Early detection of cysteine rich protein 61 (CYR61, CCN1) in urine following renal ischemic reperfusion injury

YASUNARI MURAMATSU,1 MICHIKO TSUJIE,1 YUKIMASA KOHDA,1 BERTHA PHAM, ALAN O. PERANTONI, HONG ZHAO, SANG-KYUNG JO, PETER S.T. YUEN, LEONARD CRAIG, XUZHEN HU, and ROBERT A. STAR

Renal Diagnostic and Therapeutic Unit, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, and Laboratory of Comparative Carcinogenesis, National Cancer Institute, National Institutes of Health, Frederick, Maryland; and Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA

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Background. Acute renal failure (ARF) has a high morbidity and mortality. Many therapies have worked in animals but were unsuccessful in clinical trials. The inability to diagnose ARF early may have impeded the success of these trials.

Method. We screened a subtraction library to search for potential disease markers that would be induced rapidly after renal injury. Mice and rats were subjected to 30 to 40 minutes of bilateral ischemia.

Results. mRNA for Cyr61, a secreted growth factor-inducible immediate early gene, was markedly up-regulated at two hours in the kidney but not other organs following renal ischemia. In situ hybridization studies suggested Cyr61 was synthesized in the proximal straight tubule. Cyr61 protein was analyzed by capture with heparin beads followed by Western blotting. Induction of Cyr61 protein could be detected in the kidney within one hour, peaked at four to eight hours, and remained elevated for at least 24 hours following ischemia. Cyr61 protein was detected in urine at three to six hours and peaked at six to nine hours after renal injury. Cyr61 was not detected after volume depletion, which is often difficult to differentiate from ARF.

Conclusions. The secreted, cysteine-rich, heparin binding protein Cyr61 is rapidly induced in proximal straight tubules following renal ischemia, and excreted in the urine where it might serve as an early biomarker of renal injury.

Acute renal failure (ARF) is a devastating disease that has a mortality rate of 25 to 70% [1]. Although much is known about the pathogenesis of acute ischemic and toxic injury in animals, translation of an effective drug therapy for ARF from laboratory bench to bedside has been difficult [1–4]. Numerous agents are effective in animals [for example, atrial natriuretic peptide (ANP), insulin-like growth factor-1 (IGF-1), thyroid hormone, endothelin antagonists], but have failed in clinical trials [2, 3, 5, 6]. Only one agent, N-acetylcysteine, may prevent glomerular filtration rate (GFR) from decreasing after contrast administration [7]. The slow translation of results from bench to bedside, especially in the treatment of existing ARF, might be caused by a heterogeneous patient population, multiple and diverse types of renal insults, incorrect choice of culprit, or delayed detection of ARF [2, 3]. Early detection of ARF is difficult since, in contrast to 30 to 40 years ago, it is no longer heralded by a fall in urine output. Instead, ARF is typically diagnosed by a progressive rise in serum creatinine over several days. Many patients with ARF, especially those in the intensive care unit (ICU), are volume overloaded and that may dilute serum creatinine values and thus delay diagnosis [3]. Unless patients are enrolled at the time of entry to the ICU [8], the mean plasma creatinine in most ARF clinical trials is 3 to 4 mg/dL (for example in [9]), indicating enrollment two to three days after the original injury. Therapeutic agents are more effective in animals when given early after injury, suggesting a narrow “window of opportunity” such as that which occurs for myocardial infarction and stroke [3]. Therefore, it is imperative to diagnose ARF as early as possible [2]. Detection of ARF by direct measurement of GFR is difficult because it requires a carefully timed urine collection or injection of a tracer [2]. An ARF disease marker that could be measured in blood or urine would be extremely useful in identifying patients with ARF so nephrotoxic agents can be avoided, and enrollment into clinical trials hastened [1–3].

Key words: ischemia, reperfusion, cyr61, biomarker, kidney injury.

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1Drs. Muramatsu, Tsujie and Kohda performed equal work for this study.

We hypothesized that genes induced very early following renal injury might serve as markers of injury. Representational difference analysis was used to screen for genes that are induced rapidly following renal ischemia/reperfusion, since human ARF is caused or aggravated by hypotension or renal ischemia in about 50% of patients [1, 10]. To increase the yield of native kidney proteins, the screen was performed at two hours after ischemia, when only sparse leukocyte infiltration has occurred. We confirmed that several intermediate early genes (HSP70, EGR-1) were rapidly and transiently induced following renal ischemia [11, 12]. We also detected cysteine rich protein 61 gene (Cyr61), which was originally identified as a growth factor-inducible immediate early gene in fibroblasts [13]. Cy6r1 is a secreted heparin-binding protein that may be involved in tissue growth and repair [14, 15]. Since Cy6r1 is a secreted protein, we hypothesized that it might be detected in the urine following injury. We took advantage of its heparin affinity to develop an assay for Cy6r1 protein. Since ARF (clinically diagnosed as acute tubular necrosis) and pre-renal azotemia (reversible volume depletion) can be difficult to distinguish clinically, Cy6r1 also was measured after volume depletion.

**METHODS**

**Animal models**

Male Sprague-Dawley rats and Balb/c mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) or the National Institutes of Health (NIH; Frederick, MD, USA). All animals had free access to water and food (4% mouse-rat diet; Harlan Sprague Dawley Inc.). Animal care followed the criteria of the National Institutes of Health for the care and use of laboratory animals in research. For the representational difference analysis, a rat was anesthetized with an IM injection of 100 mg/kg ketamine, 10 mg/kg xylazine, and 1 mg/kg acepromazine, and placed on a heating table kept at 39°C to maintain body temperature. The left kidney was removed, and the right renal pedicle was cross-clamped for 40 minutes. After removing the clamp, 6 mL of prewarmed (37°C) normal saline was instilled into the abdominal cavity. The abdomen was closed, and the rat was placed in a 29°C incubator. The rat was sacrificed at two hours.

Additional mice and rats were subjected to bilateral ischemia. Animals were sacrificed from 0.5 to 72 hours after injury. Sham-treated animals went through the same surgical procedure as the other animals, including blunt dissection of the renal pedicle; however, renal clamps were not applied.

A rat model of volume depletion was induced by two 20 mg/kg intraperitoneal injections of furosemide (at 0 and 8 hours), and maintained by placing the animals on a sodium deficient diet. Urine was collected from 24 to 27 hours for protein analysis (see below) and from 27 to 30 hours for sodium excretion. The animals were sacrificed at 30 hours, at which time the animals had lost greater than 8% of body weight, plasma creatinine was modestly increased (0.4 mg/dL in volume depletion vs. 0.2 in normal rats), urine sodium was <10 mmol/L, and the fractional excretion of sodium was <0.05%.

**Representational difference analysis of cDNA**

The outer medullary was hand dissected from normal and ischemic kidney, total RNA extracted by RNAzol (ISO-TEX Diagnostics, Friendswood, TX, USA), mRNA purified, cDNA synthesized, and polymerase chain reaction (PCR)-based subtractive hybridization (PCR-select cDNA subtraction kit; Clontech, Palo Alto, CA, USA) was performed. The final PCR product was subcloned into a cloning vector (AdvantAge; Clontech) and used to transform bacteria. One hundred and twenty colonies were randomly picked, and DNA was purified (Wizard Minipreps; Promega, Madison, WI, USA). One hundred clones were screened for up-regulation using a cDNA dot blot probed with non-radioactively labeled mRNA from a sham and ischemic kidneys from an independent experiment. The clones exhibiting the most dramatic up-regulation were sequenced, PCR primers were synthesized, and temporal regulation confirmed by reverse transcription (RT)-PCR (data not shown). Several clones exhibited large increases (EGR-1, HSP70, and Cyr61). Since the former are known to increase following renal ischemia [11, 12], we focused on Cyr61.

**RNA blot analysis**

Total RNA (7 μg) was fractionated via electrophoresis on a 0.9% agarose-formaldehyde gel and transferred onto a nylon membrane. The equality of RNA samples after transfer to the membrane was substantiated by staining with ethidium bromide. Membranes were fixed by baking at 80°C, then prehybridized at 42°C in 50% formamide, 0.5% sodium dodecyl sulfate (SDS) and 5 × Denhardt’s solution, 5 × standard sodium citrate (SSC), and 0.5 mg/mL salmon sperm DNA. The membranes were hybridized with 32P-dCTP-labeled cDNA clones. Portions (0.5 to 2 kb) of mouse Cyr61 were generated by PCR, and the products confirmed by sequencing. The hybridized membranes were washed twice in 0.1× SSC and 0.1% SDS at 50°C. Loading of RNA was normalized by rehybridizing with glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

**Antibody production**

A peptide corresponding to the C-terminus of mouse/rat Cyr61 (CPHPNEASFRLYSLFDNHIKFRD) was synthesized and conjugated to keyhole limpet hemocyanin by cross-linking with m-maleimidobenzoyl-N-hydroxy-
succinimide ester (Quality Controlled Biochemicals, Inc., Hopkinton, MA, USA). Rabbits were immunized and boosted subcutaneously with the peptide-KLH conjugate. Rabbit sera were purified by affinity chromatography, using the immunizing peptide coupled to thiol-reactive beads (Quality Controlled Biochemicals, Inc.). In some studies, the antibody was preabsorbed with antigenic peptide coupled to cyanogen bromide-activated Sepharose 4B beads (Amersham Pharmacia, Piscataway, NJ, USA).

**Western blot analysis**

Kidney tissue was homogenized in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL, USA) containing protease inhibitors (Complete mini protease inhibitor cocktail tablets; Roche, Mannheim, Germany) at 4°C. The homogenate was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was equilibrated with RIPA buffer [150 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)], and then incubated with heparin beads (Heparin Sepharose 6 Fast Flow, Amersham Pharmacia) at a volume ratio of 1:20 at 4°C for one hour. The beads were washed twice with excess RIPA buffer containing 400 mmol/L NaCl. Proteins were eluted with RIPA buffer at 800 mmol/L NaCl and then the eluate was incubated in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) with 200 mmol/L dithiothreitol (DTT) at 60°C for 15 minutes. The samples were incubated at 4°C overnight, briefly centrifuged, and the supernatant stored in 4°C. The protein content of the homogenate (before bead purification) was determined according to the Bradford method using bovine serum albumin (BSA) as a standard. Heparin bead purified cell lysate was fractionated on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membrane was blocked by incubation overnight in 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). The membrane was incubated with 0.75 μg/mL primary antibody for two hours, washed, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins antibody (Dako A/S, Glostrup, Denmark) for one hour at a dilution of 1:2000. The reaction products were visualized by chemiluminescence (ECL-plus; Amersham) using BioMax MR film (Eastman Kodak, Rochester, NY, USA).

**In situ hybridization**

Rat kidneys were perfused with cold saline, then 4% cold paraformaldehyde, and removed. The resected kidneys were cut in half, fixed overnight in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), dehydrated through a graded ethanol series, and embedded in paraffin. Tissue sections (3 μm) were processed for in situ hybridization and evaluated with 35S-labeled sense and anti-sense riboprobes according to Wilkinson and Green [16].

For nonradioactive in situ hybridization, tissues were fixed and pretreated as for 35S-labeled probes. Digoxigenin-labeled single-stranded RNA probes were prepared by Lofstrand Labs using the same probe as for 35S-labeling, diluted to 2 μg/mL in hybridization buffer, heated to 95°C for three minutes, and immediately placed on ice until used. Probes (100 μL/slide) were added to ethanol-dehydrated tissues, sections covered with paraffin, and hybridizations performed at 60°C overnight in a humidified chamber. The next morning, tissues were rinsed with PBS and digested with RNase A (20 μg/mL) for 30 minutes at 37°C. Slides were then rinsed with PBS and washed twice with 2 × SSC for five minutes at 55°C, twice with 1 × SSC for five minutes at 55°C, twice with 0.5 × SSC for five minutes at 55°C, twice with 0.1 × SSC in 0.1% SDS for five minutes at 55°C, and three times with PBS for five minutes at room temperature. For visualization, sections were first blocked with maleic acid buffer (MAB; 0.1 mol/L maleic acid and 0.15 mol/L NaCl adjusted to pH 7.5 with NaOH) containing Tween-20 (0.1%), levamisole (0.5 mg/mL), blocking reagent (2%, Roche), and heat-inactivated sheep serum (10%) for two hours. Blocking buffer was removed and replaced with MAB blocking buffer containing antibody (anti-digoxigenin-AP Fab fragments; Roche), which was diluted 1:4000. Sections were incubated overnight at 4°C in a humidified chamber. Slides were then washed three times for 15 minutes each in MAB containing Tween-20 (0.1%) and then once in water with Tween-20 (0.1%) for five minutes. Slides were then drained, immediately covered with BM purple AP substrate (Roche), and incubated for three days at room temperature in a humidified chamber. Reactions were stopped with ethylenediaminetetraacetic acid (EDTA; 1 mmol/L) in PBS, and tissues mounted.

**Urinary collection**

Male Sprague-Dawley rats were placed in metabolic cages (Nalgene, Rochester, NY, USA), and urine was collected at specified time intervals before or after 30 minutes of bilateral renal ischemia. Urine was collected into vials containing ice-cold 1 mol/L Tris-HCl (pH 6.8), 0.1 mg/mL PMSF, 1 μg/mL leupeptin, and 1 mmol/L sodium azide [17]. The urine samples were centrifuged at 1000 × g at 4°C for 20 minutes to remove cells, casts, and other debris, and the supernatant was used for the assay. In preliminary studies, we could not detect Cyr61 protein in the urinary pellet (data not shown). Urinary creatinine was measured by an Astra 8 autoanalyzer (Beckman Instruments, Inc., Fullerton, CA, USA) to standardize the analysis of urine samples. To control for
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Fig. 2. Northern blot analysis of Cyr61 expression in adult rat tissues following renal ischemia. Organs were obtained from rats subjected to sham surgery or 40 minutes of bilateral ischemia and four hours of reperfusion. Northern blotting and hybridization procedures are the same as described in the legend to Figure 1. Abbreviations are: H, heart; Lu, lung; K, kidney; Sp, spleen; M, skeletal muscle (pretibial muscle); Li, liver.

Fig. 3. Heparin bead capture for Cyr61 protein detection. Kidney tissues were obtained from normal mice or four hours after 40 minutes of bilateral renal ischemia (I/R). Kidney tissue homogenates were run as is (10 μg per lane; lanes 1), or were purified by incubating 200 μg protein with heparin beads, washing with 150 mmol/L (lanes 2) or 400 mmol/L (lanes 3) NaCl-RIPA buffer, then eluting with 800 mmol/L NaCl-RIPA buffer followed by Laemmli sample buffer (see Methods section). Samples were analyzed by SDS-PAGE and Western blotting, using an affinity-purified antibody to mouse/rat Cyr61.

RESULTS

Temporal expression of Cyr61 mRNA

The results of the representational difference analysis were confirmed by Northern blotting (Fig. 1). There was a dramatic up-regulation of rat Cyr61 mRNA abundance in the whole kidney and the renal outer medulla at two hours following bilateral renal ischemia, with a decline by four hours, and a return toward normal at 24 to 72 hours of reperfusion.

Organ specific expression of Cyr61 mRNA

Cyr61 mRNA has been detected by RNAse protection assays [18] or Northern blotting [19–21] in normal lung, heart, skeletal muscle, and kidney, but not in liver. Expression in spleen is controversial [18, 20]. We analyzed the expression of Cyr61 in adult rat tissues (Fig. 2). In animals subjected to sham surgery, Cyr61 mRNA abundance was highest in lung as previously described [18], but also detected at low levels in heart, kidney, and muscle. Four hours following bilateral renal ischemia, the abundance of Cyr61 mRNA increased markedly in the kidney, but not in the other organs sampled.

Optimization of Cyr61 protein assay

Western blotting analysis using an affinity-purified antibody to Cyr61 detected several non-specific bands in urinary concentration, urine samples containing 0.15 mg creatinine were purified by binding to heparin-sepharose beads and analyzed as described above (Western blot analysis). The fractional excretion of sodium (\(\text{FE}_{\text{Na}}\)) was measured using a standard formula \(\text{FE}_{\text{Na}} = \frac{\text{UNa} \times \text{PNa}}{\text{UNa} \times \text{PNa} + \text{UNa} \times \text{Cr}}\), where \(\text{FE}\) is fractional excretion, \(\text{U}\) and \(\text{P}\) are urine and plasma, respectively, and \(\text{Cr}\) is creatinine.
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Fig. 4. Time course of Cyr61 protein expression following renal ischemia. Balb/c mice were subjected to 40 minutes of bilateral ischemia then 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 hours of reperfusion. Kidney tissue homogenates (200 µg protein per lane) were incubated with heparin beads, washed with 400 mmol/L NaCl RIPA buffer, then eluted with 800 mmol/L NaCl-RIPA buffer followed by Laemmli sample buffer. Samples were analyzed as described in the legend to Figure 3. Abbreviations are: N, normal kidney. S, sham operated kidney obtained four hours after the surgery.

Fig. 5. Organ specificity of Cyr61 protein expression following renal ischemia. Organs were obtained from male Sprague-Dawley rats subjected to 40 minutes of bilateral ischemia and six hours of reperfusion (I/R 6 h) or 6 hours after sham surgery (Sham). Tissue homogenates (300 µg total protein per lane) were purified as described in Figure 4, and analyzed by SDS-PAGE and Western blotting. Abbreviations are: H, heart; Lu, lung; K, kidney; Sp, spleen; M, skeletal muscle (pretibial muscle); Li, liver. For comparison, 1/2, 1/5, 1/10, or 1/20 of the I/R 6 h kidney homogenates were loaded to the right three lanes.

Time course of Cyr61 protein expression

Using these optimized assay conditions, the appearance of Cyr61 was studied following 40 minutes of bilateral renal ischemia. Cyr61 protein was faintly detectable in normal and sham-operated kidney, which in previous studies caused peak elution of Cyr61 [19, 22, 23], and Laemmli buffer. Washing with a low stringency buffer decreased the non-specific bands and allowed detection of a doublet near the expected size of 42 kD. (Fig. 3; lane 2). The lower band, but not the higher band, was regulated by renal ischemia. By washing the beads under more stringent conditions (400 mmol/L NaCl), we obtained a single band (Fig. 3; lane 3) that was regulated by ischemia, and corresponded to Cyr61 (documented by peptide competition; see Fig. 8). Subsequent studies were performed using a 400 mmol/L NaCl wash.

Expression of Cyr61 protein in other organs

The assay was used to examine the tissue distribution of Cyr61 protein after renal ischemia. In animals subjected to sham surgery, Cyr61 protein was expressed in heart, skeletal muscle, and lung, and at very low levels in kidney and spleen (Fig. 5). Following bilateral renal ischemia, there was a dramatic up-regulation of Cyr61 protein in the kidney (≥20-fold), with more modest increases in lung and heart. The band was blocked by preabsorption with antigenic peptide coupled to Sepharose beads (data not shown).

Location of Cyr61 mRNA

Cyr61 mRNA was localized using in situ hybridization with both 35S- and digoxigenin-labeled probes in tissue sections from adult Sprague-Dawley rat kidneys (Fig. 6). Serial sections were probed with anti-sense and sense probe; sections labeled with sense probes were at background levels (data not shown). We did not detect any Cyr61 mRNA in kidney from a normal (Fig. 6A) or sham-operated rat (Fig. 6B). Two hours after bilateral ischemia, signal appeared in the outer stripe of the outer medulla and medullary rays (Fig. 6C), the location of maximal tubular injury following renal ischemia/reperfusion [24]. We also performed digoxigenin-labeled in situ hybridization to examine the cellular localization of Cyr61. Digoxigenin-labeled probe was detected in the outer stripe of the outer medulla and medullary rays (Fig. 6D), the same location as the 35S-labeled probe.
Fig. 6. Localization of renal Cyr61 mRNA by in situ hybridization. Kidneys were obtained from male Sprague-Dawley rats subjected to sham surgery or 40 minutes of bilateral ischemia and two hours of reperfusion. Each block was probed with anti-sense and sense probe on serial sections. Detection of the sense probe was at background levels for each treatment (data not shown). (A-C) 35S-labeled probe. (D-E) Digoxigenin-labeled probe. (A) Control normal kidney; (B) 2 hours after sham surgery; (C) 2 hours after renal ischemia. The positive signals for Cyr61 (white granules) were localized in the outer stripe of the outer medulla and medullary rays. (D) Digoxigenin-labeled probe (black stain) 2 hours after renal ischemia. (Magnification, ×25) E, higher magnification of the square shown in panel D (×200).

(Fig. 6C). Under higher magnification (Fig. 6E), Cyr61 mRNA was primarily expressed in the cytoplasm of proximal straight tubules that showed mild injury, although we cannot rule out labeling in other tubular cells. No signals were detected when using digoxigenin-labeled sense probes (data not shown). The distribution suggests labeling of the proximal straight tubules in the outer stripe and medullary rays, although other structures in that region also might express Cyr61.

Detection of Cyr61 in urine after ischemia

Since Cyr61 is a secreted protein and produced in tubules following renal ischemia (Fig. 6), we next determined if Cyr61 could be detected in the urine following renal injury. The length of bilateral renal ischemia was reduced to 30 minutes to obtain sufficient quantities of urine; however, renal Cyr61 protein was still up-regulated under these conditions (Fig. 8A). Cyr61 protein was not detected in normal urine, but was detected 24 hours after ischemia (Fig. 7). We then studied the time course of appearance of Cyr61. Cyr61 protein was detected as early as three to six hours after renal ischemia (Fig. 8A), peaked at six to nine hours after ischemia, and decreased thereafter. Urine also contained a number of non-specific higher molecular weight bands; however, only the 42 kD band was absent after preabsorption of the Cyr61 antiserum with excess Cyr61 peptide (Fig. 8B). In contrast, neither renal nor urinary Cyr61 was detected in rats subjected to volume depletion (Fig. 8A).

DISCUSSION

Representational difference analysis was used to search for renal genes whose protein products might serve as
Diagnostic tests for ARF. Since the role of leukocytes in human ARF is uncertain and controversial, we wished to avoid leukocyte genes. Therefore, to examine native kidney proteins, kidney tissue obtained two hours after ischemia was used, at which time only sparse leukocyte infiltration has occurred. Using this strategy, we found that (1) Cyr61 mRNA was induced in the renal outer medulla very early after renal ischemia, (2) Cyr61 protein increased rapidly after ischemia but not volume depletion, and (3) that Cyr61 protein could be detected in the urine from animals early after ischemia.

Cyr61 was first identified as a gene that was activated transcriptionally in the early-intermediate period after growth factor stimulation of mouse fibroblasts [13]. Subsequent studies found that Cyr61 is a secreted, cysteine-rich, heparin-binding protein that acts as a matrix-associated signaling molecule capable of multiple functions [reviewed in 25]. Cyr61 is a member of the CCN family, a term that refers to the three original members [Cyr61/ connective tissue growth factor/nephroblastoma overexpressed (NOV)] of a family of secreted extracellular growth factors that now includes Wnt-induced secreted proteins [25, 26]. Cyr61 can promote the proliferation, migration, and adhesion of endothelial cells and fibroblasts [23], stimulate growth and differentiation of chondrocytes [27], and induce neovascularization of the cornea [28]. Cyr61 stimulates migration and adhesion of endothelial cells, platelets, fibroblasts, and vascular smooth muscle via α5β1 [28], αvβ3 [29], and α5β1 [30, 31] integrins, respectively. Cyr61 also is induced by bladder stretch [32] and hypertonicity [33]. More central to our findings, Cyr61 promotes healing of a wounded fibroblast monolayer [14]; increases fibroblast adherence [30]; causes cytoskeleton reorganization, formation of filopodia and focal adhesive complexes [34]; and activates a genetic program for wound healing [35]. Cyr61 has been detected locally after wounding in cultured cells and rat tail [14], vascular injury [36], and bone fractures [37], suggesting a role in wound healing or tissue remodeling.

In this study, Cyr61 was measured following renal ischemia and reperfusion. Cyr61 mRNA was easily detected by Northern blotting (Figs. 1 and 2); however, detection of Cyr61 protein was challenging since several bands obscured renal Cyr61 (Fig. 3). We exploited the heparin-binding ability of Cyr61 to develop a sensitive assay for Cyr61 in tissue homogenates and biological fluids. At baseline, Cyr61 mRNA abundance was highest in lung, with lower levels in heart, kidney and muscle (Fig. 2), whereas Cyr61 protein was expressed in heart, with successively lower levels in muscle, lung, kidney, liver, and spleen. This RNA expression pattern is similar to previous studies using RNAse protection assays [18] and Northern blotting [19, 21, 23], although we did not detect Cyr61 mRNA in spleen [20]. Cyr61 protein has been detected previously in skeletal muscle, cardiac muscle, and smooth muscle of bronchioles and blood vessels [38, 39]. The reason for the difference between mRNA and protein expression is not known, but could be caused by differences in mRNA stability and/or protein turnover in different organs [18, 40]. Induction after renal ischemia was rapid, with peak induction of mRNA at two hours (Fig. 1) and protein by four hours (Fig. 4). Following renal ischemia, Cyr61 mRNA was induced in kidney and not in other organs (Fig. 2), although increased protein also could be detected in heart and lung. Cyr61 up-regulation following injury is similar to that previously described following wound healing in cultured cells and rat tail [14], vascular balloon injury [36], and bone fractures [37]. Induction of Cyr61 in the lung may be caused by the lung injury that results from renal ischemia [41].

In situ hybridization was used to localize sites of Cyr61 mRNA accumulation, a marker of net Cyr61 synthesis under these conditions of rapid induction. We did not detect Cyr61 expression in the control kidney, but did see enhanced expression primarily in proximal straight tubules in medullary rays and the outer stripe of the outer medulla (Fig. 6) at two hours after injury. This portion of the kidney sustains the most injury in the ischemia reperfusion model, and contains proximal straight tubules that undergo apoptosis and necrosis, which can be

Fig. 7. Urine Cyr61 before and after renal ischemia. Twenty-four-hour urine collections were obtained from three rats (1–3) before and after 30 minutes of bilateral renal ischemia. The urine samples were collected into T-PER buffer containing protease inhibitors. Portions of the urine supernatant (containing a constant amount of creatinine, see Methods) were purified by binding to heparin beads and analyzed by SDS-PAGE and Western blotting, as described in Figure 4. Two duplicate membranes were probed with affinity purified anti-Cyr61 antibody (A) or primary antibody that had been pre-incubated with excess peptide (B).
Fig. 8. Temporal excretion of urinary Cyr61 protein after renal ischemia and during volume depletion. Rats were subjected to volume depletion (VD) or 30 minutes of bilateral ischemia and reperfusion (I/R). Kidney homogenates (200 μg total protein per lane) were purified and analyzed as described in Figure 4. Abbreviations are: N, normal; I/R, 30 minutes of bilateral ischemia and 24 hours of reperfusion; P, positive control, 40 minutes of bilateral ischemia and six hours of reperfusion. Urine was collected from the VD and I/R rats into the collection buffer (Methods), and analyzed for Cyr61 as in Figure 7. C represents control urine collected before making VD or I/R models. Urine from the I/R rat was collected during 3–6, 6–9, 9–12, 12–18, and 18–24 hours after 30 minutes of bilateral ischemia. No urine was produced during the first three hours. The specific signals for Cyr61 protein (arrows) obtained with the anti-rat/mouse Cyr61 antiserum (A) were prevented by preabsorption with the corresponding peptide (B).

easily detected at two and four hours after reperfusion [24, 42, 43]. One previous study has detected weak expression of Cyr61 in proximal tubules of the newborn mouse kidney [38]. Nahm et al have recently shown that hypertonicity increases Cyr61 mRNA in cultured inner medullary collecting duct cells [33]. We did not detect Cyr61 mRNA in the inner medulla of normal or ischemic kidneys (Fig. 6), or Cyr61 protein in the kidney [3] following volume depletion (Fig. 8), which increases medullary toxicity. The different results may be caused by differences in experimental models, assays, or timing of assays. Nahm et al used cultured cells very early after hypertonic stress, whereas we waited 24 hours for the animals to equilibrate into steady state after volume depletion.

Early detection of ARF might be aided by discovery of a serum marker akin to troponin for myocardial infarction. Serum creatinine, while used clinically, is a suboptimal marker because it is influenced by non-renal factors [3]. The classic ARF marker is the fractional excretion of sodium (FE\textsubscript{Na}), which was originally described as <1% in volume depletion and >1% in ARF [44]. However, FE\textsubscript{Na} is also low in nonoliguric ARF, sepsis, urinary tract obstruction, acute glomerulonephritis, hepatorenal syndrome, and renal allograft rejection [45]. Attempts to use structural components of the kidney, such as N-acetyl-beta-D-glucosamine, as injury markers have failed because these markers are too sensitive and cannot differentiate normal from disease states [46]. Since Cyr61 is a secreted protein that is rapidly induced in the outer medulla following renal injury, we tested if Cyr61 could be detected in urine. We detected an increase in urinary Cyr61 (Figs. 7 and 8) after renal injury. Urinary Cyr61 was increased within three to six hours, peaked at six to nine hours, but remained detectable from nine to 24 hours after 30 minutes of bilateral renal ischemia. The clinical differentiation of ARF (that is, acute tubular necrosis) from volume depletion may be difficult, requiring measurement of FE\textsubscript{Na} and/or fluid administration. Therefore, we examined the effect of volume depletion on Cyr61. Neither renal nor urinary Cyr61 was increased following volume depletion. Recently, hepatocyte growth factor (HGF), kidney injury molecule-1 (KIM-1), IL-18, pro-inflammatory cytokines, and actin have been studied as potential ARF disease markers [47–49]. All are elevated in the urine following human ARF, and are currently being evaluated for sensitivity and specificity. HGF is stored in the normal kidney and released upon injury, whereas KIM-1 is markedly up-regulated in the proximal tubule of post-ischemic regenerating kidneys, although not as rapidly as Cyr61. How early these urinary markers increase following renal damage is unknown. Since Cyr61 is induced rapidly following renal injury, but not following pre-renal azotemia, it has the characteristics of an early disease marker, perhaps similar to troponin following myocardial infarction.

Previous studies have suggested that Cyr61 acts locally because secreted Cyr61 is tightly bound to the cell surface and extracellular matrix and was not detected in conditioned media [22, 38]. Hence, it was surprising to find Cyr61 in urine. The role of urinary Cyr61 is unknown, but we speculate that it might provide an important signal to a downstream nephron segment.

In conclusion, Cyr61 is rapidly induced in the kidney, primarily in proximal straight tubules, by renal ischemia but not volume depletion. Cyr61 is secreted into the urine early after renal ischemia, but not volume depletion. Because of its rapid induction, Cyr61 might serve as an
Early disease marker for renal injury. Thus, urinary Cyr61 might detect subtle renal injury after contrast administration, chemotherapy, transplantation, vascular surgery, or in kidney donors. Cyr61 might also detect early ARF in high-risk patients, for example, with multi-organ failure in the ICU. It is hoped that early detection of ARF will lead to earlier treatment, thus enhancing our ability to develop beneficial therapies.

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Reprint requests to Robert A. Star, M.D., National Institutes of Health, Building 10, Room 3N108, 10 Center Drive MSC 1268, Bethesda, Maryland 20892-1268, USA.

E-mail: Robert_Star@nih.gov

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