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Platelet-Derived Growth Factor-BB Induces Renal Tubulointerstitial Myofibroblast Formation And Tubulointerstitial Fibrosis

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Tubulointerstitial fibrosis correlates closely with renal function, although the mechanism regulating tubulointerstitial fibrogenesis remains poorly understood. Since platelet-derived growth factor (PDGF) is a growth factor for fibroblasts, we examined the effect of daily (for 7 days) PDGF-AA or PDGF-BB administration on renal tubulointerstitial architecture in rats. PDGF-AA administration at a dose of 5 mg/kg did not affect the renal tubulointerstitum. By comparison, PDGF-BB induced dose-dependent renal tubulointerstitial cell proliferation and fibrosis. At 5 mg/kg, PDGF-BB increased BrdU labeling in tubulointerstitial fibroblasts at 24 hours (19-fold), which peaked at 72 hours (23-fold) with bromodeoxyuridine uptake returning to control values by 7 days. Tubulointerstitial proliferation was associated with the differentiation of these cells into myofibroblasts as evidenced by α-smooth muscle actin expression beginning on day 3. The expression of α-smooth muscle actin peaked on day 5 and had markedly declined by day 21. In addition, apoptotic cells were identified within the tubulointerstitum on day 3 and progressively increased through day 7, suggesting that the disappearance of myofibroblasts may have occurred through apoptosis. These changes were accompanied by increased expression of α1 (III) collagen mRNA and interstitial accumulation of type III collagen within the renal cortex. By morphometric analysis, an approximately twofold increase in collagen III immunolabeling within the interstitial compartment was evident at 24 hours and peaked on days 5 to 7 (approximately fourfold). These data suggest that PDGF-BB may be an important mediator of tubulointerstitial hyperplasia and fibrosis. (Am J Pathol 1996, 148:1169–1180)

Tubulointerstitial injury is recognized increasingly as an important determinant of renal function in patients with glomerular disease of diverse etiology.[1–4] Glomerular filtration rate (GFR) or creatinine clearance correlates better with indices of tubulointerstitial damage than with those of glomerular damage. Tubular damage has been suggested to decrease GFR through a tubuloglomerular feedback mechanism involving the disruption and/or fibrosis of peritubular capillaries leading to reduced sodium reabsorption, which in turn leads to a fall in GFR.[5]

Growth factors have been demonstrated to be important mediators of extracellular matrix (ECM) accumulation in glomerulonephritis (GN). Transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and basic fibroblast growth factor have been shown to promote ECM accumulation in experimental glomerulonephritis.[6–8] However, the role of growth factors in the progression of tubulointerstitial fibrosis remains poorly understood. An approximate threefold increase in the expression of PDGF-B, TGF-α, and TGF-β mRNAs was reported in a rat model of antibody-associated tubulointerstitial nephritis.[9] Interestingly, there was no change in the expression of PDGF-A mRNA. The increase in TGF-β and TGF-α mRNAs was associated with tubulointerstitial expression of these proteins as determined by immunohistochemistry.[9]

PDGF exists in three mitogenically active isoforms resulting from the dimeric combination of homolo-

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gous but distinct A and B chains.[10] PDGF may be an important mediator of tubulointerstitial fibrosis, since it is chemotactic for fibroblasts[11] and smooth muscle cells[12] *in vitro*. In addition, it is also mitogenic for fibroblasts and smooth muscle cells,[13] although it does not appear to stimulate the proliferation of cultured proximal tubular cells.[14] Furthermore, PDGF increases type V collagen[15] and hyaluronate synthesis but decreases that of sulfated glycosaminoglycans[16] by fibroblasts *in vitro*.

The present study was undertaken to examine the potential role of PDGF-A and -B in the pathogenesis of tubulointerstitial injury/fibrosis by administering PDGF-AA or PDGF-BB to rats intravenously daily for up to 7 days. PDGF-AA did not affect the renal tubulointerstitium, but PDGF-BB stimulated fibroblast proliferation, induced the appearance of myofibroblast, and increased accumulation of interstitial collagen within the tubulointerstitium. Finally, preliminary data suggest that the tubulointerstitial hyperplasia may resolve through apoptotic cell death.

Materials and Methods

Experimental Design

In a preliminary study, female Lewis rats (Charles River Laboratories, Wilmington, MA) were injected intravenously daily with 5 mg/kg of recombinant human PDGF-AA, PDGF-BB, or saline (n = 5) for 7 days. Based on the results of the pilot study, 90 rats were divided into four groups. Groups II-IV received daily intravenous injections of PDGF-BB in varying doses via a tail vein, while group I rats (n = 25) received only the saline vehicle for a total of 7 days. Rats in groups II (n = 25), III (n = 20), and IV (n = 20) received daily injections of 5 mg/kg, 1 mg/kg, and 0.1 mg/kg body weight of PDGF-BB, respectively. Five rats from each group were euthanized with carbon dioxide on days 1, 3, 5, and 7. The remaining five rats in groups I (saline) and II (5 mg/kg PDGF-BB) were euthanized on day 21. The rats at the latter time point had not received either PDGF-BB or saline for the 14 days before euthanasia.

Morphological Studies

All rats were injected with bromodeoxyuridine (BrdU) (40 mg/kg) intravenously 1 hour before euthanasia. Kidney sections were fixed in 10% formalin solution or snap frozen in liquid nitrogen with Tissue-Tek (Miles, Elkhart, IN). Light microscopy was performed on paraffin sections stained with the periodic acid-Schiff reaction and hematoxylin counterstain. In addition, the kidneys of group I and II rats on day 7 were stained with Masson's trichrome.

Immunohistochemical studies were performed on paraffin- or acetone-fixed cryostat sections (3 to 4 μm), which had been blocked with a protein blocking agent (Lipshaw Immunon, Pittsburgh, PA). Cryostat sections were sequentially incubated with 3% H_2O_2 in CH_3OH, a polyclonal rabbit anti-rat type III collagen (Chemicon International, Temecula, CA) antibody, swine anti-rabbit immunoglobulin, and horseradish peroxidase rabbit anti-peroxidase complex (DAKO, Carpinteria, CA). The sections were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide (DAKO). Paraffin sections were immunolabeled with monoclonal antibodies to rat monocytes/macrophages (ED-1, Harlan Bioproducts for Science, Indianapolis, IN), α-smooth muscle actin (DAKO), or BrdU (Amersham, Arlington Heights, IL). For the latter antibody, deparaffinized sections were microwaved for 5 minutes in BioTek microwaving buffer and then incubated with a murine anti-BrdU antibody that was detected with a biotinylated horse anti-mouse immunoglobulin G (IgG) antibody (BioTek Solutions, Santa Barbara, CA) and the ABC-alkaline phosphatase reagents with the BioTek red chromagen (BioTek Solutions). Rat monocyte/macrophage and α-smooth muscle actin were detected using horseradish peroxidase ABC reagents from BioTek with DAB as the chromagen. To determine whether cells expressing α-smooth muscle actin were proliferating, tissue sections were doublestained for α-smooth muscle actin followed by anti-BrdU with DAB and BioTek red serving as the chromagens, respectively.

Interstitial cell proliferation was determined by counting the number of cells within the cortical interstitium that were labeled with the anti-BrdU antibody. Cells within tubules that had labeled were specifically excluded. Twenty-five consecutive fields of a uniform area (0.17 mm^2) were counted. In double labeling studies, 100 consecutive interstitial cells that had labeled with the anti-BrdU antibody were examined for the coexpression of α-smooth muscle actin. Mesangial proliferation was assessed by counting the number of cells expressing BrdU within 25 consecutive glomeruli of similar cross-section diameter.

Electron Microscopy

Kidney specimens for routine electron microscopy were fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.3). After overnight fixation, the specimens were rinsed in buffer, post-fixed in 1%

aqueous osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Medcast epoxy resin (Ted Pella, Redding, CA). The blocks were polymerized for 48 hours at 60°C. Semithin sections were cut from the epoxy-embedded materials and stained with toluidine blue. Selected areas of the tissue were trimmed, ultrathin sectioned, contrast enhanced with uranyl acetate and lead citrate, and then examined on a Phillips CM120 transmission electron microscope.

For immunoelectron microscopy, kidney sections were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered water (pH 7.3).[17] After overnight fixation, the tissues were washed in buffer, dehydrated in a graded ethanol series and embedded into LR White acrylic resin (Ted Pella). These specimens were polymerized at 55°C for 24 hours, semithin sectioned, and then stained with toluidine blue. Ultrathin sections from selected areas were cut and collected onto nickel grids for immunoelectron microscopy. The sections were rehydrated in phosphate-buffered saline (PBS) for 30 minutes and incubated for 2 hours with either a mouse monoclonal anti-human α-smooth muscle actin antibody (DAKO) or an IgG2a isotype control in a diluent consisting of 1% bovine serum albumin in PBS with 0.05% Tween and 0.02% NaN_3. After washing three times in PBS the tissue was blocked for 1 hour in 10% goat serum in diluent. The sections were then directly reacted for 1 hour with a 5% solution of 10 nm gold-goat α mouse secondary antibody conjugate (Amersham, Little Chalfont, UK) in diluent. The sections were rinsed in PBS, then in distilled water and subsequently contrast enhanced with aqueous uranyl acetate prior to examination on the transmission electron microscope.

Quantitation of Interstitial Collagen Accumulation

Quantitation of Steady State mRNA Expression

A riboprobe for rat α1(III) collagen (304 nucleotides) was prepared by the reverse transcriptase polymerase chain reaction (RT-PCR) from primers based on the published cDNA sequence.[18]

α1(III) 5′-primer: 5′-GGACCACCAGGGCCTC-GAGGTAAC-3′
3′-primer: 5′-GGTCTCTGCAATTGCGAG-CAGGGT-3′

The primers were synthesized on an Applied Biosystems (Perkin Elmer, Foster City, CA) automated DNA synthesizer. RT-PCR was performed for a total of 40 cycles with RNA extracted from rat liver serving as the template. After reverse transcription, PCR was performed under the following conditions: denaturing at 94°C, annealing at 54°C, and elongating at 72°C for a total of 40 cycles. The RT-PCR product was purified on agarose gel, ligated into a pGEMT vector (Promega, Madison, WI) and then sequenced with a Taq DyeDeoxy Terminator cycle sequencer kit (Applied Biosystems, Inc.) on an automated DNA sequenator (Applied Biosystems, 373A) according to the instructions of the manufacturer. The preparation of the 104 nucleotide glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA has been described previously.[19]

Total RNA was prepared from the renal cortex of rats administered PDGF-BB (5 mg/kg) or saline by acid phenol/chloroform extraction. The details of the RNAse protection assay were described previously.[19] Anti-sense riboprobes were prepared by *in vitro* transcription with either T7 (GAPDH) or SP6 (α1(III)) RNA polymerases (Ambion, Austin, TX) and the incorporation of [α-^{32}P]UTP (Amersham) at 37°C. The riboprobes were purified by electrophoresis on 6% polyacrylamide gel and then eluted with 0.5 mol/L NH_4OAc. Two μg of glomerular RNA was hybridized with 1×10^5 counts of each [^{32}P]UTP-labeled riboprobe for 14 to 16 hours at 56°C. The unhybridized RNA was digested with RNAse A and T1 (Ambion) at 30°C for 1 hour. The RNAses were then digested with proteinase K (Boehringer Mannheim) at 37°C for 30 minutes. After phenol/chloroform extraction and sodium acetate/ethanol precipitation, the samples were electrophoresed on 6% polyacrylamide gel. The radioactivity of the protected [^{32}P]UTP-labeled riboprobes was quantitated by a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The blots were computer generated on the monitor and rectangles were circumscribed around the bands of interest. The radioactivity within each rectangle was calculated by the computer and then normalized for each pixel. To ensure that there was a constant quantity of RNA within each sample, the final values were factored relative to the GAPDH mRNA levels.

Morphometric Analysis

Collagen III immunoperoxidase labeling was quantitated by morphometric analysis on a Leica Quantimet 520 system (Leica, Deerfield, IL). Input signal for analysis was obtained from a Nikon FXA microscope (Nikon Inc., Garden City, NY) via a Cohu camera (Cohu Inc., San Diego, CA). A 540 nm narrow band pass filter (Oriel Corp., Stratford, CT) was used to enhance the collagen staining for image thresholding. Data are presented as the percentage

of tubulointerstitial area which labeled with the collagen III antibody relative to the total tubulointerstitial area (labeled area/total tublointerstitial area × 100). The total tubulointerstitial area within each field was 0.02 mm^2. Twenty-five consecutive fields were evaluated with glomeruli and vascular structures excluded from the analysis.

In Situ *End Labeling of Fragmented DNA*

Apoptotic cells were detected by *in situ* labeling of the 3′-OH end of fragmented DNA according to the instructions of the manufacturer (Oncor, Gaithersburg, MD). Briefly, cryostat sections were fixed in 10% formalin and then quenched with 2% H_2O_2. Digoxigenin-labeled nucleotides were incorporated into the 3′-OH end catalyzed by terminal deoxynucleotidyl transferase. This was followed by incubation with peroxidase conjugated anti-digoxigenin antibodies, developed with H_2O_2 and DAB, and then counterstained with PAS. The number of apoptotic nuclei within the tubulointerstitum were counted by light microscopy with the area determined by the MetaMorph Imaging System (Universal Imaging System, Westchester, PA). The data are presented as the number of tubulointerstitial apoptotic cells/mm^2. To determine the origin of the apoptotic cells, cryostat sections were immunolabeled with anti-α-smooth muscle actin and BioTek red and then subjected to *in situ* end labeling.

Statistical Methods

Data are presented as the mean ± standard deviation or mean ± sum of variances. Statistical analyses were performed by analysis of variance with significant differences further analyzed by post-hoc testing with the Boneferroni/Dunn procedure (StatView, Abacus Concepts, Berkeley, CA). A $P < 0.05$ was defined as statistically different.

Results

Morphological Studies

Tubular structures were well preserved within the kidney of rats administered saline, PDGF-AA or the three doses of PDGF-BB. There was no evidence of atrophic or necrotic tubules, and the tubular basement membrane remained intact. However, interstitial expansion with disruption of the normal tubular and capillary apposition in the renal cortex was seen 24 hours after the administration of 5 mg/kg and 1 mg/kg of PDGF-BB. This expansion resulted from an increase in both the cellularity and matrix of the interstitum that progressively increased through day 7. The matrix expansion within the tubulointerstitum resulted from an increase in collagen accumulation as assessed by trichrome staining. In contrast, the cortical interstitum of rats which had received saline, PDGF-AA, or 0.1 mg/kg PDGF-BB were normal.

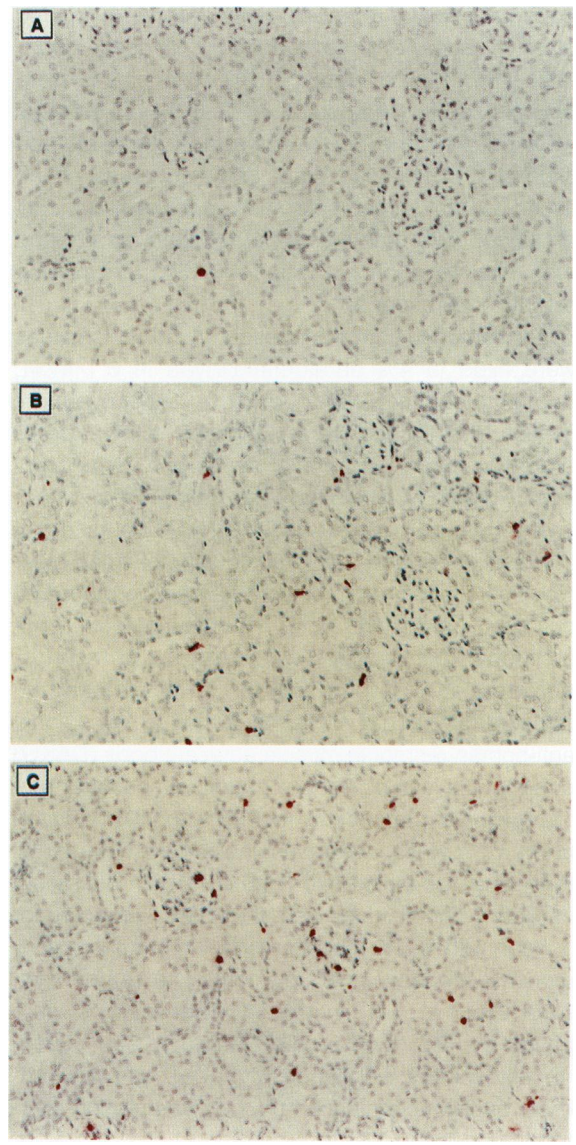

Figure 1. *Representative photomicrographs demonstrating cell proliferation within the renal tubulointerstitium as assessed by BrdU immunoperoxidase labeling. In control rats,* (**A**) *actively proliferating cells were rare. By comparison, there were numerous proliferating cells within the interstitium and glomeruli 24* (**B**) *and 72* (**C**) *hours after PDGF-BB (5 mg/kg) administration.*

The marked cellularity within the tubulointerstitum suggested cellular proliferation, which was investigated by BrdU labeling (Figure 1). There were 0.4 ± 0.04 BrdU cells/field within the tubulointerstitum of saline-treated rats at the five time points studied. By comparison, there were 7.6 ± 1.5 and 9.3 ± 1.3

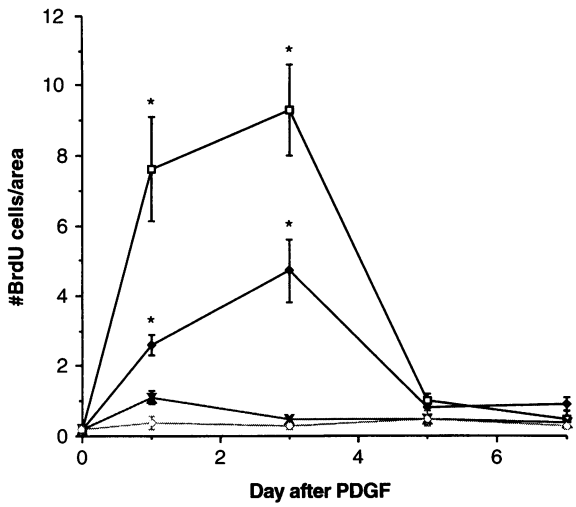

Figure 2. *Summary of renal tubulointerstitial BrdU uptake after PDGF-BB (5 mg/kg, ■; 1 mg/kg, ◆; 0.1 mg/kg, X) or saline (○) administration. Each value represents the mean ± sum of variance of five rats in each group at each time point.* *P < *0.01* versus *control.*

BrdU cells/field after PDGF-BB (5 mg/kg) administration at 24 ($P < 0.0001$) and 72 hours ($P < 0.0001$), respectively (Figure 2). The number of proliferating cells declined thereafter on days 5 and 7 to 1.0 ± 0.2 ($P < 0.01$) and 0.5 ± 0.2 BrdU cells/field, respectively. The proliferative effect of PDGF-BB was dose-dependent, since a lesser effect was seen at 1 mg/kg which also peaked on day 3 (Figure 2), whereas the 0.1 mg/kg dose did not produce an effect. Consistent with previous studies,[20,21] there was an increase in mesangial proliferation beginning 3 days (3.3 ± 0.5 BrdU cells/glomerulus) after PDGF-BB (5 mg/kg) administration which continued through day 7 (2.8 ± 0.8 BrdU cells/glomerulus) relative to control rats (0.1 ± 0.02 BrdU cells/glomerulus). A less pronounced mesangial proliferation was seen with the 1 mg/kg dose (2.3 ± 0.4 BrdU cells/glomerulus) on day 3.

To characterize the proliferating cell, tissue sections were immunostained with monoclonal antibodies to monocyte/macrophages or α-smooth muscle actin. Occasional monocytes/macrophages were detected focally within the renal interstitum of rats treated with PDGF-BB. By comparison, expression of α-smooth muscle actin was limited to the vascular smooth muscle cells of the arteries and arterioles and rarely, a tubulointerstitial cell in control rats at the five time points studied (Figure 3A). Similarly, immu-

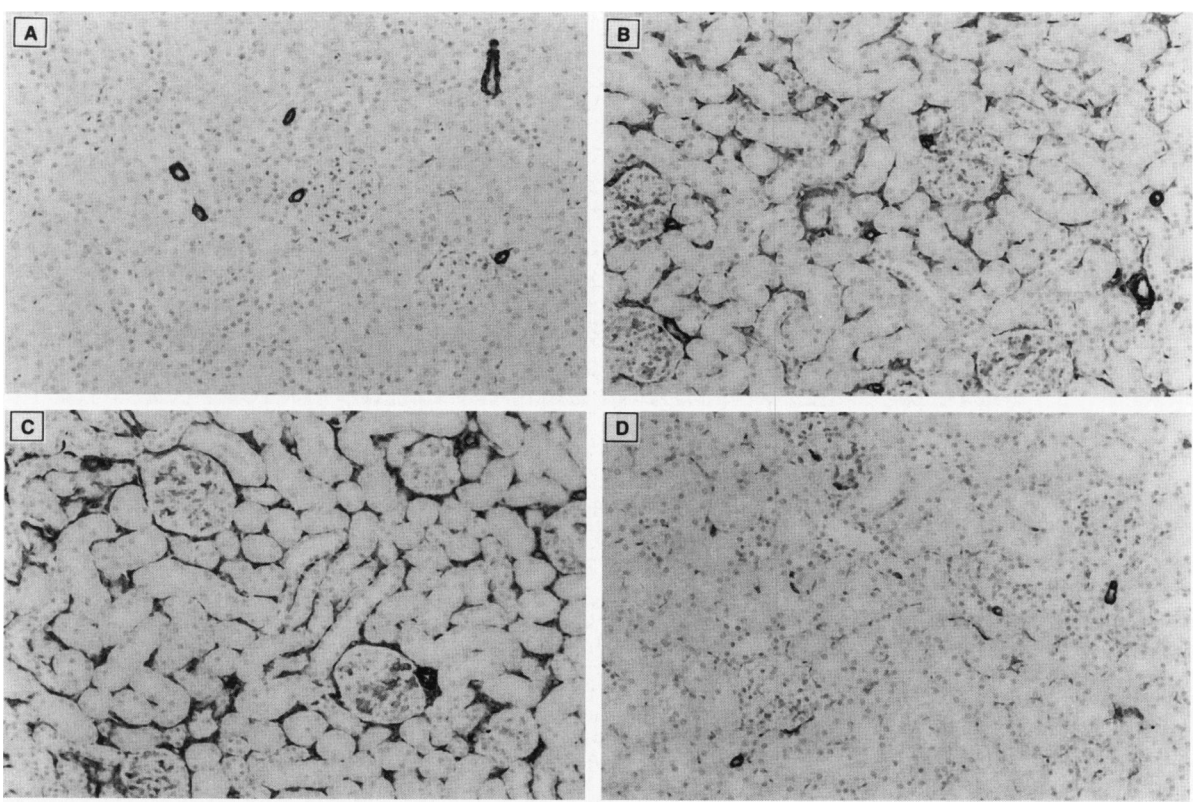

Figure 3. *Representative photomicrographs demonstrating α-smooth muscle actin expression within the glomeruli and tubulointerstitium. In rats that had received 0.9% saline* (A), *α-smooth muscle actin expression was limited to the arterioles and arteries. In contrast, rats that had been administered PDGF-BB (5 mg/kg) demonstrate marked expression of α-smooth muscle actin beginning on day 3* (B), *which peaked on day 5* (C) *and declined by day 21* (D).

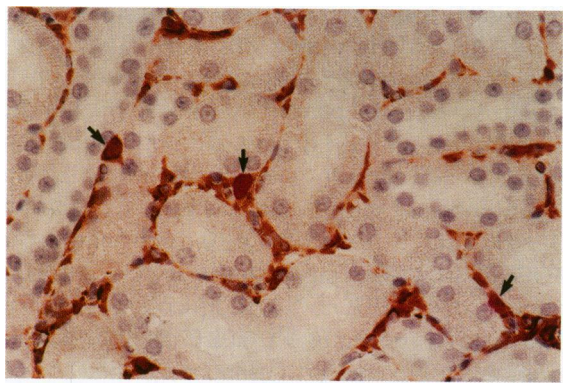

Figure 4. *Representative photomicrograph with arrows indicating tubulointerstitial cells double immunolabeling with anti-α-smooth muscle actin* (**brown**) *and anti-BrdU* (**red**) *antibodies. The majority (86 ± 2%) of the cells that labeled with BrdU coexpressed α-smooth muscle actin.*

nolabeling for α-smooth muscle actin was present primarily within vascular structures 24 hours after PDGF-BB treatment (5 mg/kg). However, a marked increase in α-smooth muscle expression was noted within the glomeruli and extravascular cells of the tubulointerstitium beginning on day 3 (Figure 3B) and peaking on day 5 (Figure 3C). Thereafter, the number of cells expressing α-smooth muscle actin declined so that by day 21, only occasional cells were labeled (Figure 2D). To demonstrate conclusively that the cells expressing α-smooth muscle were proliferating, kidney sections were double labeled with BrdU. In rats treated with PDGF-BB (5 mg/kg) for 3 days, 86 ± 2% of the cells that labeled with BrdU also expressed α-smooth muscle actin demonstrating that cells expressing this cytoskeleton component comprised the majority of the proliferative interstitial compartment (Figure 4).

Electron Microscopy

Figure 5 A and B shows transmission electron micrographs demonstrating the well developed cytoplasmic actin microfilaments (stress fibers) which are characteristic of myofibroblast. These actin microfilaments are located beneath the plasmalemma and lie parallel to the main axis of the cell. Dense bodies can be seen interspersed among the microfilaments and are in continuity with dense bodies within the plasma membrane. The force generated from the contraction of microfilaments is transmitted to the cell membrane via these dense bands. Figure 5C is an immu-

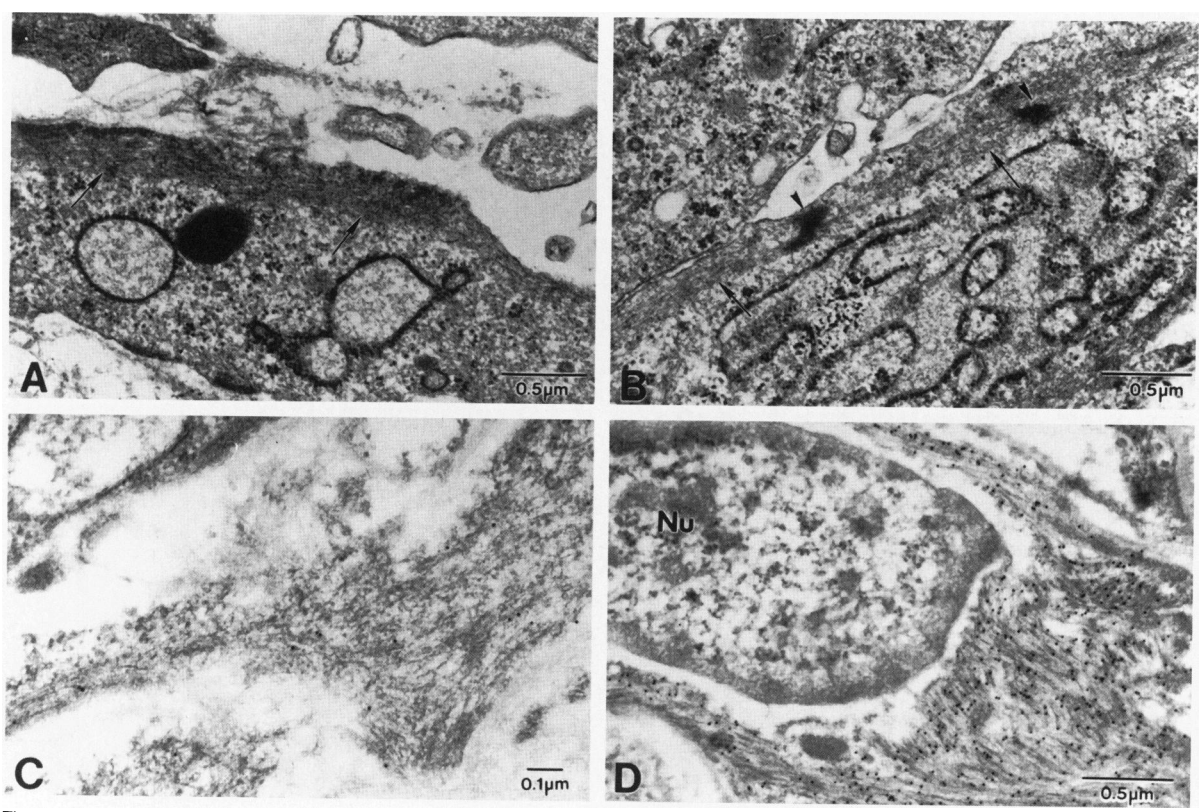

Figure 5. *Transmission electron micrographs demonstrating myofibroblasts after 7 days of PDGF-BB (5 mg/kg) administration.* **A** *and* **B** *show the characteristic bundles of actin microfilaments that lie parallel to the main axis of the cell* (**arrow**)*, among which are interspersed numerous dense bodies* (**arrowhead**)*. By immunoelectron microscopy, these microfilaments express α-smooth muscle actin* (**C**) *although to a lesser extent than that in vascular smooth muscle cells* (**D**).

noelectron micrograph indicating that these actin microfilaments labeled for α-smooth muscle actin, although to a lesser degree than that seen for vascular smooth muscle cells (Figure 5D). In contrast, kidney sections incubated with murine IgG2a did not demonstrate any labeling.

Quantitation of Interstitial Collagen Accumulation

There were increases in trichrome staining within the tubulointerstitum on day 7 in rats treated with PDGF-BB (5 mg/kg), suggesting increased collagen accumulation. To further identify the trichrome-positive material, kidneys were assayed for the presence of α1(III) collagen mRNA. In a preliminary survey, the steady state expression of α1(III) collagen mRNA relative to GAPDH mRNA increased fourfold relative to control rats on day 1 and peaked at eightfold greater than control values on days 3 to 5. Based on this survey, the steady state expression of α_1(III) mRNA was examined in all rats treated with PDGF-BB (5 mg/kg) for 3 days. There was a 17-fold increase in the α_1(III) collagen/GAPDH mRNA ratio in PDGF-BB treated rats (1.01 ± 0.67) relative to rats administered saline (0.06 ± 0.01, $P = 0.01$). The translation of α1(III) collagen mRNA to protein was confirmed by immunolabeling with a specific antibody to type III collagen (Figure 6). The results of the morphometric analysis of type III collagen immunostaining are summarized in Figure 7. There was a more than twofold increase in collagen III expression after PDGF-BB administration (5 mg/kg) at 24 hours (9.0 ± 1.9%; Figure 6B) as compared with control rats (3.9 ± 0.8%; Figure 6A). The immunolabeling for collagen III further increased on day 3 (11.6 ± 1.1%, $P = 0.001$ *versus* control) and peaked on days 5 (15.3 ± 1.5%, $P = 0.002$ *versus* control) to 7 (14.0 ± 3.7%, $P < 0.0001$ *versus* control; Figure 6C). Thereafter, collagen III accumulation declined to 10.0 ± 1.4% of the tubulointerstitial area ($P < 0.005$ *versus* control) 14 days after cessation of PDGF-BB infusion. However, there were no statistical differences in collagen III immunolabeling between days 3, 5, 7, or 21 in the PDGF-BB treated rats. The accumulation of collagen III was dose-related with a smaller increase seen with the 1 mg/kg dose (8.7 ± 0.7%, $P <$ 0.005 *versus* control) on day 7 and absence of an effect at the lowest dose (3.9 ± 0.8%).

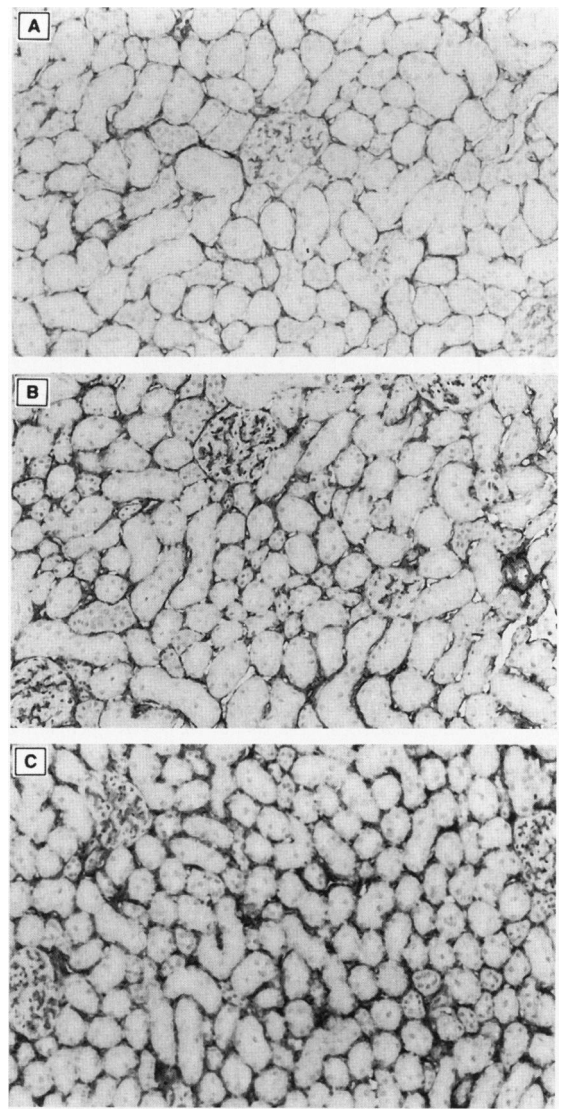

Figure 6. *Representative photomicrograph demonstrating immunoperoxidase labeling with an antibody to rat collagen III.* **A** *demonstrates collagen III expression in control rats while increased interstitial collagen III accumulation can be detected 24 hours* (**B**) *and 7* (**C**) *days after PDGF-BB infusion (5 mg/kg).*

In Situ *End Labeling of Fragmented DNA*

Apoptotic nuclei were uncommon (0.6 ± 0.2 apoptotic cells/mm^2) within the renal tubulointerstitum of rats administered saline. However, after 3 days of PDGF-BB treatment, there was a greater than twofold increase in the number of apoptotic cells within the interstitum (1.6 ± 0.4 apoptotic cells/mm^2, $P =$ 0.002). The number of apoptotic cells within the interstitum progressively increased to 3.0 ± 0.5/mm^2 on day 5 ($P < 0.003$) and 4.4 ± 1.5/mm^2 by day 7 ($P < 0.001$) although they still constituted <1% of the total tubulointerstitial cells (Figure 8). The number of apoptotic cells returned toward control values by day 21 (0.9 ± 0.4/mm^2). By double-labeling, occasional apoptotic nuclei were surrounded by cytoplasm which labeled for α-smooth muscle actin.

However, it should be noted that it is extremely difficult to determine the lineage of an apoptotic cell with absolute certainty.

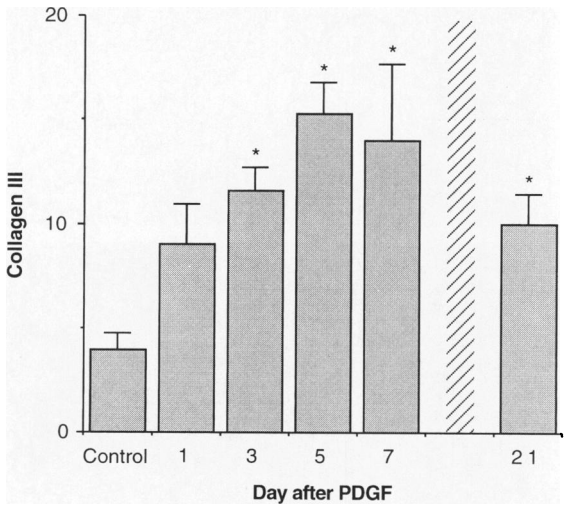

Figure 7. *Effect of PDGF-BB (5 mg/kg) on tubulointerstitial collagen III expression as quantitated by morphometric analysis. Data are presented as the percentage of a tubulointerstitial field measuring 0.02 mm² expressing collagen III. The control value was calculated by analyzing the kidney of saline-treated rats on day 7.* *P < *0.005* versus *control.*

Discussion

The present study demonstrates that exogenous administration of PDGF-BB, but not PDGF-AA, induces renal tubulointerstitial fibroblast hyperplasia and fibrosis. An increase in tubulointerstitial cell proliferation began at 24 hours and peaked at 72 hours as determined by BrdU labeling. The increase in proliferation was dose-dependent with no discernible increase in proliferation at the low dose (0.1 mg/kg) and progressive increases with 1 mg/kg and 5 mg/kg of PDGF-BB. However, despite the continued administration of PDGF-BB for 4 additional days, the number of proliferating cells returned toward the baseline level exhibited by control rats, which had received only saline. This suggests that the proliferating cells, after an initial wave of proliferation, became refractory to the mitogenic effects of PDGF-BB. The mechanism underlying this refractoriness is currently under investigation but may relate to PDGF receptor downregulation. Alternatively, PDGF-BB may induce terminal differentiation of tubulointerstitial cells and render them incapable of further proliferation.

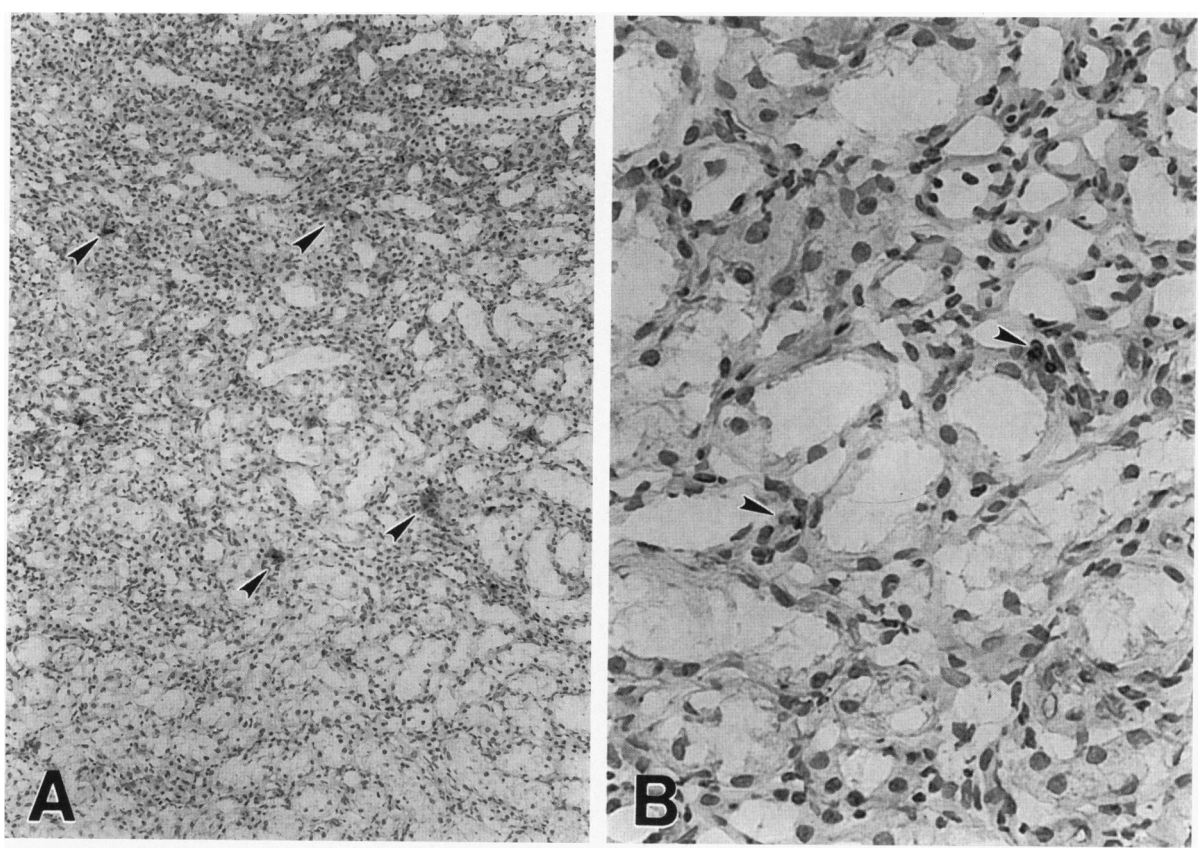

Figure 8. *Representative photomicrograph demonstrating apoptotic nuclei* (arrowhead) *labeled by the* in situ *end labeling technique in rats treated with PDGF-BB (5 mg/kg) for 7 days at ×10* (A) *and ×40* (B) *magnification.*

In addition to stimulating tubulointerstitial proliferation, PDGF-BB also increased tubulointerstitial matrix accumulation. Increased steady state expression of α1(III) collagen mRNA and accumulation of type III collagen within the tubulointerstitium was evident beginning 24 hours after PDGF-BB administration in rats receiving 5 mg/kg PDGF-BB. There was a 2.3-fold increase in tubulointerstitial type III collagen immunolabeling in PDGF-BB-treated rats when compared with that of control rats at 24 hours, which increased to an approximate fourfold difference by days 5 and 7. With withdrawal of the PDGF-BB stimulus for 14 days, immunolabeling for type III collagen decreased so that it was only 2.6-fold greater than that present in control kidneys by day 21. However, the collagen III immunolabeling between days 7 and 21 was not significantly different. Nonetheless, these data suggest that renal tubulointerstitial fibrosis will not progress if the stimulus, in this case PDGF-BB, is removed. The fibrogenic effect of PDGF-BB did not appear to be mediated through the induction of other growth factors such as TGF-β. In preliminary experiments, the kidneys of PDGF-BB treated rats did not express TGF-β mRNA.

The nature of the cells proliferating within the tubulointerstitum was further investigated. Renal interstitial cells have been classified into three main types, although it is likely that other cell types exist.[22] Within the renal cortex, these are the fibroblast-like cell (type I interstitial cell), macrophage (type II cell), and the dendritic cell, with the latter cell type being difficult to distinguish morphologically from the fibroblast-like cell.[22,23] The cells proliferating in response to PDGF-BB were presumed to be tubulointerstitial fibroblasts, because these cells are thought to be the cells principally responsible for ECM synthesis. However, strong expression of α-smooth muscle actin was noted in the majority of cells within the tubulointerstitum beginning 3 days after PDGF-BB identifying these cells as myofibroblasts. This was confirmed by identification of stress fibers which expressed α-smooth muscle actin within these tubulointerstitial cells on immunoelectron microscopy.

Myofibroblasts are believed to be terminally differentiated cells arising from the differentiation of fibroblast during wound healing/fibrosis, although the precise origin of myofibroblasts remain uncertain.[24,25] The morphological features of the myofibroblast are intermediate between those of fibroblast and smooth muscle cell, although the cell retains biological properties of fibroblast. Thus, like fibroblasts, myofibroblasts have been shown to synthesize interstitial collagens I and III.[26] However, myofibroblasts retain α-smooth muscle expression during proliferation, whereas vascular smooth muscle cells generally do not.[27] Myofibroblasts stimulated with PDGF-BB were shown by double labeling with α-smooth muscle actin and BrdU to constitute the majority (86% on day 3) of the proliferating cells *in vivo*. The acquisition of the myofibroblast phenotype may in part explain the cessation of proliferation after 3 days of PDGF-BB administration, since the capacity of myofibroblasts to proliferate relative to fibroblasts *in vitro* are significantly reduced.[28]

The significance of myofibroblast and α-smooth muscle actin expression is likely related to its role in collagen contraction during matrix remodeling.[29] These data are consistent with previous studies in models of pulmonary, biliary, and hepatic injury in which the appearance of myofibroblasts were associated with the development of fibrosis.[30,31] Within the kidney, myofibroblasts have been detected within the glomerulus and/or tubulointerstitum after ureteral obstruction, infusion of angiotensin II, renal allograft rejection, and in a variety of glomerulonephritides.[32–36] However, the present study expands upon these previous works by demonstrating that PDGF-BB induces, either directly or indirectly through as yet unidentified intermediaries, myofibroblast formation. These data conflict with previous studies which suggest that PDGF does not induce α-smooth muscle actin expression.[37] However, the dose of PDGF infused (28 ng/day) was substantially less than our lowest dose (~25 μg/day), which also did not produce an effect. Alternatively, the absence of an effect may reflect the heterogeneity of fibroblasts. Although the source of the myofibroblasts was not determined in this study, it is likely that they arose from tubulointerstitial fibroblast as had been reported in granulation tissue.[25] The close temporal association between the appearance of cells expressing α-smooth muscle actin 2 days after initiation of tubulointerstitial fibroblast proliferation suggests that the latter cells may have differentiated into myofibroblasts.

The disappearance of myofibroblast by day 21, 14 days after the withdrawal of PDGF-BB, was of interest, since withdrawal of growth factors is known to induce apoptosis *in vitro*.[38] By *in situ* end labeling, a greater than twofold increase in cells undergoing apoptosis was demonstrated in the tubulointerstitum of PDGF-BB treated rats on day 3. The number of apoptotic cells progressively increased through day 7 (sevenfold), although they still accounted for <1% of the tubulointerstitial cells. Despite the relative paucity of apoptotic cells, the increases noted are significant, given that apoptosis is a rapid (30 minutes to 3 hours) and inconspicuous phenomenon.[39,40]

Indeed, Bursch et al[40] estimated a 25% loss of hepatocyte mass within days if hepatocytes were to undergo apoptosis at a rate of 0.5%/hour with the mitotic rate remaining constant. Thus, apparent small changes in the absolute number of apoptotic cells may have profound consequences. By day 21, when only occasional myofibroblasts were present within the tubulointerstitial compartment, the number of apoptotic cells had returned to control values. These data suggest that the resolution of the myofibroblast infiltration may be mediated through apoptotic cell death and would be consistent with previous studies in granulation tissue.[41] Interestingly, the presence of apoptotic cells on day 3, at the peak of cell proliferation, suggests that homeostatic mechanism(s) are activated, which serves to counteract the effect of PDGF-BB. These factor(s) may include c-*myc* and interleukin-1β converting enzyme, which have been shown to induce apoptosis in fibroblasts.[42,43]

Numerous cytokines and growth factors, including interleukin-1, tumor necrosis factor, PDGF, and TGF-α and -β, have been suggested to be important in the induction of tubulointerstitial fibrosis.[44,45] An association between TGF-β1 and tubulointerstitial fibrosis has been suggested previously but remains unproven.[46] The importance of PDGF was suggested by numerous studies demonstrating PDGF-α and -β receptor expression within the tubulointersitum of normal kidneys with upregulation of the both receptors in tubulointerstitial nephritis.[47,48] In addition, increased PDGF-B mRNA expression by tubulointerstitial cells has been demonstrated in angiotensin II-mediated tubulointerstitial fibrosis.[34] The data reported in this communication would appear to confirm the importance of these previous observations.

In conclusion, PDGF-BB but not PDGF-AA infusion induces renal tubulointerstitial fibroblast proliferation *in vivo* with subsequent differentiation of these cells into myofibroblasts. Interestingly, proliferation in response to PDGF-BB is self-limited, despite the continued administration of PDGF-BB. The detection of apoptosis at the peak of cellular proliferation suggests that factor(s) which induce apoptotic cell death are activated in response to the tubulointerstitial hyperplasia. It would be important to determine whether a similar pattern of proliferation/apoptosis occurs in pathological conditions, and studies are currently in progress. Tubulointerstitial proliferation and myofibroblast formation are associated with increased tubulointerstitial fibrosis as assessed by collagen III accumulation. Targeting growth factors such as PDGF-BB with specific antagonists may offer a potential therapeutic strategy for the treatment of tubulointerstitial diseases.

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