Antiapoptotic properties of erythropoiesis-stimulating proteins in models of cisplatin-induced acute kidney injury

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CP is a very effective anticancer drug, but its full clinical utility is limited because of toxicity. Administration of CP to rats produces renal injury in many ways analogous to that in humans, and apoptosis and necrosis of the proximal tubular cells represent a prominent part of CP-induced nephrotoxicity (16, 21). Cell culture studies suggest that CP concentration is an important determinant of the mode of cell death, in that incubation of cells with a higher concentration of CP is often associated with necrotic cell death (16). In the present study, experiments were conducted using CP-nephrotoxicity models in the rat and human renal proximal tubular (RPTE) cells in culture. Our data provide new evidence that a significant part of rHuEPO’s protection against CP is associated with EpoR-mediated antiapoptotic properties. Moreover, the long-acting rHuEpo analog DA was found to be significantly protective against CP toxicity. The salutary effect of DA was persistent, independent of hematocrit (Hct) effects, and even when it was administered after the onset of CP-induced renal injury.

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**MATERIALS AND METHODS**

**Cell Culture**

Human primary RPTE cells (Cambrex, East Rutherford, NJ) were cultured as recommended by the supplier and prepared for experiments as described in our previous work (23). Both processes essentially involved standard cell culture techniques. These cells are well characterized for their origin and function and have the ability to proliferate up to five to seven passages.

**In Vitro Effect of rHuEpo and I-rHuEpo on CP-Induced RPTE Cell Apoptosis**

The CP toxicity dose range was determined by incubation of RPTE cells with 0, 5, 10, or 20 μM CP in basal medium Eagle (BME) at 37°C for 72 h. Floating cells in the supernatant were retrieved and added to adherent cells. Cells were then subjected to TdT-mediated dUTP nick-end labeling (TUNEL) using the Fluorescein-FragEL kit (Oncogene Research Products, San Diego, CA) and flow cytometry as described previously (24). Briefly, experimental RPTE cells and the positive and negative apoptotic controls from the assay kit were prepared and labeled with exogenous TdT and fluorescein-labeled dNTP according to the manufacturer’s directions. Fluorescent dNTP-labeled cells were sorted by flow cytometry using Flowscan (Becton-Dickinson, Franklin Lakes, NJ) and presented as percentage of total.

In other experiments, the effect of HuEpo or I-rHuEpo on CP (15 μM)-induced apoptosis was examined by incubation of RPTE cells with 50, 100, or 200 U/ml of rHuEpo or 1 μg/ml of I-rHuEpo (equivalent to 200 U/ml of rHuEpo) for 48 h. In addition, cells incubated with 15 μM CP with or without 200 U/ml of rHuEpo or 1 μg/ml of I-rHuEpo were exposed to Hoechst stain and examined under a UV microscope. The cells were fixed in 4% formaldehyde and allowed to attach to poly-L-lysine-treated cover slips, incubated with Hoechst stain (bisbenzimide) at 2.5 μg/ml for 1 h, and then washed and mounted on slides using glycerol-PBS (1:1) and observed under a fluorescent microscope. The Hoechst nuclei, which were identified by altered morphology of condensed and intact staining, were counted and presented as percentage of total. I-rHuEpo is an HuEpo analog that has two mutations in the receptor-binding domain, Lys45/Asp and Ser100/Glu. I-rHuEpo has no detectable binding to an I-rHuEpo analog that has two mutations in the receptor-binding domains, counted and presented as percentage of total. I-rHuEpo is an HuEpo fluorescent microscope (Leitz DMRX, Leica, Heidelberg, Germany) and mounted on slides using glycerol-PBS (1:1) and observed under a Hoechst stain [bis(benzimide) at 2.5 μg/ml].

**In Vitro Effect of rHuEpo on CP-Induced RPTE Cell Necrosis**

Since tubular necrosis is an important component of CP-induced nephrotoxicity, we were also interested in examining the effect of rHuEpo on RPTE cell necrosis. Cells were incubated with 15 μM CP in the presence of 200 U/ml of rHuEpo or 1 μg/ml of I-rHuEpo for 48 h, and the cellular release of lactate dehydrogenase (LDH) as a measure of necrotic injury was measured using the kinetic method of Wahlefeld (32). LDH release (supernatant) is presented as percentage of total LDH: supernatant LDH/(supernatant LDH + cell-associated LDH) (22).

**Simultaneous Measurement of CP-Induced RPTE Cell Apoptosis and Necrosis: Effect of rHuEpo and I-rHuEpo**

To ascertain the specificity of our necrotic and apoptotic cell death findings, as well as to gauge the extent of each type of cell death in the same experimental setting, we repeated the study by incubating RPTE cells with CP, rHuEpo, and I-rHuEpo for 48 h and then subjected the cells to dual staining for apoptosis and necrosis. We followed the method suggested by the kit manufacturer (Apoptotic and Necrotic Quantification Kit, Biotium, Hayward, CA) for dual staining. Euhidium homodimer III, a highly positively charged nucleic acid probe, is impermeant to live or apoptotic cells but stains necrotic cells with red fluorescence. The fluorescein-labeled annexin V stains apoptotic cells in green fluorescence by binding to phosphatidylserine exposed on the outer leaflet of the cell membrane. The cells were sorted by flow cytometry within 1 h of staining. The data are presented as percentage of total cells.

**Effect of CP, rHuEpo, and I-rHuEpo on STAT5 and Akt1/PKB Phosphorylation of RPTE Cells In Vitro**

RPTE cell layers were treated with 15 μM CP for 48 h in BME in the presence or absence of 200 U/ml of rHuEpo or an equivalent microgram concentration of receptor binding sites-mutated I-rHuEpo (1 μg/ml). In a control experiment, normal RPTE cells were incubated with rHuEpo or I-rHuEpo for examination of the effect on phosphorylation. After incubation, cells were washed and lysed in a buffer containing a cocktail of protease inhibitors. Total cellular protein (10 μg) was subjected to gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed for total and phosphorylated STAT5 (phospho-STAT5) and Akt1/PKB, along with β-actin control. The antibodies for Akt1 and phosphorylated (Ser473) Akt1 (phospho-Akt1) and STAT5 and phospho-STAT5 (Tyro694) were purchased from Cell Signaling Technology (Beverly, MA). The primary mouse monoclonal antibodies were diluted 1:400 (catalog no. SC-7382, Santa Cruz Biotechnology, Santa Cruz, CA) and 1:200 (catalog no. SC-7480, Santa Cruz Biotechnology), respectively. The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (catalog no. SC-2005, Santa Cruz Biotechnology) was diluted 1:1,000. Enhanced chemiluminescence (ECL, Amersham Life Science, Buckinghamshire, UK) was used for detection.

**Effect of JAK2 Tyrosine Kinase Inhibitor on rHuEpo Protection Against CP-Induced Apoptosis In Vitro**

To delineate the role of JAK-STAT signaling in HuEpo protection against CP-induced apoptosis, cells were incubated with 15 μM CP with and without 200 U/ml of rHuEpo, and an additional group of cells were incubated with an inhibitor of JAK2, tyrphostin AG–490 (Upstate.com) in three concentrations, as well as with CP and rHuEpo. After 72 h of incubation, the extent of apoptosis was determined by TUNEL assay. In a control experiment, the ability of AG–490 to induce apoptosis in normal RPTE cells and cells treated with CP was examined.

**Rat Model of CP Nephrotoxicity**

Male Sprague-Dawley rats (250–275 g body wt) were used for all our in vivo experiments, which were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi Medical Center, where the animal studies were conducted. The choice and doses of anesthetics and analgesics were standard and based on the IACUC guidelines. All the drugs were diluted in normal saline and administered by tail vein injection. From a preliminary dose-response study, CP at a single 5.5 mg/kg dose was chosen, inasmuch as it produced a reproducible acute kidney injury (AKI) with minimal systemic toxicity. ESP was administered as rHuEpo or DA. Hct was measured by the capillary method, kidney function was measured by serum creatinine, and, in one set of experiments, renal hemodynamics were also measured.

**Effect of rHuEpo, DA, and I-rHuEpo on CP-Induced AKI**

In a three-group study (n = 5 in each group), CP was administered as a single tail vein injection of 5.5 mg/kg to the CP + saline group. This group also received the rHuEpo vehicle (0.9% saline) in the same volume (0.3 ml) and frequency as rHuEpo. The CP + rHuEpo group received rHuEpo (5,000 U/kg) by tail vein injection 2 days before CP was administered. The rHuEpo injection was repeated on the day of CP administration and again 2 days later. The saline control group received 0.9% saline, instead of CP and rHuEpo, by tail vein injection.
Because rHuEpo reduced CP-induced renal injury, we next used the mutant I-rHuEpo in an experiment similar to that described above to examine whether the effect of rHuEpo was receptor mediated. The treatment groups (n = 5 in each group) were CP + saline, CP + rHuEpo, and CP + I-rHuEpo. The first two groups were treated as described above. In addition to CP, the CP + I-rHuEpo group received 25 μg/kg of I-rHuEpo, a molar quantity equivalent to the dose of rHuEpo.

We next examined the effect of DA, the long-acting and relatively new analog of rHuEpo, on CP-induced renal injury. The experimental design was identical to that of the previous experiment with rHuEpo. The three treatment groups were saline control (n = 5), CP + saline (n = 6), and CP + DA (n = 6). DA was administered at 25 μg/kg, a peptide mass equivalent to 5,000 U/kg of rHuEpo, by three injections into the tail vein at 2-day intervals beginning 2 days before CP. Rats in the CP + saline and saline control groups received saline, instead of DA.

**Effect of Venesection on DA Protection Against CP-Induced AKI**

Administration of ESPs, including DA, is associated with an increase in Hct. To determine whether a renoprotective property of DA is independent of Hct levels, we repeated the above-described study while controlling for Hct in the DA-treated animals by tail venesection of 0.25–0.5 ml/day, each lasting 15 min. Blood volume was immediately replaced by normal saline, which was well tolerated by the rats. One of the CP-treated groups received DA as described above (n = 4). A second CP + DA group (n = 4) was subjected to tail venesection to maintain Hct at the level of the CP + saline group (n = 4). On average, three sessions of venesection were required to maintain Hct equivalence.

**Effect of Late Administration of DA on CP-Induced AKI**

In a two-group experiment (n = 3 in each group), one of the CP groups received a single dose of 25 μg/kg of DA by tail vein 2 days after CP injection, and serial Hct and serum creatinine were obtained.

**Physiological and Histological Measurements**

In all the animal studies, body weight, Hct, and serum creatinine were measured serially for 10 days. Terminal renal hemodynamic measurements were obtained while the rats were under anesthesia, as described in detail previously, and the DA study was terminated (11). Briefly, after anesthesia, rats underwent femoral arterial, internal jugular vein, and bladder catheterization. The fluid loss during the study was replaced by an initial intravenous infusion of normal saline (1% of body weight) for 30 min followed by a maintenance infusion of 0.5 ml/h. Femoral arterial pressure via a pressure transducer (Micro-med, Louisville, KY) and body temperature via a rectal probe were continuously monitored. Hct was determined by the microcapillary method, and serum protein was determined by refractometry at the start and end of the clearance periods. Glomerular filtration rate (GFR) was determined by measurement of creatinine clearance and renal blood flow (RBF) by ultrasonic flow probing of the renal artery (Transonic Systems, Ithaca, NY). Plasma and urine creatinine levels were measured using a creatinine autoanalyzer (Beckman, Somerset, NJ). The means of two consecutive clearance periods were used to obtain these calculations. At the end of the clearance measurements, kidneys were removed and flushed with 10 ml of heparinized cold normal saline via the renal artery. Kidneys sampled in a systematic manner were sectioned coronally at 2-mm intervals and stored in buffered formaldehyde until histological processing.

For in situ detection of apoptosis, the 4-μm paraffin sections of formaldehyde-fixed kidney tissue were deparaffinized, dehydrated, and stained with an apoptosis detection kit (ApopTag, Serological, Norcross, GA) following the manufacturer’s instructions. Briefly, sections were incubated with 30% TdT, proteins were digested, and endogenous peroxidase was blocked using 3% H2O2. Sections were then incubated with a peroxidase-conjugated antibody, visualized with diaminobenzidine, and counterstained with methyl green. The specificity of the apoptotic staining was verified by inclusion of appropriate positive and negative controls. For quantification of apoptosis, digital images were obtained from five coronal sections per kidney. The images were obtained at ×200 magnification, and the extent of tubular apoptosis in the cortical and medullary areas was quantified using computer-assisted image analysis of the apoptotic stain (ImagePro software, MediaCybernetics, Carlsbad, CA). The mean score from each kidney was used for statistical analysis. The extent of tubular necrosis was also quantified using ImagePro. Digital images were taken from five locations spaced equally across the coronal section of slides magnified at ×200. Tubular sections with frank necrosis were point counted with a line grid containing 30 squares and 80 points, which was created in the ImagePro program. As a second method to verify the extent of apoptosis, the tubular sections were also counted for cells with apoptotic nuclei on the basis of the presence of nuclear condensation and fragmentation in ×200 microscopic fields. A final mean score obtained for each kidney was used for statistical analysis.

**Materials**

The cell culture solutions and media were purchased from Life Technologies (Grand Island, NY), and culture flasks and plates were obtained from Costar (Acton, MA). All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade or the highest grade available. DA and I-rHuEpo were supplied by Amgen (Thousand Oaks, CA).

**Statistical Analysis**

Statistical analyses were conducted using the StatView program (Abacus Concepts, Berkeley, CA). Results are presented as means ± SE. For in vitro studies, three to four experiments, performed at least in duplicates, were conducted. For in vivo studies, the differences between two groups were determined by Student’s t-test, and the differences among more than two groups were determined by ANOVA followed by Fisher’s post hoc test. P < 0.05 was considered statistically significant.

**RESULTS**

**In Vitro Experiments**

**Effect of rHuEpo and I-rHuEpo on CP-induced RPTE cell apoptosis.** Incubation of RPTE cells with CP caused a dose-dependent increase in apoptosis, as shown by the percentage of TUNEL-stained cells detected by flow cytometry in Fig. 1A. rHuEpo suppressed apoptosis induced by 15 μM CP, which was significant for 100 and 200 U/ml of rHuEpo (Fig. 1B). In contrast, I-rHuEpo at 1 μg/ml, a peptide mass equivalent to 200 U of rHuEpo, did not inhibit CP-induced apoptosis. The Hoechst nuclear staining method showed that rHuEpo, but not I-rHuEpo, caused an appreciable reduction in CP-induced apoptosis (Fig. 1, C and D).

**Effect of HuEpo, I-HuEpo, and CP on STAT5 and Akt1/PKB phosphorylation of RPTE cells.** Densitometric analysis showed a threefold increase in STAT5 and Akt1/PKB phosphorylation in RPTE cells incubated with rHuEpo compared with control cells treated with vehicle saline (Fig. 2A). Levels of phospho-STAT5 and phospho-Akt1/PKB appear to be lower in cells incubated with CP than in control cells, but the levels were severalfold higher in cells incubated with CP in the presence of rHuEpo than in the saline control cells (Fig. 2A). I-rHuEpo-mutated receptor binding sites did not induce phosphorylation,
confirming lack of I-rHuEpo activity. In the control experiment, incubation of normal RPTE cells with rHuEpo, but not I-rHuEpo, caused phosphorylation of STAT5 and Akt/PKB (Fig. 2, B and C).

Effect of JAK2 tyrosine kinase inhibitor on rHuEpo protection against CP-induced apoptosis. As in the previous experiments, rHuEpo significantly suppressed CP-induced RPTE cell apoptosis (Fig. 3A). However, in the presence of the JAK2 inhibitor AG-490, rHuEpo protection was reversed, suggesting that JAK2 activation is a prerequisite for rHuEpo protection (Fig. 3A). In a control experiment, AG-490, alone or in the presence of CP, did not increase RPTE cell apoptosis (Fig. 3B).

Effect of rHuEpo on CP-induced RPTE cell necrosis. Incubation of cells with 100 μM CP for 24 h caused a marked increase in LDH release (Fig. 3C). rHuEpo at 200 U/ml had no effect on 100 μM CP-induced LDH release (Fig. 3C).

Simultaneous measurement of CP-induced RPTE cell apoptosis and necrosis: effect of rHuEpo and I-rHuEpo. The percentage of cells that were positive for annexin V but negative for ethidium (apoptotic cells) and the percentage of cells that were positive for ethidium but negative for annexin V (necrotic cells) are presented in Fig. 3D. Cells that stained for both (~10%), which could represent apoptotic or necrotic cells, were not included. The results confirm our earlier findings that rHuEpo, but not I-rHuEpo, suppresses apoptotic cell death. The results also demonstrate that rHuEpo and I-rHuEpo had no significant effect on normal cell viability and, importantly, confirm our finding that rHuEpo had no significant effect on CP-induced cell necrosis (Fig. 3C).

In Vivo Experiments

Effect of rHuEpo on CP-induced AKI. In the first in vivo study, we examined the effect of rHuEpo on CP-induced acute renal failure. As expected, Hct was increased in the rHuEpo-injected rats compared with the saline-injected rats. This increased Hct was statistically significant on days 6 and 8 after initiation of rHuEpo injections (Fig. 4A). Serum creatinine was significantly higher in the CP group than in the saline group on day 4 of CP injection (Fig. 4B) but was significantly lower in the CP + rHuEpo group than in the CP + saline group on day 6: 2.5 ± 0.3 vs. 3.1 ± 0.3 mg/dl (P < 0.05).
with CP, one group received saline injection, the second rHuEpo-EpoR interactions. Among the three groups treated beneficial effects on CP-induced renal injury were mediated by a previous experiment using I-rHuEpo to determine whether the effect of rHuEpo on CP-induced renal injury is receptor mediated.

**Effect of rHuEpo on CP-induced AKI.** rHuEpo, but not I-rHuEpo, reverses CP-induced reduction in signal transducers and activators of transcription-5 (STAT5) and Akt1/PKB phosphorylation. RPTE cells were incubated with 15 μM CP, 200 U/ml of rHuEpo, I-rHuEpo at the mass equivalent of rHuEpo, or a combination for 24 h, and 10 μg of each cell lysate were electrophoresed on an SDS-polyacrylamide gel and immunoblotted for control β-actin and total and phosphorylated STAT5 and Akt1/PKB. Western blotting were carried out 3 times with similar results; Western blot represents results from 3 experiments. A: ability of rHuEpo to phosphorylate STAT5 and Akt1. B and C: control experiments demonstrating inability of I-rHuEpo to phosphorylate STAT5 and Akt1, thus demonstrating specificity of rHuEpo in induction of STAT5 and Akt phosphorylation. Values are means ± SE; n = 3. *P < 0.05 vs. others.

**Renal histological profiles.** TUNEL staining profiles are presented in Fig. 8A at a low (×20) magnification to facilitate visualization of the staining distribution in the whole kidney. The staining, based on a highly specific staining method verified by appropriate controls, was primarily confined to the tubules and was readily apparent in the CP + saline group. The staining was, however, appreciably and markedly reduced in kidneys of rats treated with DA. TUNEL staining scored by a pathologist in a blinded fashion was strikingly higher in kidneys of CP- than saline-injected rats (16,677 ± 2,788 vs. 117 ± 26 pixels, P < 0.05) and was markedly and significantly reduced by DA administration (8,962 ± 2,629 pixels, P < 0.05 vs. CP + saline). The apoptosis-reducing effect of DA was evident in the medulla and cortex but was statistically significant for the cortical area (Fig. 8B). An additional method used to identify apoptotic cells on the basis of microscopic assessment of apoptotic nuclear morphology demonstrated a twofold reduction in apoptotic cells in the kidney sections of the DA + CP group compared with the group treated with CP alone (Fig. 8C).

We also determined the extent of renal tubular necrosis histologically (Fig. 8D). The percentage of tubules with necrosis was significantly lower in the DA + CP group than in the CP + saline group: 13 ± 3% vs. 32 ± 7% (P < 0.05). The necrosis score of the saline control group was 1 ± 3%. In addition to a lower necrotic tubular score, tubular sections with increased cellular regeneration were also more frequent in the DA + CP group than in the CP + saline group.
Effect of venesection on DA protection from CP-induced AKI.

The beneficial effects of ESP on CP-induced renal injury could possibly be explained as an indirect or secondary effect of the increased Hct or intravascular volume expansion, rather than an effect mediated through EpoR in the kidney. The most direct way to determine whether the increased red cell mass was responsible for renal preservation is to bleed ESP-treated animals to an Hct level comparable to that of animals treated with CP alone. We therefore treated one group of animals with CP/rHuEpo saline and CP/rHuEpo venesection to determine whether venesection resulted in a worse renal outcome, similar to CP saline alone. In both treatment groups, femoral artery pressures were maintained within the physiological range to equalize intravascular volume status. As shown in Fig. 9A, we were able to bleed the DA-treated animals to an Hct level similar to that of CP saline animals. Nevertheless, serum creatinine levels were lower in these animals than in those treated with CP saline without DA (Fig. 9B).

Effect of late administration of DA on CP-induced AKI.

Because, in most instances, acute injury to the native kidney is unpredictable, the prophylactic administration of any potential therapy is not an option. Therefore, we determined whether administration of DA after the initiation of CP-induced AKI might mitigate the renal injury. At 2 days after the single 5.5 mg/kg tail vein CP injection, serum creatinine doubled (from 0.4 ± 0.1 to 0.8 ± 0.1 mg/dl; Fig. 10B), without a significant change in Hct (Fig. 10A). Hct increased with DA administration 2 days after the CP injection (Fig. 10A). Serum creatinine peaked earlier in DA-treated animals and was lower than in non-DA-treated animals (Fig. 10B). Serum creatinine was 1.8 mg/dl in the CP + DA group and 2.8 ± 0.1 mg/dl in the CP + saline group (P < 0.05) on day 4 and remained statistically lower to the end of the study on day 10. In this experiment, DA was administered only once, in contrast to the three doses of DA begun before CP injection in the above-described experiment. However, both schedules of administration resulted in a lower peak serum creatinine that was statistically significant and remained significant through the end of the study.

DISCUSSION

Our in vivo experiments demonstrate that ESP reduces CP-induced renal toxicity via the Epo-EpoR interaction, and our in vitro experiments suggest that this may be mediated at
least in part through the antiapoptotic property of ESP via the JAK-STAT signaling pathway.

Apoptosis is an important component of many models of renal injury, such as ischemia-reperfusion of the native and transplanted kidneys and cyclosporine- and CP-induced toxic nephropathy (6, 16, 28). The notion that rHuEpo or DA administration might be protective against these forms of renal injury is suggested by the finding of EpoR expression in human and rat kidneys, distributed in cortex, medulla, and papilla, and in human and mouse proximal tubular cell lines (34). A few earlier studies examined whether administration of rHuEpo would protect kidneys against injury, but their conclusions differed (18, 29, 30). In a discerning study, Vaziri et al. (29) reported for the first time that rHuEpo reduced the severity of kidney failure and increased tubular regeneration in the CP-induced AKI model. However, the antiapoptotic role of rHuEpo was not investigated; perhaps the antiapoptotic role of rHuEpo was not well known over a decade ago (29). Results from studies of the ischemia-reperfusion AKI model have been mixed (18, 30, 35). In the study by Nemoto et al. (18), administration of rHuEpo was not associated with a reduction in native-kidney ischemia-reperfusion injury. In contrast, in two recent studies, administration of rHuEpo before ischemia was reported to significantly reduce renal injury, but again the

Fig. 4. rHuEpo inhibits CP-induced acute kidney injury (AKI). Rats in the CP + rHuEpo group were injected through their tail veins with 5,000 U/kg of rHuEpo on days 0, 2, and 4. On day 2, they also received 5.5 mg/kg of CP as a single tail vein injection. CP + saline group received CP as in the CP + rHuEpo group, but they received saline, instead of rHuEpo. Saline control group received saline, which is the vehicle for CP and rHuEpo. A: hematocrit (Hct). *P < 0.05 vs. other groups on days 6 and 8. B: serum creatinine levels. *P < 0.05 vs. other groups on day 6.

Fig. 5. I-rHuEpo with mutated receptor binding sites does not inhibit CP-induced AKI: role of erythropoietin (Epo)-Epo receptor (EpoR) interaction in renal protection. rHuEpo and I-rHuEpo were injected at 5,000 U/kg and 25 μg/kg, respectively, through the tail vein on days 0, 2, and 4. On day 2, rats also received 5.5 mg/kg of CP as a single tail vein injection. A: Hct increased in the CP + rHuEpo, but not the CP + I-rHuEpo, group. *P < 0.05 vs. other groups on days 4, 6, and 8 of rHuEpo and I-rHuEpo injections. B: serum creatinine was lower in the CP + rHuEpo, but not the CP + I-rHuEpo, group, suggesting that Epo-EpoR interaction is a necessary step (as in the in vitro study) for renal protection provided by rHuEpo. *P < 0.05 vs. other groups on days 4 and 6 after rHuEpo and I-rHuEpo.
underlying mechanisms were not explored (30, 35). In contrast to these two studies, late administration of ESP in our study was attended by a significant protection against CP-induced nephrotoxicity. A potential explanation may be that the tempo of the development of renal injury in CP-induced nephrotoxicity is slow and accruing compared with the explosive nature of injury during ischemia-reperfusion. In contrast to native-kidney ischemia-reperfusion, most of the settings of organ transplantation provide an opportunity to precondition the organs with rHuEpo or DA. Although we did not directly compare the protective activity of rHuEpo with that of DA, our study suggests that DA may have a more potent effect than rHuEpo. RBF and GFR were two- and threefold higher in the DA-treated group than in the group treated with CP alone, which, if proven to be the case in the clinical setting, can offer significantly better clinical outcomes. DA, which is a glycosylation analog of Epo, has approximately a threefold longer serum half-life than rHuEpo (17). This allows persistently higher serum levels of DA and, possibly, longer and more continuous receptor stimulation. A direct dose comparison study between rHuEpo and DA is required to confirm this preliminary observation. We have, in general, used supratherapeutic doses of ESP, as were also used in the literature (5, 12, 27). However, the finding in one of our studies that a single dose of DA can afford significant protection against CP suggests that frequent and larger doses used by us and others in other models may not be necessary to recruit the anti-injury properties of rHuEpo proteins. Consistent with this finding is our preliminary work that a far lower dose of rHuEpo, such as 50 U/kg (compared with 5,000 U/kg in the present study), may afford tissue protection. Formal dose-response studies are required to establish the best dose.

Our in vitro studies have attempted to explore the underlying mechanism of protection provided by ESP against injury. Although interstitial cells in the renal peritubular capillary bed are the primary source for Epo, EpoRs are present on several cell types. EpoR density is highest on the erythroid progenitor cells, but it is also present on neuronal, endothelial, and renal tubular cells (34). The collective findings in our study that...
1) CP reduced phosphorylation of human renal tubular cell STAT5 and Akt1/PKB, 2) rHuEpo, but not I-rHuEpo, increased phosphorylation of these proteins, and 3) JAK2 inhibitor reversed the antiapoptotic property of rHuEpo against CP. These findings suggest that the rHuEpo-EpoR interaction and downstream JAK-STAT signaling pathways may play a role in our observation that rHuEPO protects against CP-induced apoptosis. We have extended our in vitro findings that the Epo-EpoR interaction is required for rHuEpo protection against CP-induced injury in vivo. However, the role of JAK-STAT in the cytoprotection provided by Epo is yet to be confirmed, inasmuch as the JAK2 inhibitor used in the present study is not specific for JAK (33). Additional in vivo studies, preferably utilizing genetically modified animals, are required to verify whether the role of JAK-STAT observed in cell culture applies to the renal protection observed in the intact animals.

In the present study, renal protection with ESP was consistently present in four separate in vivo experiments, in which ESP treatment resulted in a twofold improvement in renal protection, which was better appreciated by GFR and histological measurement. ESP therapy did not completely abrogate CP-induced injury; however, ESP caused a significant decline in serum creatinine levels, which peaked ~4 days after CP administration in our models. rHuEpo did not also prevent the early rise in serum creatinine but appeared to prevent more severe renal injury or enhance recovery or both; these observations are interesting from a mechanistic standpoint. These findings suggest that, despite the initial hit and injury, ESP is likely to reduce renal tubular cell death, in essence reducing the severity and duration of renal injury. The ESP protection appears not to be contingent on any preconditioning, and the significant improvement in renal function with ESP treatment persisted for 8–10 days after CP injection. Recent clinical reports suggest that even a slight decrement in renal function can be associated with higher morbidity and mortality in critically ill patients. Thus any yet to be proven clinical benefits of NSP in AKI have the potential to translate to significant improvement in patient outcomes.

Although rHuEpo suppressed apoptosis in our study, it did not suppress CP-induced necrotic cell injury in our cell culture experiments. Administration of DA in vivo, however, reduced tubular apoptosis and necrosis in the CP-nephrotoxicity model, and this disparity must be explained. One possibility is that many of the purported nonantiapoptotic effects of rHuEpo, such as limitation of the production of tissue-injuring molecules, modulation of inflammation, reversal of vasospasm, enhancement of cell replication and proliferation, and even recruitment of stem cells to the site of injury to facilitate
regeneration (31), may account for the antinecrotic effect of rHuEpo or DA therapy observed in our in vivo study. Erythropoiesis is an invariable effect of ESP administration. However, increased Hct or an associated increase in oxygen delivery does not seem to be an important mechanism in the tissue protection provided by ESP. Furthermore, in cell culture studies in which Hct is not an issue, rHuEpo directly suppressed CP-induced apoptosis. This suggests that rHuEpo and DA mediate renal protective and/or regenerative effects that are independent of erythropoiesis. A recent report that desialylation and carbamylation variants of rHuEpo do not induce erythropoiesis but protect against injury in experimental models also supports the view that the erythropoietic and protective property can be dissociated (14). However, in several clinical situations, such as chemotherapy-induced nephrotoxicity and other forms of renal damage, anemia may ensue and would be treated with rHuEpo or DA. The ESP therapeutics would therefore potentially have a dual mechanism of action in preventing tissue damage and promoting tissue regeneration in addition to treating the associated anemia. On the other hand, recent controversy over the potential of NSP in worsening the survival of cancer patients gives a pause for the study of NSP in cancer patients as a renal-protective agent (15, 26). Recent experimental studies have, however, shown that neuronal protection with Epo is not associated with tumor cell growth or reduction of CP cytotoxicity against tumor cells (2, 19). Moreover, any potential use of ESP for protection against kidney injury is likely to be a one-time use and, thus, may not be clinically relevant for the alleged adverse effects following prolonged use for correction of anemia in patients with cancer.

Some issues remain unresolved in the present study. Although DA administration was accompanied by a striking reduction in tubular cell apoptosis and necrosis, our study cannot exclude other mechanisms of tissue protection, such as

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**Fig. 9.** Effect of venesection on DA-mediated protection against CP-induced AKI. The CP + DA group was injected with 25 μg/kg of DA on days 0, 2, and 4 and subjected to repeated venesection to keep Hct similar to that of the CP group. A: Hct was maintained at similar levels in both groups. B: Serum creatinine was lower in the CP + DA + venesection group. *P < 0.05 vs. CP group on days 7 and 8.

**Fig. 10.** Effect of late administration of DA on CP-induced AKI. One of the CP groups received a single dose (25 μg/kg) of DA by tail vein 2 days after CP injection. A: Hct was higher in the CP + DA group. *P < 0.05 vs. CP group. B: Serum creatinine was lower in the CP + DA group, despite administration of a single dose of DA 2 days after CP injection, when serum creatinine was already 2-fold higher than baseline. *P < 0.05 vs. CP group.
improved tissue oxygenation (e.g., based on better blood flow and angiogenesis) or better systemic status. In the present study, body weight was maintained in the ESP-treated CP group, but serum albumin was still lower; one possible reason for the latter could be the toxic effect of platinum on the synthetic function of the liver. Furthermore, in our studies, the effect of rHuEpo was not seen immediately after its administration. At least 48 h were required for rHuEpo and DA to exhibit any significant functional protection, which may suggest that ESP-mediated protection may involve inhibition of secondary tissue damage and/or promotion of renal regeneration. A broader basis for ESP-mediated tissue protection is suggested by very recent clinical trials in which mortality was significantly lower in patients admitted to the critical care unit and treated with Epo than in untreated controls (9). On the other hand, increased tubular regeneration with ESP treatment could still be due to reduced apoptosis. It is possible that Epo, through certain systemic salutary effects, might have improved renal function. Again, detailed in vivo studies, e.g., utilizing animals with tissue-specific gene knockout of EpoR or the JAK-STAT pathway, are required to discern the specific role of antiapoptosis in ESP-mediated tissue protection.

In summary, our new findings are as follows: 1) in vitro and in vivo renal protection by ESP is mediated by the Epo-EpoR interaction, 2) the relatively new ESP DA affords protection, 3) ESP administered after injury affords protection, 4) the JAK-STAT pathway has a role in in vitro protection, and 5) Epo may have in vivo antinecrotic properties through a yet to be defined mechanism. These findings have implications for the broader use of ESP in the settings of renal and other organ injury.

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