Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury

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Kidney injury molecule-1 (Kim-1) is a type 1 membrane protein with extracellular immunoglobulin and mucin domains. The mRNA and protein for Kim-1 are expressed at very low levels in normal rodent kidney, but expression increases dramatically after injury in proximal tubule epithelial cells in postischemic rodent kidney and in humans during ischemic acute renal failure. To evaluate the utility of Kim-1 as a biomarker for other types of renal injury, we analyzed tissue and urinary expression in response to three different types of nephrotoxicanants in the rat: S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), folic acid, and cisplatin. Marked increases in Kim-1 expression were confirmed by immunoblotting in all three models. The protein was shown to be localized to the proximal tubule epithelial cell by immunofluorescence. Furthermore, Kim-1 protein was detected in urine of toxicant-treated rats. The temporal pattern of expression in response to TFEC is similar to the Kim-1 expression pattern in the postischemic kidney. In folic acid-treated kidneys, Kim-1 is clearly localized to the apical brush border of the well-differentiated proximal tubular epithelial cells. After folic acid treatment, expression of Kim-1 is present in the urine despite no significant increase in serum creatinine. Cisplatin treatment results in early detection of urinary Kim-1 protein and diffuse Kim-1 expression in S3 cells of the proximal tubule. Kim-1 can be detected in the tissue and urine on days 1 and 2 after cisplatin administration, occurring before an increase in serum creatinine. The upregulation of expression of Kim-1 and its presence in the urine in response to exposure to various types of nephrotoxicanants suggest that this protein may serve as a general biomarker for tubular injury and repair processes.

Cisplatin; S-(1,1,2,2-tetrafluoroethyl)-L-cysteine; folic acid; acute renal failure; kidney toxicity

The renal proximal tubule epithelium is particularly sensitive to the toxic effects of ischemia, chemicals, and drugs (3, 4, 12, 37). Identifying renal tubular injury at an early stage has important implications for early intervention, clinical trials of therapeutic agents, and evaluation of potential nephrotoxicity of pharmaceuticals in animals and humans. In response to an ischemic or toxic insult, the kidney proximal tubule epithelium undergoes a complex series of events involving a temporal progression through 1) loss of polarity and cytoskeletal integrity; 2) necrosis and apoptosis; 3) dedifferentiation of surviving epithelial cells; 4) migration of this poorly differentiated (simplified) regenerative epithelium over the denuded basement membrane; 5) proliferation of surviving proximal tubule epithelial cells; and 6) differentiation of the regenerating epithelial cell to form a fully functional proximal tubule epithelium (16, 37, 39, 40).

A number of genes are altered in expression in the early phase of ischemia and nephrotoxic injury including fos, egr-1, hsp70, Kid-1, PCNA, clusterin, vimentin, HB-EGF, FGF-1, and KGF (13, 15, 16, 30, 31, 33, 40, 41). Despite the importance of the repair process for reestablishment of a functional nephron, the molecular mechanisms of repair are poorly understood. One approach to the understanding of the regulatory events in injury and repair is to identify genes that encode proteins that play roles in these processes. Such proteins might also serve as biomarkers of injury. We previously reported that the kidney injury molecule-1 (Kim-1) gene is markedly upregulated in the postischemic rat kidney (14). The Kim-1 gene product is a type 1 membrane protein with an ectodomain that contains immunoglobulin (Ig) and highly O-glycosylated mucin subdomains as well as multiple N-glycosylation sites. mRNA and protein are expressed at very low levels in normal human and rodent kidney (9, 14). After ischemia-reperfusion, Kim-1 expression is markedly upregulated in the proximal tubule epithelial cell. At later time points of reperfusion (24–48 h), cells that express Kim-1 also express vimentin, a marker of dedifferentiation and sometimes BrdU as a marker of proliferation. The structure of Kim-1 suggests that it may be involved in surface adhesion interactions of the proximal tubule epithelial cells in postischemic kidney. We demonstrated in cell cultures that the human KIM-11 ectodomain is cleaved by a metalloprotease and shed into the culture media (2). In a clinical study, we detected KIM-1 protein in the urine of patients with acute tubular necrosis (9).

Nephrotoxicity is a common side effect of therapeutic interventions and environmental exposure to toxicants in the workplace. Although early biomarkers for nephrotoxicity are available, they often lack sensitivity and are not specific as indicators of epithelial cell injury (27, 32). Thus improved biomarkers could contribute greatly to the monitoring of renal
injury and repair in clinical studies, drug safety evaluation, and exposure to environmental toxins. Although it is clear that Kim-1 is upregulated in the postischemic kidney, it is not clear whether its expression serves as a general biomarker for tubular injury and processes that lead to repair. Therefore, we investigated Kim-1 expression following injury induced by three well-characterized nephrotoxins with different mechanisms of injury: S-(1,1,2,2-tetrafluoroethyl)-L-cystein (TFEC), cisplatin, and high-dose folic acid. We found that the Kim-1 protein is expressed at high levels in the kidney proximal tubule in response to all three nephrotoxins and localizes to the proximal tubules. The temporal pattern of expression in response to TFEC is similar to the Kim-1 expression pattern in the postischemic kidney. In folic acid-treated kidneys, Kim-1 is clearly localized to the apical brush border of the well-differentiated proximal tubular epithelial cells despite no change in serum creatinine concentration. Cisplatin treatment results in detection of tissue and urine Kim-1 protein before a measurable increase in serum creatinine levels. These results suggest that Kim-1 may be a general biomarker for nephrotoxicity. Despite the fact that, in all cases other than in humans (9), our current detection methods in urine are limited to Western blot analysis, an increase in Kim-1 can be found when there is no significant increase in serum creatinine (folic acid) or before a significant increase in serum creatinine (cisp].

METHODS

Treatment of Animals and Preparation of Kidney Tissue Samples

TFEC preparation and administration were carried out as described (16). Male Fischer 344 or Sprague-Dawley rats weighing 200–440 g received 20 mg/kg TFEC in water or saline by intraperitoneal injection. At the appropriate time point, treated animals were killed by CO2 asphyxiation and their kidneys were removed. A 2- to 4-mm-thick piece of tissue was trimmed into a core of kidney tissue spanning the outer cortex to the papilla. To prepare tissue enriched for injured and regenerating proximal tubules in the toxicant-exposed kidney, the outer stripe of the outer medulla (OSOM) region was dissected out from this tissue sample and processed for immunoblot analysis. This technique has been employed for analysis of growth factor protein and mRNA in prior studies (15, 16). Tissue was also harvested from the same or the contralateral kidney of each animal for immunohistochemical analysis performed as described below. In some cases, the kidney was perfusion fixed as described below.

Folic acid treatment was carried out as previously described with a modification (8). Briefly, male Sprague-Dawley rats (280–410 g) were administrated intraperitoneally 250 mg/kg folic acid in 0.3 M sodium bicarbonate. At the appropriate time point, one kidney from each animal was harvested for extraction of proteins and the other kidney was perfusion-fixed as described below.

For cis-diaminedichloroplatinum (II) (cisplatin) treatment, male Sprague-Dawley rats (280–410 g) received 10 mg/kg cisplatin by intraperitoneal injection. Animals were killed at various time points. One kidney from each animal was harvested for extraction of proteins, and the other was perfusion-fixed as described below.

To prepare kidneys for tissue protein analysis and immunocytochemistry, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg). The renal artery and vein of one kidney were ligated before perfusion of the other kidney with paraformaldehyde-Lysine-periodate fixative in situ via the abdominal aorta. The suprarenal aorta and superior mesenteric and hepatic arteries were also ligated, and vena cava was opened to facilitate the perfusion of the fixative into the kidney as described (10). The ligated unfixed kidney to be used for biochemical analyses was removed immediately after perfusion was started. After an initial 20- to 40-min period of perfusion fixation, the kidneys were kept in the fixative overnight at 4°C, rinsed with PBS, and kept in PBS at 4°C before cryosectioning. Pieces of fixed kidneys were equilibrated for at least 1 h at room temperature in PBS containing 30% sucrose, embedded in OCT medium, frozen in liquid nitrogen, and cryosectioned (5 μm). Sections on glass slides were kept in −20°C until further processing. The kidney harvested for protein sample preparation was washed with PBS, hemisected, and snap-frozen in liquid nitrogen; tissue was stored at −70°C.

Affinity-Purified Antibodies

The antigen used to raise antibodies against the cytoplasmic domain of rat Kim-1 protein was peptide 9 (HPRAEDNIYIEIDRSRGC), cross-linked to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce) according to procedures described by the manufacturer (14). To raise anti-Kim-1 ectodomain antibodies, a fusion protein of glutathione S-transferase (GST) linked to the NH2-terminal Ig domain of the rat Kim-1 protein (amino acids 1–134) was used as an antigen, as described previously (14). The peptide was conjugated to KLH. The antibodies raised against the peptide-KLH conjugates were affinity-purified on a Sepharose matrix (SulphoLink coupling gel, Pierce) to which the peptide was coupled as described by the manufacturer. Anti-GST-Kim-1 ectodomain (amino acids 1–134 in rat Kid-1 ectodomain) antibodies were affinity-purified from the antiserum on gel-purified nitrocellulose-bound denatured GST-Kim-1 (amino acids 1–134).

Serum and Urine Creatinine Measurement

Animals were anesthetized by an intraperitoneal injection of 65 mg/kg pentobarbital sodium. Approximately 0.2 ml of blood was collected before nephrotoxicant administration and at each time point during the experiment by nicking the tail vein of the anesthetized animal 3–4 mm from the tip of the tail with a blade. Serum was prepared from the collected blood sample and stored at −70°C for creatinine analysis, using a Beckman Creatinine Analyzer II. Collected urine samples were stored at −20 or −70°C. Samples were thawed and centrifuged at 14,000 rpm for 15 min. Supernatants were collected and diluted 1:10 with deionized water. Diluted urine samples were used for creatinine analysis as above.

Immunohistochemistry

Immunoperoxidase staining. Immunohistochemical staining for Kim-1 was conducted using a Vectastain Elite ABC kit (Vector, Burlingame, CA) and rabbit polyclonal antibody R9, the affinity-purified anti-peptide antibody raised against Kim-1, as described previously (14), with modification. Briefly, kidney tissue was immersion fixed in Carnoy’s solution (60% absolute ethanol, 30% chloroform, and 10% acetic acid), embedded in paraffin, and cut into 5-μm sections. The sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation in 2% hydrogen peroxide in methanol for 20 min. The sections were blocked with diluted normal goat serum (1:67) for 1 h at room temperature or overnight at 4°C, followed by incubation with affinity-purified antibody or control IgG at a concentration of 10 μg/ml. After 1 h, the sections were washed in PBS and incubated with biotinylated goat anti-rabbit IgG for 30–60 min. After further washes with PBS, the sections were incubated with an avidin-biotinylated horseradish peroxidase complex for 1–2.5 h. Finally, the sections were washed in PBS and color was developed by the addition of 50 mM sodium phosphate buffer, pH 7.6, containing 0.12% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO), 0.0075% nickel chloride, 0.0075% cobalt chloride, and 0.0075% hydrogen peroxide for 4 min. The sections were counterstained with 0.01% toluidine blue. Staining of kidney tissue from untreated kidney or staining with nonimmune serum or rabbit IgGs...
was performed for controls. Staining with control reagents was negative in all cases.

**Immunofluorescence staining of Kim-1 protein.** Sections were thawed, washed with PBS, and blocked in 1.5% normal donkey serum in PBS (blocking solution) for 30 min. Sections were then incubated for 1 h with the primary antibodies: rabbit anti-rat Kim-1 R9 polyclonal antibody (4 μg/ml) diluted in the blocking solution. The sections were washed with PBS and incubated with anti-rabbit Cy3 conjugated (1:800 dilution, Jackson) in PBS for 30 min. At the final step, sections were washed with PBS and mounted with Vector Shield mounting reagent (Vector) containing 12.5 μg/ml DAPI to identify nuclei. As a negative control, the same concentration of rabbit IgG (4 μg/ml) was used for the primary antibody.

**Double-immunofluorescence staining of Kim-1 and vimentin proteins.** Sections were thawed and washed with PBS, treated with 1% SDS in PBS for 5 min for antigen retrieval, washed with PBS, and blocked in 1.5% normal donkey serum in PBS (blocking solution) for 30 min. Sections were then incubated for 1 h with the primary antibodies: rabbit anti-rat Kim-1 R9 polyclonal antibody (4 μg/ml) and anti-swine vimentin monoclonal antibody (1:100 dilution, V9 antibody, Sigma) diluted in the blocking solution. The sections were washed with PBS and incubated with a mixture of anti-rabbit Cy3 conjugated (1:800 dilution, Jackson) and anti-mouse FITC (1:100 dilution, Jackson) in PBS for 30 min. At the final step, sections were washed with PBS and mounted with Vector Shield mounting reagent (Vector) containing 12.5 μg/ml DAPI. As a negative control, the same concentration of rabbit IgG (4 μg/ml) was used for the primary antibody.

**Immunoblot Analysis of Tissue Kim-1**

For immunoblotting of Kim-1 protein from TFEC-treated rat kidneys, frozen core samples from the OSOM (16) were directly homogenized in 1X Laemmli loading buffer containing 5 μg/ml each of the protease inhibitors: leupeptin, aprotinin and antipain, and 2-mercaptoethanol. After being boiled, the insoluble fraction was separated by centrifugation at 14,000 rpm for 5 min in a microcentrifuge and the protein concentration was determined using a Bio-Rad Protein assay kit (Bio-Rad, Richmond, CA). Interference by SDS was avoided by keeping the sample concentration low in the protein assay.

For immunoblotting of Kim-1 protein in folic acid- and cisplatin-treated rat kidneys, the hemisected frozen kidneys, without dissection of the outer medulla, were homogenized in RIPA buffer (1X PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors (1 mM PMSF, 20 μM leupeptin, 20 μg/ml aprotinin). The insoluble material was pelleted by centrifugation (12,000 g for 15 min), and the supernatant was collected. Protein concentration was determined by using the Bio-Rad Protein assay reagent.

An aliquot of tissue lysate was mixed with Laemmli loading buffer in the presence of 2-mercaptoethanol. Protein samples (25 or 40 μg per lane) were applied to 10% SDS-polyacrylamide gels for electrophoresis. Separated proteins were transferred to PVDF membranes by standard techniques. Anti-rat Kim-1 antibody R9 and horseradish peroxidase-conjugated anti-rabbit IgG were used as primary and secondary antibodies, respectively. For detection of the Kim-1 ectodomain cleavage product, a 15% gel was used. To quantitate expression of Kim-1 protein in the kidney lysates, 80- to 90-kDa mature Kim-1 protein bands were scanned and their integrated optical density was quantitated by the National Institutes of Health Image 1.61 densitometry program. The integrated optical density of the Kim-1 bands was normalized to the signal derived from an aliquot of a single pool of rat Kim-1 in lysates of Chinese hamster ovary (CHO) cells stably over-expressing Kim-1. The same lysate pool was used as the standard for all immunoblots to allow comparison between immunoblots. The data are expressed as the ratio of the integrated optical density using the value for the standard as the denominator.

**Rat Urine Kim-1 Immunoblot Analysis**

Rat urine samples were collected either directly from the bladder using a needle and syringe when kidneys were harvested or after spontaneous voiding. Samples collected from TFEC- or folic acid-treated rats were stored at 4°C overnight and then at −20°C. Urine samples were thawed, and the supernatant was separated using a microcentrifuge operating at 14,000 rpm for 15 min. PMSF (1 mM) was added to urine samples in some cases but found not to be necessary for preservation of urine Kim-1. Urine samples harvested from animals were centrifugated at 14,000 rpm for 15 min, after which the supernatant was collected and stored at −70°C. Protein fractions of the urine supernatant samples were concentrated by ultrafiltration. Urine samples were applied to Microcon 30 or Microcon 30 YM ultrafiltration membrane units (Amicon) and ultrafiltered according to company instructions. After urine sample volume was significantly reduced, the concentrated urine was diluted with 10 mM Tris-HCl buffer (pH 7.4) and ultrafiltered for two times to remove residual small molecules including salts. Protein concentration was measured, and an aliquot of protein was mixed with Laemmli loading buffer in the presence of 2-mercaptoethanol and applied to 7.5% SDS-polyacrylamide gels for electrophoresis followed by immunoblot analysis with affinity-purified rabbit anti-Kim-1 GST fusion protein antibody, which is ectodomain specific (amino acids 1-134 in rat Kim-1 ectodomain) (14). Protein concentrations of original urine samples were estimated based on protein concentration of concentrated fraction, measured by Bio-Rad protein assay, and initial and final urine volumes after ultrafiltration.

**Statistical Analysis**

Treatment means were compared with control means by ANOVA and subsequent Student’s t-test. The level of significance was set at P < 0.05 in all cases.

**RESULTS**

**Expression of Kim-1 in Kidney Tissue and Urine from TFEC-Treated Rats**

**Renal function change in rats after TFEC administration.** TFEC is a proximal tubule S3 segment-specific nephrotoxic haloalkane cysteine conjugate (15, 16). After 20 mg/kg single administration of TFEC, renal function was monitored by measurement of serum creatinine concentration at 1, 2, 3, 5, and 7 days after treatment (Fig. 1A). Serum creatinine was increased markedly 24 h after the toxicant administration and peaked at 2 days after the dose. It then declined and reached baseline levels by 5 days after the treatment.

**Immunoblot analysis of Kim-1 protein in kidneys of TFEC-treated rats.** Kim-1 protein levels in lysates of kidney tissue dissected from the outer medulla were analyzed by immunoblotting over a 14-day period after TFEC administration (Fig. 1, B and C). Kim-1 protein was not detectable in the kidney tissue from control rats. Kim-1 protein levels increased significantly 1 day after TFEC administration and remained increased for 14 days. There are at least two forms of Kim-1 protein in the lysates, a 45-kDa (band B) and 80- to 85-kDa (band A) forms (Fig. 1B). The presence of the multiple forms of Kim-1 protein is most likely due to a difference in the O-linked glycosylation of the mucin domain (14). The expression of both forms, particularly the 80- to 85-kDa (band A) form, increased markedly at day 2 and persisted at high levels for 7 days after TFEC treatment (Fig. 1, B and C). The 80- to 85-kDa form was still detectable at day 14, although there was a clear decline of Kim-1 protein expression from days 7 to 14.
sodium, and blood was withdrawn from the tip of the tail. Serum creatinine was determined as described in METHODS. Each point is the means ± SE of at least 3 animals (n = 3). Significant differences (*P < 0.05) between TFEC treatment vs. vehicle (veh) treatment at various time points are indicated. B: representative immunoblot of Kim-1 protein in the OSOM at various time points. The large size 80- to 85-kDa band apparently shifts to a slightly smaller size with progression over time after TFEC administration. At early time points, there was a good correlation between the increase in serum creatinine levels and the up-regulation of Kim-1 protein in the kidneys. High levels of Kim-1 protein persist in tissue, however, even after kidneys recover renal function, consistent with expression during tissue repair during the process of recovery of function.

**Detection of Kim-1 protein in the urine of TFEC-treated rats.** We demonstrated in cell cultures that the human KIM-1 ectodomain is cleaved and shed into the culture media (2). In a clinical study, KIM-1 protein was detected in the urine of patients with acute tubular necrosis (9). To evaluate whether Kim-1 may be a useful general biomarker for renal injury or repair in animal models of nephrotoxicant-induced injury, we investigated whether Kim-1 protein is present in urine samples collected from toxicant-exposed animals. Urine samples were collected for 7 days after TFEC administration. Collected urine samples were concentrated, and Kim-1 protein was analyzed by immunoblotting with anti-ectodomain antibodies that recognize both intact and shed forms of Kim-1 protein (Fig. 1D). There is no detectable Kim-1 band in the urine samples from untreated rats. Two forms of Kim-1, reflected by bands A and B, were detected in urine collected as early as 1 day after TFEC administration. The larger protein is detected in the urine samples collected on day 1 through day 7, and it has a size smaller than the mature Kim-1 protein seen in the kidney tissue lysates. The smaller urine Kim-1 protein (band B) can be detected at 3 to 4 days after TFEC treatment. Thus Kim-1 protein is upregulated in kidney tissue after TFEC-induced renal injury and Kim-1 protein is shed into the urine after TFEC treatment.

**Immunohistochemical analysis of Kim-1 after TFEC treatment.** The Kim-1 temporal expression pattern was characterized by immunohistochemistry using anti-peptide antibody R9 on kidney sections over a 14-day period after TFEC administration. In the untreated kidney, there was no detectable Kim-1 protein (Fig. 2A). Twenty-four hours after the administration of TFEC, Kim-1-positive cells, as indicated by black/dark brown peroxidase staining on the tissue section, were clearly present in the S3 segment of proximal tubule in the OSOM near the peritubular capillary. Kim-1-positive cells were observed in the S3 segment of proximal tubule in the untreated kidney. Kim-1-positive cells were observed in the S3 segment of proximal tubule in the OSOM near the peritubular capillary.

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Fig. 2. Immunohistochemical analysis of Kim-1 expression in kidneys of TFEC-treated rats. Affinity-purified antibody against peptide R9 was used for immunostaining of Kim-1 in paraffin sections taken at various times after TFEC administration as described in METHODS. All photomicrographs were taken by using a ×40 objective (A-G; scale bar = 20 μm; H; scale bar = 10 μm). All fields were chosen from the OSOM.

A: day 0 vehicle control kidney: Kim-1 is not detectable. B: day 1: Kim-1-positive tubules are present with epithelial cell staining (arrowheads). Many tubules show dilated and flattened morphology with casts and cell debris in the outer medulla region. C: day 2: more Kim-1-positive tubules are found at this time point (arrowheads). These tubules, containing casts and cell debris, are dilated. Some of the Kim-1 staining is apical (arrowheads), and the pattern in some cells is diffuse. D: day 3: Kim-1 protein is detected in many tubules in the injured outer medulla region. Kim-1 staining is localized primarily to the apical side of the regenerating epithelium with some occasional diffuse cytoplasmic staining (arrowheads). These tubules still contain abundant cell debris and casts in the lumen. E: day 5: Kim-1 immunoreactivity remains detectable in most of the tubules in the injured area. The staining pattern is apical in these cells, many of which have thin morphology. Casts and cell debris are reduced in the lumen. Notice the presence of a Kim-1-positive spherical cell cluster in the middle of a tubular lumen (arrowheads). The number of interstitial cells in the peritubular space is increased. F: day 14: number of Kim-1-positive tubules is clearly decreased. Kim-1 staining is apical in the thin positive tubular cells (*). G: rabbit IgG-negative control on day 5 kidney sections shows negative staining. H: double-immunofluorescence staining for Kim-1 (small arrowheads, red: Cy3) and vimentin (large arrowheads, green: FITC) in the OSOM of day 3 kidney. No significant staining was observed using rabbit IgG and omitting primary mouse antibody as a negative control (data not shown).

The number of Kim-1-positive proximal tubule cells in the OSOM increased at 2 days after the toxicant administration (Fig. 2C). Kim-1 immunoreactivity is clearly present in apical regions and some times in cytoplasm of cast-containing tubules (Fig. 2C, arrowheads). Apical localization of Kim-1 staining is clearly recognized on day 3 (Fig. 2D) but there is also some diffuse cytoplasmic staining, along with the presence of cell debris and casts in the dilated lumen (Fig. 2D). Most of the viable cells, identified by intact nuclei, in the injured tubules are Kim-1 positive (Fig. 2D, arrowheads). There is a dramatic further increase in the number of Kim-1-positive cells and tubules between days 3 and 5, when expression peaks (Fig. 2, D and E). Five days after the toxicant administration, there were fewer casts and less debris in the proximal tubules in the injured region. Significant tubular dilatation, however, was still seen at this time. Kim-1 staining is present in most of the regenerating flat S3 segment cells that contain scanty cytoplasm in these dilated tubules in the outer medulla and cortical medullary ray (Fig. 2E). At this time, the subcellular localization of Kim-1 is predominantly apical, but there is also some diffuse cytoplasmic staining (Fig. 2E). There is intense Kim-1 expression in a cell cluster in the lumen of the dilated tubule.
Expression of Kim-1 in Kidney Tissue and Urine from Folic Acid-Treated Rats

To test the generality of the results with TFEC for other forms of injury, we evaluated the high-dose folic acid nephrotoxicity model (Fig. 3). This model differs from the TFEC model in that folic acid causes a massive increase in cell proliferation in the proximal tubules (8). Although folic acid can induce large increases in plasma creatinine levels in the rat (35), under our experimental conditions there was no statistically significant increase in serum creatinine concentration, as shown in Fig. 3A. Despite no increase in serum creatinine, Kim-1 protein upregulation was detected by immunoblot analysis of the kidney lysates extracted from the hemisected folic acid-treated kidney tissues at days 1, 4, and 7 (Fig. 3B). Only a weak 45-kDa Kim-1 protein (band B) was detectable in the vehicle control kidney, whereas both 45- and 80- to 85-kDa (band A) proteins were detected after folic acid administration. A 60-kDa nonspecific band does not change during treatment or over the time course. Compared with TFEC-treated animals, however, the intensity of Kim-1 bands is weaker compared with Kim-1 CHO cell lysate standards, and there was relatively less of the 80- to 85-kDa and more of the 45-kDa band present. In the concentrated urine protein fractions from days 1, 2, and 3 folic acid-treated rats, two sizes of Kim-1 bands were detected at each time point, albeit faintly on day 1 (Fig. 3C). The sizes of these forms were similar to the sizes of proteins detected in the TFEC urine sample. Thus, although there was no change in serum creatinine, Kim-1 protein was upregulated in kidney tissue and present in the urine after folic acid treatment.

Immunohistochemical analysis of Kim-1 after folic acid treatment. The spatial expression pattern of the Kim-1 protein in the folic acid-treated kidneys and its relationship to vimentin expression were then analyzed by double-immunofluorescence staining for both proteins (Fig. 4). There was no detectable Kim-1 or epithelial cell vimentin immunoreactivity in the...
control kidneys (Fig. 4A). Twenty-four hours after folic acid administration, some proximal tubules in the OSOM and medullary rays of the cortex were mildly dilated and stained positively for Kim-1 (Fig. 4B). Kim-1 staining was localized to the brush border in these Kim-1-positive epithelial cells (small arrowheads). Morphologically, most of proximal tubule epithelial cells still retained a brush border, suggesting these cells were relatively well differentiated. In some tubules, Kim-1 immunoreactivity was limited to only a few of the epithelial cells (Fig. 4B, outside boundaries of two of the tubules are indicated by large arrowheads), and there was minimal luminal cast formation in the OSOM proximal tubules. Unlike the ischemia and TFEC models, no vimentin staining was detectable in any Kim-1-positive tubules after folic acid. Upregulation of Kim-1 immunoreactivity persisted 2 days after treatment with folic acid (Fig. 4C). The staining pattern remained the same in the OSOM; however, some cell debris, presumably exfoliated cells, were found in the diluted lumen of the proximal tubules, suggesting progression of the injury (Fig. 4C; large arrowhead). Nonetheless, the extent of the injury was minimal compared with that seen in the TFEC model described above. At 3 days after folic acid administration, the apical staining pattern of Kim-1 in the proximal tubules was the same as on days 1 and 2; however, staining among cells in the same tubules was more uniform and the tubules contained more casts and cell debris with dilation of the lumen (Fig. 4D). The cells remained negative for vimentin. At day 4 after folic acid (Fig. 4E), many Kim-1-expressing tubules showed a mosaic pattern; i.e., a mixture of Kim-1-positive and -negative cells was observed. There was also an increase in vimentin-positive interstitial cells (large arrowhead) surrounded the Kim-1-positive tubules, indicating expansion of the interstitium. Apical Kim-1 immunoreactivity persisted for at least 7 days after folic acid administration (Fig. 4F), when a few cells coexpressed Kim-1 and vimentin (large arrowhead). In some Kim-1-positive tubules, cell debris was present in the lumen. Thus, despite the lack of significant changes in serum creatinine or extensive necrosis or vimentin expression in the folic acid model, upregulation of Kim-1 protein occurred in the apical part of proximal tubule epithelial cells, and Kim-1 was shed into the urine.

Expression of Kim-1 in Kidney Tissue and Urine from Cisplatin-Treated Rats

Cisplatin is one of the most effective chemotherapeutic agents for treatment of solid tumors, but its utility is compromised by its nephrotoxicity. Cisplatin differs from folic acid in that it causes extensive necrosis. Unlike TFEC, however, cisplatin also induces apoptosis. Indeed, it is likely that the extensive necrosis observed after cisplatin treatment in vivo may occur subsequent to considerable loss of cells through apoptosis (19, 23, 34). In addition, the sloughing of necrotic cells and formation of tubular casts are delayed by several days compared with ischemic and TFEC-induced injury. Given this delay, we considered that this model of nephrotoxicity might
be the best of three models studied to evaluate whether urinary Kim-1 might be present before significant changes in serum creatinine.

In the initial 2-day period after exposure to cisplatin, serum creatinine levels remained below 1.0 mg/dl (Fig. 5A). At 3 days and longer, creatinine levels consistently increased and plateaued from days 6 to 8. Marked upregulation of kidney tissue Kim-1 protein was found by immunoblot analysis of hemisected kidney lysates on days 4 and 8 after exposure (Fig. 5, B and C). Only very small amounts of the 45-kDa Kim-1 protein (band B) are detectable in the kidney from vehicle-treated animals, whereas both the 45- and 80- to 85-kDa (band A) proteins are highly upregulated 4 days after cisplatin treatment and further increased at day 8. In contrast to the folic acid model, in which the 45-kDa band predominates, both the 45- and 80- to 85-kDa bands are abundant after cisplatin. The 80-to 85-kDa band clearly shifts to a smaller size with progression of the time course as seen in the two other models. During the first day, despite no increase in serum creatinine, urinary 75-kDa Kim-1 protein was detectable (Fig. 5D), indicating that Kim-1 protein expression was already upregulated in the tubules and the ectodomain shed into the urine. A smaller size (~40 kDa) immunoreactive band was also observed in the urine 2 days after cisplatin administration. Note that urinary Kim-1 was detectable on days 1 and 2, whereas whole kidney lysate Kim-1 was below the level of detectability on days 1 and 2. Urinary protein concentration increased from 0.50 (t = 0) and 0.46 (t = 1 day) to 2.07 mg/ml at 2 days after cisplatin. Urinary creatinine concentrations were 25, 46, and 69 mg/dl, respectively.

Shedding of the human KIM-1 ectodomain generates a remnant membrane and cytosolic fragment polypeptide of 14 kDa (2). To determine whether the 14-kDa peptide is present in the rat kidney after nephrotoxicant administration, a 15% polyacrylamide was used for analysis of the tissue lysates. A band of ~14 kDa is present 3 days after cisplatin, and this band is markedly upregulated 6 days after administration of cisplatin (Fig. 5E), reflecting the proteolytic cleavage of the Kim-1 protein resulting in the larger ectodomain fragment and a 14-kDa fragment.

Fig. 5. Time course of serum creatinine and immunoblot analysis of Kim-1 protein in the kidney and urine from cisplatin-treated rats. A: serum creatinine time course. Rats received 10 mg/kg ip cisplatin. At various times after treatment, animals were anesthetized with pentobarbital sodium, and blood was withdrawn from the tip of the tail. Serum creatinine was determined as described in METHODS. Each point is the means ± SE of at least 3 animals. Significant differences (***P < 0.05) between cisplatin treatment vs. vehicle treatment at various time points are indicated. B: representative immunoblot analysis of Kim-1 protein in the cisplatin-treated rat kidney. Twenty-five micrograms of sample lysates were prepared from hemisected rat kidneys at various times after cisplatin treatment and loaded onto each lane. Proteins were analyzed by SDS-PAGE, and immunoblot analysis was carried out using R9 anti-rat Kim-1 antibody. Two bands (A and B) were recognized at 80 to 85 and 45 kDa, respectively, by the antibody. Rat Kim-1 stable CHO cell lysate (0.5 μg) was used as a standard for Kim-1 protein bands. C: quantitative analysis of Kim-1 protein levels in the cisplatin-treated rat kidneys by densitometry. The mature form 80- to 85-kDa bands were scanned, and the integrated optical density was analyzed. The signals were normalized to Kim-1-expressing CHO cell standard, and the data are expressed as the ratio of the signal to the standard. Each time point is means ± SE of data from 3 animals. Significant differences (**P < 0.05) between cisplatin treatment vs. vehicle treatment at various time points are indicated. D: immunoblot analysis of Kim-1 protein in the urine. Twenty-five micrograms of proteins were prepared from normal urine and urine samples collected at 1 and 2 days after treatment. Protein samples were loaded onto each lane. Proteins were probed with anti-rat Kim-1 ectodomain antibody. A 75-kDa band was present at day 1, and 2 bands (A and B) are noted at 75 and 45 kDa, respectively, at day 2 after treatment. E: immunoblot analysis of Kim-1 remnant peptides in the kidney. Twenty-five micrograms of tissue protein lysates, prepared from cisplatin-treated rat kidneys, were loaded onto the each lane. Proteins were separated by 15% SDS-PAGE and immunoblot analysis was carried out using R9 anti-rat Kim-1 antibody.
Immunohistochemical analysis of Kim-1 after cisplatin treatment. On days 1 and 2 after cisplatin, Kim-1 tissue immunoreactivity was detected but not prominent in the proximal tubules in the OSOM (Fig. 6A). Cellular staining was more diffuse than apical (Fig. 6A, day 1, arrowhead). This early upregulation of Kim-1 protein is consistent with the detection of Kim-1 in the urine. At day 3, Kim-1 expression is increased and localized to the apical part of many of the proximal tubule cells with some expressing diffuse cytoplasmic staining in the OSOM and medullary rays of the cortex (Fig. 6B). Some Kim-1-positive tubules are dilated with thinned epithelium and occasionally contain casts at this time point. A given tubule may have only a fraction of its cells Kim-1 positive (day 3, large arrowheads). Vimentin expression was low and coexpression with Kim-1 was very rare at this time point. At day 4 (Fig. 6B), many dilated proximal tubules in the OSOM and medullary rays have cells with apical Kim-1 immunoreactivity. Some tubules have cells expressing diffuse cytoplasmic staining (day 4, small arrowhead), and many contain cell debris and casts. At this time point, some Kim-1-positive tubules were also vimentin positive (day 4, large arrowhead). At day 6, there was a pronounced increase in Kim-1 in S3 segments that characteristically coexpress Kim-1 and vimentin (Fig. 6B, large arrowheads). Thus Kim-1 is detected in the tissue and urine of cisplatin-treated rats. Urine Kim-1 can be detected before any significant increase in serum creatinine and at a time when tissue expression is modest.

DISCUSSION

Kim-1 is a type I membrane protein that has Ig and mucin domains in its ectodomain and a relatively short cytoplasmic tail. We first identified the protein, by subtractive cloning, as the product of a gene that is highly induced in the postischemic kidney (9, 14). Although it is clear that Kim-1 is induced in the postischemic kidney, the generality of this response to other forms of nephrotoxic injury was not clear. Therefore, we investigated Kim-1 protein expression in three distinct models of nephrotoxicant-induced renal injury: TFEC, folic acid, and cisplatin. Although the mechanism of TFEC injury involves production of reactive acylating species (11), the pattern and timing of injury in the nephron are similar to that observed following ischemic injury. High-dose folic acid, which injures the nephron due to the precipitation of folate crystals in the tubule lumen, causes an intense proliferative response in the absence of significant necrosis (8). Cisplatin also produces...
necrosis in the rodent kidney, but, unlike ischemic injury and TFEC, the response is delayed and is likely to involve apoptosis to a greater extent (19, 23, 34). Despite the diverse nature of the mechanisms underlying these three models of injury and the differences in the timing and progression of the injury, tissue Kim-1 expression increases in all three models. In addition, the Kim-1 ectodomain and fragments of this domain were found in urine in each model, indicating that expression in the injured nephron is associated with shedding of the ectodomain, allowing for noninvasive monitoring of nephrotoxicity. In this study, we used immunoblot analysis to identify tissue and urinary Kim-1 as well as immunofluorescence analysis of Kim-1 expressed in tubular epithelial cells. We detected Kim-1 in the tissue and urine in each model of nephrotoxicity even though these techniques are much less sensitive than an ELISA such as the one we used previously in human urine (9). At this point, however, we do not have the antibodies necessary for an ELISA on rodent urine.

After TFEC administration, TFEC metabolite adducts are localized in the proximal tubules at 12 h. Proximal tubule epithelial cell proliferation, as measured with BrdU labeling, is upregulated at 1 day after injection and peaks at days 2 to 3 (11, 16). A proximal tubule marker of dedifferentiation, vimentin, is also upregulated at day 2 and high levels of expression of this protein persist for at least 14 days (16). There is coordinated expression of Kim-1 protein at the time of onset of TFEC-induced renal injury in the early phases. This time course of proliferation, dedifferentiation, elevation in serum creatinine levels, and Kim-1 expression in TFEC-induced injury is similar to the time course of proliferation, differentiation, and elevation in serum creatinine levels in postischemic kidneys (1, 40). Both TFEC and ischemia-reperfusion result in cell death and cast formation, followed by dedifferentiation and cell proliferation of the proximal tubule epithelium, particularly in the OSOM. Kim-1 temporal expression patterns are reminiscent of vimentin and FGF-1 expression in TFEC-treated kidneys (16). Kim-1 expression in vimentin-expressing tubular cells in various models indicates that Kim-1 is expressed in dedifferentiated cells. It is possible that Kim-1 plays an important role in the repair process by which the surviving proximal tubule epithelial cells undergo dedifferentiation, migration, proliferation, and then restoration of morphological and functional integrity to the epithelium. From these studies, however, it is clear that detectable epithelial vimentin expression is not a prerequisite for Kim-1 staining because Kim-1 staining is observed early in the course of folic acid and cisplatin-induced injury in the absence of detectable vimentin staining.

An increase is observed in the number of BrdU-labeled nuclei in the proximal tubules between 6 and 24 h after folic acid administration (8). Several genes are known to be altered in expression in this model (5–7, 17, 21, 24, 28, 29, 35, 36, 41). Kim-1 protein is significantly increased after folic acid injection, and increased expression persists up to day 7, documented by both immunofluorescence and immunoblot analyses. Despite this increase in expression, there was no significant increase in serum creatinine. In contrast to the TFEC model, however, after folic acid Kim-1 immunolocalizes to cells with relatively well-retained epithelial morphology including an apparently intact brush border in mildly dilated proximal tubules. Tubules containing positively stained cells occasionally have some cell debris and casts in their lumen. These data suggest that Kim-1 can be induced early under conditions of relatively moderate injury to proximal tubules that maintain a differentiated morphology. Folic acid is known to result in obstruction by crystallization and precipitation in the outer medulla. The Kim-1-positive proximal tubules might be exposed to the stress induced by obstruction and/or the direct mechanical stress imposed by the folic acid crystals. Our laboratory recently showed that obstruction induces Kim-1 expression in mice (20). Despite no significant increase of serum creatinine levels, kidney tissue Kim-1 upregulation and detection in urine from folic acid-treated rats suggest that Kim-1 may be a sensitive biomarker for detection of low-level renal injury or early stages of repair.

After cisplatin treatment, kidneys show Kim-1 staining patterns similar to those seen with TFEC and folic acid in the proximal tubules in the injured area. The increase in plasma creatinine levels after cisplatin treatment occurs later than the increases seen with TFEC. Despite the delayed increase in serum creatinine with cisplatin, tubule Kim-1 expression and urine Kim-1 can be detected as early as 1 day after cisplatin exposure. This detection of Kim-1 in tubule and urine implies that Kim-1 may be an early biomarker for cisplatin-induced renal injury. In cisplatin-treated animals’ kidneys, Kim-1 expression continues to increase as serum creatinine levels increase over an 8-day period. By comparison, Kim-1 levels peaked at days 3 to 5 in TFEC-treated kidneys.

The ectodomain of the Kim-1 protein includes an Ig and mucin domain. Our previous study demonstrated that this ectodomain is cleaved with proteases and the soluble ectodomain is constitutively shed into the culture media of human KIM-1-expressing cells (2). Released soluble KIM-1 protein is also detected in urine collected from patients with acute tubular necrosis (ATN), and we proposed this molecule as a new useful renal injury biomarker in humans with ATN (9). As in humans, we now report detection of rat Kim-1 protein in the tissue and urine samples, suggesting that Kim-1 may be a sensitive general renal injury or early repair biomarker in animals exposed to nephrotoxicants. The appearance of tubule-expressed and urinary Kim-1 in cisplatin-treated rats before significant functional injury is in good agreement with our finding in humans in which urine KIM-1 is detected before urinary casts are seen (9). Because animal models are commonly used for evaluation of toxicity of new candidate therapeutics and assessment of potential chemical hazards in industry and environmental exposure, Kim-1 may serve as a useful general biomarker for drug safety and chemical hazard-related renal injury. The finding that the Kim-1 ectodomain is shed with toxicity in animals also implies that animal models may be useful tools for investigating the mechanisms involved in Kim-1 shedding and the role of shedding in vivo in the injury/repair processes. Measurement of Kim-1 protein expression may also be beneficial for detection of subtle abnormalities of tubular epithelial cells during injury/repair in the kidney at a time distant from an insult when gross morphology and physiological parameters have already returned to baseline. We observed that Kim-1 expression can be detected by immunohistochemical analysis in the kidney up to 64 days after TFEC administration (Ichimura T, Bowes RC III, and Bonventre JV, unpublished data).
Taken together, the data suggest that Kim-1 is a general marker for a variety of nephrotoxic injuries in rodents and humans; however, the function of Kim-1 protein is unclear. Recent reports show that Kim-1 is a prototypical member of a larger family of proteins that may function as an extracellular sensor or a receptor for adhesion/signaling in a variety of processes involving cell-cell or cell-pathogen interactions (18, 22, 25, 26). Although the gene family has been proposed to be involved in T cell function and immune response, the roles of Kim family members, particularly the functions of Kim-1 in injury and repairing kidney, remain unknown. Although T cells have been implicated in the pathophysiology of postischemic injury to the kidney, we have not observed that infiltrating cells express Kim-1 either in ischemic kidneys or after nephrotoxins (42). The protein structure of this molecule suggests that Kim-1 may be an adhesion and/or protective molecule for the cell surface (14). Therefore, we speculate that Kim-1 might alter cellular adhesion and/or modulate interactions between the injured epithelial cell and the luminal contents that include casts, debris, and viable epithelial cells that have become dislodged from the basement membrane (38). Kim-1 may enhance mobility and proliferation of surviving epithelial cells.

In summary, Kim-1 expression was analyzed in three forms of nephrotoxicant-induced rat kidney injury. Kim-1 expression is not detectable in normal kidney but is upregulated in response to each of three different toxicants: TFEC, folic acid, and cisplatin, which exert injury by different mechanisms. Kim-1 is present in the urine in each case. Notably, after cisplatin treatment, Kim-1 appears in the tissue and urine before there is obvious functional impairment as measured by an increase in creatinine. Kim-1 may serve as a biomarker of kidney injury and/or early repair and may be useful in detection and monitoring of nephrotoxins. Kim-1 may be useful in preclinical and clinical studies vital to drug development and evaluation. It may also serve in the monitoring of disease states that manifest as injury to the proximal tubule and be useful in guiding interventional strategies.

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