Keratinocyte-derived chemokine is an early biomarker of ischemic acute kidney injury

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Am J Physiol Renal Physiol 290: F1187–F1193, 2006. First published December 20, 2005; doi:10.1152/ajprenal.00342.2005.—Renal ischemia-reperfusion injury (IRI) is the leading cause of acute kidney injury (AKI; acute renal failure [ARF]) in native kidneys and delayed graft function in deceased donor kidney transplants. Serum creatinine rises late after renal IRI, which results in delayed diagnosis. There is an important need to identify novel biomarkers for early diagnosis and prognosis in renal IRI. Given the inflammatory pathophysiology of renal IRI, we used a protein array to measure 18 cytokines and chemokines in a mouse model of renal IRI at 3, 24, and 72 h postischemia. A rise in renal keratinocyte-derived chemokine (KC) was the earliest and most consistent compared with other molecules, with 3-h postischemia values being 9- and 13-fold greater than sham and normal animals, respectively. Histological changes were evident within 1 h of IRI but serum creatinine only increased 24 h after IRI. With the use of an ELISA, KC levels in serum and urine were highest 3 h postischemia, well before a significant rise in serum creatinine. The human analog of KC, Gro-α, was markedly elevated in urine from humans who received deceased donor kidney transplants that required dialysis, compared with deceased donor kidney recipients with good graft function and live donor recipients with minimal ischemia. Measurement of KC and its human analog, Gro-α, could serve as a useful new biomarker for ischemic ARF.

There are significant data that early inflammatory changes underlie the pathogenesis of renal IRI (3, 8, 11, 15, 21). We therefore used a mouse model of renal IRI to examine kidney, blood, and urine for evidence of changes in cytokine and chemokine expression using a protein array adapted to small volume samples. The earliest and most striking change was an increase in keratinocyte-derived chemokine (KC), a CXC chemokine that is structurally homologous to rat cytokine-induced neutrophil chemoattractant (CINC) and human growth-related oncogene-α (Gro-α) (19). KC levels were elevated in serum within 1 h of renal ischemia in the mouse and increased in kidney tissue and urine within 3 h. To begin to evaluate the clinical significance, we examined humans with ischemic injury following a renal transplant and found a correlation of urine Gro-α levels with kidney injury that required dialysis. These studies provide evidence that KC and its human analog, Gro-α, could be a valuable biomarker for early diagnosis and prognosis of ARF.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under pathogen-free conditions, according to National Institutes of Health guidelines.

Surgical Procedures

An established model of renal IRI was used (14), and sterile techniques were followed at all times. Briefly, mice weighing 25–30 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (75 mg/kg). Bilateral flank incisions were made, and the kidneys were exposed. Following abdominal incisions, renal pedicles were bluntly dissected and a nontraumatic vascular clamp (Roboz microaneurysm clamp; Roboz Surgical Instruments, Washington, DC) was applied across each of the pedicles for 30 min. During the procedure, animals were kept well hydrated with warm saline and at a constant temperature (37°C). After 30 min of ischemia, the clamps were removed, the wounds sutured, and the animals were allowed to recover with free access to food and water. Sham animals underwent similar surgical procedures without renal pedicle clamping. IRI and sham animals were killed at 1, 3, 24, and 72 h after ischemia or sham surgery, using intraperitoneal injection of pentobarbital sodium (150 mg/kg). Normal animals were also killed at time 0.

Sample Harvesting and Collection

At death, mice were euthanized, 0.5 ml of blood were collected from the inferior vena cava, and urine was collected by bladder...
puncture. Blood and urine were centrifuged to obtain serum and supernatant, respectively, and stored at −80°C for ELISA. One kidney was snap-frozen and stored at −80°C. The other kidney was fixed in formalin for histology.

Pathological Evaluation of Postischemic Kidneys

Upon death, one kidney from each mouse was dissected and cut coronally. These sections were fixed in 10% formalin and processed for histology using standard techniques as previously described (14).

Renal Function

Blood samples were obtained at death from all mice at various time points, and serum creatinine (Scr) levels were measured as a marker of renal function, using a 557A Creatinine kit (Sigma Diagnostics, St. Louis, MO) and analyzed on a Cobas Mira S Plus automated analyzer (Roche Diagnostics, Indianapolis, IN).

Protein Array for Cytokines in Kidney Tissue from Mice

Total protein analysis. Kidney samples were thawed over ice. Part of each kidney was homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL). The homogenate was centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was collected and used for the cytokine multiplex bead-based kit (Bio-Rad Laboratories, Hercules, CA). Tissue homogenates were centrifuged at 12,000 rpm for 15 min at 4°C, and supernatants were collected and used for the cytokine multiplex bead-based kit (Bio-Rad Laboratories). Proteins assayed included KC, macrophage inflammatory protein-1α (MIP-1α), RANTES, tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, granulocyte colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interferon-γ (IFN-γ), IL-12 (p40), IL-12 (p70), and IL-17. Cytokine assay plate layout consisted of eight standards in duplicate (32,000 to 1.95 pg/ml), two blank wells (for background fluorescence subtraction), and each sample in duplicate wells. The cytokine multiplex assay uses microbead and flow-based protein detection system (Bio-Plex Suspension Array System, Bio-Rad Laboratories) based on the Luminex xMAP technology. In this quantitative assay, surfaces of fluorescence-coded microbeads were conjugated to specific antibodies directed against each cytokine or chemokine. Each fluorescence-coded microbead type was conjugated to one specific capture antibody and consequently one specific target analyte. Each supernatant sample was first incubated with a mixture of all microbead types for 90 min at room temperature. Samples were then washed, incubated with a mixture of secondary biotinylated detection antibodies also directed against each target for 30 min at room temperature, washed again, and incubated with a streptavidin-coupled phycoerythrin reporter system for 10 min at room temperature. After a final wash, the samples were resuspended in buffer and subjected to flow cytometric analysis. The Bio-Plex system instrument uses fluids, laser excitation, fluorescence detection, and digital signal processing for individual scanning and microbead identification. Each bead taken from the sample was identified based on its internal fluorescence signature, and the phycoerythrin reporter signal associated with that bead was quantified. The data were analyzed using Bio-Plex Manager 3.0 software (Bio-Rad).

ELISA for KC in Mice Urine and Serum and for Gro-α in Human Urine

All samples were thawed over ice. KC was determined in mice urine and serum using a Quantikine mouse KC kit (MKC00, R&D Systems, Minneapolis, MN) as per the manufacturer’s instructions. The minimum detection limit for the KC assay is <2 pg/ml. Similarly, Gro-α was determined in human urine using a Quantikine human Gro-α kit (DGR00, R&D Systems) as per the manufacturer’s instructions. The minimum detection limit for the Gro-α assay is <10 pg/ml. For both assays, each plate included eight standards (1,000 to 7.8 pg/ml), blank, and samples in duplicate.

Human Urine Collections

After obtaining institutional IRB approval, urine samples from adult patients were analyzed on postoperative day 2 after living and deceased donor kidney transplants. The samples were collected and immediately placed on ice. The samples were centrifuged for 15 min at 3,000 rpm at 4°C. The supernatant was decanted and stored at −80°C until analysis. Kidney biopsies were performed 1 wk post-transplant as clinically indicated for those considered having delayed (requiring dialysis) or slow improvement graft function.

Comparison, Quantification, and Statistical Analysis of Data

All data involving animal experimentation were collected in a double-blind fashion. Sigma Stat 8.0, Sigma Plot 8.0, and MS Excel software were used for the statistical analysis and data presentation. The experimental data are expressed as means ± SE. Statistical analysis included one-way ANOVA with appropriate post hoc analysis to test differences between means of various groups, and P < 0.05 was considered to be statistically significant.

RESULTS

Scr and Kidney Histology in Mice after IRI

We evaluated Scr at various times following renal ischemia to define the severity and timing of renal failure in our model to correlate this with changes in potential biomarkers (Fig. 1). Compared with baseline values (0.64 ± 0.86; n = 10), Scr was significantly increased in mice 24 h (2.88 ± 0.4; n = 10) and 72 h (2.4 ± 0.87; n = 10) after ischemia. Kidney histology evaluated at intervals postischemia revealed that even by 1 h postischemia, red blood cell congestion and slight cast formation at the outer medulla were present compared with sham-operated animals. Furthermore, the cytoplasm of some tubular epithelial cells was condensed and the endothelium was slightly edematous (Fig. 2B). At 3 h postischemia, the histological changes were more pronounced, with increased erythrocyte sludging in capillaries, condensation of tubular epithe-
lium cytoplasm, epithelial cell necrosis, swelling of endothelial cells with basal membrane widening, and moderate cast formation (Fig. 2D). At 24 and 72 h postischemia, there was significant tubular injury, characterized by extensive tubular necrosis and cast formation. Endothelial cells were markedly edematous, basal membranes were widened immensely, and capillaries were filled with erythrocytes (Fig. 2, F and H).

Cytokines and Chemokines in Murine Renal Tissue

We utilized a cytokine multiplex assay to determine protein levels of 18 cytokines/chemokines in kidney tissue from normal (no surgery) and sham-operated (surgery but no renal artery clamping) or IRI mice at intervals after surgery. KC levels in ischemic kidney tissues were significantly increased 3 h postischemia compared with normal and sham groups (P < 0.05; Fig. 3). There were also significant early increases in IL-6 and IL-12 (p40) at 3 h after ischemia in IRI mice compared with sham-operated mice (Fig. 4). TNF-α showed a trend to increase with time postischemia. Meanwhile, IL-1α, IL-2, IL-4, and IFN-γ showed significantly decreased decline trends during time course in IRI mice compared with sham group. MIP-1α was significantly higher 24 h after IRI compared with all groups (P < 0.05). No differences were noted across groups.
for levels of IL-1β, IL-3, IL-5, IL-10, IL-12 (p70), IL-17, GM-CSF, and RANTES (data not shown).

**KC in Mice Serum**

After identifying an increase in KC with the multiplex assay, we measured KC levels in serum taken from normal, sham-surgery, or IRI mice using an ELISA assay. Compared with normal or sham-operated mice, KC levels in serum were significantly elevated at 1 and 3 h postischemia and dropped to baseline levels by 24 h (Fig. 5).

**KC in Mice Urine**

To examine whether urine KC could be a useful noninvasive marker of renal IRI, we measured KC in urine from normal mice, as well as at early time points in urine from sham surgery or IRI mice. Urine KC levels were comparable in normal, sham, and ischemic mice at 1 h IRI; however, they were significantly increased at 3 h posts ischemia in ischemic mice compared with sham-operated mice (Fig. 6).

**Gro-α in Human Urine During Renal IRI**

A major goal of this work is to identify pathways or markers using controlled models that have future relevance to human ischemic renal injury. To examine whether the elevation in urine KC in mice with renal IRI was translatable to humans, we hypothesized that the human analog of KC, Gro-α, correlates with AKI in patients during kidney transplantation. We performed a pilot study in patients after kidney transplantation measuring day 2 urine after they had come out of the ICU. Recipients of deceased donor kidneys had higher SCr than those with live donor kidneys on day 2 after transplantation (Fig. 7). Of the four groups represented in Fig. 7, only patients who received deceased donor kidneys progressed to requiring short-term dialysis until resolution of the ARF. This group had significantly higher urine Gro-α levels than did the groups who received deceased or live donor kidneys with good graft function (Fig. 8). Those with elevated day 2 urine Gro-α levels also had evidence of ATN on the day 7-10 biopsies (data not shown). Three of five recipients of deceased donor kidneys with prompt graft function had biopsies: two had focal ATN with isometric vacuolization, and one had moderate ATN and acute antibody-mediated rejection. Renal biopsy from only one of six patients who received deceased donor kidneys with delayed graft function showed severe ATN with isometric vacuolization. From the recipients of live donors, one of four had graft function initially but then deteriorated, and was found to have mild focal ATN. Of the two patients with live donor kidneys with slow graft function (suboptimal improvement in graft function but not serious enough to require dialysis), one had mild acute tubular injury with focal interstitial hemorrhage, and one had severe acute cellular rejection with arteritis.

**DISCUSSION**

Ischemic AKI remains a major diagnostic and therapeutic problem in native kidneys and allografts. A major barrier to clinical trials in renal IRI has been the late start of therapy, in part, due to a lack of early biomarkers. We utilized a new protein array technique to identify potential pathophysiological mediators of the inflammatory injury following renal IRI and unexpectedly observed a very early rise in KC in kidney and blood, indicating that KC may be useful as an early biomarker for renal IRI. Preliminary studies translating this to humans demonstrated that the human analog of KC, Gro-α, can be measured in urine early after kidney transplantation and correlates with increased early injury.

The crucial role of inflammation and the immune response in the pathogenesis of ischemic ARF are well established, with
many proinflammatory cytokines/chemokines being upregulated in the kidney at the protein or mRNA level after renal IRI (3, 8, 11, 15, 21). We therefore used a cytokine/chemokine protein array to analyze the protein expression patterns in postischemic murine kidney at early and later times after IRI. We observed a time-dependent increase in several potential mediators in the postischemic kidney, including KC, MIP-1α, IL-6, IL-1α, and IL-12 (p40). Of these, we focused on KC for several reasons. First, the marked upregulation of KC in ischemic kidney was relatively selective. Second, the increase in KC preceded the changes seen for other potential mediators. Finally, the increase in KC occurred well before a rise in SCr.

KC can be secreted in most organs by a variety of cells including neutrophils, epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, macrophages, platelets, and lymphocytes (19). The main stimuli for KC production identified to date are IL-1, TNF-α, bacterial products, radical oxygen species, and LPS, as well as the T cell products IL-4 and IFN-γ which are often present at the site of injury (19). One study found renal KC mRNA to be increased between 1–4 h after IRI (16). In another study, kidney KC mRNA and protein levels...
peaked at 9 h postischemia in C57BL/6 mice after renal IRI, and antibodies to KC administered just before reperfusion produced a decrease in neutrophil infiltration and lower SCr compared with mice treated with control serum (11). Unilateral clamping of the left renal pedicle for 45 min in Swiss mice produced a peak in KC mRNA levels in left kidney at 6 h (2). Our findings are consistent with the results of those studies but derived from a broader-based search for potential inflammatory mediators using the multiplex cytokine assay. Armed with this information, we hypothesized that KC protein could be a useful early biomarker following renal IRI. Using an ELISA technique, we found that serum KC was elevated as early as 1 h postischemia and decreased to baseline by 24 h. Because the composition of urine can provide insights into pathophysiological events in the kidney, and in humans can be noninvasively collected, we measured urine KC as a potential early indicator of AKI. KC was markedly increased in the urine by 3 h after IRI in mice. To extend this novel finding, we performed preliminary studies in humans to examine whether these findings were translatable for potential diagnosis and prognosis of renal injury in patients. There was a significant increase in the human KC analog, Gro-α, in the urine samples from deceased donor kidney transplant patients with delayed graft function requiring dialysis compared with those with prompt graft function or live donor kidney transplant recipients, and this correlated with ischemic ATN on biopsy. These findings indicate that urine Gro-α levels may be a useful biomarker for detection of significant ischemic ATN, thereby facilitating noninvasive identification of patients who are candidates for intervention trials for ischemic ARF. However, we recognize that early allograft injury is more complex than ischemia reperfusion alone and reflects an interplay of additional immunological and toxic factors.

Fig. 6. Increased urine KC early after IRI. KC in urine from normal mice (0 h-no surgery) and mice that underwent IRI or sham surgery and killed at 1 and 3 h after surgery. **P < 0.02; ***P < 0.0001, n = 8 per group.

Fig. 7. Elevated day 2 serum creatinines in humans with deceased donor kidney transplants compared with those with live donor kidney transplants. Groups include patients who received deceased donor kidneys with (gray bar) prompt or (open bar) delayed graft function, or live donor kidneys with (filled bar) prompt or (hatched bar) slow graft function. *Deceased kidney recipients with delayed graft function have significantly higher serum creatinines than living kidney recipients with slow graft function. **Living kidney recipients with prompt graft function have significantly lower serum creatinines than all groups.

Fig. 8. Elevated day 2 urine Gro-α patients with deceased donor kidney allografts and delayed graft function. Groups include patients who received deceased donor kidneys with (gray bar) prompt or (open bar) delayed graft function, or live donor kidneys with (filled bar) prompt or (hatched bar) slow graft function. *Deceased kidney recipients with delayed graft function have significantly higher Gro-α than deceased kidney recipients with prompt function and living with slow graft function.

There are a number of new potential biomarkers for AKI. Lipocalin has recently been shown to be highly sensitive of kidney injury in a recent study of children after cardiac bypass (10). KIM-1 has been shown to correlate closely with various forms of kidney injury and may well be a pathogenic molecule in the development of ARF as well (6). IL-18 has shown increasing promise, as have other cytokines (7, 12). Urinary enzymes are also potentially valuable biomarkers (20). Despite the promise for clinical use of these markers, there is still an important need to develop and validate new markers of ARF.

Our current study focused on biomarker discovery and initial validation of KC and its human analog Gro-α. However, the role of KC in the injury or repair process has not been fully worked out. Despite studies demonstrating a protective role of KC blockade (11), blocking KC has enhanced plaque formation in apoE-deficient mice (9). Thus KC appears to have multifaceted roles. In addition, immunodetection of KC in serum and urine may not correlate with function, and further studies including examining chemokinesis-inducing activity of KC in these fluids are required (18).

These data, starting with a protein array in mice, then validating with ELISA, followed by pilot studies in humans,
suggest that murine KC, as well as its human analog Gro-α, is a novel early biomarker for ischemic AKI. Future studies specifically designed to test this in humans are required to validate this. In addition to being a biomarker, KC/Gro-α may play a causative role in the pathophysiology of ischemic renal injury and therefore is a potential target for novel therapies to treat this clinically important problem.

REFERENCES


