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, translation of these findings to therapeutics useful in clinical trials and into postmarketing studies has been hindered by 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.

Routinely used measures of renal function, such as levels of blood urea nitrogen (BUN) and serum creatinine, increase significantly only after substantial kidney injury occurs and then 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 π-glutathione-S-transferases (α- and π-GST; see Ref. 7), neutrophil gelatinase-associated lipocalin (NGAL; see Ref. 24), cysteine-rich protein 61 (CYR-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 dediffer-entiated 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 intraperitonea1ly 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 days 1, 2, 3, 4, or 5. Blood was collected from the dorsal aorta in heparinized tubes for measurement of BUN and creatinine. One kidney was perfused via the left ventricle with PBS and then with PLP for 10 min. The other kidney pedicle was clipped with hemostatic forceps and allowed to ischemically perfuse for 0, 10, 20, 30, or 45 min. Reperfusion commenced when the clips were removed. Occlusion 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 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. Occlusion 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; Thermotrace). 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 polyacrylamide 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’-CGGTGCCCTGTGAGTAAATAGATG-3’ and antisense 3’-CTGGCCATGACACAAATAGAC-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 1× 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 1× 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 electrophoresed 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 semiquantitative 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 analysis, FAM and VIC probes, respectively, have been designed (4331182 and 4319413E; Applied Biosystems). FAM and VIC were used as fluorescent reporter dyes and conjugated to 5′ ends of probes to detect amplification products. The amount of FAM or VIC fluorescence in each reaction liberated by the exonuclease degradation of the TaqMan probe during PCR amplification was measured as a function of PCR cycle number using an ABI 7000 Prism (Applied Biosystems). PCR was carried out in 96-well plates on cDNA equivalent to 2.5 ng total RNA isolated individually from each renal cortex.

Thermal cycling conditions were 10 min at 95°C followed with 40 cycles at 95°C for 1 min and 60°C for 1 min. Data were collected using the ABI PRISM 7000 SDS analytical thermal cycler (Applied Biosystems). Each individual sample was tested in triplicate to ensure statistical significance. The relative quantification of Kim-1 gene expression was performed using the comparative threshold cycle (Ct) method (21). The Ct value is defined as the point where a statistically significant increase in the fluorescence has occurred. The number of PCR cycles (Ct) required for the FAM and VIC intensities to exceed a threshold just above background was calculated for the test and reference reactions. In all experiments, 18S rRNA was used as control. Results were analyzed in a relative quantification study by the ratio Kim-1/18S rRNA. Negative controls were included in the reaction plate.

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)-Fc] was made and encoded the extracellular domain of rat Kim-1 (residues 1–234) attached to the Fc portion of human IgG1 hinge, calponin homology (CH2 + CH3) domains and cloned into an expression vector PEA3347, 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. 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). 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-PEO₄-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
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 quantifying 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

Characteristics of the Kim-1 ELISA

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 three 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 non-denatured 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.
Thus a Kim-1 ELISA was constructed using MARKE-Trap as the primary antibody and MARKE-1 as a secondary antibody. The evaluation of the performance characteristics of the Kim-1 ELISA is as follows (Table 1).

**Sensitivity** (<39 pg/ml). A standard curve was generated using serial dilutions of Kim-1-Fc protein in sample diluent starting with 5,000 pg/ml (Fig. 2A). The sensitivity of this assay, or lower limit of detection (LLD), was defined as the lowest Kim-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 Kim-1 ELISAs were 5,000, 2,500, 1,250, 625, 312.5, 156.3, and 78.13 pg/ml.

**Specificity.** This ELISA is specific for the measurement of natural and recombinant rat Kim-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 Kim-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 Kim-1 (732, 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 Kim-1-Fc to vehicle-treated or 5 mg cisplatin/kg-treated rat urine samples containing 300–2,100 pg/ml Kim-1. Recoveries of Kim-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 Kim-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 Kim-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 Kim-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 Kim-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 Kim-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

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Fig. 2. Monoclonal antibodies to rat Kim-1, principle of Kim-1 ELISA, standard curve, and precision profile. A: monoclonal anti-rat Kim-1 ectodomain (MARKE)-1, MARKE-2, and MARKE-Trap detect Kim-1 ectodomain fusion protein by Western blot analysis. Purified Kim-1-Fc (25 ng/lane) was loaded on 3 different lanes for PAGE and transferred onto a polyvinylidene difluoride membrane that was then cut into 3 strips. Each strip was incubated with 1 μg/ml of either MARKE-1, -2 or MARKE-Trap. Rabbit anti-mouse polyclonal antibody was used as a secondary antibody. B: Kim-1 ELISA is based on a typical sandwich ELISA assay protocol using 96-well plates coated with MARKE-Trap, which traps the Kim-1 antigen present in the urine. Biotinylated MARKE-1 is used as a secondary antibody, and this complex is colorimetrically detected using a spectrophotometer at 450 nm. C: typical standard curve using recombinant Kim-1 ectodomain fusion protein was made using serial dilutions from a 10 ng/ml stock concentration. The standard curves were repeated several times. The intra- and interassay variability was <5%; R² = 0.99 represents a very high correlation coefficient. The equation of the line (y = 0.0006x + 0.0532) can be used to calculate the unknown concentration of Kim-1 (x) by substituting the absorbance value (y). D: precision profile for Kim-1 ELISA. The intra-assay and interassay reproducibility of the Kim-1 ELISA was calculated by adding 5 different concentrations of Kim-1 (750, 1,000, 1,500, 2,000, and 2,500 pg/ml) to control urine samples. For the intra-assay variability, each sample was measured in n = 6 replicates within one plate. For the interassay variability, each sample was measured 3 times in three different plates. CV, coefficient of variation; OT, overnight; TMB, 3,3,5,5-tetramethylbenzidine.
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...
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 con-

Fig. 6. Comparison of Kim-1 with routinely used biomarkers as an early diagnostic indicator of kidney injury. Urinary glucose (A), protein (B), and N-acetyl-β-glucosaminidase (C), and urine Kim-1 (D) were measured on days 0, 1, and 2 after 2.5, 5, or 7.5 mg cisplatin/kg administration. BUN (E) and plasma creatinine (F) were measured on these days after 5 and 7.5 mg/kg cisplatin administration. *P ≤ 0.05 compared with value on day 0.

Fig. 7. Functional renal injury and urine Kim-1 levels after different times of renal ischemia. Male Wistar rats were subjected to 0 (sham), 10, 20, 30, or 45 min of bilateral ischemia by clamping the renal pedicles for the stipulated time and then removing the clamps and confirming reperfusion. After reperfusion (2 h), the rats were placed in metabolic cages, and urine, blood, and tissue were collected at 24 h. BUN (A) and plasma creatinine (B) concentrations were measured, and creatinine clearance (D) was calculated as per MATERIALS AND METHODS. Urine protein (C) and Kim-1 (E) concentrations were also measured. *P ≤ 0.05 compared with value in sham-treated group.
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...
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 was made against rat Kim-1-Fc fusion proteins from which three were purified, dialyzed, and concentrated to ~2.5 mg/ml. Two of these were used in the ELISA, and all three were used for immunocytochemistry. We found that just 500 ng/well MARKE-trap and 100 ng/well biotinylated MARKE-1 were sufficient to efficiently bind Kim-1 antigen in 100 μl urine.

Investigators have relied on a set of tests to detect AKI in rodent models that include serum creatinine, BUN, urine volume, urinalysis (including pH, specific gravity, glycosuria, proteinuria), kidney weight (wet wt-to-dry wt ratio), and histopathology. A significant disadvantage of these tests is the time delay between injury and detection, making the tests insensitive and unreliable for diagnostic and prognostic measurements (36). Specific proteins excreted in the urine after injury to particular segments of the nephron can serve as biomarkers for assessing the site and severity of renal damage (44). Previously used biomarkers can be broadly classified into the following three categories (13): 1) enzymes: e.g., alanine aminopeptidase (AA), alkaline phosphatase (AP), γ-glutamyltransferase (γ-GT), NAG, cathepsin B, lysozyme, and lactate dehydrogenase (LDH); 2) low-molecular-weight proteins: e.g., β2-microglobulin, α1-microglobulins, and retinol-binding protein; and 3) kidney-derived antigens: e.g., α-GST, clusterin, CYR-61, NGAL, and F-actin. These biomarkers have been studied in various models of nephrotoxicity, but a number of disadvantages have been identified. AA, AP, and γ-GT are stable only for 4 h after urine collection, and urine samples have to be gel filtered to remove potentially interfering substances. β2-Microglobulin is unstable in acidic pH; therefore, the urine has to be neutralized immediately after collection (44). The α-GST assay requires that the urine be stored with a specific preservative. α-GST is localized to the proximal tubule and is readily and rapidly released in the urine when renal tubular injury occurs (40). NAG, a proximal tubular brush-border enzyme, is a sensitive and persistent indicator of renal proximal tubule injury, but some metals and other nephrotoxicants directly inhibit NAG activity and therefore in such cases NAG cannot be used as a biomarker (41, 43). Other markers suffer from lack of specificity in measuring kidney injury. Serum, biliary, and urinary levels of γ-GT and LDH also increase after hepatic parenchymal injury (20). Elevation of urinary IL-6, IL-8, and IL-18 has been implicated as biomarkers for AKI, but these are also nonspecific since they are elevated with sepsis involving liver or lung in the absence of renal injury (8, 19, 28). Another problem with currently identified biomarkers is the onset of elevation and sustainability of the increase. AA and CYR-61 are significantly elevated in the urine at day 1 after renal damage but fail to remain elevated at later time points in spite of persistent renal injury (26, 37). Finally, most of the assays used for measuring urinary biomarkers are either enzymatic or colorimetric assays, thereby preventing high-throughput detection of samples, which is required when handling a large number of samples over extensive time courses.

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 in the 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 nephrotoxic compounds early in the drug development process.

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