Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury

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ALTHOUGH THERE HAS BEEN a significant progress in understanding the biochemical and molecular mechanisms of ischemic or toxic forms of acute kidney injury (AKI) in animal models, the slow translation of the results from bench to bedside, especially in the treatment of existing AKI, is the absence of reliable biomarkers of injury in animal and human studies (36, 39). The Food and Drug Administration’s critical path initiative has reinforced the need for additional biomarkers to predict drug toxicity in preclinical studies. These biomarkers can act as surrogate endpoints and/or aid in making efficacious and cost-saving decisions including terminating drug development more quickly (39a). Easily quantifiable and sensitive biomarkers can be influential in every phase of therapeutics, from drug discovery and preclinical evaluation through each phase of clinical trials and into postmarketing studies.

Routine use of measures of renal function, such as levels of blood urea nitrogen (BUN) and serum creatinine, increase significantly only after substantial kidney injury occurs and with a time delay (15). In drug development, minimizing nephrotoxicity is highly desirable. Insensitivity of tests affects the evaluation of toxicity in preclinical studies by allowing drug candidates, which have low but nevertheless important nephrotoxic side effects in animals, to pass the preclinical safety criteria only to be found to be clinically nephrotoxic in humans at great costs to patients. The kidney is one of the primary sites of drug toxicity. Despite this, the tests available to detect toxicity and early ischemic renal injury are either invasive and difficult to quantitate or noninvasive, nonspecific, and insensitive. Acute kidney failure is a common disease and is associated with a high mortality rate (5, 38). It has been recognized that better biomarkers for kidney injury are needed both for animal studies and for use in humans where early detection of kidney injury will influence therapy and potentially morbidity and mortality.

Urine has been examined as a source for biomarkers given its easy availability and reduced complexity when compared with serum. Many urinary proteins such as α- and β-glutathione-S-transferases (α- and β-GST; see Ref. 7), neutrophil gelatinase-associated lipocalin (NGAL; see Ref. 24), cysteinereich protein 61 (CYP-61; see Refs. 26, interleukin (IL)-18 (28), clusterin (2), F-actin (34), N-acetyl-β-D-glucosaminidase (NAG; see Ref. 42), etc., have been evaluated as noninvasive indicators of renal injury. However, problems with reliable use of these proteins to identify and monitor kidney injury includes instability in the urine, modification due to physicochemical properties of the urine, delayed appearance, inconsistency of upregulation with different models of nephrotoxicity, absence of sustained elevation throughout the time course of renal injury to monitor progression and regression of injury, and lack of a high-throughput detection method.

We have cloned a potential biomarker for AKI named kidney injury molecule-1 (Kim-1; see Ref. 16). Kim-1 is a type 1 transmembrane protein that is not detectable in normal

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kidney tissue but is expressed at very high levels in dedifferentiated proximal tubule epithelial cells in human and rodent kidneys after ischemic or toxic injury (12, 16). The Kim-1 ectodomain is stable in the urine for prolonged periods of time and can be detected in the kidney and urine in a number of nephrotoxic models in animals (1, 17) and in humans with AKI (13). Although we have previously developed a sensitive ELISA assay to measure human Kim-1 in the urine (12), this unfortunately does not detect either rat or mouse Kim-1.

The present study was designed to develop, evaluate, and validate a high-throughput detection method for quantitating Kim-1 in rat urine and to test the sensitivity of this assay to detect kidney injury before changes in other tests used routinely to detect kidney injury. The test was applied and validated in two well-established and widely used mechanistically distinct animal models of renal injury: cisplatin-induced nephrotoxicity and bilateral renal ischemia-reperfusion (I/R; see Ref. 32).

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley rats (275–300 g) were purchased from Charles River laboratories (Wilmington, MA), and male Wistar rats (275–300 g) were purchased from Harlan (Indianapolis, IN). Rats were maintained in central animal facilities in Boston and Mexico City under conditions of 21 ± 1°C and 50–80% relative humidity at all times in a 12:12-h light-dark cycle over wood chips free of any known chemical contaminants. The rats were fed with commercial rodent chow (Teklad rodent diet no. 7012), given water ad libitum, and acclimated for 1 wk before use. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health and were approved by our Institutional Animal Care and Use Committees.

**Cisplatin-Induced Nephrotoxicity Studies**

Male Sprague-Dawley rats were administered 2.5, 5, or 7.5 mg cisplatin/kg intraperitoneally in 0.9% saline or the same volume of vehicle as controls (n = 4/dose for each time point). Animals were euthanized by an overdose of pentobarbital sodium (200 mg/kg ip) at 4°C, rinsed with PBS, and kept in PBS containing 0.2% sodium azide 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 optimum cutting temperature medium, frozen in liquid nitrogen, and cryosectioned (5 μm). Sections on glass slides were kept in −20°C until further processing. The other half was fixed with 10% phosphate-buffered formaldehyde for 48 h. The tissues were then transferred to 70% ethyl alcohol, processed, and embedded in paraffin wax. Kidney sections (5 μm) were stained with hematoxylin and eosin for histological examination under a light microscope. The kidney harvested for biochemical analysis was washed with PBS, hemisected, and snap-frozen in liquid nitrogen; tissue was stored at −70°C.

**Renal I/R Studies**

Twenty-five male Wistar rats weighing ~300 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37°C, by means of a rectal probe attached to a temperature regulator that was in turn attached to a homeothermic blanket. A midline laparotomy was made, renal pedicles were isolated, and bilateral renal ischemia was induced by clamping the renal pedicles for 0, 10, 20, 30, or 45 min. Reperfusion commenced when the clips were removed. Occulsion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush.

After reperfusion (2 h), the rats were placed in metabolic cages at 22°C with a 12:12-h light-dark cycle and allowed free access to water. Individual 24-h urine samples were collected.

**Renal Functional Parameters**

In the cisplatin studies, plasma and urine creatinine concentrations were measured using a Beckman Creatinine Analyzer II. BUN was spectrophotometrically measured at 340 nm using a commercially available kit (catalog no. TR12421; Thermodac). Collected urine samples were centrifuged at 6,000 rpm for 15 min. Supernatants were collected and diluted 1:10 with deionized water. Diluted urine samples were used for analysis of creatinine. Urine glucose (catalog no. 1530–500; Thermo-DMA), protein (catalog no. TP0400; Sigma), and NAG (catalog no. 875406; Roche Diagnostics) were measured spectrophotometrically (41) according to the manufacturers’ protocols.

In the I/R studies, urinary protein excretion was measured by the TCA turbidimetric method (14). Serum and urine creatinine concentration and BUN were measured with an autoanalyzer (Technicon RA-1000; Bayer, Tarrytown, NY), and renal creatinine clearance was calculated by the standard formula C = (U × V)/P, where U is the concentration in urine, V is urine flow rate, and P is the plasma concentration.

**RNA Isolation**

Total RNA was isolated from cortices of each group using the guanidine isothiocyanate-cesium chloride method (33). Integrity of isolated total RNA was examined by 1% agarose gel electrophoresis, and RNA concentration was determined by ultraviolet light absorbance at 260 nm (model DU640; Beckman, Brea, CA).

**Kim-1 Semiquantitative RT-PCR**

Relative levels of Kim-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were assessed in renal cortex and medulla by semiquantitative RT-PCR, as previously described (4, 10). Briefly, primer sequences were custom made by GIBCO-BRL (Gaithersburg, MD). Kim-1 primers were designed from the rat Kim-1 sequence. The sense primer was 5’-CCGTCGCCATTGAATTGAT-3’ and antisense 3’-CAGCCATGACCAAAATAAGAC-5’, which amplified a fragment of 418 bp, bases 1 to 418. To evaluate or reduce nonspecific effects of experimental treatment and to semiquan-
tify Kim-1 expression, we amplified a fragment of GAPDH using primers previously described (31). Genomic DNA contamination was checked by treating all RNA samples with DNase and by carrying samples through the PCR procedure without adding reverse transcriptase (RT).

RT was carried out using 2.5 μg total RNA from renal cortices. Before RT reaction, total RNA was heated at 65°C for 10 min. RT was performed at 37°C for 60 min in a total volume of 20 μl using 200 units of the Moloney murine leukemia virus RT (GIBCO-BRL), 100 pmol random hexamers (GIBCO-BRL), 0.5 mM of each dNTP (Sigma Chemical, St. Louis, MO), and 1X RT buffer (75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl₂, and 10 mM dithiothreitol, pH 8.3). Samples were heated at 95°C for 5 min to inactivate the RT and diluted to 40 μl with PCR-grade water. One-tenth of RT individual samples of each group was used for Kim-1 or GAPDH amplification in 20 μl final volume reactions containing 1X PCR buffer (10 mM Tris·HCl, 1.5 mM MgCl₂, and 50 mM KCl, pH 8.3), 0.1 mM of each dNTP, 0.2 μM [α-32P]dCTP (~3,000 Ci/mmol; 9.25 MBq, 250 μCi), 10 μM of each primer, and 1 unit Taq DNA polymerase (GIBCO-BRL). Samples were overlaid with 30 μl mineral oil, and PCR cycles were performed in a DNA thermal cycler (Whatman; Biometra), with the following profile: denaturing for 1 min at 94°C, annealing for 1 min at 57°C, and 1 min extension step at 72°C. The last cycle was followed by a final extension step of 5 min at 72°C. The control gene was coamplified simultaneously in each reaction. The optimal number of cycles for each primer pair was determined through kinetic amplification following our standard procedure (4, 10). To routinely semi-quantify Kim-1 and the control gene, 23 and 18 cycles were used. All reactions were performed individually from each total RNA isolated from each renal cortex. Genomic DNA contamination was checked by treating all RNA samples with RNase-free DNase I and by carrying samples through the PCR procedure without adding RT.

To analyze PCR products, one-half of each reaction was electro-phoresed in a 5% acrylamide gel. Bands were ethidium bromide stained and visualized under ultraviolet light, cut out, suspended in 1 ml scintillation cocktail (Ecolume; ICN, Aurora, OH), and counted by liquid scintillation (model LS6500; Beckman, Fullerton, CA). Kim-1 mRNA levels are expressed as the ratio of the radioactivity amount recovered from the excised Kim-1 bands over the radioactivity of the respective gene control bands. The RT-PCR semi quantitative analysis was performed at least in quadruplicate.

Kim-1 Real-time PCR

PCR primers and 6-carboxyfluorescein (FAM) or VIC dye-labeled TaqMan minor groove binder (MGB) probes sets were selected from the Applied Biosystems Assays-on-demand product line and were specifically used to detect and quantify cDNA sequences without detecting genomic DNA. For Kim-1 and 18S rRNA expression, we amplified a fragment of GAPDH using primers previously described (31). Genomic DNA contamination was checked by treating all RNA samples with DNase and by carrying samples through the PCR procedure without adding reverse transcriptase (RT).

RT was carried out using 2.5 μg total RNA from renal cortices. Before RT reaction, total RNA was heated at 65°C for 10 min. RT was performed at 37°C for 60 min in a total volume of 20 μl using 200 units of the Moloney murine leukemia virus RT (GIBCO-BRL), 100 pmol random hexamers (GIBCO-BRL), 0.5 mM of each dNTP (Sigma Chemical, St. Louis, MO), and 1X RT buffer (75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl₂, and 10 mM dithiothreitol, pH 8.3). Samples were heated at 95°C for 5 min to inactivate the RT and diluted to 40 μl with PCR-grade water. One-tenth of RT individual samples of each group was used for Kim-1 or GAPDH amplification in 20 μl final volume reactions containing 1X PCR buffer (10 mM Tris·HCl, 1.5 mM MgCl₂, and 50 mM KCl, pH 8.3), 0.1 mM of each dNTP, 0.2 μM [α-32P]dCTP (~3,000 Ci/mmol; 9.25 MBq, 250 μCi), 10 μM of each primer, and 1 unit Taq DNA polymerase (GIBCO-BRL). Samples were overlaid with 30 μl mineral oil, and PCR cycles were performed in a DNA thermal cycler (Whatman; Biometra), with the following profile: denaturing for 1 min at 94°C, annealing for 1 min at 57°C, and 1 min extension step at 72°C. The last cycle was followed by a final extension step of 5 min at 72°C. The control gene was coamplified simultaneously in each reaction. The optimal number of cycles for each primer pair was determined through kinetic amplification following our standard procedure (4, 10). To routinely semi-quantify Kim-1 and the control gene, 23 and 18 cycles were used. All reactions were performed individually from each total RNA isolated from each renal cortex. Genomic DNA contamination was checked by treating all RNA samples with RNase-free DNase I and by carrying samples through the PCR procedure without adding RT.

To analyze PCR products, one-half of each reaction was electro-phoresed in a 5% acrylamide gel. Bands were ethidium bromide stained and visualized under ultraviolet light, cut out, suspended in 1 ml scintillation cocktail (Ecolume; ICN, Aurora, OH), and counted by liquid scintillation (model LS6500; Beckman, Fullerton, CA). Kim-1 mRNA levels are expressed as the ratio of the radioactivity amount recovered from the excised Kim-1 bands over the radioactivity of the respective gene control bands. The RT-PCR semi quantitative analysis was performed at least in quadruplicate.

Recombinant Rat Kim-1 Ectodomain Protein

The soluble form of rat Kim-1 was obtained as described earlier (3). Briefly, a construct [rat Kim-1 (mucin)-Ig] was made that encoded the extracellular domain of rat Kim-1 (residues 1–234) attached to theFc portion of human IgG1 hinge, calponin homology (CH2 + CH3) domains and cloned into an expression vector PEAG347, obtained from Biogen Idec. Transfected Chinese hamster ovary (CHO) cell lines expressing the fusion protein were selected, adapted in suspension with the hybridoma serum-free media (BD Biosciences), and grown in a cell factory (Fisher Scientific). The rat Kim-1-Fc protein was purified from the conditioned media by chromatography on a protein A-Sepharose column (Amersham Biosciences). In brief, the protein A-Sepharose column (2 × 20 cm) was equilibrated with 20 mM sodium phosphate and 0.15 M NaCl (pH 7). The conditioned media was applied, and the column was washed with the equilibration buffer. The column was eluted with 25 mM sodium phosphate and 0.1 M NaCl (pH 2.8). Fractions (1 ml) were collected in 1.5-ml Eppendorf tubes containing 50 μl neutralizing buffer (0.5 M sodium phosphate, pH 8.6). The concentration of each fraction was measured by a sandwich ELISA using rabbit anti-human-IgG-Fc as a trapping antibody and horseradish peroxidase (HRP)-labeled goat anti-human IgG-Fc as a secondary antibody. This was further confirmed by measuring the Kim-1 protein concentration using a protein assay reagent (Bio-Rad). Fractions of similar concentrations were combined and dialyzed [mol wt cut off (MWCO) = 30,000] against PBS overnight at 4°C and concentrated using 30,000 MWCO amicon centrifugal filters (Millipore).

Mouse Monoclonal Antibodies Against Rat Kim-1 Ectodomain

Mice were immunized with purified rat Kim-1-Fc protein to generate monoclonal antibodies using standard antibody production techniques by contract to Dr. Jun Hayashi’s laboratory at the University of Maryland (Baltimore, MD). Eighteen hybridoma clones were selected that were positive for Kim-1-Fc and negative for human IgG-Fc. These clones were adapted in suspension with the serum-free hybridoma media (BD Biosciences) grown in 175-cm² tissue culture flasks. These clones were adapted in suspension with the hybridoma serum-free media (BD Biosciences), and the monoclonal anti-rat Kim-1 ectodomain (MARKE) antibodies were purified from conditioned media using a protein G-Sepharose column (Amersham Biosciences). In brief, the protein G-Sepharose column (2 × 20 cm) was equilibrated with 20 mM sodium phosphate and 0.15 M NaCl (pH 7). The conditioned media was applied, and the column was washed with the equilibration buffer. The column was eluted with 100 mM glycine buffer and 0.1 M NaCl (pH 2.7). Fractions (1 ml) were collected in 1.5-ml Eppendorf tubes containing 50 μl neutralizing buffer (1 M Tris-Cl, pH 9). Absorbance of each fraction was measured in a quartz cuvette at 280 nm (absorbance of 1 = 0.8 mg/ml) using a spectrophotometer (Molecular Devices). Fractions of similar concentrations were combined and dialyzed against PBS overnight at 4°C and concentrated using amicon centrifugal filters with 30,000 MWCO (Millipore).

Rat Kim-1 Sandwich ELISA

Biotinylation of MARKE-1. One MARKE antibody (MARKE-Trap), at a concentration ~1.5 mg/ml, served as a trapping/primary antibody. The other MARKE antibody (concentration ~2.2 mg/ml) was biotinylated using the Pierce EZ-Link NHS-PEO₂-Biotin conjugating kit (Pierce) to act as a detecting/secondary antibody. The number of moles of biotin/mole of protein was determined to be six (corresponding to very high efficiency of biotinylation) using an
EZ-Biotin Quantitation Kit. The nonreacted and hydrolyzed biotinylation reagent was removed by dialyzing with PBS, pH 7, overnight at 4°C.

Construction of Kim-1 sandwich ELISA. The wells of an ELISA plate (MaxiSorp; Nunc, Naperville, IL) were coated with MARKE-Trap [overnight incubation at 4°C with 100 µl antibody and 10 µg/ml in coating buffer (0.1 M potassium phosphate buffer, pH 9)]. The plate was washed three times using a squirt bottle with wash solution (PBS-0.05% Tween, PBST). The wells were then blocked for 1 h at 37°C with a blocking solution (3% BSA/PBS with 0.02% sodium azide), and after the incubation period the plate was washed again three times with PBST. One hundred microliters of either standard rat Kim-1-Fc pure protein in serial dilutions (0–5 ng/ml) or urine samples from control and treated groups were added to the plate in duplicate, and the plate was incubated for 1.5 h at 37°C. All of the dilutions of Kim-1 standards, urine samples, MARKE-1, and HRP-conjugated streptavidin antibody were done in a sample diluent (2.38% HEPES, 0.6% NaCl, 1% BSA, and 0.1% Tween, pH 7.4). After three washes with PBST, biotinylated MARKE-1 antibody was added, followed by HRP-conjugated streptavidin. Color was developed by adding 3,3',5,5'-tetramethyl benzidine substrate (Sigma), and the reaction was stopped after 15 min by adding 1 N HCl. The absorbance was measured at 450 nm using a plate reader (Molecular Devices). The urinary Kim-1 concentration was calculated based on the standard curve and expressed in absolute terms (ng/ml).

Evaluation of the Kim-1 ELISA. The performance characteristics of the Kim-1 ELISA were evaluated by measuring the sensitivity, assay range, specificity, reproducibility, recovery, dilutional linearity, and interference. The analytical recovery was determined by adding a known amount of 750 or 1,500 pg/ml recombinant Kim-1-Fc into vehicle-treated rat urine samples and quantitating the Kim-1 levels in these spiked samples. To assess the recovery of Kim-1 in urine from animals with kidney injury, a known amount of 750 or 1,500 pg/ml recombinant Kim-1-Fc was added to 5 mg cisplatin/kg treated rat urine samples containing 300–2,100 pg/ml Kim-1. This was done to verify that there was no interfering substance in the urine of animals with AKI and to demonstrate that cisplatin, which is in the urine of these animals, does not interfere with the assay (27). Dilutional linearity was evaluated in normal and cisplatin-treated rat urine to justify sample dilution to fit the concentrations of Kim-1 in the linear range of the standard curve (0–5,000 pg/ml). Cisplatin-treated rat urine samples containing 6, 8, 12, or 18 ng/ml (as measured by the Kim-1 ELISA) corresponding to medium and high concentration of Kim-1 were diluted 1:5, 1:10, 1:20 using sample diluent, and the Kim-1 levels were measured by ELISA.

Immunofluorescence Microscopy

Immunocytochemistry for Kim-1 was performed as previously described (17). Sections were thawed, washed with PBS, and blocked in 1.5% normal goat serum in PBS (blocking solution) for 30 min. Sections were then incubated for 1 h with the primary antibodies [MARKE-1, MARKE-2, and MARKE-Trap (5 µg/ml) diluted in the blocking solution]. The sections were washed with PBS and incubated with anti-mouse 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 mouse IgG (5 µg/ml) was used for the primary antibody.

Statistics

All results are expressed as means ± SE. 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

Construction and Purification of Rat Kim-1 Ectodomain Fusion Protein

Rat Kim-1 maps to chromosome 10 (10q21) of the rat genome and is conserved across species with 77% identity to mouse Kim-1, 30% identity to chimpanzee, and 38% identity with human KIM-1. The protein structure of rat Kim-1 consists of a total of 307 amino acids (aa) with a signal peptide (1–21 aa), a highly conserved 6-cysteine-rich Ig-like domain (22–130), a serine-threonine- and proline-rich mucin domain containing several N- and O-linked glycosylation sites (131–235), a transmembrane domain (236–256), and a short cytoplasmic tail (257–307) containing a tyrosine kinase phosphorylation site from 291 to 297 aa (Fig. 1A). The Kim-1 ectodomain is shed from the proximal tubule after injury and has been detected by Western blot analysis in the urine of rats treated with nephrotoxins (17). To quantitate the release of Kim-1 into the urine, monoclonal antibodies were generated that are specific to the ectodomain of rat Kim-1. A plasmid encoding a fusion protein of the rat Kim-1 ectodomain (1–234 aa) with the Fc portion of human IgG was generated (Fig. 1B), stably transfected into CHO cells, and purified from the conditioned media as described in MATERIALS AND METHODS. Three different batches of purified and dialyzed rat Kim-1-Fc were loaded on the gel, and the purity was assessed by Commassie staining (Fig. 1C) and Western blot analysis (Fig. 1D) using HRP-labeled anti-human IgG-Fc antibody. In the Commassie-stained blot, the strongest band was of the Kim-1-Fc protein at 85 kDa that corresponds to the predicted molecular weight of the glycosylated Kim-1 ectodomain (1–234 aa) fused to the Fc portion of human IgG. Similar results were obtained in the Western blot with only one band at 85 kDa in all three batches of purified Kim-1-Fc, attesting to its purity.

Monoclonal Antibodies and Evaluation of Performance Characteristics of the Kim-1 ELISA

To obtain highly specific MARKE antibodies, mouse monoclonal antibodies were generated against the pure recombinant Kim-1-Fc protein. The hybridoma cells were screened and selected in such a way that the conditioned media recognized Kim-1-Fc but did not recognize human IgG-Fc. Three such MARKE antibodies were purified using protein G-Sepharose, and the efficiency of these antibodies (MARKE-1, MARKE-2, and MARKE-Trap) to detect Kim-1-Fc was estimated by Western blot analysis (Fig. 2A). All three purified antibodies at the concentration of 1 µg/ml recognized 25 ng/lane of Kim-1-Fc without any nonspecific binding. This suggested that all three MARKEs were able to detect the denatured Kim-1-Fc protein. To further evaluate whether these antibodies would detect nondenatured three-dimensional Kim-1-Fc protein in a sandwich ELISA (Fig. 2B), MARKE-1 and -2 (2 µg/ml) were biotinylated to act as secondary antibodies, and MARKE-Trap (10 µg/ml) was used as a trapping antibody. Both MARKE-1 and -2 were able to detect Kim-1 when MARKE-Trap was used as a primary trapping antibody. When either MARKE-1 or -2 was used as the trapping antibody and the other as the detecting antibody, these two antibodies could not be made to effectively identify Kim-1, suggesting that they may be binding to the same or overlapping epitopes of Kim-1.
A standard curve was generated using serial dilutions of Kin-1-Fc protein in sample diluent starting with 5,000 pg/ml (Fig. 2C). The sensitivity of this assay, or lower limit of detection (LLD), was defined as the lowest Kin-1-Fc concentration that could be differentiated from zero (assay blank/sample diluent) by Student’s t-test. The LLD was 39 pg/ml sample diluent (n = 5; P < 0.001).

Assay range (0–5,000 pg/ml). The assay range was estimated by calculating the coefficient of variation (CV) of each standard constructing five independent standard curves. The CV obtained for each standard from 78.1 to 5,000 pg/ml sample diluent was <10%. The standard curve concentrations used for all the Kin-1 ELISAs were 5,000, 2,500, 1,250, 625, 312.5, 156.3, and 78.1 pg/ml.

Specificity. This ELISA is specific for the measurement of natural and recombinant rat Kin-1. It does not detect mouse and human KIM-1 (data not shown). It also does not detect human IgG-Fc or an irrelevant protein (C-RET, a protooncogene reported to be involved in sporadic papillary thyroid carcinoma) fused with human IgG (C-RETFc), attesting to the specificity of the antibodies to bind Kin-1.

Reproducibility. The precision profile was assessed by use of urine samples collected on days 1 and 2 after 5 mg cisplatin/kg that contained different concentrations of Kin-1 (752, 1,203, 1,692, 2,120, 2,630, and 4,250 pg/ml). Intra- and interassay CVs ranged from 2 to 4.9% (n = 6) and from 2.7 to 9.6% (n = 3 in 3 different plates), respectively (Fig. 2D).

Recovery. The analytical recovery was determined by adding 750 or 1,500 pg/ml recombinant Kin-1-Fc to vehicle-treated or 5 mg cisplatin/kg-treated rat urine samples containing 300–2,100 pg/ml Kin-1. Recoveries of Kin-1 ranged from 90 to 110%.

Dilution linearity. When cisplatin-treated rat urine samples containing 6, 8, 12, or 18 ng/ml corresponding to medium and high concentrations of Kin-1 were diluted 1.5, 1.10, and 1.20, each sample gave results close to linearity (r = 0.95–0.99), confirming parallelism between the standards and the urine samples (data not shown).

Interference. Urine samples containing 732 or 2,630 pg/ml Kin-1 were supplemented with potentially interfering agents, including cisplatin and mercuric chloride, at various concentrations. We tested cisplatin, since we used it as a model nephrotoxicant in this study, and mercuric chloride to assess the ability of metals to potentially interfere with the Kin-1 ELISA. There was no significant interference from ascorbic acid (<56.8 mmol/l), albumin (<5 g/l), globin (<0.1 g/l), creatinine (<132.6 mmol/l), creatine (<38.1 mmol/l), mercuric chloride (<3 mM), urea (<1.0 mol/l), or sodium chloride (<0.5 mol/l), indicating that the estimation of Kin-1 was not affected by interference from a wide range of urinary constituents that are expected to be present in the urine after AKI.

Nephrotoxicity of Cisplatin as Measured by BUN, Plasma Creatinine, and Histopathology

We used a widely established and well-studied cisplatin-induced AKI model (1, 17, 18) to induce nephrotoxicity and to evaluate whether Kin-1 could serve as an early predictor of nephrotoxicity. Low (2.5 mg/kg), medium (5 mg/kg), or high (7.5 mg/kg) doses of cisplatin were administered intraperitoneally in 0.9% saline or the same volume of vehicle to male Sprague-Dawley rats. Because previously we had shown only mild changes in BUN, serum creatinine, and histopathology after a low dose of 2.5 mg cisplatin/kg (1, 17, 18), we only collected urine from this group. After 5 or 7.5 mg cisplatin/kg, BUN or plasma creatinine did not increase significantly until day 2 but increased ~4- and 10-fold, respectively, over base-
line (day 0) on day 3 (Fig. 3, A and B). BUN and creatinine further increased on day 4 by 5- to 7-fold and 20-fold over baseline, respectively, reflecting significant renal dysfunction and kidney injury (Fig. 3, A and B). After 7.5 mg cisplatin/kg, 50% mortality was observed on day 4, whereas no mortality was observed with the 5 mg/kg dose until day 5. Histological examination of the kidney sections revealed no significant alterations on day 1 after 5 (Fig. 3D) or 7.5 (Fig. 3E) mg cisplatin/kg compared with the controls (Fig. 3C). On day 2 after either dose, there was some single cell necrosis, tubular dilation, and sloughing of cells in the lumen of the S3 segment of proximal tubules into the outer stripe of the outer medulla (OSOM; Fig. 3, F and G). On day 3 after either dose, there was significant proximal tubular necrosis, particularly in the S3 segment, and intratubular casts were clearly seen (Fig. 3, H and I). The inflammation, edema, tubular degeneration, dead cells, necrosis, and apoptosis were most severe in the 7.5-mg cisplatin/kg-treated group.

Urinary Kim-1 Levels Over Time in Response to Varying Doses of Cisplatin

Previous studies from our laboratory have shown Kim-1 protein levels to be upregulated in the kidney by immunocytochemistry and ectodomain shedding into the urine by immunoblot analysis from day 1 to 6 after 10 mg cisplatin/kg in rats (17). We have previously lacked, however, a sensitive and high-throughput method of detecting Kim-1 in the urine. With the highly specific and sensitive ELISA we constructed to quantitate Kim-1, we found an approximate three- to fivefold
increase in urinary Kim-1 on day 1 after 2.5, 5, or 7.5 mg cisplatin/kg compared with the vehicle-treated controls (day 0). Urinary Kim-1 levels further increased to 9- to 12-fold baseline on day 2 (Fig. 4). Kim-1 levels peaked on day 3 after administration of 7.5 mg cisplatin/kg, reaching 26 ng/ml in the animals that subsequently succumbed (50%). The remaining 50% of the rats that survived this high dose had Kim-1 levels 22 ng/ml on days 4 and 5. Kim-1 levels remained between 15 and 25 ng/ml for 2.5 and 5 mg cisplatin-treated groups from days 3 to 5, indicating a plateau in the excretion of Kim-1 associated with severe kidney injury. Thus there was a time-dependent progressive increase in Kim-1 after 2.5, 5, or 7.5 mg cisplatin/kg administration from day 0 to day 3 after which there was a plateau of urinary Kim-1 levels.

Immunocytochemical Localization of Kim-1 in the Kidney after 5 mg/kg Cisplatin Administration using Three Different Monoclonal Antibodies

As indicated previously, MARKE-Trap was used as a primary antibody, and biotinylated MARKE-1 or -2 was used as secondary antibody in the Kim-1 ELISA. We tested by immunocytochemistry the efficacy of these three monoclonal anti-Kim-1 ectodomain antibodies (including MARKE-2) to localize Kim-1 in tissue from animals treated with 5 mg/kg cisplatin (Fig. 5). With the use of the three MARKEs, there was no Kim-1 staining on day 0, indicating undetectable expression of Kim-1 under normal conditions (Fig. 5, A, E, and I). On day 1 after cisplatin, all three MARKEs showed a very similar pattern of positive Kim-1 staining in an apical membrane distribution in the OSOM in isolated epithelial cells of the S3 segments of the proximal tubules (Fig. 5, B, F, and J). The expression significantly increased on day 3 after 5 mg cisplatin/kg administration, consistent with the high urinary Kim-1 levels (13.4 ± 4.4 ng/ml). Kim-1 was localized on the apical surface of cells in the proximal tubules, indicative of tubular injury.

Figure 3. Plasma blood urea nitrogen (BUN), creatinine, and renal histopathology over a time course after administration of 5 or 7.5 mg cisplatin/kg. Male Sprague-Dawley rats (n = 10 for each) were administered 5 or 7.5 mg cisplatin/kg ip in 0.9% saline. Animals were kept in Nalgene rat metabolic cages overnight, and 12-h urine samples were collected in the morning from 0 to 5 days. Urine was centrifuged at 6,000 rpm for 15 min and stored in aliquots at −80°C for further analysis. Measurement of Kim-1 in the urine samples was performed by ELISA, as described in MATERIALS AND METHODS. Each sample was measured in triplicate diluted 1:5, 1:10, and 1:20 in sample diluent to fit in the linear range of the standard curve. The urinary Kim-1 content is expressed as ng/ml. *P ≤ 0.05 compared with the value at time 0.

Figure 4. Measurement of urinary Kim-1 by ELISA over time after cisplatin administration. Male Sprague-Dawley rats (n = 10 for each) were administered 2.5, 5, or 7.5 mg cisplatin/kg ip in 0.9% saline. Animals were kept in Nalgene rat metabolic cages overnight, and 12-h urine samples were collected in the morning from 0 to 5 days. Urine was centrifuged at 6,000 rpm for 15 min and stored in aliquots at −80°C for further analysis. Measurement of Kim-1 in the urine samples was performed by ELISA, as described in MATERIALS AND METHODS. Each sample was measured in triplicate diluted 1:5, 1:10, and 1:20 in sample diluent to fit in the linear range of the standard curve. The urinary Kim-1 content is expressed as ng/ml. *P ≤ 0.05 compared with the value at time 0.

Table 1. Performance characteristics of Kim-1 ELISA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
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<tbody>
<tr>
<td>Sensitivity</td>
<td>Lowest limit of detection: 39 pg/ml</td>
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<tr>
<td>Assay range</td>
<td>78.1–5,000 pg/ml</td>
</tr>
<tr>
<td>Specificity</td>
<td>Does not cross-react with mouse or human KIM-1</td>
</tr>
<tr>
<td>Intra-assay variability</td>
<td>2–4.9%</td>
</tr>
<tr>
<td>Inter-assay variability</td>
<td>2.7–9.6%</td>
</tr>
<tr>
<td>Recovery</td>
<td>90–110%</td>
</tr>
<tr>
<td>Dilution linearity</td>
<td>1:5, 1:10, and 1:20</td>
</tr>
<tr>
<td>Interference</td>
<td>None identified</td>
</tr>
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Kim-1, kidney injury molecule 1.
membranes of the injured tubules, which were dilated and also had protein casts. In some parts of the OSOM region, the Kim-1 staining pattern appeared diffusely cytoplasmic. On day 5 after the 5 mg/kg dose, although the Kim-1 levels in the urine (17.8 ± 4.6 ng/ml) did not increase very dramatically from day 3 (13.38 ± 4.37 ng/ml), there was a significantly greater Kim-1 protein expression in the kidneys as detected by all three MARKEs (Fig. 5, D, H, and L). The localization was apical and diffusely cytoplasmic but was much more widespread expressed throughout the injured region of the OSOM. Thus all three antibodies can very sensitively detect a progressive injury-dependent expression of denatured (Fig. 2A) or undenatured (Figs. 4 and 5) Kim-1 in the kidney after cisplatin nephrotoxicity.

Kim-1 as an Early Diagnostic Indicator of AKI After Cisplatin Nephrotoxicity

BUN, plasma creatinine, urinalysis, glycosuria, and proteinuria are currently the most widely used biomarkers for renal dysfunction and injury. In the urine samples collected on days 0, 1, and 2 from rats treated with 2.5, 5, or 7.5 mg cisplatin/kg glucose (Fig. 6A), protein (Fig. 6B) and NAG (Fig. 6C) were measured and compared with urinary Kim-1 (Fig. 6D) to evaluate the efficiency of Kim-1 as an early diagnostic indicator of kidney injury. In the 2.5 mg cisplatin/kg group, there was no significant increase in urine glucose (Fig. 6A), protein (Fig. 6B), or NAG (Fig. 6C) on days 1 and 2; however, by contrast, there was an ~5-fold increase in Kim-1 on day 1 that further increased up to ~12-fold on day 2 (Fig. 6D). Thus the concentrations of urinary Kim-1 were significantly higher on days 1 and 2, indicating renal injury when none of the conventionally used biomarkers of renal injury, including glycosuria, proteinuria, or urinary NAG levels, were able to predict this. Similarly, in the 5 and 7.5 mg cisplatin/kg groups, there were no significant differences in the urinary glucose, protein, NAG (Fig. 6, A–C), BUN (Fig. 6E), or plasma creatinine (Fig. 6F) values on day 1, but there was an approximate three- to fivefold increase in urinary Kim-1 levels, indicating significant renal injury as early as day 1. The ~7- to 10-fold increase in Kim-1 on day 2 was accompanied by increases in glycosuria, proteinuria, and NAG values after 5 or 7.5 mg cisplatin/kg (Fig. 6, A–D). These results suggest that Kim-1 is a highly sensitive and early indicator of renal dysfunction useful for detection of even minor alterations in renal structural and functional integrity, as demonstrated by its significant upregulation on day 1 after 2.5 mg/kg cisplatin.

Comparison of Kim-1 with other Biomarkers of Kidney Injury in Renal I/R Model

To generalize the effectiveness of Kim-1 as a sensitive and early diagnostic biomarker for AKI, we quantitated the Kim-1 ectodomain in the urine of male Wistar rats after different
periods of bilateral I/R injury. There was no significant increase in BUN (Fig. 7A) or plasma creatinine (Fig. 7B) on day 1 after 10 or 20 min of bilateral I/R injury. By contrast, 1 day after 30 or 45 min bilateral I/R injury, BUN increased approximately four- to fivefold, and plasma creatinine increased approximately five- to sixfold, indicating severe renal dysfunction. Twenty-four hours after 10 min of bilateral I/R injury, Kim-1 was fivefold higher (Fig. 7E) than sham-operated control.
trols, whereas there was no significant decrease in the creatinine clearance (Fig. 7D) nor an increase in proteinuria (Fig. 7C) with this length of ischemia. With 20 min bilateral I/R injury, there was a 50% reduction in creatinine clearance (Fig. 7D) with no significant change in proteinuria (Fig. 7C). Urine was collected for 24 h starting after initiation of reperfusion. Urinary Kim-1 was ~50-fold higher than controls in this collection (Fig. 7E). As seen previously in the cisplatin-induced nephrotoxicity model (Fig. 4), the urinary Kim-1 levels were quantitatively in the 25–35 ng/ml range in the 20, 30, or 45 min bilateral I/R groups after 24 h. Creatinine clearance decreased by 75 and 85%, respectively, after 30 or 45 min bilateral I/R (Fig. 7D), with a significant increase in proteinuria occurring only in the 45-min bilateral I/R group (Fig. 7C). Thus, in the bilateral I/R injury model, as in the cisplatin model, Kim-1 was a highly sensitive indicator of renal dysfunction, with fivefold higher Kim-1 levels in the 24-h collection of urine after 10-min bilateral I/R injury at a time when BUN, plasma creatinine, creatinine clearance, and urinary protein were unchanged from controls.

**Renal I/R-Induced Injury-Dependent Changes in Kim-1 Gene Expression and its Correlation with Creatinine Clearance**

To correlate Kim-1 gene expression with the degree of injury, Kim-1 mRNA was quantitated using semiquantitative (Fig. 8, A and B) and quantitative (Fig. 8C) RT-PCR after different periods of bilateral I/R injury. In concordance with the urinary Kim-1 protein levels, which increased approximately fivefold after 10 min bilateral I/R injury, Kim-1 mRNA levels were also significantly upregulated at 24 h in this group (Fig. 8, A–C). With the use of quantitative real-time PCR, a much more sensitive measure of gene expression (Fig. 8C), Kim-1 mRNA was ~25-fold higher than controls after 10 min bilateral I/R injury at 24 h. There was an injury-dependent increase in Kim-1 mRNA, as seen in both semiquantitative and quantitative real-time PCR, with an ~160-, 280-, and 350-fold increase (Fig. 8C) in Kim-1 mRNA at 24 h after 20, 30, or 45 min bilateral I/R injury, respectively. These data complement the findings of an injury-dependent increase in Kim-1 protein expression (Fig. 5) after 5 mg/kg cisplatin. Collectively, these results indicate that both Kim-1 mRNA and protein expression increase as a function of injury. The correlation coefficient relating creatinine clearance as a parameter of renal function to Kim-1 mRNA levels was 0.732, with a statistical significance of $P < 0.0001$ (Fig. 8D).

**DISCUSSION**

We report the development of a quantitative assay for measuring Kim-1 in the rat urine as a biomarker for AKI and demonstrate that marked increases in urinary Kim-1 can be detected with semiquantitative (Fig. 8, A and B) and quantitative (Fig. 8C) RT-PCR after different periods of bilateral I/R injury. In concordance with the urinary Kim-1 protein levels, which increased approximately fivefold after 10 min bilateral I/R injury, Kim-1 mRNA levels were also significantly upregulated at 24 h in this group (Fig. 8, A–C). With the use of quantitative real-time PCR, a much more sensitive measure of gene expression (Fig. 8C), Kim-1 mRNA was ~25-fold higher than controls after 10 min bilateral I/R injury at 24 h. There was an injury-dependent increase in Kim-1 mRNA, as seen in both semiquantitative and quantitative real-time PCR, with an ~160-, 280-, and 350-fold increase (Fig. 8C) in Kim-1 mRNA at 24 h after 20, 30, or 45 min bilateral I/R injury, respectively. These data complement the findings of an injury-dependent increase in Kim-1 protein expression (Fig. 5) after 5 mg/kg cisplatin. Collectively, these results indicate that both Kim-1 mRNA and protein expression increase as a function of injury. The correlation coefficient relating creatinine clearance as a parameter of renal function to Kim-1 mRNA levels was 0.732, with a statistical significance of $P < 0.0001$ (Fig. 8D).

**Fig. 8.** Kim-1 mRNA levels in kidney after different periods of ischemia and correlation of Kim-1 mRNA in kidney with creatinine clearance. Kim-1 mRNA levels were measured by semiquantitative (A and B) and quantitative (C) real-time RT-PCR in renal cortex of rats at 24 h after 0, 10, 20, 30, and 45 min of bilateral ischemia [sham control (N), I-10, I-20, I-30, and I-45, respectively]. A: mRNA was isolated from renal cortex, and the RT-PCR products were separated on a 5% ethidium bromide acrylamide gel. Top: Kim-1 mRNA after different periods of renal ischemia. Bottom: renal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA values, which were used as internal standard. B: Kim-1 mRNA level was semiquantitated by excising the bands from the ethidium bromide-stained gel and counted by liquid scintillation. The results are presented as the ratio of PCR products (cpm) of Kim-1 and GAPDH ± SE. C: real-time PCR was carried out in 96-well plates on cDNA derived from 2.5 ng total RNA isolated individually from each renal cortex. The amount of fluorescence in each reaction liberated by the exonuclease degradation of the TaqMan probe during PCR amplification was measured as a function of the PCR cycle number using an ABI 7000 Prism (Applied Biosystems). Results are presented as relative Kim-1-to-18S rRNA ratios compared with the sham value. D: values of creatinine clearance (Cl) are plotted vs. Kim-1-to-GAPDH mRNA ratios. The $r$ value of 0.732 indicates a high degree of correlation between creatinine clearance and Kim-1 mRNA expression with a significance of $P < 0.0001$. *$P < 0.05$ compared with value in the sham-treated group.
measured under conditions where changes in other standard indicators are not measurable. These results have direct implications for evaluation of nephrotoxicity in animals where sensitive and specific measures of toxicity have been elusive. Nephrotoxicity often is evaluated by reliance on serum creatinine levels, an insensitive measure of kidney injury, or histological analysis, which requires killing a large number of animals and is inherently difficult to quantitate. The Kim-1 assay is a very sensitive and robust system with minimal interference from other components of the diseased urine and is not affected by variation in physicochemical properties of the urine. The assay has a dynamic range from 0 to 5,000 pg/ml with the lowest limit of detection being <38 pg/ml and an inter- and intra-assay variability <10% (Fig. 2, C and D).

The performance characteristics of the developed ELISA are comparable to the commercially available ELISAs to measure cytokines in rat serum (e.g., tumor necrosis factor-α, IL-6) as markers of inflammation. Although an extensive performance characteristic evaluation has not been reported for the human Kim-1 ELISA, both the rat Kim-1 and human KIM-1 ELISAs are highly comparable in terms of sensitivity, inter- and intra-assay variability, and recovery (12). For the development of the rat Kim-1 ELISA, a panel of 18 mouse monoclonal antibodies were made against rat Kim-1-Fc fusion proteins from which three were purified, dialyzed, and concentrated to 10% (Fig. 2, C). Two of these were used in the ELISA, and all three were used as in the urine. The assay has a dynamic range from 0 to 5,000 pg/ml with the lowest limit of detection being <38 pg/ml and an inter- and intra-assay variability <10% (Fig. 2, C and D).

NGAL is upregulated and can be detected in the kidney (22) and urine of mice three h after cisplatin (20 mg/kg) and has been proposed as an early biomarker for diagnosing AKI (24, 25). These rodent studies have been extended to humans in a recent study indicating that NGAL levels at 2 h is a very powerful predictor of AKI in children after cardio pulmonary bypass (23). Similarly, CYR-61, a secreted protein, is present early in the urine after I/R but currently can only be detected by immunoblotting the urine and kidney extracts at 3–6 h after 30 min bilateral I/R injury (26). Urinary CYR-61 levels are reduced over time despite the continuous progression of injury. Clusterin, a protein associated with programmed cell death and tissue reorganization, as measured by RIA, has been correlated with the elevation of creatinine and NAG in the gentamicin-induced renal injury model in rats (2). Clusterin mRNA and protein levels, however, did not increase until day 5 in the cisplatin-induced renal injury model (35). F-actin is released in the urine in rats at 1 h after 25 min renal ischemia because of actin depolymerizing factor-mediated disruption of microvillar microfilament and apical membrane (34). Increased urinary actin along with IL-6 and IL-8 have also been suggested as useful markers for the prediction of sustained acute renal failure after ischemia in humans (19).

An ideal biomarker to detect AKI in animal studies should be organ and site specific, sensitive to detect minor perturbations in renal function, correlate with the degree of tubular injury, persist throughout the time course of renal injury to indicate progression and regression of injury, be noninvasive, and be quantifiable by a high-throughput method to facilitate large sample size measurements. In addition, it would also add to the merit of the biomarker if it were conserved across species from fish to rodents to humans, allowing for the extrapolation of the results and use of the same marker in preclinical and clinical studies. Kim-1 is expressed predominantly on the apical membrane of the epithelial cells of proximal tubules after injury. An upregulation of Kim-1 in the urine indicates kidney proximal tubular injury (Fig. 5) except when proximal cells have also become dedifferentiated for another reason, such as in renal cell carcinoma (11). Urinary Kim-1 levels increase at least fivefold on day 1 after 5 and 7.5 mg/kg cisplatin, whereas BUN and plasma creatinine increases were seen only from day 3 onward (Fig. 3, A and B). Similar results were obtained in the I/R injury model where 10 min bilateral I/R injury caused a significant increase in urinary Kim-1 without any changes in plasma creatinine, BUN, creatinine
clearance, or proteinuria. Thus Kim-1 is upregulated and shed in the urine with mild insults, which result in minimal injury, thus attesting to the sensitivity and early diagnostic ability of Kim-1 to serve as a biomarker for AKI. An injury-dependent increase in Kim-1 mRNA is observed in the I/R model with progressively increasing Kim-1 gene expression levels after 10, 20, 30, or 45 min bilateral I/R injury (Fig. 8, A–C). A similar trend in the injury-dependent increase in Kim-1 protein expression is evident from the immunohistochemical staining of Kim-1 on days 0–5 after 5 mg/kg cisplatin (Fig. 5). Thus Kim-1 gene expression and surface protein expression correlate very well with the degree of renal tubular injury (Fig. 7C).

A significant advantage of measuring Kim-1 as a biomarker for AKI is that it is conserved across species and is upregulated after renal injury in zebrafish (unpublished observations), mice (29), rats (17), nonhuman primates (9), and humans (12) encompassing a complete array of preclinical to clinical risk assessment models. Thus quantitation of urinary Kim-1 is likely to be very useful for the evaluation of kidney injury in animal pathophysiological studies and predictive toxicology and may improve our ability to identify effective therapeutic agents for kidney injury and eliminate nephrotic compounders early in the drug development process.

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