Cisplatin-induced cell death is EGFR/src/ERK signaling dependent in mouse proximal tubule cells

Istvan Arany,1 Judit K. Megyesi,1 Hideaki Kaneto,2 Peter M. Price, and Robert L. Saffirstein1
1Department of Internal Medicine, University of Arkansas for Medical Sciences and Central Arkansas Veterans Healthcare System, Little Rock, Arkansas 72205; and 2Department of Internal Medicine and Therapeutics, Osaka University, Graduate School of Medicine, Osaka 545, Japan

Cisplatin-induced cell death is EGFR/src/ERK signaling dependent in mouse proximal tubule cells. Am J Physiol Renal Physiol 287: F543–F549, 2004. First published May 18, 2004; 10.1152/ajprenal.00112.2004.—Cisplatin treatment induces extensive death of the proximal tubules in mice. We also demonstrated that treatment of immortalized mouse proximal tubule cells (TKPTS) with 25 μM cisplatin induces apoptotic death in vitro. Here, we demonstrate that members of the MAPKs such as ERK, JNK, and p38 are all activated after cisplatin treatment both in vivo and in vitro. Because MAPKs mediate cell survival and death, we studied their role in cisplatin-induced cell death in vitro. Apoptosis was confirmed by cell morphology, fluorescence-activated cell-sorting analysis, annexin V/propidium iodide binding, and caspase-3 activation in TKPTS cells. Inhibition of ERK, but not JNK or p38, abolished caspase-3 activation and apoptotic death, suggesting a prodeath role of ERK in cisplatin-induced injury. We also determined that cisplatin-induced ERK as well as caspase-3 activation are epidermal growth factor receptor (EGFR) and c-src dependent because inhibition of these genes inhibited ERK and caspase-3 activation and attenuated apoptotic death. These results suggest that caspase-3 mediates cisplatin-induced cell death in TKPTS cells via an EGFR/src/ERK-dependent pathway. We also suggest that the prodeath effect of ERK is injury type dependent because during oxidant injury, ERK supports survival rather than death in the same cells. We propose that injury-specific outcome diverges downstream from ERK in cisplatin- or H2O2-mediated cell survival and death.

THE CHEMOTHERAPEUTIC AGENT cisplatin is commonly used against various solid tumors. Despite its potent antitumor activity, its clinical use is hampered by nephrotoxicity. Recent surveys indicate that ~20% of acute renal failure (ARF) cases among hospitalized patients is due to cisplatin nephrotoxicity (5).

The cisplatin-related nephrotoxicity is most probably due to its high accumulation in the kidney, especially in the S3 segment, which undergoes extensive necrosis (3). Mice injected with cisplatin undergo ARF (21), and immortalized mouse proximal tubule cells (TKPTS) undergo extensive death 24 h after cisplatin treatment (27).

Various types of stress signals activate MAPKs that serve to coordinate cellular responses to those stimuli (8, 19). Generally, activation of ERK confers survival advantage to cells during most stress conditions (6, 8), whereas activation of JNK or p38MAPK is associated with cell death (9, 37). Cisplatin treatment has been found to activate ERK, JNK, and p38 in various systems (25, 29, 35, 38) including primary cultures of mouse proximal tubule cells (23). The protective role of ERK in cisplatin-induced apoptosis was suggested by studies that demonstrated pharmacological inhibition of ERK-sensitized cells to cisplatin (20, 25, 26), although other studies demonstrated a prodeath function of ERK as well (23, 35). Still, other groups proposed that activation of JNK, but not ERK, was responsible for apoptotic effects of cisplatin (15, 16, 29, 38), although its protective role has also been demonstrated (15). These studies relied heavily on pharmacological inhibition of MAPKs and thus may not have the necessary specificity.

Accordingly, the aim of this study was to reexamine the role of MAPKs in the death of mouse proximal tubule cells following cisplatin treatment by using additional direct approaches. We also sought to define the upstream regulation of MAPK activation.

MATERIALS AND METHODS

Animals and treatment. Six- to eight-week-old male 129Sv mice received a single intraperitoneal injection of 20 mg/kg cisplatin (27). Control and treated mice were killed 1, 2, and 3 days after treatment. The animals used in these studies (IACUC protocol 5-02-8) were housed at the Veterinary Medical Unit at the John McClellan Veterans Hospital at Little Rock. Animals were painlessly killed in accordance with methods of euthanasia approved by the Panel of Euthanasia of the American Veterinary Medical Association.

Cells and treatment. The immortalized mouse proximal tubule cell line (TKPTS) was a gift from Dr. Bello-Reuss (11) and was maintained as described previously (2, 10). Subconfluent cultures were treated with 25 μM cisplatin for the time points described in RESULTS.

Western blotting. Kidneys were removed and homogenized in a RIPA buffer that contained 100 μg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 100 mM sodium orthovanadate (Sigma), and 50 μg/ml of proteinase inhibitor cocktail (Sigma) as described earlier (2). The total lysates were used for Western blotting. Similarly, monolayers of TKPTS cells were lysed in a RIPA buffer. Protein content was determined by using a Bio-Rad Protein Determination Assay (Bio-Rad, Hercules, CA) as described earlier (2). One-hundred micrograms of protein from cell or tissue lysates were separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane (Bio-Rad). The filters were hybridized with the appropriate primary antibodies followed by a horseradish peroxidase (HRP)-conjugated
secondary antibody. The bands were visualized by an ECL method (Amersham) and quantified by densitometry (UnScan-It, Silk Scientific, Ore, UT). Antibodies against ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), JNK, p38, phospho-p38 (Thr180/Tyr182), and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-JNK (Thr183/Tyr185), phospho-epidermal growth factor receptor (EGFR) (Tyr1173), and EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit HRP-conjugated secondary antibody was purchased from Cell Signaling Technology (Beverly, MA), whereas anti-mouse and anti-goat HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

Cell cycle analysis and quantitation of apoptosis. TKPTS cells were collected after trypsinization and fixed in 70% ethanol. After RNase treatment, cells were incubated with 5 μg/ml propidium iodide (PI) and analyzed with a Becton Dickinson FACSCalibur analyzer. The cell cycle distribution was determined using the CellQuest software. Cells in the subG1 phase were considered apoptotic (27).

Apoptosis in TKPTS cells was further confirmed using an annexin V/PI binding assay (BD Biosciences). PI and annexin V-FITC fluoro...
**Table 1. Percentage of apoptotic (subG1) cells after CP treatment**

<table>
<thead>
<tr>
<th></th>
<th>% of Cells</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.96±0.5</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>45.3±5.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>U-0126</td>
<td>4.1±0.9</td>
<td>N.S.*</td>
</tr>
<tr>
<td>CP + U-0126</td>
<td>28.6±2.1</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Ad-dnJNK</td>
<td>4.3±1.2</td>
<td>N.S.*</td>
</tr>
<tr>
<td>CP + Ad-dnJNK</td>
<td>50.1±4.0</td>
<td>N.S.*</td>
</tr>
<tr>
<td>AG-1478</td>
<td>3.7±0.8</td>
<td>N.S.*</td>
</tr>
<tr>
<td>CP + AG-1478</td>
<td>24.1±3.8</td>
<td>0.004†</td>
</tr>
<tr>
<td>PP1</td>
<td>5.5±1.2</td>
<td>N.S.*</td>
</tr>
<tr>
<td>CP + PP1</td>
<td>26.6±5.8</td>
<td>0.014†</td>
</tr>
</tbody>
</table>

Results are means ± SD (n = 3). TKPTS cells were treated with 25 μM cisplatin (CP) for 24 h. Some cells were pretreated with various inhibitors alone or before CP treatments as listed and described in MATERIALS AND METHODS. Percentage of subG1 cells was determined by FACS analysis. Significance of differences was computed by a paired Student’s t-test. *Compared with the control. †Compared with the CP-treated group. N.S., not significant.

**RESULTS**

Cisplatin activates MAPKs in the kidney of cisplatin-treated mice. Mice treated intraperitoneally with a single dose of 20 mg/kg cisplatin were killed 1, 2, or 3 days after treatment. Phosphorylation status of ERK, JNK, and p38 was determined by Western blotting (Fig. 1). Our results show that all three MAPKs are activated by cisplatin compared with the control animals. The activation was sustained during the observation period and preceded the development of ARF (21). It is important to note that both inhibition of GFR and extensive death of proximal tubules occur only after day 3 of cisplatin treatment (21).

Cisplatin induces apoptosis and activates MAPKs in TKPTS cells. Cisplatin treatment damaged proximal tubule cells in vitro as well (27). Cisplatin induced apoptosis of TKPTS cells 24 h after exposure (Fig. 2A) as was demonstrated previously (27). The number of apoptotic cells (subG1 fraction) increased from 2.96 to 45.3% 24 h after cisplatin treatment as determined by FACS analysis (Fig. 2B and Table 1). Also, cisplatin treatment significantly increased the number of annexin V-stained (apoptotic) cells (from 8 to 43%; Fig. 2C) and activated caspase-3 (Fig. 2D) 24 h after treatment. Cisplatin treatment, similar to the kidney, significantly increased phosphorylation of all three major MAPKs (Fig. 3), although with different kinetics. A high level of ERK phosphorylation was already apparent at 1 h after treatment, whereas JNK and p38 phosphorylation was elevated later at 6 h after treatment. The elevated phosphorylation was maintained at 24 h posttreatment in all cases. Thus MAPK activation occurred well before obvious cell death in a similar manner both in vivo and in vitro. This allowed us to study the role of MAPKs in cisplatin-induced cell death in vitro.

**Effects of inhibition of various MAPKs on cisplatin-induced apoptosis and caspase-3 activation in vitro.** TKPTS cells were pretreated with 50 μM U-0126 for 1 h before cisplatin treatment to inhibit ERK activation. Previously, we demonstrated...
complete inhibition of endogenous and injury-induced ERK phosphorylation using the MEK inhibitor U-0126. Also, activation of JNK and p38 was prevented by infection of TKPTS cells with dominant-negative JNK or p38 adenoviruses (data not shown) 24 h before cisplatin treatment. After treatment of these pretreated cells with 25 \( \mu \)M cisplatin for 24 h, inhibition of ERK, but not JNK or p38, ameliorated cisplatin-induced morphological changes 24 h after treatment (Fig. 4). FACS analysis demonstrated that the percentage of cisplatin-induced apoptotic cells (45.3%) was significantly reduced in U-0126-pretreated (28.6%) cells but not in cells that were pretreated with either adeno-dn-JNK or adeno-dn-p38 (Table 1). Neither U-0126, adeno-dnJNK, nor adeno-dnp38 induced apoptosis when applied alone (Table 1). U-0126 significantly decreased annexin V-positive cells from 43 to 18% (Fig. 5A). Neither adeno-dnJNK nor adeno-dnp38 infection affected the annexin V-positive cell number. Furthermore, ERK inhibition, but not JNK or p38, also inhibited caspase-3 expression and activation (Fig. 5B).

**Effects of inhibition of EGFR or c-src on cisplatin-induced apoptosis as well as caspase-3 and ERK activation in vitro.** Because cisplatin treatment is capable of activating the EGFR/src pathway upstream from ERK (4), we determined the role of this pathway in survival of TKPTS cells treated with cisplatin. First, we determined that cisplatin treatment activates (phosphorylates) EGFR both in vivo and in vitro (Fig. 6). Second, we determined that application of the EGFR inhibitor AG-1478 (20 \( \mu \)M AG-1478 1 h before cisplatin treatment), c-src inhibitor PP1 (20 \( \mu \)M 1 h before cisplatin treatment), or MEK inhibitor U-0126 (50 \( \mu \)M 1 h before cisplatin treatment) efficiently inhibited endogenous phosphorylation of ERK before cisplatin treatment. In contrast, phosphorylation of JNK or p38 was unchanged under the same conditions (data not shown). Accordingly, inhibition of EGFR activation by AG-1478 significantly improved cell morphology after cisplatin treatment (Fig. 7). FACS analysis of AG-1478-pretreated cells showed reduced numbers of apoptotic cells (subG1 fraction from 45.3 to 24.1%) after cisplatin administration (Table 1). Annexin V binding was also lower in cells that were pretreated with AG-1478 (from 43 to 23%) before 24-h cisplatin treatment (Fig. 8A). Moreover, cisplatin-induced caspase-3 activation was also, albeit partially, inhibited by AG-1478 pretreatment (Fig. 8B). Similarly, pretreatment with the c-src inhibitor PP1 significantly improved cell morphology (Fig. 7), apoptosis...
DISCUSSION

Cisplatin treatment damages the proximal tubules both in vivo (21) and in vitro (23, 27). Morphological changes in the proximal tubules of the kidney are observed 3 days after cisplatin administration (21). Immortalized mouse proximal tubule cells (TKPTS) treated with 25 μM cisplatin for 24 h undergo apoptosis (27) as demonstrated by the morphological changes (Fig. 2A), FACS analysis (Fig. 2B), annexin V-binding (Fig. 2C), as well as caspase-3 activation (Fig. 2D). These changes were preceded by activation of ERK, JNK, and p38 both in vivo and in vitro (Figs. 1 and 3). We next sought to determine the role of each member of the MAPK cascade in either survival or death of these cells exposed to cisplatin.

Using specific inhibitors of those MAPKs in vitro, we found that inhibition of ERK, but not JNK or p38, significantly improved survival of cells 24 h by inhibiting apoptosis after cisplatin treatment (Figs. 4 and 5, A and B, and Table 1). These data suggested that ERK, but not JNK or p38, mediates cisplatin-induced cell death.

Because cisplatin activates EGFR in various types of cells (4) including the kidney (18), we next sought to determine whether EGFR mediates some of these effects evoked by cisplatin. Indeed, cisplatin treatment enhanced phosphorylation of EGFR both in vivo and in vitro (Fig. 6A). Pretreatment of TKPTS cells with the EGFR inhibitor AG-1478 before cisplatin treatment significantly improved cell survival (Fig. 7) and attenuated apoptosis (Table 1 and Fig. 8A). Also, inhibition of EGFR diminished cisplatin-induced ERK activation (Fig. 8B).

These results suggest that cisplatin-induced cell death is EGFR dependent. Interestingly, this receptor activation is ligand independent (4) but involves activation of c-src and ERK. Such activation of ERK is usually prosurvival (4) but has been shown recently to participate in cisplatin-mediated ERK activation in the death of HeLa cells (35).

EGFR is present in the proximal tubules (7, 24) but not in the thick ascending limb or distal convoluted tubules of the kidney. Also, in the regenerating proximal tubules, EGFR expression is lost in cisplatin-treated animals (18). Similarly,
cells derived from the proximal tubules are significantly more sensitive to cisplatin cytotoxicity than those derived from the distal convoluted tubules in vitro (17). As the proximal tubules, but not the thick ascending limb or distal convoluted tubules, undergo extensive cell death after cisplatin treatment (31), these data together suggest a role of EGFR in cisplatin-induced cell death. Small-molecule tyrosine kinase inhibitors, monoclonal antibodies to EGFR or pan-EGFR inhibitors, are safe and well tolerated by patients with cancer (33). Thus targeting EGFR could be a useful measure in preventing cisplatin-induced renal cytotoxicity in addition to the treatment of cancer.

Inhibition of c-src by the inhibitor PP1 before cisplatin treatment also abolished cell death (Figs. 6 and 7A and Table 1) and inhibited cisplatin-induced ERK activation (Fig. 8B). Thus cisplatin-induced ERK activation, and thus cell death, is EGFR/src dependent. A recent study suggested that c-src functions as a kinase in EGFR phosphorylation after cisplatin treatment (4), although c-src could be downstream from the EGFR (12). It will be necessary to perform additional studies to determine whether c-src is downstream or upstream of the EGFR in cisplatin-induced cell death signaling.

However, ERK activation is not sufficient to stimulate the death pathway in TKPTS cells. Our earlier studies showed that the survival of TKPTS cells exposed to moderate levels of H2O2 was ERK dependent (2). Obviously, cisplatin activates additional signal(s) that render ERK to stimulate the death rather than the prosurvival pathway. Wang et al. (35) proposed that sustained ERK activation by cisplatin might be responsible for the prodeath function of ERK in HeLa cells. In at least one study, the duration of ERK activity and resultant biological responses were traced to the effects of prolonged ERK activity on the stability and phosphorylation of immediate early gene products such as c-fos (22). Consistent with this view, cisplatin induced a longer-lasting ERK activation than H2O2 treatment in these cells. Revealing the mechanism of this phenomenon, however, will require additional studies.

Our data showed that inhibition of ERK also inhibited caspase-3 activation (Fig. 8B). A link between ERK and caspase-3 activation has been described before (14, 28, 30), although the mechanism of this link is not fully understood. It is still unknown if ERK activates caspase-3 directly or indirectly. Generally, ERK inhibits rather than activates caspase-3; this inhibition has been associated with NF-κB activation (32), activation of the X-linked inhibitor of apoptosis (34), or with direct phosphorylation of caspase-9, and subsequent inhibition of caspase-3 activation (1). Determining whether any of these factors is responsible for the opposing effects of ERK activation in cell survival in those two conditions is also an area of future attention.

In summary, our data suggest that the cisplatin-induced ERK activation is prodeath in nature and that this pathway is activated by EGFR and c-src. Thus manipulation of this pathway might be useful in ameliorating the outcome of cisplatin-induced ARF.

ACKNOWLEDGMENTS

The authors acknowledge Dr. E. Bello-Reuss (Dept. of Internal Medicine, UTMB, Galveston, TX) for the TKPTS cells. We also thank Dr. J. Han (Department of Immunology, The Scripps Research Institute, La Jolla, CA) for the dominant-negative p38 adenovirus.

GRANTS

This material is the result of work supported in part with resources and the use of facilities at the Central Arkansas Veterans Healthcare Center (Little Rock, AR) and was supported by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases Grant PO1-DK-58324-01A1 to R. L. Safrstein.

REFERENCES