Kidney injury molecule-1 expression in IgA nephropathy and its correlation with hypoxia and tubulointerstitial inflammation

Qiongzen Lin,1,2,3 Ying Chen,1,2,3 Jicheng Lv,1,2,3 Hong Zhang,1,2,3 Jiawei Tang,1,2,3 Lakshman Gunaratnam,4 Xiaomei Li,1,2,3 and Li Yang1,2,3

1Renal Division, Department of Medicine, Peking University First Hospital, Beijing, People’s Republic of China; 2Institute of Nephrology, Peking University, Beijing, People’s Republic of China; 3Key Laboratory of Renal Disease, Ministry of Health of China, Beijing, People’s Republic of China; and 4Department of Medicine, Department of Microbiology and Immunology, Western University, London, Ontario, Canada

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Although the clinical course of kidney disease is highly variable, many patients with chronic kidney disease (CKD) experience a progressive decline in their renal function with time and may eventually develop end-stage renal disease (ESRD). Studies on the pathogenetic mechanisms of CKD have stressed a contributory role for the tubulointerstitium in the progression of the disease, especially given the sensitivity of tubular epithelial cells to injury (14, 23). The prevailing theory is that the severity of tubulointerstitial damage rather than that of the glomerulus correlates more closely with the decline in kidney function in CKD patients (25). In CKD patients, severe tubulointerstitial damage is often associated with a rapid progression to ESRD (28). Chronic hypoxia and tubulointerstitial inflammation are common pathways leading to renal interstitial fibrosis (13, 27, 44). As the most abundant cell type in the renal cortex, tubular epithelial cells play an active role in the development of chronic tubulointerstitial hypoxia and inflammation in the progression of CKD (37).

Kidney injury molecule (KIM)-1 is a tubular protein that is induced and markedly upregulated in proximal tubular epithelial cells after ischemic kidney injury (1, 18, 34). KIM-1 is a type I transmembrane glycoprotein (17) whose extracellular domain can be shed into the tubular lumen and detected in the urine by biochemical assays (2). A previous study (16) has shown that KIM-1 is a phosphatidylserine receptor that recognizes apoptotic cells and may play an important role in limiting the autoimmune response to injury through the clearance of apoptotic debris from the tubular lumen in acute kidney injury (AKI). In recent years, KIM-1 has been largely studied as a putative biomarker for early diagnosis and a predictor of the progression of renal dysfunction in AKI (6, 39). In an ischemia-reperfusion injury mouse animal model, 24,600 genes were analyzed during the repair stage after AKI. Among them, KIM-1 (murine KIM-1 gene) was one of the most highly upregulated genes, indicating that KIM-1 may be a possible biomarker of the AKI-to-CKD transition (22). Data from various animal models of CKD and CKD patients suggest that KIM-1 is upregulated in proximal tubules and associated with its expression in renal interstitial fibrosis and inflammation (38, 39, 42). A recent study (15) using a genetic approach has shown that sustained KIM-1 expression in renal epithelial cells in mice resulted in kidney inflammation and fibrosis.

To further assess the role of KIM-1 in tubulointerstitial injury in human CKD, we studied KIM-1 expression in patients with IgA nephropathy (IgAN), one of the most common causes of CKD in Asia. Two groups of patients with similar clinical manifestation at renal biopsy but with divergent rates of disease progression during follow-up were selected. We observed that the level of tubular KIM-1 correlated positively with the decline in renal function and the inflammatory infiltrate on renal biopsy. To test whether KIM-1 could actively regulate inflammation and fibrosis in CKD, we used in vitro cell culture models to assess KIM-1 expression under long-term cellular stress conditions and studied its effects on cytokine production and inflammatory cell differentiation.

MATERIALS AND METHODS

Patients and follow-up. This study was approved by the Ethical Review Committee of Peking University First Hospital (PUFH).
Patients seen at PUFH between 2002 and 2006 with biopsy-proven IgAN were included in this study. The enrollment criteria were as follows: first, patients at CKD stages 1–2 with urine total protein between 1 and 3.5 g/24 h. According to this criterion, 203 patients were selected from a larger cohort of 638 IgAN patients at PUFH. Second, no previous treatment with corticosteroids or immunosuppressive drugs and no history of hypertension. According to this criterion, 114 patients were selected from the above 203 patients. Third, patients were between the ages of 16 and 45 yr old at renal biopsy, were followed up for 3–7 yr, and had sufficient renal biopsy tissue (minimum of 10 glomeruli) available for histological evaluation. Only 49 patients were left who met the above criteria. Finally, patients who had AKI, systemic diseases, severe infection, or any other factors that might exacerbate the progression of CKD were excluded from the study. All patients were regularly seen at PUFH at intervals of 6 mo. Baseline clinical and demographic data, including age, sex, blood pressure, protein excretion, serum creatinine, and estimated glomerular filtration rate (eGFR) were collected at the time of renal biopsy.

The progression of the disease was defined by two criteria. The first criterion used the final event as the development of irreversible ESRD requiring dialysis or transplantation (or death before dialysis), and the second criterion was based on final eGFR. For the latter, patients were considered as disease progressors if the decrease in eGFR was >50% from the time of renal biopsy. Patients who did not meet this criterion were defined as nonprogressors. Using these criteria, only 11 patients of the above 49 patients were left as progressive IgAN. Eleven nonprogressors who had baseline clinical-pathological data matched to progressive IgAN but did not reach the end point during the same follow-up time period were selected as the control group. The study selection process is shown in Fig. 1.

Histological parameters. Light microscopic evaluation of glomerular, vascular, tubular, and interstitial changes in the biopsy specimen was done using the previously described semiquantitative scoring system for IgAN (19). Six indexes, covering typical lesions found in biopsy specimen IgAN, including the extracapillary glomerular activity index, endothelial proliferation index, mesangial proliferation index, glomerular, tubular, and interstitial inflammation index, and tubulointerstitial chronicity index, were used and evaluated by one pathologist who was blinded to patient clinical information.

Briefly, the extracapillary glomerular activity index was applied to assess the severity of cellular/fibrocellular crescents. Segmental and circumferential lesions were both scored (by the percentage of glomeruli with these lesions: 0, 0%; 1, <10%; 2, 10–24%; 3, 25–49%; and 4, ≥50%). In reference to the scoring system for lupus nephritis, which emphasizes cellular crescents and necrosis, the circumferential score was weighted by a factor of two before it was added to the segmental score. The endothelial proliferation index was used to assess the severity of glomerular endothelial cell proliferation (0, no proliferation; 1, <50% of glomeruli proliferated; 2, ≥50% of glomeruli proliferated but most were segmental; and 3, ≥50% of glomeruli globally proliferated). Endothelial proliferation was defined as more than one nucleus in one glomerular capillary loop. The mesangial proliferation index was used to assess the severity of mesangial proliferative lesions: 0, no proliferation, with less than three mesangial cells per mesangial area; 1, focal mild proliferation, <50% of glomeruli with three to five mesangial cells per mesangial area; 2, diffuse mild proliferation or focal segmental prominent proliferation, the latter with more than five mesangial cells per mesangial area; and 3, diffuse global prominent proliferation. The glomerular chronicity index was used to assess the severity of lesions containing fibrous crescents and glomerular sclerosis. Global sclerosis was defined as sclerosis involving the entire glomerular tuft, whereas segmental sclerosis was defined as any other amount of the tuft involved with sclerosis. Global and segmental lesions were both scored (by the percentage of glomeruli with these lesions: 0, 0%; 1, <10%; 2, 10–24%; 3, 25–49%; and 4, ≥50%) before they were added together. The interstitial inflammation index and tubulointerstitial chronicity index were used to assess the severity of tubule-interstitial lesions according to the proportion of inflammatory cell infiltration and tubular atrophy and interstitial fibrosis (0, 0% area involved; 1, <10%; 2, 10–24%; 3, 25–49%; and 4, ≥50%).

Immunostaining of kidney sections. Immunohistochemical staining for CD3, CD68, CD34, and hypoxia-inducible factor (HIF)-1α and immunofluorescence staining for KIM-1 were performed on 2-μm paraffin sections of formaldehyde-fixed renal tissue from IgAN patients. Normal renal tissues adjacent to neoplastic areas (paraneoplastic) within nephrectomy specimens for malignancy were used as controls (3 samples). Briefly, after being dewaxed and rehydrated at room temperature, sections were incubated in H2O2 (3%) in 100% methanol for 15 min at room temperature to quench endogenous peroxidase activity. Epitopes were retrieved either in citrate buffer for CD3, CD68, CD34, and KIM-1 or in 0.25% trypsin for HIF-1α, Rabbit anti-human monoclonal antibody against CD3 (ZETA, mouse anti-human monoclonal antibody against CD68 (Invitrogen), mouse anti-human monoclonal antibody against CD34 (Zymed), HIF-1α (R&D), and KIM-1 (kindly provided to use by Prof. J. V. Bonventre, Brigham and Women’s Hospital, Harvard Medical School) were added to each section directly and incubated overnight at 4°C. Thereafter, sections were washed and incubated with horseradish peroxidase-conjugated anti-IgG/Fab polymer (polymer detection system, Zymed) or Cy3-labeled goat anti-mouse IgG antibody (1:500, Jackson) for 20 min at 37°C. After sections had been washed twice with PBS, a color reaction to peroxidase was performed in 3’,4’-diaminobenzidine solution for immunohistochemical staining. For immunofluorescence staining, sections were counterstained with 4’,6-diamidino-2-phenylindole for 20 min. PBS instead of primary antibody was used as the negative control in each group.

To evaluate the spatial relationship between KIM-1 and inflammatory cells, a double-staining immunofluorescence method was performed on 4-μm paraffin sections. After antigen retrieval with citrate buffer, sections were incubated in 1% BSA at 37°C for 1 h. Goat anti-human monoclonal antibody against CD3 (ZETA), mouse anti-human monoclonal antibody against CD68, mouse anti-human monoclonal antibody against CD34 (Zymed), HIF-1α, Rabbit anti-human monoclonal antibody against CD3 (ZETA), mouse anti-human monoclonal antibody against CD68 (Invitrogen), mouse anti-human monoclonal antibody against CD34 (Zymed), HIF-1α (R&D), and KIM-1 (kindly provided to use by Prof. J. V. Bonventre, Brigham and Women’s Hospital, Harvard Medical School) were added to each section directly and incubated overnight at 4°C. Thereafter, sections were washed and incubated with horseradish peroxidase-conjugated anti-IgG/Fab polymer (polymer detection system, Zymed) or Cy3-labeled goat anti-mouse IgG antibody (1:500, Jackson) for 20 min at 37°C. After sections had been washed twice with PBS, a color reaction to peroxidase was performed in 3’,4’-diaminobenzidine solution for immunohistochemical staining. For immunofluorescence staining, sections were counterstained with 4’,6-diamidino-2-phenylindole for 20 min. PBS instead of primary antibody was used as the negative control in each group.

Immunostaining results were evaluated using the single-blind method. For immunohistochemical staining, all nonoverlapping mi-
concentrations of CoCl2, recombinant human TNF-
mRNA was calculated using the comparative threshold cycle (Ct) GoTaq qPCR Master Mix (A6001, Promega). The relative amount of on an Applied Biosystems 750 fast real-time PCR System using transcription kit. Gene primer pairs were designed using Primer3 software template for cDNA synthesis using a Promega A3500 reverse tran-

Western blot analysis. HK-2 cells were treated with different concentrations of CoCl2, recombinant human TNF-α, BSA, and human serum albumin for 24 – 48 h. Cells were lysed in RIPA buffer containing 1% Triton X-100 and protease inhibitor cocktail. After centrifugation at 12,000 rpm for 10 min, supernatants were collected and analyzed for protein concentration with a DC protein assay kit. Total protein (30–50 µg) was diluted in sample buffer and boiled for 5 min for denaturation. Proteins in each sample were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. After being blocked with 5% dry milk in Tris-buffered saline-Tween, the membrane was probed with primary antibody against KIM-1 (kind gift of Prof. J. V. Bonventre) or the pcDNA- empty vector were kind gifts of Prof. J. V. Bonventre (Brigham and Wom-

Real-time PCR. Total RNA was isolated using the RNA simple extraction kit from Tiangen. Total RNA (500 ng) was used as a template for cDNA synthesis using a Promega A3500 reverse transcrip-
tion kit. Gene primer pairs were designed using Primer3 software and are shown in Table 1. Real-time quantitative PCR was performed on an Applied Biosystems 7500 fast real-time PCR System using GoTag qPCR Master Mix (A6001, Promega). The relative amount of mRNA was calculated using the comparative threshold cycle (Ct) method. The amplification efficiencies of the target and reference were shown to be approximately equal with a slope of log input method. The amplification efficiencies of the target and reference

<table>
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<th>Target Gene</th>
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<th>Reverse</th>
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<tbody>
<tr>
<td>Sus scrofa (pig)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_001206359</td>
<td>5'-ATCCCCATCACCATGCTTCA-3'</td>
<td>5'-GGTGATGCACATGACAAAC-3'</td>
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number of peritubular capillaries with CD34-positive staining was greatly decreased in IgAN patients with a more pronounced decrease in the progressive group than in the nonprogressive group (Fig. 2, G–I). Tissue from IgAN patients had increased HIF-1α staining in the renal tubulointerstitial region compared with normal kidney tissue. The increased staining of HIF-1α was more obvious in the progressive group than in the nonprogressive group (Fig. 2, J–L).

Correlation of KIM-1 with histological parameters and inflammatory cell infiltration. There was no detectable KIM-1 expression in control kidney tissue. IgAN patients showed extensive tubular KIM-1 expression involving almost the entire cortex, with more KIM-1-positive tubules and more strong KIM-1 expression in the progressive group than in the nonprogressive group (Fig. 3, A–C). Double immunostaining showed that KIM-1-positive tubules were surrounded by T cells (CD3 positive) and macrophages (CD68 positive). Occasionally, positive staining for macrophages was found in the dilated tubular lumen, indicating the presence of denuded epithelium (Fig. 3, D and E).

The level of KIM-1 expression in IgAN patients did not correlate with baseline serum creatinine, proteinuria, or eGFR at the time of renal biopsy. However, it positively correlated with time-averaged proteinuria ($r = 0.470$, $P < 0.05$) and negatively correlated with the slope of the annual eGFR decline ($r = -0.599$, $P < 0.01$) during follow-up. In renal specimens, the level of KIM-1 expression correlated with scores for the interstitial inflammation index ($r = 0.627$, $P < 0.01$) and tubulointerstitial chronicity index ($r = 0.636$, $P < 0.01$). However, the extracapillary glomerular activity index, endothelial proliferation index, mesangial proliferation index, and glomerular chronicity index did not correlate with the level of KIM-1 expression (Table 4).

The fraction of KIM-1-expressing tubules positively correlated with the number of infiltrated CD68-positive monocytes/macrophages ($r = 0.679$, $P < 0.01$) and CD3-positive T cells ($r = 0.673$, $P < 0.01$). However, the expression level of KIM-1 had no significant correlation with peritubular capillary density (Table 5).

Cellular stress induces KIM-1 expression in human proximal tubule epithelial cells. In vitro, KIM-1 expression increased in cultured human proximal tubule epithelial cells in response to cellular stressors, including chemically induced hypoxia (CoCl$_2$), oxidative stress (H$_2$O$_2$), and exposure to excessive proteins, but not to TNF-α-induced cell damage (Fig. 4). HIF-1α expression was greatly increased after 24 and 48 h of exposure to 150 μM CoCl$_2$, indicating that CoCl$_2$ had its expected effect. Western blot analysis revealed that KIM-1 was detectable in untreated HK-2 cells. KIM-1 expression under hypoxic conditions was slightly decreased at 24 h but then significantly increased above baseline levels, peaking at 48 h (Fig. 4A). This result indicates that prolonged CoCl$_2$ stimulation could significantly upregulate KIM-1 expression in HK-2 cells. These data were consistent with results from murine proximal tubule epithelial cells, where KIM-1 expression in hypoxic conditions was downregulated at 6 h and
then progressively increased, peaking at 48 h (41). Simulation of oxidative stress with H$_2$O$_2$ also stimulated KIM-1 expression in HK-2 cells at 24 h in a dose-dependent fashion at concentrations between 0.5 and 50 M. This effect persisted up to 48 h (Fig. 4B). Exposure to human serum albumin increased KIM-1 protein expression in a dosage-dependent manner, peaking at 48 h (Fig. 4C). BSA also induced KIM-1 expression in HK-2 cells, but with a lower dosage at 0.2 and 1.0 mg/ml. This effect also peaked at 48 h (data not shown). As shown in Fig. 4D, the proinflammatory cytokine TNF-$\alpha$ did not affect KIM-1 expression in HK-2 cells at the time points or doses we tested.

Effects of chemically simulated hypoxia on TNF-$\alpha$, IL-6, and MCP-1 gene expression in KIM-1-overexpressing LLC-PK1 cells. Since hypoxic conditions could induce KIM-1 expression in kidney epithelial cells, we examined the effect of KIM-1 overexpression on cytokine production after CoCl$_2$-induced...
hypoxia in LLC-PK1 cells overexpressing KIM-1. IL-6 and MCP-1 mRNA expression were greatly increased in KIM1-PK1 cells compared with control pcDNA-PK1 cells after 150 μM CoCl2 treatment for 24 h. However, there was no significant difference of TNF-α mRNA expression between KIM1-PK1 and pcDNA-PK1 cells after CoCl2 treatment (Fig. 5).

Table 3. Comparison of the average number of KIM-1-, CD68-, CD3-, and CD34-positive staining under ×400 fields in two groups of IgAN patients

<table>
<thead>
<tr>
<th>Variants</th>
<th>Nonprogressive Group</th>
<th>Progressive Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM-1-positive tubule fraction</td>
<td>0.04 (0.01–0.09)</td>
<td>0.14 (0.06–0.18)</td>
<td>0.001</td>
</tr>
<tr>
<td>CD68-positive cell number</td>
<td>5.82 (0.25–16.29)</td>
<td>17.09 (3.50–71.80)</td>
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<tr>
<td>CD3-positive cell number</td>
<td>5.67 (1.00–27.18)</td>
<td>32.83 (7.33–88.50)</td>
<td>0.003</td>
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<tr>
<td>CD34-positive capillary number</td>
<td>64.83 (38.17–72.00)</td>
<td>43.30 (28.18–67.57)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

All data are presented as medians (range); n = 11 patients/group. KIM-1, kidney injury molecule-1.

Effect of KIM1-PK1 conditioned medium on MCP-1 mRNA and protein expression in RAW264.7 cells. Given that macrophages play a key role in mediating kidney injury and repair, we investigated whether hypoxic tubular cells might affect their ability to potentiate inflammation. RAW264.7 cells were cultured in the conditional medium from either KIM1-PK1 or

Fig. 3. Kidney injury molecule (KIM)-1-positive tubules were surrounded by infiltrated inflammatory cells. A–C: sections stained for KIM-1 (red) in kidney sections from paraneoplastic normal renal tissue (A), kidney tissue from a nonprogressive IgAN patient (B), or kidney tissue from a progressive IgAN patient (C). More strong KIM-1 expression was observed in the IgAN progressive group than in the nonprogressive group. Double immunostaining for KIM-1 (green) and the T cell marker CD3 (red in D) and the monocyte/macrophage marker CD68 (red in E) was performed in kidney sections from progressive IgAN patients. KIM-1-positive tubules were surrounded by infiltrated T cells (D) and macrophages (E). The arrow in D indicates CD3-positive T cells (red) infiltrated in the KIM-1-positive (green) tubule epithelium. The dashed line in E shows the basement membrane of KIM-1-positive tubule. As shown in E, CD68-positive macrophages (red) infiltrated the KIM-1-positive (green) tubule and entered the tubule lumen.
KIM-1 IN TUBULOINTERSTITIAL INFLAMMATION

Table 4. Correlation of renal tubular KIM-1 expression and clinical-pathological indexes

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Spearman Correlation Coefficient</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Baseline serum creatinine</td>
<td>0.306</td>
<td>0.177</td>
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<tr>
<td>Baseline eGFR</td>
<td>−0.405</td>
<td>0.068</td>
</tr>
<tr>
<td>Baseline proteinuria</td>
<td>−0.021</td>
<td>0.930</td>
</tr>
<tr>
<td>Time-averaged MAP</td>
<td>0.276</td>
<td>0.227</td>
</tr>
<tr>
<td>Time-averaged proteinuria</td>
<td>0.470</td>
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<tr>
<td>eGFR declined rate</td>
<td>−0.399</td>
<td>0.004</td>
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<td>Pathological scores</td>
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<tr>
<td>Extracapillary glomerular activity index</td>
<td>−0.193</td>
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<tr>
<td>Endothelial proliferation index</td>
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<td>Mesangial proliferation index</td>
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<td>Glomerular chronicity index</td>
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</tr>
<tr>
<td>Interstitial inflammation index</td>
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<td>0.002</td>
</tr>
<tr>
<td>Tubulointerstitial chronicity index</td>
<td>0.636</td>
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DISCUSSION

The extreme variability in the clinical course of idiopathic IgAN is appreciated by many physicians (10). Patients in this study were biopsied at an early stage of the disease but had significantly different outcomes during the follow-up period despite having similar baseline clinical characteristics and treatment plans. For these reasons, the two cohorts were ideal for investigating the potential mechanisms of disease progression despite the limited number of participants. Recently, a cross-sectional study (39) showed that KIM-1 is upregulated in a variety of human renal diseases, including diabetic nephropathy, focal segmental glomerulosclerosis, hypertension, IgAN, membranous nephropathy, lupus nephritis, acute allograft rejection, chronic allograft nephropathy, and Wegener’s granulomatosis (39). Independent of the disease, renal KIM-1 correlated positively with baseline serum creatinine and the creatinine clearance rate but negatively with baseline eGFR. There was no correlation with baseline proteinuria. The only two studies (32, 42) conducted on patients with IgAN with long-term follow-up data showed that high urinary KIM-1 excretion positively correlated with poor kidney outcome. We found increase tubular KIM-1 expression in both our cohorts of patients with IgAN. Its expression also significantly correlated with time-averaged urinary protein excretion and eGFR decline but not with baseline eGFR, although there was some tendency toward it \( r = −0.405, P = 0.068 \). Thus, higher KIM-1 expression might be a marker for worse proteinuria and a faster decline of renal function.

Various mechanisms have been proposed as the triggers of KIM-1 induction after ischemia or renal toxicity. First, it has been reported that sustained proteinuria is an independent risk factor in many different kinds of kidney diseases, especially in diabetic nephropathy and IgAN (7, 29). KIM-1 protein expression has been found to be markedly induced in the damaged tubules of uninephrectomized rats with renal injury caused by protein overload (38). Consistent with this in vivo study, we found that exposure of human proximal tubule cells (HK-2 cells) to human serum albumin or BSA increased KIM-1 expression in a dose-dependent manner, peaking at 48 h. Protein overload may lead to a tubular misbalance in energy expenditure and availability due to increased cellular lysosome processing, affecting O2 demand (33). Thus, a relative lack of O2 might also be involved in KIM-1 induction. Under chemically induced hypoxia, KIM-1 expression was downregulated at 24 h and then progressively increased, peaking at 48 h. The upregulation of KIM-1 expression after chemically induced hypoxia was more pronounced compared with albumin and \( \text{H}_2\text{O}_2 \) treatment of HK-2 cells. However, the stimulation of inflammation with TNF-\( \alpha \) had no significant effect on KIM-1 expression in HK-2 cells. Thus, we speculate that chronic tubular interstitial hypoxia might be the major simulating factor for the upregulation of KIM-1 expression in CKD. We also found that the increased KIM-1 expression in the IgAN progressive group was most pronounced at the site of reduced capillary density around the renal tubule, perhaps suggesting a relationship between hypoxia and KIM-1 expression.

In recent years, collective data from the kidney injury literature have demonstrated that tubular epithelial cells can drive inflammatory by producing a wide variety of inflammatory mediators that attract inflammatory cells to their vicinity (8, 11, 24). The existing notion is that this sets the stage for a positive feedback loop of activation between proximal tubular epithelial cells and infiltrated inflammatory cells, leading to an overproduction of extracellular matrix components, resulting in fibrosis and, ultimately, loss of kidney function (21, 35, 36). Although the exact function of KIM-1 in this context is still unclear, our in vitro data showed that KIM-1-overexpressing LLC-PK1 cells expressed more chemokines/cytokines, such as IL-6 and MCP-1, under chemically induced hypoxia conditions. MCP-1 is a powerful chemotactic factor for monocytes/macrophages (43). Studies (3, 9, 20, 26, 31, 43) of human and animal models of kidney diseases have shown that MCP-1 levels in renal tissues closely correlate with the number of infiltrated monocytes/macrophages. Renal tubular epithelial cells are one of the important sources of MCP-1 production in the kidney (30). MCP-1 acts as a potent monocyte chemotactic factor that not only causes the migration and aggregation of inflammatory cells but also changes their functional status. In
Fig. 4. KIM-1 expression in response to different cellular stress stimulations in HK-2 cells. A, left: 150 μM CoCl2 was used to stimulate HK-2 cells for 24 and 48 h. HIF-1α and KIM-1 expression were detected by Western blot analysis. Right, quantitative results of 24- and 48-h treatment. B, left: increasing concentrations of H2O2 (0.5, 5, and 50 μM) were used to treat HK-2 cells for 24 h. KIM-1 expression was detected by Western blot analysis. H2O2 (0.5 μM) was then used to treat HK-2 cells for 24 and 48 h. Right, quantitative results of 24- and 48-h treatment. C, left: increasing concentrations of human serum albumin (HSA; 0, 0.2, 1, 5, and 10 mg/ml) were used to treat HK-2 cells for 24 and 48 h. KIM-1 expression was detected by Western blot analysis. Right, quantitative results of 24- and 48-h treatment. D, left: increasing concentrations of TNF-α (5, 10, 20, and 40, ng/ml) were used to treat HK-2 cells for 24 h. TNF-α (10 ng/ml) was then used to treat HK-2 cells for 24 and 48 h. KIM-1 expression was detected by Western blot analysis. Right, quantitative results of 24- and 48-h treatment. In all experiments, GAPDH was used as a loading control. KIM-1 expression was normalized to the control condition (Con). All quantitative data are from the results of three experiments. *P < 0.05 was accepted as statistically significant.
CoCl2 treatment for 24 h. The gene expression level at the control condition overexpressing KIM1-PK1 cells and control pcDNA-PK1 cells after 150 chemokine protein (MCP)-1 mRNA expression were detected in KIM-1-overexpressing KIM1-PK1 cells and control pcDNA-PK1 cells after 150 μM CoCl2 treatment for 24 h. The gene expression level at the control condition without CoCl2 treatment was set as reference number 1. The gene expression level under CoCl2-treated conditions was normalized to the control condition. *p < 0.05, comparison of pcDNA-PK1 cells or KIM1-PK1 cells with or without CoCl2 treatment; #p < 0.05, comparison between pcDNA-PK1 and KIM1-PK1 cells after CoCl2 treatment.

Fig. 5. Hypoxia stimulates inflammatory cytokine production in KIM-1-overexpressing cells (n = 5 experiments). TNF-α (TNFA), IL-6, and monocyte chemotactic protein (MCP)-1 mRNA expression were detected in KIM-1-overexpressing KIM1-PK1 cells and control pcDNA-PK1 cells after 150 μM CoCl2 treatment for 24 h. The gene expression level at the control condition without CoCl2 treatment was set as reference number 1. The gene expression level under CoCl2-treated conditions was normalized to the control condition. *p < 0.05, comparison of pcDNA-PK1 cells or KIM1-PK1 cells with or without CoCl2 treatment; #p < 0.05, comparison between pcDNA-PK1 and KIM1-PK1 cells after CoCl2 treatment.

In vitro studies (4, 5) have shown that MCP-1 can stimulate monocytes/macrophages to secrete more nitric oxide, TNF-α, and IL-1 and exhibit proinflammatory effects. In addition, MCP-1 can stimulate renal tubular epithelial cells, mesangial cells, and smooth muscle cells to secrete IL-6 and ICAM-1 and promote renal inflammation and vascular diseases (12, 40).

Our data showed that “conditioned” supernatants from KIM1-PK1 cells stimulated the production of MCP-1 in macrophages, providing experimental evidence to suggest a positive feedback loop between epithelial cells and inflammatory cells, where KIM-1 expression may play an important role in regulating the immune response of infiltrated inflammatory cells in the tubulointerstitial area. Consistent with in vitro data, in IgAN patient renal biopsy specimens, tubular KIM-1 was predominantly expressed in areas with tubulointerstitial damage, characterized by the presence of T cells (CD3 positive) and macrophages (CD68 positive), tubular dilatation, degeneration of peritubular capillaries, and upregulation of the hypoxia marker HIF-1α. KIM-1 displayed a heterogeneous pattern of expression with localization primarily to the apical part of the cell and occasionally in the cytoplasm within one tubular cross section. The level of KIM-1 expression also positively correlated with the number of infiltrated macrophage and T cells, perhaps indicating the active involvement of KIM-1 in attracting inflammatory cells to the region. Based on the results of the present study, we suggest that KIM-1 expression during CKD is not just in response to tubular injury but also plays an active role in the progression of CKD by amplifying the proinflammatory effect of sustained hypoxia on tubular cells.

The major limitation and strength of the present study is the small sample size. While the study sample size limited the wide applicability of the results, the strict enrollment criteria allowed us to avoid multiple confounding factors, including initial clinical manifestations and subsequent interventions and treatments that may have affected the validity of the results. Therefore, this study population was invaluable for studying the influencing factors and related mechanism of IgAN progression.

In conclusion, KIM-1 protein expression is markedly induced in IgAN. Higher KIM-1 expression is associated with faster disease progression. Patients with progressive IgAN exhibited stronger KIM-1 staining in individual tubules and more KIM-1-positive tubules compared with nonprogressors. Based on its colocalization with infiltrated inflammatory cells, we hypothesize that KIM-1 might drive tubulointerstitial damage. Our in vitro data indicate that several stimuli can induce KIM-1 expression, with chronic hypoxia being the major factor. Although our data reveal that prolonged hypoxia can significantly induce the upregulation of peritubular capillaries, and upregulation of the hypoxia marker HIF-1α; KIM-1 displayed a heterogeneous pattern of expression with localization primarily to the apical part of the cell and occasionally in the cytoplasm within one tubular cross section. The level of KIM-1 expression also positively correlated with the number of infiltrated macrophage and T cells, perhaps indicating the active involvement of KIM-1 in attracting inflammatory cells to the region. Based on the results of the present study, we suggest that KIM-1 expression during CKD is not just in response to tubular injury but also plays an active role in the progression of CKD by amplifying the proinflammatory effect of sustained hypoxia on tubular cells.

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ulation of KIM-1 expression in renal proximal tubular epithelial cells, leading to the secretion of chemoattractants and activation of monocytes/macrophages, the exact role of KIM-1 during chronic tubulointerstitial injury needs to be further investigated.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: Q.L. and J.T. performed experiments; Q.L. analyzed data; Q.L., Y.C., and L.Y. interpreted results of experiments; Q.L., Y.C., J.L., H.Z., L.G., X.L., and L.Y. edited and revised manuscript; Y.C. prepared figures; Y.C. and L.Y. drafted manuscript; L.Y. conception and design of research; L.Y. approved final version of manuscript.

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