Blockade of cysteine-rich protein 61 attenuates renal inflammation and fibrosis after ischemic kidney injury

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Submitted 20 December 2013; accepted in final form 10 June 2014

ACUTE KIDNEY INJURY (AKI) is a common clinical syndrome that increases the risk of adverse outcomes (13). Recent long-term followup studies have demonstrated that AKI significantly increases the risk of chronic kidney disease (CKD) development and renal function deterioration and causes a more rapid onset of end-stage renal disease (ESRD) (16, 28, 37, 47, 49). These clinical data indicate that we should not view AKI merely as an innocent event but rather a long-lasting, progressive disorder. Because the burden of AKI and its consequences are still growing, there is an urgent need to prevent or retard the renal function decline after AKI (30).

AKI results in tubular damage through the processes of necrosis and apoptosis and leads to inflammation and the secretion of cytokines. Damaged cells may undergo dedifferentiation and redifferentiation to regenerate and recover their structural and functional integrity. However, recent studies have shown that AKI can also result in incomplete tubular repair, which is characterized by the failure of differentiation and persistently high signaling activity (17, 18, 45). This drives downstream events involving tubulointerstitial inflammation, macrophage activation, capillary rarefaction, fibroblast proliferation, and aberrant extracellular matrix deposition, a common feature and the primary determinant of the progression to CKD and ESRD (8, 36, 46, 50). Because the exact cellular mechanisms linking AKI to CKD remain obscure, an understanding of the pathophysiology of the AKI-to-CKD transition and identifying potential modifiable factors warrant further study.

Cysteine-rich protein 61 (Cyr61) has been reported to control the cell cycle, stimulate chemostasis, regulate fibroblasts, remodel the cell matrix, and participate in angiogenesis (9–11, 22). Emerging data have suggested that Cyr61 promotes inflammation and regulates the trafficking of immune cells (2, 9, 38). All of the aforementioned mechanisms can contribute to cellular pathophysiologies after ischemic AKI (7, 46). Using a kidney ischemia-reperfusion injury (IRI) animal model, Cyr61 has been found to be rapidly upregulated in the renal outer medulla and urine after injury (40). Based on evidence in the literature, we hypothesized that Cyr61 plays a functional role in contributing to the pathogenesis of the AKI-to-CKD transition.

In the present study, we investigated the expression of Cyr61 in unilateral kidney IRI in mice and in cultured proximal tubular epithelial cells. Our results demonstrate that Cyr61 was significantly induced in post-IRI kidneys. Cyr61 blockade attenuated kidney inflammation and fibrosis and prevented capillary rarefaction 14 days after a severe event of ischemic AKI.
MATERIALS AND METHODS

Reagents. Normal rabbit IgG and anti-α-smooth muscle actin (α-SMA) antibodies were purchased from Sigma (St. Louis, MO), and the antibody against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-mouse Cyr61 polyclonal antibody was produced by immunizing rabbits with the synthesized peptide, which was selected from the Cyr61 gene sequence (GenBank Accession No. AAH66019.1). This anti-Cyr61 antibody has structure specificity and neutralizing activity for Cyr61 (27). The anti-cabin antibody was a generous gift from Dr. Timothy A. Sutton (Indiana University, Indianapolis, IN). This antibody is highly preferential to labeling renal endothelial cells in the peritubular microvasculature rather than capillaries of the glomerulus or small arterioles (5). Recombinant Cyr61 protein was from PeproTec (Rocky Hill, NJ). DMEM, HBSS, FBS, and other cell culture reagents were obtained from Gibco-BRL (Rockville, MD).

Cell culture experiments. Normal rat kidney proximal tubular epithelial cells (NRK-52E cells) were pretreated as previously described (34). Cells were cultured in DMEM supplemented with 10% FBS. Subconfluent cells were made quiescent by placing them in DMEM with 0.1% FBS for 16 h before the experiments. To simulate an ischemic event, cells were incubated in medium without nutrients (HBSS) in a hypoxic incubator (1% O2-5% CO2-94% N2) for 6 h. Reoxygenation was performed in complete DMEM in a regular incubator (21% O2-5% CO2-balance N2) for 3 h. Starved cells incubated in HBSS under normoxic conditions for 6 h served as controls.

Animal model. Eight-week-old male ICR mice weighing 25–30 g were obtained from the laboratory animal center of the National Taiwan University College of Medicine. Unilateral IRI of the kidney was performed under anesthesia, as previously described (36). In brief, the left kidney and renal vessels were exposed through surgical flank incisions, and a vascular clamp (Micro-Clips ST-M110, Karl Klappenecker) was placed across the renal arteries and veins. The kidneys were confirmed to become dusky and were replaced in the retroperitoneum for 45 min at a core temperature of 36.8–37.3°C. The clamp was removed, and the return of perfusion to the kidney was confirmed before wound closure. Mice were euthanized at 1, 3, 5, 7, or 14 days after the surgery (n = 4 mice at each time point). The kidneys were removed and divided into three parts, as previously described (33). The first part was fixed in 10% neutral buffered formalin, the second part was fixed in 4% paraformaldehyde and PBS at 4°C, and the third part was snap frozen in liquid N2 and stored at −70°C. Age-matched, sham-operated mice underwent left renal artery manipulation but not clamping. Sham-operated mice were euthanized 24 h after the operation, and their kidneys served as controls (n = 4). In other experiments, mice were treated with 10 μg/g body wt of anti-Cyr61 antibody or control IgG intraperitoneally 2 h before surgery. Two groups of mice were euthanized 24 h after IRI (n = 4 mice/group). In other groups, one dose of anti-Cyr61 antibody or control IgG was injected per day until animals were euthanized 7 or 14 days after the surgery (n = 6–8 mice/group). All surgeries were performed under 2,2,2-tribromoethanol (Avertin; 0.25 mg/g body wt) intraperitoneal anesthesia, and all efforts were made to minimize the animals’ suffering. Animal experimenters were performing using a protocol approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine.

Histological examination and immunofluorescence staining. For histological analysis, paraffin-embedded kidneys were sectioned at 5 μm and stained with hematoxylin and eosin. In the assessment of kidneys harvested 24 h after IRI, tubular injury was scored semiquantitatively by estimating the percentage of tubules in the cortex and outer medulla that displayed epithelial cell sloughing, necrosis, and case formation as follows: 0 = no changes, 1 = changes affecting <10% of the sample, 2 = changes affecting 10–25% of the sample, 3 = changes affecting 25–50% of the sample, and 4 = changes affecting 50–75% of the sample, and 5 = changes affecting >75% of the sample. To study the distribution of the renal collagen matrix, picrosirius red staining of paraffin sections was performed according to established methods (48).

Snap-frozen mouse kidneys were prepared and cut as cryostat sections for immunofluorescence staining. Primary antibodies against the following proteins were used for immunolabeling: Cyr61, 1:500; Lotus tetragonolobus lectin (LTL), 1:200 (Vector Lab, Burlingame, CA); kidney injury molecule-1 (KIM-1), 1:200 (eBioscience, San Diego, CA); Dolichos biflorus agglutinin, 1:50 (Vector Lab); F4/80, 1:100 (Invitrogen, Frederick, MD); and cablin, 1:200. The following fluorescent-conjugated secondary antibodies were used: anti-rabbit FITC, anti-rat FITC, and anti-rabbit rhodamine at 1:200 (Jackson ImmunoResearch, West Grove, PA). Sections were colabeled with 4′,6-diamidino-2-phenylindole (1:1,000, Invitrogen) and mounted with Prolong Gold (Invitrogen), as previously described (35). Images were captured and processed using a confocal microscope (LSM 510 META, Carl Zeiss, Jena, Germany). To quantify the area of tissue occupied by positive staining for F4/80 or picrosirius red, sections were assessed morphometrically using Povea Pro 4.0 software (Reindeer Graphics, Asheville, NC) (8, 36, 48). For the quantification of vascular density, cablin-immunostained sections were examined through a 10 × 10 grid under a ×40 objective. Each square within the grid that contained cablin-positive vascular cells was counted. Vascular density data were normalized and expressed as percentages relative to sham-operated mice. For all measurements, at least 10 randomly selected cortical interstitial field images were taken, and the mean value was calculated for each animal. Examinations of sections were performed on a blinded basis.

Human specimens. All patients were registered in the National Taiwan University Hospital Study Group on Acute Renal Failure database. The database was constructed for quality assurance at one medical center (National Taiwan University Hospital, Taipei, Taiwan) and its three branch hospitals in different cities (21). AKI patients in the present study were consecutively enrolled when dialysis-requiring AKI developed between September and November 2013. The definition of AKI was based on Acute Kidney Injury Network criteria (19). Samples of urine (5 ml) were collected from each patient just before the initiation of renal replacement therapy. In addition, we included 10 patients who underwent open-heart surgery but did not develop AKI as controls. Urine samples collected at 48 h after operation from control patients were used for comparison. This study was approved by the Institutional Review Board of the National Taiwan University Hospital (ClinicalTrials.gov identifiers: NCT00451373 and NCT01876134). All participants signed written informed consent before inclusion in the study.

Immunoenasys for urinary KIM-1 and Cyr61. Urinary samples were collected from mice 24 h after IRI surgery and were used for KIM-1 measurements using a commercially available ELISA kit (Quantikine, MKM100, R&D, Minneapolis, MN). Urinary samples from patients with AKI or control individuals were measured for Cyr61 using a commercially available ELISA kit (EIA-5108, DRG, Marburg, Germany) according to the manufacturer’s instructions. All measurements were made in duplicate and in a blinded fashion. The urine creatinine concentration was measured by the Jaffe reaction using a urine creatinine assay kit (no. 500701, Cayman Chemical, Ann Arbor, MI). Measured urinary KIM-1 and Cyr61 values were normalized to the urinary creatinine concentration and expressed as micrograms per gram of creatinine.

RNA extraction and quantitative PCR. Total tissue or cell RNA was isolated using TRIzol reagent (Invitrogen). cDNA was then synthesized using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. Quantitative PCR was performed using standard methods as previously described (35). The specific primer pairs used for quantitative PCR are shown in Table 1.
Table 1. Primer sequences used for quantitative PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Cyr61</td>
<td>5'-ATTCTCTGAGTATGACATTAGG-3'</td>
<td>5'-GTACACTGGAACGGGAGCTC-3'</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-GTAAGGCTGACCGCTTAGAATC-3'</td>
<td>5'-CTCTCACAGAAGGCTTGA-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-ATACCTGTTGCTGGTGAACG-3'</td>
<td>5'-TGTACCTTTCTGTTTCTG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5 '-ATAGCGAAAGCGGAGAACAGA-3'</td>
<td>5 '-TTTCTGGAAGGAGATGCTG-3'</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5'-GACAATCCGGACACTTTCAAC-3'</td>
<td>5'-CACTTACAGCCACACAGC-3'</td>
</tr>
<tr>
<td>MIP-2</td>
<td>5'-GACTTGATCGTGGTGAACAGA-3'</td>
<td>5'-GTGAATGTTGATAGCAGGCTG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GACCTGGATCTGCTGGTGAACAGA-3'</td>
<td>5'-ACCCTGGCAACATGAGCTG-3'</td>
</tr>
<tr>
<td>IL-12</td>
<td>5'-CCCTTTCGCTGCTGGTGAACAGA-3'</td>
<td>5'-TTTCTTACAACCTTTGCCAAAGTA-3'</td>
</tr>
<tr>
<td>Collagen type I-α1</td>
<td>5'-AGCAAGGGGAAAGAAGGCTA-3'</td>
<td>5'-GTTGACCTTGTTTATAGGTA-3'</td>
</tr>
<tr>
<td>Collagen type III-α1</td>
<td>5'-GCAAAGGAAAGAAGGCTA-3'</td>
<td>5'-GATTGTATTAGCTGCTGTCAG-3'</td>
</tr>
<tr>
<td>Transforming growth factor-β1</td>
<td>5'-TTATGTTTCCTCACAAGG-3'</td>
<td>5'-TATATTTCGTTGATAGGT-3'</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-I</td>
<td>5'-TCTTCTCCCTTGGACACAG-3'</td>
<td>5'-TTTCTTACACCCCTTTGCCAAAGTA-3'</td>
</tr>
<tr>
<td>18S</td>
<td>5'-GGTTGGTTTGAGGTTGAGGC-3'</td>
<td>5'-GTCGACCTGTTTATAGGTA-3'</td>
</tr>
</tbody>
</table>

Cyr61, cysteine-rich protein 61; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein.

Protein extraction and Western blot analyses. Total proteins from kidney tissues were dissolved in RIPA buffer (Millipore, Temecula, CA) and subjected to Western blot analysis using previously stated methods (12). Blots were incubated with primary antibodies at 4°C overnight, further incubated with peroxidase-conjugated antibodies, and then visualized using chemiluminescence reagents (Millipore) according to the manufacturer’s instructions.

Statistical analysis. Results are expressed as means ± SD and were prepared using GraphPad Prism software (version 4.0, GraphPad Software, San Diego, CA). Statistical comparisons were performed using Kruskal-Wallis one-way ANOVA or a Mann-Whitney U-test with Stata 10.0 software (StataCorp, College Station, TX). P values of <0.05 were considered to be statistically significant.

RESULTS

Cyr61 expression increases after kidney IRI. Basal Cyr61 expression was low in sham-operated kidneys (Fig. 1, A–C). In response to kidney IRI, the level of Cyr61 transcripts in the kidneys increased significantly starting from 1 day after surgery and remained elevated at subsequent time points (Fig. 1A). Accompanying the increased transcripts, Cyr61 protein expression gradually increased in IRI kidneys, with a peak observed at 5 days after surgery (Fig. 1, B and C). By immunohistochemistry, Cyr61 was found to be predominantly localized in renal tubular epithelial cells in post-IRI kidneys (Fig. 1D). Dual-staining images showed that Cyr61-positive cells were colabeled with LTL or KIM-1 but not with Dolichos biflorus agglutinin. These findings suggest that Cyr61 is expressed mainly in injured and noninjured proximal tubular epithelial cells after ischemic kidney injury.

Cyr61 expression in cultured renal tubular epithelial cells. To simulate ischemia-reperfusion conditions in vitro, NRK-52E cells were incubated in nutrient-free medium and exposed to low-O2 conditions (1% O2) followed by reoxygenation and nutrients repletion to reproduce reperfusion. As shown in Fig. 2A, we found a 1.75-fold increase of Cyr61 transcript expression in NRK-52E cells under hypoxic conditions for 6 h. Expression was further enhanced to 4.27-fold higher after reoxygenation for 3 h.

In a previous study (27), we observed that Cyr61 induces monocyte chemoattractant protein (MCP)-1 expression in NRK-52E cells. Here, we examined whether Cyr61 affects the expression of proinflammatory cytokines and chemokines in renal tubular epithelial cells. As shown in Fig. 2B, recombinant Cyr61 protein treatment elicited an increase in MCP-1 and IL-6 expression of TNF-α. Expression levels of IL-1β, macrophage inflammatory protein (MIP)-2, MIP-1α, and IL-12 were not significantly affected by Cyr61.

Elevated urinary Cyr61 during AKI. To validate the expression of Cyr61 in kidneys after IRI, we measured the levels of urinary Cyr61 in patients with AKI. Urine samples were collected from 10 patients with dialysis-requiring AKI classified according to Acute Kidney Injury Network criteria (serum creatinine: 3.27 ± 2.14 mg/dl). Among them, seven patients developed AKI due to ischemia, two patients due to sepsis, and one patient due to hepatic failure. During the same study period, 10 patients undergoing cardiac surgery but not having AKI were included as controls (serum creatinine: 0.84 ± 0.27 mg/dl). Characteristics of AKI and control patients are shown in Table 2. As shown in Fig. 3, urinary Cyr61 concentrations in the AKI group (8.53 ± 5.9 μg/g creatinine) were significantly higher than those in the control group (0.38 ± 0.7 μg/g creatinine, P < 0.001). This indicates that urinary Cyr61 excretion is increased in patients with AKI.

Cyr61 blockade reduces indicators of inflammation in kidneys after IRI. To clarify the functional role of Cyr61 after ischemic kidney injury, we examined the effect of Cyr61 blockade in the unilateral kidney IRI model. In this model, the
Fig. 1. Increased cysteine-rich protein 61 (Cyr61) expression in kidneys after unilateral ischemia-reperfusion injury (IRI). A: quantitative PCR time course showing the expression of kidney Cyr61 mRNA after unilateral IRI. Expression levels were normalized to 18S rRNA. B: representative images of the time-course Western blots showing Cyr61 protein expression in kidney lysates after IRI. GAPDH was used as a loading control. C: bar graph showing the time course of Cyr61 protein expression normalized to GAPDH. n = 4 animals/time point. Values are means ± SE. *P < 0.05 vs. sham operation (sham); #P < 0.05 vs. the contralateral kidney (CLK). D: representative immunofluorescence micrographs (magnification: ×400, scale bar = 50 μm) of kidneys from sham mice and mice at 5 days after IRI. Cyr61-positive cells were colabeled with *Lotus tetragonolobus* lectin (LTL; apical staining) but not with *Dolichos biflorus* agglutinin (DBA; basolateral staining). Cyr61 staining was also present in kidney injury molecule (KIM)-1-positive cells (yellow arrows) as well as KIM-1-negative cells (white arrowheads).
level of serum creatinine does not increase due to compensation by the contralateral kidney (50). Therefore, we measured urinary KIM-1 excretion to confirm the development and severity of AKI (20, 31). The results showed that urinary KIM-1 levels were significantly elevated at 24 h after kidney IRI. The increased levels did not differ between IRI mice treated with either control IgG or anti-Cyr61 antibody (Fig. 4A). Histological evaluation of paraffin-embedded kidneys harvest 24 h after IRI disclosed significant tissue damage, with the presence of tubular epithelial sloughing, necrosis, and cast formation. Semiquantitative analysis revealed that the severity of tubular injury was similar between mice treated with control IgG and anti-Cyr61 antibody (Fig. 4B). These results indicate a similar degree of severity of initial kidney injury between groups.

Previous studies have indicated that Cyr61 can provoke proinflammatory signaling and regulate immune cell migration (2, 9, 38). Here, we investigated the effect of Cyr61 antagonism on renal inflammation in the unilateral kidney IRI model. After IRI, transcript levels of MCP-1, IL-6, IL-1β, MIP-2, MIP-1α, IL-12, and TNF-α all increased significantly. Compared with mice treated with control IgG, anti-Cyr61 antibody treatment resulted in significant reductions in the levels of all these proinflammatory gene transcripts on day 14 (Fig. 4, C–I). Cyr61 blockade also significantly attenuated the upregulation of MCP-1, IL-6, IL-1β, and MIP-2 on day 7 (Fig. 4, C–F). Immunostaining with the mouse macrophage-/monocyte-specific cell surface marker F4/80 showed the infiltration of macrophages in the renal interstitium after IRI. Blockade of Cyr61 resulted in a significant reduction in the infiltration of F4/80-positive macrophages on days 7 and 14 after IRI (Fig. 5A). These results support the proinflammatory and chemokinetic effects of Cyr61 in post-IRI kidneys.

Cyr61 blockade attenuates the severity of fibrosis in mouse IRI kidneys. Severe unilateral IRI leads to undesirable profibrotic effects in the injured kidney (2). Next, we examined the effect of Cyr61 blockade on fibrosis in the unilateral kidney IRI model. Compared with mice treated with control IgG, anti-Cyr61 antibody treatment markedly attenuated the expected increased levels of collagen type I-α1, collagen type III-α1, transforming growth factor (TGF)-β, and plasminogen activator inhibitor (PAI)-1 transcripts in post-IRI kidneys by 87.8%, 92.3%, 77.2%, and 90.2%, respectively, on day 14 (Fig. 6, A–D). Similarly, the elevated expression of α-SMA protein in treatment.

| Table 2. Clinical characteristics of control patients and patients with dialysis-requiring AKI |
|-----------------|-----------------|-----------------|-----------------|
|           | Control Patients | AKI Patients | P Value |
| n     | 10               | 10             | 0.9       |
| Age, yr | 63.12 ± 8.06     | 62.41 ± 24.70  | 1.0       |
| Female patients, n (%) | 5 (50) | 5 (50) | 1.0        |
| Patients with diabetes mellitus, n (%) | 4 (40) | 3 (30) | 1.0        |
| Patients with hypertension, n (%) | 9 (90) | 6 (60) | 1.0        |
| Patients with coronary artery disease, n (%) | 7 (70) | 2 (20) | 1.0        |
| Baseline serum creatinine, mg/dl | 0.9 ± 0.25 | 1.5 ± 1.45 | 1.0        |
| Serum creatinine at urine collection, mg/dl | 0.84 ± 0.27 | 3.27 ± 2.14 | 1.0        |
| Patients who received surgery, n (%) | 10 (100) | 8 (80) | 1.0        |
| Patients with concurrent sepsis, n (%) | 1 (10) | 3 (30) | 1.0        |

Data are presented as means ± SD or as numbers of patients (n) with corresponding percentages. AKI, acute kidney injury.
Cyr61 has been identified as a potential biomarker of AKI (6, 40). Muramatsu et al. (40) previously reported that the Cyr61 gene was significantly upregulated in proximal tubules very early after bilateral renal ischemia. Cyr61 protein was induced in the kidney and detectable in the urine within 1 day after injury (40). Using a severe unilateral kidney IRI model, we confirmed that Cyr61 was rapidly upregulated in proximal tubular cells after injury. This observation was further supported by in vitro experiments showing that hypoxia-reoxygenation stimulated Cyr61 expression in cultured proximal tubular epithelial cells. Notably, we validated this finding by examining clinical samples that showed significantly higher urinary Cyr61 excretion in patients with AKI. In contrast to Muramatsu et al.’s results, we found that kidney Cyr61 protein production increased progressively after day 1 and peaked on day 5 after injury. This may be due to the difference in animal models used because severe unilateral IRI leads to progressive interstitial fibrosis, whereas moderate bilateral IRI does not (25, 44, 50). Whether Cyr61 is a practical AKI biomarker and whether its level in urine reflects progressive kidney injury deserve further large-scale clinical studies.

We also sought to determine the role of Cyr61 in post-IRI kidneys. Previous reports have shown that injury in proximal tubules with incomplete regeneration and cell cycle G2/M arrest was capable of triggering an inflammatory response and gave rise to tubulointerstitial fibrosis (17, 18, 45, 50). Injured, dedifferentiated tubular cells play a central role in activating a myriad of proinflammatory and profibrotic factors with autocrine and paracrine functions, which contribute to pathological fibrosis (7, 46). As demonstrated by our immunofluorescence staining, the majority of Cyr61 expression in post-IRI kidneys was in LTL-positive and/or KIM-1-positive proximal tubular epithelial cells (Fig. 1D). Our data also suggested that blockade of Cyr61 was beneficial for kidney inflammation and fibrosis after IRI. Therefore, we speculate that Cyr61 is one of the crucial regulators that connect tubular epithelial injury, maladaptive repair, and progressive kidney fibrosis after IRI.

As shown in Figs. 5B and 6, A–F, anti-Cyr61 antibody treatment ameliorated interstitial fibrosis after severe ischemic AKI. Expression of the PAI-1 gene, an important fibrosis-promoting factor (39), was suppressed, and collagen fibril accumulation was attenuated on days 7 and 14. Suppression of collagen and TGF-β gene expression and α-SMA protein production was also evident on day 14. Intuitively, the antifibrotic effect of Cyr61 blockade might be due to the inhibition of fibroblasts. However, previous studies have reported that Cyr61 can suppress collagen expression and induce apoptosis in skin fibroblasts (10, 22). We have previously demonstrated that Cyr61 can directly inhibit the expression of collagen types I and III in kidney fibroblasts in vitro (27). Furthermore, one recent study (23) using a carbon tetrachloride liver injury model in hepatocyte-specific Cyr61 deletion mice reported that Cyr61 attenuates liver fibrosis by inducing myofibroblast senescence. These results suggest that without cell-cell or cell-microenvironment interactions in post-IRI kidneys, Cyr61 blockade is not able to suppress fibroblasts and collagen production. As such, the antifibrotic effect we observed in this study is likely due to other mechanisms rather than direct inhibition of fibroblasts.

Accumulating evidence indicates that the culprit of interstitial fibrosis after AKI is inflammation (7, 8, 29, 46). After post-IRI kidneys was reduced in mice treated with anti-Cyr61 antibodies on day 14 (Fig. 6, E and F). On day 7 after IRI, there were no discernible differences in the expression of these profibrotic markers, except for PAI-I transcripts, between control IgG and anti-Cyr61 antibody groups (Fig. 6D).

This antifibrotic effect of Cyr61 antagonism in post-IRI kidneys was further verified by picrosirius red staining for collagen fibrils. The extent of renal interstitial fibrosis was evident and progressive after IRI in mice treated with control IgG. Anti-Cyr61 antibody treatment reduced the area of fibrosis by 27.0% and 57.1% by days 7 and 14 after IRI, respectively (Fig. 5B).

Cyr61 blockade ameliorates peritubular capillary rarefaction in kidneys after IRI. Current evidence suggests that capillary loss after AKI is an important factor driving interstitial fibrosis (3, 5, 46). Thus, we examined whether Cyr61 blockade affects capillary rarefaction in post-IRI kidneys. Peritubular microvascular density was evaluated based on the numbers of grids containing vascular cells positively immunostained for cavin (Fig. 5, C and D). By day 7 after IRI, vascular density decreased to levels of 70.4% and 86.8% relative to sham-operated controls in mice treated with control IgG and anti-Cyr61 antibody, respectively (P = 0.248). By day 14 after IRI, the vascular density was more preserved by anti-Cyr61 antibody treatment (control IgG vs. anti-Cyr61 antibody: 49.8% vs. 70.0%, P = 0.028). In contrast, there were no significant differences in the levels of kidney VEGF-A transcripts between the two groups on days 7 and 14 (Fig. 6G).

**DISCUSSION**

In this report, we demonstrated the upregulation of Cyr61 in proximal tubular epithelial cells after severe ischemic AKI. We also noted that Cyr61 blockade using a neutralizing antibody had a protective effect and significantly attenuated the severity of renal inflammation and macrophage infiltration after injury. Furthermore, blockade of Cyr61 was associated with reduced renal fibrosis and the amelioration of peritubular capillary rarefaction. Collectively, these data suggest that the proinflammatory effect of Cyr61 is an important cause mediating progressive renal fibrosis after an acute ischemic kidney injury.
ischemic injury, renal tubular cells generate proinflammatory and chemotactic cytokines, such as MCP-1, IL-1β, IL-6, and TNF-α, which activate inflammatory cells (7). This results in the exudation of neutrophils in the early stages and infiltration of monocytes/macrophages in subsequent stages (15, 36, 46). Recruited inflammatory macrophages further release a broad range of proinflammatory cytokines, which potentiate further injury and fibrosis through cellular cross-talk (8, 15, 24, 46). In
A)

- Sham Day 1
- Control IgG
- Anti-Cyr61 Ab

Day 7
Day 7
Day 14
Day 14

Blue: DAPI
Green: F4/80

B)

- Control IgG
- Anti-Cyr61 Ab

Day 7
Day 7
Day 14
Day 14

C)

- Sham Day 1
- Control IgG
- Anti-Cyr61 Ab

Day 7
Day 7
Day 14
Day 14

Blue: DAPI
Red: Cablin

D)

- Microvascular rarefaction
- Vessel density (% of sham)

Day 7
Day 7
Day 14
Day 14

IgG
Anti-Cyr61
IgG
Anti-Cyr61

IgG
Anti-Cyr61
IgG
Anti-Cyr61

IgG
Anti-Cyr61
IgG
Anti-Cyr61

#
the present study, treatment with anti-Cyr61 antibody led to a significant reduction in the transcripts of MCP-1, IL-6, and IL-1β as well as macrophage-derived proinflammatory chemo-kine MIP-2 on days 7 and 14 after IRI (Fig. 4). Cyr61 blockade also reduced the levels of MIP-1α, IL-12, and TNF-α transcripts 14 days after injury. Furthermore, targeting Cyr61 attenuated the infiltration of F4/80-positive macrophages into post-IRI kidneys (Fig. 5A). The amelioration of inflammation and macrophages infiltration appeared earlier (day 7) than the suppression of renal collagen type I-α1, collagen type III-α1, and TGF-β expression (day 14). In vitro experiments revealed that Cyr61 enhances MCP-1 and IL-6 expression in renal tubular epithelial cells (Fig. 2B). These findings are compatible with recent reports suggesting that Cyr61 is involved in regu-

Fig. 5. Cyr61 blockade reduces the recruitment of macrophages, decreases collagen fibril accumulation, and ameliorates microvascular rarefaction in kidneys after unilateral IRI. A: representative immunofluorescence micrographs (magnification: ×400, scale bar = 50 μm) showing interstitial F4/80-positive macrophages (green) in kidneys on days 7 and 14 after unilateral IRI. The bar graph shows the percentage of positive F4/80-stained areas in kidneys from mice treated with control IgG or anti-Cyr61 antibody. DAPI, 4’,6-diamidino-2-phenylindole. B: representative images of picrosirius red staining for interstitial fibrillar collagen (red) in post-IRI kidneys (magnification: ×200, scale bar = 100 μm). The bar graph shows picrosirius red-stained percentages in the field. C: representative cavin immunofluorescence micrographs showing the peritubular microvasculature (red) in post-IRI kidneys (magnification: ×400, scale bar = 50 μm). D: graph showing morphometric quantification of the peritubular microvasculature after IRI. Data are expressed as percentages of vascular density in sham-operated kidneys. n = 6–8 animals/group. All values are means ± SE. *P < 0.05 vs. sham; #P < 0.05 vs. control IgG.

Fig. 6. Cyr61 blockade attenuates fibrosis in kidneys after unilateral IRI. A–D: quantitative PCR analysis showing that the increased expression of renal collagen type I-α1 (Col 1-α1; A), collagen type III-α1 (Col 3-α1; B), transforming growth factor (TGF)-β (C), and plasminogen activator inhibitor (PAI)-1 (D) transcripts after unilateral IRI was inhibited by anti-Cyr61 antibody treatment. Data are expressed as fold differences compared with sham kidneys. E and F: representative images (E) and bar graph (F) of the Western blot analysis showing that blockade of Cyr61 decreased α-smooth muscle actin (α-SMA) expression in the kidneys 14 days after unilateral IRI. G: quantitative PCR analysis showing that VEGF-A expression was similar between control IgG and anti-Cyr61 antibody groups. n = 6–8 animals/group. All values are means ± SE. *P < 0.05 vs. sham; #P < 0.05 vs. control IgG.
lating immune cells trafficking and inducing proinflammatory signaling (2, 26, 27, 38). Recently, a microarray study (25) suggested that persistent activated inflammation after AKI could contribute to the pathogenesis of progressive renal fibrosis. Early anti-inflammatory treatments or depletion of macrophages may attenuate renal fibrosis after IRI (8, 24, 44). It is likely that the antifibrotic effect of Cyr61 blockade comes from the attenuation of post-IRI kidney inflammation.

There is a discrepancy between the effect of Cyr61 on TNF-α expression in NRK-52E cells (Fig. 2B) and the effect of blockade of Cyr61 in vivo (Fig. 4I). It has been shown that many biological functions of Cyr61 are cell type specific (11). Bai et al. (2) demonstrated that Cyr61 induces the expression of many proinflammatory factors (including TNF-α) in murine macrophages. To confirm this, we treated cultured mouse leukemic monocyte/macrophage cells (RAW 264.7 cells) with recombinant Cyr61 protein. Cyr61 elicited an increase in TNF-α expression in RAW 264.7 cells (Fig. 7). Anti-Cyr61 antibody treatment can therefore reduce renal TNF-α expression in vivo through its direct action on macrophages and through its effect of attenuating macrophage infiltration.

Using an animal model of unilateral ureteral obstruction that results in progressive inflammation and fibrosis, we had previously observed that Cyr61 blockade attenuated kidney fibrosis in the early phase but that this antifibrotic effect was not sustained in the later phase (27). In unilateral ureteral obstructed kidneys, the offending influence (obstruction) is not eliminated, and thus proinflammatory macrophages are exquisitively activated, inflammation escalates, tubular apoptosis persists, and fibroblast activation progresses (14). As such, in repetitive or chronic injury states, sole blockade of Cyr61 is not sufficient to prevent these deleterious outcomes. This differs from the present study, in which we showed that Cyr61 blockade led to durable anti-inflammatory and antifibrotic effects. In animal models of single kidney injury and repair, such as IRI, the direct targeting of proximal proinflammatory signaling events in renal tubules can potentially ameliorate entire pathological processes that lead to tubulointerstitial fibrosis.

We further performed a supplementary experiment in which daily injection of control IgG or anti-Cyr61 antibody was
delayed to be initiated until 72 h after kidney IRI (n = 3–4 animals/group). As shown in Fig. 8, delayed Cyr61 blockade was not able to reduce α-SMA protein production at 7 and 14 days after severe unilateral IRI. We infer that the timing of Cyr61 blockade accounts for the different responses. If we delay anti-Cyr61 antibody treatment until 72 h after IRI, the initial kidney inflammation will not be affected. Therefore, the antifibrotic effect could not be observed.

Rother et al. (42) used adenoviral transfection to overexpress Cyr61 in a murine model of autoimmune myocarditis. They found that Cyr61 gene transfer inhibited immune cell migration/infiltration and attenuates cardiac injury (42). This seems contradict our present findings in post-IRI kidneys and those of others (2, 26, 27). Interestingly, they observed that recombinant Cyr61 proteins bind to spleen macrophages but not to cardiac cells. A further investigation by this group (38) found that Cyr61 is a biphasic immune cell migration modulator: acute short-term treatment enhances the migration of immune cells, whereas prolonged/pretreatment inhibits migration. It is possible that continuous overexpression of Cyr61 before an acute insult inhibits the ability of spleen macrophages to migrate toward the upcoming inflammatory cardiac disease. In contrast, locally produced Cyr61, such as in post-IRI kidneys, attracts immune cells to the injured area and leads to secondary local immobilization at the site of inflammation (38). As a matrix-associated protein, Cyr61 may act variable biological properties, depending on the subtypes of integrin receptor and their interacting molecules (11). The precise-cell specific and time-specific effects of Cyr61 require further investigation.

Capillary rarefaction is recognized as an important consequence of ischemic AKI (3, 32). Possible mechanisms accounting for the capillary loss that have been proposed include diminished angiogenic factors, such as VEGF (4), loss of pericytes from peritubular capillaries (1, 43), macrophage-dependent vascular regression (41), pericyte-endothelium interactions, and VEGF isotype switching (32). A reduction in macrophage-derived angiostrophic factors may not be a crucial mechanism responsible for the capillary loss that have been proposed include diminished angiogenic factors, such as VEGF (4), loss of pericytes from peritubular capillaries (1, 43), macrophage-dependent vascular regression (41), pericyte-endothelium interactions, and VEGF isotype switching (32). A reduction in macrophage-derived angiostrophic factors may not be a crucial mechanism responsible for vascular regression in kidneys and those of others (2, 26, 27). Interestingly, they observed that recombinant Cyr61 proteins bind to spleen macrophages but not to cardiac cells. A further investigation by this group (38) found that Cyr61 is a biphasic immune cell migration modulator: acute short-term treatment enhances the migration of immune cells, whereas prolonged/pretreatment inhibits migration. It is possible that continuous overexpression of Cyr61 before an acute insult inhibits the ability of spleen macrophages to migrate toward the upcoming inflammatory cardiac disease. In contrast, locally produced Cyr61, such as in post-IRI kidneys, attracts immune cells to the injured area and leads to secondary local immobilization at the site of inflammation (38). As a matrix-associated protein, Cyr61 may act variable biological properties, depending on the subtypes of integrin receptor and their interacting molecules (11). The precise-cell specific and time-specific effects of Cyr61 require further investigation.

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In conclusion, we showed that Cyr61 was upregulated in proximal tubular epithelial cells after ischemic kidney injury. These observations were supported by culture experiments and clinical measurements. Targeting Cyr61-mediated proinflammatory signaling after severe ischemic AKI inhibits the triad of inflammation, interstitial fibrosis, and capillary rarefaction. Figure 9 shows the putative model for the role of Cyr61 after ischemic kidney injury. The results of this study expand the knowledge of the mechanisms of the AKI-to-CKD transition and suggest that Cyr61 may be a potential therapeutic target.

ACKNOWLEDGMENTS

The authors thank Dr. Timothy A. Sutton (Indiana University, Indianapolis, IN) for the kind gift of anti-cabilin antibody. The authors also thank the staff of the Second and Seventh Core Labs, National Taiwan University College of Medicine, for technical assistance.

Some results have been previously presented as abstracts at the 50th European Renal Association-European Dialysis and Transplant Association Congress in Istanbul, Turkey, on May 18–21, 2013.

GRANTS

This work was supported by National Science Council Grant 101-2314-B-002-149-MY3, Aim For Top University Program 102R-7620 of the National Taiwan University, National Taiwan University Hospital Grants 100-1667 and 100-1688, the Ta-Tung Kidney Foundation, and the Mrs. Hsiu-Chin Lee Kidney Research Fund.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

Cyr61 in ischemic AKI


