J Am Soc Nephrol 14: 2544–2555, 2003

Obstructive Uropathy in Mice and Humans: Potential Role for PDGF-D in the Progression of Tubulointerstitial Injury

SEKIKO TANEDA,* KELLY L. HUDKINS,* STAVROS TOPOUZIS,‡ DEBRA G. GILBERTSON,‡ VUDDHIDEJ OPHASCHAROENSUK,† LUAN TRUONG,§ RICHARD J. JOHNSON,‖ and CHARLES E. ALPERS*

**Department of Pathology, †Division of Nephrology, University of Washington, and ‡ZymoGenetics Inc., Seattle, Washington; and §Department of Pathology and ‖Division of Nephrology, Baylor College of Medicine, Houston, Texas.*

Abstract. Tubulointerstitial fibrosis is a major characteristic of progressive renal diseases. Platelet-derived growth factor (PDGF) is a family of growth regulatory molecules consisting of PDGF-A and -B, along with the newly discovered PDGF-C and -D. They signal through cell membrane receptors, PDGF receptor α (PDGF-Rα) and receptor β (PDGF-Rβ). Involvement of PDGF-B and PDGF-Rβ in the initiation and progression of renal fibrosis has been well documented. The authors studied the localization of PDGF ligands and receptors by immunohistochemistry, with emphasis on the role of PDGF-D in murine renal fibrosis induced by unilateral ureteral obstruction (UUO). In mice with UUO, *de novo* expression of PDGF-D was detected in interstitial cells at day 4, which increased to maximal expression at day 14. Increased expression of PDGF-B by interstitial cells and in some tubules was observed after day 4. The diseased mice did not show augmentation of PDGF-A or PDGF-C proteins in the areas of fibrosis. PDGF-Rα and -Rβ protein expression was increased in interstitial cells after day 4 and reached maximal expression at day 14. Human renal nephrectomies ($n = 10$) of chronic obstructive nephropathy demonstrated similar *de novo* expression of PDGF-D in interstitial cells, correlating with expression of PDGF-Rβ and PDGF-B, as it did in the murine model. These observations suggest that PDGF-D plays an important role in the pathogenesis of tubulointerstitial injury through binding of PDGF-Rβ in both human obstructive nephropathy and the corresponding murine model of UUO.

Tubulointerstitial inflammation and fibrosis are critical determinants of renal function and prognosis for patients with a variety of renal diseases. Although many studies have been conducted to elucidate the underlying mechanisms of this injury, the role of growth factors involved in the progression of tubulointerstitial fibrosis still remains incompletely understood.

Platelet-derived growth factor (PDGF) is a family of growth regulatory molecules that has 4 identified members, PDGF-A and -B, and the newly discovered PDGF-C and -D. The original members of the PDGF family are secreted as disulfide bonded homo- or heterodimers (PDGF-AA, -AB, and -BB) (1,2), whereas PDGF-C and -D are secreted in a latent form and require extracellular proteolytic cleavage to release the active growth factor domain (3,4). The C-terminal growth factor domain of PDGF-C and -D has homology to vascular endothelial growth factor (3–5). The amino acid sequence of PDGF-D is closely related to PDGF-C (approximately 50%) (3,4) and to PDGF-A and PDGF-B (approximately 25%) (4). Functional differences between PDGF-C and -D are based on their binding properties to PDGF receptors. There are two tyrosine kinase receptors for PDGF: PDGF receptor alpha (PDGF-Rα) and receptor beta (PDGF-Rβ). Each PDGF receptor consists of α and β subunits that are brought together to form one of three isoforms on binding PDGF, PDGF R-$\alpha\alpha$, -$\alpha\beta$, and -$\beta\beta$ (6). PDGF-A can bind PDGF R-$\alpha\alpha$ with a high affinity, and PDGF-B can bind all isoforms of receptors, PDGF R-$\beta\beta$, -$\alpha\beta$ and -$\alpha\alpha$ (7). It has been recently reported that PDGF-CC binds both PDGF R-$\alpha\alpha$ and PDGF R-$\alpha\beta$ (5), whereas PDGF-D can bind only PDGF-Rβ and is the only PDGF-βR–specific ligand (4,8).

Of these PDGF ligand/receptor systems, PDGF-B, signaling through PDGF-Rβ, is considered to be an important mediator in the initiation and progression of renal fibrosis as a result of its biologic activity as a mitogen and chemoattractant for fibroblasts (9). Several studies have demonstrated the involvement of this PDGF-B/PDGF-Rβ signaling pathway in both experimental and human interstitial injuries (10–13). It has also been demonstrated that PDGF-D, the second major ligand of PDGF-Rβ, is involved in renal development in mice and humans (4,8,14) and is normally expressed by visceral epithelial cells and vascular smooth muscle cells in adult human kidneys (14). However, little is known of the role of PDGF-D in the progression of tubulointerstitial fibrosis.

Received April 4, 2003.Accepted July 19, 2003.

Correspondence to Dr. Charles E. Alpers, University of Washington Medical Center, Department of Pathology, Box 356100, 1959 NE Pacific Street, Seattle, WA 98195. Phone: 206-543-5616; Fax: 206-543-3644; E-mail: calp@u.washington.edu

1046-6673/1410-2544
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000089828.73014.C8

Our study was undertaken to examine the localization of all PDGF ligands and their receptors, with emphasis on the role of PDGF-D, in the murine model of unilateral ureteral obstruction (UUO), a well accepted experimental model for the study of the mechanisms of tubulointerstitial injury. We report that PDGF-B, -D, -Rα, and -Rβ, but not PDGF-C and -A, were upregulated in areas of renal fibrosis, and that the distribution pattern of PDGF-D was congruent with that of PDGF-Rβ. PDGF-D was expressed by α smooth muscle actin (αSMA) expressing interstitial fibroblasts (myofibroblasts), cells that play a major role in fibrosing renal injuries (15).

Materials and Methods

Animal Model and Experimental Design

Unilateral ureteral ligation resulting in UUO was performed in 15 male 129/SvJ × Black Swiss mice (10 to 12 wk old) by ligation of the left ureter of each animal at the ureteropelvic junction. Tissue from animals reported in the previous study (16) was used in the present investigation. Animals were killed at days 4, 7, and 14 after induction of the disease ($n = 5$ at each time point), and renal samples were obtained via biopsy from each animal. Tissue samples were fixed in either 10% neutral-buffered formalin or methyl Carnoy solution and were embedded in paraffin. Kidneys obtained from uninjured mice (day 0, $n = 4$) were used as controls. Tissue samples were studied for all PDGF ligands (PDGF-A, -B, -C, and -D) and receptors (PDGF-αR and -βR), interstitial proliferating cells (defined by the nuclear cell proliferation-associated antigen, Ki-67), αSMA (a marker for interstitial myofibroblast), type I collagen, and endothelial cells (MECA-32) by immunohistochemistry.

Tissue Samples

Residual paraffin-embedded, formalin-fixed renal nephrectomy tissue no longer required for diagnosis from ten patients with chronic obstructive nephropathy were studied. Included were samples from seven patients from Baylor College of Medicine and three from the University of Washington. Tissues were studied for expression of PDGF-B and -D, PDGF-βR, αSMA, and type I and type IV collagen by immunohistochemistry.

Immunohistochemistry

Four-micron sections of formalin- and methyl Carnoy–fixed, paraffin-embedded tissue were processed by an indirect immunoperoxidase technique as described previously (17). Primary antibodies included the following.

PDGF-A (N-30)

Rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) raised against the epitope mapping at the carboxy terminus of PDGF-A of human origin specific for PDGF-A was used. Absorption studies and Western blot tests demonstrating the specificity of this reagent have been published previously (17).

PDGF-B (Ab-1)

Rabbit polyclonal antibody (Oncogene Research Products, La Jolla, CA) is an affinity-purified antibody raised against the peptide corresponding amino acid residues 101 to 116 of human PDGF B chain (18).

PDGF-C

Rabbit polyclonal antibody (Lot no. E2243; ZymoGenetics, Seattle, WA) is an affinity-purified antibody raised against the PDGF-C molecule of human origin (5).

PDGF-D

Rabbit polyclonal antibody (Lot no. E3812; ZymoGenetics) is an affinity-purified antibody raised against the middle part of PDGF-D molecule. It recognizes the full-length human PDGF-D peptide. The specificity of this reagent has been previously characterized by Western blot analysis of tissue-extracted protein from human kidneys and a control preparation of PDGF-D full-length protein (14). The specificity of this antibody was also confirmed by immunoblotting, which demonstrated that neither PDGF-C growth factor domain nor full-length protein cross-reacted with anti–PDGF-D antibody (14). This reagent has been used by our laboratory for immunohistochemistry procedures (14).

PDGF-Rα (C-20)

Rabbit polyclonal antibody (Santa Cruz Biotechnology) raised against the epitope mapping at the carboxy terminus of human PDGF receptor type α (identical to corresponding mouse sequence) was used. This antibody has been characterized by Western blotting of normal mouse kidneys (19) and used for immunohistochemistry procedures in murine and rat kidneys (19,20).

PDGF-Rβ (958)

Rabbit polyclonal antibody (Santa Cruz Biotechnology) is an affinity-purified antibody raised against a recombinant protein corresponding to amino acids 958 to 1106 mapping at the carboxy terminus of PDGF receptor type β of human origin. The specificity of this reagent has been demonstrated by Western blotting and immunohistochemistry of glomerular mesangial cells of normal mouse (19).

Ki-67 (Ab-1)

Mouse monoclonal antibody (clone B56; BD Biosciences, San Diego, CA) reacts with a human nuclear cell proliferation–associated antigen expressed in all active stages of the cell cycle. Epitope analysis of this antibody has been conducted previously (21)

αSMA

Mouse monoclonal antibody (clone 1A4, DAKO, Carpinteria, CA) has been well characterized by Western blot test (22) and used as a marker of myofibroblasts as described previously (15).

Mouse Endothelial Cell

Rat monoclonal antibody (clone MECA-32; Developmental Studies Hybridoma Bank, Iowa City, IA), a marker of endothelial cells (23), was used to identify peritubular capillaries and vascular endothelial cells as described previously (24).

Type I Collagen

Goat polyclonal antibody (Southern Biotechnology, Birmingham, AL) is a purified antibody raised against human type I collagen and does not react with type II, III, IV, V, and VI collagen.

Type IV Collagen

Goat polyclonal antibody (Southern Biotechnology) is a purified antibody raised against human type IV collagen and does not react with type I, II, III, V, and VI collagen.

Analysis

For all samples, negative controls for the immunohistochemistry included substituting for the primary antibody an irrelevant IgG from the same species, or PBS. The number of proliferating cells labeled with Ki-67 in the cortical interstitial cells was counted in 20 sequentially selected fields of renal cortex at a magnification of ×400. The results were expressed as the mean number ± SEM per high-power field (25). αSMA expression in each biopsy sample was calculated by scoring 20 sequentially selected fields of renal cortex at ×400 magnification with a semiquantitative scale of 0 to 4 as follows: 0 = absent staining, 1 = minimal expression (1% to 9% of interstitial), 2 = mild expression (10% to 49%), 3 = moderate expression (50% to 89%), and 4 = marked expression (more than 95%), as described previously (25).

Cell Culture, Protein Preparation, and Western Blot Test

Mouse mesangial cells derived from normal mouse glomeruli (provided by Dr. S.J. Shankland, University of Washington, Seattle) (26) and mouse endothelial cells (provided by Dr. GE Striker, University of Miami School of Medicine, Miami, FL) were passaged in appropriate growth medium as described previously (26). Growth medium composed of 20% (vol/vol) of FBS was used for endothelial cells and 10% (vol/vol) of FBS was used for mesangial cells. Mesangial cells were identified by positive staining for mesangial cells (αSMA), and the absence of markers for endothelial cells (factor VIII staining) and podocytes (secreted protein, acidic, and rich in cysteine; SPARC) (27). Similarly, endothelial cells were identified by positive staining for factor VIII and the absence of immunostaining for αSMA and SPARC. Total proteins were extracted from each type of cells lysed in buffer as described previously (28).

The protein samples were electrophoresed on 8% to 16% polyacrylamide gels, transferred to nitrocellulose membranes, and then blocked with 5% nonfat dry milk in TBS containing 2.5% BSA as described previously (28). The membranes were then incubated overnight with a 1/1000 dilution of rabbit polyclonal anti–PDGF-D antibody or rabbit polyclonal anti–PDGF-C antibody, or a 1/50 dilution of PDGF-B antibody diluted in PBS containing 1% BSA and 5% nonfat dry milk at 4°C. After thoroughly washing in TBS with 0.3% Tween-20, the membranes were sequentially incubated with goat anti-rabbit IgG alkaline phosphatase conjugated for 30 min, washed, developed with a chemiluminescent substrate (CSPD; Applied Biosystems, Norwalk, CT) and exposed to film. Full-length PDGF-C and -D proteins (provided by ZymoGenetics), and a purified, recombinant human PDGF-BB (Upstate Biotechnology, Lake Placid, NY) were used as positive protein controls. As a negative antibody control, the primary antibody was replaced by normal rabbit IgG at an equivalent concentration.

Double-Labeling Immunohistochemistry

Four-micron sections of methyl Carnoy–fixed, paraffin-embedded tissues were prepared for immunohistochemistry as described previously (29). Double immunolabeling for PDGF-D and αSMA was performed to determine the cell types expressing PDGF-D in this mouse model. The slides were sequentially incubated with a rabbit polyclonal PDGF-D antibody, biotinylated goat anti-rabbit IgG, the ABC-Elite reagent, and 3,3'-diaminobenzidine to give a brown reaction. Peroxidase activity was blocked again with 3% hydrogen peroxide. Then the sections were incubated with mouse monoclonal antibody against αSMA, followed by biotinylated anti-mouse IgG, the ABC-Elite reagent, and finally a purple substrate kit (Vector VIP SK-4600, Peroxidase Substrate Kit; Vector Laboratories, Burlingame, CA) to yield a purple reaction product. Cells with overlapping staining by both antibodies were identified as myofibroblasts expressing PDGF-D protein.

Double immunolabeling for PDGF-D and type I collagen was performed to determine whether type I collagen was produced by the same cells that produce PDGF-D. Ki-67/αSMA double immunostaining was also performed to assess the number of myofibroblasts undergoing proliferation. Sections were first stained with a murine monoclonal antibody to Ki-67, followed by biotinylated anti-mouse IgG antibody, the ABC-Elite reagent, and 3,3'-diaminobenzidine with nickel as the chromogen to give a black reaction. Then sections were incubated with αSMA as described above without nickel, to give a brown reaction product.

Statistical Analyses

All values are expressed as the mean ± SEM. For data analysis, we used the SPSS program, version 10.0 for Windows (SPSS, Chicago, IL). Statistical significance, defined as $P < 0.05$, was evaluated by either the nonparametric Mann-Whitney U test or the parametric one-way ANOVA with Tukey's post hoc test.

Results

PDGF-A Is Detected in Infiltrating Cells in Mice with UUO

In normal mice, PDGF-A expression was primarily localized in papillary regions with presumable expression by tubular cells comprising the loop of Henle (Figure 1A). In the renal cortex, there was widespread expression of this protein by the vascular smooth muscle cells (Figure 1B) and barely detectable expression in interstitial cells (Figure 1B). In mice with UUO, expression of PDGF-A was found in vascular smooth muscle cells and was absent in the cortical interstitial cells (Figure 1C),

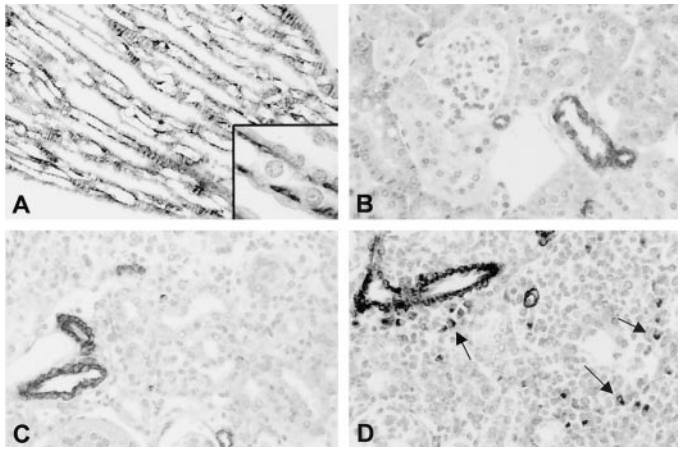

Figure 1. Platelet-derived growth factor A (PDGF-A) is detected in infiltrating cells in mice with unilateral ureteral obstruction (UUO). In normal mice, PDGF-A expression is most highly expressed by interstitial cells of the papillary region (A). In the renal cortex, immunostaining of PDGF-A is normally observed in vascular smooth muscle cells but barely detected in interstitial cells (B). In the mice with UUO, PDGF-A protein is absent in the cortical interstitial cells (C), although occasional infiltrating cells express PDGF-A (D) at day 14 (arrows). Original magnification: ×400; A, inset, ×1000.

generally corresponding to the pattern seen in normal mice. Infiltrating cells occasionally expressed PDGF-A protein in the diseased mice (Figure 1D).

Tubulointerstitial Upregulation of PDGF-B Is Observed in Mice with UUO

The presence of PDGF-B in mouse mesangial cells was confirmed by Western blot analysis that used the rabbit polyclonal antibody directed against human PDGF-B (Ab-1, Oncogene research product). Specific immunoreaction was localized to an approximately 15-kD band in mouse mesangial cell extracts when run under reducing condition (data not shown), which was consistent with the biochemical properties of PDGF-B in other reports (30,31).

In normal mice, immunostaining for PDGF-B was localized to vascular smooth muscle cells (Figure 2A). PDGF-B protein was also weakly expressed by cortical interstitial cells but was absent in tubules (Figure 2A). In the mice with UUO, increased immunostaining for PDGF-B was observed in cortical interstitial cells at day 4 (Figure 2B), which did not show a significant change until day 14. PDGF-B expression was also detected in occasional proximal tubules (Figure 2C) and distal tubules (Figure 2D), which was most apparent at day 4. Immunostaining for PDGF-B was also present in interstitial cells in the areas containing inflammatory infiltration but was not detected in the infiltrating cells themselves (Figure 2E).

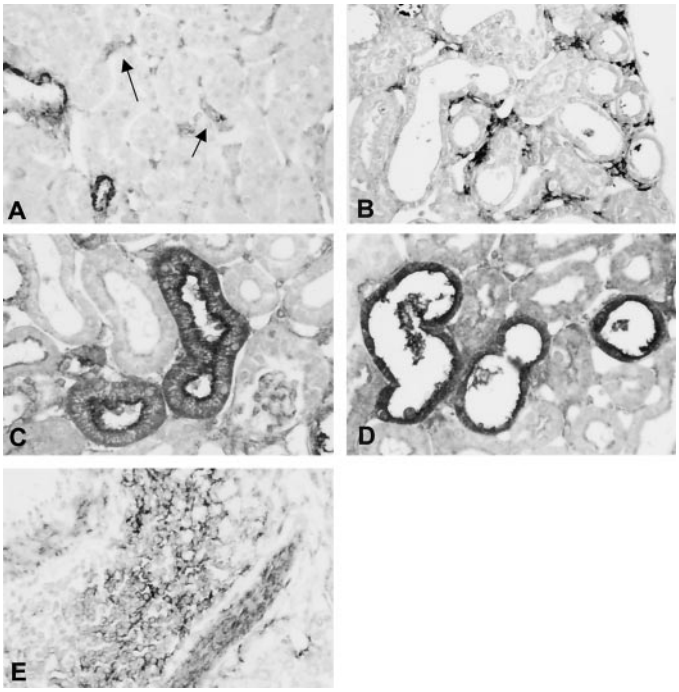

Figure 2. Tubulointerstitial overproduction of platelet-derived growth factor B (PDGF-B) is observed in areas of tubulointerstitial fibrosis. In normal mice (A), expression of PDGF-B is observed in vascular smooth muscle cells and weakly detected in the interstitial cells (arrows) but is absent in tubular cells. In mice with unilateral ureteral obstruction (UUO), increased expression of PDGF-B is observed in interstitial cells (B) and in some proximal tubules (C) and distal tubules (D) at day 4. PDGF-B protein is present in interstitial cells in the areas containing inflammatory infiltrates but is not detected in the infiltrating cells themselves at day 14 (E). Original magnification: ×400.

PDGF-C Is Expressed by Peritubular Capillaries and Was Not Upregulated in Mice with UUO

The presence of PDGF-C protein in mouse endothelial cells was confirmed by Western blot analysis that used the polyclonal anti–PDGF-C antibody. An approximately 50-kD monospecific band corresponding to the size of the PDGF-C monomer as indicated by using a control preparation of PDGF-C full-length protein was identified under reducing conditions (Figure 3A). In the same conditions, we also detected PDGF-C full-length protein at approximately 100 kD, which was thought to be a PDGF-CC dimer (Figure 3A). These results were consistent with the previously characterized biochemical properties of PDGF-C (3).

Immunostaining of PDGF-C was normally expressed by glomerular endothelial cells (Figure 3B), vascular smooth muscle and vascular endothelial cells (Figure 3, C and D), and peritubular capillary endothelial cells (Figure 3D). No expression of PDGF-C by either tubular or interstitial cells was detected. In the mice with UUO at day 4, the expression of PDGF-C in peritubular capillaries persisted (Figure 3E). At day 14, PDGF-C expression was somewhat diminished (Figure 3F). Serial sections labeled with MECA-32, an antibody that labeled mouse endothelial cells including peritubular capillaries (24), demonstrated an expression pattern similar to PDGF-C (Figure 3, F and G). This observation indicates that although PDGF-C appeared to be downregulated in some peritubular capillaries, it was not because peritubular capillaries did not produce PDGF-C protein, but because of damage or loss of portions of the capillary network resulting from increased fibrosis and cellular infiltration. There was no expression of PDGF-C in interstitial areas where MECA-32 protein was undetectable (data not shown).

De Novo *Production of PDGF-D in Interstitial Cells Was Observed in Areas of Tubulointerstitial Fibrosis in Mice with UUO*

The presence of PDGF-D protein in mouse mesangial cells was confirmed by Western blot analysis that used the polyclonal anti–PDGF-D antibody. An approximately 50-kD monospecific band corresponding to the size of the PDGF-D monomer as indicated by using a control preparation of PDGF-D full-length protein was identified under reducing conditions (Figure 4A). Under the same conditions, we also detected PDGF-D full-length protein at approximately 100 kD, which was thought to be the dimer PDGF-DD (Figure 4A). These results were consistent with the previously characterized biochemical properties of PDGF-D (4,8,14).

Immunostaining of PDGF-D in normal mice was localized to glomerular mesangial cells and vascular smooth muscle cells (Figure 4B). In contrast, the mice with UUO demonstrated *de novo* expression of PDGF-D in interstitial cells (Figure 4C).

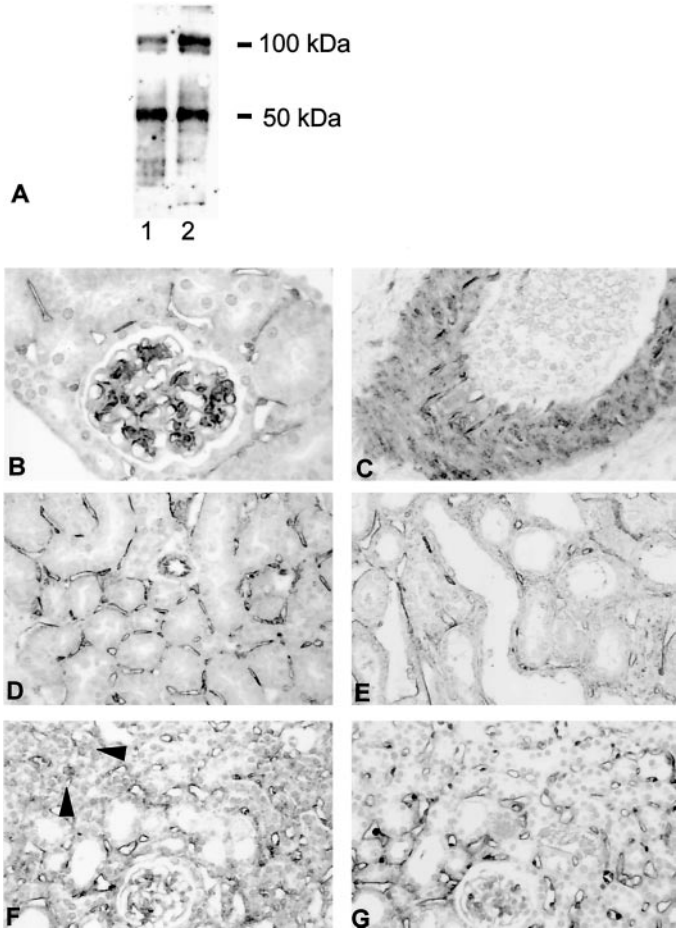

Figure 3. Platelet-derived growth factor C (PDGF-C) is expressed by peritubular capillaries and was unchanged in mice with unilateral ureteral obstruction (UUO). Western blot analysis utilizing anti–PDGF-C antibody demonstrating an approximately 50-kD monospecific band corresponding to the size of the PDGF-C monomer under reducing conditions (A; lane 1). In the same condition, PDGF-C full-length protein at approximately 100 kD was also detected, which was thought to be a dimer of PDGF-CC (A; lane 1). The antibody reacts with mouse endothelial cells (A; lane 2). In normal mouse, PDGF-C protein is expressed by glomerular endothelial cells (B), by vascular smooth muscle and vascular endothelial cells (C, D), and by peritubular capillary endothelium (D). In mouse with UUO, expression of PDGF-C in peritubular capillaries persists at day 4 (E), and its expression was somewhat diminished at day 14 (arrowheads) (F). In a serial section immunolabeled with MECA-2 (G), a marker for mouse endothelial cells, expression patterns of PDGF-C and MECA-2 staining are seen to be congruent (cf. F and G). Original magnification: B and C, ×600; others, ×400.

PDGF-D expression was focally detected in interstitial cells at day 4, which was particularly prominent around dilated and atrophic tubules (Figure 4C). Expression of PDGF-D increased over the time course studied and was maximal at day 14 (Figure 4D). PDGF-D was expressed by spindle-shaped interstitial fibroblasts (Figure 4D). Expression of this protein was not detected in tubules in either the normal or diseased mice (or in infiltrating inflammatory cells) (Figure 4, B through E).

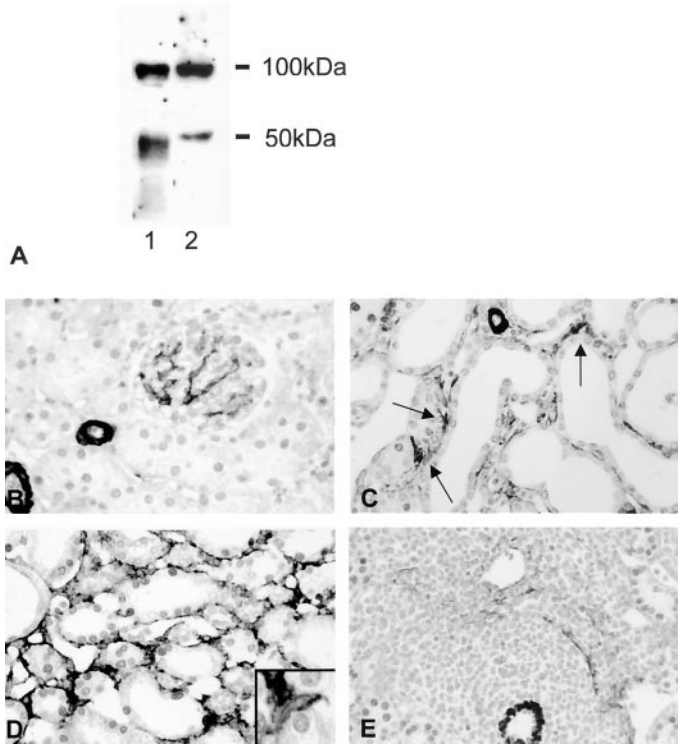

Figure 4. De novo production of platelet-derived growth factor D (PDGF-D) is detected in interstitial cells in areas of tubulointerstitial fibrosis. Western blot analysis that uses anti–PDGF-D antibody demonstrated an approximately 50-kD monospecific band corresponding to the size of the PDGF-D monomer under reducing conditions (A; lane 1). In the same condition, PDGF-D full-length protein at approximately 100 kD was also detected, which was thought to be a dimer of PDGF-DD (A; lane 1). The antibody reacts with mouse mesangial cells (A; lane 2). In normal mouse, PDGF-D is expressed by glomerular mesangial cells and by vascular smooth muscle cells (B). *De novo* expression of PDGF-D is focally observed in interstitial cells around diluted and atrophic tubules at day 4 (arrows) (C), which was increased at day 14 (D). Occasional spindle-shaped interstitial cells express PDGF-D (inset, D). No PDGF-D expression is observed in infiltrating cells at day 14 (E). Original magnification: ×400; E, inset, ×1000.

Interstitial Upregulation of PDGF-Rα Is Detected in Areas of Tubulointerstitial Fibrosis in Mice with UUO

In normal mice, PDGF-Rα was very focally expressed in cortical interstitial cells (Figure 5A), and it was highly expressed in the interstitium of the papillary region (Figure 5B). This protein was also expressed by adventitial cells in arterial vessels (Figure 5C). No other glomerular or interstitial structures expressed PDGF-Rα (Figure 5A). In contrast, in mice with UUO, PDGF-Rα expression in cortical interstitial cells became prominent at day 4 (Figure 5D), which increased progressively until day 14 (Figure 5E). No staining for PDGF-Rα was noted in tubules or in infiltrating inflammatory cells (Figure 5F).

Upregulation of PDGF-Rβ Is Detected in Areas of Tubulointerstitial Fibrosis in Mice with UUO

Normal mice showed weak expression of PDGF-Rβ in glomerular mesangial cells and interstitial cells (Figure 6, A and

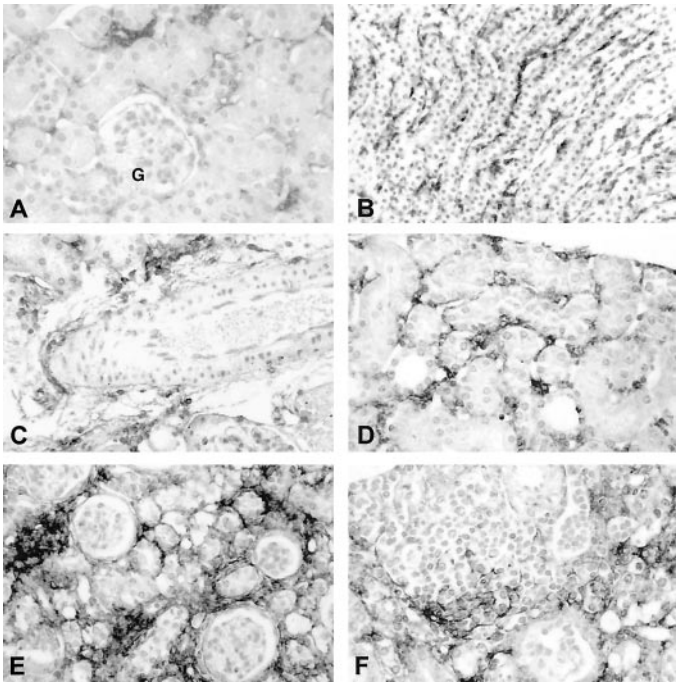

Figure 5. Platelet-derived growth factor Rα (PDGF-Rα) is expressed in areas of tubulointerstitial fibrosis in mice with unilateral ureteral obstruction (UUO). G indicates glomerulus. In the normal mice, PDGF-Rα expression is barely detected in cortical interstitial cells (A) and highly expressed in interstitial cells in the papillary region (B). PDGF-αR is also observed in vascular adventitial cells (C). In the mice with UUO, PDGF-Rα expression in interstitial cells becomes prominent at day 4 (D) and is increased at day 14 (E). No infiltrating inflammatory cells express PDGF-Rα (F). Original magnification: ×400.

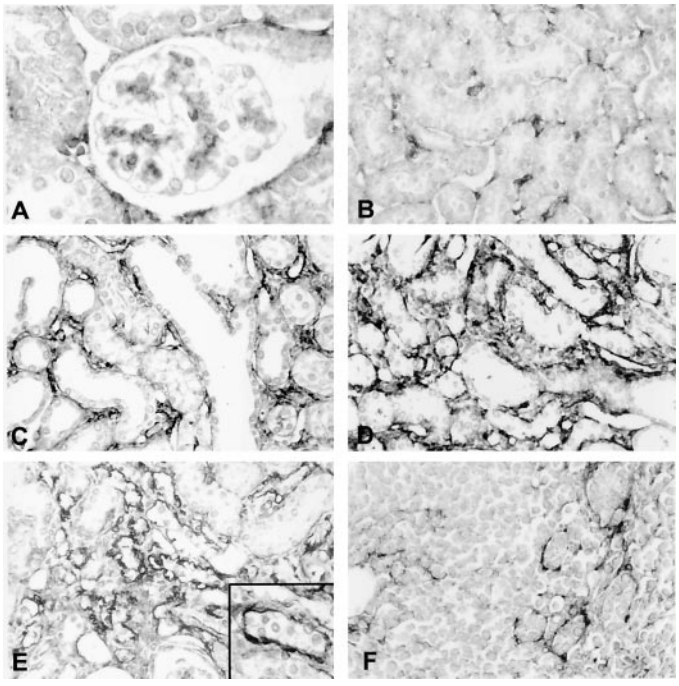

Figure 6. Platelet-derived growth factor Rβ (PDGF-Rβ) is expressed in areas of tubulointerstitial fibrosis in mice with unilateral ureteral obstruction (UUO). In normal mouse, PDGF-Rβ expression is weakly detected in glomerular mesangial cells (A) and interstitial cells (B). PDGF-Rβ expression in interstitial cells becomes prominent at day 4 (C) and markedly elevated at day 7(D). PDGF-Rβ protein is also detected in tubular basement membranes of atrophic tubules surrounded by massive fibrosis and inflammatory cells at day 14 (E). PDGF-Rβ expression is absent in inflammatory cells at day 14 (F). Original magnification: A and F, ×600; B through E, ×400; E, inset, ×1000.

B). PDGF-Rβ protein was not detected in either tubules or blood vessels (Figure 6A). In contrast, the mice with UUO demonstrated increased immunostaining of PDGF-Rβ in interstitial cells at day 4 (Figure 6C). PDGF-Rβ expression was markedly elevated at day 7 (Figure 6D), and was maximal at day 14. PDGF-Rβ expression was noted in interstitial cells that surrounded the tubules in areas containing inflammatory infiltrates, but was absent in inflammatory cells themselves (Figure 6E). Furthermore, atrophic tubules surrounded by massive fibrosis and inflammatory cells demonstrated strong expression of PDGF-Rβ in their tubular basement membranes at day 14 (Figure 6F).

Upregulation of PDGF-D Corresponded to PDGF-Rβ Expression in Mice with UUO

Because PDGF-Rβ is reported to be the only receptor for PDGF-D (4,8), and PDGF-BB also has been known to bind PDGF-Rβ with high affinity (7,32), we immunolocalized PDGF-B, -D and PDGF-Rβ on serial sections in the mice with UUO to assess the distribution pattern of each molecule. In areas of tubulointerstitial fibrosis, the distribution pattern of PDGF-Rβ expression most closely corresponded to that of PDGF-D expression (Figure 7, A and B), and demonstrated less congruence with PDGF-B expression (Figure 7, A and C). The interstitial region illustrated in Figure 7, D and E as immunostained for PDGF-Rβ and PDGF-D, respectively, on consecutive sections, and the distribution patterns of both proteins were highly congruent.

The Majority of Interstitial Cells Expressing PDGF-D Also Expressed αSMA and Are the Principal Proliferating Cell Population

Interstitial cell proliferation was assessed by labeling with the Ki-67 antibody. In normal mice, low-grade proliferative activity was noted in the cortical tubulointerstitium, whereas a diffuse increase in the number of interstitial proliferating cells was observed in mice with UUO at all time points studied (Figure 8A). Proliferative activity peaked at day 4, with 13.7 Ki-67 positive cells per high-power field *versus* 1.7 proliferating cells in control mice ($P < 0.01$), then decreased abruptly at day 7 ($P < 0.05$), and increased again at day 14 ($P < 0.01$) (Figure 8A). Double labeling for Ki-67 and αSMA was performed to assess the contribution of myofibroblasts to the proliferative activity in the cortical tubulointerstitium. Proliferating myofibroblasts (that is, Ki-67+/αSMA+) could not be demonstrated in the normal mice. In contrast, the majority of interstitial proliferating cells (72.5% ± 6.8% at day 4, 63.0%

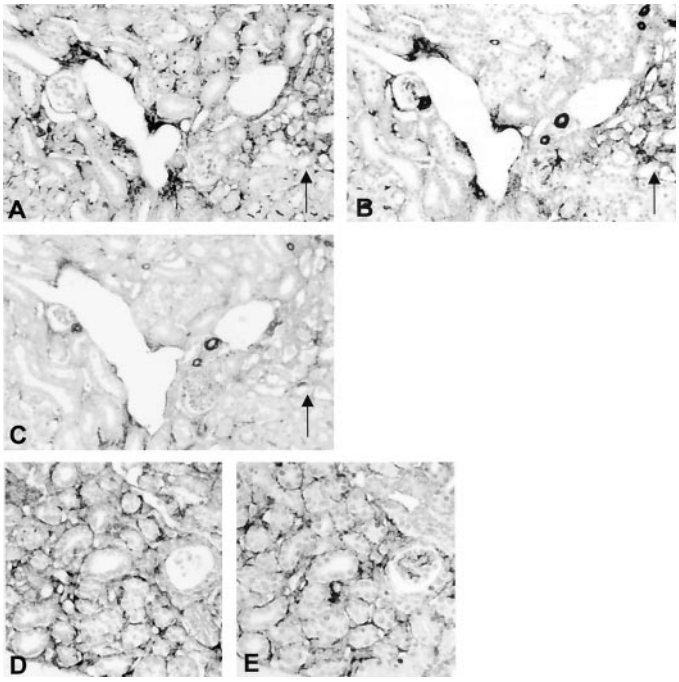

Figure 7. Distribution pattern for platelet-derived growth factor Rβ (PDGF-Rβ) is closely similar to that of PDGF-D in mice with unilateral ureteral obstruction (UUO). On consecutive sections obtained from a mouse with UUO at day 14, the distribution pattern of PDGF-Rβ expression in areas of tubulointerstitial fibrosis (arrow) (A) closely corresponded to that of PDGF-D expression (arrow) (B), and it demonstrated less congruent with PDGF-B expression (arrow) (C). On consecutive sections, the distribution pattern of PDGF-Rβ (D) and PDGF-D (E) is highly congruent. Original magnification: A through C $\times$200; D and E, $\times$400.

$\pm$ 4.9% at day 7, and 66.0% $\pm$ 7.7% at day 14) also stained positive for αSMA (Figure 8B), indicating that the myofibroblast was the predominant cell type proliferating in the mice after obstruction.

Induction of αSMA in interstitial cells is considered to be due to the myofibroblastic phenotypic change of interstitial fibroblasts (33). In normal mice, αSMA expression was confined to vessels, and interstitial cells expressing αSMA were not detected. In contrast, the mice with UUO demonstrated increased staining for αSMA in interstitial cells at day 4, which was maximal at day 14 (Figure 8C). To identify the cell type expressing PDGF-D in the interstitium, we performed double immunolabeling for PDGF-D and αSMA. Interstitial myofibroblasts expressing PDGF-D could not be demonstrated in normal mice. In contrast, approximately half of interstitial cells ($<$45% up to 70%) expressing PDGF-D also expressed αSMA (myofibroblast) at day 14, a time point when both PDGF-D and αSMA proteins demonstrated maximal expression (Figure 8D).

Interstitial PDGF-D Expression Colocalizes with Type I Interstitial Collagen Deposition in Mice with UUO

Progressive interstitial accumulation of type I collagen was previously demonstrated in the mice with UUO (16). No evidence of renal interstitial fibrosis as judged by type I collagen deposition was detected in the normal mice or the mice with UUO at day 4 (16). A marked accumulation of type I collagen in the mice with UUO was observed in areas of interstitial fibrosis after day 7 and increased progressively until day 14 (16). Immunohistochemistry on serial sections demonstrated a highly similar distribution pattern of type I collagen deposition compared with that of PDGF-D expression in areas of tubulointerstitial fibrosis, although type I collagen was present primarily in extracellular matrix rather than the cellular expression of PDGF-D (Figure 9, A and B). Furthermore, we performed double immunolabeling for PDGF-D and type I collagen in the mice with UUO to determine their colocalization. The majority of PDGF-D expressing cells also expressed produced type I collagen (Figure 9C).

Interstitial Expression of PDGF-D in Humans with Chronic Obstructive Nephropathy Is Similar to that in Mice with UUO

Renal nephrectomy specimens with chronic obstructive nephropathy generally had variable degrees of atrophic or dilated tubules separated by fibrous tissue, variable amounts of interstitial mononuclear inflammation, and periglomerular fibrosis. A range of glomerular changes were observed from generally normal with abnormal features limited to periglomerular fibrosis, to focal and segmental and focal global sclerosis. Arterial changes of medial and intimal thickening were frequently observed in the nephrectomies.

In normal human adult kidneys, PDGF-D was uniformly expressed by visceral epithelial cells but not by other glomerular structures, and by smooth muscle cells of arteries and arterioles (14). PDGF-D expression was not detected in normal tubular and interstitial cells (14). In human specimens with chronic obstructive nephropathy, there was persistent expression of PDGF-D by glomerular visceral epithelial cells and vascular smooth muscle cells (Figure 10A), as well as *de novo* expression by periglomerular interstitial cells and by some neointimal cells of arteriosclerotic vessels (Figure 10A). *De novo* expression of PDGF-D was focally detected in interstitial cells but not in infiltrating leukocytes (Figure 10, A and B), which was similar to the expression pattern observed in mice with UUO. On consecutive sections, the distribution pattern of PDGF-D (Figure 10C) in areas of tubulointerstitial fibrosis closely matched that of PDGF-B (Figure 10D) and PDGF-Rβ (Figure 10E). The tubulointerstitial areas in humans with chronic obstructive nephropathy demonstrated progressive interstitial accumulation of type IV collagen (Figure 10F), type I collagen (Figure 10G), and αSMA (Figure 10H), in agreement with our previous data in mice with UUO (16,25). Furthermore, the distribution pattern of PDGF-D (Figure 10C) closely corresponded to that of type I collagen and αSMA (Figure 10, G and H) on consecutive sections obtained from human with chronic obstructive nephropathy. Those findings closely matched those observed in mice with UUO.

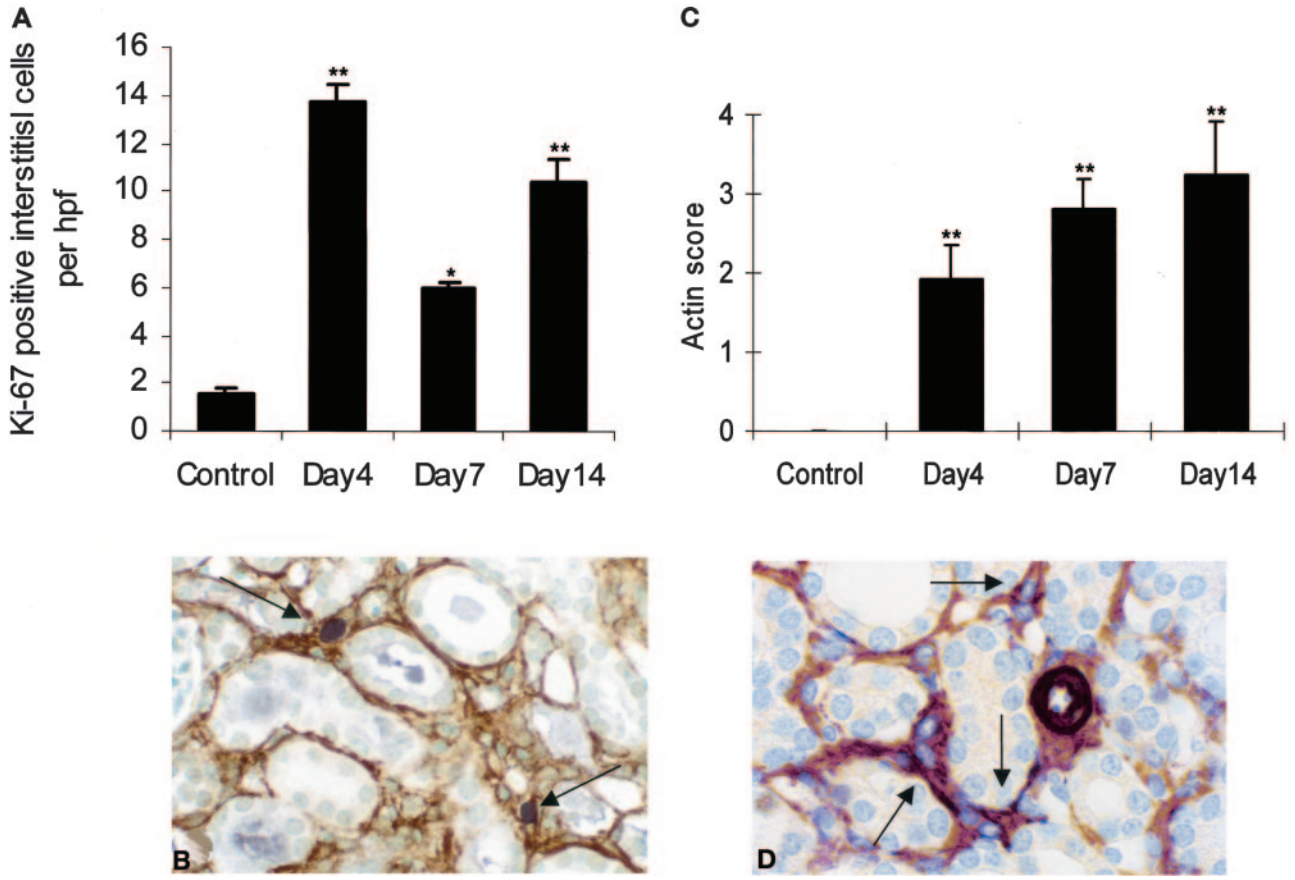

Figure 8. (A) Quantitation of interstitial cell proliferation assessed by Ki-67 immunostaining. Data are expressed as mean ± SEM. $*P < 0.05$; $**P < 0.01$ *versus* control mice. (B) Double staining for Ki-67 (black) and α smooth muscle actin (αSMA) (brown) detects interstitial myofibroblasts undergoing cell proliferation (arrows). (C) Semiquantitative assessment of interstitial expression of αSMA demonstrates significantly increased myofibroblast accumulation in unilateral ureteral obstruction (UUO) at all time points studied. Data are means ± SEM. $**P < 0.01$ *versus* control mice. (D) Double immunolabeling for αSMA (purple) and platelet-derived growth factor D (PDGF-D) (brown) in a mouse with UUO at day 14. A PDGF-D expressing myofibroblast is indicated by arrows in (D). Original magnification: ×600.

Discussion

PDGF plays an important role in the development of a variety of renal diseases because of its ability to act as a potent mitogen and chemoattractant for fibroblasts and mesenchymal cells including smooth muscle cells (9,34,35). This study provides the first evidence that PDGF-D, a newly recognized member of PDGF family, likely is an important contributor to interstitial fibrosing injury.

In the mouse, PDGF-D was found to be normally expressed by vascular smooth muscle cells and glomerular mesangial cells but was not detected in any other interstitial structures. In contrast, in mice with UUO, we detected *de novo* induction of PDGF-D expression in interstitial cells in areas of interstitial fibrosis. Fibroblasts are the main effector cells in fibrogenesis and contribute to the deposition of matrix components in the interstitium. We demonstrated that PDGF-D was expressed by fibroblasts in areas of tubulointerstitial injury. In agreement with the previous data from other groups (25), the number of interstitial proliferating cells was increased in the UUO model, with myofibroblasts being the predominant proliferating cell type. Furthermore, the majority of interstitial cells expressing PDGF-D were myofibroblasts, and interstitial expression of PDGF-D colocalized with type I collagen deposition. Furthermore, the distribution pattern of PDGF-Rβ was highly congruent with that of the PDGF-D in the areas of renal fibrosis, indicating that PDGF-D exerts its fibrogenic effect through its ability to engage this receptor.

Increased expression of PDGF-B in addition to PDGF-D was observed in the areas of tubulointerstitial injury, in agreement with previous data from our group (12). It has been well documented that PDGF-B, signaling through PDGF-Rβ, is a key mediator of cell proliferation and plays an important role in interstitial fibroblast proliferation (11,35,36). Sustained elevation of PDGF-B and PDGF-Rβ mRNA has been demonstrated by Northern blot analysis in rat kidneys with UUO (10). Expression of PDGF-B mRNA and PDGF receptors by proliferating interstitial cells have been documented by *in situ* hybridization and immunohistochemistry (11,36). Furthermore, it has been demonstrated that PDGF-B has the ability to convert fibroblasts to myofibroblasts (35,37), and to increase tubulointerstitial accumulation of type III collagen (35). Our observations are consistent with a role for both PDGF-B and

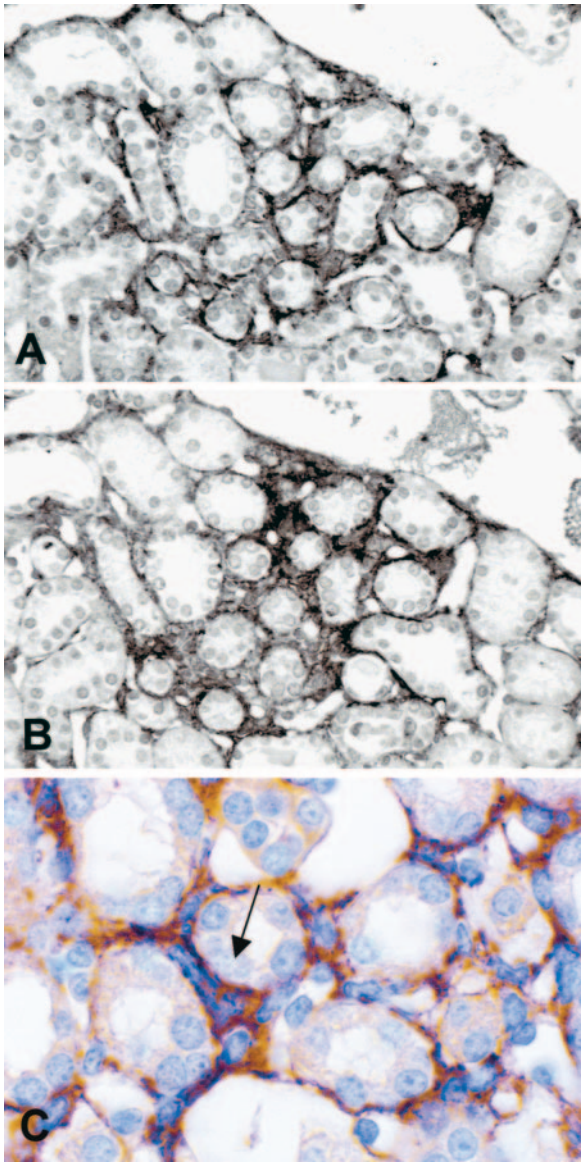

Figure 9. Interstitial platelet-derived growth factor D (PDGF-D) expression colocalizes with interstitial collagen type I deposition in mice with unilateral ureteral obstruction (UUO). On consecutive sections, the distribution pattern of PDGF-D (A) in areas of tubulointerstitial fibrosis highly corresponds to that of type I collagen (B) in a mouse with UUO at day 14. (C) Double immunolabeling for PDGF-D (purple) and type I collagen (brown) in the mouse with UUO at day 14 demonstrates that a PDGF-D expressing interstitial cell also produces type I collagen (arrows). Original magnification: ×600.

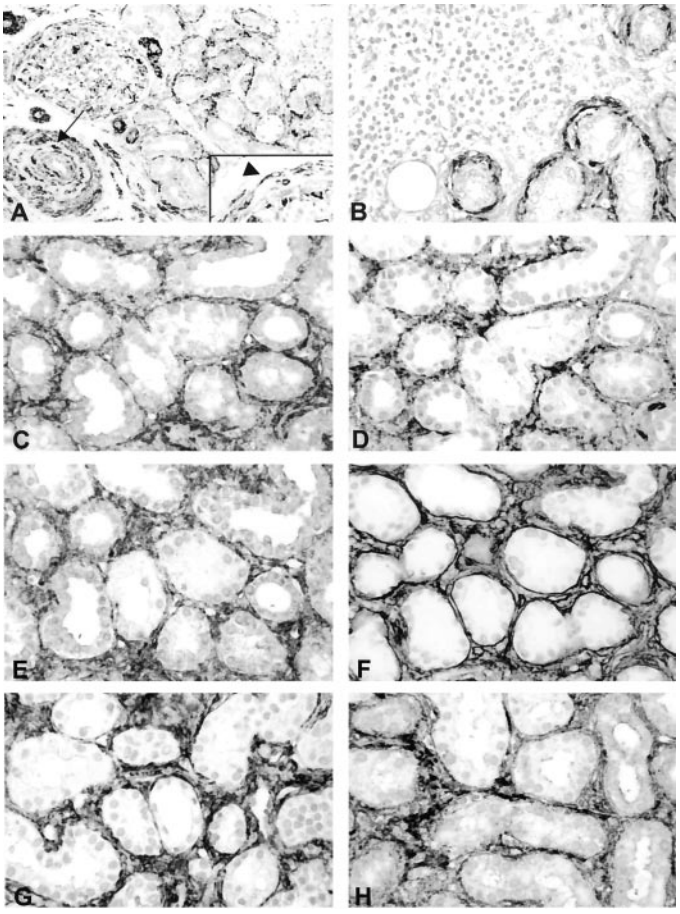

Figure 10. Interstitial expression pattern for interstitial platelet-derived growth factor D (PDGF-D) in human with chronic obstructive nephropathy is similar to that in mice with unilateral ureteral obstruction (UUO). In humans with chronic obstructive nephropathy, PDGF-D is expressed *de novo* by some neointimal cells of vessels (arrow) and by periglomerular interstitial cells (arrowhead, inset) (A). Expression by glomerular visceral epithelial cells and by vascular smooth muscle cells was similar to that previously observed in normal human adult kidneys (14). *De novo* expression of PDGF-D is observed in interstitial cells (A). No PDGF-D expression is observed in infiltrating leukocytes (B). On consecutive sections, the distribution pattern of PDGF-D (C) in areas of tubulointerstitial fibrosis highly corresponds to that of PDGF-Rβ (D) and PDGF-B (E), as well as that of type IV collagen (F), of type I collagen (G), and of α smooth muscle actin (αSMA) (H). These findings were similar to those observed in mice with UUO. Original magnification: A, ×200; A, inset, ×600; others, ×400.

PDGF-D growth factors, signaling through PDGF-Rβ expressed by interstitial cells, in the pathogenesis of renal interstitial fibrosis through induction of myofibroblast phenotype of interstitial cells, stimulation of proliferation of these cells, and the production of matrix by these cells.

Increased expression of PDGF-Rα in interstitial cells in the areas of renal fibrosis was an unexpected finding in this study. In normal mice, PDGF-Rα is most highly expressed in the papillary regions and weakly expressed by cortical interstitial cells, in agreement with our previous *in situ* hybridization data localizing sites of PDGF-Rα synthesis in developing and mature murine kidneys (38). Unlike the mouse, human kidneys normally express PDGF-Rα widely in the interstitium and in a small subset of glomeruli (39,40). In diseased human kidneys, interstitial upregulation of PDGF-Rα has been documented (40), which suggests the upregulated expression of PDGF-Rα in murine renal fibrosis might have important correlates with human renal fibrosis.

Despite increased expression of PDGF-Rα, the only receptor for PDGF-A ligand, we were not able to detect upregulation of

interstitial PDGF-A in this study. In normal mice, PDGF-A is most highly expressed in papillary regions, and barely detected in cortical interstitial cells, in agreement with our previous data on sites of PDGF-A synthesis in murine kidneys (38). Compared with PDGF-B, a role of PDGF-A in the pathogenesis of tubulointerstitial fibrosis injury remains undefined. The few studies of renal interstitial injuries and PDGF-A expression provide little support for a role for PDGF-A in this setting (35,41). No increase in expression of PDGF-A mRNA was documented in rat kidneys with tubulointerstitial nephritis, in settings where an approximately threefold increase in expression of PDGF-B and TGF-β mRNA was observed (41). Furthermore, PDGF-BB but not PDGF-AA infusion induces renal tubulointerstitial fibroblast proliferation *in vivo* (35). Given these findings, we speculate that PDGF-A is unlikely to be an important mediator of tubulointerstitial fibrosis.

The mice with UUO in this study did not show augmentation of PDGF-C in areas of renal fibrosis. It has been recently demonstrated that PDGF-C expression is increased at sites of fibrosing tubulointerstitial injury of rats, and that infiltrating monocytes/macrophages are a potential source of PDGF-C in this setting, although the possibility that a small subset of fibroblasts or myofibroblasts expressed PDGF-C could not be excluded (42). It has been shown that PDGF-C is a potent mitogen for cultured fibroblasts (3), which is supported by the observation that transgenic mice overexpressing PDGF-C in the heart demonstrate cardiac fibroblast proliferation and interstitial fibrosis (3). On the other hand, our group recently demonstrated that mice with elevated systemic circulating levels of PDGF-C after administration of an adenoviral vector containing the PDGF-C showed no glomerular or renal interstitial alterations *in vivo* (43). Although available evidence is limited, we speculate on the basis of the systemic overexpression studies that PDGF-C, which acts mainly through engagement of PDGF-Rα, has a limited role at best in the development of interstitial fibrosis in the mouse.

To establish the significance of our findings in the murine model, we localized expression of PDGF-D, -B, and PDGF-Rβ in human nephrectomies with chronic obstructive nephropathy. The diseased human kidneys were characterized by interstitial expression of αSMA (myofibroblastic transformation), and accumulation of type I and type IV collagen in the interstitium, in agreement with the findings observed in mice with UUO (16,25). *De novo* expression of PDGF-D was detected in interstitial cells in diseased human kidneys in patterns similar to those of mice with UUO. The expression pattern of this protein corresponded to that of PDGF-Rβ, PDGF-B, αSMA both in humans with chronic obstructive nephropathy and in mice with UUO, indicating myofibroblastic interstitial cells were a principal site of PDGF-D production or binding. Distinguishing between these two possibilities awaits development of a probe suitable for detection of PDGF mRNA production by *in situ* hybridization in the injured tissues.

In summary, we have shown that PDGF-D is expressed by interstitial cells and αSMA expressing fibroblasts in a well characterized model of interstitial fibrosis. We have also shown that the majority of interstitial cells expressing PDGF-D were present at sites where type I collagen is produced and deposited. The distribution pattern of PDGF-Rβ, currently the only known receptor for PDGF-D, was highly congruent with that of PDGF-D. These observations indicate that PDGF-D, in conjunction with PDGF-B, plays an important role in the pathogenesis of tubulointerstitial injury, most likely through direct engagement of PDGF-Rβ expressed at the surface of interstitial fibroblasts. Furthermore, *de novo* expression of PDGF-D in interstitial cells was also detected in human kidneys with chronic obstructive nephropathy, and the expression pattern corresponded to that of PDGF-B and -Rβ. The similarities between patterns of growth factor expression in mice with UUO and human chronic obstructive nephropathy indicate the utility of this murine model for studies that would test the efficacy of modulating the PDGF-D ligand/receptor system to ameliorate human disease.

Acknowledgments

Support was provided by NIH grant DK47959 and by a grant from ZymoGenetics.

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See related editorial, "PDGF-D and Renal Disease: Yet Another One of those Growth Factors," on pages 2690–2691.