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

Vishal S. Vaidya,1 Victoria Ramirez,2 Takaharu Ichimura,1 Norma A. Bobadilla,2 and Joseph V. Bonventre1

1Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; and 2Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México and Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico

Submitted 15 July 2005; accepted in final form 13 September 2005

Vaidya, Vishal S., Victoria Ramirez, Takaharu Ichimura, Norma A. Bobadilla, and Joseph V. Bonventre. Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. Am J Physiol Renal Physiol 290: F517–F529, 2006. First published September 20, 2005; doi:10.1152/ajprenal.00291.2005.—Sensitive and specific biomarkers are needed to detect early kidney injury. The objective of the present work was to develop a sensitive quantitative urinary test to identify renal injury in the rodent to facilitate early assessment of pathophysiological influences and drug toxicity. Two mouse monoclonal antibodies were made against the purified ectodomain of kidney injury molecule-1 (Kim-1), and these were used to construct a sandwich Kim-1 ELISA. The assay range of this ELISA was 50 pg/ml to 5 ng/ml, with inter- and intra-assay variability of <10%. Urine samples were collected from rats treated with one of three doses of cisplatin (2.5, 5, or 7.5 mg/kg). At one day after each of the doses, there was an approximately three- to fivefold increase in the urine Kim-1 detected. After only 10 min of bilateral ischemia, Kim-1 levels on day 1 were 10-fold higher (5 ng/ml) than control levels, whereas plasma creatinine and BUN were not increased and there was no glycosuria, increased proteinuria, or increased urinary NAG levels. Thus urinary Kim-1 levels serve as a noninvasive, rapid, sensitive, reproducible, and potentially high-throughput method to detect early kidney injury in pathophysiological studies and in preclinical drug development studies for risk-benefit profiling of pharmaceutical agents.

biomarkers; nephrotoxicity; enzyme-linked immunosorbent assay; cisplatin; ischemia; acute renal failure

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 practice remains challenging (13, 38). One of the predominant reasons for 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.

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 β-glutathione-S-transferases (α- and β-GST; see Ref. 7), neutrophil gelatinase-associated lipocalin (NGAL; see Ref. 24), cysteine-rich protein 61 (CysR61; 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 kidney...
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 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 paraformaldehyde lysine periodate (PLP) for 10 min for histology and immunostaining, as described below. The other kidney pedicle was clamped before PLP perfusion, and that kidney was snap-frozen for immunoblotting and RNA extraction. Another set of rats (n = 10/dose for each time point) was kept in Nalgene rat metabolic cages (Fisher) before and subsequent to administration of cisplatin. Urine was collected daily for 5 days and stored in aliquots at −80°C. Urine was centrifuged at 6,000 g for 15 min, and the supernatant was used to measure creatinine, glucose, protein, NAG, and Kim-1 as described below.

Tissue Collection, Preparation, and Histology

Kidneys from control and treated rats at various time points were perfused via the left ventricle with PBS at 37°C and then with PLP for 10 min (17, 30). The right contralateral pedicle-clamped unfixxed kidney, to be used for biochemical analyses, was removed immediately after perfusion was started. After an initial 10- to 15-min period of perfusion fixation, the left kidney was removed, washed with ice-cold normal saline (0.9% NaCl), and cut sagittally into two halves. One half of the left kidney was kept in the PLP fixative overnight 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, hemisedected, 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. 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 $C = (U \times V)/P$, 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′-CGGTGCTCTTGAGTAGTAATAG-3′ and antisense 3′-CTGCGCATGACACAAATAGAC-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 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 semiquantify 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 etidium 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 amplification, the PCR procedure was carried out in a final volume of 20 μl 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) each primer, and 1 unit Taq DNA polymerase (GIBCO-BRL). 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 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 semiquantify 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 etidium 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.
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 nephrotoxicants (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 dialedyzed rat Kim-1-Fc were loaded on the gel, and the purity was assessed by Comassie staining (Fig. 1C) and Western blot analysis (Fig. 1D) using HRP-labeled anti-human IgG-Fc antibody. In the Comassie-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 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 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.
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 (<3.3 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 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

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**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>
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<tr>
<td>Assay range</td>
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<td>Specificity</td>
<td>Does not cross-react with mouse or human KIM-1</td>
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<tr>
<td>Intra-assay variability</td>
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<tr>
<td>Interference</td>
<td>None identified</td>
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</tbody>
</table>

Kim-1, kidney injury molecule 1.

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**Fig. 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 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.

**Fig. 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.

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**Fig. 5.** Immunocytochemical Localizatin of Kim-1 in the Kidney after 5 mg/kg Cisplatin Administration using Three Different Monoclonal Antibodies. A: representative photomicrographs of hematoxylin and eosin-stained kidney sections from respective treatment groups. C: vehicle-treated control; D, F, and H: days 1, 2, and 3, respectively, after 5 mg cisplatin/kg; E, G, and I: days 1, 2, and 3 after 7.5 mg cisplatin/kg, respectively. All fields were chosen from outer stripe of outer medulla (OSOM). Arrowheads indicate sloughing of cells, tubular dilation, and necrosis.
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 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.
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-

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**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 (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-glutamyltranseptidase (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 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 depolymerization 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.

ACKNOWLEDGMENTS

Part of this work was presented at the 44th Annual Society of Toxicology meeting March (5–10, 2005) in New Orleans, LA, and Experimental Biology Meeting (April 2–5, 2005) in San Diego CA.

GRANTS

This work was supported by a postdoctoral fellowship grant from the National Kidney Foundation to V. S. Vaida, National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-39773 and DK-72381 to J. V. Bonventre, by research Grants G34511M and CO1–40182A-1 from the Mexican Council of Science and Technology, and Academic Support Program (DGAPA) IN208602–3 of the National University of Mexico to N. A. Bobadilla.

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