and roscovitine could prevent caspase-dependent and -independent pathways or that they could inhibit a process common to both pathways.

In a recent study, Deverman et al. (6) used cisplatin to induce apoptosis in mouse fibroblasts. Using a lower concentration of cisplatin (1–5 μM) than in our study, they found that p53-dependent induction of p21 could significantly protect the cells from apoptosis by prevention of Bcl-xL deamidation, which in turn prevented caspase-9 activation. This, however, does not seem to be the pathway of protection from cisplatin-induced apoptosis in TKPTS kidney cells. First, we found no evidence of Bcl-xL deamidation (data not shown), and, second, we found no protection using olomoucine, which Deverman et al. found could protect as well as p21 under conditions (using p53-negative cells) where p21 was not induced.

These studies show that the kinase inhibitors p21 and roscovitine are capable of protecting kidney cells from cisplatin-induced cell death and that this protection is not merely dependent on cell cycle inhibition. The mechanism of this protection is likely to be at least partially the result of the inhibition of caspase activation. However, because of reports that pan-caspase inhibitors do not totally protect from apoptosis, it is likely that other mechanisms are also involved. Because of the complete protection from cisplatin-induced apoptosis afforded by p21, while at the same time p21 has only...
a minimal effect on the cell cycle, the upregulation of p21 is a fruitful target for the treatment of acute renal failure.

ACKNOWLEDGMENTS
We thank Dr. Wafik El-Deiry (University of Pennsylvania Medical School) for providing the p21 adenovirus, Dr. Bert Vogelstein (Johns Hopkins University School of Medicine) for providing the control GFP adenovirus, and Dr. Elisa Bello-Reuss (University of Texas Medical Branch) for providing the TKP7S cells.

DISCLOSURES
This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-54471 and a grant from the Biomolecular Research Foundation and with resources and the use of facilities at the John L. McClenan Memorial Veterans Hospital (Little Rock, AR).

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