Chronic epithelial kidney injury molecule-1 expression causes murine kidney fibrosis

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Acute kidney injury predisposes patients to the development of both chronic kidney disease and end-stage renal failure, but the molecular details underlying this important clinical association remain obscure. We report that kidney injury molecule-1 (KIM-1), an epithelial phosphatidylserine receptor expressed transiently after acute injury and chronically in fibrotic renal disease, promotes kidney fibrosis. Conditional expression of KIM-1 in renal epithelial cells (Kim1RECtg) in the absence of an injury stimulus resulted in focal epithelial vacuolization at birth, but otherwise normal tubule histology and kidney function. By 4 weeks of age, Kim1RECtg mice developed spontaneous and progressive interstitial kidney inflammation with fibrosis, leading to renal failure with anemia, proteinuria, hyperphosphatemia, hypertension, cardiac hypertrophy, and death, analogous to progressive kidney disease in humans. Kim1RECtg kidneys had elevated expression of proinflammatory monocyte chemotactic protein-1 (MCP-1) at early time points. Heterologous expression of KIM-1 in an immortalized proximal tubule cell line triggered MCP-1 secretion and increased MCP-1–dependent macrophage chemotaxis. In mice expressing a mutant, truncated KIM-1 polypeptide, experimental kidney fibrosis was ameliorated with reduced levels of MCP-1, consistent with a profibrotic role for native KIM-1. Thus, sustained KIM-1 expression promotes kidney fibrosis and provides a link between acute and recurrent injury with progressive chronic kidney disease.

Introduction

Acute kidney injury (AKI) is characterized by a rapid decline in kidney function, often triggered by an ischemic or toxic insult. This clinical syndrome is associated with substantial short-term morbidity, mortality, and cost, but it had previously been assumed that patients surviving the episode made a full renal recovery (1). However, AKI is now appreciated to be markedly associated with increased risk of future chronic kidney disease (CKD), end-stage renal disease (ESRD) (2, 3), and long-term mortality (4). The population rate of AKI is increasing at greater than 7% per year (5, 6), and some estimates indicate that the incidence of AKI-related ESRD is equal to the incidence of ESRD from diabetes (7). The mechanisms that might explain the link between AKI and future CKD/ESRD are poorly understood, but peritubular capillary loss, a known consequence of AKI (8), is proposed to lead to chronic hypoxia and later development of tubulointerstitial fibrosis and CKD (9, 10). How chronic ischemia might trigger parenchymal loss at a molecular level is unresolved.

Kidney injury molecule-1 (KIM-1), originally identified as hepatitis A virus receptor (HAVCR1, also known as Tim-1), is a type 1 transmembrane protein strongly induced by ischemic and toxic insults to kidney. It also plays diverse roles in T and B cell biology (11). In healthy kidney, KIM-1 is undetectable, but after injury, it is induced more than any other protein, in which case it localizes to the apical surface of surviving proximal tubule epithelial cells (12). The extracellular KIM-1 Ig variable domain binds and internalizes oxidized lipid as well as phosphatidylserine exposed on the outer leaflet of luminal apoptotic cells (13, 14), thereby aiding in nephron repair and tissue remodeling through phagocytosis of cells and debris (15). KIM-1 is expressed in CKD (16–20) where it colocalizes with areas of fibrosis and inflammation (21), and its expression correlates directly with interstitial fibrosis in human allografts (22). Increased urinary KIM-1 is an independent predictor of long-term renal graft loss and is also elevated in human nondiabetic, proteinuric CKD (23, 24). The expression of KIM-1 in chronic and progressive kidney disease, settings without significant numbers of apoptotic cells in the tubule lumen, the epidemiologic association of AKI with future CKD (25), and the temporal and spatial association of KIM-1 with inflammation and fibrosis suggest that it might play a pathogenic role in linking AKI to CKD and renal fibrosis.

In this study, we examined the functional consequences of chronic KIM-1 expression in renal epithelial cells. To dissociate the effects of KIM-1 expression from the pleiotropic effects of...
ischemic kidney injury used to induce KIM-1, we created a genetic model in which KIM-1 is expressed chronically in the absence of any injury stimulus. Using this model, we demonstrate here that chronic KIM-1 expression leads to inflammation, tubulointerstitial fibrosis characterized by elevated monocyte chemotactic protein-1 (MCP-1) levels and a murine CKD phenotype. In contrast, mice with mutant endogenous KIM-1 were protected from fibrosis in a mouse model of CKD and had a reduced level of MCP-1. Together, these results indicate that persistent KIM-1 expression after AKI promotes interstitial fibrosis and correlates with MCP-1 expression and further suggest that KIM-1 may represent a novel therapeutic target in CKD (26). The mouse model we have developed also recapitulates the renal and extrarenal manifestations of CKD seen in humans. These studies provide insight into how recurrent tubular injury, as reflected by persistent KIM-1 expression, might facilitate progressive CKD and lead to ESRD.

Results
To determine the kinetics of KIM-1 induction during fibrotic disease, we examined the time course for KIM-1 expression in a rodent model of renal fibrosis, unilateral ureteral obstruction (UUO). KIM-1 protein was strongly upregulated 2 days after ureteral ligation and fell thereafter, but remained significantly elevated at day 14 (Figure 1A). KIM-1 was expressed on the apical aspect of proximal tubule epithelia, in tubules surrounded by expanded interstitium with abundant interstitial smooth muscle actin–positive myofibroblasts (Figure 1B). To distinguish between KIM-1 expression as a cause or consequence of epithelial injury and fibrosis in vivo, we created a conditional Z/Kim1-AP transgene enabling Cre recombinase-dependent activation of KIM-1 and alkaline phosphatase (AP) expression (Figure 2, A–E). Crossing the Z/Kim1-AP mouse with Six2-GFPCre mice (hereafter referred to as Six2-GC) (27), generated bigenic Kim1RECtg (Kim1 renal epithelial cell transgenic) mice with KIM-1 and AP expression in metanephric mesenchyme–derived kidney epithelia. Kim1RECtg kidneys expressed the Z/Kim1-AP transgene primarily in cortical and outer medullary epithelia, with rare transgene expression in inner medulla (Figure 2E and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI45361DS1). Mosaic transgene activity was observed with 10%–20% of renal tubules positive for AP activity, with a similar fraction of positive podocytes. Note, however, that despite the AP expression pattern, KIM-1 protein was only seen in tubules and never in podocytes (Figure 3C and data not shown). A comparison of the distribution of endogenous KIM-1 after UUO versus AP expression in Kim1RECtg kidneys is presented in Table 1.

Kim1RECtg mice were born at expected Mendelian ratios and expressed Kim1 mRNA at birth (Figure 3A). KIM-1 protein was properly sorted to the apical membrane of cortical proximal tubule epithelia (Figure 3, B and C). There was no difference in the birth weights of transgenic versus littermate control mice (n = 3 Kim1RECtg or 7 littermate controls), but Kim1RECtg mice did not gain weight as quickly as littermate controls (Supplemental Figure 2). At birth, kidneys from Kim1RECtg mice were 23% smaller by weight, however, than those of littermate controls (Figure 3, D and E, P < 0.05, n = 5 Kim1RECtg or 13 control kidneys). This was associated with 43% fewer nephrons in Kim1RECtg kidneys without significant differences in glomerular diameter at P14 (Figure 3, F and G). Kidney histology at P1 showed a mild reduction in cortical thickness, with occasional microcysts that appeared to be glomerular (about 10% of total glomeruli; Figure 3H) and rare large cysts (fewer than 1 per section). A detailed histologic analysis at P15 revealed normal glomeruli including foot processes, however (Figure 3, I and J), as well as normal interstitium and vasculature. Focal coarse vacuolization and focal epithelial degeneration were noted only in Kim1RECtg mouse kidneys (n = 3 Kim1RECtg and 3 control kidneys; Tables 2 and 3). These coarse vacuoles, suggestive of local injury, were found in about 1% of tubules (Figure 3, K and L). There were no histologic differences in other organs of Kim1RECtg mice when compared with organs from littermate controls (data not shown). Thus, P15 kidneys from Kim1RECtg mice were characterized by reduced nephron endowment and rare tubular epithelial vacuolization, but kidney histology was otherwise normal.

At 5 weeks, kidneys from Kim1RECtg mice developed a patchy mononuclear interstitial infiltrate with occasional hyaline casts and focal tubular damage. KIM-1 continued to be expressed in a subset of tubular epithelial cells along the apical membrane (Supplemental Figure 3). By 12 weeks, interstitial infiltrates were extensive, together with tubular dedifferentiation, microcystic tubular dilatation, hyaline casts, and fibrosis. This inflammatory, tubular injury, and fibrotic phenotype was observed in all Kim1RECtg kidneys (n = 43% fewer nephrons in Kim1RECtg with 43% fewer nephrons in Kim1RECtg kidneys and 3 control kidneys). However, there were no histologic differences in other organs of Kim1RECtg mice when compared with organs from littermate controls (data not shown). Thus, P15 kidneys from Kim1RECtg mice were characterized by reduced nephron endowment and rare tubular epithelial vacuolization, but kidney histology was otherwise normal.

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rose progressively thereafter (Figure 4C), and kidneys from aged mice were shrunken with a cobblestone appearance typical of end-stage renal fibrosis (Figure 4D). *Kim1RECtg* mice died spontaneously of progressive renal failure at a median age of 11 weeks (Figure 4E).

Immunohistochemistry and collagen stains confirmed early focal fibrosis surrounding isolated tubules beginning at 4 weeks, whereas older mice exhibited extensive fibrosis, with abundant αSMA-positive interstitial myofibroblasts and collagen fiber deposition (Figure 5A). In *Kim1RECtg* mice with established fibrotic disease, AP transgene expression was expressed in some, but not all, damaged and dilated tubules, consistent with transgene expression in 10%–20% of tubules (Figure 5B). We did not detect any interstitial cells that expressed the AP transgene, arguing against any direct contribution of injured epithelial cells to the myofibroblast population through epithelial-to-mesenchymal transition and consistent with the notion that epithelia are not capable of contributing directly to the interstitial myofibroblast pool (28, 29).

Given the progressive kidney disease exhibited by *Kim1RECtg* mice, we looked for extrarenal manifestations of CKD. Cardiac hypertrophy often accompanies CKD in humans and is linked to the very high cardiac mortality associated with CKD (30). We performed cardiac ultrasound in *Kim1RECtg* mice at age 10 to 12 weeks, a time when the renal phenotype is well established. At this time point, there was no increase in the systolic blood pressure (Figure 5, E–I), nor was there an increase in the interventricular septal thickness, left ventricular end-diastolic diameter, or left ventricular posterior wall dimensions in *Kim1RECtg* compared with littermate controls (Table 4). There was a significantly increased percentage fractional shortening, consistent with increased cardiac contractility, which is likely a consequence of the anemia that the mice develop (Figure 5J). In contrast, *Kim1RECtg* mice that survived past 6 months of age had clear evidence of left ventricular hypertrophy, measured as ventricular wall thickness-to-diameter ratio (Figure 5, C and D). If hypertension were the primary cause of the renal fibrosis in

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**Figure 2**

Generation and initial characterization of *Kim1RECtg* mice. (A) Schematic of the Z/Kim1-AP transgene used for generation of transgenic mice. (B) Transient transfection of Cos7 cells with the Z/Kim1-AP (Z/Kim1) plasmid in the absence or presence of a plasmid directing expression of a GFPCre fusion protein verifies that LacZ is expressed before Cre-dependent recombination, and AP after. Original magnification, ×400. (C) KIM-1 protein is detected only in lysates of Cos7 cells cotransfected with Z/Kim1-AP and GFPCre plasmids. (D) Wholemount X-gal stain of a Z/Kim1-AP-positive mouse and littermate control (age 4 weeks) shows mosaic LacZ activity throughout cortex. (E) WT mice express neither LacZ nor AP, whereas Z/Kim1-AP mice (age 4 weeks) exhibit mosaic LacZ expression but no AP expression. Kidney sections from bigenic *Kim1RECtg* mice show reduced LacZ expression and activation of AP expression in the cortex. Scale bar: 250 μm.
Kim1RECtg mice then cardiac hypertrophy would have been expected to occur by the 10- to 12-week time point when renal injury and dysfunction were severe.

The presence of a normal blood pressure at 10 to 12 weeks is also consistent with the notion that hypertension was not simply a consequence of reduced nephron endowment, because it did not precede the renal phenotype, but rather that hypertension was a consequence of severe reduction in glomerular filtration rate (Figure 5E). The severe and progressive anemia developed by the Kim1RECtg mice (Figure 5F) was normocytic (mean corpuscular volume was not different between control and Kim1RECtg mice; data not shown). Late stages of CKD are characterized by hyperkalemia, hyperphosphatemia, and hypoalbuminemia. Thus, Kim1RECtg mice displayed all 3 of these characteristics in a progressive fashion over time (Table 5).

The phenotype is not a result of primary proteinuria, podocyte expression of KIM-1, or general toxicity of AP. Chronic proteinuria of any cause has been proposed to drive renal fibrosis. Kim1RECtg mice developed proteinuria after 4 weeks of age, subsequent to tubular damage and leukocyte influx (Figure 5G and Supplemental Figure 5). Importantly, there was no proteinuria in Kim1RECtg mice at P14, when podocyte foot processes and glomerular capillary endothelium were normal (Figure 3J). Since KIM-1 is not normally expressed in podocytes, we investigated whether the Kim1RECtg phenotype was

![Figure 3](http://www.jci.org)

**Figure 3** Tubular KIM-1 expression and phenotype of Kim1RECtg kidneys. (A) Kim1 mRNA is present in P1 kidneys only in bigenic Kim1RECtg mice. (B and C) At P14, kidneys from control mice do not express KIM-1 protein, whereas kidneys from Kim1RECtg mice exhibit appropriate apical expression of KIM-1 in proximal tubules. KIM-1, red; Dolichos biflorus lectin (DBA), green. Scale bar: 20 μm. (D and E) At birth, Kim1RECtg kidneys are smaller in size and 23% smaller in weight compared with those of littermate controls. (F) Kim1RECtg mice (n = 8) had 46% fewer nephrons than controls at P14 (n = 9); *P = 0.0001. (G) Glomerular diameter was not different between controls and Kim1RECtg mice. (H) Low- and high-power views of P1 kidneys from control or Kim1RECtg kidneys reveal thinned cortex and occasional cystic glomerular changes (*) in Kim1RECtg. Scale bar: 25 μm. (I) At P15, glomeruli of control and Kim1RECtg kidneys were similar, without cystic dilation. Original magnification, x400. (J) Electron microscopy of P14 Kim1RECtg kidneys revealed normal glomerular architecture. Scale bar: 2 μm. In tubules, occasional focal coarse epithelial vacuoles were visible (K and L), suggestive of epithelial injury. Scale bars: 10 μm.

<table>
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<tr>
<th>Table 1</th>
<th>Comparison of endogenous Kim1 expression after UUO and AP expression in Kim1RECtg mice</th>
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<tr>
<td><strong>Podocytes</strong></td>
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<tr>
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<tr>
<td><strong>Kim1RECtg (AP)</strong></td>
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a consequence of delayed toxicity to podocytes from glomerular KIM-1 expression. Mice with KIM-1–AP expression exclusively in podocytes (bigenic Podocin-Cre/Z/Kim1-AP) had expression of AP activity in 55% of glomeruli in a focal pattern (Supplemental Figure 6) with histologically normal glomeruli (data not shown). There was no effect on kidney size, and mice did not develop proteinuria even up to 6 months of age. Serum creatinine and hematocrit had the same values as in littermate controls (Figure 5, I and J). Taken together, these observations show that renal fibrosis was not a secondary consequence of abnormal glomerular development, early hypertension, or podocyte expression of KIM-1. We also evaluated the possibility that AP expression alone in KIM-1–AP mice might mediate kidney damage independently of KIM-1 (31). However, bigenic Six2-GCZ/AP mice that expressed the AP transgene (without KIM-1) in nearly 100% of renal epithelia (Supplemental Figure 5) had normal renal histology, no proteinuria, and normal serum creatinine, even in the case of aged mice (Figure S1 and data not shown).

Characterization of KIM-1–induced kidney inflammation. Since early inflammation was a prominent histologic feature in KIM1RECtg mice, we next sought to characterize this infiltrate at the earliest time that disease appeared, 4 weeks. There was no interstitial infiltrate at 2 weeks, but CD3+ lymphocytes and F4/80+ macrophages and dendritic cells migrated into KIM1RECtg kidneys by 4 weeks, and their appearance correlated with increased interstitial cell proliferation at this time point (Figure 6, A–C). KIM-1 has recently been identified as an endogenous ligand for the activating receptor leukocyte monokine immunoglobulin-like receptor 5 (LMIR5) (also known as CD300b), and binding of KIM-1 to LMIR5 promotes neutrophil influx regulated by myeloid cells (32). However, there were no neutrophils at 4 weeks in KIM1RECtg kidneys, and they were only observed at late stages of disease (Figure 6C and data not shown). CD3+ lymphocytes were located in a focal pattern at early stages, often adjacent to KIM-1–positive tubules (Figure 6D), suggesting that secreted factors from KIM-1–expressing cells might be responsible for recruiting inflammatory cells to the kidney in KIM1RECtg mice.

Tubule damage is known to contribute to and be exacerbated by inflammation. We therefore tested whether tubular damage could be detected using urinary biomarkers at 4 weeks. Since KIM-1 itself undergoes proteolytic cleavage, resulting in release of the soluble ectodomain that might have the capacity to send a proinflammatory signal itself (32), we measured urinary KIM-1 levels. Even at 4 weeks, when disease was mild, urinary KIM-1 levels were higher in KIM1RECtg mice compared with controls, as expected (Figure 7A). Consistent with early epithelial damage, urinary N-acetylβ-D-glucosaminidase (NAG) was also increased at 4 weeks and increased further at 8 weeks (Figure 7B). We further measured the levels of cytokines and chemokines known to be activated by epithelial pattern recognition receptors that might mediate leukocyte recruitment. We detected a strong upregulation of mRNA encoding a panel of cytokines capable of being secreted by epithelial cells at 4 weeks (Figure 7C). Three of these cytokines, CXCL-1, MCP-1, and TGF-β, were upregulated at 2 weeks—a time point without evident histologic damage.

The inflammation observed in KIM1RECtg mice coupled with increased proinflammatory cytokine expression suggested the possibility that KIM-1 might directly regulate epithelial cytokine expression. To test this possibility, we stably expressed either vector alone (pcDNA-LLC) or KIM-1 (KIM-1–LLC) in LLC-PK1 porcine proximal tubule cells. The supernatant from these cultures was collected and assessed for cytokines. The supernatant from KIM-1–expressing, but not control, cultures showed significant upregulation of mRNA encoding CXCL-1, MCP-1, and TGF-β (Figure 7D–F). This effect could be largely abrogated by addition of a neutralizing anti–MCP-1 antibody (Figure 7I), consistent with the increased expression of MCP-1 detected in KIM-1–LLC supernatant.

### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tubule histology</th>
<th>Vacuolization</th>
<th>Epithelial degeneration</th>
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<tr>
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### Table 3

<table>
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<tr>
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<th>Podocyte effacement</th>
<th>Vacuolization</th>
<th>Reabsorption granules</th>
<th>GBM thickening</th>
<th>Endo fenestration loss</th>
<th>Mes expansion</th>
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GBM, glomerular basement membrane; Mes., mesangial.
Soluble fibronectin is a known inducer of proinflammatory cytokines in renal epithelial cells (33), and the KIM-1–expressing cell line had substantially elevated fibronectin expression compared with control cells. Moreover, in a third cell line that expressed a point mutant of a cytoplasmic tyrosine within a consensus phosphorylation sequence (Y350F), fibronectin levels were increased in comparison with native KIM-1, reflecting the absence of the heavily glycosylated mucin domain encoded by exon 3 (Figure 8A).

These findings indicate that KIM-1 expression drives proinflammatory cytokine expression, suggesting a mechanism to explain enhanced leukocyte infiltration at early time points in the Kim1RECtg mouse model. KIM-1 also drives fibronectin expression in LLC-PK1 cells through a mechanism that requires cytoplasmic tyrosine Y350, suggesting that fibronectin upregulation may underlie the proinflammatory cytokine expression observed.

**KIM-1 mutant mice are protected from renal fibrosis in the UUO model.** The Kim1RECtg phenotype suggested that mice with mutant KIM-1 might be protected from kidney fibrosis. To test this hypothesis, we analyzed mice carrying a deletion of exon 3 of the KIM-1 locus (Kim1Δmuc), resulting in an in-frame deletion of the extracellular mucin domain. This mutation results in a smaller KIM-1 polyprotein that is defective in KIM-1–dependent phagocytic function (34). When subjected to UUO injury, KIM-1Δmuc protein was upregulated, but as expected, it migrated faster on Western blot than native KIM-1, reflecting the absence of the heavily glycosylated mucin domain encoded by exon 3 (Figure 8A).

Both control and Kim1Δmuc mice (n = 10 each) were subjected to UUO and sacrificed on day 10. Kim1Δmuc mice had significantly reduced interstitial collagen deposition as well as reduced tubular injury scores (Figure 8, B–D). mRNA levels of the myofibroblast marker αSMA were also reduced in Kim1Δmuc mice, in addition to levels of collagen 1α1 and fibronectin (Figure 8, E and F). Taken together, these findings indicate that Kim1Δmuc mice are protected from renal fibrosis in the UUO model. Since proinflammatory cytokine levels were increased in Kim1RECtg mice and KIM-1 expression regulates MCP-1, TGF-β, and IL-6 secretion in vitro, we analyzed the same panel of cytokines in Kim1Δmuc kidney samples before and after UUO. MCP-1 levels were significantly reduced in Kim1Δmuc mice compared with control at day 10 of UUO (Figure 8H), while other cytokine mRNAs were unchanged (data not shown). This result, combined with our previous data, strongly implicates MCP-1 as a mediator of KIM-1–dependent fibrosis in the mouse kidney.

**Discussion**

Resident kidney epithelial cells play an important role in detection of injury, regulation of the inflammatory and tissue repair responses, and mediation of interstitial fibrosis through paracrine mechanisms (35, 36). In this study, we hypothesized that KIM-1 might regulate kidney inflammation and fibrosis when its expression is prolonged because: (a) it is upregulated very early after kidney injury and is thus poised to serve as a sentinel of damage; (b) it is expressed in chronic fibrosing kidney disease, where it colocalizes with areas of fibrosis and inflammation (21, 22); (c) it is a phosphatidylserine receptor and may function in a manner similar to that of Toll-like receptors that are known to regulate innate immu-
Figure 5
CKD phenotype in Kim1RECtg. (A) Focal fibrotic changes at 4 weeks in Kim1RECtg that become progressively more severe with time. Scale bar: 50 μm. (B) AP expression identifies cells that have undergone Cre-mediated recombination. No AP-positive cells were found in fibrotic interstitium. Scale bar: 50 μm. (C) Concentric left ventricular hypertrophy in aged Kim1RECtg, trichrome stain. (D) Ventricular wall ratio (outer to inner diameter) was increased in aged (range, 12–45 weeks) Kim1RECtg (n = 5 for each group). *P = 0.03. (E) Younger Kim1RECtg (n = 3) do not have hypertension, but older Kim1RECtg (n = 5) do develop hypertension. *P = 0.0002. (F) Hematocrit in control (n = 10) or Kim1RECtg (n = 5) mice between 6 and 10 weeks or control (n = 13) and Kim1RECtg (n = 7) mice measured between 10 and 20 weeks of age. *P = 0.001; **P = 0.0001. (G) Total urinary protein is elevated in 8-week-old Kim1RECtg (n = 3–5) but not at 2 or 4 weeks compared with littermate controls (n = 4–6). *P = 0.01. (H) Urinary protein is not elevated in mice with expression of KIM-1 in podocytes alone at either 2 or 4 weeks. (I) Serum creatinine 13- to 20-week-old mice comparing control (n = 12) and Kim1RECtg (n = 8), control (n = 6) and Six2-GC;Z/AP (n = 7); or control (n = 4) and Podocin-Cre;Z/Kim1-AP (n = 4). *P = 0.006, NS. (J) Anemia was seen in Kim1RECtg but not control mice or mice in which KIM-1 was expressed in podocytes. Control refers to mice with neither transgene or 1 transgene for all groups. *P < 0.0001, NS.
nity (15); and (d) blockade of KIM-1 by monoclonal antibodies in other inflammatory conditions reduces disease pathology (37–40). The progressive kidney inflammation and fibrosis observed in Kim1RECtg mice are consistent with this hypothesis and, combined with the extrarenal manifestations described here, establish the Kim1RECtg mouse as what we believe to be a novel rodent model of progressive CKD and implicate the KIM-1 protein as a target for antifibrotic therapy in man (26).

The lower nephron endowment in our Kim1RECtg mouse model most likely reflects activation of KIM-1 expression in metanephric mesenchyme with effects on kidney development. Low nephron number is a recognized risk factor for the development of hypertension and possibly CKD (41). However, reduction in nephron number by 28%–40% results in no, or very mild, interstitial fibrosis, even at late time points of greater than 1 year in a number of models (42–45). In contrast, Kim1RECtg mice described here had a comparable degree of nephron reduction (43%), but all developed spontaneous fibrosis starting at 4 weeks of age, with progressive renal insufficiency, proteinuria, renal fibrosis, and death at a median age of 19 weeks. Further arguing against a direct role of reduced nephron endowment in the observed phenotype, hypertension, proteinuria, and cardiac hypertrophy did not develop until well after the onset of renal fibrosis, and directed expression of the Z/Kim1-AP transgene in podocytes alone had no phenotype.

The primary finding of the current report is that chronic KIM-1 expression in renal epithelial cells directly causes interstitial inflammation followed by progressive fibrotic renal disease. Our data suggest that epithelial cells that express KIM-1 chronically may act in a paracrine fashion to recruit mononuclear cells to the renal interstitium, setting up a proinflammatory cascade, ultimately leading to further tubule damage and loss of renal function. We observed an early influx of leukocytes in the Kim1RECtg mouse model associated with early elevation in the proinflammatory cytokine MCP-1, a potent cytokine that mediates mononuclear cell recruitment and parenchymal cell activation. In contrast, in the Kim1homo mouse model, in which KIM-1–induced phagocytosis is impaired, we observed reduced levels of MCP-1 and a reduction in renal fibrosis. Finally, our observation that stable expression of KIM-1 in proximal tubule cells in vitro triggers MCP-1 release and macrophage chemotaxis is consistent with a model in which chronic epithelial KIM-1 expression causes epithelial MCP-1 release, triggering leukocyte influx and ultimately kidney fibrosis. The results provided do not offer proof that MCP-1 is most important in triggering leukocyte influx, though an important role for MCP-1 is supported by the observation that mice deficient in the major MCP-1 receptor, CCR2, are protected from renal fibrosis (46).

The recent finding that soluble KIM-1 ectodomain serves as a ligand for the LMIR5/CD300b-activating receptor on resident macrophages provides indirect support for a model in which KIM-1 regulates inflammation (32); however, it is unclear whether soluble KIM-1 is required in our model, since we observed proinflammatory cytokine secretion from epithelia themselves. Similarly, further investigation is required to determine whether ligation of KIM-1 by apoptotic bodies, oxidized lipid, or some other unidentified KIM-1 ligand is required for proinflammatory signaling, analogous to the requirement of Toll-like receptor ligands for induction of inflammatory responses in macrophages (47).

The stimulus for sustained KIM-1 expression after AKI or during CKD requires further investigation. The most likely explanation is that AKI itself causes peritubular capillary rarefaction (8), leading to chronic tubular hypoxia, which is a potent stimulus for KIM-1 expression (12). Chronic KIM-1 expression will promote further tubulointerstitial inflammation, capillary loss, and hypoxia, further inducing KIM-1 and creating a positive feedback loop of hypoxia and inflammation that culminate in tubulointerstitial fibrosis.

Our study suggests what we believe to be a novel role for chronic KIM-1 expression in the pathogenesis of renal fibrosis and through activation of the innate immune system and leukocyte recruitment. In contrast, very early induction of KIM-1 after AKI may serve an adaptive function to clear apoptotic and necrotic cells and debris and thereby decrease the early response of the immune system at a site of tissue damage. Persistent KIM-1 expression is perhaps maladaptive through chronic uptake of cell toxic components of the tubular lumen, thereby promoting chronic inflammation and ultimately renal fibrosis. Thus KIM-1 may represent a novel therapeutic target in fibrotic kidney disease, and antagonizing KIM-1 signaling might ameliorate renal fibrosis in CKDs.

Methods

Mouse strains. A KIM-1 cDNA was inserted into the NotI site of the Z/AP plasmid (48), and linearized Z/Kim1-AP transgene was introduced into FVB zygotes (Charles River Laboratories) by pronuclear injection. Three independent founder lines were obtained; all exhibited Six2-GC–dependent transgene expression, and the line with highest outer medulla and cortex transgene expression was selected for further analysis. The Z/Kim1-AP transgenic was maintained on an FVB × C57BL/6J (Jackson Laboratory) mixed background.

Z/Kim1-AP transgenic mice were crossed with the Six2-GC Cre driver line (27) maintained on a CD-1 × Swiss Webster (Taconic) × C57BL/6J (Jackson Laboratory) mixed background. In other experiments, the Z/Kim1-AP

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<th>Table 4</th>
<th>Echocardiography in Kim1RECtg mice at 2.5–3 months</th>
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<tbody>
<tr>
<td></td>
<td>Control (n = 7)</td>
</tr>
<tr>
<td>Intervertricular septal thickness (mm)</td>
<td>0.69 ± 0.06</td>
</tr>
<tr>
<td>Left ventricular end-diastolic diameter (mm)</td>
<td>3.40 ± 0.33</td>
</tr>
<tr>
<td>Left ventricular posterior wall thickness (mm)</td>
<td>0.76 ± 0.05</td>
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<tr>
<td>Fractional shortening (%)</td>
<td>38.73 ± 3.55</td>
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\*P < 0.05.

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<tr>
<th>Table 5</th>
<th>Biochemical parameters in Kim1RECtg mice</th>
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<tr>
<td></td>
<td>Control (n = 10)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>23.4 ± 1.1</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Na (mM)</td>
<td>149 ± 0.9</td>
</tr>
<tr>
<td>K (mM)</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Cl (mM)</td>
<td>104.3 ± 0.7</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.2 ± 0.1</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>7.8 ± 0.3</td>
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\*P < 0.05.
transgenic mice were crossed with the Podocin-Cre driver line (49). The Z/AP reporter line was from JAX and was maintained on a 129/BL6 background. The Kim1REC gene has been described (34). The Z/AP and Z/Kim1-AP mice were genotyped using β-Geo—specific primers. The Six2-GC allele was genotyped as described (29). The Podocin-Cre allele was genotyped using Cre primers. In all cases, littermate control phenotypes were compared. Littermate controls were used for all mouse experiments.

**Induction of renal fibrosis by UUO.** Male BALB/c mice aged 8 to 10 weeks weighing 20–22 g were purchased from Charles River Laboratories. Mice were anesthetized and the left kidney exposed by flank incision. The ureter was ligated at 2 points proximal to the kidney with 6-0 silk. Sham animals had kidney exposed, but ureter was not tied.

**Tissue preparation and histology.** Mice were anesthetized, sacrificed, and immediately perfused via the left ventricle with ice-cold PBS for 2 minutes. Kidneys were semi-sectioned, and portions were snap frozen in liquid nitrogen. Other kidneys were fixed in 10% neutral buffered formalin at 4°C for 12 hours, processed, embedded in paraffin wax, sectioned, and stained with PAS using standard procedures. Some kidneys were prepared for electron microscopy by glutaraldehyde fixation. Other kidneys were fixed in 4% PLP fixative (4% paraformaldehyde, 75 mM L-lysine, 10 mM sodium periodate) for 2 hours at 4°C, cryoprotected in 30% sucrose and fixed in PLP for 2 hours and was carried out for 12–24 hours at 37°C; then

Immunofluorescence and immunohistochemical staining. Cryosections of 7-μm were mounted on Fisher Superfrost Plus (Fisher) microscope slides, air dried, and treated for immunofluorescence as described (50). Primary antibodies against the following proteins were used: KIM-1 was detected with rabbit polyclonal anti-peptide antibody R9 exactly as described for both immunofluorescence and Western analysis (16); F4/80 (rat, 1:100, cat. no. 6640; Abcam); SMA (mouse FITC coupled, 1:200, cat. no. F3777, Sigma-Aldrich); DBA (lectin, 1:500, cat. no. L-1030; Vector Laboratories); Ki67 (rabbit monoclonal, 1:1000, cat. no. VP-RM04; Vector Laboratories); CD3 (rabbit monoclonal, 1:500 cat. no. VP-RM01; Vector Laboratories); and neutrophil (rat monoclonal, 1:1000, cat. no. sc-71674; Santa Cruz Biotechnology Inc.). Secondary antibodies were obtained from Dako. Sections were mounted in Vectashield containing 4,6-diamino-2-phenylindole (Vector Labs). Images were taken with a Nikon TE2000 microscope CoolSnap camera (Roper Scientific) and processed using IP Lab Software (BD Biosciences). Immunofluorescence images were obtained on a Nikon TE2000 or a Nikon C1 D-Eclipse confocal microscope using standard procedures.

Immunohistochemical stains (αSMA, CD3, neutrophil, KIM-1) were performed on formalin-fixed, paraffin-embedded 4-μm sections. Sections were rehydrated and antigens retrieved using heated citrate. Staining was visualized using horseradish peroxidase–coupled secondary antibodies (Vectastain Elite; Vector Labs). LacZ activity was measured by standard X-gal staining protocol on 7-μm frozen kidney sections that had been fixed in PLP for 2 hours and was carried out for 12–24 hours at 37°C; then
sections were counterstained with eosin (Sigma-Aldrich) and mounted. PAS, H&E, and Masson’s trichrome stains were performed using standard techniques. AP activity was measured using NBT/BCPIP, with a standard protocol that included a 30-minute incubation at 60°C to inactivate endogenous AP activity.

**Histologic analysis.** Histological analysis was performed on paraffin-embedded and serially cut kidney sections (3 μm) stained with H&E, PAS, and Masson’s trichrome. Analysis of tubules included the evaluation of epithelial histology. The degree of injury was scored semiquantitatively on a 0 to 4 scale for reabsorption granules, vacuolization, and epithelial degeneration as follows: 0, no lesion; 1, minimal (minor focal changes); 2, mild; 3, moderate; 4, severe. The distribution was graded as focal if 49% or less and diffuse if 50% or more of the tubule showed these changes. Semiquantitative analysis of glomeruli included glomerular histology as well as foot process morphology assessed by electron microscopy and was graded as follows: minimal: 1 (involving < 5% of glomerulus); mild: 2 (5%–24%); moderate: 3 (25%–49%); and severe: 4 (≥ 50%). For assessment of glomerular involvement, an average of 80–120 glomeruli per section were examined on multiple levels. All scoring was done in a blinded manner by an experienced renal pathologist. Semiquantitative analysis of tubular morphology in Kim1Δmuc mice was also performed in a blinded fashion exactly as described (51).

**Electron microscopy.** Portions of kidneys were fixed in Karnovsky’s fixative and processed for electron microscopic studies by standard procedures. Semi-thin sections of each block were stained with toluidine blue sections were counterstained with eosin (Sigma-Aldrich) and mounted. PAS, H&E, and Masson’s trichrome stains were performed using standard techniques. AP activity was measured using NBT/BCPIP, with a standard protocol that included a 30-minute incubation at 60°C to inactivate endogenous AP activity.

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transcribed with the M-MLV reverse transcriptase kit and Oligo dT primers (Promega). Real-time PCR was performed by TaqMan gene expression assays (Applied Biosystems) for detection of mRNA expression using GAPDH as the internal control. Qualitative RT-PCR was performed on 1/200th of the RT product using the following primer pairs (from 5′ to 3′):

Cre forward: TTCCCGCAGAACCTGAAGATG, reverse: CCCCAGAAATGCCAGAT-TACG; KIM-1 forward: ATGAATCAGATTCAAGTCTTC, reverse: TCTGGTTGTAGTCCATGTG; GAPDH forward: TGGAGAAACCTGCCAAGTA, reverse: AAGAGTGGGAGTTGCTGTTG. The cycling conditions were as follows: Cre: melting temperature (Tm) 57°C and 35 cycles, KIM-1: Tm 55°C and 30 cycles; GAPDH: Tm 53°C and 25 cycles. PCR products were visualized on ethidium bromide containing, 2% agarose gels and photographed.

Quantitative PCR was performed using a Bio-Rad iCycler and the following primers (from 5′ to 3′):

Col-1\(\alpha_1\) forward: TGACTGGAAGAGCGGAGAGT, reverse: GTTCGGGCTGATGTACCAGT; CXCL1

Figure 8
The functional mutant K\(\text{im1}^{-}\text{muc}\) is protected from kidney fibrosis. (A) Control and K\(\text{im1}^{-}\text{muc}\) mice were subjected to UUO and sacrificed at day 2. Kidney lysates reveal KIM-1 protein at 75 kDa in the control, but at 55 kDa in the K\(\text{im1}^{-}\text{muc}\), corresponding to the deletion of exon 3 (arrows). The arrowhead identifies a nonspecific Ig band. Proliferating cell nuclear antigen (PCNA) staining reflects increased cell proliferation after UUO. (B) Control or K\(\text{im1}^{-}\text{muc}\) kidney sections before or after UUO. There is reduced interstitial collagen in K\(\text{im1}^{-}\text{muc}\) reflected by Masson's trichrome stain, and reduced tubular injury (PAS). (C and D) Quantification of tubular atrophy and fibrosis index, respectively (\(n = 5\) kidneys each condition). (E–G) qPCR of kidney cortex fibrosis, presented as fold increase of K\(\text{im1REC}^{-}\text{tg}\) (\(n = 5\)) compared with control (\(n = 5\)) at 10 days after UUO. *\(P < 0.05\). (H) Reduced MCP-1 mRNA by qPCR in K\(\text{im1}^{-}\text{muc}\) compared with control (\(n = 5\)). *\(P < 0.05\).

Western blot analysis. Kidney tissues were lysed, and lysates were prepared as previously described (50). Membranes were incubated with 1 or more of the following primary antibodies: rabbit antibody to LacZ (1 in 5,000; Cappel), chicken antibody to GFP (1 in 1,000; AVES), rabbit antibody to KIM-1 (1 in 250) (12), rabbit anti-fibronectin (1 in 1,000; Abcam), proliferating nuclear cell antigen (1 in 1,000; Abcam) and ERK (1 in 1,000; Cell Signaling). Horseradish peroxidase–conjugated secondary antibodies were applied, and enhanced chemiluminescence (Amersham Biosciences) was used to detect proteins.

Quantification of mRNA by reverse transcription PCR. Total RNA was isolated from snap-frozen kidneys with RNeasy columns (QIAGEN). Five micrograms of total RNA was treated with DNase I (Invitrogen) and reverse transcribed with the M-MLV reverse transcriptase kit and Oligo dT primers (Promega). Real-time PCR was performed by TaqMan gene expression assays (Applied Biosystems) for detection of mRNA expression using GAPDH as the internal control. Qualitative RT-PCR was performed on 1/200th of the RT product using the following primer pairs (from 5′ to 3′): Cre forward: TTCCCGCAGAACCTGAAGATG, reverse: CCCCAGAAATGCCAGAT-TACG; KIM-1 forward: ATGAATCAGATTCAAGTCTTC, reverse: TCTGGTTGTAGTCCATGTG; GAPDH forward: TGGAGAAACCTGCCAAGTA, reverse: AAGAGTGGGAGTTGCTGTTG. The cycling conditions were as follows: Cre: melting temperature (Tm) 57°C and 35 cycles, KIM-1: Tm 55°C and 30 cycles; GAPDH: Tm 53°C and 25 cycles. PCR products were visualized on ethidium bromide containing, 2% agarose gels and photographed.

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Figure 8
The functional mutant K\(\text{im1}^{-}\text{muc}\) is protected from kidney fibrosis. (A) Control and K\(\text{im1}^{-}\text{muc}\) mice were subjected to UUO and sacrificed at day 2. Kidney lysates reveal KIM-1 protein at 75 kDa in the control, but at 55 kDa in the K\(\text{im1}^{-}\text{muc}\), corresponding to the deletion of exon 3 (arrows). The arrowhead identifies a nonspecific Ig band. Proliferating cell nuclear antigen (PCNA) staining reflects increased cell proliferation after UUO. (B) Control or K\(\text{im1}^{-}\text{muc}\) kidney sections before or after UUO. There is reduced interstitial collagen in K\(\text{im1}^{-}\text{muc}\) reflected by Masson's trichrome stain, and reduced tubular injury (PAS). (C and D) Quantification of tubular atrophy and fibrosis index, respectively (\(n = 5\) kidneys each condition). (E–G) qPCR of kidney cortex fibrosis, presented as fold increase of K\(\text{im1REC}^{-}\text{tg}\) (\(n = 5\)) compared with control (\(n = 5\)) at 10 days after UUO. *\(P < 0.05\). (H) Reduced MCP-1 mRNA by qPCR in K\(\text{im1}^{-}\text{muc}\) compared with control (\(n = 5\)). *\(P < 0.05\).
forward: CTGGGATTCCTCAGAAGCCTC, reverse: CAGGGT- CAAGCCAAGGCGTCTG; CCLX2 forward: CCACACCGAAGCTGCTC, reverse: GGCTCAGTGAAGCCTTG; CCLX10 forward: CGAATGTGCTGCTGCTGCTG; GAPDH forward: CTTTGCCAGATGTTTCTC, reverse: GGCTC- GCCACCCCTTTGCT; IL-1β forward: CTTGGCCATTTTCCGAACTGG; reverse: GAGAAAGGGTTTCTTCAGGTT; TGF-β forward: GCCAAATTCTCTGGCTTCC; reverse: CAGGAGCCGCTATTTGTA; TNF-α forward: GCTCGCAGCAGTCTCTTC; reverse: CATCTC- CAGAGTCCACGACA; fibronectin forward: ATGGCAGCCCTCCT- GTAAGT; reverse: GGCCAGGTATTCGACAGG.

**Physiologic measurements.** Serum creatinine was measured using a Beckman Creatinine Analyzer 2 by the Jaffe rate method (total protein kit; Sigma-Aldrich) or by the CO2. To create stable cell lines expressing full-length KIM-1 (KIM-1–LLC), the human monomyelocytic cell line, was cultured in RPMI 1640 medium DMEM/F12 medium with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) removed from 20- to 25-g BALB/c mice. BM was isolated from these by standard sterile techniques and matured for 7 days in unspaced Petri dishes using DMEM/F12 medium with 10% FCS, penicillin (100 μg/ml), and streptomycin (100 mg/ml) and conditioned with M-CSF from L929. The U937 cell line, a human monomyelocytic cell line, was cultured in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 2 mM L-glutamine. Cells were subcultured 3 times a week and maintained at a concentration of 0.5–1.0 × 10^6 cells/ml. Monocytic differentiation of U937 cells was achieved by adding 10 nM PMA for 48 hours. PMA-differentiated U937 was washed 3 times by sterile PBS before experiments.

**Cytokine measurement.** Both KIM-1–LLC and pcDNA-LLC cells were grown to confluence, and supernatant was collected, centrifuged, and washed 3 times by sterile PBS before experiments. Approximately 106 cells/ml. Monocytic differentiation of U937 cells was achieved by adding 10 nM PMA for 48 hours. PMA-differentiated U937 was washed 3 times by sterile PBS before experiments.

**Cytokine measurement.** Both KIM-1–LLC and pcDNA-LLC cells were grown to confluence, and supernatant was collected, centrifuged, and stored at –80°C until further analysis. Alternatively, mouse urine was collected with a metabolic cage and processed in a similar fashion. MCP-1, IL-6, and TGF-β microbead-based assays were developed and validated in the lab. Approximately 6000 beads/50 μl were incubated with 30 μl of sample or recombinant proteins (R&D Systems) for 1 hour, washed 3 times with PBS, and incubated in corresponding biotinylated antibodies (R&D Systems) for 45 minutes on an orbital shaker at 300 rpm. Beads were washed again with PBS and incubated for 15 minutes with streptavidin–PE solution (Invitrogen). The signal from the fluorochrome, which is directly proportional to the amount of antigen bound at the micro-bead surface, was captured using the Bio-Plex 200 system (Bio-Rad). Data were generated and interpreted using parametric logistic regression analysis.

**Boyden chamber assay.** Cell chemotaxis assay was performed in a modified Boyden chamber using 24-well flat-bottom tissue plates with 5-μm polyethylene/terephthylan membrane inserts (BD). The lower compartment of each chamber was filled with 500 μl of the conditioned medium. Membrane inserts were filled with 300 μl of cell suspension and placed in the pre-filled lower compartments. The chambers were then incubated for 3 hours in 37°C, 5% CO2. After incubation, nonmigrated cells in the upper wells were removed by scraping and the migrated cells were stained with eosin on the membrane. Adherent cells on the lower surface of the membrane were counted from 3 high power fields by light microscopy (×60). Data are presented as cells per high-power field. Neutralizing antibody against MCP-1 was from Sigma-Aldrich.

**Statistics.** All results are reported as mean ± SEM. All error bars on graphs represent SEM. Statistical tests are 2-tailed, unequal t tests except for survival analysis (Figure 4E), which used the log rank test, and Figures 7B and Supplemental Figure 2, which used a repeated measures t test.

**Study approval.** All animal studies were approved by the Harvard Institutional Animal Care and Use Committee.

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11. Rennert PD. Novel roles for TIM-1 in immunity..