Reduction of proteinuria in adriamycin-induced nephropathy is associated with reduction of renal kidney injury molecule (Kim-1) over time

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Kramer AB, van Timmeren MM, Schuurs TA, Vaidya VS, Bonventre JV, van Goor H, Navis G. Reduction of proteinuria in adriamycin-induced nephropathy is associated with reduction of renal kidney injury molecule (Kim-1) over time. Am J Physiol Renal Physiol 296: F1136–F1145, 2009. First published February 25, 2009; doi:10.1152/ajprenal.00541.2007.—Tubulointerstitial lesions are important in the progression of proteinuric renal disease. Tubular kidney injury molecule-1 (Kim-1) is induced in acute renal injury and reversible as a natural course. Kim-1 is also present in chronic renal damage; however, the dynamics of Kim-1 in chronic renal damage and effects of antiproteinuric treatment on Kim-1 are unknown. We studied Kim-1 in adriamycin nephrosis (AN) before and after renin-angiotensin system blockade. A renal biopsy was taken 6 wk after adriamycin injection to study renal damage and Kim-1 expression. Subsequently, ACE inhibition (ACEi; n = 23), angiotensin II antagonist (AT1A; n = 23), or vehicle (n = 10) was given for 6 wk; healthy rats served as controls (CON; n = 8). In AN, renal Kim-1 mRNA was induced 26-fold vs. CON at week 6, with further increase in vehicle to week 12 (40-fold) but was reduced by ACEi and AT1A to 10- and 12-fold vs. CON (P < 0.05 vs. week 6). Kim-1 protein was undetectable in CON; in AN, it was present in brush border of dilated tubules in areas with adjacent interstitial lesions. Renal Kim-1 protein levels increased from weeks 6–12 in vehicle and decreased in ACEi- and AT1A-treated groups (P < 0.05). In vehicle, urinary Kim-1 was increased (P < 0.05 vs. CON), with a reduction by ACEi and AT1A (P < 0.05 vs. vehicle). Renal and urinary Kim-1 correlated with proteinuria and interstitial damage cross-sectionally. Reductions in proteinuria and renal Kim-1 correlated, which was not associated by corresponding changes in tubulointerstitial fibrosis. In conclusion, on longitudinal follow-up during antiproteinuric treatment increased renal Kim-1 expression is reversible in proportion to proteinuria reduction, likely reflecting reversibility of early tubular injury, supporting its potential as a biomarker for tubulointerstitial processes of damage and repair.

ACE inhibitors; chronic kidney disease; fibrosis; proximal tubule; proteinuria

CHRONIC PROTEINURIA ELICITS tubulointerstitial damage, which plays an important role in proteinuria-induced progressive renal function loss. The severity of tubulointerstitial lesions can predict the clinical course (12, 29, 38, 39) as well as the efficacy of antiproteinuric treatment (20, 23) in experimental and clinical renal conditions. Intervention in the renin-angiotensin system (RAS) provides renoprotection through effects on blood pressure and proteinuria. (17, 27). In particular, the antiproteinuric effects are involved in protection against progressive tubulointerstitial injury (26, 28).

Kidney injury molecule-1 (Kim-1), a type 1 membrane protein that is expressed at negligible levels in normal rat kidneys, is massively induced in tubules after ischemic or toxic injury in rats (14, 15), with resolution during the restoration of structural and functional integrity (14). After the initial observations in acute renal injury, it has become increasingly clear that Kim-1 is also expressed in chronic renal conditions. This has been established in experimental models such as protein-overload nephropathy (33), adriamycin-induced nephropathy (21), angiotensin II-induced renal damage in homozygous Ren2 rats (9), and murine polycystic kidney disease (22). In renal patients, Kim-1 is upregulated in a variety of conditions, including chronic proteinuric kidney disease and acute and chronic renal transplant dysfunction (35, 40). Kim-1 is localized to proximal tubular cells, and, in acute renal injury, particularly expressed in cells with characteristics of injury and regeneration, such as loss of brush border, a flat cell structure, and luminal debris (14, 15, 22). In chronic renal disease, Kim-1 is also mainly expressed in dedifferentiated proximal tubuli, in areas of fibrosis and inflammation (35). The Kim-1 protein has a short cytoplasmic domain and extracellular Ig and mucin domains (14). The functional significance of renal Kim-1 expression during conditions of renal injury has not been established as yet, but recent findings by Ichimura et al. (13) in acute renal injury demonstrate a main role for Kim-1 role in phagocytosis of apoptotic cells. These findings corroborate earlier studies showing that Kim-1 is a functional phosphatidyserine receptor that can induce phagocytosis of apoptotic cells (18). Moreover, Kim-1 was also shown to have properties of a type B macrophage scavenger receptor and to bind oxidized LDL (13). These recent data suggest a functional role of Kim-1 in the repair capacity of the kidney.

Interestingly, the ectodomain of Kim-1 can be shed into urine after cleavage by a matrix metalloproteinase (1), regulated by MAP kinase signaling pathways in response to cell stress (41). Urinary Kim-1 levels are associated with Kim-1 protein expression in injured nephrons in experimental and clinical renal disease (15, 33, 35). Moreover, urinary Kim-1 levels were associated with a worse renal prognosis at long term follow-up after renal transplantation (34). Together, these findings fueled interest in the potential of urinary Kim-1 as a noninvasive biomarker for processes of tubular injury associated with tubulointerstitial damage (5, 24). This would be of interest for several renal conditions, including proteinuric renal disease where tubulointerstitial injury is a main determinant of outcome (24). Recent data from our group in patients with...
nephrotic range proteinuria showed that nonpharmacological and pharmacological reduction in proteinuria were accompanied by a reduction in urinary KIM-1 (36). However, whether proteinuria reduction is associated with a reduction in renal Kim-1 expression, and corresponding tubulointerstitial damage, has not been established.

Therefore, we studied renal and urinary Kim-1 in adriamycin-induced nephropathy, a rat model of chronic proteinuria-induced renal damage, before and after antiproteinuric intervention with RAS blockade. By taking a renal biopsy during the phase of established proteinuria before renoprotective intervention, we could obtain longitudinal within-individual data on renal Kim-1. Parameters of interstitial inflammation and fibrosis were measured to establish a possible association with Kim-1 expression and its changes over time.

**METHODS**

**Animals.** Sixty-four male Wistar rats (HsdCpb-Wu; Harlan, Zeist, The Netherlands), weighing 336 ± 17 g at disease induction, were studied. Rats were housed in a temperature-controlled room with a 12:12-h light-dark cycle and free access to food and water. Urine was collected over a 24-h period each week in metabolic cages and stored at −20°C.

All surgical procedures took place under isoflurane anesthesia in N2O/oxygen (1:2). Adriamycin nephrosis (AN) was induced by injection of 2 mg/kg adriamycin (doxorubicin) into the tail vein. Six weeks thereafter, a renal biopsy was performed via dorsolateral incision to study pretreatment renal damage. Immediately after surgical removal of a small part of the lower pole from the left kidney, gelfoam was applied to achieve hemostasis. Renal tissue samples were snap-frozen in liquid nitrogen and stored at −80°C; another part was fixed in 4% paraformaldehyde and embedded in paraffin.

After recovery from the biopsy, groups were treated with vehicle (VEH; n = 10), ACE inhibitor (ACEi; lisinopril, 75 mg/l in the drinking water, equal to 5 mg·kg⁻¹·day⁻¹, n = 23) or angiotensin type 1 receptor antagonist (AT1A; L158,809, 150 mg/l drinking water, equal to 5 mg·kg⁻¹·day⁻¹, n = 23) (6). Healthy control animals (CON; n = 8) were used as time controls. In prior experiments, we showed that the biopsy procedure does not affect the course of renal damage (37). Treatment was continued for 6 wk until death at week 12. Immediately after surgery for the biopsy, four animals died in the VEH group, one in the ACEi group, and three in the AT1A group; these animals were not included in the analyses. At the end of the study, a 2-ml blood sample was taken by cannulation of the abdominal aorta; kidneys were perfused with saline, removed, and further processed as with the biopsy. The protocol was approved by the Committee for Animal Experiments of the University of Groningen, Groningen, The Netherlands.

**Clinical parameters.** Proteinuria was measured by the Biuret method (Bioquem, Merck, Darmstadt, Germany). Plasma and urine creatinine levels were determined colorimetrically (Sigma, St. Louis, MO). Systolic blood pressure was measured weekly by the tail-cuff method (Biotronex). Clinical parameters were studied. Rats were housed in a temperature-controlled room with a 12:12-h light-dark cycle and free access to food and water. Urine was collected over a 24-h period each week in metabolic cages and stored at −20°C.

**Microbeam-based assay for quantitation of urinary Kim-1.** Kim-1 protein in urine was measured using Microsphere-based Luminox XMAP technology with monoclonal antibodies raised against rat Kim-1 in the Vaidya/Bonventre laboratory (32). This technique is an adaptation of the recently developed and validated sandwich ELISA assay described previously (31). For measurement, 30 µl of urine samples were analyzed in duplicate.

**Quantitative PCR for Kim-1.** Total RNA was isolated using an adaptation of the standard guanidine thiocyanate lysis (10). First-strand cDNA was synthesized from 1 µg total RNA (RT-PCR Core kit, PerkinElmer). TaqMan real-time PCR was performed in 38-well plates on an ABI Prism 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). Kim-1 gene-specific Taqman probe and primer sets were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. The AOD identification number for Kim-1 was Rn00597703 m1. Sequences of the primers and probe for housekeeping gene GAPDH mRNA are as follows: 5’-GAA CAT CAT CCC TGC ATC CA-3’ (forward), 5’-CCA GTG AGC TTC CCG TTC A-3’ (reverse), and 5’-CTT GCC CAC AGC CTT GCC AGC-3’ (probe).

For GAPDH, the quantitative (q) PCR mixture contained 5 µl of cDNA, 10 µl of 2X TaqMan Universal PCR Master Mix (Eurogentec, Seraing, Belgium), 900 nmol/l of each primer, and 200 nmol/l probe in a total reaction volume of 20 µl. The qPCR mixture for the AOD product Kim-1 contained 10 µl of 2X TaqMan Universal PCR Master Mix (Eurogentec), 1 µl of 20x AOD Gene Expression Assay Mix, and 5 µl of cDNA in a total reaction volume of 20 µl. All assays were performed in triplicate. Reaction tubes without template cDNA served as negative controls. The PCR plate was incubated for 2 min at 50°C to optimize the uracil-N-glycosylase enzyme activity and at 95°C for 10 min to activate the Taq polymerase. The reaction was then subjected to 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 1 min. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passed a preset threshold. The samples with Ct >37 were considered not to express the given mRNA. The Ct is inversely proportional to the logarithmic scale of the starting quantity of template cDNA. Consequently, the gene dosage was deduced by calculation of the difference in Ct of the Ct of the reference gene GAPDH. The average Ct values for Kim-1 were subtracted from the average housekeeping gene Ct values to yield ΔCt. Results were finally expressed as 2ΔCt, which is an index of the relative amount of renal Kim-1 mRNA expression.

**In situ hybridization.** The primer used for Kim-1 in situ hybridization was forward: 5’-AAC GCA GCG ATT GTG CAT CC-3’ and reverse: 5’-GTC CAC TCA CCA TGG TAA CC-3’. The 969-bp Kim-1 PCR product was subcloned into the pCR II TOPO vector (Invitrogen, Carlsbad, CA). RNA probes were labeled with a DIG RNA labeling kit (Sp6/T7, Roche, Mannheim, Germany). In situ hybridization was performed on routinely fixed paraffin-embedded tissue sections using standard laboratory protocols. Briefly, deparaffinized sections were air-dried, treated with Triton X-100, followed by proteinase K. Thereafter, slides were incubated with DIG-labeled probe in a hybridization solution consisting of 1 ml 20× SSC, 50 µl 100× Denhardt’s solution, 1 ml 50% dextran sulfate, 2.5 ml formamide, 200 µl t-RNA, 50 µl 1 M DTT, and 125 µl salmon sperm DNA overnight at 55°C. After a washing, slides were treated with 2 U/ml RNase T1 in 1 mM EDTA and 2× SSC at 37°C for 30 min. Positive cells were visualized with anti-DIG-labeled alkaline phosphatase for 1 h at 37°C in 0.1 M maleic acid buffer containing 0.15 M NaCl, 1% blocking buffer, and 2% normal sheep serum. The staining reaction was performed for 48 h at 4°C with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in TBS with MgCl₂ and levamisole.

**Immunohistochemistry.** Paraffin sections (4 µm) were stained with periodic acid-Schiff to evaluate focal glomerulosclerosis (FGS) and interstitial fibrosis. Immunostaining was performed on paraffin sections for Kim-1 (antibody against the intracellular domain of Kim-1: peptide 9; dilution 1:1,000, Biogen, Cambridge, MA), α-smooth muscle actin (α-SMA; clone 1A4, dilution 1:15,000, Sigma), collagen type III (dilution 1:100, Biogenesis, Poole, UK), and macrophages (ED1; dilution 1:1,000, Serotec, Oxford, UK). After dewaxing with xylol and alcohol, antigen retrieval was performed by overnight incubation (80°C) in 0.1 M Tris-HCl buffer. After blocking of endogenous peroxidase activity, sections were incubated for 1 h with diluted primary antibodies in PBS with 1% bovine serum albumin. Binding for antibodies was detected using two sequential incubations (30 min) with peroxidase-labeled secondary antibodies. Peroxidase activity was developed using 3,3'-diaminobenzidine (DAB; Sigma).
solution for 10 min, to which hydrogen peroxide was added. An automated staining system (DAKO Autostainer, Edition 4.0, DAKO, Carpinteria, CA) was used to obtain comparable staining results for all slides.

To study the colocalization of Kim-1 with renal interstitial damage, double staining with ED1 (macrophages), α-SMA, or collagen III was performed. Slides were incubated with a mixture of primary antibodies for 1 h at room temperature: anti-Kim-1 and anti-ED1 at dilution 1:400, α-SMA at 1:500. After washing with PBS, secondary antibodies were added. Kim-1 was detected with peroxidase-labeled goat anti-rabbit antibodies and ED1 and α-SMA with alkaline phosphatase-labeled goat anti-mouse antibodies. First, peroxidase activity was developed with DAB for 10 min. Subsequently, alkaline phosphatase activity was developed with Naphthol AS-MX, and color was developed with Fast Blue BB combined with levamisol and MgSO₄ for 30 min. To combine collagen III and Kim-1 (both polyclonal), staining for Kim-1 with peroxidase-labeled secondary antibodies and DAB was first performed, followed by incubation with glycine/HCl, pH 2.0, for 30 min. After blocking of endogenous biotin and streptavidin, sections were incubated with anti-collagen III (dilution 1:50) followed by a biotin-labeled goat anti-rabbit antibody and alkaline phosphatase-labeled streptavidin. Appropriate isotype and PBS controls were consistently negative for all antibodies.

Quantification of renal damage. FGS was scored semiquantitatively on a scale of 0–4 in 50 glomeruli/kidney, moving from outer to inner cortex. FGS lesions were defined as glomerular areas with mesangial expansion and adhesion formation simultaneously present in one segment (25). Interstitial fibrosis was scored semiquantitatively on a scale of 0–3 (30). Interstitial Kim-1, α-SMA, and collagen type III staining was measured by a blinded observer using computer image analysis (Advanced QUIPS, Leica Imaging Systems, Cambridge, UK). The proportional area of immunostaining was measured in 50 randomly selected cortical interstitial images/kidney, with exclusion of large vessels and glomeruli. The area of immunostaining for Kim-1, α-SMA, and collagen III was divided by the total surface of the image. The number of glomerular macrophages was determined in 50 glomeruli/slide. Interstitial macrophages were counted in 50 consecutive fields.

Statistical analysis. Data are expressed as means ± SD when a normal distribution was present, or as median and 95% confidence interval when the data distribution was not normal. When normal distribution was present, ANOVA with a post hoc test (differences between groups) or paired samples t-test (differences between weeks 6 and 12) were used. When abnormal distribution was present, the nonparametric Kruskal-Wallis (differences between groups) and Wilcoxon signed rank test (differences between weeks 6 and 12) were used. To perform linear regression, data were transformed with natural logarithms. Spearman’s Rho correlation coefficients are given. To calculate the predictive value of the different parameters at week 6 for outcomes at week 12 in the ACEi- and AT₁A-treated animals, linear regression analysis was performed, using the backward method. Dependent variables were, respectively, FGS, interstitial fibrosis, and proteinuria at week 12. As input variables, we entered blood pressure, proteinuria, FGS, interstitial fibrosis, macrophages, α-SMA, Kim-1 protein (immunohistochemistry), and Kim-1 mRNA (all variables at week 6). Statistical analyses were performed using SPSS statistical software, version 12.0, and GraphPad Prism, version 3.02. Statistical significance was assumed at the 5% level.

RESULTS

Clinical parameters. At week 6, overt proteinuria was present in the adriamycin rats (Fig. 1A). During ACEi treatment (75 mg/l drinking water), proteinuria decreased from week 6 to week 12 by 78 ± 15% and with AT₁A by 59 ± 24% (both P < 0.05 vs. week 6). In the VEH animals, proteinuria stabilized between the 6- and 12-wk time points. Blood pressure was significantly reduced by ACEi and by AT₁A but was stable during VEH (Table 1). Compared with healthy controls, creatinine clearance was significantly reduced in all adriamycin groups.

Quantification of renal damage. Macrophage influx was high in adriamycin rats at week 6 and decreased in all groups from week 6 to week 12 (Table 1). In adriamycin-treated rats,
α-SMA expression was present at week 6. It was increased compared with control rats and was not different between weeks 6 and 12 in all groups. Collagen type III staining and interstitial fibrosis were increased in adriamycin rats compared with healthy controls, without an apparent effect of ACEi and AT1A. Mild FGS was present at week 6 compared with control rats and was not different between groups (from 0.23 ± 0.25 to 0.10 ± 0.10 and 0.23 ± 0.16 to 0.13 ± 0.10% of tissue volume, respectively, for week 6 compared with week 12, both P < 0.05, Fig. 2F and G). In the VEH-treated group, Kim-1 protein increased (from 0.23 ± 0.20 to 0.35 ± 0.23% of tissue volume for week 6 compared with week 12, P < 0.05, Fig. 2E). In healthy controls, virtually no Kim-1 protein was present (0.01 ± 0.01% tissue volume, Fig. 2H).

Colocalization studies. To investigate whether renal Kim-1 expression was associated with classic markers of tubulointerstitial damage, we performed double staining for Kim-1 with a macrophage marker (ED1, inflammation), α-SMA expression (myofibroblast transformation, indicating the presence of pre-fibrotic changes), and collagen type III (fibrotic lesions). The double staining of Kim-1 with ED1 showed that Kim-1-positive tubules were often surrounded by interstitial macrophages (Fig. 3A), whereas Kim-1-negative (and morphologically normal) tubules were not associated with local macrophage infiltration. However, in more advanced fibrotic lesions, macrophages were also present in areas with severely dilated Kim-1-negative tubules. The Kim-1-positive tubules were surrounded by α-SMA-positive fibroblasts, indicating the presence of pre-fibrotic changes (Fig. 3B). In this double staining, also severely diluted, but Kim-1-negative, tubules were surrounded by α-SMA-positive fibroblasts. Kim-1-positive tubules were often surrounded by collagen III deposits (Fig. 3C).

Urinary Kim-1. The shedded ectodomain of Kim-1 in urine was measured by a microbead (Luminex)-based assay at the end of the study (32). The concentration of urinary Kim-1 was significantly increased in all adriamycin-treated animals at week 12. In the VEH-treated group, urinary Kim-1 was 257 ± 10 pg/ml (below the limits of detection of the assay) in controls (P < 0.05). Treatment with ACEi or AT1A for 6 wk significantly reduced urinary Kim-1 to 53 ± 47 and 91 ± 111 pg/ml, respectively (both P < 0.01 vs. VEH). In Fig. 4, urinary Kim-1 levels are normalized to urinary creatinine concentration.

Table 1. Clinical parameters and renal damage

<table>
<thead>
<tr>
<th>Week</th>
<th>VEH (n = 6)</th>
<th>ACEi (n = 22)</th>
<th>AT1A (n = 21)</th>
<th>CON (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure, mmHg</td>
<td>6</td>
<td>151 ± 11</td>
<td>146 ± 9</td>
<td>144 ± 11</td>
</tr>
<tr>
<td>12</td>
<td>139 ± 24</td>
<td>103 ± 20†</td>
<td>118 ± 23*</td>
<td>118 ± 14</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>12</td>
<td>13.7 ± 0.88‡</td>
<td>1.20 ± 0.39‡</td>
<td>1.43 ± 0.37‡</td>
</tr>
<tr>
<td>Macrophages/interstitial field</td>
<td>6</td>
<td>73 (49–231)</td>
<td>88 (36–138)</td>
<td>79 (60–118)</td>
</tr>
<tr>
<td>12</td>
<td>9 (2–44)*</td>
<td>13 (7–18)*</td>
<td>18 (15–60)*</td>
<td>9 (2–16)</td>
</tr>
<tr>
<td>α-SMA, % interstitial staining</td>
<td>6</td>
<td>7.7 (4–11)</td>
<td>7.5 (5.6–8.4)</td>
<td>6.9 (5.1–8)</td>
</tr>
<tr>
<td>12</td>
<td>9.6 (3.7–16.1)‡</td>
<td>5.5 (4.4–8.5)‡</td>
<td>7.1 (5.3–10.6)‡</td>
<td>0 (0–2)</td>
</tr>
<tr>
<td>Collagen type III, % interstitial staining</td>
<td>6</td>
<td>0.41 (0.19–0.64)</td>
<td>0.56 (0.42–0.63)</td>
<td>0.52 (0.45–0.75)</td>
</tr>
<tr>
<td>12</td>
<td>0.55 (0.25–1.1)‡</td>
<td>0.31 (0.28–0.71)‡</td>
<td>0.5 (0.37–0.85)‡</td>
<td>0.12 (0–0)</td>
</tr>
<tr>
<td>Interstitial fibrosis, score 0–3</td>
<td>6</td>
<td>0.5 (0–2)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>12</td>
<td>1 (0–3)*‡</td>
<td>1 (1–2)*‡</td>
<td>1 (0–2)*‡</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>FGS, score 0–400</td>
<td>6</td>
<td>20 (5–28)</td>
<td>30 (22–59)</td>
<td>12 (8–32)</td>
</tr>
<tr>
<td>12</td>
<td>107 (45–160)*‡</td>
<td>28 (26–68)</td>
<td>36 (23–88)*‡</td>
<td>4 (0–16)</td>
</tr>
</tbody>
</table>

Values are means ± SD for blood pressure and creatinine clearance and median and 95% confidence interval (in parentheses) for macrophages, α-SMA, positive tubules/interstitial field expression was significantly attenuated in the ACEi and AT1A groups (from 0.23 ± 0.25 to 0.10 ± 0.10 and 0.23 ± 0.16 to 0.13 ± 0.10% of tissue volume, respectively, for week 6 compared with week 12, both P < 0.05, Fig. 2F and G). In the VEH-treated group, Kim-1 protein increased (from 0.23 ± 0.20 to 0.35 ± 0.23% of tissue volume for week 6 compared with week 12, P < 0.05, Fig. 2E). In healthy controls, virtually no Kim-1 protein was present (0.01 ± 0.01% tissue volume, Fig. 2H).
Correlations among urinary and renal Kim-1, proteinuria, and renal damage. On individual analysis, urinary Kim-1 and renal Kim-1 were strongly associated ($R^2 = 0.56, P < 0.001$, Fig. 5). Taking the parameters separately, renal Kim-1 (mRNA) was strongly associated with proteinuria ($R^2 = 0.42, P < 0.001$) and with markers of renal damage. The association with $\alpha$-SMA was strong as well ($R^2 = 0.52, P < 0.001$). Kim-1 mRNA was also associated with other parameters of renal damage, namely, tissue macrophages ($R^2 = 0.15, P < 0.05$), interstitial fibrosis ($R^2 = 0.44, P < 0.01$), and FGS ($R^2 = 0.49, P < 0.01$). Also, urinary Kim-1 was associated with proteinuria ($R^2 = 0.55, P < 0.001$) and with renal damage: $\alpha$-SMA ($R^2 = 0.32, P < 0.001$), macrophages ($R^2 = 0.23, P < 0.01$), interstitial fibrosis ($R^2 = 0.30, P < 0.01$), and FGS ($R^2 = 0.55, P < 0.01$). There was no association with blood pressure ($R^2 = 0.04$). Thus, on cross-sectional analysis, animals with more proteinuria had higher levels of renal Kim-1 and urinary Kim-1. Higher levels of Kim-1 mRNA and urinary Kim-1 were...
also associated with more renal damage. The association between the change in proteinuria with the change in Kim-1 during antiproteinuric treatment is shown in Fig. 6, giving an $R^2$ of 0.33, $P < 0.001$, whereas the association between reduction in blood pressure and reversibility of Kim-1 was of borderline significance with a $R^2$ of 0.12, $P = 0.05$. On multivariate analysis, the reduction of Kim-1 was explained by the reduction in proteinuria but not blood pressure. The changes in Kim-1 expression were not correlated with changes in interstitial fibrosis (prefibrotic changes as apparent from α-SMA) or changes in FGS (Fig. 6, bottom).

To analyze whether pretreatment renal Kim-1 expression predicts renal outcome after treatment, we performed univariate analysis, showing for ACEi- and AT1A-treated rats, renal Kim-1 mRNA at week 6 measured by real-time PCR predicts proteinuria at week 12 ($R^2 = 0.62$, $P < 0.05$), focal glomerulosclerosis ($R^2 = 0.53$), and interstitial fibrosis ($R^2 = 0.41$).

When linear regression analysis is used, the best predicting model for FGS at week 12 contains FGS, macrophages, Kim-1 protein (immunohistochemistry), and proteinuria. In an analysis for proteinuria at week 12, Kim-1 mRNA at week 6 is the best predictor. For interstitial fibrosis at week 12, only α-SMA at week 6 is the best predictor.

**DISCUSSION**

The major findings of this paper are the reversibility of renal tubular Kim-1 expression during antiproteinuric treatment by RAS blockade, in proportion to the reduction in proteinuria. This is the first study with longitudinal data on renal Kim-1 expression before and after antiproteinuric treatment. Interest-
ingly, renal Kim-1 expression before treatment predicts the outcome of renal damage after treatment. In line with earlier studies, Kim-1 expression was present in injured and dilated tubules in areas with interstitial inflammation and fibrosis, but less so in tubuli with advanced lesions. Moreover, the levels of urinary Kim-1 were strongly associated with renal tissue Kim-1.

This study describes the expression of Kim-1 in adriamycin-induced nephropathy and its reversibility during treatment with RAS blockade. Adriamycin nephropathy is a well-established model of for interstitial fibrosis, characterized by the gradual development of proteinuria, due to direct toxicity on glomerular structure responsible for changes in glomerular permeability (3, 4, 16). The direct toxicity is due to oxygen free radicals, as demonstrated by increased ATPase activity (2). Proteinuria usually stabilizes after 4–5 wk (4). During the subsequent phase, persistent proteinuria causes tubular cell activation and injury with attraction of interstitial inflammatory cells (3, 43) and the development of interstitial fibrosis. Both macrophages and tubular cells contribute to fibrosis through the production of growth factors (3). Although the pathophysiology of adriamycin nephropathy has not been elucidated in full detail, by the sequence of events with proteinuria preceding the development of renal fibrotic lesions, adriamycin is well suited to study the effects of antiproteinuric intervention on the subsequent development of renal structural damage and on the reversibility of tubular damage markers.

In chronic kidney disease, RAS blockade improves the long-term outcome, but in many patients renoprotection is incomplete, prompting better identification of patients that need intensified therapy. In adriamycin nephropathy, we previously showed that more severe pretreatment tubulointerstitial damage predicts a worse long-term outcome of renoprotective intervention (20), which can be ameliorated by intensified treatment (19). For clinical purposes, therefore, it would be important to have a biomarker that reflects the severity of pretreatment tubulointerstitial renal damage better than proteinuria alone to identify patients that need intensified treatment (24). Here, pretreatment renal Kim-1 levels predicted antiproteinuric response and outcome at an end point with a worse outcome in individuals with higher Kim-1 levels. Also, in line with other studies (33, 35), urinary Kim-1 was strongly associated with renal Kim-1 expression, although unfortunately urinary Kim-1 was available only at the end point. Future studies therefore should further explore this issue and investigate whether pretreatment urinary Kim-1 can identify
individuals that need intensified therapy to obtain effective long-term renoprotection.

The mechanism of Kim-1 induction by adriamycin nephropathy, and its reversibility during antiproteinuric treatment, was not specifically studied here, but several inferences can be made. Kim-1 expression occurs in a wide array of renal conditions, in association with early tubular damage. It is plausible that in our study proteinuria was the trigger for tubular damage, as supported by the between-group differences in proteinuria and renal Kim-1 expression, the cross-sectional correlation between renal Kim-1 and proteinuria, and the corresponding changes in proteinuria and Kim-1 during treatment. Reabsorption of leaked proteins is followed by tubular activation characterized by the production of growth factors and cytokines (43). This may lead to tubular injury characterized by proliferation, apoptosis, inflammation, and increased extracellular matrix production (42). A role for proteinuria would be in line with the abundant expression of Kim-1 in protein overload-induced proteinuria (33). The functional consequences of Kim-1 expression have not been established so far, but recently, Ichimura et al. (13) demonstrated that epithelial cells expressing Kim-1 are capable of phagocytosis of apoptotic and necrotic cells of injured tubule lumen in cultured primary rat tubule epithelial cells. Kim-1 recognizes apoptotic cells by surface-specific epitopes (phosphatidylserine and oxidized lipoproteins) (13). Thus Kim-1 may play a role in the remodeling after injury and renal repair capacity. However, it cannot be established from our study whether Kim-1 is actively involved in the process of damage and repair, or, alternatively, is just a consequence of renal injury. Unraveling cause and consequence would require specific intervention in Kim-1, for instance, by Kim-1 knockout or overexpression models.

After antiproteinuric treatment with RAS blockade, the increase in renal and urinary Kim-1 was significantly attenuated. This reduction in Kim-1 levels is probably due to reduction of proteinuria and its intrarenal sequelae. Based on the current data, we cannot exclude the possibility that it is due to a direct pharmacological effect of the intervention by amelioration of angiotensin II-dependent activation of tubular cells and the subsequent cytokine and growth factor release (7). Recent human data from our group may allow the partial dissection between effects of pharmacological intervention and the effects of proteinuria reduction per se. In nondiabetic proteinuric patients, nonpharmacological reduction of proteinuria was associated with a decrease in urinary KIM-1 excretion (36). For the current study, we assume that the RAS blockade contributed particularly by reducing proteinuria, whereas a role for blood pressure cannot be fully excluded. Also, RAS blockade can act by direct reduction of angiotensin II effects and improve medullary blood flow and tubular oxygenation, which may support restoration of tubular integrity (11) by restoring ischemia. This is also in line with data on Kim-1 expression in homozygous Ren-2 rats, a model of angiotensin-mediated renal damage, where treatment with RAS blockers reduced Kim-1 expression compared with untreated Ren-2 rats. The latter effect is not blood pressure dependent, demonstrated by the reduction of Kim-1 expression in the same study with homozygous Ren-2 rats after treatment with p38 MAP kinase inhibitors (9). In the present study, a weak association was present between the reduction in blood pressure and reduction in Kim-1, which, nevertheless, was no longer significant after correction for the reduction in proteinuria by multivariate analysis.

We also studied the cellular localization of Kim-1 in relation to the presence of tubulointerstitial damage. Kim-1 was localized in the apical membrane of dilated tubules, which corresponds to the localization of Kim-1 in ischemic and toxic injury (14, 15). In ischemic injury, Kim-1 expression is most prominent in the S3 segment (i.e., the segment most susceptible to ischemic injury), whereas in our model of proteinuria-induced renal damage Kim-1 expression was also prominent in the midcortical and superficial tubules, which is in congruence with the Kim-1 expression in folic acid-induced renal injury and polycystic kidney disease, models where damage is not predominantly in the S3 segment (15, 22). Therefore, localization of Kim-1 expression appears to be related to the susceptibility of the specific tubular segments to different types of injury.

When related to localization of tubulointerstitial damage, we found that Kim-1 was expressed in areas that display interstitial inflammation, tubular dilation, and (pre-)fibrotic changes. Interestingly, Kim-1 expression was mainly apparent at the apical membrane of dilated tubular cells, whereas it was absent in advanced stages of tubular damage. We observed a mosaic staining pattern of Kim-1 within one tubule: cells with preserved morphology within the tubule showed Kim-1 expression, and the more flattened cells were Kim-1 negative, using our technique. In ischemia-reperfusion, Kim-1 is colocalized with vimentin (dedifferentiation) and BrdU (proliferation) in regenerating tubular cells (14). In polycystic kidney disease (22), Kim-1-positive cells demonstrate partial loss of polarity but preserved staining for actin, villin, and E-cadherin. Together, these data suggest that Kim-1 is expressed early in the sequence of events of dedifferentiation of an injured tubular cell. It should be noted that the reversibility of renal Kim-1 during antiproteinuric treatment was not accompanied by reversibility of interstitial fibrosis, reinforcing the notion that the dynamics of Kim-1 expression predominantly reflect early tubular injury rather than late fibrosis. This is in line with recent data in renal transplant recipients (40) and with the study by Ichimura et al. (13), demonstrating phagocytosis of necrotic and apoptotic cells by epithelial cells expressing Kim-1. By analogy, in our proteinuric model, Kim-1 might protect the renal interstitium from toxic mediators and apoptotic cells induced by the proteinuric ultrafiltrate.

Kim-1 is shed into urine, and, in accord with other studies, we found that its urinary excretion corresponded to intrarenal Kim-1 expression. Urinary Kim-1 was increased in untreated proteinuric animals, whereas animals after antiproteinuric treatment showed lower levels of urinary Kim-1. As urinary Kim-1 correlates with proteinuria and renal damage (and renal Kim-1 levels), it is a potential noninvasive marker for interstitial injury. Recently, we found that urinary KIM-1 reflects not only tubular KIM-1 expression in human renal disease but also other markers of renal damage (35). Also, in renal transplant recipients, urinary KIM-1 is an independent predictor of long-term graft loss (34). Thus KIM-1 is a promising new biomarker of renal damage and graft loss. Therefore, it would be of great interest to see whether the effects of treatment on urinary Kim-1 could prospectively predict renal outcome of treatment. In this study, however, we did not measure urinary Kim-1 levels before treatment and cannot draw conclusions on this
issue. In nondiabetic proteinuric patients, we prospectively studied urinary KIM-1 levels before and after antiproteinuric treatment. Urinary KIM-1 levels are markedly elevated in these patients and decrease with antiproteinuric treatment, by angiotensin II blockade, as well as by a low-sodium diet and the combination with diuretic therapy [36]. Our current data suggest that urinary KIM-1 levels may be used to monitor the effect of antiproteinuric intervention on the tubulointerstitial sequelae of proteinuria, which could be of prognostic relevance. However, prospective long-term studies would be needed to test this assumption.

In conclusion, the increase in renal KIM-1 expression in adriamycin nephropathy is reversible by antiproteinuric treatment by RAS blockade in proportion to the reduction in proteinuria. This reversibility however, is not paralleled by reversibility of established fibrotic lesions, in line with the association of renal KIM-1 with early injury in particular. Pretreatment renal KIM-1 levels predict antiproteinuric response and the subsequent renal outcome of treatment. Moreover, renal KIM-1 is closely related to urinary KIM-1 levels. These data provide further support for KIM-1 as a marker for early tubular injury, which could be used to guide and individualize renoprotective intervention.

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