Endogenous urokinase lacks antifibrotic activity during progressive renal injury

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Submitted 23 September 2006; accepted in final form 8 March 2007

Yamaguchi I, Lopez-Guisa JM, Cai X, Collins SJ, Okamura DM, Eddy AA. Endogenous urokinase lacks antifibrotic activity during progressive renal injury. Am J Physiol Renal Physiol 293: F12–F19, 2007. First published March 13, 2007; doi:10.1152/ajprenal.00380.2006.—Interstitial fibrosis is a universal feature of progressive kidney disease. Urokinase-type plasminogen activator (uPA) is thought to participate in this process because it promotes matrix protein degradation. However, the antifibrotic activity of uPA has not been fully elucidated. The present study tested the hypothesis that endogenous uPA reduces fibrosis severity during a unilateral ureteral obstruction (UUO) model, which is the most established animal model for human fibrosis.

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shown to promote renal tubular membrane degradation via MMP-9 activation, facilitating migration of transdifferentiated tubular epithelial cells to the interstitium (10, 29). The net effect was more severe fibrosis in WT mice compared with tPA-deficient mice (29). Second, endogenous plasmin activity was found to modestly but significantly increase, rather than decrease, renal fibrosis (3, 31). These results suggest that the profibrotic effect of PAI-1 and the antifibrotic action of uPAR may be plasmin independent. However, they do not clarify the role of endogenous renal uPA during fibrogenesis. Results from other experimental model systems fail to answer this question, as uPA has been reported to promote or attenuate fibrosis, depending on the organ and disease model that is investigated (2, 7, 9, 13, 18, 21, 26, 27). To determine the role of uPA in the pathogenesis of chronic renal interstitial fibrosis, disease severity in response to UUO was compared between WT and uPA−/− mice. Our findings suggest that uPA does not function as an endogenous modulator of renal scarring.

MATERIALS AND METHODS

Animals and experimental protocol. The uPA+/− mice were originally generated by Dr. Peter Carmeliet et al. (1). The experiments were performed on phenotypically normal, healthy, and fertile uPA+/− and WT C57BL/6 male mice. The WT C57BL/6 mice were purchased from Harlan Laboratories (Kent, WA). Breeding pairs of uPA+/− mice (B6.129S2-Plauim1Mlg/j) were purchased from the Jackson Laboratory (Bar Harbor, ME). Although the original uPA+/− mice were made in the 129S2 background (1), they were back-crossed for at least eight generations with C57BL/6 as of September 2004. Theoretically, this back-cross gives ~98% C57BL/6 background, so C57BL/6 mice were used as control. UUO or sham surgery was performed on 9- to 11-wk-old mice (n = 8–10 per group). The obstructed kidneys were harvested at 3, 7, 14, and 21 days. All procedures were performed in accordance with the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals.

Genotyping. Genotyping was performed by PCR using genomic DNA isolated from tails. PCR primer sequences were obtained from Jackson Laboratory. The neomycin primers oIMR0162–5'-CCGGT-TCTTTTTTGCAAGACCG-3' and oMR0163–5'-CGGCAGGAG-CAGGTTGAG-3' produce a 197-bp fragment of the null allele. The WT uPA primers oIMR0432–5'-TCTGGAGGACCGCT-TATCTG-3' and oIMR0433–5'-CTCTTCTCCAATGTGGGATTG-3' produce a 153-bp fragment. PCR conditions were as follows: 95°C for 5 min, then 94, 55, and 72°C for 1 min each for 30 cycles, and final extension at 72°C for 10 min. The PCR products were run on 3% agarose gels to identify the predicted bands.

Phenotyping. Mouse kidney uPA activity was quantified using a mouse Urokinase ELISA kit (Molecular Innovations, Southfield, MI). Briefly, the active PAI-1-coated microtiter plates bind only functionally active uPAR. The uPA was detected by a horseradish peroxidase-
conjugated anti-mouse uPA antibody, and oxidized tetramethylbenzidine substrate was measured by a spectrophotometer as optical density at 450 nm. A standard calibration curve was generated using dilutions of purified mouse uPA. Samples were measured in duplicate.

**Blood pressure measurement.** Because it was previously reported that blood pressure is lower in uPA−/− mice (23), systolic blood pressure was measured under light isoflurane anesthesia using a tail cuff connected to a PowerLab system and Chart Software (ADInstruments, Colorado Springs, CO). Measurements were taken on days 3 and 14 after UUO in WT and uPA−/− mice. The blood pressure of each mouse was measured at least three times, and average readings were reported (n = 8–10 each group).

**Histological examination.** Picrosirius red staining and immunohistochemical staining were performed on paraffin-embedded sections or cryosections using procedures established in our laboratory (16, 24, 32, 33). For picrosirius red staining, sections were deparaffinized by baking at 55°C for 1 h, hydrated, and stained with picrosirius red solution (0.1% Sirius red in saturated picric acid) for 18 h, followed by treatment with 0.01 N HCl for 2 min, dehydration, and coverslip mounting. Sections were examined by polarized light microscopy. Interstitial monocytes/macrophages were detected by staining paraffin-embedded tissue sections with F4/80 rat anti-mouse macrophage monoclonal antibody (Serotec, Oxford, UK) followed by peroxidase-conjugated, mouse plasma-absorbed F(ab')2 goat anti-rat IgG (Accurate Chemical & Scientific, Westbury, NY) using 3,3'-diaminobenzidine (DAB; Dako, Carpinteria, CA) as the chromogen. Interstitial myofibroblasts were quantified by staining using peroxidase-conjugated murine anti-human α-smooth muscle actin (α-SMA) 1A4 monoclonal antibody (Dako). The 1A4 antibody was detected using the Enhanced Polymer One-Step Staining (EPOS) reagent (Dako) as described previously (16, 24, 32, 33).

The images (×400 magnification) of five random, nonoverlapping cortical fields per slide were captured using a SPOT digital camera (Diagnostic Instruction, Sterling Heights, MI) and the stained tubulointerstitial area was quantified using a computer-assisted image analysis system (Image-Pro Plus software, Media Cybernetics, Silver Spring, MD) as described previously (16). Results were expressed as percentage of total tubulointerstitial area stained.

**Western blotting.** Pieces of frozen kidney were homogenized in 50 mM Tris, pH 7.5, 1% SDS or RIPA buffer with PMSF, protease inhibitor cocktail, and sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA). The protein concentration was determined by the bicinchoninic acid method (Beyotime, Beijing, China). Pieces of renal cortex were homogenized in 50 mM Tris, pH 7.5, 1% SDS or RIPA buffer with PMSF, protease inhibitor cocktail, and sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA).

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**Fig. 3. Interstitial F4/80+ macrophages after UUO.** A: representative photomicrographs illustrate the increased density of interstitial macrophages 7 days after UUO in mice of both genotypes (×400). B: representative Western blot illustrates F4/80 protein (110 kDa) and β-actin protein bands 3 and 7 days after UUO (42 kDa; n = 4 each group). C: graph summarizes the results of band density measurement normalized by β-actin (n = 4 each group) and expressed relative to the mean WT protein level on each day. F4/80 protein expression did not differ between WT and uPA−/− mice. Results are means ± SD.
using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Protein samples (20 μg) were separated by 4–15% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The primary antibodies used were F4/80 rat anti-mouse macrophage monoclonal antibody, goat anti-mouse E-cadherin (R&D Systems, Minneapolis, MN), mouse anti-Ksp-cadherin (Zymed Laboratories, South San Francisco, CA), sheep anti-mouse PAI-1 (American Diagnostica, Greenwich, CT), goat anti-mouse HGF antibody (R&D systems), Met mouse monoclonal antibody, phospho-Met rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA), mouse anti-human fibrinogen β-chain monoclonal antibody (American Diagnostica, Stamford, CT), mouse monoclonal anti-β-actin (Sigma, St. Louis, MO), and mouse monoclonal anti-beta tubulin-1 antibody (Abcam, Cambridge, MA). The secondary antibodies were hors eradish peroxidase-conjugated antibodies (Chemicon International, Temecula, CA), IRDye 800 infrared dye-labeled antibodies (Rockland, Gilbertsville, PA), and Alexa Fluor 680-labeled antibodies (Molecular Probes, Eugene, OR). Protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology) or the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) for fluorescent Western blots.

**Total collagen assay.** Total collagen was calculated based on measurement of the hydroxyproline concentration in hydrolysates of protein extracted from frozen kidney samples as described previously (16, 24, 32). Total collagen was calculated on the assumption that collagen contains 12.7% hydroxyproline by weight.

**Renal protease activity.** Renal protease activity was measured by plasminogen gel zymography as described previously (16, 24, 32). In brief, protein samples were separated by electrophoresis in 10% SDS-polyacrylamide gels containing 1 U/ml human plasminogen (Sigma) and 1% casein. The gel was soaked in 2.5% Triton X-100 solution and incubated for 16 h at 37°C in 0.1 M glycine-NaOH, pH 8.3, and stained with Coomassie blue. The gel was dried and scanned, and the size of each lytic band was measured using image analysis software (Quantity One, Bio-Rad, Hercules, CA).

**Expression of uPAR mRNA.** Total kidney uPAR mRNA levels were measured by real-time reverse transcriptase (qRT) PCR. Total RNA was extracted from kidney tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). The first-strand cDNA was generated by reverse transcriptase (iScript cDNA Synthesis kit, Bio-Rad). QPCR was performed using uPAR-specific primers [forward primer: 5′-AGCCAACGGACCTCTTCATTCTCTCT-3′, reverse primer: 5′-TTCGCTGGAAAGCTCTGAAGA-3′ (17) using the iCycler (Bio-Rad) standard protocol]. The housekeeping gene GAPDH was amplified using the forward primer 5′-ACTTTGTCAGCTTACATTTCC-3′ and the reverse primer 5′-TCGAGGAATCTTTATTTATGAG-3′. Standard curves were obtained for both uPAR and GAPDH primers. Reactions were run in triplicate, and uPAR mRNA was quantified relative to GAPDH.

**HGF activity and phosphorylation of Met.** The expression of pro-HGF, active HGF (α-chain), the HGF receptor Met, and active, phosphorylated Met were analyzed by Western blotting, normalizing each band to β-actin levels.

**Statistical analysis.** All data are expressed as means ± SD. Results were analyzed by ANOVA or t-test using STATA or Excel software. A P value <0.05 was considered statistically significant.

**RESULTS**

**uPA genotype/phenotype confirmation.** By PCR analysis, all WT mice were shown to have an intact uPA gene, identified by a 153-bp product, whereas the mutant uPA gene was identified by a 197-bp neomycin gene product. Urokinase activity, measured by ELISA, confirmed that the kidneys of the uPA−/− mice had minimal activity compared with WT (uPA−/−, 14 ± 5 ng/g protein, n = 32 vs. WT, 271 ± 113 ng/g protein, n = 8; Fig. 1). Systolic blood pressure measured by tail cuff with light anesthesia did not differ significantly from baseline at 3 and 14 days after UUO. Preoperative and day 14 post-UUO systolic blood pressures in WT mice were 180 ± 28 and 191 ± 28 mmHg, respectively, (n = 10 each time point). Preoperative and day 14 post-UUO systolic blood pressure readings in uPA−/− mice were 178 ± 24 and 189 ± 22 mmHg, respectively, (n = 8 each time point). Differences between WT and uPA−/− mice were not statistically significant at any time point.

**Renal fibrosis severity in WT and uPA−/− mice.** Picrosirius red staining showed similar levels of interstitial collagen accumulation in WT and uPA−/− mice (Fig. 2A). Total kidney collagen measured biochemically increased linearly from day 3 to day 21 after UUO in both WT and uPA-deficient mice (Fig. 2B). The sham operation did not change total collagen; levels were not significantly different from day 3 to day 14 after sham surgery.
surgery. Differences between the UUO groups of WT and uPA−/− mice were also not statistically significant at any time point (n = 8–10).

**Interstitial inflammation.** The renal response to UUO is characterized by development of an interstitial infiltrate of F4/80 expressing monocytes/macrophages. The F4/80-positive cells were localized in the interstitium, as confirmed by immunohistochemistry (Fig. 3A). By Western blot analysis there was no difference in F4/80 protein levels between WT and uPA−/− either 3 or 7 days after UUO (Fig. 3, B and C).

**Interstitial myofibroblasts.** After injury, fibroblasts are activated by fibrogenic signaling molecules such as transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) and are often identified as interstitial cells that express α-SMA. The density of α-SMA expressing interstitial myofibroblasts, expressed as the percentage of tubulointerstitial area stained for α-SMA, increased from 3 to 7 days after UUO; the area declined slightly from 14 to 21 days (Fig. 4). The percent α-SMA+ tubulointerstitial area was greater after UUO than after sham surgery but differences between WT and uPA−/− mice did not differ significantly at any time point.

**Tubular epithelial damage.** Renal function cannot be measured in the UUO model due to compensation by the normal contralateral kidney. Therefore, loss of expression of the normal tubular epithelial adhesion molecules E-cadherin and Ksp-cadherin was evaluated as surrogate measures of tubular injury as described previously (16, 32). Renal E-cadherin and Ksp-cadherin protein levels measured by Western blotting at 14 days were significantly decreased in the UUO kidneys compared with day 14 sham kidneys (Fig. 5). However, the differences between WT and uPA−/− mice on day 7 and day 14 after UUO were not significant (day 7 data not shown).

**Profile of renal plasminogen activators.** To determine whether the lack of uPA resulted in a compensatory increase in tPA activity, renal uPA and tPA activities were quantified by plasminogen gel zymography on day 14 after sham or UUO surgery (Fig. 6). The proteolytic bands were found at molecular weights appropriate for uPA (40 kDa) and tPA (65 kDa). In the WT mice, both uPA and tPA activity levels were increased significantly 14 days after UUO compared with sham surgery mice. In uPA−/− mice, tPA activity was increased while uPA activity remained negligible 14 days after UUO. The differences in tPA activity between WT and uPA−/− mice 14 days after sham or UUO were not statistically significant.

**Renal uPAR and PAI-1 expression.** The possibility was considered that the lack of uPA might result in compensatory changes in expression of the uPAR receptor that might influence the outcome by basal signaling activity or via activation by one of its other ligands (vitrorectine or kininogen). By semiquantitative real-time PCR, renal uPAR mRNA levels were found to be increased 14 days after UUO, but levels did not differ between WT and uPA−/− mice (Fig. 7).

Given that PAI-1 is thought to be degraded by a process that involves interaction with the uPA/uPAR complex, we postulated that PAI-1 levels might be elevated in uPA−/− mice, leading to secondary effects on fibrosis severity. To test this hypothesis, renal PAI-1 protein levels were evaluated by Western blotting and found to be similar in WT and uPA−/− mice on days 7 and 14 after UUO (Fig. 8).

**Active HGF and phosphorylated HGF receptor (Met) levels.** HGF has been shown to have impressive antifibrotic effects in experimental models of kidney injury (6, 14, 15, 19, 28). Because uPA is known to activate latent HGF (22), levels of the active HGF α-chain protein and the inactive pro-HGF protein were measured by Western blot analysis. HGF α-chain protein increased after UUO, peaking at day 7, while pro-HGF protein remained the same (data not shown). However, levels of pro-HGF protein, α-chain HGF protein, and the ratio of α-chain/pro-HGF in the WT and uPA−/− mice after UUO were not significantly different at 7 days (Fig. 9). To verify these results, activation of the HGF receptor (Met) was examined by Western blot analysis of total Met and its active, phosphorylated form (phospho-Met). Differences between the WT and uPA−/− mice were not significant (Fig. 10). Together, these data suggest that endogenous uPA does not
contribute substantially to HGF activation during chronic injury induced by UUO.

Renal fibrin(ogen) deposition. Fibrin is considered an important early provisional matrix protein in other solid organs, especially the lung (4, 5). To determine whether fibrin(ogen) accumulated in chronic tubulointerstitial disease and whether levels are regulated by endogenous uPA activity, fibrin(ogen) levels were evaluated by Western blot analysis. Renal fibrin B/β-fibrinogen protein levels on day 14 were significantly increased in the UUO kidneys compared with sham kidneys. However, there was no difference between WT and uPA−/− mice (Fig. 11).

DISCUSSION

The present study reports the unexpected finding that genetic uPA deficiency does not alter interstitial macrophage infiltration, myofibroblast recruitment, matrix accumulation, or tubular integrity when progressive injury is induced by UUO. These findings provide further evidence that the impressive fibrosis-promoting effects of PAI-1 in the renal interstitium appear to occur independent of its ability to block the activity of the endogenous serine proteases tPA, plasmin, and now uPA. The finding that uPA deficiency had no effect is remarkable given that the kidney expresses very high levels of this serine protease and that total enzyme activity increases after UUO. It further suggests that the effects of uPA are organ specific, as it has previously been shown that bleomycin-induced pulmonary fibrosis was attenuated by recombinant...
human urokinase in a rat model (7) and by adenovirus-mediated uPA−/− transfer in mice (27). In the liver, uPA gene therapy attenuates the severity of hepatic fibrosis (13). In the liver and the lung, fibrin forms an early provisional matrix that may be a key substrate for uPA. In the present study, kidney levels of fibrin B/fibrinogen were increased after UUO, but the levels were not influenced by the presence or absence of uPA activity.

Although it is always a concern that genetic uPA deficiency might result in compensatory changes in other genes that regulate serine protease activity, the present study failed to detect differences in renal tPA activity, PAI-1 protein levels, and mRNA levels of the receptor uPAR. An alternative consideration is the possibility that the antifibrotic activities of uPA might be redundant in the kidney. In particular, the ability of uPA to activate latent HGF has been proposed as its primary antifibrotic effects, at least in the lung (8). In the present study, we found that levels of the active HGF α-chain were similar in uPA−/− and WT mice, suggesting that an alternative pathway can activate latent HGF in the kidney.

In addition to extracellular effects, uPA is known to bind uPAR and possibly other cellular receptors (12, 30). Previous studies from our laboratory found that the fibrosis-modulating effects of uPAR were associated with several significant differences including enhanced uPA activity and PAI-1 degradation (30, 32, 33). The present study suggests that the greater uPA activity detected in the WT kidneys relative to uPAR−/− may not account for the renoprotective effects associated with uPAR expression.

It has been suggested that uPA might alter fibrosis by promoting cell migration via interactions that involve uPAR, integrins, low-density lipoprotein receptor-associated protein, and perhaps other cellular receptors (20). The pathogenesis of chronic kidney disease involves the recruitment of two important cell populations: monocytes/macrophages and (myo)fibroblasts. The finding that the densities of F/4/80+ macrophages and αSMA+ myofibroblasts were similar in uPA−/− and WT mice indicates that uPA does not play an essential role in recruitment of these cells. This finding differs from results obtained in an acute glomerulonephritis model that is characterized by significant fibrin deposition. Compared with nephritic WT mice, the number of glomerular macrophages was reduced in nephritic uPA−/− mice, although, curiously, other features of glomerular injury were similar (11).

An issue that deserves further consideration is whether sufficient quantities of endogenously generated uPA gain access to the interstitial space, where it would be needed if it were to modulate pathogenetic pathways that cause tubulointerstitial fibrosis. By contrast, apical uPA secretion into tubular lumina is clearly established (25). In situ zymography was not sufficiently sensitive to distinguish between interstitial and tubular uPA activity (data not shown). Whether increasing interstitial uPA activity would change fibrosis severity after UUO remains to be determined. In summary, data from the present study indicate that endogenous uPA activity does not regulate the renal fibrogenic response that is triggered by ureteral obstruction. It should be noted that UUO is a rather rapid model of progressive renal fibrosis. It remains possible...
that some effect of uPA might be observed in other, more slowly progressing models such as 5/6 nephrectomy and radiation nephropathy. Our findings provide further evidence that the predominant fibrosis-promoting actions of PAI-1 are mediated by direct cellular effects that are independent of its ability to inhibit extracellular serine protease activity. It is remarkable that the phenotype of uPA-deficient mice appears to be normal during development, postnatally, and even after severe renal injury. Why the kidney produces large quantities of uPA is a question that remains to be answered.

DISCLOSURES

A. A. Eddy is a member of the Amgen Nephrology Scientific Advisory Board.

REFERENCES


