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

Vishal S. Vaidya*, Victoria Ramirez†, Takaharu Ichimura*, Norma A. Bobadilla†, and Joseph V. Bonventre*.

*Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, †Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autonoma de México and Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico.

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Address correspondence to:

Joseph V. Bonventre
Renal Division
Brigham and Women’s Hospital, Harvard Medical School
4 Blackfan circle, Harvard Institutes of Medicine, Rm 550
Boston, MA 02115
Tel. 617-525-5960
Fax. 617-525-5965
E-Mail: joseph_bonventre@hms.harvard.edu
ABSTRACT

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 influence 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 the inter- and intraassay variability of less than 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 ~ 3-5-fold increase in urine Kim-1 whereas other routinely used biomarkers measured in this study {plasma creatinine, BUN, urinary N-acetyl-β-glucosaminidase (NAG), glycosuria, proteinuria} lacked the sensitivity to show any sign of renal damage at this time point. When rats were subjected to increasing periods (10, 20, 30 or 45 min) of bilateral ischemia there was increasing amount of urinary 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 non-invasive, 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.

Key Words: Biomarkers, Kim-1, Nephrotoxicity, ELISA, Cisplatin, Ischemia, Acute Renal Failure
INTRODUCTION

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 FDA’s critical path initiative has reinforced the need for additional biomarkers to predict drug toxicity in preclinical studies which can act as surrogate endpoints, and/or aid in making efficacious and cost-saving decisions or terminating drug development more quickly (1). 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 post-marketing studies.

Routinely used measures of renal function, such as levels of blood urea nitrogen 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 non-invasive and non-specific and insensitive. Acute kidney failure is a common disease and is associated with high mortality rate (6, 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 will influence therapy and potentially morbidity and mortality.
The urine has been examined as a source for biomarkers given its easy availability and reduced complexity when compared to serum. Many urinary proteins such as α and π glutathione-S-transferases (α and π GST) (7), neutrophil gelatinase-associated lipocalin (NGAL) (24), cysteine rich protein 61 (CYR61) (26), interleukin-18 (IL-18) (28), clusterin (3), F-actin (34), N-acetyl-β-D-glucosaminidase (NAG) (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) (16). Kim-1 is a type 1 transmembrane protein that is not detectable in normal 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). Kim-1 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 (2, 17) and in humans with acute kidney injury (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 prior to 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) (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 h light-dark cycle over wood chips free of any known chemical contaminants. The rats were fed with commercial rodent chow (Teklad rodent diet #7012), given water ad lib, and were acclimated for 1-week prior to 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 U. S. National Institutes of Health and were approved by our Institutional Animal Care and Use Committees (IACUC).

Cisplatin-Induced Nephrotoxicity Studies. Male Sprague Dawley rats were administered 2.5, 5, or 7.5 mg cisplatin/kg intraperitoneally (ip) in 0.9 % saline or the same volume of vehicle as controls (n=4/dose/time point). Animals were sacrificed by overdose of pentobarbital (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 blood urea nitrogen (BUN) and creatinine. One kidney was perfused via the left ventricle with phosphate-buffered saline 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/time point) was kept in Nalgene® rat metabolic cages (Fisher) prior to and subsequent to administration of cisplatin. Daily urine was collected for 5 days and stored in aliquots at −80°C for urinalysis. Urine was centrifuged at 6000 g for 15 min and the supernatant was used to measure creatinine, glucose, protein, N-acetyl-β-glucosaminidase (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 (17, 30) with phosphate-buffered saline at 37 °C and then with paraformaldehyde lysine periodate (PLP) for 10 min. The right contralateral pedicle-clamped unfixed 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 sagitally 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 % Na 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 OCT 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 (H & E) for histological examination under a light microscope. The kidney harvested for biochemical analysis was washed with PBS, hemisected, and snap-frozen in liquid nitrogen; tissue was stored at -70°C.

**Renal Ischemia-Reperfusion Studies.** Twenty-five male Wistar rats weighing approximately 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 which is 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.
Two hours after renal ischemia/reperfusion, the rats were placed in metabolic cages at 22°C with 12:12 h light-dark cycle and allowed free access to water. Individual 24-hour urine samples were collected.

*Renal Functional Parameters.* In the cisplatin studies plasma and urine creatinine concentrations were measured using a Beckman Creatinine Analyzer II. Blood urea nitrogen (BUN) was spectrophotometrically measured at 340 nm using a commercially available kit (cat # 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 (Thermo-DMA, cat # 1530-500), protein (Sigma, cat # TP0400), and NAG (Roche diagnostics, cat # 875406) were measured spectrophotometrically (41) according to the manufacturers’ protocols.

In the ischemia/reperfusion studies urinary protein excretion was measured by trichloroacetic acid (TCA) turbidimetric method (14). Serum and urine creatinine concentration, as well as blood urea nitrogen were measured with an autoanalyzer (Technicon RA-1000, Bayer Co. 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 UV-light absorbance at 260 nm (Beckman DU640, Brea CA).
**Km-1 Semiquantitative RT-PCR.** Relative levels of Kim-1 and GADPH mRNA expression were assessed in renal cortex and medulla by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), as previously described (5, 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’ CGGTGCCTGTGAGTAAATAGAT3’ and antisense: 3’ CTGGCCATGACACAAATAAGAC 5’, which amplified a fragment of 418 bp, bases 1 to 418. To evaluate or reduce nonspecific effects of experimental treatment and to semi-quantify Kim-1 expression we amplified a fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using primers previously described (31). Genomic DNA contamination was checked by treating all RNA samples with DNAase and by carrying samples through the PCR procedure without adding reverse transcriptase.

Reverse transcription (RT) was carried out using 2.5 μg of total RNA from renal cortices. Prior to 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 U of the Moloney murine leukemia virus reverse transcriptase (Gibco BRL), 100 pmol of random hexamers (Gibco BRL), 0.5 mM of each dNTP (Sigma Chemical Co., St. Louis MO), and 1X RT buffer (75 mM KCl; 50 mM Tris-HCl; 3 mM MgCl₂; 10 mM DTT, pH 8.3). Samples were heated at 95°C for 5 min to inactivate the reverse transcriptase and diluted to 40 µl with PCR grade water. One tenth of RT individual samples of each group was used for Kim-1 or GAPDH amplification in 20 µl final volume reactions containing 1X PCR buffer (10 mM Tris-HCl; 1.5 mM MgCl₂; 50 mM KCl, pH 8.3), 0.1 mM of each dNTP, 0.2 µCi of [α³²P]-dCTP (~ 3000 Ci/mmol, 9.25 MBq, 250 µCi), 10 µM of each primer and one unit of 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, Germany), with the following profile: denaturation 1 min at 94°C, annealing 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 co-amplified simultaneously in each reaction. The optimal number of cycles for each primer pair was determined through kinetic amplification following our standard procedure (5, 10). To routinely semi-quantify Kim-1 and the control gene, 23 and 18 cycles respectively 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 DNAase I and by carrying samples through the PCR procedure without adding reverse transcriptase.

To analyze PCR products, one-half of each reaction was electrophoresed in a 5% acrylamide gel. Bands were ethidium bromide stained and visualized under UV light, cut out, suspended in 1 ml of scintillation cocktail (Ecolume, ICN, Aurora, OH), and counted by liquid scintillation (Beckman LS6500, 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 FAM or VIC dye-labeled TaqMan 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). The FAM (6-carboxyfluorescein) 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 of 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 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 ratio between 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 (4). Briefly, a construct {rat Kim1 (mucin)-Ig} was made which encoded the extracellular domain of rat Kim-1 (residues 1-234) attached to the Fc portion of human IgG1 (hinge, CH2 + CH3 domains). 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-1Fc protein was purified from the conditioned media by chromatography on protein A-sepharose column (Amersham Biosciences). In brief the protein A-sepharose column (2 X 20 cm) was equilibrated with 20mM sodium phosphate, 0.15M NaCl, (pH 7). The conditioned media was applied and the column was washed with the equilibration buffer. The column was eluted with 25mM sodium phosphate, 0.1M NaCl, (pH 2.8). One ml fractions were collected in 1.5 ml Eppendorf tubes containing 50 µl of neutralizing buffer (0.5M Na 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 horse raddish 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 (Biorad). Fractions of similar concentrations were combined and dialyzed \{Molecular Weight Cut-Off (MWCO) = 30,000\} against PBS overnight at $4^\circ$C and concentrated using a 30,000 MWCO amicon centrifugal filters (Millipore).

*Mouse Monoclonal Antibodies Against Rat Kim-1 Ectodomain.* Mice were immunized with purified rat Kim-1Fc protein to generate monoclonal antibodies using standard antibody production techniques by contract to Dr. Jun Hayashi’s laboratory at the University of Maryland, Baltimore. Eighteen hybridoma clones were selected that were positive for Kim-1Fc and negative for human IgG-Fc. These clones were adapted in suspension with the serum free hybridoma media (BD Biosciences) grown in 175 cm$^2$ 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 X 20 cm) was equilibrated with 20mM sodium phosphate, 0.15M NaCl, (pH 7). The conditioned media was applied and the column was washed with the equilibration buffer. The column was eluted with 100mM glycine buffer, 0.1M NaCl, (pH 2.7). One ml fractions were collected in 1.5 ml Eppendorf tubes containing 50 µl of neutralizing buffer (1M 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 Device). Fractions of similar concentrations were combined and dialyzed (MWCO = 30,000) against PBS overnight at $4^\circ$C and concentrated using an amicon centrifugal filters with 30,000 MWCO (Millipore).

*Rat Kim-1 Sandwich ELISA.*

*Biotinylation of MARKE-1:* One MARKE antibody (MARKE-Trap), at concentration ~ 1.5 mg/ml, served as a trapping/primary antibody. The other MARKE antibody (conc. ~2.2 mg/ml) was biotinylated using the Pierce EZ$^\text{®}$-Link NHS-PEO$_4$-Biotin conjugating kit (Pierce) to act as a
detecting/secondary antibody. The number of moles of biotin/mole of protein was determined to be 6 (corresponding to very high efficiency of biotinylation) using an EZ®-Biotin Quantitation Kit. The nonreacted and hydrolyzed biotinylation reagent was removed by dialyzing with PBS, pH 7 overnight at 4°C.

Construction of Kim-1 Sandwich ELISA: The wells of an ELISA plate (MaxiSorp; Nunc, Naperville, IL, USA) were coated with MARKE-Trap (overnight incubation at 4°C with 100μl of antibody 10μg/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% Na azide) and after the incubation period the plate was washed again three times with PBST. One hundred microliters of either standard rat Kim-1Fc pure protein in serial dilutions (0-5 ng/ml) or urine samples from control and treated groups were added to the plate in duplicates and the plate was incubated for 1.5 h at 37°C. All 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 (TMB) 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 was 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 pg/ml or 1500 pg/ml recombinant Kim-1Fc into vehicle-treated rat urine samples and quantitating the Kim-1 levels in these spiked samples. In order to assess the recovery of Kim-1 in urine from animals
with kidney injury, a known amount of 750 pg/ml or 1500 pg/ml recombinant Kim-1Fc was added to 5 mg cisplatin/kg-treated rat urine samples containing 300 to 2100 pg/ml Kim-1. This was done to verify that there were 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 in order to fit the concentrations of Kim-1 in the linear range of the standard curve (0 to 5000 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: Monoclonal Anti-Rat Kim-1 Ectodomain (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 ± S.E. Treatment means were compared with control means by ANOVA and subsequent Student's t-test. The level of significance was set at P < 0.05 in all cases.
RESULTS

*Construction and Purification of Rat Kim-1 Ectodomain Fusion Protein.* Rat Kim-1 maps to chromosome 10 (10q21) of the rat genome and is conserved across species with 77 % identity to mouse Kim-1, 30 % identity to chimpanzee, and 38 % identity with human Kim-1. The protein structure of rat Kim-1 consists of a total of 307 amino acids (aa) with a signal peptide (1-21 aa), a highly conserved 6-cysteine-rich Ig-like domain (22-130), a serine-threonine-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 291-297 amino acids (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 which are specific to the ectodomain of rat Kim-1. A fusion protein of 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 Methods. Three different batches of purified and dialyzed rat Kim-1Fc were loaded on the gel and the purity was assessed by commassie staining (Fig. 1C) and western blot analysis (Fig. 1D) using horseraddish peroxidase (HRP) labeled anti human IgG-Fc antibody. In the commassie-stained blot the strongest band was of the Kim-1Fc protein at 85 kDa that corresponds to the predicted molecular weight of the glycosylated Kim-1Fc 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-1Fc, attesting to its purity.

*Monoclonal Antibodies and Evaluation of Performance Characteristics of the Kim-1 ELISA.* To obtain highly specific Monoclonal Anti Rat Kim-1 Ectodomain (MARKE) antibodies, mouse mABs
were generated against the pure recombinant Kim-1Fc protein. The hybridoma cells were screened and selected in such a way that the conditioned media recognized Kim-1Fc 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, 2 and MARKE-Trap) to detect Kim-1Fc was estimated by western blot analysis (Fig. 2A). All three purified antibodies at the concentration of 1 µg/ml recognized 25 ng per lane of Kim-1Fc without any non-specific binding. This suggested that all three MARKE’s were able to detect the denatured Kim-1Fc protein. To further evaluate whether these antibodies would detect non-denatured three-dimensional Kim-1Fc 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 were used as trapping antibody and the other as detecting, 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):

A. Sensitivity: < 39 pg/ml. A standard curve was generated using serial dilutions of Kim-1Fc protein in sample diluent starting with 5000 pg/ml (Fig. 2C). The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest Kim-1Fc concentration that could be differentiated from zero (assay blank/sample diluent) by the Student’s t-test. The LLD was 39 pg/ml of sample diluent (n=5; P<0.001).

B. Assay Range: 0-5000 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 78.1-5000 pg/ml of sample diluent was < 10 %. The standard curve concentrations used for all the Kim-1 ELISA’s were 5000, 2500, 1250, 625, 312.5, 156.3, and 78.13 pg/ml.

C. 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.

D. 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, 1203, 1692, 2120, 2630, and 4250 pg/ml). Intra- and interassay CVs ranged from 2 % to 4.9 % (n=6) and from 2.7 % to 9.6 % (n=3 in three different plates), respectively (Fig. 2D).

E. Recovery: The analytical recovery was determined by adding 750 pg/ml or 1500 pg/ml recombinant Kim-1Fc into vehicle-treated or 5 mg cisplatin/kg-treated rat urine samples containing 300 to 2100 pg/ml Kim-1. Recoveries of Kim-1 ranged from of 90 % to 110 %.

F. Dilution linearity: When cisplatin treated rat urine samples containing 6, 8, 12, or 18 ng/ml corresponding to medium and high concentration of Kim-1 were diluted 1:5, 1:10, 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).

G. Interference: Urine samples containing 732 or 2630 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), cisplatin (>5 mM), 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 Blood Urea Nitrogen, Plasma Creatinine and Histopathology.** We used a widely established and well-studied cisplatin-induced acute kidney injury model (2, 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 ip in 0.9 % saline or the same volume of vehicle to male Sprague Dawley rats. Since previously we had shown only mild changes in blood urea nitrogen (BUN), serum creatinine, and histopathology following a low dose of 2.5 mg cisplatin/kg (2, 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 approximately four and ten-fold respectively over baseline (day 0) on day 3 (Fig. 3A 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. 3A and B). Following 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 mg (Fig. 3E) cisplatin/kg as compared to the controls (Fig. 3C). On day 2 after either dose there was some single cell necrosis, tubular dilation and sloughing of cells into the lumen of S3 segment of proximal tubules in the outer stripe of the outer medulla (Fig. 3F and G). On day 3, after either dose there was significant proximal tubular necrosis, particularly in the S3 segment, and intratubular casts clearly seen. The inflammation, edema, tubular
degeneration, dead cells, necrosis and apoptosis were most severe in the 7.5 mg cisplatin/kg treated group (Fig. 3H and I).

**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 assay we constructed to quantitate Kim-1, we found ~ 3-5 fold increase in urinary Kim-1 on day 1 following 2.5, 5 or 7.5 mg cisplatin/kg when compared to 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 7.5mg 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 15-25 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 were used as detecting secondary antibodies in the Kim-1 ELISA. We tested 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, by immunocytochemistry (Fig. 5). Using each of the three MARKE’s there was no Kim-1 staining on day
0 indicating undetectable expression of Kim-1 under normal conditions (Fig. 5A, E, and I). On day 1 after cisplatin all three MARKE’s showed a very similar pattern of positive Kim-1 staining in an apical membrane distribution in the outer stripe of outer medulla (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 following 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 5mg/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 MARKE’s (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 following cisplatin nephrotoxicity.

*Kim-1 as an Early Diagnostic Indicator of Acute Kidney Injury following Cisplatin Nephrotoxicity.*

Blood urea nitrogen, 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 N-acetyl-β-glucosaminidase (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 ~5-fold increase in Kim-1 on day 1
which 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. 6A to C), or blood urea nitrogen (Fig. 6E) or plasma creatinine (Fig. 6F) values on day 1 but there was approximately a ~ 3 to 5 fold increase in urinary Kim-1 levels indicating significant renal injury as early as day 1. The ~ 7-10 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. 6A to 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 following 2.5 mg/kg cisplatin.

**Comparison of Kim-1 with other Biomarkers of Kidney Injury in Renal Ischemia/Reperfusion Model.** In order to generalize the effectiveness of Kim-1 as a sensitive and early diagnostic biomarker for acute kidney injury we quantitated Kim-1 in the urine of male Wistar rats following different periods of bilateral ischemia/reperfusion (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, one day after 30 or 45 min bilateral I/R injury, BUN increased ~4-5 fold and plasma creatinine increased ~5-6 fold indicating severe renal dysfunction. Twenty-four hours after 10 min of bilateral I/R injury, Kim-1 was 5-fold higher (Fig. 7C) than sham operated controls (N) whereas there was no significant decrease in the creatinine clearance (Fig. 7D) nor increase in proteinuria (Fig. 7E) 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. 7E). Urine was collected for 24 hrs starting after initiation of reperfusion. Urinary Kim-1 was ~50-fold higher than controls in this collection (Fig. 7C). 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. 7E). Thus, in the bilateral I/R injury model, as in the cisplatin model, Kim-1 was a highly sensitive indicator of renal dysfunction with 5-fold higher Kim-1 levels in the 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.* In order to correlate Kim-1 gene expression with the degree of injury, Kim-1 mRNA was quantitated using semiquantitative and quantitative RT-PCR following different periods of bilateral I/R injury. In concordance with the urinary Kim-1 protein levels which increased ~ 5-fold after 10 min bilateral I/R injury, Kim-1 mRNA levels were also significantly upregulated at 24 h in this group (Fig. 8A, B, and C). Using 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 semi quantitative and quantitative real time PCR with ~ 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 injury-dependent increase in Kim-1 protein expression (Fig. 5) following 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 acute kidney injury 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 sacrificing 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 5000 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 ELISA’s to measure cytokines in rat serum (TNF-α, IL-6 available from Pierce-Endogen) as markers of inflammation. Although an extensive performance characteristic evaluation has not been reported for the human KIM-1 ELISA, both the rat and human Kim-1 ELISA’s are highly comparable in terms of sensitivity, inter- and intra-assay variability, and recovery (12). For the development of the rat Kim-1 ELISA, a panel of 18 mouse monoclonal antibodies were made against rat Kim-1Fc 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 of MARKE-trap and 100 ng/well of biotinylated MARKE-1 was sufficient to efficiently bind Kim-1 antigen in 100 µl of urine.
Investigators have relied on a set of tests to detect acute kidney injury in rodent models that include serum creatinine, blood urea nitrogen (BUN), urine volume, urinalysis (including pH, specific gravity, glycosuria, proteinuria), kidney weight (wet weight to dry weight 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 into the urine following 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 three categories (13): I. Enzymes: e.g. alanine aminopeptidase (AA), alkaline phosphatase (AP), γ-glutamyltranspeptidase (γ-GT), N-acetyl β-D glucosaminidase (NAG), cathepsin B, lysozyme, lactate dehydrogenase (LDH); II. Low molecular weight proteins: e.g. β2-microglobulin, α1-microglobulins, retinol-binding protein (RBP); III. Kidney derived antigens: e.g. α-glutathione-S-transferase (α-GST), clusterin, cysteine rich protein (CYR-61), neutrophil gelatinase-associated lipocalin (NGAL), F-actin. These biomarkers have been studied in various models of nephrotoxicity but a number of disadvantages have been identified. AA, AP, 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 and therefore the urine has to be neutralized immediately after collection (44). The α-glutathione-S-transferase (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 into 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, IL-18 have been implicated as biomarkers for AKI but these are also non specific 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 large number of samples over extensive time courses.

NGAL is upregulated and can be detected in the kidney (22) and urine of mice three hours 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 hours is a very powerful predictor of acute kidney injury in children after cardio pulmonary bypass (23). Similarly, CYR-61, a secreted protein is present early in the urine after ischemia/reperfusion but currently can only be detected by immunoblotting the urine and kidney extracts at 3-6 hours after 30 min bilateral ischemia/reperfusion 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 radioimmunoassay, has been correlated with the elevation of creatinine and NAG in the gentamicin-induced renal injury model in rats (3). Clusterin mRNA and protein levels, however, did not increase until day 5 in the cisplatin-induced renal injury model (35). F-actin is released into the urine in rats at 1 hour after 25 min of renal ischemia due to 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 ARF after ischemia in humans (19).
An ideal biomarker to detect acute kidney injury 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 non invasive, and quantifiable by 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 5-fold on day 1 after 5 and 7.5 mg/kg cisplatin whereas blood urea nitrogen (BUN) and plasma creatinine increases were seen only from day 3 onwards (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 into 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 following 10, 20, 30 or 45 min bilateral I/R injury (Fig. 8A, B, and C). A similar trend in injury-dependent increase in Kim-1 protein expression is evident from the immunohistochemical staining of Kim-1 on days 0 to 5 after 5 mg/kg cisplatin (Fig. 5). Thus Kim-1 gene expression and surface protein expression correlates very well with the degree of renal tubular injury (Fig. 7C).

A significant advantage of measuring Kim-1 as a biomarker for acute kidney injury is that it is conserved across species and is upregulated after renal injury in zebrafish (unpublished data), mice (29), rats (17), non-human 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.
ACKNOWLEDGEMENT

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DISCLOSURES

The authors declare no conflict of interest with third parties.
REFERENCES


FIGURE LEGENDS

Fig. 1. Structure of Kim-1 and construction and purity of the rat Kim-1 ectodomain-Fc fusion protein. A. Structure of rat Kim-1 protein consists of 307 amino acids (aa) with a signal peptide, Ig-like domain, mucin region, transmembrane domain (TM) and a short cytoplasmic tail with a tyrosine kinase phosphorylation site from 291-297 amino acids. B. Kim-1 ectodomain fusion protein was produced by creating an expression construct which encoded the extracellular domain of Kim-1 (1 to 234) fused to the Fc portion of human IgG1 as described in methods. Purity of the Kim-1-Fc fusion protein was assessed by PAGE of three different batches of purified and dialyzed Kim-1Fc and detecting by (C) commassie reagents and (D) western blot using HRP labeled anti human IgG-Fc antibody.

Fig. 2. Monoclonal antibodies to rat Kim-1, principle of Kim-1 ELISA, standard curve, and precision profile. A. MARKE-1, 2 and MARKE-Trap detect Kim-1 ectodomain fusion protein by western blot analysis. Purified Kim-1Fc (25 ng/lane) was loaded onto three different lanes for PAGE, and transferred on to a PVDF membrane, which was then cut, into three 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 inter assay variability was less than 5 %. R² = 0.99 representing 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 inter-assay reproducibility of the Kim-1 ELISA was calculated by adding five different concentrations of Kim-1 (750, 1000, 1500, 2000, and 2500 pg/ml) to control urine samples. For the intra-assay variability each sample was measured in \(n=6\) replicates within one plate. For the inter-assay variability each sample was measured three times in three different plates.

Fig. 3. Plasma blood urea nitrogen, creatinine and renal histopathology over a time course after administration of 5 or 7.5 mg cisplatin/kg. Male Sprague Dawley rats \((n = 4)\) were administered 5 or 7.5 mg cisplatin/kg ip in 0.9 % saline. Blood and kidney tissue were taken on days 0 through 5. Since the administration of 7.5 mg cisplatin/kg resulted in 50 % mortality by day 4, eight rats were treated in this group to get 4 survivors on day 4 and 5 each. Blood urea nitrogen (A) and creatinine (B) were measured. *, \(p \leq 0.05\) compared to 0 h time point. C to I: Representative photomicrographs of H & E 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). Original magnification X 20. Arrows indicate sloughing of cells, tubular dilation and necrosis.

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 d. 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 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. 5. Immunofluorescence staining to detect Kim-1 expression in the kidney after 5 mg cisplatin/kg administration using the MARKE antibodies. Purified and concentrated MARKE-1 (A to D), MARKE-2 (E to H) or MARKE-Trap (I to L) 5µg/ml respectively were used for immunohistochemical localization of Kim-1 in the PLP-fixed frozen kidney sections from day 0 to 5 after 5 mg cisplatin/kg administration as described in ‘Methods’. All photomicrographs were taken using X 40 objective. Arrows indicate the progressive increase of Kim-1 staining primarily on the apical membrane over time. No staining was observed on day 0 or when using mouse IgG as a negative control (data not shown).

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. Blood urea nitrogen (E) and plasma creatinine (F) were measured on these days after 5 and 7.5 mg/kg cisplatin administration. *, p ≤ 0.05 compared to 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. Two hours after reperfusion the rats were placed in metabolic cages and urine, blood and tissue collected at 24 h. Blood urea nitrogen (A), serum creatinine (B) and urine creatinine (C) concentrations were measured and creatinine clearance (C) was calculated as per methods. Urine protein (D) and Kim-1 (E) concentrations were also measured. *, p ≤ 0.05 compared to value of sham treated group.

Fig. 8. Kim-1 mRNA levels in kidney following different periods of ischemia and correlation of Kim-1 mRNA in kidney with creatinine clearance. Kim-1 mRNA levels were measured by semiquantitative (A, B) and quantitative real time (C) RT-PCR in renal cortex of rats at 24 h after 0, 10, 20, 30 and 45 min of bilateral ischemia. A) mRNA was isolated from renal cortex and the RT-PCR products were separated on a 5 % ethidium bromide acrylamide gel. Upper row of bands represent Kim-1 mRNA following different periods of renal ischemia. Lower row of bands represent the renal GAPDH mRNA values, which was used as an internal standard. B) Kim-1 mRNA level was semiquantitated by excising the bands from the ethidium bromide stained gel and counted by liquid scintillation. The results are presented as the ratio of PCR products (cpm) of Kim-1 and GAPDH ± SE. C) Real time PCR was carried out in 96-well plates on cDNA derived from 2.5 ng of total RNA isolated individually from each renal cortex. The amount of fluorescence in each reaction liberated by the exonuclease degradation of the TaqMan probe during PCR amplification was measured as a function of PCR cycle number using an ABI 7000 Prism (Applied Biosystems). Results are presented as relative Kim-1/18S rRNA ratios compared to the sham value. D) The value of creatinine clearance are plotted versus Kim-1/GAPDH mRNA ratios. The r value of 0.732
indicates a high degree of correlation between creatinine clearance and Kim-1 mRNA expression with a significance of $p<0.0001$. 
Table 1. Performance characteristics of Kim-1 ELISA

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<td>Sensitivity</td>
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<td>Assay Range</td>
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<tr>
<td>Specificity</td>
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<td>Intra-assay variability</td>
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Figure 1

A  Rat Kim-1 protein

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B

1-234 aa

C 1 2 3

D 1 2 3

Rat Kim-1Fc

Fusion protein
Figure 2

A

MARKE-1  MARKE-2  MARKE-Trap

190  115  85  66  59  37  31

B

Kim-1 in rat urine  Biotinylated MARKE-1

1.5 h  1.5 h

Streptavidin-HRP

MARKE-Trap  OT

96 well plate

15 min

+ TMB substrate + Stop Solution

C

\[ y = 0.0006x + 0.0532 \]
\[ R^2 = 0.9919 \]

Absorbance (450 nm)

Kim-1 (pg/ml)

D

Precision profile

\% CV

Kim-1 (pg/ml)

Intra-Assay  Inter-Assay
Figure 3

A: BUN

![Graph showing BUN levels with columns for Cisplatin (mg/kg): 5 and 7.5 mg/kg.](image)

B: Plasma Creatinine

![Graph showing Plasma Creatinine levels with columns for Days after cisplatin administration: 0, 1, 2, 3, 4, 5.](image)

Control

5 mg/kg

7.5 mg/kg

C: Control

D: 5 mg/kg

E: 7.5 mg/kg

F: Arrowheads indicate...

G: Arrowheads indicate...

H: Arrowheads indicate...

I: Arrowheads indicate...
Figure 5

Days after 5 mg cisplatin/kg administration

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Figure 6

A. Glycosuria

B. Proteinuria

C. N-acetyl-β-glucosaminidase

D. Kim-1

E. BUN

F. Plasma Creatinine

Days after cisplatin administration
Figure 8

A  
Kim-1  
GAPDH  
N  I-10  I-20  I-30  I-45

B  
Kim-1/GAPDH

C  
Relative mRNA levels

D  
Creatinine CI (ml/min)

Ischemia/Reperfusion

$r = 0.732; p < 0.0001$