

Transforming Growth Factor- β -Dependent and -Independent Pathways of Induction of Tubulointerstitial Fibrosis in $\beta 6^{-/-}$ Mice

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Transforming growth factor- $\beta 1$ (TGF- $\beta 1$) and the renin-angiotensin-aldosterone system are key mediators in kidney fibrosis. Integrin $\alpha v\beta 6$, a heterodimeric matrix receptor expressed in epithelia, binds and activates latent TGF- $\beta 1$. We used $\beta 6$ integrin-null mice ($\beta 6^{-/-}$) to determine the role of local TGF- $\beta 1$ activation in renal fibrosis in the unilateral ureteral obstruction (UO) model. Obstructed kidneys from $\beta 6^{-/-}$ mice showed less injury than obstructed kidneys from wild-type (WT) mice, associated with lower collagen I, collagen III, plasminogen activator inhibitor (PAI-1), and TGF- $\beta 1$ mRNA levels and lower collagen content. Infusion with either angiotensin II (Ang II) or aldosterone (Aldo) or combination in $\beta 6^{-/-}$ UO mice significantly increased collagen contents to levels comparable to those in identically treated WT. Active TGF- β protein expression in $\beta 6^{-/-}$ mice was less in UO kidneys with or without Ang II infusion compared to matched WT mice. Activated Smad 2 levels in $\beta 6^{-/-}$ obstructed kidneys were lower than in WT UO mice, and did not increase when fibrosis was induced in $\beta 6^{-/-}$ UO mice by Ang II infusion. Anti-TGF- β antibody only partially decreased this Ang II-stimulated fibrosis in $\beta 6^{-/-}$ UO kidneys. *In situ* hybridization and immunostaining showed low expression of PAI-1 mRNA and protein in tubular epithelium in $\beta 6^{-/-}$ UO kidneys, with increased PAI-1 expression in response to Ang II, Aldo, or both. Our results indicate that interruption of $\alpha v\beta 6$ -mediated activation of TGF- $\beta 1$ can protect against tubulointerstitial fibrosis. Further, the robust induction of tubulointerstitial fibrosis without increase in activated Smad 2 levels in obstructed $\beta 6^{-/-}$ mice by Ang II suggests the existence of a TGF- $\beta 1$ -independent pathway of induction of fibrosis through angiotensin. (*Am J Pathol* 2003, 163:1261-1273)

Transforming growth factor (TGF)- β has been implicated as a key molecule in fibrosis and immune regulation, and its expression is increased in numerous fibrotic conditions.¹ Mammals express three isoforms (TGF- $\beta 1$, TGF- $\beta 2$, and TGF- $\beta 3$) that are synthesized in the latent form and must be activated before they can bind to their receptors and induce TGF- β -mediated effects. The non-covalent association of mature TGF- β with an N-terminal fragment of the same gene product [termed latency-associated protein (LAP)] is responsible for maintaining TGF- β isoforms in latent form.¹

Integrins are composed of α and β subunits that govern cell-cell and cell-extracellular matrix interactions, thus influencing growth, differentiation, and development. The integrin $\alpha v\beta 6$ is expressed principally on epithelial cells and serves as a receptor for the RGD motif of the extracellular matrix proteins fibronectin, tenascin, and vitronectin.² The $\alpha v\beta 6$ integrin is highly expressed in the lung, skin, and kidney during organogenesis.³ In the mouse, $\beta 6$ integrin subunit mRNA expression is very low at day 11 of gestation, increasing dramatically by E14 and E17, and plateauing by 2 weeks after birth. Expression of $\beta 6$ integrin mRNA in the adult rat is present in proximal tubules, cortical thick ascending limb, inner and outer medullary collecting duct, and macula densa.⁴ Expression of $\alpha v\beta 6$ integrin is increased in response to inflammation or repair in the kidney, such as in chronic pyelonephritis or transplant rejection.³ Mice devoid of $\alpha v\beta 6$ integrin show significant inflammation in skin and lungs, but do not have renal structural abnormalities.⁵

TGF- $\beta 1$ LAP is a ligand for the integrin $\alpha v\beta 6$, and its binding induces activation of TGF- $\beta 1$.⁶ Further, in a bleomycin model of lung epithelial cell injury, knockout mice devoid of $\beta 6$ integrin were completely protected against the fibrosis that developed in the wild type (WT).⁶ Interestingly, this protection against injury occurred despite persistent macrophage infiltration in the knockout that was similar in magnitude to that seen in the WT. This

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protection was linked to prevention of TGF- β 1 activation by absence of α v β 6 integrin.

Activated TGF- β facilitates matrix accumulation in at least four ways, including increased synthesis of matrix proteins, increased synthesis of integrins involved in matrix assembly at the cell surface, decreased synthesis of matrix metalloproteinases, and increased synthesis of proteinase inhibitors, including plasminogen activator inhibitor-1 (PAI-1), thereby decreasing matrix degradation. PAI-1 is the major physiological inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-like plasminogen activator (u-PA), both of which activate plasminogen to plasmin, thus promoting fibrinolysis and proteolysis.^{7,8} Plasmin also activates other latent matrix metalloproteinases. Important interactions of the renin-angiotensin-aldosterone system (RAAS) with PAI-1 have been demonstrated *in vitro* and *in vivo*, supporting a direct induction of PAI-1 by angiotensin, and enhancement of this induction by aldosterone (Aldo).⁹⁻¹² However, *in vitro* data suggest that angiotensin induction of PAI-1 has an early TGF- β -independent stage with later TGF- β -dependent increase of PAI-1.¹³ Thus, the RAAS, TGF- β , and PAI-1 have been intricately linked in injury response and fibrosis.¹⁴ In this study, we used β 6^{-/-} mice to examine possible TGF- β -dependent and -independent mechanisms of renal fibrosis.

Materials and Methods

Animals

Adult male WT and β 6 integrin-null mice (β 6^{-/-}) with C57BL/6 or 129Sv background were used. Mice were housed in microisolator cages in a pathogen-free barrier facility. All protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Experimental Protocol

Groups of WT and β 6^{-/-} mice, age 10 to 12 weeks, underwent unilateral ureteral obstruction (UUO) (WT UUO, $n = 12$; β 6^{-/-} UUO, $n = 12$) under sterile conditions as described previously.¹⁵ Left ureters of mice were double-ligated with 6-0 silk and cut between the two ligated points. The upper ligation was consistently placed at the level of lower pole of the kidney. In pilot studies, identical degree of fibrosis developed on 129 and C57BL/6 background after UUO, and results are therefore combined. To investigate the effects of RAAS on fibrosis, angiotensin II (Ang II), Aldo, or both were administered to additional mice (WT UUO + Ang II, $n = 6$; β 6^{-/-} UUO + Ang II, $n = 6$; WT UUO + Aldo, $n = 4$; β 6^{-/-} UUO + Aldo, $n = 3$; WT UUO + Ang II/Aldo, $n = 6$; β 6^{-/-} UUO + Ang II/Aldo, $n = 6$). Miniosmotic pumps (Alzet, model 1002; Durect Corp., Cupertino, CA) filled with Ang II (concentration of 40 μ g/kg/hour; Bachem Bioscience Inc., Torrance, CA) were inserted into the peritoneal space. The contents of the miniosmotic pump were delivered at a rate of 0.25 μ l/hour for 2 weeks. Aldo implantable pellets (Innovative Research of America,

Sarasota, FL) were inserted subcutaneously for 2 weeks releasing at 140 μ g/kg/day.¹⁶ Doses for Ang II or Aldo were based on previous long-term studies showing only moderate hypertension with mild fibrosis in the rat with these levels, and our unpublished data of mild hypertension, induction of PAI-1 but only minimal fibrosis after up to 8 weeks with these doses in the mouse.¹⁷

To determine the TGF- β -dependent and -independent pathways for inducing fibrosis, monoclonal anti-TGF- β antibody (Ab) (1D11; Genzyme Corp., Framingham, MA) or isotype-matched irrelevant control antibody (13C4, Genzyme Corp.) were given to WT or β 6^{-/-} mice with UUO with or without Ang II infusion. Low and high doses of 1D11 (low, 0.5 mg/kg/body weight; high, 5 mg/kg/body weight) and control antibody (13C4, 5 mg/kg BW) were administered every other day by intraperitoneal injections. The doses of anti-TGF- β antibody were chosen based on inhibition of biological activity of TGF- β in the mouse without producing histopathology in any organs of the body.¹⁸ The first TGF- β antibody injection was given 1 day before UUO. WT UUO mice were treated with antibodies as follows: control antibody 13C4 (WT UUO + CONT Ab, $n = 5$; WT UUO + Ang II + CONT Ab, $n = 3$), high-dose anti-TGF- β antibody (WT UUO + H-TGF- β Ab, $n = 5$; WT UUO + Ang II + H-TGF- β Ab, $n = 5$), or low-dose anti-TGF- β antibody (WT UUO + L-TGF- β Ab, $n = 4$; WT UUO + Ang II + L-TGF- β Ab, $n = 4$). β 6^{-/-} UUO mice infused with Ang II were treated with antibodies as follows: control antibody 13C4 (β 6^{-/-} UUO + Ang II + CONT Ab, $n = 4$), high-dose anti-TGF- β antibody (β 6^{-/-} UUO + Ang II + H-TGF- β Ab, $n = 8$), or low-dose anti-TGF- β antibody (β 6^{-/-} UUO + Ang II + L-TGF- β Ab, $n = 5$). At 5 or 14 days after UUO surgery, mice for the above groups were sacrificed and both obstructed and nonobstructed contralateral kidneys (used as normal control) were harvested for morphological, immunostaining, biochemical, and molecular assessment.

Morphological Assessment

The degree of tubulointerstitial fibrosis was scored on Masson's trichrome-stained coronal sections. All cortical and medullary fields were scored from 0 to 4+ for each field, with an average for each kidney calculated. Fibrosis was assessed as 0 for 0%, 1 for <25%, 2 for 25 to 50%, 3 for >50 to 75%, and 4 for >75% of each field occupied by tubulointerstitial fibrosis. All sections were examined without knowledge of the treatment protocol.

Blood Pressure Measurement

Systolic blood pressure was measured using a tail-cuff blood pressure monitor for rats and mice (model 2000; Muromachi Kikai Co., Ltd., Tokyo, Japan) in conscious, trained mice at room temperature.

Immunohistochemistry

Kidney tissues were fixed overnight in 4% paraformaldehyde at 4°C, and embedded in paraffin using standard

techniques. Four- μm sections were dewaxed and rehydrated. Endogenous peroxidase was quenched with 3% hydrogen peroxidase for 10 minutes and exposed to Power Block (BioGenex Laboratories, San Ramon, CA) for 45 minutes.

Proliferating cells were identified by proliferating cell nuclear antigen (PCNA) cyclin polypeptide immunohistochemistry using the Mouse on Mouse kit (Innogenex, San Ramon, CA). Monoclonal mouse anti-human PCNA (1:100; DAKO Corp., Carpinteria, CA) was applied, followed by rabbit anti-mouse antibody (DAKO), biotinylated goat anti-rabbit Ig (BioGenex), and peroxidase-conjugated streptavidin. PCNA-positive tubulointerstitial cells in the cortex were semiquantified using a 0 to 4+ scale by scoring all high-power cortical fields per section (0 to 4 represent: 0%, <25%, 25 to 50%, >50 to 75%, and >75% of the tubulointerstitial nuclei in the field showing positive nuclear staining).

For immunostaining of PAI-1, rabbit anti-rat PAI-1 antibody (1:50; American Diagnostica, Inc., Greenwich, CT) was used at 4°C overnight followed by biotinylated goat anti-rabbit Ig (BioGenex) and peroxidase-conjugated streptavidin.

For detection of active TGF- β , sections were treated with hyaluronidase (Sigma Chemical Co., St. Louis, MO) at 1 mg/ml in 0.1 mol/L sodium acetate, pH 5.5, containing 0.85% NaCl at 37°C for 30 minutes. After blocking with 5% normal goat serum, a rabbit polyclonal antibody to active TGF- β (LC 1-30-1; gift from Dr. Kathy Flanders, National Cancer Institute) at 6.5 $\mu\text{g}/\mu\text{l}$ in Tris-buffered saline/0.4% Triton buffer was used overnight at 4°C.¹⁹ Biotinylated goat anti-rabbit Ig (BioGenex) was added and followed by peroxidase-conjugated streptavidin.

TSP-1 was stained with primary goat anti-human TSP-1 (1:100, N-20, sc12312; Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C and secondary horseradish peroxidase-conjugated anti-goat antibody (1:100). Diaminobenzidine was added as a chromogen.

Infiltration of macrophages was detected by rat anti-mouse F4/80 antibody (1:20; Serotec Inc., Raleigh, NC). Biotinylated anti-rat secondary antibody (Vector Laboratories, Burlingame, CA) was used and followed by streptavidin-conjugated alkaline phosphatase (Innogenex). After blocking with 0.25% levamisole for 10 minutes, Sigma Fast Red TR/Naphtol AS-Mx tablets (Sigma) were added as a chromogen. Slides were counterstained with hematoxylin. Infiltrating macrophages in the interstitium were counted and data expressed as macrophage number per high-power field. Control slides treated with nonspecific antisera instead of primary antibodies showed no staining.

Total Kidney Collagen Content

Part of the kidney tissue was frozen, and relative collagen content as a percentage of total protein was calculated from the concentration of hydroxyproline and proline, measured as their phenylisothiocyanate derivatives by reverse phase-high performance liquid chromatography.²⁰ Briefly, tissue homogenates were hydrolyzed *in*

vacuo in constant boiling HCl (110°C, 16 hours), dried, taken up in 1 ml of redrying solution [ethanol/water/(C₂H₅)₃N, 2:2:1], and 100 μl were redried under nitrogen. Twenty μl of derivatization reagent [methanol/water/(C₂H₅)₃N-phenylisothiocyanate, 7:1:1:1] were added, and the reaction was allowed to proceed at room temperature for 20 minutes. The sample was then lyophilized during centrifugation to remove the reagents. Samples were either frozen at this stage or diluted to 1 ml with buffer (5% acetonitrile in 5 mmol/L disodium phosphate, pH 7.4). A solution containing amino acid standards was dried and coupled in the same manner. The coupling efficiency was >99% under these conditions. Recovery and efficiency were normalized by including norleucine in each homogenate.

Northern Blot Hybridization

Northern blot was performed as described.²¹ Total RNA from kidney was extracted by Trizol reagent (Life Technologies, Grand Island, NY). Twenty μg of total RNA were loaded and fractionated by electrophoresis in 1% agarose gel and transferred to a nylon membrane. The blots were hybridized with the following cDNA probes: mouse PAI-1 (365 bp),²¹ mouse TGF- β 1 (974 bp), human collagen I (American type Culture Collection, Manassas, VA), and mouse collagen III (355 bp). To evaluate RNA loading, blots were reprobated with rat housekeeping gene GAPDH cDNA. The ratio of specific message to GAPDH was used to quantify expression for each tissue sample.

In Situ Hybridization

[³⁵S]-labeled sense and anti-sense riboprobes for PAI-1 were prepared as previously described by transcription of the pCR II plasmid (Invitrogen, Carlsbad, CA) combining a PAI-1 cDNA insert by SP6 or T7 RNA polymerase (Ambion Inc., Austin, TX).²² After treatment by proteinase K and triethanolamine/acetic anhydride, sections were dehydrated in ethanol and air-dried. Hybridization was done as described previously. A control *in situ* hybridization done with sense probes showed no specific signal.

Western Blot Analysis of Smad 2

Frozen kidney tissues were transferred in RIPA plus buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L ethylenediamine tetraacetic acid, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 $\mu\text{g}/\text{ml}$ phenylmethyl sulfonyl fluoride, 1:100 phosphatase inhibitor cocktail I, 1:100 phosphatase inhibitor cocktail II (Sigma), 1:100 proteinase inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). Tissue samples were homogenized in 1.5 ml of homogenizer (Pellet Pestle Dips; Kontes Glass Company, Vineland, NJ) on ice, and centrifuged at 13,000 rpm for 15 minutes at 4°C. The protein concentration was measured using Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty μg of protein from samples were loaded and separated on 10% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis and transferred onto a 0.2 $\mu\text{mol/L}$ nitrocellulose membrane. Phosphorylated-Smad 2 (P-Smad 2) was detected using a specific rabbit anti-phosphorylated-Smad2 (P-Smad 2) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C, 1:500 dilutions. After washing in Tris-buffered saline with 0.1% Tween 20 (TBS-T), horseradish peroxidase-labeled donkey anti-rabbit IgG secondary antibody (1:2500 dilution in 5% milk TBS-T) was added and incubated at room temperature for 50 minutes. Protein bands on Western blots were visualized by ECL Plus (Amersham, Arlington Heights, IL) according to the manufacturer's instructions and were developed on film. The membranes were stripped with 100 mmol/L β -mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCL, pH 6.7. Total Smad 2 (T-Smad 2) was detected using rabbit anti-Smad 2 polyclonal antibody (Zymed Laboratories Inc., San Francisco, CA). Cell lysates obtained from human colon carcinoma cell line FET stimulated with TGF- β 1 (12 ng/ml for 1 hour, kindly provided by Dr. Pran Datta, Vanderbilt University Medical Center) were used as a positive control.

Statistical Analysis

Results are expressed as mean \pm SEM. Statistical difference was assessed by a single factor variance (analysis of variance) followed by unpaired Student's *t*-test as appropriate. Nonparametric data were compared by Mann-Whitney *U*-test. A *P* value <0.05 was considered to be significant.

Results

Tubulointerstitial Fibrosis in $\beta 6^{-/-}$ UUO Kidney

Tubular dilation, tubular atrophy, and widening of interstitial space were present in obstructed kidneys in WT mice at 5 days. Tubulointerstitial damage progressed in both cortex and medulla by day 14 after obstruction in WT mice. In contrast, obstructed kidneys from $\beta 6^{-/-}$ mice had significantly less injury (Figure 1). Nonobstructed kidneys in both WT and $\beta 6^{-/-}$ mice showed few PCNA-positive cells. There was significant tubular epithelial cell proliferation in WT, but not $\beta 6^{-/-}$ mice, after obstruction (Figure 2). Macrophage infiltration after UUO was more prominent in $\beta 6^{-/-}$ than in WT, but this difference was not statistically significant (Figure 3). Nonobstructed control kidneys in both WT and $\beta 6^{-/-}$ mice expressed similar trace to low levels of mRNA for collagen I, collagen III, PAI-1, and TGF- β 1 (Figure 4). Renal mRNA levels for collagen I (Figure 4, A and B), collagen III (Figure 4, A and C), PAI-1 (Figure 4, A and D), and TGF- β 1 (Figure 4, A and E) were increased in response to UUO in WT mice. In contrast, $\beta 6^{-/-}$ mice showed mRNA expression levels only one-third to one-half of those in obstructed WT at day 14 (collagen I: WT UUO 1.2 ± 0.1 versus $\beta 6^{-/-}$ UUO 0.3 ± 0.02 , $P < 0.01$; collagen III: WT UUO 1.3 ± 0.1 versus $\beta 6^{-/-}$ 0.4 ± 0.1 , $P < 0.01$; PAI-1: WT UUO 1.1 ± 0.1 versus $\beta 6^{-/-}$ 0.03 ± 0.1 , $P < 0.01$,

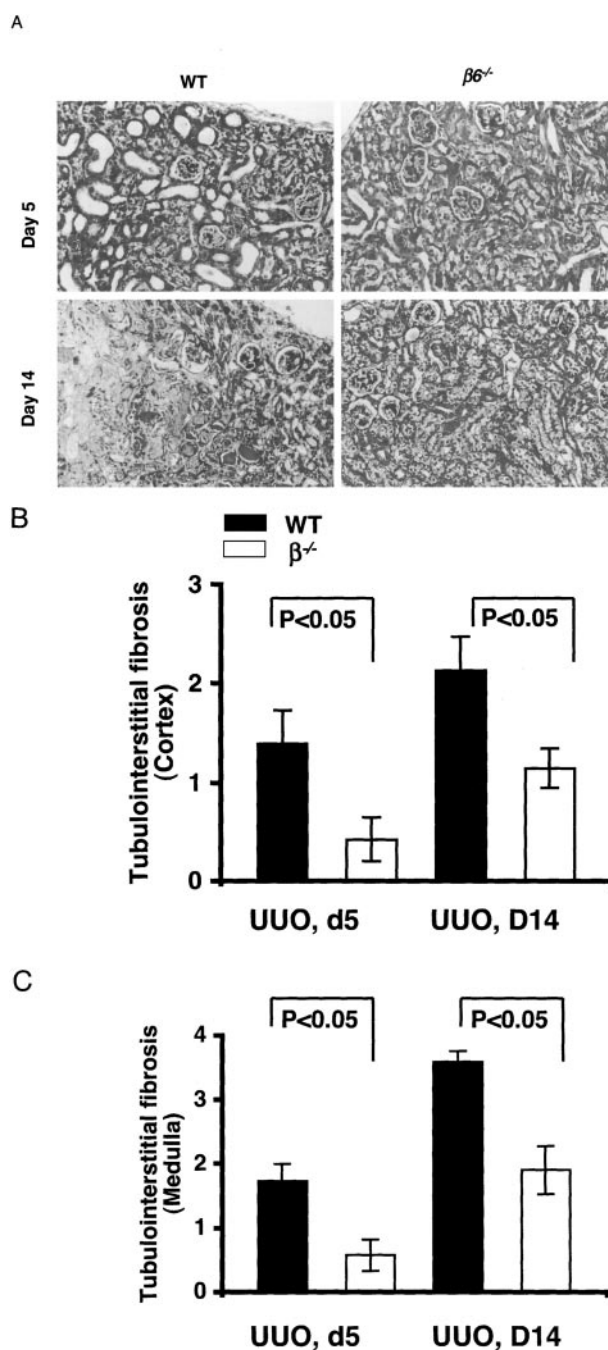


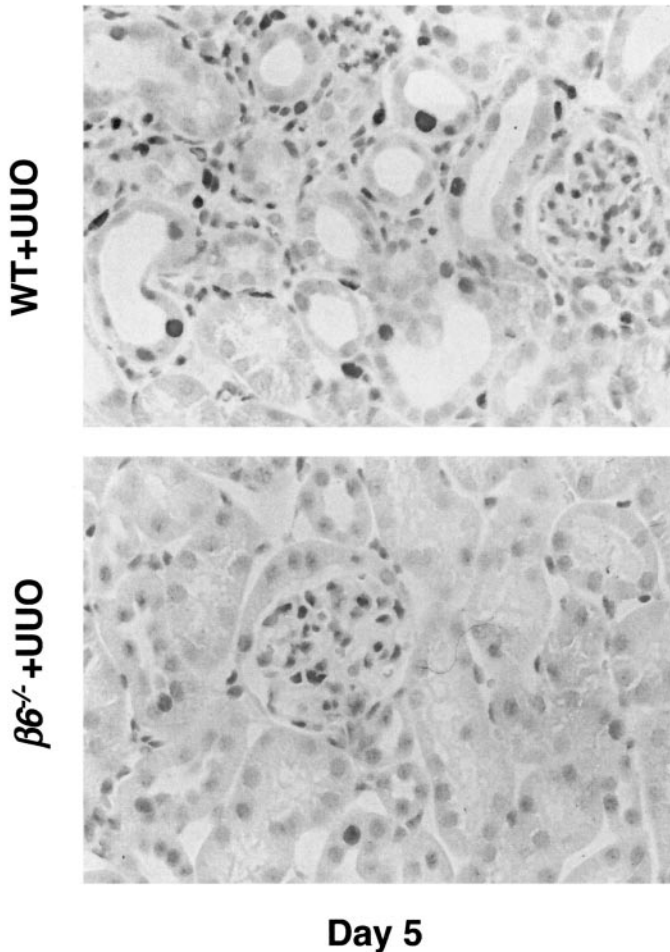
Figure 1. Kidney morphological changes in obstructed kidneys. **A:** $\beta 6^{-/-}$ mice had less fibrosis at days 5 and 14 after UUO than time-matched WT. **B** and **C:** Semiquantitative scoring of fibrosis (0 to 4+ scale) showed less injury in $\beta 6^{-/-}$ UUO in cortex (**B**) and medulla (**C**) compared to WT UUO ($n = 4$ for each group at day 5, $n = 8$ for each group at day 14). Data are shown as mean \pm SEM. Masson's trichrome stain; original magnifications, $\times 200$.

TGF- β 1: WT + UUO 3.6 ± 0.2 versus $\beta 6^{-/-}$ 1.9 ± 0.2 , all expressed as density ratios relative to GAPDH mRNA) (Figure 4).

Systolic Blood Pressure

Blood pressure remained normal in sham-operated control mice (104 ± 6 mmHg) and all other animals with UUO alone

A



B

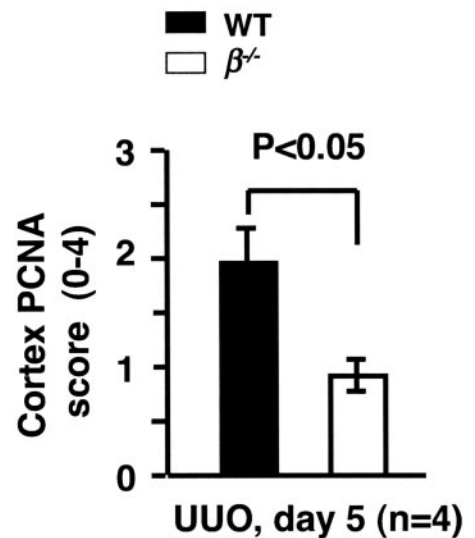


Figure 2. Tubular cell proliferation after UUO. **A:** PCNA immunostaining in tubules and interstitium in WT and $\beta 6^{-/-}$ UUO kidneys at day 5. **B:** Semi-quantitative scoring of PCNA-positive tubulointerstitial cells in cortex at day 5 ($n = 4$ for each group). Data are shown as mean \pm SEM. Original magnifications, $\times 400$.

(Figure 5). Blood pressure increased significantly and comparably in both UUO WT and UUO $\beta 6^{-/-}$ mice infused with Ang II for 14 days (Figure 5). Infusion with Aldo alone led to a slight, but not significant, increase in blood pressure compared to mice with UUO alone. Blood pressure in both WT and $\beta 6^{-/-}$ mice with UUO was further augmented in response to a combination infusion of angiotensin and Aldo compared to either alone (Figure 5).

Induction of Fibrosis in $\beta 6^{-/-}$ UUO Kidney by the RAAS

Collagen content of obstructed kidneys in WT UUO was significantly increased compared to $\beta 6^{-/-}$ UUO at day 14 (percent collagen/total protein 8.8 ± 0.9 versus 2.7 ± 0.8 , $P < 0.01$; Figure 6). In contrast, obstructed $\beta 6^{-/-}$ showed no significant increase in collagen content compared to baseline (Figure 6). In $\beta 6^{-/-}$ UUO mice, infusion of either Ang II or Aldo or combination for 14 days significantly increased collagen contents to levels comparable to those in infused WT UUO (Figure 6). Ang II or Aldo infusion alone

in $\beta 6^{-/-}$ UUO mice did not affect the macrophage infiltration. Combined Ang II and Aldo infusion in $\beta 6^{-/-}$ UUO mice significantly decreased macrophage infiltration. In contrast, in WT UUO, Ang II infusion led to increased macrophage infiltration to a level similar to obstructed $\beta 6^{-/-}$ kidney (macrophages/hpf: $\beta 6^{-/-}$ UUO 41 ± 7 versus WT UUO 23 ± 9 , pNS; $\beta 6^{-/-}$ UUO + Ang II 32 ± 9 , $\beta 6^{-/-}$ UUO + Aldo 36 ± 8 , $\beta 6^{-/-}$ UUO + Ang II/Aldo 21 ± 6 ; $\beta 6^{-/-}$ UUO versus $\beta 6^{-/-}$ UUO + Ang II/Aldo, $P < 0.05$; WT UUO + Ang II 51 ± 14 , WT UUO + Aldo 39 ± 6 , WT UUO + Ang II/Aldo 34 ± 1 , WT UUO versus WT UUO + Ang II, $P < 0.05$).

Thrombospondin (TSP)-1 Expression

TSP-1 protein, a potential activator of TGF- β , was similarly abundant in smooth muscle cells of arterioles and arteries in nonobstructed kidneys in both WT and $\beta 6^{-/-}$ mice. TSP-1 was markedly increased in obstructed WT in cortical tubular epithelial cells, with a diffuse and strong granular pattern. In contrast, less TSP-1 was present in

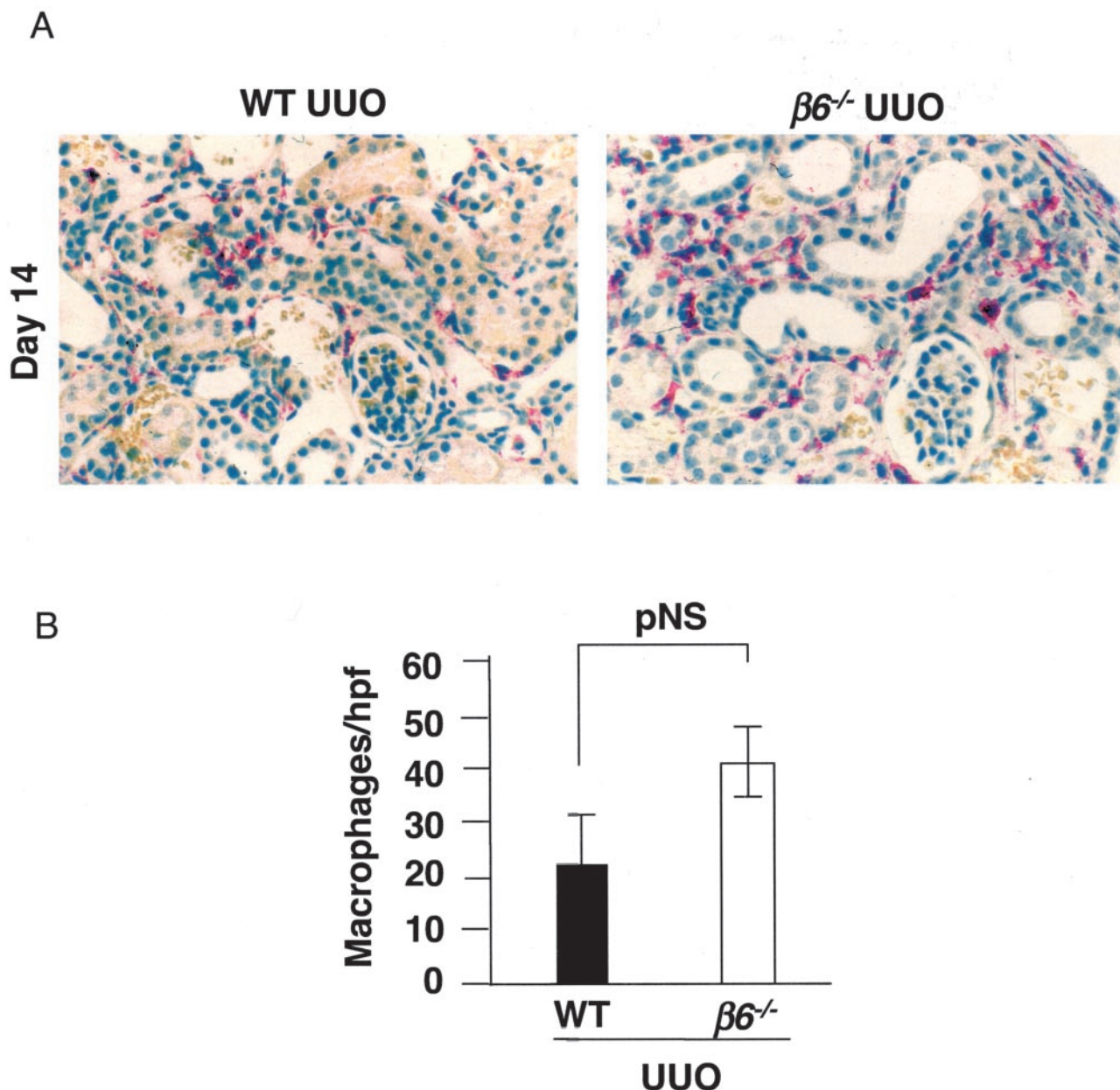


Figure 3. F4/80 + interstitial macrophages in obstructed kidneys at day 14. **A:** Macrophages (in red) in WT and $\beta 6^{-/-}$ UUU kidneys. **B:** Quantitation of average macrophage number per high-power field ($n = 8$ for each group). Data are shown as mean \pm SEM. Original magnification, $\times 400$.

injured tubules in obstructed $\beta 6^{-/-}$ kidneys. Infusion with Ang II in obstructed $\beta 6^{-/-}$ mice led to a stronger and more diffuse granular pattern of TSP-1 expression, similar to that seen in Ang II-infused WT UUU (Figure 7).

Activity of TGF- β and Fibrosis

Active TGF- β immunostaining was present at low level in nonobstructed kidneys in both WT (Figure 8A) and $\beta 6^{-/-}$ mice (Figure 8B). Active TGF- β , mostly expressed in tubular epithelial cells, as well as interstitial cells and glomerular parietal cells, was markedly increased in WT UUU kidneys (Figure 8C). In contrast, active TGF- β was only focally present in some tubules in $\beta 6^{-/-}$ UUU kid-

neys (Figure 8D), indicating dampened local activation of TGF- β from these parenchymal tubular epithelial and interstitial cells. Angiotensin infusion substantially increased active TGF- β expression in WT UUU kidney (Figure 8E), but only mildly increased active TGF- β in $\beta 6^{-/-}$ UUU mice (Figure 8F).

Total Smad 2 was equally expressed at high levels in nonobstructed contralateral and obstructed kidneys at day 5 and day 14 after UUU in WT mice (Figure 9A). Phosphorylated Smad 2, a marker of TGF- β signaling activation, was dramatically increased from day 5 to 14 after obstruction in WT (Figure 9A). In contrast, phosphorylated Smad 2 was significantly lower in $\beta 6^{-/-}$ obstructed kidneys and did not increase when fibrosis was

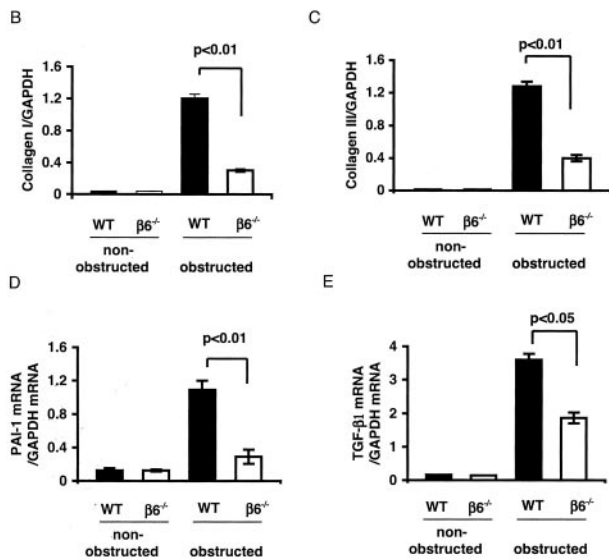
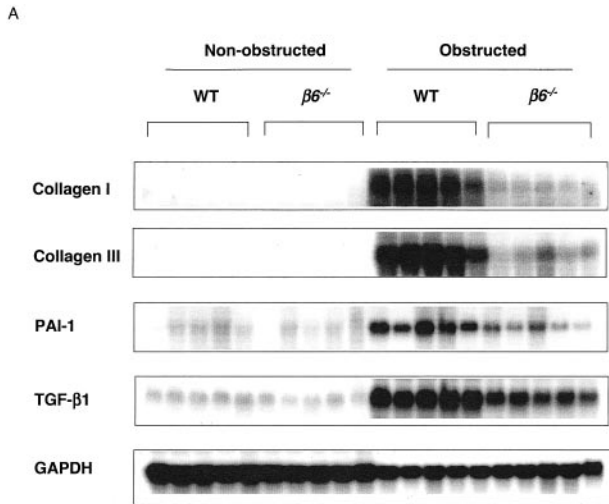


Figure 4. Northern blot analysis of kidney mRNA expression. Collagen I (A, B), collagen III (A, C), PAI-1 (A, D), and TGF- β 1 (A, E) mRNA expressions in kidneys in $\beta 6^{-/-}$ UUU versus WT UUU at day 14 ($n = 5$ in each group). Data are shown as mean \pm SEM.

induced by infusing Ang II in obstructed $\beta 6^{-/-}$ kidneys (Figure 9, B and C). Abundantly expressed phosphorylated-Smad 2 in PET cell lysate stimulated with TGF- β 1 served as a positive control (Figure 9B).

To further examine mechanisms of kidney fibrosis, we treated mice with anti-TGF- β antibody. The effects of anti-TGF- β antibody on fibrosis were first examined in WT mice with UUU alone. Collagen content in obstructed WT kidneys was significantly decreased by low doses of anti-TGF- β antibody and further decreased with high dose of anti-TGF- β antibody compared to control antibody (Figure 10). However, anti-TGF- β antibody alone, even with high dose, only partially inhibited (less than 50%) increased collagen in WT UUU kidneys (Figure 10), with or without Ang II infusion (Figure 11), suggesting other mechanisms may play a role in fibrosis. Similarly, both low and high doses of anti-TGF- β antibody, but not control antibody, significantly but only partially decreased the Ang II-stimulated collagen increase in

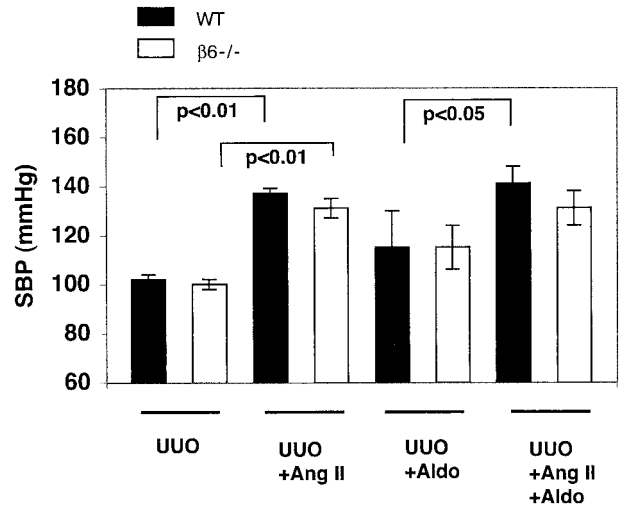


Figure 5. Systolic blood pressure (SBP). Ang II infusion alone or in combination with Aldo for 14 days increased systolic blood pressure significantly in both UUU WT and UUU $\beta 6^{-/-}$ mice. Infusion with Aldo alone slightly increased systolic blood pressure ($n = 3$ to 8 in each group). Data are shown as mean \pm SEM.

$\beta 6^{-/-}$ UUU kidneys (Figure 11). Anti-TGF- β antibody treatment in $\beta 6^{-/-}$ UUU mice infused with Ang II only numerically decreased phosphorylated Smad 2 (Figure 9, B and C).

PAI-1 Localization and Expression

WT UUU showed moderate PAI-1 mRNA expression in tubules in cortex and medulla and in transitional epithelial cells by *in situ* hybridization, compared to low-level PAI-1

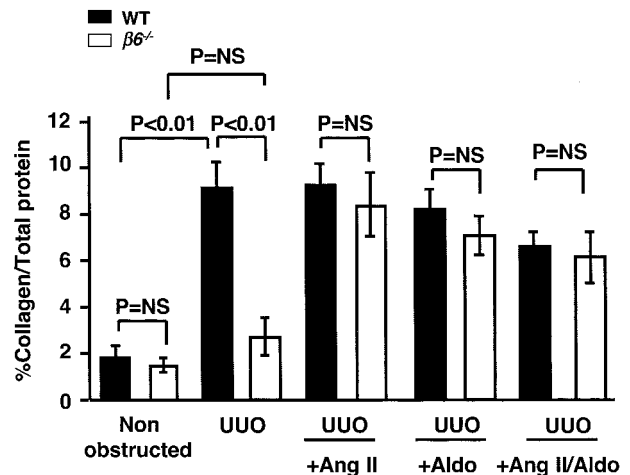


Figure 6. Kidney collagen contents. Total collagen content (expressed as percent total protein) was increased in WT UUU at day 14 compared to the nonobstructed, contralateral kidney, in contrast to no significant change in $\beta 6^{-/-}$ in response to UUU. Infusion of Ang II or Aldo or combination restored full fibrotic response in $\beta 6^{-/-}$ UUU ($n = 8$ each for WT and $\beta 6^{-/-}$ with UUU only; $n = 3$ to 8 each for WT and $\beta 6^{-/-}$ with UUU plus infusion). Data are shown as mean \pm SEM.

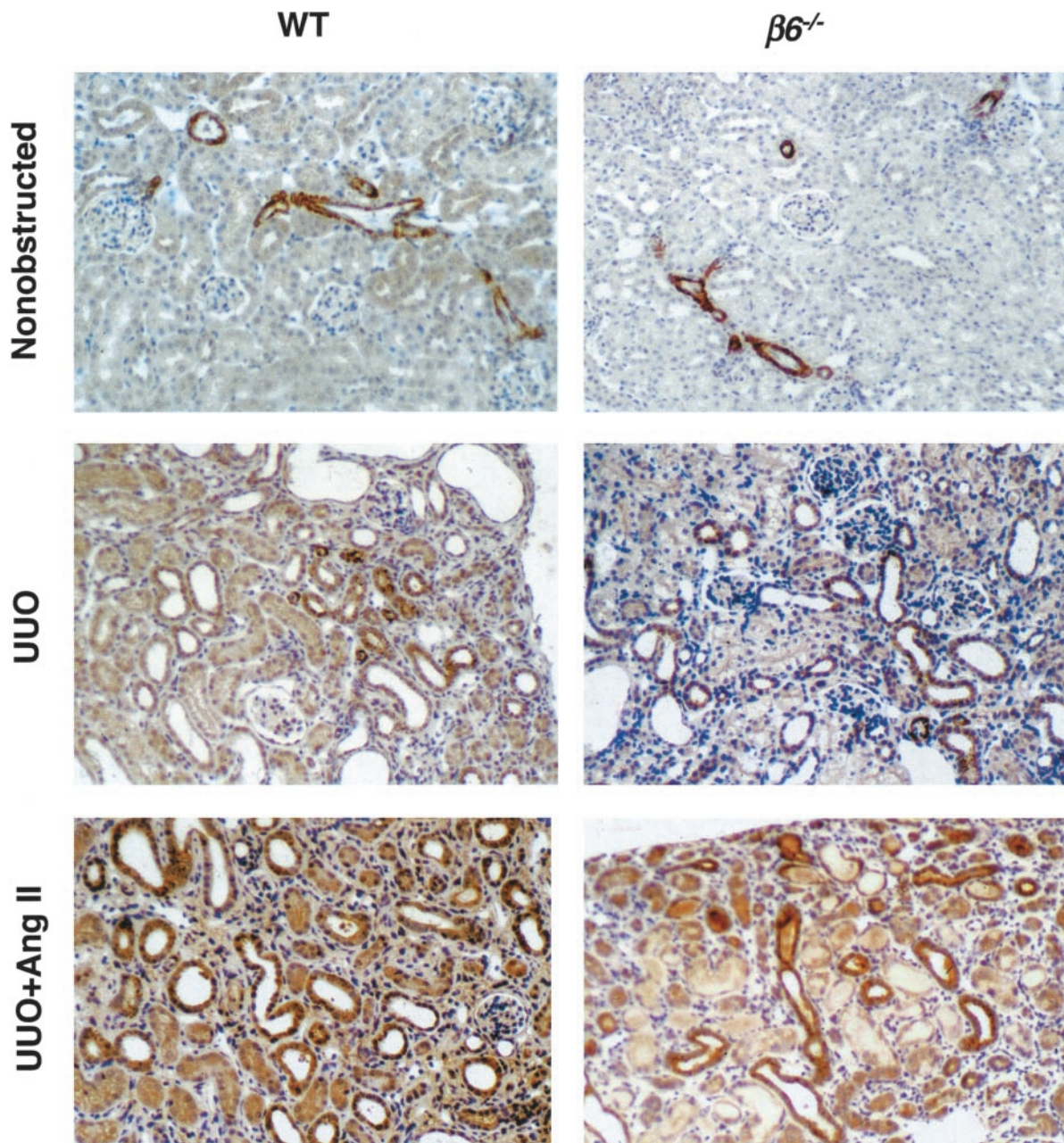


Figure 7. TSP-1 protein expression. TSP-1 protein was identified in nonobstructed kidneys in smooth muscle cells of arterioles and arteries in both WT and $\beta 6^{-/-}$ mice. TSP-1 was markedly increased in obstructed WT kidneys in cortical tubular epithelial cells, with a diffuse and strong granular pattern at day 14. In contrast, less TSP-1 was present in injured tubules in obstructed $\beta 6^{-/-}$. Infusion with Ang II in obstructed $\beta 6^{-/-}$ lead to a stronger, more diffuse granular pattern of TSP-1 expression, similar to that seen in Ang II-infused WT UUO ($n = 3$ to 6 for each group). Data are shown as mean \pm SEM. Original magnifications, $\times 200$.

mRNA expression in obstructed $\beta 6^{-/-}$ kidneys. PAI-1 mRNA expression was increased in obstructed $\beta 6^{-/-}$ kidney after infusion with Ang II, Aldo, or combination, with similar intensities and locations as in identically treated WT mice (Figure 12). There was no change in PAI-1 mRNA expression, as detected by Northern blot and *in situ* hybridization, in response to TGF- β antibody treatments compared to treatment with control antibody (data not shown).

PAI-1 protein was not present in nonobstructed WT kidneys (Figure 13A). However, PAI-1 was highly expressed in WT UUO kidney at day 14, primarily in cortical

proximal tubular epithelial cells (Figure 13B) and glomerular parietal epithelial cells (data not shown). Infusion of Ang II or Aldo (Figure 13, C and D) in obstructed WT did not alter PAI-1 staining intensity or distribution compared to WT UUO alone (Figure 13B). In contrast, $\beta 6^{-/-}$ UUO mice at day 14 showed much less PAI-1 in proximal tubules and none in glomeruli (Figure 13E), with focal strong PAI-1 staining in inner medullary tubules (not shown). However, infusion of Ang II in $\beta 6^{-/-}$ UUO mice markedly increased PAI-1 expression to a pattern similar to that seen in WT, with increased proximal tubule PAI-1 expression (Figure 13F).

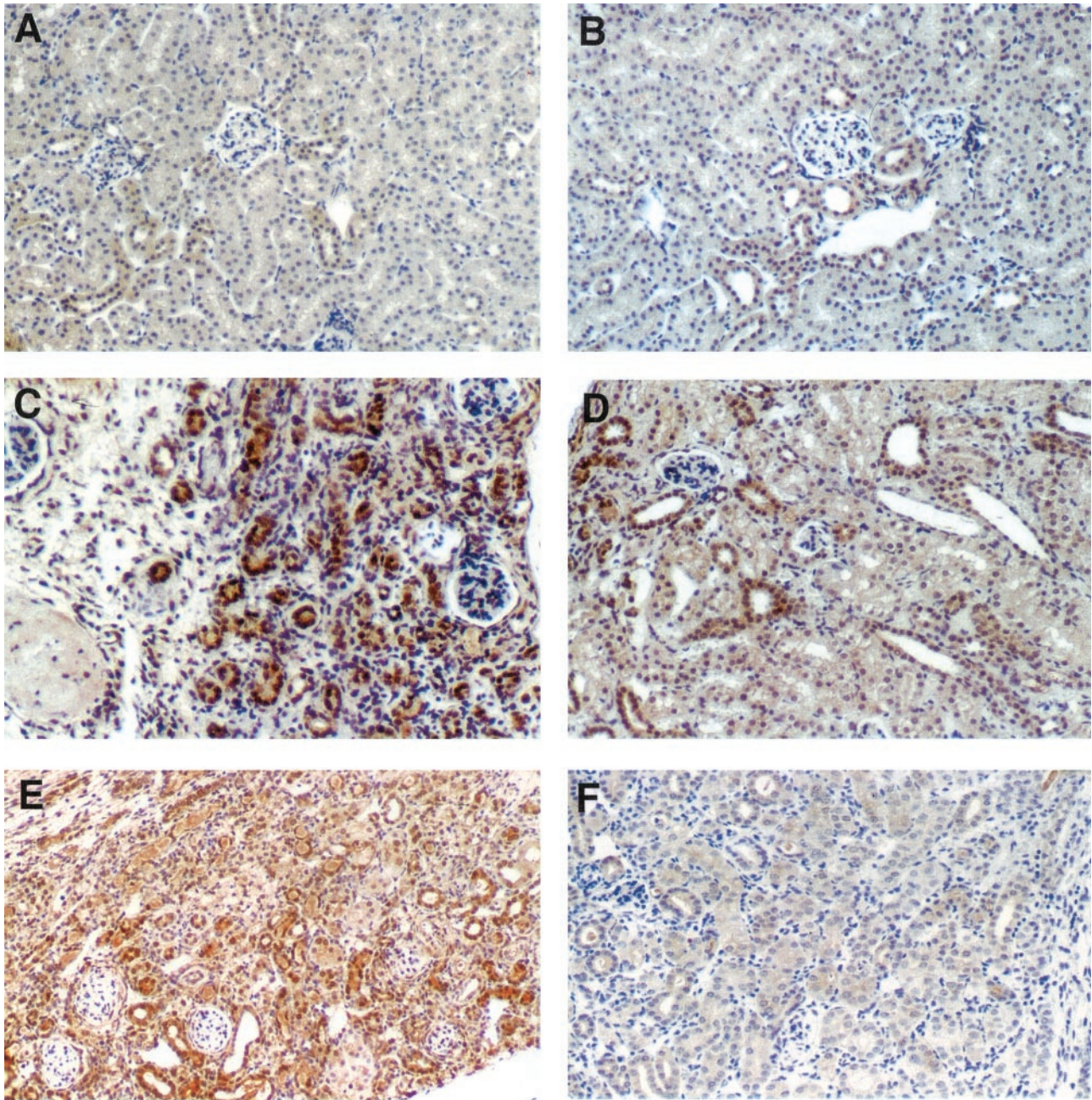


Figure 8. Active TGF- β expression. Active TGF- β was expressed in low levels in nonobstructed kidneys from both WT (A) and $\beta 6^{-/-}$ mice (B). At day 14, active TGF- β , mostly expressed in tubular epithelial cells, as well as interstitial cells and glomerular parietal cells, was markedly increased in WT UUO kidney (C). In contrast, much less active TGF- β was detected in tubules in $\beta 6^{-/-}$ UUO kidney (D). Ang II infusion resulted in fully activated TGF- β expression in WT UUO kidney (E), but only mild increase of active TGF- β in $\beta 6^{-/-}$ UUO mice at day 14 (F). $n = 3$ to 6 for each group. Original magnifications, $\times 200$.

Discussion

TGF- β and the RAAS have been intricately linked to development of fibrosis, and they are interrelated. Both activate fibrosis in part by inducing matrix synthesis, and in part by inhibiting matrix degradation. A powerful mediator of the latter mechanism is induction of PAI-1.²³ Although angiotensin can directly induce PAI-1 through the type 1 receptor (AT1) *in vivo* and *in vitro*, some *in vitro* evidence points to a later, TGF- β -dependent phase of this induction of PAI-1 by angiotensin.¹³ We therefore used $\beta 6^{-/-}$ mice to determine fibrosis mechanisms that

are dependent on local TGF- β activation and those that are independent of these TGF- β -mediated events. We chose the $\beta 6^{-/-}$ mouse because it has a normal phenotype, unlike TGF- $\beta 1$ knockout mice, which succumb to disease related to marked immune cell infiltration. Because $\alpha v\beta 6$ integrin is only one mechanism of activation of TGF- $\beta 1$, other mechanisms of TGF- $\beta 1$ activation remain potentially active, and thus, the $\beta 6^{-/-}$ mice do not have baseline renal abnormalities. We further chose to use the $\beta 6^{-/-}$ mice particularly for the study of development of tubulointerstitial fibrosis. $\beta 6$ integrin is expressed in epithelia

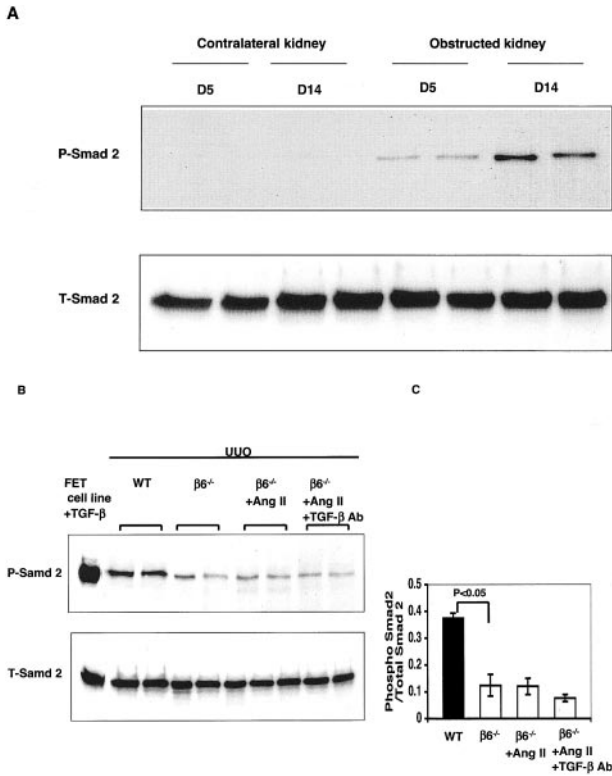


Figure 9. Representative Western blot analysis of Smad 2 in UVO kidneys. **A:** Phosphorylated Smad 2 (P-Smad 2) and total Smad 2 (T-Smad 2) in nonobstructed contralateral and obstructed kidneys at day 5 and day 14 after UVO in WT mice. P-Smad 2 in obstructed WT kidneys was dramatically increased from day 5 to day 14, contrasting with lower P-Smad 2 in obstructed $\beta 6^{-/-}$ kidneys (**B, C**). Ang II infusion in $\beta 6^{-/-}$ UVO mice did not increase P-Smad 2 levels (**B, C**). Anti-TGF- β antibody treatment in $\beta 6^{-/-}$ UVO mice infused with Ang II only numerically decreased P-Smad 2 (**B, C**). PET cell lysate stimulated with TGF- $\beta 1$ served as a positive control (**B**) ($n = 5$ for each animal group). Data are shown as mean \pm SEM.

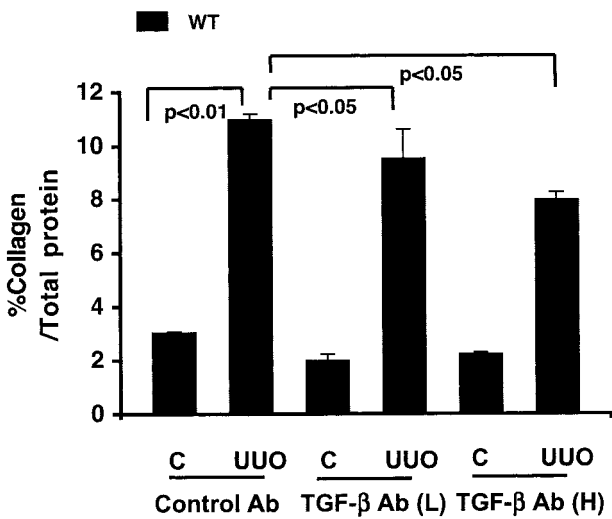


Figure 10. Effect of TGF- β antibody on kidney collagen content in WT UVO mice. Control antibody had no effect on total collagen content (expressed as percent total protein) increased in obstructed (UVO) WT kidney versus nonobstructed contralateral kidney (C). Total collagen content was partially but significantly inhibited after either low (L) or high (H) doses of TGF- β antibody (Ab) treatment ($n = 4$ to 5 for each group). Data are shown as mean \pm SEM.

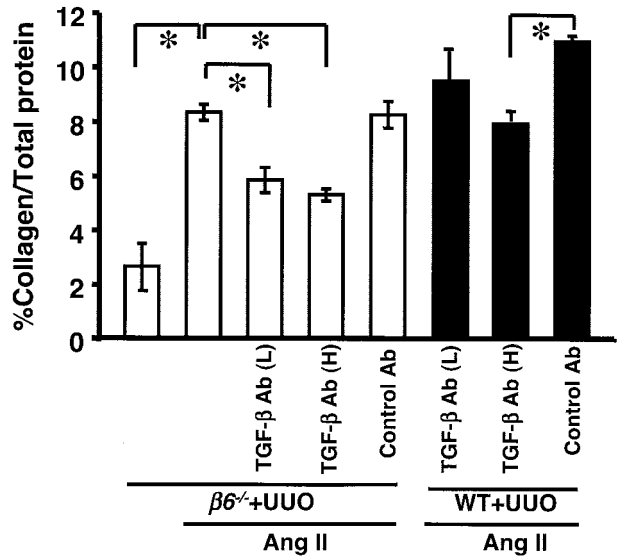


Figure 11. Effect of TGF- β antibody on kidney collagen content in Ang II-infused UVO mice. Total collagen content (expressed as percent total protein) in $\beta 6^{-/-}$ UVO kidney was increased after Ang II infusion, and was only partially inhibited after either high (H) or low (L) doses of TGF- β antibody (Ab) treatment. High dose of TGF- β antibody had similar effects on kidney collagen content in Ang II-infused WT UVO kidney ($n = 3$ to 8 for each group). *, $P < 0.01$. Data are shown as mean \pm SEM.

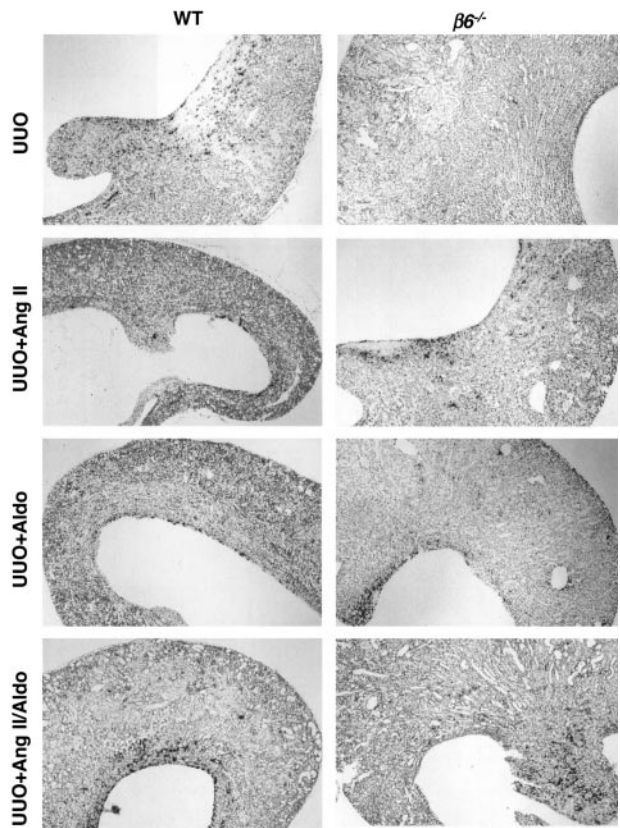


Figure 12. PAI-1 mRNA expression by *in situ* hybridization. PAI-1 mRNA expression was marked in WT UVO, contrasting very little expression in $\beta 6^{-/-}$ UVO. After Ang II or Aldo or combination infusion, PAI-1 mRNA expression was similar in $\beta 6^{-/-}$ UVO and WT UVO, correlating with similar degree of collagen accumulation ($n = 3$ to 6 for each group). Original magnifications, $\times 40$.

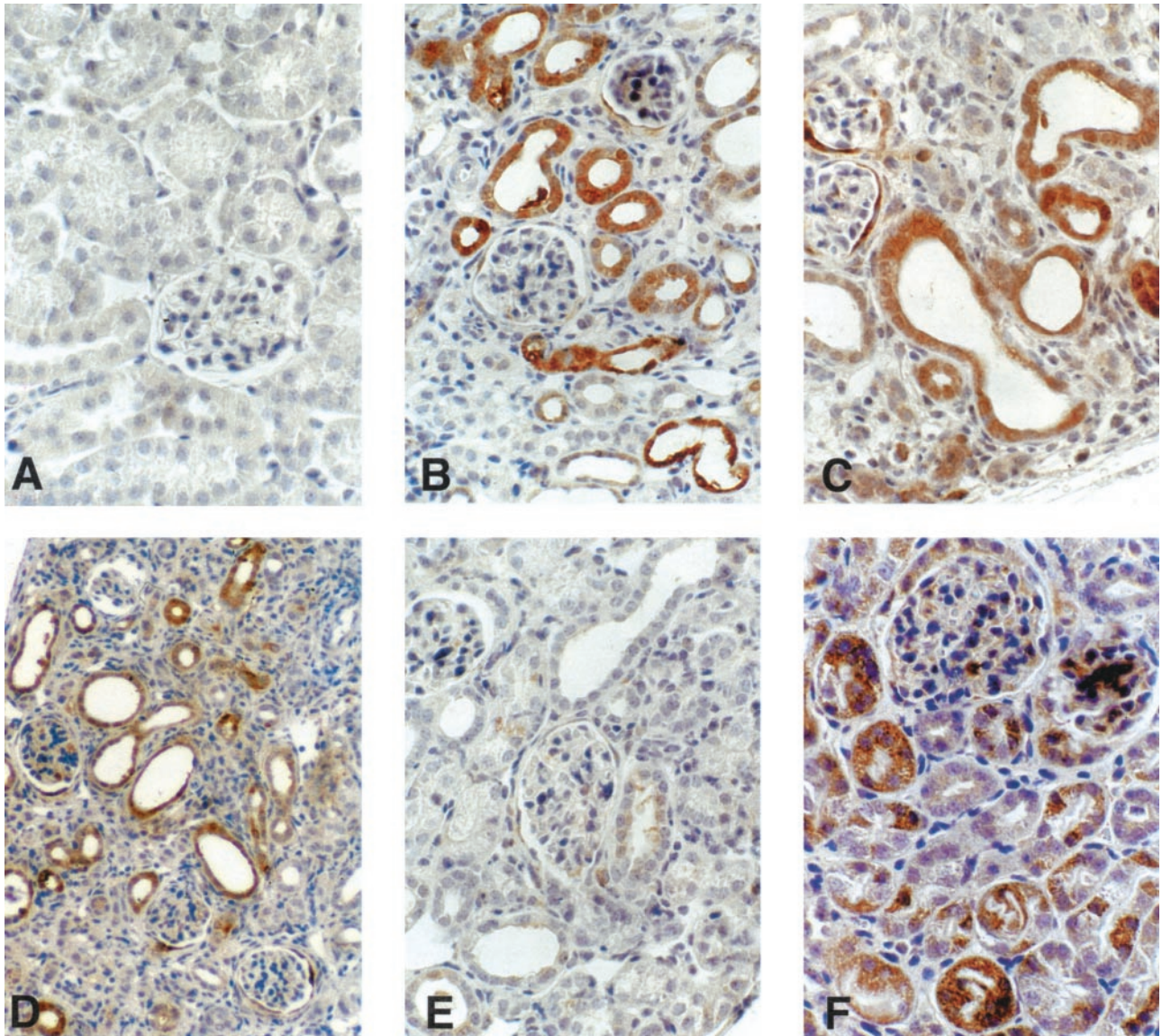


Figure 13. PAI-1 expression in UUO kidney. PAI-1 immunoreactivity in WT nonobstructed kidneys (**A**); WT UUO kidneys (**B**); WT UUO kidneys after infusion with Ang II (**C**) or Aldo (**D**); and in $\beta 6^{-/-}$ UUO kidney without (**E**) or with (**F**) Ang II infusion ($n = 3$ to 6 for each group). Original magnifications: $\times 400$ (**A-C**, **E**, **F**); $\times 200$ (**D**).

of skin, lung, and tubular epithelium of kidney.⁴ No expression has been detected in the glomerulus. Thus, the local, renal epithelial cell activation of TGF- $\beta 1$ via $\beta 6$ and its importance for fibrosis can be determined by inducing tubulointerstitial injury in these mice.

This study indeed demonstrated that $\alpha \nu \beta 6$ -mediated TGF- β activation is pivotal for development of full injury after UUO. Tubulointerstitial fibrosis in response to UUO was markedly less in $\beta 6^{-/-}$ mice compared to WT mice, indicating that interruption of $\beta 6$ mechanisms can protect against tubulointerstitial fibrosis.

Activation of TGF- β can occur through several mechanisms, including plasmin cleavage.²⁴ However, additional mechanisms must exist *in vivo* for activation of TGF- β , because knockout of plasminogen, the substrate yielding plasmin, results in mice that do not share the phenotype of TGF- $\beta 1$ knockout mice. Other mechanisms

for activating TGF- β *in vitro* including reactive oxygen species and TSP-1.²⁵ Indeed TSP-1 knockout mice have a similar pattern of inflammation as TGF- $\beta 1$ knockout mice, supporting the concept that thrombospondin plays a major role in activation of TGF- β *in vivo*.²⁶ In the present study, there was increased TSP-1 in tubular epithelial cells in WT UUO kidneys. However, in response to angiotensin infusion in obstructed $\beta 6^{-/-}$ mice, there was increased TSP-1 but less activated TGF- β by immunostaining, suggesting that TSP-1-dependent TGF- β activation does not explain the induction of fibrosis by angiotensin in obstructed $\beta 6^{-/-}$ kidneys.

We then examined potential downstream mechanisms for the protective effect of $\beta 6$ deletion. We examined local, active TGF- $\beta 1$ expression in WT and $\beta 6^{-/-}$ mice UUO kidney using a specific antibody for detection of active TGF- β ¹⁹ and found markedly increased active

TGF- β in tubular epithelial cells and some interstitial cells in WT UUO kidneys, contrasting with very little active TGF- β expression in $\beta 6^{-/-}$ UUO. This observation supports that knockout of $\beta 6$ integrin dampens the local activation of TGF- β in these parenchymal cells in the tubulointerstitium. We also investigated TGF- β activation by assessing its downstream signaling pathways, the Smad proteins.^{27,28} Smad-2 in its phosphorylated state has been used as a specific marker of TGF- β activation. Further evidence of the key role of Smad 2 in TGF- β signaling is seen by inhibition of TGF- β -induced fibrosis when it is inactivated by Smad 7.²⁹ In the obstructed, fibrotic kidneys in the WT mice, we observed, as expected, concomitant enhancement of active TGF- $\beta 1$ as indirectly assessed by immunostaining and activation of Smad 2. Low expression of phosphorylated Smad 2 in the obstructed, but nonfibrotic kidneys in $\beta 6^{-/-}$ mice indicates that prevention of TGF- $\beta 1$ activation is a key protective mechanism against fibrosis in $\beta 6^{-/-}$ mice, as previously also observed in the bleomycin pulmonary fibrosis model.

We next determined whether direct RAAS stimuli could induce fibrosis in $\beta 6^{-/-}$ UUO mice. A contribution of angiotensin in interstitial fibrosis in obstructive nephropathy was recently documented in angiotensin type 1 receptor (AT1a) knockout mice.³⁰ In our model, blood pressure effects may have augmented and contributed to the fibrotic effects of RAAS stimulation. However, fibrosis was not induced by Ang II or Aldo infusion in nonobstructed kidneys, indicating that systemic hemodynamic effects alone were not sufficient to cause injury. In this regard, studies of mice with 0 to 4 copies of the angiotensinogen gene demonstrated that angiotensin regulates at least 50% of the renal interstitial fibrotic response in obstructive nephropathy through nonhemodynamic effects.³¹ Indeed, we observed that infusion with either Ang II, Aldo, or their combination for 14 days restored fibrosis in $\beta 6^{-/-}$ UUO mice. The lack of complete prevention of Ang II- or Aldo-induced fibrosis in these mice by treatment with neutralizing antibody to TGF- β supports the existence of a component of fibrosis that is TGF- β -independent. This fibrosis in $\beta 6^{-/-}$ UUO mice with angiotensin was not linked to increased TGF- β activation as assessed by phospho-Smad 2, further supporting that angiotensin can induce TGF- β -independent mechanisms of tubulointerstitial fibrosis in the obstructed $\beta 6^{-/-}$ mice.

Angiotensin is a strong inducer of PAI-1. Increased local PAI-1 expression is directly linked to sites of sclerosis and tubulointerstitial fibrosis.^{10,21,23,32} In addition, PAI-1 knockout mice are protected against fibrosis induced by ureteral obstruction.³³ These results suggest that both decreased extracellular matrix synthesis, because of reduced TGF- β activation, and increased matrix degradation, mediated by decreased PAI-1, contribute to the lesser collagen accumulation in $\beta 6^{-/-}$ mice. Indeed, the protected $\beta 6^{-/-}$ UUO mice had very low levels of PAI-1 compared to WT with UUO. Further, the fibrotic response in $\beta 6^{-/-}$ UUO mice treated with RAAS stimuli correlated with increased PAI-1 expression, suggesting that this pathway could be in part TGF- β -independent. Importantly, despite treatment with antibody to TGF- β ,

both fibrosis and PAI-1 expression were primarily maintained in RAAS-stimulated $\beta 6^{-/-}$ UUO mice. We postulate that modulation of matrix degradation via PAI-1 is an important component of the fibrotic response. PAI-1 increases were similar in intensity and location in both RAAS-stimulated obstructed WT and $\beta 6^{-/-}$ mice. These findings support our hypothesis that the development of fibrosis is tightly linked to local PAI-1 expression that can, in the absence of $\alpha v\beta 6$, be TGF- β -independent.

Macrophages have diverse role in tissue injury, mediating repair and remodeling after injury. Persistence of activated macrophages is tightly correlated with progressive fibrosis in the kidney.³⁴ However, the macrophage may not mediate fibrosis per se. Our data show that despite robust macrophage infiltration after UUO, interstitial fibrosis was blunted in the absence of parenchymal cell responsiveness in $\beta 6^{-/-}$ mice, in parallel to previous observations in the lung bleomycin model.⁶

Macrophages bear AT1 receptors, and they respond to angiotensin with chemotaxis and activation.³⁵ However, we have shown that AT1 receptor-mediated actions of macrophages may have a protective role in some settings of fibrosis. Adoptive transfer of AT1a-null bone marrow resulted in more injury after UUO although the number of macrophages was not altered.³⁶ In the current study, we also observed lack of correlation of macrophages with fibrosis in $\beta 6^{-/-}$ UUO mice given RAAS stimulation. This lack of correlation of macrophages and fibrosis was also previously observed in UUO mice treated with AT1 receptor antagonist.³⁷ In the current study, macrophage infiltration was reduced although fibrosis was fully restored by angiotensin infusion, pointing to nonmacrophage-dependent fibrosis. We speculate that parenchymal cell PAI-1 expression could be one such mechanism.

In summary, we have shown that integrin $\alpha v\beta 6$ is a critical determinant of kidney fibrosis. Absence of $\beta 6$ confers remarkable protection against tubulointerstitial fibrosis despite robust macrophage infiltration. Both angiotensin and Aldo induce fibrosis in $\beta 6^{-/-}$ mice with UUO without increased TGF- β activation. This fibrosis was linked to increased PAI-1. These data suggest a TGF- β -independent component of induction of PAI-1. Our findings raise the possibility that targeting integrin $\alpha v\beta 6$ and/or PAI-1 may provide novel clinical strategies in treatment of kidney fibrosis.

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