Urokinase Receptor Modulates Cellular and Angiogenic Responses in Obstructive Nephropathy

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Abstract. Interstitial cells have been implicated in the pathogenesis of renal fibrosis. Given that the urokinase receptor (uPAR) is known to play a role in cell adhesion, migration, and angiogenesis, the present study was designed to evaluate the role of uPAR in the regulation of the phenotypic conversion of interstitial cells (macrophages, myofibroblasts, capillaries) in response to chronic renal injury. Groups of uPAR wild-type (+/+) and knockout (−/−) mice were investigated between 3 and 14 d after unilateral ureteral obstruction (UUO) or sham surgery (n = 8 mice per group). The density of F4/80+ interstitial macrophages (Mφ) was significantly lower in the −/− mice (3.3 ± 0.4 versus 6.9 ± 1.7% area at day 3 UUO; 10.8 ± 1.6 versus 15.7 ± 1.0% at day 14 UUO; −/− versus +/+). In contrast, in the −/− mice there were significantly more α smooth muscle actin (αSMA)–positive cells (12.9 ± 3.2 versus 7.8 ± 1.5% area at day 3 UUO; 21.0 ± 4.7 versus 9.7 ± 1.9% at day 14 UUO) and CD34-positive endothelial cells (8.4 ± 1.9 versus 4.0 ± 1.1% area at day 14 UUO). These differences were associated with significantly more interstitial fibrosis in the −/− mice based on Sirius red staining (4.6 ± 0.9 versus 2.3 ± 0.9% area at 14 d UUO). Absence of the uPAR scavenger receptor was associated with significantly greater accumulation of plasminogen activator inhibitor-1 protein (PAI-1) (20.5 ± 3.5 versus 9.1 ± 2.9% area, day 14 UUO) and vitronectin protein (2.4 ± 1.1 versus 0.9 ± 0.4% area, day 14 UUO). By immunostaining αSMA+ cells, CD34+ cells, vitronectin and PAI-1 co-localized to the same tubulointerstitial area. The number of apoptotic cells increased in response to UUO but was significantly higher in the −/− mice (2.0 ± 0.2 versus 1.2 ± 0.2 per 100 tubulointerstitial cells, day 14 UUO) while the number of proliferating cells was significantly lower in the uPAR−/− mice. These data suggest that uPAR deficiency suppresses renal Mφ recruitment, but the absence of this scavenger receptor actually accentuates the fibrogenic response, likely due in part to the delayed clearance of angiogenic/profibrotic molecules such as PAI-1 and decreased receptor-associated uPA activity.

Interstitial fibrosis and subsequent tubular atrophy are pivotal pathologic changes that lead to end-stage kidney disease (1). Renal fibrosis is the end result of a series of events often initiated by the recruitment of monocytes from the circulation, their activation, and differentiation into macrophages. Macrophage activities are numerous and may include trophic effects (growth promoting, cellular differentiation, tissue repair, angiogenesis), cytotoxic tissue injury, and scavenging activities. Tubulointerstitial infiltration by macrophages is thought to play an active role in tissue fibrogenic reactions (1,2). Several molecules, including macrophage chemoattractant protein-1 (MCP-1), osteopontin, transforming growth factor-β (TGF-β), RANTES, integrins, and the urokinase-plasmin cascade, have been implicated in the processes of macrophage adhesion and migration (3). Myofibroblasts usually become the predominant interstitial cell type with chronic inflammation. These cells are considered to be the major source of the extracellular matrix components that accumulate during renal fibrosis. Myofibroblasts appear de novo in areas of future fibrosis in response to stimuli such as basic fibroblast growth factor, platelet-derived growth factor, TGF-β1, metalloproteinase-2 (MMP-2), and plasminogen activator inhibitor-1 (PAI-1). Despite significant advances, knowledge about the regulation and function of macrophages and myofibroblasts during renal fibrosis remains incomplete, a fact that has hampered the development of effective therapy for patients with progressive renal disease. A high-affinity cellular receptor for urokinase-type plasminogen activator (uPAR or CD87) has been identified on the plasma membrane of a variety of cell types, including monocytes, neutrophils, activated T cells, endothelial cells, glomerular epithelial and mesangial cells, tubular epithelial cells, fibroblasts, and myofibroblasts (4–10). First identified in 1985 (11), uPAR is a highly glycosylated 50-kD to 60-kD protein. Its currently known ligands are uPA, vitronectin, and kininogen (12). uPAR itself lacks a transmembrane domain. It is...
anchored to the plasma membrane by a glycosyl phosphatidylinositol (GPI) moiety. Soluble forms also exist. Tubular uPAR immunoreactivity has been reported in normal human kidneys (10). Changes in the pattern of uPAR expression have not yet been extensively investigated in renal disease. Increased renal production of uPAR has been reported in humans and mice with endotoxemia and in human kidneys with chronic pyelonephritis, acute tubular necrosis, and chronic allograft rejection (7,8,13,14).

It is now clear that uPAR is a multifunctional protein (15,16,17). The glycolipid-anchored uPAR co-localizes peri-cellularly with components of the urokinase-plasmin activation system, including uPA and PAI-1, and endocytosis receptors such as members of the LDL receptor related protein (LRP) family. uPAR is frequently co-expressed with caveolin and members of the beta integrin superfamily (12). The formation of functional units with these cellular proteins allows uPAR to generate cell surface concentrated proteolysis required for cell migration and also to contribute to non-proteolytic cellular adhesion by interacting with β2-leukocyte integrins or αvβ3 or αvβ5 integrins and vitronectin (18–20). Due to these cellular functions, uPAR is thought to regulate cellular responses during angiogenesis, inflammation, wound repair, and tumor metastasis (12,19,21,22). In addition to its ability to modulate cell migration, uPAR may mediate molecular crosstalk at cellular surfaces, cytoskeletal reorganization, endocytosis-dependent scavenging, and cellular apoptosis (12,17,23).

Given these important functions for uPAR together with the observation that PAI-1 and uPA may be upregulated during renal fibrosis, the present study was designed to investigate the role of uPAR in the renal cellular response that follows ureteral obstruction.

Figure 1. Urokinase receptor (uPAR) genotype and its renal expression. (A) Southern blot analysis demonstrating the 5-kb band of the wild-type uPAR gene in the uPAR+/+ mice and the 3.5-kb mutant band in the uPAR−/− mice. (B) Northern blot analysis shows specific uPAR1 mRNA bands in the kidneys of uPAR+/+ mice 7 d after unilateral ureteral obstruction (UUO).

Figure 2. uPAR expression. After 7 d of UUO, uPAR protein was not detected in uPAR−/− kidneys (A). At this time, uPAR was expressed by interstitial cells (B) and tubular epithelial cells (C). Magnification: ×400.
were processed for cryostat and microtome sectioning. The day 7 UUO kidneys and their sham controls were processed for protein and RNA extraction. Pieces to be embedded in paraffin were fixed in 10% buffered formalin, and those for cryostat sectioning were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrence, CA) and snap-frozen in pre-chilled 2-methylbutane. Tissues for protein and mRNA extraction were snap-frozen in liquid nitrogen and stored at \(-80^\circ\mathrm{C}\) for subsequent use. For protein isolation, frozen kidney tissue from each animal was individually ground into a fine powder using a pre-chilled mortar and pestle, homogenized in extraction buffer (0.05 M Tris, 0.01 M CaCl\(_2\), 2.0 M guanidine HCl, 0.2% Triton X-100, pH 7.5), and dialyzed using dialysis membrane Spectra/Por\textsuperscript{R} 1 (Spectrum Medical Industries, Inc., Houston, Texas) against 0.05 M Tris, 0.2% Triton X-100, pH 7.5, for 48 h at 4\(^\circ\)C. The samples were centrifuged for 5 min (14,000 \(\times\) g). The supernatant was aliquoted on the basis of the protein concentration measured using the Bradford protein assay (Bio-Rad, Hercules, CA). The aliquoted samples were stored at \(-80^\circ\)C.

**Northern Blot Analysis**

Total kidney RNA was isolated by a modified phenol and guanidine isothiocyanate method using TRIZol reagent (Life Technologies BRL, Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Total kidney RNA (15 \(\mu\)g) from each animal was separated by a 1% agarose formaldehyde gel electrophoresis. After a photomicrograph of the ethidium bromide-stained gel was obtained to evaluate RNA loading equality, the RNA was transferred to a hybridization membrane (GeneScreen Plus, New England Nuclear Life Science Products, Boston, MA) and fixed by ultraviolet cross-linking (UV Crosslinker, Hoeffer Scientific Instruments, San Francisco, CA). Complementary DNA probes for mouse uPAR1 (a 1.5-kb fragment), provided by Dr. Niels Behrendt, Finsen Laboratory, Copenhagen, Denmark (25), rat osteopontin, provided by Dr. C. Giachelli, University of Washington, Seattle, WA (26), and mouse MCP-1 provided by Dr. B. Rollins, Dana Farber Cancer Institute, Boston, MA (27), were labeled with \(^{32}\)P dCTP (3,000 Ci/mmol) by random priming with the T7 Quick Prime kit (Pharmacia Biotech, Piscataway, NJ). The membranes were hybridized with the radiolabeled cDNA probe using the QuickHyb hybridization solution (Stratagene, La Jolla, CA). Autoradiographs were developed and the density of each band quantified using the NIH Image program. The density of the 18 s ribosomal bands in the formaldehyde gels were used for RNA loading control.

**Histologic Studies**

Immunohistochemical studies were performed on frozen or paraffin-embedded renal tissue sections (4 \(\mu\)m). Immunoperoxidase staining using ABC ELITE kits (Vector Laboratories Inc, Burlingame, CA) was performed on paraffin sections. Primary antibodies (Ab1) included anti-mouse uPAR (R&D Systems, Minneapolis, MN), rat anti-mouse F4/80, rat anti-mouse CD11b monoclonal antibodies (Serotec Ltd., Oxford, UK), goat anti-mouse MCP-1 (Santa Cruz Biotechnology, CA), anti-mouse osteopontin (Santa Cruz Biotechnology), horseradish peroxidase (HRP)-conjugated mouse anti-human smooth muscle actin (SMA) monoclonal antibody (DAKO Corp., Carpinteria, CA), HRP-conjugated mouse-anti-polymerizing cell nuclear antigen (PCNA; DAKO), murine anti-mouse PAI-1 monoclonal antibody (MA-33H1F7, a generous gift from Dr. Declerck, Katholieke University, Belgium) (28), and rat anti-mouse CD34 antibody (Pharmingen, San Diego, CA). For PAI-1 staining, Ab1 and Ab2 were pre-complexed before incubation with the tissue sections to minimize cross-reactivity between Ab2 and murine tissue IgG (29). Cryosec-

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**Materials and Methods**

**Animals and Experimental Protocol**

UPAR-deficient (uPAR\(-/-\)) and wild-type (uPAR\(+/+\)) mice on a C57BL/6 genetic background used for this study have been previously described (24). Mice were bred in our animal facility and allowed to grow to a minimum weight of 20 g before the study began. The genotype of the mice was confirmed by Southern blot analysis of DNA extracted from tails. Five groups of gender-matched, age-matched, and weight-matched uPAR\(+/+\) mice were studied: 3 d after UUO (female, \(n=8\) each), 7 d after UUO (male, \(n=8\) each), 14 d after UUO (female, \(n=8\) each), and 7 d after sham surgery (\(n=8\) males and \(8\) females). UUO surgery was performed under general anesthesia. The left ureter was ligated with 4.0 silk at two separate locations in the UUO groups. Mice were killed by exsanguination under general anesthesia. All procedures were performed in compliance with the guidelines established by National Research Council Guide for the Care and Use of Laboratory Animals.

**Kidney Tissue Preparation**

Following exsanguination, the obstructed left kidney was harvested and the capsule removed. Day 3 and day 14 UUO and sham kidneys were processed for cryostat and microtome sectioning. The day 7 UUO kidneys and their sham controls were processed for protein and RNA extraction. Pieces to be embedded in paraffin were fixed in 10% buffered formalin, and those for cryostat sectioning were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrence, CA) and snap-frozen in pre-chilled 2-methylbutane. Tissues for protein and mRNA extraction were snap-frozen in liquid nitrogen and stored at \(-80^\circ\)C for subsequent use. For protein isolation, frozen kidney tissue from each animal was individually ground into a fine powder using a pre-chilled mortar and pestle, homogenized in extraction buffer (0.05 M Tris, 0.01 M CaCl\(_2\), 2.0 M guanidine HCl, 0.2% Triton X-100, pH 7.5), and dialyzed using dialysis membrane Spectra/Por\textsuperscript{R} 1 (Spectrum Medical Industries, Inc., Houston, Texas) against 0.05 M Tris, 0.2% Triton X-100, pH 7.5, for 48 h at 4\(^\circ\)C. The samples were centrifuged for 5 min (14,000 \(\times\) g). The supernatant was aliquoted on the basis of the protein concentration measured using the Bradford protein assay (Bio-Rad, Hercules, CA). The aliquoted samples were stored at \(-80^\circ\)C.

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tions were stained with antibodies to vitronectin (rabbit anti-mouse polyclonal antibody; a generous gift from Dr. David Loskutoff, The Scripps Research Institute, CA) (30) and αv integrin (goat anti-mouse αv integrin antiserum; Santa Cruz Biotechnology). The Ab2 used were FITC-conjugated goat anti-rabbit IgG (Organon Teknika Corp., West Chester, PA) or FITC-conjugated rabbit anti-goat IgG (Southern Biotechnology Associates). For co-localization studies, a few additional serial UUO paraffin sections were stained for αSMA and CD34.

Figure 4. CD11b-positive interstitial macrophages. (A through D) Photomicrographs of CD11b immunohistochemical staining in 3 d UUO (A and B) and 14 day UUO (C and D) kidneys. At both times, there are significantly fewer macrophages in the uPAR−/− mice (B and D) compared with the uPAR+/+ mice (A and C). Magnification: ×750. (E) The CD11b-positive cells per 100 tubulointerstitial cells, expressed as mean ± 1 SD. Open bars are uPAR−/−; closed bars are uPAR+/+. * P < 0.05, uPAR−/− versus uPAR+/+. † P < 0.01, UUO compared with sham group.
vitronectin, or PAI-1. αSMA and CD34 double-staining were performed with HRP-conjugated and alkaline phosphatase-conjugated secondary antibodies. Sections stained with Ab2 only were run in parallel as a negative control. The stained tubulointerstitial area was measured using a computerized image analysis system and Optimas software (Optimas version 6.5, Optimas Corp., Bothell, WA) as previously reported (31). A point-counting method was used to quantify F4/80+ macrophage staining. Results were expressed as percentage of total measured tubulointerstitial area. CD11b+ interstitial cells and PCNA-positive tubular and interstitial cells were counted manu-

Figure 5. Renal αv integrin expression. (A through D) Photomicrographs of αv integrin immunofluorescence staining in sham (A and B) and 14 day UUO (C and D) kidneys. Weak tubular staining is present in a few tubules of sham uPAR+/+ kidneys (A) and uPAR−/− kidneys (B). Tubular staining is increased on day 14 UUO but to a greater degree in the +/+ kidneys (C) than the −/− kidneys (D). Magnification: ×400. (E) The αv integrin-positive tubulointerstitial area expressed as mean ± 1 SD. Open bars are uPAR−/−. Closed bars are uPAR+/+. * P < 0.001, uPAR−/− versus uPAR+/+. + P < 0.01, UUO compared with sham group.
ally using an eyepiece grid and expressed as the percent positive tubulointerstitial cells as described previously (32).

**Western Blot Analysis**

Protein samples (20 or 80 μg) were separated by 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and the immunoreactive protein visualized using ECL-enhanced chemiluminescence (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Ab1 used were rabbit anti-mouse CD14 antiserum (Santa Cruz Biotechnology), mouse anti-murine PAI-1 monoclonal antibody, HRP-conjugated mouse anti-PCNA monoclonal antibody, and HRP-conjugated mouse anti-human α-SMA monoclonal antibody. Ab2 were HRP-conjugated goat anti-rabbit IgG antiserum (Chemicon International Inc.) and HRP-conjugated goat anti-mouse IgG antiserum (Sigma Chemical Co.). For PAI-1 probing, Ab1 and Ab2 were premixed before incubation with the blots to block the crossreaction of Ab2 to tissue mouse IgG (29). Ponceau S red or amido black staining of the blots was performed to determine loading equality.

**In Situ End Labeling of Apoptosis**

Apoptotic cell nuclei were detected by in situ end labeling of endonuclease-cleaved DNA as described previously (33). Briefly, sections were deparaffinized and stripped of proteins by incubation with 20 μg/ml proteinase K in PBS at 37°C for 15 min. Endogenous peroxidase was inactivated by immersing the sections in 3% H2O2 for 5 min. After pre-incubation with equilibration buffer, the samples were incubated with TdT in reaction buffer (containing bio-14-dUTP) at 37°C for 60 min. Rinsed with PBS, the tissue sections were stained with the ABC ELITE kit (Vector Lab. Inc.). Color was developed with AEC Substrates Chromogen (DAKO Corp.) and counterstained with hematoxylin. Negative controls were obtained by eliminating TdT in reaction buffer while other steps were run in parallel. Apoptotic tubulointerstitial cell nuclei were counted in 10 random cortical fields (×400 magnification). Results were expressed as the number positive per 100 nuclei. For day 3 UUO kidneys, the number of apoptotic nuclei within tubular cross-sections and the interstitium were evaluated separately.

**Interstitial Fibrosis Evaluated by Sirius Red Staining**

Picrosirius red staining was performed to evaluate histologically the interstitial area occupied by collagen fibrils as described previously (31). Sections were examined by polarized light microscopy. Photographs of six random cortical fields (×400) from each animal...
Figure 7. Osteopontin (OPN) expression. A Northern blot autoradiograph 7 d after UUO demonstrates significantly increased renal osteopontin (OPN) mRNA compared with sham kidneys. OPN mRNA was significantly less abundant in the uPAR−/− compared with the uPAR+/+ kidneys (A). Immunostaining detected OPN protein in renal tubules; the lower expression levels in the uPAR−/− animals appeared to be attributed to more extensive tubular destruction (B: uPAR+/+ day 7 UUO; C: uPAR−/− day 7 UUO; D: uPAR+/+ day 14 UUO; E: uPAR−/− day 14 UUO; F: uPAR+/+ sham). Magnification: ×400. The histograms illustrate the day 7 results expressed as mean ± 1 SD. Open bars are uPAR+/+; closed bars are uPAR−/−. * P <0.05, uPAR−/− versus uPAR+/+ mice. † P < 0.05 UUO compared with sham mice of the same genotype.
Figure 8. Alpha smooth muscle actin (α-SMA) expression. (A through D) Photomicrographs of α-SMA immunohistochemical staining in 3 d (A and B) and 14 d UUO (C and D) kidneys. Significantly less staining is present in the interstitium of uPAR+/− kidneys (A and C) than in the uPAR−/− kidneys (B and D). Magnification: ×400. (E) The α-SMA-positive tubulointerstitial area expressed as mean ± 1 SD. Open bars are uPAR−/−; closed bars are uPAR+/+. * P < 0.05, ** P < 0.01, uPAR−/− versus uPAR+/+. † P < 0.01, UUO compared with correspondent sham.
Figure 9. CD34-positive microvasculature. (A through D) Photomicrographs of CD34 immunohistochemical staining in sham (A and B) and 14 d UUO (C and D) kidneys. In the sham uPAR+/+ (A) and −/− (B) kidneys, CD34 antigen is expressed by peritubular capillaries and glomerular endothelial cells (arrow). Following ureteral obstruction, the CD34-positive interstitial area is reorganized with expansion in fibrotic areas (stars). These changes are more marked in the uPAR−/− mice (D) compared with the uPAR+/+ (C) mice. Magnification: ×400. (E) The CD34 tubulointerstitial area expressed as mean ± 1 SD. Open bars: uPAR−/−; closed bars: uPAR+/+. * P < 0.01, uPAR−/− versus uPAR+/+. 
demonstrated undetectable uPAR message in the kidneys of uPAR−/− mice and the sham uPAR+/+ mice. In the kidneys of the uPAR+/+ mice, uPAR mRNA was present 7 d after UUO (Figure 1B). uPAR immunohistochemical staining was negative on sham and all uPAR−/− kidneys. In response to UUO, uPAR expression was detected on interstitial and tubular cells in the uPAR+/+ kidneys (Figure 2).

**Interstitial Macrophages and α Integrin Expression**

In response to UUO and compared with the sham-operated kidneys, the number of F4/80+ interstitial macrophages was significantly increased at 3 d only in the uPAR+/+ mice; by day 14, the number of F4/80+ interstitial macrophages was significantly increased in the mice of both genotypes (Figure 3). However, at both time points, there were significantly fewer F4/80+ cells in the uPAR−/− mice compared with the +/+ mice. This difference in the number of renal macrophages was reconfirmed when CD11b+ interstitial cells were counted (Figure 4). Western blot analysis for CD14, another murine monocyte/macrophage antigen, on day 7, also showed that CD14 protein levels were 1.5-fold higher in uPAR+/+ mice (1.00 ± 0.11 versus 0.63 ± 0.02 arbitrary units).

Given that previous studies have suggested that uPAR may interact with αvβ3 and αvβ5 integrin receptors to facilitate leukocyte adhesion and migration, expression of the αv integrin chain was examined (18,19,22). In the +/+ mice αv integrin was expressed at low levels on a few cortical tubules in the sham control kidneys. αv protein was increased tenfold in the day 14 UUO group, but its expression was primarily restricted to tubules (Figure 5). Although tubular expression of αv integrin also increased in uPAR−/− mice during UUO, this adhesive molecule was expressed at a significantly lower level in uPAR−/− mice on days 14 UUO compared with +/+ mice.

Several chemoattractant molecules are also known to participate in renal monocyte recruitment triggered by ureteral obstruction such as monocyte chemoattractant protein-1 (MCP-1) and osteopontin. In response to 7 d of UUO, renal MCP-1 and osteopontin mRNA levels were significantly increased, but only osteopontin expression was attenuated in the uPAR−/− mice compared with uPAR+/+ mice (Figures 6 and 7). In fact, MCP-1 levels were higher in the uPAR−/− mice on day 7. Western blot analysis reconfirmed the difference in MCP-1 expression between the uPAR+/+ and −/− mice (Figure 6). Immunostaining of the obstructed kidneys detected MCP-1 protein mainly in renal tubules but some interstitial reactivity was also present. Osteopontin expression in the UUO kidneys was limited to a subpopulation of renal tubules, and the difference between the genotypes appeared to be due to greater tubular destruction in the uPAR−/− mice, especially on day 14 when several of the osteopontin-positive cells appeared as tubular remnants within the interstitium (Figure 7).

**Results**

**uPAR Genotype and Renal Expression**

The genotypes of the experimental mice were confirmed by Southern blot analysis (Figure 1A). Northern blot analysis was taken using a SPOT camera and the percent positive tubuloin- terstitial area measured using the Optimas program.

**Statistical Analyses**

All data were expressed as mean ± 1 SD unless otherwise stated. Results were analyzed by the Mann-Whitney U test or t test using the SPSS or Excel software. A P value < 0.05 was considered statistically significant.

**Results**

**uPAR Genotype and Renal Expression**

The genotypes of the experimental mice were confirmed by Southern blot analysis (Figure 1A). Northern blot analysis...
of positive interstitial cells significantly increased with time, but the response was more intense in the uPAR−/− mice (Figure 8). The mean number of interstitial myofibroblasts was 1.8-fold and 2.1-fold higher in uPAR−/− compared with the +/+ mice on days 3 and 14 UUO, respectively. This difference between the two genotypes was reconfirmed on day 7 UUO by Western blot analysis that detected 2.4-fold more α-SMA protein in the kidneys of the uPAR−/− mice (data not shown).

The interstitial cellular response to UUO was also characterized by increased angiogenesis, as defined by the density of CD34+ microvascular endothelial cells (34). The degree of neovascularization was significantly greater in the uPAR−/− group compared with the +/+ group (Figure 9). These CD34+ microvessels co-localized with α-SMA-positive interstitial myofibroblasts (Figure 10). There was a significant positive correlation between the numerical values for the area of the tubulointerstitium stained for CD34 and αSMA (r = 0.81; P < 0.05; Spearman rank correlation).

Figure 11. Tubulointerstitial cell apoptosis. Photomicrographs illustrating in situ end labeling (ISEL) of apoptotic nuclei after 14 day UUO in uPAR+/+ (A) and −/− (B) kidneys. Arrows indicate apoptotic cells with typical condensed nuclei within the tubules and interstitial area. Sections were counterstained with hematoxylin. Magnification: ×400. The graphs show the total number of apoptotic tubulointerstitial cells (C) and the number of apoptotic tubular cells (TC) and interstitial cells (IC) counted separately at 3 day UUO (D) expressed as mean ± 1 SD. Open bars are uPAR−/−; closed bars are uPAR+/+. * P < 0.05, uPAR−/− versus uPAR+/+. † P < 0.05, UUO versus sham (C).
versus expression levels as an estimate of mitotic activity, Western

affected the proliferative response to injury. Using PCNA

and interstitial cells on day 3 and day 14 UUO. Genotype also

resulted in the appearance of significantly more apoptotic tubular

with time following UUO (Figure 11). uPAR deficiency re-

sham kidneys of both genotypes, but the number increased

Tubulointerstitial Cell Apoptosis and Proliferation

Figure 12. Tubulointerstitial cell proliferation. Western blot analysis
detected a significant increase in PCNA expression 3 d (A) and 14 d
(B) after UUO between uPAR+/+ and −/− mice. Densitometric
analysis of the blots showed significantly less PCNA protein, reflect-
ing lesser mitotic activity, in the uPAR−/− mice (open bars) com-
pared with the uPAR+/+ mice (closed bars) (C). PCNA immunohis-
tochemical staining identified most PCNA-positive (black) nuclei as
tubular epithelial cells (TC) with lesser numbers of positive interstitial
cells (IC). Fewer proliferating TC and IC were detected in the
uPAR−/− kidneys (D; day 3 UUO). Photomicrographs are represen-
tative fields 3 d (E: uPAR+/+; G: uPAR−/−) and 14 d after UUO
(F: uPAR+/+; H: uPAR−/−). Magnification: ×400. Results are
shown graphically as mean ± 1 SD. Open bars are uPAR−/−; closed
bars are uPAR+/+. * P < 0.05, uPAR−/− versus uPAR+/+. † P <
0.05, UUO versus sham mice of the same genotype.

Tubulointerstitial Cell Apoptosis and Proliferation

Apoptotic tubulointerstitial cells were rarely detected in
sham kidneys of both genotypes, but the number increased
with time following UUO (Figure 11). uPAR deficiency re-
sulted in the appearance of significantly more apoptotic tubular
and interstitial cells on day 3 and day 14 UUO. Genotype also
affected the proliferative response to injury. Using PCNA
expression levels as an estimate of mitotic activity, Western

blot studies indicated that proliferation was an early response
to UUO (Figure 12). However, at both 3 and 14 d, PCNA
protein levels were significantly higher in the uPAR+/+ kid-
neys compared with the uPAR−/− kidneys. By semiquantita-
tive immunohistochemistry it was determined that most
PCNA-positive cells in the UUO kidneys were tubular epithe-
lial cells although rare positive interstitial cells were also
detected (Figure 12).

Accumulation of Vitronectin and PAI-1

Vitronectin, a PAI-1-binding extracellular matrix protein
that is a ligand of αvβ3 and αvβ5 integrins (18,19), was
restricted to the vasculature and glomeruli in the sham-oper-
ated kidneys. After UUO, vitronectin was deposited within
atrophic tubules and the interstitium to a greater extent in the
uPAR−/− mice compared with +/+ mice (Figure 13). By
immunostaining PAI-1 protein, not detected in the sham kid-
ney, accumulated in interstitial areas, often co-localizing with
α-SMA-positive cells (Figure 14). Western blot analysis dem-
onstrated significantly more PAI-1 protein (1.5-fold increased)
in uPAR−/− compared with +/+ kidneys on days 7 after
UUO (Figure 15).

Interstitial Fibrosis

Sirius red staining showed an impressive increase in inter-
stitial collagen fibrils after UUO, reaching a tenfold increase in
the uPAR-deficient mice by 14 d (Figure 16). The Sirius
red-positive interstitial area was significantly less in the
uPAR+/+ mice compared with the −/− mice after 14 d of
ureteral obstruction.

Discussion

The results of this study indicate that the urokinase receptor
serves to dampen the severity of the renal fibrogenic response
that is initiated by ureteral obstruction. In this experimental
model, we have previously reported that renal uPA gene ex-
pression and enzyme activity are significantly increased (35).
From the present study, it is evident that uPAR plays a pivotal
role in the regulation of the cellular responses to ureteral
obstruction but that its role is highly cell specific. Cells of
multiple lineages may express uPAR including resident kidney
cells (epithelial, mesangial, and endothelial), inflammatory
cells (monocytes, activated T cells, and neutrophils), and fi-
broblasts/myofibroblasts. In response to ureteral obstruction,
renal uPAR gene expression was significantly upregulated.
The uPAR protein was identified on both interstitial cells and
renal tubules in wild-type kidneys after UUO. The expression
of uPAR in these regions of the kidney coupled with significant
differences in the number of interstitial monocytes, myofibro-
blasts, and interstitial endothelial cells in uPAR wild-type mice
compared with uPAR null mice suggests that recruitment and
perhaps function of these cells are modulated by uPAR.

Monocytes and macrophages that pervade the interstitium of
chronically damaged kidneys are thought to be one of the
mediators of fibrosis due their ability to synthesize several
pro-fibrotic molecules (36). The present study is one of the first
to demonstrate that the phenotype of the inflammatory renal
interstitial cells is a critical factor that determines whether the macrophages function primarily as scavengers to minimize injury or as villains that perpetrate damage. In the absence of uPAR, not only is interstitial macrophage recruitment impaired, but the absence of this "scavenging" receptor appears to delay the clearance of molecules that promote fibrosis. Although many molecules may be involved, our data suggest that delayed clearance of PAI-1 and perhaps apoptotic cells by uPAR-bearing cells may contribute to a more aggressive fibrotic response. In addition to several in vitro studies docu-

Figure 13. Vitronectin accumulation. (A through D) are photomicrographs of vitronectin immunofluorescence staining in sham (A and B) and 14 d UUO (C and D) kidneys. Vitronectin is present in glomeruli (block arrow) and vessels (single arrow) of sham uPAR+/+ kidneys (A) and uPAR−/− kidneys (B). Vitronectin accumulated in the tubulointerstitium, especially within dilated or atrophic tubules, by day 14 UUO. Compared with the −/− kidneys (D), the extent of vitronectin deposition was less in the +/+ kidneys (C) and was limited to a few fibrotic loci. Magnifications: ×250 in A and B; ×400 in C and D. (E) The vitronectin-positive tubulointerstitial area expressed as mean ± 1 SD. Open bars are uPAR−/−; closed bars are uPAR+/+. * P < 0.05, uPAR−/− versus uPAR+/+. † P < 0.05 UUO versus sham of the same genotype.
menting a role for uPAR in cellular movement, impaired migration of tumor cells and neutrophils has also been reported in uPAR-deficient mice (22,37). In the mouse model of bleomycin-induced lung fibrosis, delayed macrophage recruitment has also been observed in uPAR-deficient mice (38). The findings in the present study of significantly fewer renal macrophages in uPAR−/− mice contrasts with the results of an earlier study of acute crescentic glomerulonephritis that demonstrated that neither uPAR nor uPA-deficiency modified the severity of glomerular inflammation or renal dysfunction, possibly because increased uPA is not a significant feature in that model of acute glomerular injury (39).

The increased number of interstitial mononuclear cells in chronically damaged kidneys is thought to be the consequence of the migration of circulating monocytes into the interstitium although limited in situ proliferation of resident interstitial macrophages may also occur (40). In the present study, most of the PCNA+ cells were tubular cells, and a difference in the number of positive interstitial cells was relatively small but significant. Whether the proliferating cells were macrophages or myofibroblasts was not determined. Urokinase is known to play a role in cell migration due to its ability to facilitate cell-cell and cell-matrix interactions. uPAR is known to associate with the leukocyte integrin CD11b/CD18 and L-selectin to regulate leukocyte migration and cellular signaling (20,41,42). In addition, the uPA-uPAR complex physically cooperates with certain members of the integrin superfamily including αvβ5 and αvβ3 to direct cell adhesion to and migration along vitronectin (18,19,43). Co-clustering and resonance energy transfer between uPAR and αvβ5 or αvβ3 integrins has been observed to transduce migratory signals to cells adherent to vitronectin (44). In addition to serving as an inte-

Figure 14. Renal plasminogen activator inhibitor-1 protein (PAI-1) accumulation. Immunohistochemical staining on day 14 UUO shows greater PAI-1 accumulation in the interstitium of the uPAR−/− mice (B) compared with the uPAR+/+ mice (A). Staining of serial sections illustrates co-localization of PAI-1 protein (C) to areas of interstitial αSMA+ myofibroblasts (D). Stars highlight regions stained for both PAI-1 and α-SMA. PAI-1 protein is not detected in uPAR−/− sham kidneys (E). Magnification: ×400. The graph illustrates the PAI-1-positive tubulointerstitial area on day 14. * P < 0.01.
grin-associated protein, the cell surface glycolipid-anchored uPAR may also serve as an integrin ligand, thereby mediating direct contact with adjacent cells (45). In the present study, expression of the αv integrin chain was enhanced in response to UUO but it was most abundant on tubular cells. Expression of the αv integrin chain by tubular cells was attenuated in the uPAR-deficient mice at 14 d, perhaps an indication that, like the leukocyte integrin CD11b/CD18, clustering of uPAR may induce αv integrin expression (46) or that the two receptors are coordinately expressed (19).

Furthermore, uPA may cleave its receptor to release soluble uPAR, a molecule with monocyte chemoattractant properties (47). It has recently been reported that this chemotactic response is triggered by interactions of soluble uPAR with the FPRL1/LXA4 receptor (formyl-methionyl-leucyl-leucyl-proline[fMLP]-like receptor-1/lipoxin A4 receptor) (48). Additional studies will be necessary to determine if uPAR-integrin and/or soluble uPAR-FPRL1/LXA4 receptor interactions fully explain why the interstitial recruitment of uPAR-deficient monocytes is impaired in obstructive uropathy. Several chemokines and adhesion molecules have been implicated in the genesis of the interstitial inflammatory response to obstruction, including MCP-1 (49) and osteopontin (50). We cannot eliminate the possibility that the lower levels of osteopontin observed in the uPAR−/− mice also contributed to the blunted inflammatory response. In fact, osteopontin-induced cell migration may be dependent on uPA-uPAR activity (51).

Despite the fact that uPAR deficiency dampened the intensity of the interstitial inflammatory response to obstruction, the severity of fibrosis was worse in the uPAR-deficient mice. This outcome is likely due to the more aggressive myofibroblastic response that developed in the uPAR-deficient mice. The development of a myofibroblastic phenotype in the renal interstitium has been highly predictive of renal functional deterioration due to fibrosis (52–54). Myofibroblasts are currently considered to be a major source of the matrix proteins that accumulate in the kidney during fibrosis. The specific cellular origin of interstitial myofibroblasts remains controversial but possibilities included transformed resident interstitial fibroblasts, transdifferentiated tubular epithelial cells, migratory vascular cells and pericytes cells, circulating mesenchymal precursor cells, and perhaps even transformed monocytic cells (3,55). Given the unknown origin of the interstitial myofibroblasts, it is impossible to determine if uPAR plays a direct role in promoting or impairing their migration. Our in vivo observations argue against differences in myofibroblast proliferation and/or apoptosis as an explanation but this possibility should be investigated more carefully in vitro. Of note is the in vivo
observation that uPAR deficiency does not affect the migration of smooth muscle cells (56). Our results also suggest the possibility that impaired “scavenging” activities in the uPAR null mice may have resulted in the appearance of significantly more myofibroblasts as a secondary consequence. Parallel genotype-dependent differences in the extent of interstitial angio-

Figure 16. Interstitial collagen accumulation. (A through D) Photomicrographs illustrating picrosirius red staining of sham uPAR+/+ and −/− kidneys (A and B, respectively) and day 14 UUO uPAR+/+ and −/− kidneys (C and D, respectively). Magnification: ×400. The graph shows the Sirius red-positive tubulointerstitial area expressed as mean ± 1 SD. Open bars are uPAR−/−; closed bars are uPAR+/+. * P < 0.01, −/− versus +/+ . † P < 0.01, UUO versus sham.
genesis, as defined by the number of cells expressing the endothelial antigen CD34, and the observed co-localization of regions of neovascularization with interstitial myofibroblasts suggest a significant relationship between these two processes.

UPAR has been characterized as a scavenger receptor by virtue of its ability to work in collaboration with other scavenger receptors, especially the LDL receptor-related protein (LRP) to delete “unneeded” molecules by endocytosis (57–60). LRP-independent internalization of uPAR ligands has also been reported (61). This endocytotic pathway is the primary route of elimination of extracellular PAI-1 (4,62). UPA and PAI-1 are subsequently degraded within lysosomes while uPAR is recycled to the cell surface. Significantly more PAI-1 accumulated in the kidneys of the uPAR−/− mice after UUO. This finding, coupled with the fact that there was no difference in renal PAI-1 mRNA levels between uPAR-deficient and wild-type mice (63), suggests a key role for uPAR in PAI-1 protein turnover in the kidney. The physiologic internalization of the urokinase-PAI-1 complex is triggered by the interaction of PAI-1 with a receptor belonging to the LRP family, and involves the formation of a macro-quaternary structure of uPAR, uPA, LRP, and PAI-1 (4). In addition, an alternative internalization process has also been described whereby uPAR acts as the anchoring structure on the plasma membrane and LRP subsequently works as the endocytic trigger (64).

Over-expression of PAI-1 is a feature of most progressive renal diseases (65). We have recently reported that genetic PAI-1 deficiency resulted in significantly fewer interstitial myofibroblasts and decreased renal fibrosis in mice with obstructive nephropathy suggesting that the increased PAI-1 accumulation may be relevant to pathogenesis of the enhanced fibrosis that was observed in the uPAR null mice (35). While decreased plasmin-dependent proteolysis may partially explain the pro-fibrotic effects of PAI-1 deficiency, PAI-1 may also regulate the migration of fibroblasts along vitronectin matrices. Vitronectin (or protein-S) is an adhesive protein that accumulates within extracellular matrices during the course of injury and repair (66,67). In addition to certain integrin receptors, including αβ3, the classic vitronectin receptor, uPAR also has a vitronectin-binding site (30,68). Vitronectin, the primary PAI-1 binding protein, binds both uPAR-bound and matrix-bound PAI-1. It has been suggested that vitronectin may function as a shuttle to facilitate PAI-1 transport to and phagocytosis by uPAR (66). In the present study the extent of vitronectin accumulation in response to obstruction was greater in the kidneys of the uPAR null mice, suggesting that the delayed clearance of PAI-1 protein in these mice may be related to the absence of a functional vitronectin-uPAR pathway. While the mechanism that accounts for greater vitronectin accumulation cannot be addressed by this in vivo study, there

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**Figure 17.** Schematic summary of the potential anti-fibrotic effects of uPAR. The uPAR binds both single-chain pro-urokinase and active two-chain uPA resulting in the generation of high pericellular proteolytic activity. Plasmin has multiple effects, including the activation of certain latent matrix-degrading metalloproteinases. Both uPA and uPAR exhibit direct monocyte chemoattractant properties. Although uPAR itself is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) moiety and lacks an intracellular domain, it frequently partners with a variety of integrin receptors to promote cellular adhesion to vitronectin matrices. In addition, the uPAR-integrin complexes may collaborate in the initiation of intracellular signaling reactions although the relevance of these effects to fibrosis remains unexplored. Through interactions with scavenger receptors such the LDL-receptor related protein (LRP) and the uPAR-associated protein (uPARAP), uPAR appears to facilitate endocytosis and degradation of pro-fibrotic molecules such as PAI-1.
are reasons to speculate that it may be an indirect consequence of uPAR deficiency. Vitronectin is endocytosed and degraded by the αβ5 integrin receptor (69). Not only does uPAR interact with αβ5 integrin (18), it is also possible that, like αβ3, it may be coordinately expressed with uPAR (70).

Increased PAI-1 accumulation may also be relevant to the differences in neovascularization observed between the uPAR wild-type and deficient mice. In a study of transplanted malignant keratinocytes, genetic PAI-1 deficiency was associated with a less robust angiogenic response, resulting in less extensive local tumor invasion (71). Recent data suggest that the angiogenic effects of PAI-1 are dependent on its ability to inhibit proteolytic activity rather than due to its interactions with vitronectin and integrins (72).

The uPAR may also modify the rate of renal cell death by apoptosis. Tubular cell apoptosis is currently considered to be a major pathway leading to tubular atrophy in progressive renal disease. In the present study, uPAR deficiency resulted in the appearance of significantly more apoptotic tubular cells; at the same time proliferation-dependent tubular cell regeneration was blunted. Cultured human glioma cells exposed to uPAR anti-sense have been reported to undergo more apoptotic cell death, an observation that was associated with upregulated expression of the pro-apoptotic gene BAX (23). Interactions between uPAR and the αβ3 integrin may also enhance cell survival via anti-apoptotic mechanisms (73).

Changes in the cellular responses to ureteral obstruction were not the only differences observed in mice lacking uPAR. As predicted, renal plasminogen activator activity was significantly decreased despite similar renal mRNA levels of the plasminogen activators and their known inhibitors (63).

In summary, uPAR plays an important role in directing changes in the cellular phenotype of tubulointerstitial cells that is associated with the fibrogenic response to ureteral obstruction. Our data suggest that uPAR deficiency impairs monocyte/macrophage recruitment and diminishes scavenger receptor function resulting in delayed clearance of PAI-1 and vitronectin (Figure 17). As a consequence, ureteral obstruction induced in the absence of uPAR is characterized by a more intense myofibroblastic response, neovascularization, and tubular cell death resulting in more extensive renal destruction by fibrosis.

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