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Kidney injury molecule-1 expression in transplant biopsies is a sensitive measure of cell injury

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Kidney injury molecule-1 (KIM-1) is a specific histological biomarker for diagnosing early tubular injury on renal biopsies. In this study, KIM-1 expression was quantitated in renal transplant biopsies by immunohistochemistry and correlated with renal function. None of the 25 protocol biopsies showed detectable tubular injury on histologic examination, yet 28% had focal positive KIM-1 expression. Proximal tubule KIM-1 expression was present in all biopsies from patients with histological changes showing acute tubular damage and deterioration of kidney function. In this group, higher KIM-1 staining predicted a better outcome with improved blood urea nitrogen (BUN), serum creatinine, and estimated glomerular filtration rate (eGFR) over an ensuing 18 months. KIM-1 was expressed focally in affected tubules in 92% of kidney biopsies from patients with acute cellular rejection. By contrast, there was little positive staining for Ki-67, a cell proliferation marker, in any of the groups. KIM-1 expression significantly correlated with serum creatinine and BUN, and inversely with the eGFR on the biopsy day. Our study shows that KIM-1 staining sensitively and specifically identified proximal tubular injury and correlated with the degree of renal dysfunction. KIM-1 expression is more sensitive than histology for detecting early tubular injury, and its level of expression in transplant biopsies may indicate the potential for recovery of kidney function.

Kidney International (2008) **73,** 608–614; doi:10.1038/sj.ki.5002697; published online 26 December 2007

KEYWORDS: kidney injury molecule-1; renal tubular injury; biomarker; renal transplantation

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Received 20 December 2006; revised 27 July 2007; accepted 5 September 2007; published online 26 December 2007

Better tools are needed to identify tubular epithelial cell injury in the kidney. Even when biopsy tissue is available, it is difficult to define early signs of injury. Active tubular injury, which is a cellular pathological description, can cause acute kidney injury, a clinical syndrome, which is increasing in prevalence and is associated with significantly increased mortality and hospitalization cost.[1,2] In renal allografts, tubular injury associated with ischemia, nephrotoxicity secondary to immunosuppressive medication, and/or acute cellular rejection can lead to chronic allograft nephropathy and eventual graft failure.[3] Pre-renal and post-renal factors contributing to renal failure are not always easily distinguished from intrarenal injury in allograft recipients. Changes in kidney epithelial cell morphology in allograft biopsies can vary from no or minimal histologic changes of proximal tubules to diminished brush borders, apoptosis and/or necrosis, dilated tubules, and sloughed epithelial cells in the tubular lumina.[4,5] A pathologic diagnosis of acute tubular injury is often quite subjective. A sensitive tissue biomarker of tubular injury, which can be used to identify or confirm the presence of epithelial cell injury when pathological changes are minimal would be very helpful in the evaluation of biopsy specimens.

Kidney injury molecule-1 (KIM-1) is a type I transmembranous protein whose expression is undetectable in normal renal tissue and is markedly upregulated with injury of proximal tubule epithelial cells in rats[4–8] and has been reported to be upregulated in a limited number of native human biopsies where acute tubular injury was diagnosed histologically.[9] In this study, we used immunohistochemical techniques to characterize the expression of KIM-1 in renal transplant biopsies and to compare this staining with morphological findings of tubular injury and acute cellular rejection. We also determined whether there was a significant correlation between renal functional indices and KIM-1 staining intensity. We found that KIM-1 expression is more sensitive than histology for detecting early tubular injury in human allografts.

RESULTS

General evaluation of KIM-1 staining intensity

There was no immunohistochemical expression of KIM-1 in a large number of benign human tissues including normal

Table 1 | Negative immunohistochemical expression of KIM-1 in benign human tissues

Different types of human tissue
Pediatric and adult thymus (*n*=6, excision)
Adult livers (*n*=6, wedge biopsy)
Sinusitis (*n*=40, tissue microarray)
Tonsils (*n*=40, tissue microarray)
Uninjured fetal kidneys (*n*=3, 12–28 weeks of gestational age, without autolysis)
Benign bone marrow (*n*=10, biopsy)
Benign human tissue microarray (*n*=3 each) including adrenal gland, cerebrum, cerebellum, pituitary gland, breast, uterine cervix, colon, esophagus, heart, kidney, testis, salivary gland, liver, lung, skin, striated muscle, pancreas, placenta, prostate, small intestine, spleen, bone marrow, stomach, thymus, thyroid, parathyroid, tonsil, endometrium, nerve, ovary, fallopian tube, and ureter

KIM-1, kidney injury molecule-1.

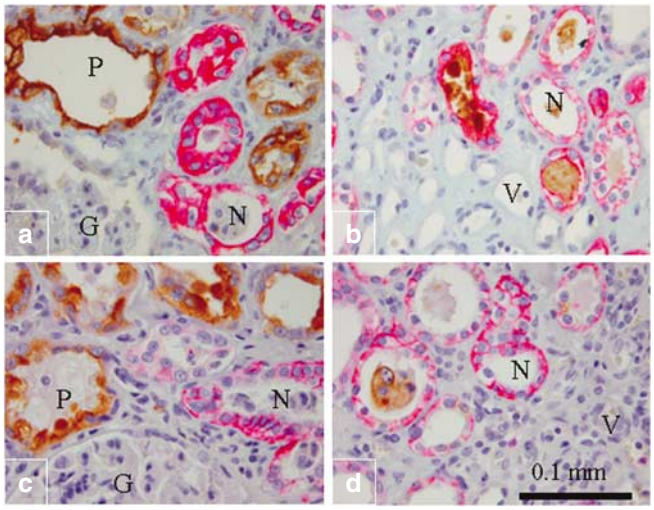

Figure 1 | Expression of KIM-1 in a positive control transplant tissue (a nephrectomy specimen following embolism in a renal transplant). Proximal tubules with prominent acute tubular injury showed dilation and flat epithelium. There was strong positive brown (peroxidase) KIM-1 staining (**a** and **c**). Cytokeratin-7 (**a**) or kidney-specific cadherin (**c**) expression in non-proximal tubules (N) is depicted in red (alkaline phosphatase). Glomerulus (G) is negative for expression of KIM-1, cytokeratin-7, or kidney-specific cadherin. In the medulla, the lumina of non-proximal tubules (N), indicated by either cytokeratin-7 (**b**) or kidney-specific cadherin (**d**), contained sloughed KIM-1-positive casts (brown color in **b** and **d**). Small vessels are negative for KIM-1 (both **b** and **d**), cytokeratin-7 (**b**), or kidney-specific cadherin stains (**d**) (original magnification $\times$600 in **a**–**d**).

human fetal and adult kidneys and lymphoid tissue, including thymus, bone marrow, and tonsils (Table 1). The positive control kidney tissue came from the viable portion of infarcted transplant kidney, where there was KIM-1 expression along the luminal plasmalemmal surface of the proximal tubular epithelium in a geographic pattern. Positive staining extended into some disrupted cellular junctional areas (Figure 1a and c). Distal nephron segments positive for cytokeratin 7 and kidney-specific cadherin were negative for KIM-1 staining, indicating specificity of KIM-1 for proximal tubules (Figure 1a and c). In the medulla, KIM-1-positive epithelial casts were present in the lumen of the distal nephron (Figure 1b and d).

Expression of KIM-1 in allograft biopsies

Kidney tissue was classified into three groups. Kidney recipients in the three groups were similar in age (Table 2). The duration of time from transplantation to the day of biopsy was significantly longer in group 3 (acute cellular rejection, ACR group) when compared with groups 1 (protocol biopsy group) and 2 (acute tubular injury, ATI group) (Table 2). In all cases in groups 1 and 2, minimal interstitial fibrosis was noted. In group 3, some interstitial spaces were occupied with inflammatory cells. No glomerulopathy was evident on light microscopy in any of the groups. The frequency of KIM-1-positive staining in each group is listed in Table 3. Immunohistochemical expression of KIM-1 in group 1 was absent in all nephron segments and vessels in 72% of cases (Figure 2a). In 7 of 25 cases, however, there was low-grade staining of proximal tubules for KIM-1 in these protocol biopsies. There was no difference in morphology between KIM-1-positive and -negative cases. This indicates that KIM-1 expression is more sensitive than normal histology for detection of low-grade proximal tubule injury. In group 2, when morphologic tubular injury was obvious on hematoxylin–eosin stained sections (with diminished brush borders on Periodic acid-Schiff sections), KIM-1 expression was seen in 100% of cases. Kidney injury molecule-1-positive staining was characteristically localized along the plasmalemmal surface of proximal luminal epithelium and extended into the lateral cellular membranes when epithelial junctions appeared disrupted (Figure 2b). The basal aspect of the epithelium was negative for KIM-1 staining even when there was marked tubular injury morphologically. It was noted that even when inflammatory infiltration and tubulitis were prominent in the group 3 biopsies, KIM-1 expression was localized to apical regions of a subset of proximal tubules (Figure 2c; Table 4). Inflammatory cells, including lymphocytes, monocytes, and plasma cells, were entirely negative for KIM-1 staining in all biopsies. Overall, group 2 had the highest intensity score of KIM-1 staining, followed by groups 3 and 1 in that order (Table 4). Proliferation of proximal tubules cells was evaluated by positive staining of nuclei for Ki-67. Ki-67 expression was minimal when compared to KIM-1 expression in groups 2 and 3, and there was nearly no positive staining for Ki-67 identified in group 1 (Table 4).

KIM-1 expression was significantly correlated with renal dysfunction

Serum creatinine and blood urea nitrogen (BUN) were all significantly higher, and estimated glomerular filtration rate (eGFR) significantly lower, in groups 2 and 3 compared with group 1 (Table 4). When groups of cases were analyzed together to correlate the KIM-1 staining score with measures of renal function, KIM-1 immunoreactivity was significantly

positively correlated with BUN and creatinine across the entire three groups as well as in each study subgroup (Figure 3). In addition, KIM-1 positivity was also inversely and significantly correlated with the eGFR of the combined three groups ($r = 0.587$, $P < 0.0001$).

KIM-1 staining is correlated with change in renal function in patients with acute tubular injury but not in patients with undetectable pathological evidence of tubular injury over 18 months of follow-up

When the renal function of patients ($n = 18$) with protocol biopsies negative for KIM-1 staining was compared to seven patients with protocol biopsies positive for KIM-1 staining (all from group 1) on the day of biopsy and 6, 12, and 18 months after biopsy, there was no significant difference in BUN, serum creatinine, or eGFR at any of the time points (Table 5). Since the tubular injury in these protocol biopsies was minimal, we then examined the change in renal function over time in those patients with acute tubular injury (group 2) and evaluated whether the extent of KIM-1 staining at the time of biopsy was correlated with changes in renal function over 18 months subsequent to biopsy. We found that the patients with greater levels of KIM-1 staining (2+ or 3+) had better recovery of function over time (Table 6; Figure 4) as reflected by reduction in BUN, serum creatinine, and increase in eGFR.

DISCUSSION

Evaluation of KIM-1 staining may serve to optimize the diagnosis of tubular injury in allograft biopsies. Our data indicate that KIM-1 protein expression in proximal tubules is significantly correlated with renal dysfunction. All patients in whom KIM-1 positivity was at a level of 1+ or greater had diminished renal function (serum creatinine levels >1.4 mg per 100 ml and serum BUN levels >20 mg per 100 ml). The

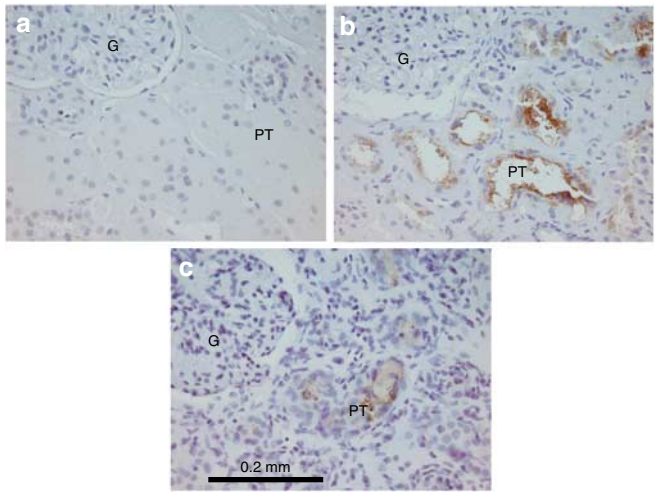

Figure 2 | KIM-1 expression in transplant biopsies. KIM-1 expression in protocol biopsy (**a**), acute tubular injury (**b**), and acute cellular rejection (**c**). (**a**) No KIM-1 staining was present in any compartment of this protocol renal transplant biopsy. (**b**) When active tubular injury was present, upregulated KIM-1 expression (3+) was seen along the luminal surface of proximal tubules. (**c**) With acute cellular rejection, a proximal tubule was positive for KIM-1 staining (1+), whereas inflammatory cells were not reactive with the AKG7 antibody (original magnification ×400 in **a**–**c**).

Table 2 | Profiles of patients with renal transplants in three groups

Groups	*N*	Pathologic description	Age (years)	Gender	Duration from Tx to Bx day (weeks)
1	25	Protocol biopsies	44.8 ± 2.9	12 female/13 male	20.3 ± 4.1
2	25	Active tubular injury	49.0 ± 2.9	9 female/14 male	16.0 ± 8.1
3	12	Acute cellular rejection	44.3 ± 4.5	6 female/6 male	78.8 ± 19.5*#

Tx, transplantation; Bx, biopsy.
*$P < 0.05$ vs group 1; #$P < 0.05$ vs group 2.

Table 3 | Distribution of KIM-1 staining intensity in each of the groups

		KIM-1 staining intensity (percentage of biopsies, %)				
Groups	Description	0	0.5 (±)	1+	2+	3+
1	Protocol biopsies	18/25 (72)	6/25 (24)	1/25 (4)	0/25 (0)	0/25 (0)
2	Active tubular injury	0/25 (0)	0/25 (0)	15/25 (60)	8/25 (32)	2/25 (8)
3	Acute cellular rejection	1/12 (8)	4/12 (33)	5/12 (42)	2/12 (17)	0/12 (0)

KIM-1, kidney injury molecule-1.

Table 4 | Renal functional indices, kidney injury molecule-1 (KIM-1), and Ki-67 staining scores

Groups	Description	BUN (mg per 100 ml)	Cr (mg per 100 ml)	BUN/Cr ratio	eGFR (ml min^{-1})	KIM-1 scores	Ki-67 scores
1	Protocol biopsies	22.8 ± 1.8	1.43 ± 0.09	16.22 ± 0.89	48.9 ± 2.2	0.16 ± 0.06	0.02 ± 0.02
2	Active tubular injury	46.2 ± 4.4*	3.43 ± 0.46*	15.42 ± 1.09	22.8 ± 3.1*	1.48 ± 0.13*	0.13 ± 0.09*
3	Acute cellular rejection	43.9 ± 4.8*	3.22 ± 0.50*	15.11 ± 1.41	26.8 ± 4.1*	1.00 ± 0.18*#	0.17 ± 0.11

BUN, blood urea nitrogen; Cr, serum creatinine; eGFR, estimated glomerular filtration rate.
*$P < 0.05$ vs protocol biopsies; #$P < 0.05$ vs active tubular injury group. Data are expressed as mean ± s.e.

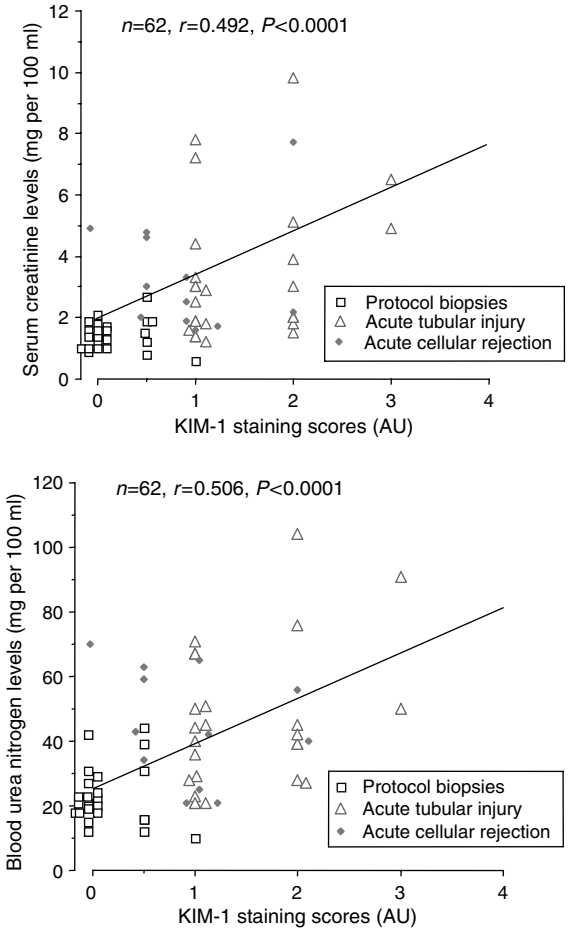

Figure 3 | KIM-1 staining was significantly correlated with serum creatinine (upper panel) and BUN (lower panel) across all groups.

Table 5 | Renal functional indices in protocol biopsies with negative or positive KIM-1 stains

Protocol biopsies	KIM-1-negative cases (*n*=18)	KIM-1-positive cases (*n*=7)
KIM staining scores (arbitrary units)	0.00 ± 0.00	0.57 ± 0.07*
BUN (mg per 100 ml)		
Biopsy day	22.39 ± 1.59	24.00 ± 5.21
6-month follow-up	21.72 ± 1.6	20.71 ± 5.17
1-year follow-up	22.39 ± 1.60	22.17 ± 1.64
1 and ½-year follow-up	22.14 ± 2.48	19.43 ± 2.26
Cr (mg per 100 ml)		
Biopsy day	1.41 ± 0.08	1.51 ± 0.27
6-month follow-up	1.44 ± 0.11	1.47 ± 0.24
1-year follow-up	1.49 ± 0.11	1.57 ± 0.21
1 and ½-year follow-up	1.44 ± 0.10	1.46 ± 0.22
eGFR (ml min^{-1})		
Biopsy day	49.68 ± 2.37	48.76 ± 5.80
6-month follow-up	48.76 ± 2.68	49.13 ± 5.13
1-year follow-up	47.26 ± 2.62	46.76 ± 4.90
1 and ½-year follow-up	49.14 ± 2.83	49.49 ± 5.14

BUN, blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate; KIM-1, kidney injury molecule-1.
*$P<0.05$.

Table 6 | Renal functional indices over 18 months in patients with ATI with either low (1+) or high (2 to 3+) level of KIM-1 kidney tissue expression by immunohistochemistry

Protocol biopsies	KIM-1 staining scores: 1+ cases (*n*=15)	KIM-1 staining scores: 2 to 3+ cases (*n*=10)
KIM staining scores (arbitrary units)	1.00 ± 0.00	2.20 ± 0.13*
BUN (mg per 100 ml)		
Biopsy day	40.93 ± 4.53	54.10 ± 8.47
6 months after biopsy	35.71 ± 5.97	29.90 ± 4.11#
12 months after biopsy	28.69 ± 2.80	26.56 ± 2.05#
18 months after biopsy	30.79 ± 2.77	26.00 ± 2.39#
Cr (mg per 100 ml)		
Biopsy day	3.02 ± 0.52	4.05 ± 0.83
6 months after biopsy	2.90 ± 0.66	1.98 ± 0.30#
12 months after biopsy	2.85 ± 0.65	1.62 ± 0.11#
18 months after biopsy	2.68 ± 0.53	1.62 ± 0.13#
eGFR (ml min^{-1})		
Biopsy day	30.76 ± 4.54	25.16 ± 5.17
6 months after biopsy	32.24 ± 4.18	43.04 ± 4.63#
12 months biopsy	31.21 ± 3.92	48.39 ± 3.05*#
18 months after biopsy	32.16 ± 4.04	49.37 ± 3.82*#

ANOVA, analysis of variance; ATI, active tubular injury; BUN, blood urea nitrogen; Cr, serum creatinine; eGFR, estimated glomerular filtration rate; KIM-1, kidney injury molecule-1.
*$P<0.05$ vs KIM-1 1+ positive group (by unpaired Student *t*-test).
#$P<0.05$ vs biopsy day value (by ANOVA).

fact that 28% of protocol biopsies had either 0.5 ± (24%) or 1 + (4%) KIM-1 staining suggests that KIM-1 staining is very sensitive as a marker for early kidney tubular injury. It could be argued that this might be a false-positive finding. Arguing against this is the exhaustive animal data carried out by a number of laboratories with agreement that Kim-1 is not expressed in normal kidney. Furthermore, we have found very good correlation between Kim-1 mRNA tissue expression and urinary Kim-1 in animals,[7] and we consistently find that urinary KIM-1 is absent in humans without kidney disease.

In animal studies, we know that many factors leading to tubular epithelial cell injury result in Kim-1 protein expression. Both gene and protein products of *Kim-1* are upregulated by 3 h after renal ischemia–reperfusion injury in animal models.[7] Cisplatin, folic acid, *S*-(1,1,2,2-tetrafluoroethyl)-l-cysteine, [5] cyclosporine,[10,11] and other nephrotoxicants[12] also result in *Kim-1* upregulation. In addition, Kim-1 protein is expressed in the kidneys of a transgenic rat that develops tubular-interstitial disease secondary to enhanced activity of the renin–angiotensin system,[6] and is also expressed in animal models of protein overload nephropathy[8] and obstruction.[13] In microarray studies of kidneys from hypotensive brain dead rats, Kim-1 was found to be highly upregulated 6 h following brain death.[14]

Our data in animal models indicate that Kim-1 expression was localized only to proximal tubules following

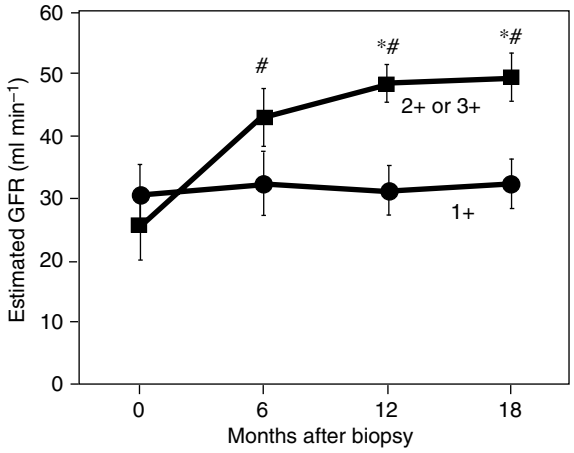

Figure 4 | For equivalent levels of renal dysfunction at the time of biopsy, a higher KIM-1 staining score, reflecting more acute injury, is predictive of improvement in eGFR over the ensuing 6, 12, and 18 months. $^{*}P<0.05$ vs KIM-1 1+ positive group; $^{\#}P<0.05$ vs biopsy day value.

ischemia–reperfusion and nephrotoxicant injury in rat kidneys.[4,5] The current study confirms that KIM-1 protein expression is localized to the injured human proximal tubular epithelia (Figure 1a and c). Since the AKG7 antibody is reactive with the metalloproteinase-released ectodomain,[15] the KIM-1-positive staining in the lumen of distal nephron tubules represents reaction of the antibody with KIM-1 ectodomain released from the proximal tubules, which then moved within the tubule to more distant segments of nephrons (Figure 1b and d). We noted KIM-1-positive staining in some atrophic tubules with surrounding fibrosis and inflammation. Although the function of KIM-1 is unclear, this finding supports our previously published hypothesis that KIM-1 might be involved in the development of interstitial fibrosis following injury.[13] If so, then KIM-1 expression in the allograft may have important implications for chronic allograft nephropathy. The data also indicate that there may be a significant difference on outcome with respect to the temporal character of KIM-1 expression. Given an equivalent level of diminished renal function in patients with allografts, as measured by eGFR, a high level of KIM-1 expression was found to be a good prognostic sign. This suggests that the reason for the reduction in renal function was more acute and involved tubules that were able to generate a robust KIM-1 response. In a patient with an allograft, a lower level of KIM-1 expression might be reflective of more chronicity and hence potentially less reversibility.

Pre-renal and post-renal factors contributing to renal dysfunction are not always easily distinguished from early tubular injury in renal transplant recipients. The absence of KIM-1 expression in human allograft biopsies would suggest that the cause of clinical renal dysfunction in these patients was related to pre-renal causes or cellular rejection with inflammation in the absence of significant epithelial cell injury. In 28% of protocol biopsies with no obvious pathological abnormalities, however, there was KIM-1-positive staining, indicating that there was injury at a time when the pathological evaluation was not sensitive enough to identify this. This could dictate therapeutic changes.

These observations indicate that KIM-1 staining is a very sensitive and specific indicator for tubular injury in human allografts as we have found in animals.[16] Furthermore, KIM-1 immunoreactivity was much more sensitive than Ki-67 positivity for detecting tubular injury (Table 4). Concurrent expression of KIM-1 and Ki-67 in groups 2 and 3 implies that tubular injury, epithelial cell dedifferentiation, and tubular regeneration processes can occur contemporaneously.

Kidney injury molecule-1-positive staining scores were significantly higher in group 2 when compared with group 3, whereas serum creatinine and BUN levels were similar in groups 2 and 3, reflecting additive detrimental functional effects of the lymphocytic infiltration in group 3. Within group 3, the KIM-1 staining score was significantly correlated with renal dysfunction, indicating that proximal tubular injury may contribute in an important way to renal functional deficit even when inflammatory cells dominate the pathology.

Kidney injury molecule-1 is identical to T-cell immunoglobulin and mucin-domain-containing molecule-1, which has been reported to be present in T lymphocytes (particularly Th2 cells) and to potentially be involved in autoimmune disorders.[17–19] The antibody, AKG7, however, has a high specificity in recognizing upregulated KIM-1 antigen in injured renal tubules and does not react with infiltrating T lymphocytes, other types of inflammatory cells, or other examined organs in humans (Table 1) using the methods we have employed. Native inflammatory cells (thymus, bone marrow, and tonsils) and inflammatory cells that have migrated into tissue (in 40 cases of sinus tissue with rhinitis including lymphocytes, plasma cells, monocytes, neutrophils, and eosinophils) were negative by AKG7 staining (Table 1).

In conclusion, tissue KIM-1 staining is a very useful biomarker to diagnose kidney epithelial cell injury in renal allografts. Positive staining in proximal tubules correlates very well with renal dysfunction. When a renal transplant recipient has renal dysfunction without acute cellular rejection present in the renal biopsy, negative staining of KIM-1 suggests that renal dysfunction is not associated with tubular injury and may be attributed to pre-renal factors. The level to which KIM-1 staining is seen in the tubules in kidneys with acute cellular rejection reveals the extent of damage to the tubules and correlates with functional deficits. On the basis of this study, we propose to use KIM-1-positive staining as a standard to limit subjective error and confirm tubular injury in the evaluation of renal transplant biopsies.

MATERIALS AND METHODS

Renal transplant biopsies

Cases were selected at random from renal transplant biopsies performed from 2003 to 2005 at Geisinger Medical Center (Danville, PA, USA). With only a few exceptions (prior to May 2003), renal allograft recipients received intraoperative Campath-1H (Alemtuzumab;

Genzyme Corporation, Cambridge, MA, USA) induction and postoperative immunosuppression. The pretreatment regimen included 1 g of methylprednisolone i.v., 650 mg of acetaminophen p.o., 50 mg of diphenhydramine i.v., and 30 mg of Campath-1H i.v. over 2 h (given before unclamping of the transplanted kidney's vessels). Postoperative immunosuppression included FK 506 (target level of 10 ng ml^{-1} from day 1) or mycophenolate mofetil, 1 g b.i.d. from day 1 (mycophenolate mofetil was preferentially given to expanded criteria donors, non-heart-beating donors, and to donors of age <10 years). In addition, all patients received prednisone 20 mg once a day from day 1, with a subsequent wean to 0 mg in 8 weeks (reduced 2.5 mg every week). Each patient also received Valgancyclovir 450–900 mg a day for cytomegalovirus prophylaxis from the first week post transplant (duration and dosing of Valgancyclovir was dependent on renal function and pretransplant donor and recipient cytomegalovirus status). Testing for cytomegalovirus antigenemia was carried out once a week for the first 3 months, once every 2 weeks from months 3 to 9, and once a month after month 9. Patients were followed with weekly laboratory tests. Renal biopsies were performed as clinically indicated to rule out acute cellular rejection and/or by protocol at 2 weeks, 3 months, and 1 year. Renal functional indices, including serum creatinine, BUN, and eGFR, were obtained from the Geisinger Epicare system. In the Geisinger clinical laboratory, the eGFR was calculated using a standard formula from the National Kidney Foundation: $(\text{eGFR} = 186 \times (S_{Cr})^{-1.15} \times (\text{Age})^{-0.20} \times (0.742 \text{ if female}) \times (1.21 \text{ if African American})$.

Grouping renal cases

Three groups were identified via a computer search based on ICD9 codes. The control group included protocol renal transplant biopsies done less than 1 year following renal transplantation (group 1). All protocol biopsies were interpreted as having no significant pathology by one of the authors (PLZ), a renal pathologist. Group 2 included cases with obvious pathological findings of proximal tubular injury including dilation of tubules, diminished brush borders, and flat and detached epithelia, whereas no acute cellular rejection was present. This group was designated the active tubular injury group. Group 3 was composed of cases where the pathology revealed mild acute cellular rejection (ACR) (Banff criteria Ia and Ib).[20]

Histology and immunohistochemical staining for KIM-1 and proliferative nuclear antigen Ki-67

The renal biopsy tissue was fixed in formalin and paraffin embedded. The tissue block was cut into 3-μm sections and underwent routine staining for hematoxylin–eosin staining (three sections), Periodic acid-Schiff staining (three sections), and Masson Trichrome staining (one section). For each block, one 3-μm section was dewaxed in xylene and rehydrated with graded ethanols to water. AKG7, a mouse monoclonal antibody against the ectodomain of human KIM-1,[15] was used in the study. The stock concentration of AKG7 was 1 mg ml^{-1}. The slides were treated with a 20 min heat-induced antigen retrieval protocol (Target Retrieval Solution, DakoCytomation, Carpinteria, CA, USA). Primary AKG7 antibody (diluted 1:8 with antibody diluent; DakoCytomation) was applied for 1 h. The secondary antibody (peroxidase-labeled goat anti-mouse; Env + kit; DakoCytomation) was applied for 30 min. DAB (3,3′-diaminobenzidine; Env + kit; DakoCytomation) was applied for 10 min. KIM-1 positivity was reflected as brown staining of the proximal tubules. KIM-1 staining was also performed on a large number of benign human tissues from Geisinger Medical Center, and on an FDA true 33 normal tissue screen tissue array (Cybrdi Inc., Gaithersburg, MD, USA).

To identify parts of the nephron distal to the proximal tubule, in some cases, after staining for KIM-1, the tissue sections were treated with Denaturing Solution (Biocare Medical, Concord, CA, USA) for 5 min, rinsed with Tris-buffered saline with Tween 20 buffer, and costained with cytokeratin-7 primary antibody (diluted 1:100; Biocare Medical) for 1 h. The slides were rinsed with Tris-buffered saline with Tween 20, and treated with MACH-2 goat anti-mouse alkaline phosphatase secondary antibody (Biocare Medical) for 30 min. Slides were rinsed with Tris-buffered saline with Tween 20, and Vulcan Fast Red Chromagen Substrate (Biocare Medical) was applied for 15 min. The slides were counterstained with Gill-II Hematoxylin (Thermo Shandon, Pittsburgh, PA, USA) and coverslips were applied. Non-proximal tubules were also positively stained red with alkaline phosphatase using a monoclonal mouse antibody to kidney-specific cadherin (Zymed, San Francisco, CA, USA), at a dilution of 1:50. Kidney-specific cadherin is known to be expressed in the distal nephron.[21] Other sections were stained with MIB-1 antibody against Ki-67 antigen (DakoCytomation) using the Dako Autostainer. Expression of the Ki-67 antigen has been reported to be low in normal tubular epithelium[22] but upregulated in injured renal tubular epithelial cells.[23]

Quantitation of immunohistochemical staining

Kidney injury molecule-1-positive staining intensity in proximal tubules of all biopsy cases from groups 1 to 3 was evaluated manually. The staining intensity score of targeted and control epithelial cells was graded from 0 to 3+ (0, no staining; 0.5±, weak fine granular staining focally present along the luminal surface of non-atrophic proximal tubules; 1+, weak fine granular staining completely surrounding the luminal surface of non-atrophic proximal tubules; 2+, moderate granular staining completely surrounding the luminal surface of non-atrophic proximal tubules and extending into intercellular junctions; and 3+, strong large granular staining completely surrounding the luminal surface of non-atrophic proximal tubules and extending into intercellular junctions).

Statistics

Results were expressed as mean ± 1 s.e.m. Data among groups were compared using one-way analysis of variance (StatView program). Correlation between KIM-1 staining scores and renal function indices was assessed using simple regression analysis (StatView program). A P-value less than 0.05 was considered significantly different.

DISCLOSURE

Dr Bonventre holds patents on KIM-1. The therapeutic rights to KIM-1 have been licensed to BiogenIdec Corp, Cambridge, MA, USA.

ACKNOWLEDGMENTS

This work has been supported by Geisinger intramural grant (to PLZ) and National Institutes of Health Awards DK 39773, DK 72381, DK 74099, and DK 38452 (to JVB).

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