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Key Words: cisplatin, TFEC, folic acid, acute renal failure, kidney toxicity.
ABSTRACT

Nephrotoxicity is a common side effect of therapeutic interventions, environmental insults and exposure to toxicants in the workplace. Although biomarkers for nephrotoxicity are available, they often lack sensitivity and are not specific as indicators of epithelial cell injury. Kidney Injury Molecule-1 (Kim-1) is a type-1 membrane protein with extracellular immunoglobulin and mucin domains. The mRNA and protein for Kim-1 are expressed at very low levels in normal rodent kidney, but expression increases dramatically after injury in proximal tubule epithelial cells in postischemic rodent kidney and in humans during ischemic acute renal failure. To evaluate the utility of Kim-1 as a biomarker for other types of renal injury, we analyzed tissue and urinary expression in response to three different types of nephrotoxicants in the rat: S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), folic acid and cisplatin. Marked increases of Kim-1 expression were confirmed by immunoblotting in all three models. The protein was shown to be localized to the proximal tubule epithelial cell by immunofluorescence. Furthermore, Kim-1 protein was detected in urine of toxicant-treated rats. The temporal pattern of expression in response to TFEC is similar to the Kim-1 expression pattern in the postischemic kidney. In folic acid-treated kidneys, Kim-1 is clearly localized to the apical brush border of the well-differentiated proximal tubular epithelial cells. After folic acid treatment, expression of Kim-1 is present in the urine despite no significant increase in serum creatinine. Cisplatin treatment results in early detection of urinary Kim-1 protein, and a delayed and diffuse Kim-1 expression in S3 cells of the proximal tubule. Kim-1 can be detected in the tissue and urine on days 1 and 2 after cisplatin administration, occurring prior to an increase in serum creatinine. The upregulation of expression of Kim-1 and its presence in the urine in response to exposure to various types of nephrotoxicants suggests that this protein may serve as a general biomarker for tubular injury and repair processes.
INTRODUCTION

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

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

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

Treatment of animals and preparation of kidney tissue samples.

S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC) preparation and administration was carried out as described (16). Male Fisher 344 rats weighing 200-440 grams received 20 mg/kg TFEC in water or saline by intraperitoneal injection. At the appropriate time point, treated animals were sacrificed by CO₂ asphyxiation and their kidneys were removed. A 2 to 4 mm thick piece of tissue was trimmed into a core of kidney tissue spanning the outer cortex to the papilla. To prepare tissue enriched for injured and regenerating proximal tubules in the toxicant-exposed kidney, the outer stripe of the outer medulla (OSOM) region was dissected out from this tissue sample and processed for immunoblot analysis. This technique has been employed for analysis of growth factor protein and mRNA in prior studies (15, 16). Tissue was also harvested from the same or the contralateral kidney of each animal for immunohistochemical analysis performed as described below. In some cases, the kidney was perfusion fixed as described below.

Folic acid treatment was carried out as previously described with a modification (8). Briefly, male Sprague Dawley rats (280-440 g) were administrated intraperitoneally (i.p.) 250 mg/kg folic acid in 0.3 M sodium bicarbonate. At the appropriate time point, one kidney from each animal was harvested for extraction of proteins and another kidney was perfusion fixed as described below.

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

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

**Affinity purified antibodies**

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

**Serum and Urine Creatinine Measurement**

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

**Immunohistochemistry**

*Immunoperoxidase staining:* Immunohistochemical staining for Kim-1 was conducted using a Vectastain Elite ABC kit (Vector, Burlingame, CA) and rabbit polyclonal antibody, R9, the affinity purified anti-peptide antibody raised against Kim-1, as described previously (14), with modification. Briefly, kidney tissue was immersion fixed in Carnoy's solution (60% absolute ethanol, 30% chloroform and 10% acetic acid), embedded in paraffin, and cut into 5 μm sections. The sections were deparaffinized and endogenous peroxidase activity was ablated by incubation in 2% hydrogen peroxide in methanol for 20 min. The sections were blocked with diluted normal goat serum (1:67) for 1 hr at room temperature or overnight at 4°C, followed by incubation with affinity purified or control IgG at a concentration of 10 μg/ml. After 1 hr, the sections were washed in PBS and incubated with biotinylated goat anti-rabbit IgG for 30-60 min. After further washes with PBS, the sections were incubated with an avidin-biotinylated horseradish peroxidase complex for 1-2.5 hr. Finally, the sections were washed in PBS and color was developed by the addition of 50 mM sodium phosphate buffer, pH 7.6, containing 0.12% 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO), 0.0075% nickel chloride, 0.0075% cobalt chloride, and 0.0075% hydrogen peroxide for 4 min. The sections were counterstained with 0.01% toluidine blue. Staining of kidney tissue from untreated kidney or staining with non immune serum or rabbit IgGs was performed for controls. Staining with control reagents was negative in all cases.
Immunofluorescence staining of Kim-1 protein: Sections were thawed, washed with PBS, and blocked in 1.5% normal donkey serum in PBS (blocking solution) for 30 min. Sections were then incubated for 1 hr with the primary antibodies: rabbit anti-rat Kim-1 R9 polyclonal antibody (4 μg/ml) diluted in the blocking solution. The sections were washed with PBS, incubated with anti-rabbit Cy3 conjugated (1:800 dilution, Jackson) in PBS for 30 min. At the final step, sections were washed with PBS, and mounted with Vector Shield mounting reagent (Vector) containing 12.5 μg/ml DAPI to identify nuclei. As a negative control, the same concentration of rabbit IgG (4 μg/ml) was used for the primary antibody.

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

Immunoblot analysis of tissue Kim-1.

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

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

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

**Rat Urine Kim-1 immunoblot analysis**

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

**Statistical analysis**

In some cases, treatment means were compared to control means by analysis of variance (ANOVA) and subsequent Student’s t-test. The level of significance was set at P<0.05 in all cases.
RESULTS

Expression of Kim-1 in kidney tissue and urine from TFEC treated rats.

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

Immunoblot analysis of Kim-1 protein in kidneys of TFEC-treated rats: Kim-1 protein levels in lysates of kidney tissue dissected from the outer medulla were analyzed by immunoblotting over a 14 day period after TFEC administration (Fig. 1B, C). Kim-1 protein was not detectable in the kidney tissue from control rats. Kim-1 protein levels increased significantly one day after the TFEC administration and remained increased for 14 days. There are at least two forms of Kim-1 protein in the lysates, a 45 kDa (band B) and 80-90 kDa (band A) forms (Fig. 1B). The presence of the multiple forms of the Kim-1 protein is most likely due to a difference in the O-linked glycosylation of the mucin domain (14). The expression of both forms, particularly the 80-90 kDa (band A) form, increased markedly at day 2 and persisted at high levels for 7 days after TFEC treatment (Fig. 1B, C). The 80-90 kDa form was still detectable at day 14, although there was a clear decline of Kim-1 protein expression from day 7 to 14. The large size 80-90 kDa bands apparently shift to slightly smaller size with progression over time after TFEC administration. At early time points, there was a good correlation between the increase of serum creatinine levels and the upregulation of Kim-1 protein in the kidneys. High levels of Kim-1 protein persist in tissue, however, even after kidneys recover renal function, consistent with expression during tissue repair leading to recovery of function.
Detection of Kim-1 protein in the urine of TFEC-treated rats: We have demonstrated in cell cultures that the human KIM-1 ectodomain is cleaved and shed into the culture media (2). In a clinical study, KIM-1 protein was detected in the urine of patients with acute tubular necrosis (9). To evaluate whether Kim-1 may be a useful general biomarker for renal injury or repair in animal models of nephrotoxicant-induced injury, we investigated whether Kim-1 protein is present in urine samples collected from toxicant-exposed animals. Urine samples were collected for 7 days after TFEC administration. Collected urine samples were concentrated and Kim-1 protein was analyzed by immunoblotting with anti-ectodomain antibodies which recognize both intact and shed forms of Kim-1 protein (Fig. 1D). There is no detectable Kim-1 band in the urine samples from untreated rats. Two forms of Kim-1, reflected by bands A and B, were detected in urine collected as early as one day after TFEC administration. The larger protein is detected in the urine samples collected on day 1 through day 7, and it has a size similar to the mature Kim-1 protein seen in the kidney tissue lysates. The smaller Kim-1 protein can be detected at 3 to 4 days after TFEC treatment. The size of the smaller protein in the urine sample is approximately 60 to 65 kDa, close to a predicted size of shed rat Kim-1 ectodomain (15 to 20 kDa less than the mature form due to cleavage from transmembrane and cytoplasmic domains assuming that cleavage occurs at a site similar to that we have identified in human KIM-1 (2)). Thus, Kim-1 protein is upregulated in kidney tissue after TFEC-induced renal injury and Kim-1 protein is shed into the urine after TFEC treatment.

Immunohistochemical analysis of Kim-1 after TFEC treatment: The Kim-1 temporal expression pattern was characterized by immunohistochemistry using anti-peptide antibody R9 on kidney sections over a 14 day period after TFEC administration. In the untreated kidney, there was no detectable Kim-1 protein (Fig. 2A). Twenty-four hr after the administration of TFEC, Kim-1 positive cells, as indicated by black/dark brown peroxidase staining on the tissue section, were clearly present in the S3 segment of proximal tubule in the outer stripe of the outer medulla (OSOM) near the OSOM / inner stripe of the outer medulla (ISOM) boundary (Fig. 2B). At this time, Kim-1 positive cells have a flattened morphology (arrowheads) with casts and cell debris
present in the region (Fig. 2B). The number of Kim-1 positive proximal tubule cells in the OSOM increased at 2 days after the toxicant administration (Fig. 2C). Kim-1 immunoreactivity is clearly present in apical regions and some times in cytoplasm of cast-containing tubules, (Fig. 2C, arrowheads). Apical localization of Kim-1 staining is clearly recognized on day 3 (Fig. 2D) but there is also some defuse cytoplasmic staining, along with the presence of cell debris and casts in the dilated lumen (Fig. 2D). Most of the viable cells, identified by intact nuclei, in the injured tubules are Kim-1 positive (Fig. 2D, arrowheads). There is a dramatic further increase in the number of Kim-1 positive cells and tubules between day 3 and day 5, when expression peaks (Fig. 2D, E). Five days after the toxicant administration, there were fewer casts and less debris in the proximal tubules in the injured region. Significant tubular dilatation, however, was still seen at this time. Kim-1 staining is present in most of the regenerating flat S3 segment cells which contain scanty cytoplasm in these dilated tubules in the outer medulla and cortical medullary ray (Fig. 2E). At this time the subcellular localization of Kim-1 is predominantly apical but there is also some diffuse cytoplasmic staining (Fig. 2E). There is intense Kim-1 expression in a cell cluster in the lumen of the dilated tubule (Fig. 2E, arrowheads). No staining was seen in interstitial cells. Fourteen days after TFEC administration, Kim-1 immunoreactivity was detectable only in the few tubules that remain dilated (Fig. 2F). At this time Kim-1 staining is localized to the apical side of the tubules which lacks a well developed brush border, a sign that these tubules have not completely restored the differentiated phenotype typical of a normal functional proximal tubule (Fig. 2F, asterisks). Kim-1 positive tubular cells (small arrowheads) also stained positively with an antibody to vimentin (large arrowheads) as shown in Fig. 2H where double immunofluorescence staining was carried out on sections from kidneys taken 3 days after TFEC administration. Thus, Kim-1 protein expression in TFEC-treated rats is clearly upregulated within 24 hr after treatment and peaks in expression at day 3 to 5, after which the levels of expression fall with time. Kim-1 remains detectable in a few tubules up to at least 14 days after exposure to the nephrotoxicant.
Expression of Kim-1 in kidney tissue and urine from folic acid treated rats.

In order to test the generality of the results with TFEC for other forms of injury, we evaluated the high dose folic acid nephrotoxicity model (Fig. 3). This model differs from the TFEC model in that folic acid causes a massive increase in cell proliferation in the proximal tubules (8). Although folic acid can induce large increases in plasma creatinine levels in the rat (35), under our experimental conditions there was no statistically significant increase in serum creatinine concentration, as shown in Fig. 3A. Despite no increase in serum creatinine, Kim-1 protein upregulation was detected by immunoblot analysis of the kidney lysates extracted from the hemisected folic acid-treated kidney tissues at days 1, 4 and 7 days (Fig. 3B). Only a weak 45 kDa Kim-1 protein (band B) was detectable in the vehicle control kidney whereas both 45 and 80-90 kDa (band A) proteins were detected after folic acid administration. A 60 kDa non-specific band does not change during with treatment or over the time course. In comparison with TFEC-treated animals, however, the intensity of Kim-1 bands is weaker compared to Kim-1-CHO cell lysate standards, and there was relatively less of the 80 kD and more of the 45 kD band present. In the concentrated urine protein fractions from day 1, 2, and 3 folic acid-treated rats, two sizes of Kim-1 bands were detected at each time point, albeit faintly on day 1 (Fig. 3C). The sizes of these forms were similar to the sizes of proteins detected in the TFEC urine sample. Thus, although there were no change in serum creatinine, Kim-1 protein was upregulated in kidney tissue and present in the urine after folic acid treatment.

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

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

In the initial 2 day period after exposure to cisplatin, serum creatinine levels remained below 1.0 mg/dl (Fig. 5A). At 3 days and longer creatinine levels consistently increased and plateaued from day 6 to 8. Marked upregulation of kidney tissue Kim-1 protein was found by immunoblot analysis of hemisected kidney lysates on days 4 and 8 after exposure (Fig. 5B, C). Only very small amounts of the 45 kDa Kim-1 protein (band B) are detectable in the kidney from vehicle treated animals whereas both the 45 and 80-90 kDa (band A) proteins are highly upregulated 4 days after cisplatin treatment, and further increased at day 8. In contrast to the folic acid model, in which the 45 kDa band predominates, both the 45 and 80-90 kDa bands are abundant after cisplatin. The 80-90 kDa band clearly shifts to a smaller size with progression of the time course as seen in the two other models. During the first day, despite no increase in serum creatinine, urinary 75 kDa Kim-1 protein was detectable (Fig. 5C), indicating that Kim-1 protein expression was already upregulated in the tubules and the ectodomain shed into the urine. A smaller size (approx. 40 kDa) immunoreactive band was also observed in the urine 2 days after cisplatin administration. Note that urinary Kim-1 was detectable on day 1 and 2 whereas whole kidney lysate Kim-1 was below the level of detectability on day 2. Urinary protein concentration increased from 0.50 mg/ml \((t = 0)\) and 0.46 mg/ml \((t = 1 \text{ day})\) to 2.07 mg/ml at 2 days after cisplatin. Urinary creatinine concentrations were 25 mg/dl, 46 mg/dl and 69 mg/dl respectively.
Shedding of the human KIM-1 ectodomain generates a remnant membrane and cytosolic fragment polypeptide of 14 kDa (2). In order to determine if the 14 kDa peptide is present in the rat kidney after nephrotoxicant administration, a 15% polyacrylamide was used for analysis of the tissue lysates. A band of approximately 14 kDa is present 3 days after cisplatin and this band is markedly upregulated 6 days after administration of cisplatin (Fig. 5C lower panel), reflecting the proteolytic cleavage of the Kim-1 protein resulting in the larger ectodomain fragment and a 14 kDa fragment.

**Immunohistochemical analysis of Kim-1 after cisplatin treatment:** On day 1 and 2 after cisplatin Kim-1 tissue immunoreactivity was detected but not prominent in the proximal tubules in the OSOM (Fig. 6A). Cellular staining was more diffuse than apical (Fig. 6A, Day 1, arrowhead). This early upregulation of Kim-1 protein is consistent with the detection of Kim-1 in the urine. At day 3, Kim-1 expression is increased and localized to the apical part of many of the proximal tubule cells with some expressing diffuse cytoplasmic staining in the OSOM and the medullary rays of the cortex (Fig. 6B). Some Kim-1 positive tubules are dilated with thinned epithelium, and occasionally contain casts at this time point. A given tubule may have only a fraction of its cells Kim-1 positive (Day 3, large arrowheads). Vimentin expression was low and co-expression with Kim-1 was very rare at this time point. At day 4 (Fig. 6B, Day 4), many dilated proximal tubules in the OSOM and medullary rays have cells with apical Kim-1 immunoreactivity. Some of tubules have cells expressing diffuse cytoplasmic staining (Day 4, small arrowhead) and many contain cell debris and casts. At this time point some Kim-1 positive tubules were also vimentin positive (Day 4, large arrowhead). At day 6 there was a pronounced increase of Kim-1 in S3 segments which characteristically co-express Kim-1 and vimentin (Fig. 6B, Day 6, large arrowheads). Thus, Kim-1 is detected in the tissue and urine of cisplatin-treated rats. Urine Kim-1 can be detected prior to any significant increase in serum creatinine and at a time when tissue expression is modest.
Kim-1 is a type-1 membrane protein which has Ig and mucin domains in its ectodomain and a relatively short cytoplasmic tail. We first identified the protein, by subtractive cloning, as the product of a gene that is highly induced in the post-ischemic kidney (9, 14). Although it is clear that Kim-1 is induced in the post-ischemic kidney, the generality of this response to other forms of nephrotoxic injury was not clear. Therefore, we investigated Kim-1 protein expression in three distinct models of nephrotoxicant-induced renal injury, TFEC, folic acid and cisplatin. Although the mechanism of TFEC injury involves production of reactive acylating species (11) the pattern and timing of injury in the nephron is similar to that observed following ischemic injury. High dose folic acid, which injures the nephron due to the precipitation of folate crystals in the tubule lumen, causes an intense proliferative response in the absence of significant necrosis (8). Cisplatin also produces necrosis in the rodent kidney, but, unlike ischemic injury and TFEC, the response is delayed and is likely to involve apoptosis to a greater extent (19, 23, 34). Despite the diverse nature of the mechanisms underlying these three models of injury and the differences in the timing and progression of the injury, tissue Kim-1 expression increases in all three models. In addition, the Kim-1 ectodomain and fragments of this domain were found in urine in each model indicating that expression in the injured nephron is associated with shedding of the ectodomain, allowing for noninvasive monitoring of nephrotoxicity. In this study we have used immunoblot analysis to identify tissue and urinary Kim-1 as well as immunofluorescence analysis of Kim-1 expressed in tubular epithelial cells. We detect Kim-1 in the tissue and urine in each model of nephrotoxicity even though these techniques are much less sensitive than an ELISA such as the one we used previously in human urine (9). At this point, however, we do not have the antibodies necessary for an ELISA on rodent urine.

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

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

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

The ectodomain of the Kim-1 protein includes an Ig and mucin domain. Our previous study demonstrated that this ectodomain is cleaved with proteases and the soluble ectodomain is constitutively shed into the culture media of human KIM-1 expressing cells (2). Released soluble KIM-1 protein is also detected in urine collected from patients with acute tubular necrosis and we have proposed this molecule as a new useful renal injury biomarker in human with ATN (9). As in humans we now report detection of rat Kim-1 protein in the tissue and urine samples suggesting that Kim-1 may be a sensitive general renal injury or early repair biomarker in animals exposed to nephrotoxicants. The appearance of tubule-expressed and urinary Kim-1 in cisplatin-treated rats prior to significant functional injury is a good agreement with our finding in humans in which urine KIM-1 is detected before urinary casts are seen (9). Since animal models are commonly used for evaluation of toxicity of new candidate therapeutics and assessment of
potential chemical hazards in industry and environmental exposure, Kim-1 may serve as a useful general biomarker for drug safety and chemical hazard-related renal injury. The finding that the Kim-1 ectodomain is shed with toxicity in animals also implies that animal models may be useful tools to investigate the mechanisms involved in Kim-1 shedding and the role of shedding \textit{in vivo} in the injury/repair processes. Measurement of Kim-1 protein expression may also be beneficial for detection of subtle abnormalities involving dedifferentiation of tubular epithelial cells during injury/repair in the kidney at a time distant from an insult when gross morphology and physiological parameters have already returned to baseline. We have observed that Kim-1 expression can be detected by immunohistochemical analysis in the kidney up to 64 days after TFEC administration (Ichimura et al, unpublished data).

Taken together, the data suggest that Kim-1 is a general marker for a variety of nephrotoxic injuries in rodents and humans; however, the function of Kim-1 protein in repair of renal injury is unclear. Recent reports show that Kim-1 is a prototypical member of a larger family of proteins which may function as an extracellular sensor or a receptor for adhesion/signaling in a variety of processes involving cell-cell or cell-pathogen interactions (18, 22, 25, 26). While the gene family has been proposed to be involved in T-cell function and immune response, the roles of Kim family members, particularly the functions of Kim-1 in injured and repairing kidney, remain unknown. While T-cells have been implicated in the pathophysiology of post-ischemic injury to the kidney we have not observed that infiltrating cells express Kim-1 either in ischemic kidneys or after nephrotoxicants (42). The protein structure of this molecule suggests that Kim-1 may be an adhesion and/or protective molecule for the cell surface (14). Therefore, we speculate that Kim-1 might alter cellular adhesion and/or prevent interactions between the injured epithelial cell and the luminal contents which include casts, debris, and viable epithelial cells that have become dislodged from the basement membrane (38).

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

This work was supported in part by National Institutes of Health grants DK 39773, DK 46267, DK 38453 and NS 10828. Antibody reagents were created with Véronique Bailly of the Biogen Inc. Cheng Chieh Hung also received support from Chang Gung Memorial Hospital.
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Figure 1. Time course of serum creatinine and immunoblot analysis of Kim-1 protein in the OSOM in kidney and the urine from TFEC-treated rats. A, Serum creatinine time course. Rats received 20 mg/kg i.p. TFEC. At various times after treatment, animals were anesthetized with pentobarbital, and blood was withdrawn from the tip of the tail. Serum creatinine was determined as described in “Methods.” Each point is the mean ± S.E. of at least three animals (n=3). Significant differences (*, p<0.05) between TFEC treatment versus vehicle treatment at various time points are indicated. B, A representative immunoblot of Kim-1 protein in the OSOM at various time points. The OSOM region of TFEC-treated rat kidney was dissected out and proteins were extracted. Thirty micrograms of sample lysates were loaded onto each lane. Proteins were analyzed by SDS-PAGE and immunoblot analysis using the R9 anti-rat Kim-1 antibody. Two bands (A and B) are noted at 80-90 and 45 kDa respectively. Rat Kim-1 stable CHO cell lysate (7.5 μg) was used as a standard (std) for Kim-1 protein bands. C, Quantitative analysis of Kim-1 protein levels in the OSOM by densitometry. The mature form 80-90 kDa Kim-1 bands were scanned and integrated optical density was determined. The signals were normalized to Kim-1 expressing CHO cell standard and the data were expressed as the ratio of the signal using the value for the standard as the denominator. Each time point is mean ± S.E. of data from three animals. Significant differences (*, p<0.05) between TFEC treatment versus vehicle treatment at various time points are indicated. D, Immunoblot analysis of Kim-1 protein in the urine. Ten micrograms of protein was prepared from urine samples collected at various time points after treatment and loaded onto each lane. The urine collected from two untreated rats were used as a negative control (C). Proteins were probed with anti-rat Kim-1 ectodomain antibody. Two bands (A and B) are noted at 75 and 60 kDa respectively.

Figure 2. Immunohistochemical analysis of Kim-1 expression in kidneys of TFEC- treated rats. Affinity purified antibody against peptide R9 was used for immunostaining of Kim-1 on
paraffin sections taken at various times after TFEC administration as described in Methods. All photomicrographs were taken by using a 40 X objectives (A-G, scale bar = 20 µm, H, scale bar = 10 µm). All fields were chosen from the outer stripe of the outer medulla. A, Day 0 vehicle control kidney: Kim-1 is not detectable. B, Day 1: the number of Kim-1 positive tubules is increased with epithelial cell staining (arrowheads). Many tubules show dilated and flattened morphology with casts and cell debris in the outer medulla region. C, Day 2: More Kim-1 positive tubules are found at this time point (arrowheads). These tubules, containing casts and cell debris, are dilated. Some of the Kim-1 staining is apical (arrowheads) and the pattern in some cells is diffuse. D, Day 3: Kim-1 protein is detected in many tubules in the injured outer medulla region. Kim-1 staining is localized primarily to the apical side of the regenerating epithelium with some occasional diffuse cytoplasmic staining (arrowheads). These tubules still contain abundant cell debris and casts in the lumen. E, Day 5: Kim-1 immunoreactivity remains detectable in most of the tubules in the injured area. The staining pattern is apical in these cells many of which have thin morphology. Casts and cell debris are reduced in the lumen. Notice the presence of a Kim-1 positive spherical cell cluster in the middle of a tubular lumen (arrowheads). The number of interstitial cells in the peritubular space is increased. F, Day 14: The number of Kim-1 positive tubules is clearly decreased. Kim-1 staining is apical in the thin positive tubular cells (asterisks). G: rabbit IgG negative control on day 5 kidney sections shows negative staining. H: Double-immunofluorescence staining for Kim-1 (small arrowheads, Red: Cy3) and vimentin (large arrowheads, Green: FITC) in the OSOM of day 3 kidney). No significant staining was observed using Rabbit IgG and omitting primary mouse IgG as a negative control (data not shown).

Figure 3. Time course of serum creatinine and immunoblot analysis of Kim-1 protein in the kidney and the urine from folic acid-treated rats. A, Serum creatinine time course. Rats received 250 mg/kg i.p. folic acid. At various times after treatment, animals were anesthetized with pentobarbital, and blood was withdrawn from the tip of tail. Serum creatinine was
determined as described in “Methods.” Each point is the mean ± S.E. of at least three animals. There was no statistically significant change in the serum creatinine time course. B, A representative immunoblot analysis of Kim-1 protein in the kidney at various time points after folic acid treatment. Forty micrograms of sample lysates, prepared from hemisected rat kidneys, were loaded onto each lane. Proteins were analyzed by SDS-PAGE and immunoblot analysis was carried out using R9 anti-rat Kim-1 antibody. Two Kim-1 bands (A and B) are noted at 80-90 and 45 kDa respectively. Rat Kim-1 stable CHO cell lysate (0.5 µg) was used as a standard (std) for Kim-1 protein bands. C, Immunoblot analysis of Kim-1 protein in the folic acid treated rat urine. Ten micrograms of protein was prepared from urine samples collected at 1, 2, and 3 days after treatment, and loaded onto each lane. Proteins were probed with anti-rat Kim-1 ectodomain antibody. Two bands (A and B) are noted at 75 and 60 kDa respectively, in urine collected at day 1, 2 and 3 after folic acid. Urines collected from two untreated rats were used as a negative controls (C).

Figure 4. Immunocytochemical analysis of Kim-1 and vimentin expression in folic acid treated rat kidney. Affinity purified antibody against peptide R9 and anti-vimentin monoclonal antibody were used for double-immunostaining of Kim-1 and vimentin on frozen sections of rat kidneys after folic acid administration as described in Methods. All photomicrographs were taken using 20 X objectives (A-F, scale bar = 50 µm). All fields were chosen from the cortical medullary ray or the OSOM region. A, Vehicle-treated control kidney. B, Day 1: apical Kim-1 staining is present in some proximal tubules which are mildly dilated in the medullary ray (small arrowheads). Positive staining of Kim-1 (red) in tubules sometimes is present only in some of the cells of a tubule (large arrowheads). C, Day 2: Kim-1 staining is seen in tubule with a great deal of debris (large arrowhead). D, Day 3, E, Day 4, F, Day 7 after folic acid: From day 2 to 7, persistent apical Kim-1 immunoreactivity is observed in the proximal tubules. There are increased number of vimentin positive cells (green) in the peritubular spaces on day 4 (4E, large arrowhead). Coexpression of Kim-1 and vimentin occurs but is rare at day 4 but more frequent at day 7 (4F, large arrowhead). Autofluorescence is present in some intra-luminal cell debris.
Figure 5. Time course of serum creatinine and immunoblot analysis of Kim-1 protein in the kidney and the urine from cisplatin-treated rats. A, Serum creatinine time course. Rats received 10 mg/kg i.p. cisplatin. At various times after treatment, animals were anesthetized with pentobarbital, and blood was withdrawn from the tip of the tail. Serum creatinine was determined as described in “Methods.” Each point is the mean ± S.E. of at least three animals. Significant differences (*, p<0.05) between cisplatin treatment versus vehicle treatment at various time points are indicated. B, A representative immunoblot analysis of Kim-1 protein in the cisplatin treated rat kidney. Twenty-five micrograms of sample lysates were prepared from hemisected rat kidneys at various times after cisplatin treatment and loaded onto each lane. Proteins were analyzed by SDS-PAGE and immunoblot analysis carried out using R9 anti-rat Kim-1 antibody. Two bands (A and B) were recognized at 80-90, 45 kDa respectively by the antibody. Rat Kim-1 stable CHO cell lysate (0.5 μg) was used as a standard (std) for kim-1 protein bands. C, Quantitative analysis of Kim-1 protein levels in the cisplatin treated rat kidneys by densitometory. The mature form 80-90 kDa bands were scanned and the integrated optical density was analyzed. The signals were normalized to Kim-1 expressing CHO cell standard and the data are expressed as the ratio of the signal to the standard. Each time point is mean ± S.E. of data from three animals. Significant differences (*, p<0.05) between cisplatin treatment versus vehicle treatment at various time points are indicated. D, Immunoblot analysis of Kim-1 protein in the urine. Twenty-five micrograms of proteins was prepared from normal urine and urine samples collected at 1 and 2 days after treatment. Protein samples were loaded onto each lane. Proteins were probed with anti-rat Kim-1 ectodomain antibody. A 75 kDa band (a) was present at day 1, and two bands (A and B) are noted at 75 and 45 kDa respectively at day 2 after treatment. E, Immunoblot analysis of Kim-1 remnant peptides in the kidney. Twenty-five micrograms of tissue protein lysates, prepared from cisplatin-treated rat kidneys, were loaded onto the each lane. Proteins were separated by 15 % SDS-PAGE and immunoblot analysis carried out using R9 anti-rat Kim-1 antibody.
Figure 6. Immunocytochemical analysis of Kim-1 and vimentin expression in cisplatin-treated rat kidney. A, Immunocytochemical analysis of Kim-1. Affinity purified antibody against peptide R9 was used for immunofluorescence staining for Kim-1 in frozen sections from the rat kidneys after cisplatin administration as described in “Methods”. Photomicrographs were taken using 20 X objectives (scale bar = 50 µm). Kim-1 protein is observed in the tubules at day 1 after cisplatin. Defuse staining is also detected at day 2. B, Double-immunostaining of Kim-1 and vimentin. Affinity purified antibody against peptide R9 and anti-vimentin monoclonal antibody were used to double-immunostain for Kim-1 and vimentin on frozen sections from the rat kidneys after cisplatin administration as described in “Methods”. All photomicrographs were taken using 20 X objectives (scale bar = 50 µm). All fields were chosen from the cortical medullary ray or the OSOM region. Day 3, Some proximal tubules in the medullary ray are positive for apical Kim-1 staining and are mildly dilated. The staining pattern is a “mosaic” (with a mixture of positive and negatively stained cells in one tubule, large arrowheads). Day 4, There is an increase in the number of Kim-1 positive cells, some of which also express vimentin, and intra-luminal cell debris is seen with some Kim-1/vimentin coexpression (large arrowhead). Note some cytoplasmic Kim-1 staining (small arrowhead). Day 6, Persistent apical Kim-1 immunoreactivity in the proximal tubules is detected with some Kim-1/vimentin coexpression (large arrowheads).
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6

A

Day 1

Day 2

B

Day 3

Day 4

Day 6

Figure 6