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Interleukin-10 inhibits ischemic and cisplatin-induced acute renal injury

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Interleukin-10 inhibits ischemic and cisplatin-induced acute renal injury.

Background. Acute renal failure (ARF) is caused by ischemic and nephrotoxic insults acting alone or in combination. Anti-inflammatory agents have been shown to decrease renal ischemia-reperfusion and cisplatin-induced injury and leukocyte infiltration. Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that inhibits inflammatory and cytotoxic pathways implicated in acute renal injury. Therefore, we sought to determine if IL-10 inhibits acute renal injury.

Methods. The effects of IL-10 were studied in mice following cisplatin administration and bilateral renal ischemia-reperfusion, in a rat model of renal transplantation, and in cultured mouse cortical tubule cells.

Results. IL-10 significantly decreased renal injury following cisplatin administration and following renal ischemia/reperfusion. Delay of IL-10 treatment for one hour after cisplatin also significantly inhibited renal damage. IL-10 and α-melanocyte stimulating hormone (α-MSH) increased recovery following transplantation of a kidney subjected to warm ischemia. To explore the mechanism of action of IL-10, its effects were measured on mediators of leukocyte trafficking and inducible nitric oxide synthase (NOS-II). IL-10 inhibited cisplatin and ischemia-induced increases in mRNA for tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), and NOS-II. IL-10 also inhibited staining for markers of apoptosis and cell cycle activity following cisplatin administration, and nitric oxide production in cultured mouse cortical tubules.

Conclusions. IL-10 protects against renal ischemic and cisplatin-induced injury. IL-10 may act, in part, by inhibiting the maladaptive activation of genes that cause leukocyte activation and adhesion, and induction of iNOS.

[1] These three authors contributed equally to this work.

Key words: ischemia-reperfusion injury, inflammation, leukocytes, renal transplantation, nitric oxide synthase, alpha-melanocyte stimulating hormone.

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Acute renal failure (ARF) is caused by ischemic and nephrotoxic insults acting alone or in combination [1, 2]. Ischemic injury is present in about 50% of patients with acute renal failure, and is thought to be responsible for much of the non-immunologic injury that occurs in the immediate days following renal transplantation [1, 3]. Methods to inhibit post-transplant ARF might allow transplantation from marginal donors, and hence, decrease the waiting time for a renal transplant.

Cisplatin is one of the most effective chemotherapeutic agents for the treatment of solid tumors including ovarian carcinomas, head and neck carcinomas, and germ cell tumors. The anti-tumor effects of cisplatin are dose-related; intensification of the dose can help patients who are refractory to lower doses of cisplatin [4]. Treatment of patients with peritoneal carcinomatosis, an incurable stage is cancer, is quite difficult. Surgical debulking, followed by cisplatin administered via continuous hyperthermic peritoneal perfusion shows promise for treatment of peritoneal mesothelioma [5]. However, dose-limiting nephrotoxicity occurs with doses above 90 mg/m^2 IV or 270 mg/m^2 IP, despite adequate hydration and the administration of systemic sodium thiosulfate to bind systemically absorbed cisplatin. Because the dose limiting toxicity of regionally administered intraperitoneal cisplatin is systemic nephrotoxicity, strategies that allow dose escalation may result in improved efficacy. Methods to prevent cisplatin-induced ARF might minimize treatment morbidity and interruptions in therapy.

Whereas *N*-acetyl-cysteine has been recently shown to decrease renal injury from radiocontrast-induced ARF [6], no therapeutic agents are available to prevent ARF following ischemia, other nephrotoxic agents, or transplantation. We recently found that the anti-inflammatory cytokine α-melanocyte stimulating hormone (α-MSH) protects against renal ischemia reperfusion injury even when started six hours after ischemia [7]. α-MSH inhibits

both maladaptive inflammatory and cytotoxic (nitric oxide) pathways activated during the reperfusion period. The anti-inflammatory cytokine interleukin-10 (IL-10) is a potent inhibitor of fever, early-phase inflammation, and has many effects similar to α-MSH including inhibition of cytokines, chemokines, neutrophil activation, and nitric oxide (NO) production [8]. IL-10 has been shown recently to inhibit ischemia-reperfusion injury to the lung [9, 10], hind limb [11], and heart [12].

Because of similarities between cisplatin and ischemic-induced renal injury [13, 14], we tested the hypothesis that IL-10 inhibits both ischemic and cisplatin-induced renal injury. We also tested the effects of IL-10 (and α-MSH) in a model that mimics transplantation of a marginal kidney.

METHODS

Animals

Male BALB/c and C57BL/6J were purchased from Jackson Labs (Bar Harbor, ME, USA), Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA) or the National Institutes of Health (NIH). All animals had free access to water and food (4% mouse-rat diet, Harlan Sprague-Dawley Inc.; or NIH-07 rodent chow, Zeigler Bros. Inc., Gardners, PA, USA) before surgery, and a lower K diet after surgery that supplied 50% the potassium of the normal diet, as previously [15]. Animal care followed the criteria of the University of Texas Southwestern Medical Center and NIH for the care and use of laboratory animals in research.

Chemicals

Interleukin-10 was purchased from PharMingen (San Diego, CA, USA); α-MSH was purchased from Phoenix Pharmaceuticals, Inc. (Mountain View, CA, USA). All other chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO, USA).

Cisplatin

The mice were anesthetized with an IM injection of 100 mg/kg ketamine, 10 mg/kg xylazine, and 1 mg/kg acepromazine. The animals were given 20 mg/kg cisplatin (Sigma) in 20 mL/kg normal saline IP, and then IL-10, α-MSH or vehicle (0.5 mL) was given IV. Blood was withdrawn at 48 and 72 hours; the animals were sacrificed at 72 to 96 hours. At the time of sacrifice, blood was collected for measurement of plasma creatinine. Both kidneys were harvested for histological and mechanistic studies.

Ischemia-reperfusion model

Surgery was performed as previously described [7, 15]. The mice were anesthetized with ketamine, xylazine, and acepromazine as above. The animals were placed on a heating table kept at 39°C to maintain constant body temperature. Both renal pedicles were cross-clamped for 40 minutes. Both kidneys were inspected for ischemia after two minutes. IL-10, α-MSH or vehicle (0.5 mL) was given at the end of the ischemic period immediately before the clamps were removed. After the clamps were removed, the kidneys were again inspected for restoration of blood flow, and 1 mL of prewarmed (37°C) normal saline was instilled into the abdominal cavity. The abdomen was closed, and the animals were placed in a 29°C incubator overnight. The animals were sacrificed at 24 hours. At the time of sacrifice, blood was collected for measurement of plasma creatinine (Beckman Astra 8 autoanalyzer; Beckman Industries, Inc.; Fullerton, CA, USA). Both kidneys were harvested for histological study or mechanistic studies. Sham-treated animals went through the same surgical procedure as the other animals, including dissection of the renal pedicle; however, renal clamps were not applied.

Histological examination

Formalin (10%)-fixed and paraffin-embedded kidney specimens were stained with hematoxylin and eosin (H&E; 2 μm) or naphthol AS-D chloroacetate esterase (Sigma Kit # 91-C, 3 μm sections). Histological changes were evaluated by semiquantitative measurements of tissue necrosis and erythrocyte congestion as described previously [7, 15]. Since the esterase stain identifies both infiltrating neutrophils and macrophages [16], the term leukocytes will be used through the rest of this article. Leukocyte infiltration was measured by counting five high power fields (×40) per section. TUNEL (Kit 1 684 809; Boehringer Mannheim, Mannheim, Germany) and PCNA (Kit 93-1143, Zymed Laboratories, Inc.) staining was carried out using the manufacturer's recommended protocol using 4% paraformaldehyde-fixed tissue; the PCNA slides were lightly counterstained with methyl green.

Measurement of mediator mRNA by RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [17, 18]. Briefly, renal total RNA was purified using guanidinium thiocyanate-phenol-chloroform extraction [19]. cDNA was generated from kidney RNA samples by Molony murine leukemia virus (MMLV) reverse transcriptase. Portions of the cDNA were amplified by PCR primers directed toward mouse tumor necrosis factor-α (TNF-α), intracellular adhesion molecule-1 (ICAM-1), or nitric oxide synthase-II (NOS-II) (Table 1). PCR conditions were Mg 2.5 mmol/L, annealing temperature 58°C, and 35 cycles. The PCR products were confirmed by sequencing. These conditions resulted in a single band with a linear relationship between cDNA and PCR product. We normalized for amount of RNA by measuring MDH in separate PCR reactions. The PCR products were electrophoresed through 1.5% agarose.

Table 1. Sequences of oligonucleotide primers

Gene	Gene Bank identifier	Oligonucleotide sequence	Location	Product size
			bp	
Mouse MDH	(M29462)			
5′ sense		GGTCATTGTTGTGGGAAACC	(456–475)	431
3′ antisense		TCGACACGAACTCTCCCTCT	(867–886)	
Mouse TNF-α	(M38296)			
5′ sense		GAACTGGCAGAAGAGGCACT	(32–50)	211
3′ antisense		AGGGTCTGGGCCATAGAACT	(242–261)	
Mouse ICAM-1	(M31585)			
5′ sense		AGATCACATTCACGGTGCTG	(533–552)	252
3′ antisense		CTTCAGAGGCAGGAAACAGG	(784–803)	
Mouse NOS-II	(M87039)			
5′ sense		TGCATGGACCAGTATAAGGCAAGC	(1948–1971)	223
3′ sense		GCTTCTGGTCGATGTCATGAGCAA	(2147–2170)	

Genes are identified by GeneBank abbreviations. Abbreviations are: bp, base pair; MDH, malate dehydrogenate; ICAM-1, intercellular adhesion molecule-1; NOS-II, inducible nitric oxide synthase-II; TNF-α, tumor necrosis factor-α.

Renal transplantation

Male Lewis rats weighing 300 g were used for both the kidney donor and transplant recipient. After the induction of anesthesia (sodium pentobarbital, 30 mg/kg IP), the donor rat was placed on a warm operating pad and the abdomen was entered via a midline incision. A small clip was placed on the left renal pedicle and the renal blood flow was completely occluded. After 40 minutes, the clip was released and the kidney was reperfused for 30 minutes. A clamp was placed on the aorta above the renal arteries and both donor kidneys were flushed with 3 mL ice-cold University of Wisconsin (UW) solution via a 27-gauge needle. The left kidney and ureter were harvested and kept in UW solution at 4°C overnight.

Recipient surgery. After anesthesia, the recipient rat's abdomen was entered via a midline incision. The abdominal aorta and vena cava were dissected free and clamped with a Sun Lee clamp. The donor kidney was implanted to the aorta and vena cava in an end-to-side fashion with 9-0 nylon suture. Then the clamp was released and homeostasis achieved. The donor ureter was anastomosed to the recipient bladder with a pull-through technique. Both kidneys of the recipient were subsequently removed. The abdomen was closed and the animal was repleted with 3 to 5 mL saline via the penile vein. The animals were kept in a 29°C incubator overnight while recovering from surgery.

Administration of drugs. Interleukin-10 was administered to the recipient rats as a single 1.5 μg IV dose immediately after the implantation of the donor kidney. Alpha-MSH was given as multiple 150 μg doses IV at 0, 6, and 16 hours after the implantation of donor kidney. The animals also received similar doses of α-MSH BID on the second post-operative day, and on the morning of the third post-operative day.

Cell culture

Mouse cortical tubule (MCT) and RAW 264.7 cells were grown as described in [15]. Briefly, MCT cells were grown in RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Atlanta Premium Select, Atlanta Biologics, Norcross, GA, USA). RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, non-essential amino acids, 4 mmol/L L-glutamine, penicillin, streptomycin, and 10% heat-inactivated FBS. MCT and RAW cells were seeded in 96-well microplates at concentrations of 1.2×10^4 and 7.5×10^4 cells per 100 μL per well. The cells were incubated for 30 hours at 37°C in 5% CO_2/air. MCT cells were treated after 30 hours with 0.35 U/mL mouse interferon-γ (IFN-γ) with vehicle, 0.35 nmol/L α-MSH, or 0.35 ng/mL IL-10 in an additional 100 μL complete-RPMI/well. The RAW cells were treated after 30 hours with 10 ng/mL lipopolysaccharide (LPS; *E. coli* 026:B6; Sigma) with vehicle, 0.35 nmol/L α-MSH, or 0.35 ng/mL IL-10 in an additional 100 μL complete DMEM/well. The treated cells were incubated for an additional 16 to 20 hours at 37°C. Nitrite concentrations were determined with Greiss reagent using $NaNO_2$ as a standard [15]. All assays were performed using three to four replicates, and repeated in at least four independent experiments.

Statistical analysis

All data presented as mean ± SEM. Different treatments were compared using ANOVA techniques (completely randomized design) followed by Dunnett's test for individual comparisons between group means. The null hypothesis was rejected when $P < 0.05$.

RESULTS

Effect of IL-10 on renal function following cisplatin administration

In preliminary studies, the dose dependency of IL-10 was investigated in male BALB/c and C57BL/6 mice

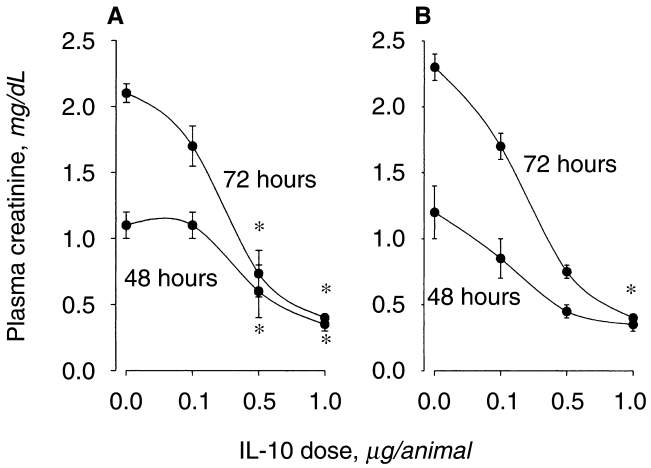

Fig. 1. Dose dependency of interleukin-10 (IL-10) on renal function following cisplatin administration in (*A*) BALB/c and (*B*) C57BL6 mice. Animals were given 20 mg/kg cisplatin IP, and either vehicle or 0.1, 0.5 or 1 μg IL-10 by IV injection at the time of cisplatin administration (3 to 5 BALB/c and 2 C57BL/6 mice per group). Blood was obtained at 48 and 72 hours. Animals were sacrificed at 72 hours. Data are mean ± 1 SE. $*P < 0.05$ vs. no IL-10 by ANOVA.

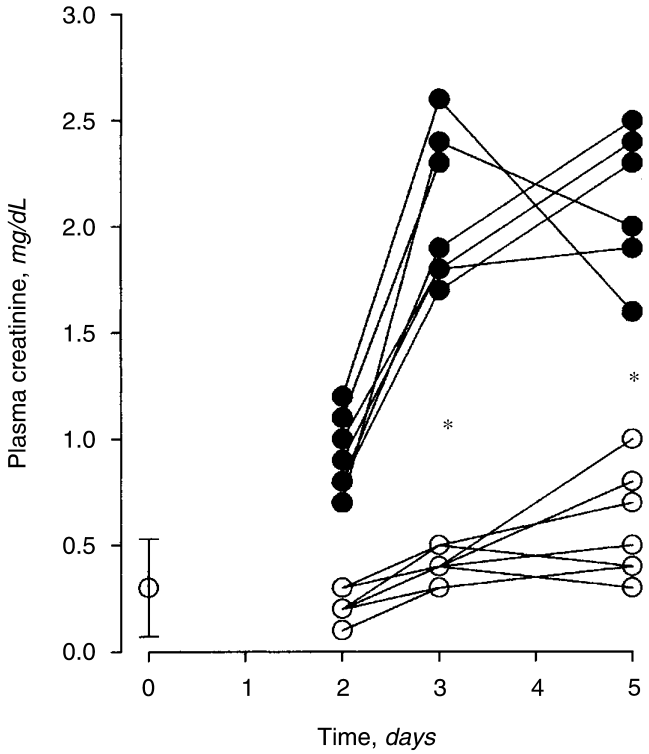

Fig. 2. Effect of IL-10 on renal function following cisplatin administration. BALB/c mice received IP cisplatin and vehicle (●) or 1 μg IL-10 by IV injection at the time of cisplatin administration (○). Data are mean ± 1 SE. $N = 8$ to 10 animals per group. $*P < 0.05$.

using plasma creatinine at 48 and 72 hours as an end point. We found that 1 μg of IL-10 given with cisplatin produced maximal inhibition of cisplatin-induced renal injury in both strains of mice (Fig. 1). A more extensive study in BALB/c mice (Fig. 2) demonstrated that 1 μg IL-10 significantly decreased renal injury when given at the time of cisplatin administration.

Effect of IL-10 histologic damage following cisplatin administration

To determine if the functional changes were associated with histologic evidence of protection, histologic changes at 72 hours after cisplatin were investigated. Cisplatin caused necrosis of proximal convoluted and straight tubules, casts, and leukocyte accumulation (Fig. 3A). IL-10 significantly decreased cisplatin-induced necrosis and intraluminal cast formation (Fig. 3B). The histologic changes were measured using semiquantitative scales (0 to 3+) for tubular necrosis, casts, and erythrocyte congestion, and quantitative counts of the number of neutrophils (Fig. 4). IL-10 inhibited tubule necrosis, cast formation, erythrocyte congestion, and leukocyte accumulation.

Delayed IL-10 administration

Most therapeutic agents have a "golden window of opportunity" beyond which they are not effective. For example, Kelly et al found that ICAM-1 antibody is not effective when therapy is delayed for one hour [13]. In contrast, IL-10 was still effective when treatment was delayed by one hour following cisplatin administration in both BALB/c and C57BL/6 mice (Fig. 5).

Effect of IL-10 following renal ischemia/reperfusion

Figure 6 shows the effect of administering 0.5 μg IL-10 or vehicle IV to BALB/c mice immediately after removal of the vascular clamps. IL-10 administration significantly reduced renal injury, as assessed by plasma creatinine 24 hours after ischemia. Ischemic kidneys showed widespread necrosis and sloughing of the proximal straight tubule with obstructing casts (Fig. 3C) and accumulation of leukocytes between tubules in the outer stripe of the outer medulla. There was also erythrocyte congestion in the inner stripe of the outer medulla (not seen in Fig. 3C). In contrast, kidneys of mice treated with IL-10 had less necrosis of the proximal straight tubule (Fig. 3D) and decreased leukocyte plugging. The histologic changes were measured by semiquantitative scores of necrosis and congestion, and by counting the number of leukocytes (Fig. 7). IL-10 significantly reduced the outer and inner stripe necrosis and outer stripe leukocyte infiltration, although there was no significant change in inner stripe congestion.

Effect of α-MSH and IL-10 following transplantation of a kidney subjected to warm ischemia

The effects of IL-10 alone and in combination with α-MSH were tested in a second species using a clinically

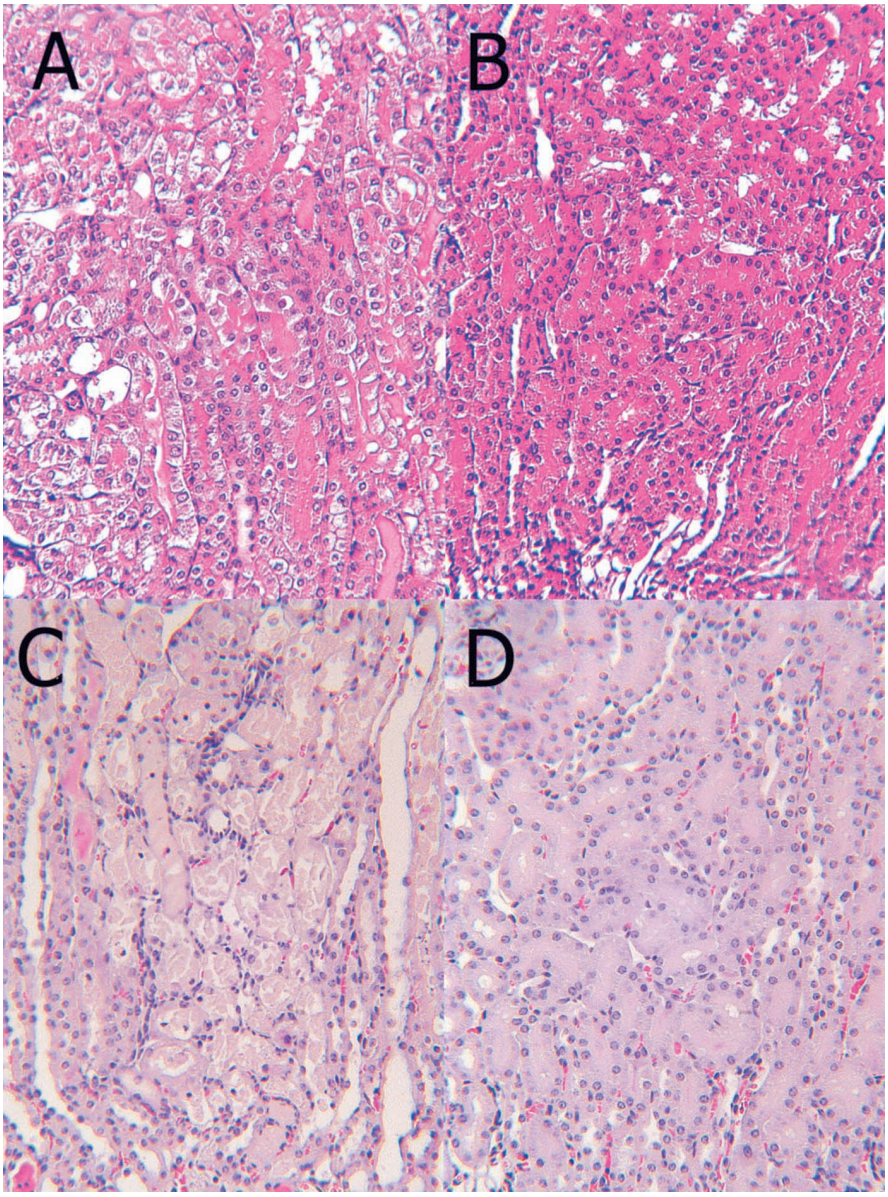

Fig. 3. Typical histology and leukocyte accumulation following cisplatin administration. Mice were studied 72 hours after cisplatin (*A, B*) or 24 hours after 40 minutes of ischemia (*C, D*). Mice were treated with vehicle (A, C) or IL-10 (B, D). Sections stained with H&E.

relevant model of renal transplantation from a marginal donor. The rat donor kidney was clamped for 40 minutes, reperfused for 30 minutes, flushed with preservation solution, stored overnight in cold preservation solution, and then transplanted into the same strain of rats. As would occur clinically, α-MSH and IL-10 were given only to the recipient rats. We found that both agents alone, and in combination, increased recovery of renal function following transplantation (Fig. 8) that was particularly evident on days 2, 3, and 5. There was no statistically significant synergy between the two agents on days 2 and 3.

Mechanism of action

Previous studies have shown that inhibition of TNF-α, ICAM-1, or NOS-II inhibits ischemia/reperfusion injury, whereas inhibition of ICAM-1 inhibits cisplatin-induced injury [13, 20–24]. We found that IL-10 inhibited the induction of TNF-α, ICAM-1, and NOS-II mRNA abundance after either cisplatin administration (Fig. 9, top) or ischemia (Fig. 9, bottom). Cisplatin and ischemia both increase apoptosis and cause proliferation of tubule cells [25–27]. IL-10 decreased the number of TUNEL (a measure of apoptosis; terminal deoxy transferase uridine triphosphate nick end-labeling), and PCNA (a measure of cell cycle activity; proliferating cell nuclear antigen) positive cells at day 3 following cisplatin administration (Fig. 4). The TUNEL positive cells were located in cortical tubules between medullary rays, presumably proximal convoluted tubules. The PCNA positive cells were located in damaged tubules in the outer stripe.

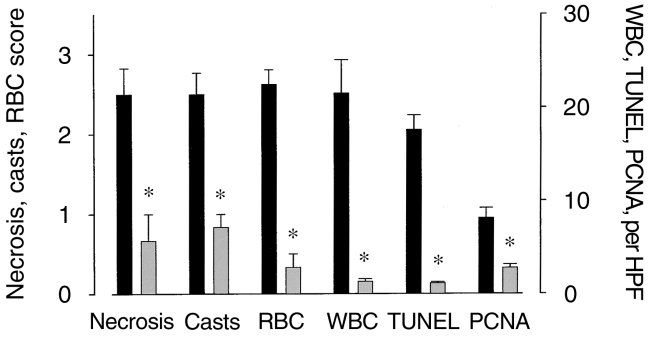

Fig. 4. Effects of IL-10 on semiquantitative histology and quantitative leukocyte accumulation, TUNEL, and PCNA staining in BALB/c mice following cisplatin administration. A description of the methodology is in the **Methods** section. Symbols are: (■) vehicle; (▩) IL-10. Data are mean ± 1 SE. Number of animals are 4 to 8 cisplatin, and 3 cisplatin + IL-10. *$P < 0.05$ vs. vehicle.

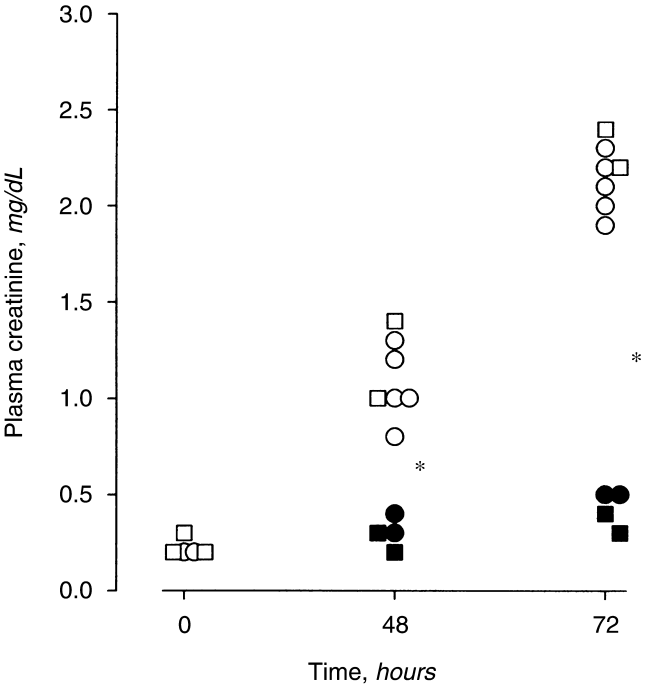

Fig. 5. Effect of delayed IL-10 on renal function following cisplatin administration. Animals received 1 μg IL-10 IV one hour after cisplatin. Data are mean ± 1 SE. Data from BALB/c mice are displayed as circles; C57BL/6 mice are shown as squares; open symbols are vehicle and closed symbols are IL-10. *$P < 0.05$.

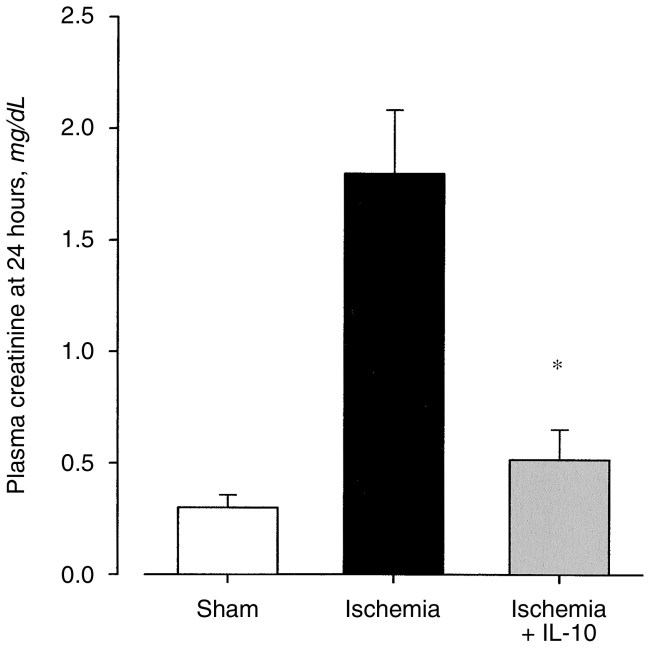

Fig. 6. Effect of IL-10 on renal function following ischemic injury. BALB/c mice were subjected to 40 minutes of ischemia, received vehicle or 0.5 μg IL-10 IP after clamp release, and sacrificed after 24 hours. Number of animals are 3 sham, 5 ischemia, and 6 ischemia + IL-10. *$P < 0.05$.

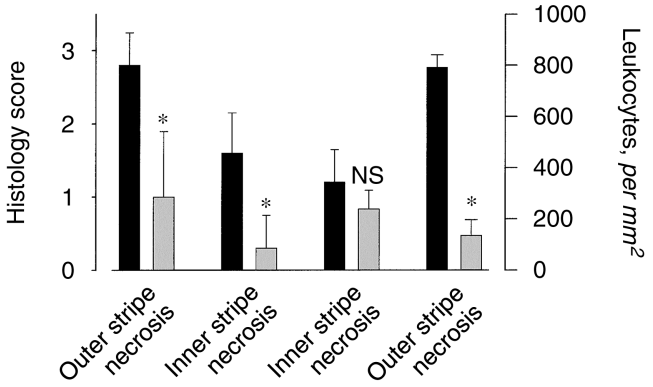

Fig. 7. Effects of IL-10 on semiquantitative histology and quantitative leukocyte counts in BALB/c mice subjected to 40 minutes of ischemia. A description of the methodology is in the **Methods** section. Data are mean ± 1 SE. Number of animals are 3 sham, 5 ischemia, and 6 ischemia + IL-10. Symbols are: (■) vehicle; (▩) IL-10; *$P < 0.05$ vs. vehicle.

Effects of IL-10 in proximal tubule cells in vitro

α-Melanocyte stimulating hormone inhibits nitric oxide production in macrophages and mouse cortical tubule (MCT) cells [15, 17]. To test if IL-10 inhibited the induction of nitric oxide in MCT cells, cells derived from mouse cortical proximal tubules were used. As a positive control, we tested IL-10 in RAW macrophage cells known to respond to IL-10 [28]. IL-10 inhibited LPS/interferon-γ–stimulated nitrite production in both RAW cells and MCT cells (Fig. 10). The inhibition was similar to that caused by α-MSH as previously described [15].

DISCUSSION

Interleukin-10, initially denoted cytokine synthesis inhibitory factor, was originally identified as an activity produced by Th2 helper T cells that inhibited cytokine synthesis by Th1 helper cells in the presence of monocytes-macrophage antigen presenting cells. This effect was found to be indirect via inhibitory actions on macro-

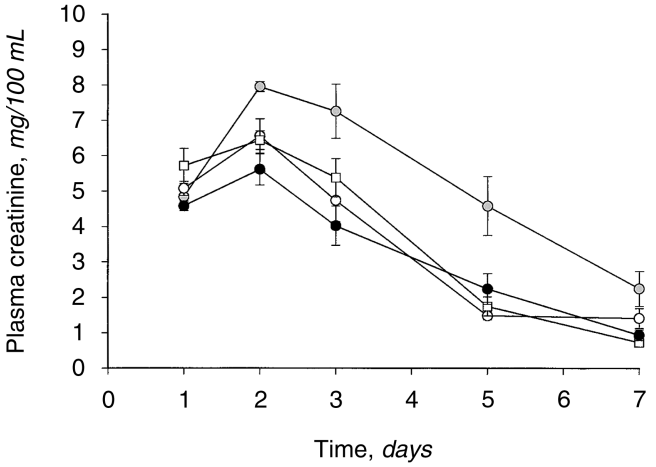

Fig. 8. Effect of α-melanocyte stimulating hormone (α-MSH), interleukin-10 (IL-10), or both agents on recovery from transplantation of a kidney subjected to 40 minutes of warm ischemia. Six to 7 animals per group; details are in the text. Symbols are: (⊜) vehicle; (○) α-MSH; (□) IL-10; (●) α-MSH + IL- 10. $P < 0.05$ vs. vehicle by ANOVA for α-MSH on days 2, 3, and 5; IL-10 on days 2, 5, 7; MSH and IL-10 on days 2, 3, and 5.

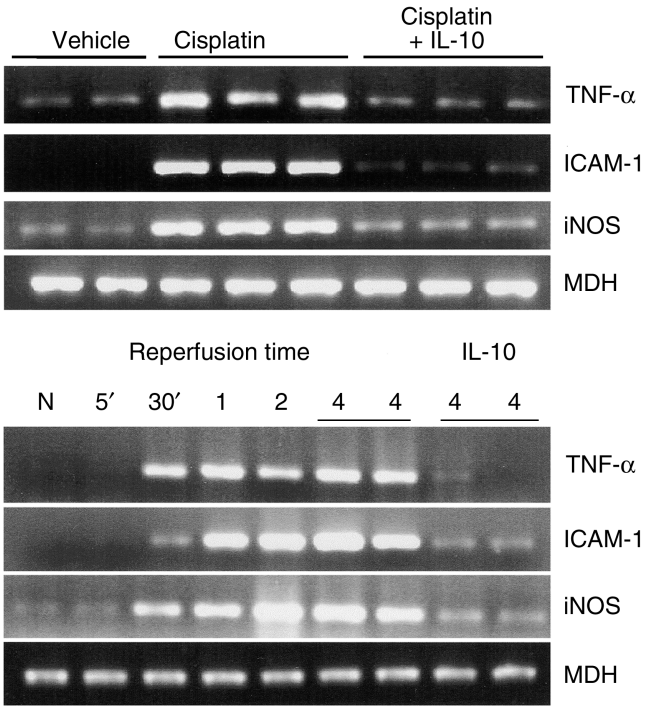

Fig. 9. Effect of IL-10 on mRNA abundance of TNF-α, intracellular adhesion molecule-1 (ICAM-1), and inducible nitric oxide synthase (NOS-II) following cisplatin administration or ischemia/reperfusion. Top: Animals were administered 20 μg/kg cisplatin IV, given vehicle or 1 μg IL-10 IV, and then sacrificed at 4 hours. The RNA was extracted, and RT/PCR performed as described in the **Methods** section. Bottom: Animals were subjected to 40 minutes of ischemia, given vehicle or 1 μg IL-10 IV, and then sacrificed at 30 minutes to 4 hours.

phages [29]. IL-10 is a pleotrophic cytokine with many immunosuppressive effects, and a few immuno-stimulatory effects [reviewed in [30]. IL-10 is also a potent anti-inflammatory cytokine that suppresses the activation of neutrophils and monocytes, and their production of chemokines, cytokines, and nitric oxide in animals and humans [8, 31–33]. Thus, IL-10 acts at multiple sites in the inflammatory cascade. IL-10 inhibits tissue injury, neutrophil mediated inflammation, and induction of iNOS. Exogenous IL-10 decreases death from endotoxin sepsis [34, 35], and prevents tissue injury in a wide variety of animal models. In humans, pretreatment with IL-10 attenuates endotoxin-induced fever, proinflammatory cytokine production, and neutrophil accumulation in lungs [33]. Unfortunately, when given 30 minutes after endotoxin, IL-10 has limited effects. IL-10 inhibits neutrophil migration and LPS-induced cytokine (IL-1, TNF-α) and chemokine (IL-8) release [32, 36, 37]. IL-10 inhibits neutrophil-mediated tissue injury in hind limb [11], visceral [38], and lung [9] ischemia models. IL-10 also decreases LPS-stimulated neutrophil accumulation in humans [33]. IL-10 inhibits the production of iNOS in whole animals and macrophages [32, 34, 39, 40]. IL-10 knockout mice have unregulated production of NO following LPS infusion, which contributes to the high mortality [34]. Taken together, there is abundant evidence that IL-10 inhibits neutrophil and iNOS pathways that cause tissue injury.

This study found that (*1*) IL-10 decreased renal injury in three different models of renal injury (cisplatin, ischemia, transplantation of a marginal kidney) in two species (mouse and rat) and two different mouse strains (BALB/c and C57BL/6). (*2*) IL-10 dramatically inhibited both inflammatory, cytotoxic, and apoptotic pathways of renal injury. (*3*) In contrast to many other agents, IL-10 was effective even when started after cisplatin. The significance of these findings is discussed in the next section.

Effect of IL-10 on renal injury

Acute renal injury from ischemia/reperfusion and cisplatin are known to share many common features, including similar histologic changes [necrosis of the proximal straight tubule, cast formation, and leukocyte accumulation], gene induction [ICAM-1, TNF-α], and gene repression [pre-pro-EGF] [13, 41, 42]. Several studies have suggested that cisplatin causes renal ischemia, because of changes in renal hemodynamics, leukocyte accumulation in the outer stripe of the outer medulla, and protection by the vasodilator verapamil [13, 14, 43]. Finally, inhibition of ICAM-1 decreases renal injury and leukocyte infiltration in both models [13, 22-24, 44]. Since IL-10 also inhibits neutrophil infiltration [8], IL-10 was tested in both cisplatin and ischemia models. We found that a single injection of IL-10 inhibited renal damage based on its ability to inhibit rises in plasma creatinine following cisplatin administration in BALB/c and C57BL/6 mice (Figs. 1 and 2). IL-10 also demonstrated marked protec-

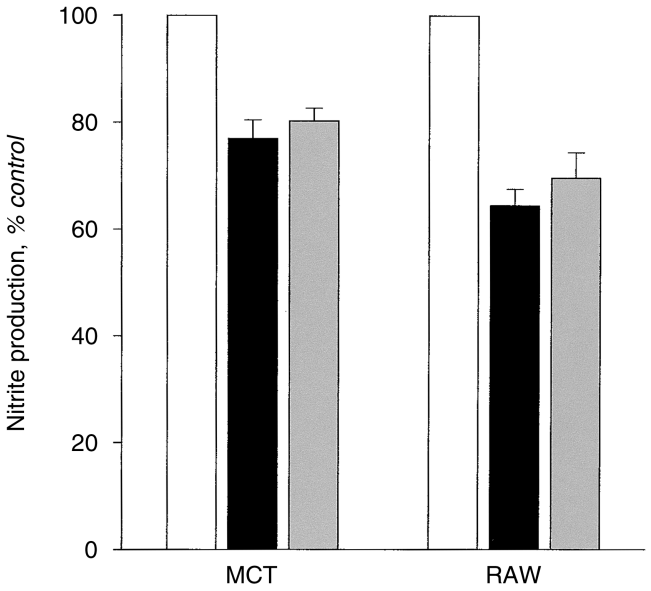

Fig. 10. Effect of IL-10 and α-MSH on nitrite production from mouse cortical tubule cells or RAW 264.7 cells. MCT cells were stimulated with IFN-γ. RAW cells were simulated with LPS. All assays were performed using 3 to 4 replicates, and repeated in at least 4 independent experiments. *$P < 0.05$ vs. control. The control and α-MSH data were published previously [15]. Symbols are: (□) control; (■) IL-10; (▩) α-MSH.

tion from renal dysfunction following ischemia reperfusion injury (Figs. 6 and 8). These effects were confirmed at the histologic level; IL-10-treated kidneys had less necrosis of proximal tubules in both models (Figs. 3, 4 and 7). IL-10 inhibited erythrocyte accumulation following cisplatin (Fig. 4) but not following ischemia (Fig. 7). The reasons and pathophysiological significance for this disparity are unknown. Erythrocyte accumulation is largely secondary to plugging of the ascending vasa recta by leukocytes, with accentuation by egress of plasma water due to high pressure or increased capillary permeability. IL-10 had a numerically greater percent inhibition of leukocyte accumulation in the cisplatin model than in the ischemia model, thus potentially leaving more blockages in the ischemic vasa recta. However, the ischemia protocol was performed using a smaller dose of IL-10; the 0.5 μg dose of IL-10 was also less effective that the 1 μg dose in the cisplatin model (Fig. 1). Also, a single dose of IL-10 may not have been sufficient for the cisplatin model, as there was a rise in creatinine from day 3 to day 5 (Fig. 2). Since we did not perform extensive dose optimization studies in either model, some of these small differences may be caused by different doses employed.

We also tested IL-10 in a second species (rat) using a more clinically relevant model of renal ischemia, that is, transplantation of a marginal kidney. A single dose of IL-10 and/or six doses of α-MSH given either individually or together increased the recovery of renal function following transplantation of a marginal donor kidney (Fig. 8). While there was a trend toward protection at 24 hours, a statistically significant protective effect may have been obscured by the intensity of renal damage (plasma creatinine near 5 mg/dL at 24 hours). Although some of the effects of α-MSH may be mediated by IL-10 [45], we used this severe model of injury to test for synergy, which might imply that the two agents have different mechanisms of action. We did not detect significant synergy; however, we did not optimize either α-MSH or IL-10 dosage regimens in this model. IL-10 and α-MSH could either inhibit a second wave of renal injury or accelerate the repair process, akin to growth factors that only minimally inhibit renal damage as assessed at 24 hours, but accelerate repair [46, 47]. This issue cannot be resolved since we did not obtain renal biopsies at 24 to 48 hours post-transplant. However, it is unlikely that IL-10 enhanced recovery following cisplatin administration, since IL-10 decreased cell cycle activity measured by PCNA labeling (Fig. 4).

Taken together, IL-10 reduces renal ischemia-reperfusion and cisplatin-induced injury in two mice strains and enhances recovery following renal transplantation in a second species (rat). The degree of renal protection of IL-10 is similar to that found in lung [9, 10], hind limb [11], and heart [12] ischemia-reperfusion models. IL-10 treatment has some advantages over several recently described therapeutic agents, either because of enhanced effectiveness or more prolonged window of opportunity. In the setting of ischemia, IL-10 is more effective than anti-ICAM-1 antibodies [22], α_1 acid glycoprotein [48], α_1-antitrypsin [48], A_{2a} adenosine receptor blockers [49], or a poly(ADP-ribose) polymerase inhibitor [50] based upon the degree of reduction in creatinine at 24 hours. IL-10 may be as effective as a soluble P-selectin ligand [51] or antisense oligonucleotides to inducible nitric oxide synthase [52]; however, these agents had to be perfused directly into the renal artery, or given 30 minutes to 8 hours before ischemia. In the cisplatin model, IL-10 is as effective as disopyramide or verapamil [53], ebselen [54], methimazole [55] or melatonin [56], although these drugs had to be started 30 to 240 minutes before cisplatin. IL-10 is more effective than glycine [57] and as effective as hepatocyte growth factor (HGF), but HGF required dosing every six hours [58].

Mechanism of action

Interleukin-10 inhibited leukocyte accumulation in both ischemia and cisplatin models (Figs. 3, 4, and 7); therefore, our study initially focused on cytokines and adhesion molecules that cause leukocyte infiltration in acute inflammation, increase rapidly after ischemia, and blockade reduces renal injury [41, 59]. For example, TNF-α activates neutrophils and monocytes-macrophages. Renal TNF-α mRNA and protein are rapidly increased

within 30 minutes and one hour after ischemia, and neutralization of TNF-α reduces damage and leukocyte accumulation after ischemia [20, 21, 24, 51]. Leukocyte adherence to endothelial cells is promoted by ICAM-1 expressed on endothelial cells. ICAM-1 mRNA is induced after both ischemia and cisplatin administration; blocking ICAM-1 by neutralizing antibody or knockout mice reduces renal damage and leukocyte infiltration after ischemia or cisplatin administration [13, 22–24, 44, 51]. After confirming that TNF-α mRNA abundance was rapidly increased after ischemia (Fig. 9, top), the previous findings were extended to show that TNF-α is also increased after cisplatin administration (Fig. 9, bottom). IL-10 inhibited induction of TNF-α mRNA abundance in both ischemia and cisplatin models, an effect seen previously in activated macrophages and mesangial cells [60, 61], and in peripheral blood monocytes from patients with psoriasis receiving IL-10 [62]. We found that ICAM-1 mRNA is dramatically increased one hour after renal ischemia, and four hours after cisplatin (Fig. 9). IL-10 inhibited induction of ICAM-1 mRNA after ischemia or cisplatin (Fig. 9), as seen previously following immune complex lung injury [63]. Induction of both TNF-α and ICAM-1 occur early after ischemia, before many leukocytes accumulate in the kidney [7, 16, 51, 64, 65], suggesting that these two mediators are produced by intrinsic kidney cells to attract leukocytes to the kidney. In addition to these indirect effects on leukocyte function, IL-10 also directly inhibits neutrophil and macrophage function (Fig. 10 and [8, 60]). Thus, IL-10 may have such dramatic effects on both ischemic- and cisplatin-induced renal injury because of its direct (leukocyte) and indirect (cytokine, adhesion molecule) effects. Taken together, the above findings indicate that IL-10 inhibits the activation of genes that promote leukocyte activation, adhesion, and function, and that this inhibits an acute inflammatory response that occurs following renal ischemia and cisplatin administration.

Ischemia induces NOS-II, and inhibition of NOS-II either by antisense oligonucleotides or knockout mice decreases renal damage following renal ischemia [52, 66]. In contrast, the role of NOS-II following cisplatin administration is unknown. Since IL-10 inhibits induction of nitric oxide production [28], we examined the effect of IL-10 on renal NOS-II following cisplatin and found that NOS-II was induced following cisplatin (Fig. 9, top). Furthermore, IL-10 inhibited both ischemic and cisplatin-induction of NOS-II (Fig. 9). Since IL-10 inhibits induction of NOS-II in cultured mouse RAW cell macrophages and mouse cortical tubules (Fig. 10), this suggests that these stresses induce NOS-II via a common pathway that is inhibited by IL-10. IL-10 is likely to inhibit the transcription of NOS-II, since NOS-II is primarily regulated by transcription. That IL-10 also inhibits NOS-II induction in vivo and NO production in vitro suggests that part of IL-10's actions occur via inhibition of NOS-II induction by ischemia or cisplatin. Whether the induction occurs by the initial injury (ischemia or cisplatin) per se or by subsequent events (that is, cytokines) initiated during the subsequent waves of injury is unknown.

Effect on apoptosis and cell cycle activity

Apoptosis and cell proliferation are increased following both ischemia and cisplatin-induced renal injury [26, 27, 67]. We found that IL-10 reduced the number of positive staining cells in both the TUNEL assay (a marker for apoptosis) and in the PCNA assay (a marker of cell cycle activity) following cisplatin administration (Fig. 4). The inhibition of TUNEL assay would be expected if there was less injury, and hence, less response of the kidney to injury, as occurs following scavenging of free radicals with dimethylthiourea [27], or prevention of ischemic injury with α-MSH [68]. IL-10 decreased the number of PCNA positive cells following cisplatin administration, suggesting that IL-10 treated kidneys have less cell cycle activity, which may be linked to its cytoprotective effects. This finding also indicates that IL-10 is unlikely to enhance tissue repair following cisplatin administration. Further studies, for example, at different time points in both models of injury, would be needed to solidify these conclusions.

IL-10 is effective when administered after cisplatin

The timing of cisplatin administration is easy to determine, and hence, there is sufficient time for patients to receive non-specific preventive strategies such as hydration, mannitol, and/or furosemide [69]. However, these therapies are not always successful and medication errors could prevent correct prophylaxis from being delivered, so an effective therapy that can be started after administration of cisplatin would be useful. Knowledge of the window of opportunity can aid in understanding the mechanism of action. IL-10 was protective even when administration is begun one hour after cisplatin (Fig. 3), reminiscent of the protective effects of α-MSH when started six hours after ischemia [7]. This suggests that injury does not occur because of cisplatin administration per se, but is a consequence of the maladaptive activation of injury pathways such as TNF-α, ICAM-1 and NOS-II. Delayed administration of ICAM-1 antibodies or glycine do not protect against cisplatin-induced renal injury [13, 57]. Each of these agents may inhibit one injury pathway that is protective if started early enough; however, evidently both α-MSH (following ischemia) and IL-10 (following cisplatin) have a broader spectrum of activity against injury pathways. If these injury pathways are not activated, as occurs with IL-10 following cisplatin-administration (or α-MSH following ischemia) then renal injury does not ensue. Alternatively, IL-10 may promote recovery of cellular function, perhaps by

preventing destruction of renal architecture that provides a scaffold for cells to repair the damage during the recovery phase of acute renal failure.

Physiological significance

The ability of IL-10 to inhibit both ischemic- and cisplatin-induced acute renal failure is likely to be clinically important since ischemia is an important cause of acute renal failure in general, and acute renal failure after renal transplantation increases the morbidity and cost of renal transplantation, the risk of acute rejection, and may diminish long-term graft survival. The risk of post-transplant acute renal failure is particularly high following transplantation of "marginal kidneys" that have been subjected to prolonged warm or cold ischemia. Therefore, better methods and drugs are desperately needed that would reduce the risk of post-transplant ARF and thus allow expansion of the donor pool. The broad mechanism of action on both inflammatory and cytotoxic pathways is reminiscent of α-MSH. However, the extent of protection afforded after cisplatin administration distinguishes IL-10 from most other agents that protect against acute renal injury.

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APPENDIX

Abbreviations are: ARF, acute renal failure; HGF, hepatocyte growth factor; ICAM-1, intracellular adhesion molecule-1; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCT, mouse cortical tubule; MSH, melanocyte-stimulating hormone; NO, nitric oxide; NOS, NO synthase; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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