Expression of PDGF α-Receptor in Renal Arteriosclerosis and Rejecting Renal Transplants

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Abstract. Platelet-derived growth factor (PDGF) plays an important role in renal disease. We have recently demonstrated that in healthy mature human kidney, PDGF α-receptor expression is largely restricted to interstitial cells. The study presented here assesses the expression of PDGF α-receptor in 18 mature adult kidneys with arteriosclerosis from individuals with no clinically evident history of renal disease other than localized neoplasia, in 13 kidneys with irreversible transplant rejection, and in a series of renal transplant biopsies composed of examples of both severe and absent rejection, by *in situ* hybridization and immunocytochemistry. Strong focal or diffuse expression of PDGF α-receptor mRNA and protein was noted in some intimal cells of intrarenal arterial vessels exhibiting signs of arteriosclerosis and/or vascular rejection. By double immunostaining, it could be shown that these cells were neither endothelial cells nor infiltrating leukocytes. The cells were most often identified as smooth muscle by colabeling for the smooth muscle cell-specific protein SM22α and less commonly for α-smooth muscle actin. There was also a population of PDGF α-receptor-expressing cells that failed to colabel with any of these markers, and hence remain of uncertain histogenesis. These intimal cells were generally negative for several other markers of differentiated smooth muscle cells, *i.e.*, calponin and desmin. Near these PDGF α-receptor-positive intimal cells, expression of PDGF A-chain, an α-receptor ligand, was demonstrated in endothelial, intimal, and/or medial cells. Prominent PDGF α-receptor mRNA and protein expression also was noted in areas of interstitial fibrosis and in some glomeruli, in particular those with segmental glomerulosclerosis or fibrotic crescents. Double immunolabeling for PDGF α-receptor and α-smooth muscle actin confirmed that most of these latter PDGF α-receptor-positive cells were interstitial myofibroblasts or mesangial cells, or both. In summary, these data demonstrate widespread expression of PDGF α-receptor in renal cell types involved in fibrotic and sclerosing processes. The data also show that PDGF α-receptor expression identifies a unique population of phenotypically altered vascular smooth muscle cells, which appear to be involved in the vascular response to injury. (J Am Soc Nephrol 9: 211–223, 1998)

Platelet-derived growth factor (PDGF) is composed of an A- and B-chain, which combine to form three possible isoforms (*i.e.*, PDGF-AA, PDGF-AB, and PDGF-BB) (reviewed in references 1 and 2). PDGF receptors consist of an α- and β-subunit, which dimerize upon binding of PDGF. The α-receptor subunit binds all PDGF isoforms with high affinity, whereas the β-receptor subunit only binds PDGF-BB with high affinity and PDGF-AB with lower affinity (3,4).

PDGF is a mitogen and chemoattractant for mesenchymal cells. It plays an important role in wound healing, organ fibrosis, and malignancy, and may play a role in atherosclerosis (1,5,6). Considerable data have emerged over the past few years to also implicate PDGF in the ontogenesis of the glomerular mesangium, where gene inactivation studies have clearly demonstrated essential roles for both PDGF B-chain and the PDGF β-receptor in this process (7,8). PDGF also plays a major role in the pathogenesis of renal diseases, especially those characterized by increased mesangial cell proliferation and matrix accumulation (8–10). However, the available information on PDGF, in particular regarding its role in renal disease, largely relates to PDGF B-chain and the β-receptor subunit (11–16). Few studies have investigated the significance of renal PDGF A-chain expression. These latter studies have shown that in mature human kidney, glomerular visceral epithelial cells and arterial smooth muscle cells of renal vessels constitutively express PDGF A-chain (17). PDGF A-chain is expressed by a subpopulation of neointimal smooth muscle cells in aging human kidney (15) and rejecting renal allografts (18), and, in acute allograft rejection, upregulated expression of PDGF A-chain by vascular endothelial cells occurs (18). Because PDGF A-chain can signal only through the PDGF α-receptor subunit, insight into the (patho-)physiologic roles of the A-chain in the kidney requires a concomitant assessment of the renal PDGF α-receptor expression.

In a recent study, we investigated the renal localization of PDGF α-receptor in developing and healthy mature human kidneys, using a combined immunohistochemistry and *in situ* hybridization approach (19). The findings of this study showed that PDGF α-receptor expression in the mature human kidney

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is confined largely to interstitial fibroblasts, but that low levels of expression can be detected in some mesangial areas. It was also found that rare vascular smooth muscle cells express the receptor (19). In the present study, we have extended these data by analyzing the synthesis and localization of PDGF α-receptor in mature adult human kidneys demonstrating arteriosclerosis, and in the damaged arterial vessels of allograft kidneys undergoing rejection. In so doing, we demonstrate some histopathologic parallels between the vasculopathies of arteriosclerosis and rejected renal transplants, and identify sites of expression of this receptor in renal allograft rejection that correspond to the enhanced expression of PDGF A-chain that previously has been documented in this setting.

Materials and Methods

Tissue Selection

Normal-appearing human kidneys (n = 16) obtained fresh from uninvolved portions of kidneys surgically resected for localized renal cell carcinoma were fixed in 10% neutral-buffered formalin and processed and embedded in paraffin according to conventional techniques. Portions of 10 of these kidneys, as well as of three additional normal-appearing portions of kidneys obtained during tumor nephrectomy, were fixed in methyl Carnoy's solution (13), paraffin-embedded or fixed in paraformaldehyde or acetone, and then snap-frozen as described (13). The age of the patients at the time of nephrectomy ranged from 41 to 85 yr (mean, 60.3). In addition to these normal-appearing kidneys from mature individuals, we also examined renal allografts excised for irreversible rejection (n = 13). Twenty-one needle biopsies from renal allograft recipients were also used and were chosen to reflect a range of diagnoses, including (*1*) no evidence of rejection (n = 5); (*2*) mild-to-moderate rejection (n = 7); and (*3*) severe rejection (n = 9), as we have previously defined these terms (18,20). Cases of moderate-to-severe rejection exhibiting an arterial component, as described previously, were identified as a separate category. Cases exhibiting moderate-to-severe rejection were the only ones in which a vascular component was identified. The distribution of these cases is provided in Table 1. Four-micrometer sections of all specimens were stained with the periodic acid-Schiff reagent, silver methenamine, and hematoxylin eosin, using conventional techniques for light microscopy and evaluation of the overall morphology.

Antibodies

PDGF α-Receptor. PDGF (R)-A 951 (Santa Cruz Biotechnology, Santa Cruz, CA) is an affinity-purified rabbit polyclonal antibody raised against a glutathione *S*-transferase fusion protein construct containing PDGF α-receptor sequences corresponding to amino acid residues 951 to 1089 of the carboxy terminus of the human PDGF α-receptor. This antibody recognizes PDGF α-receptor in acetone-fixed, frozen, and methyl Carnoy's-fixed paraffin-embedded sections. We have previously demonstrated the specificity of this antibody (19) by binding studies, Western blotting studies of kidney tissue, and correlative *in situ* hybridization for corresponding mRNA expression in selected tissues.

The second antibody used was a murine monoclonal IgG_1 antibody to human PDGF α-receptor (Genzyme Diagnostics, Cambridge, MA). The antibody specifically binds to PDGF α-receptor subunit and is not reactive with PDGF β-receptor (20). This antibody was nonreactive with paraffin-embedded material but recognized PDGF α-receptor in acetone-fixed, frozen sections.

Additional antibodies against the PDGF α-receptor included rabbit polyclonal antibodies 1890 and 2025 against the extracellular domain of the mouse PDGF α-receptor expressed as a fusion protein with glutathione-S-transferase (kindly provided by R. A. Seifert and D. Bowen-Pope, University of Washington, Seattle, Washington) (21). Both antibodies recognized PDGF α-receptor in methyl Carnoy's-fixed, paraffin-embedded sections, as well as acetone-fixed frozen sections.

Leukocyte Markers. The common leukocyte antigen CD 45, expressed on the majority of human leukocytes, was detected using a mixture of murine monoclonal antibodies PD7/26 and 2B11 (DAKO, Carpinteria, CA) (22,23). Monocytes/macrophages were identified using the murine monoclonal antibody KP-1 against the CD 68 antigen (DAKO) (23).

Endothelial Cell Marker. *Ulex europaeus* agglutinin I (Vector Laboratories, Burlingame, CA) is a lectin that labels human endothelial cell surfaces, type O red blood cells, and cells of collecting ducts, and has been used previously to delineate renal vascular endothelium (13,24,25).

Smooth Muscle/Mesenchymal Cell Markers. Murine monoclonal antibody 1A4 (DAKO) has been characterized by tissue immunohistochemistry and Western blotting (26), and has been previously demonstrated to recognize smooth muscle α-actin in methyl Carnoy's-fixed tissues (13,27). Murine monoclonal antibody D33 (DAKO) is an antibody against the intermediate filament protein desmin of human muscle (28). Murine monoclonal antibody CP-93 (Sigma, St. Louis, MO) recognizes human smooth muscle calponin and has been characterized previously (29). SM22α, a calponin-related protein, was detected using a murine monoclonal antibody (clone E-11) to porcine SM22α (a kind gift of Saverio Sartore, University of Padova, Italy, and Steven Schwartz, University of

Table 1. Cases used for analysis of PDGF α-receptor expression[a]

Diagnosis	Total No. of Cases Studied	Total No. of Cases Studied by ICC	Total No. of Cases Studied by ISH	Total No. of Cases Studied by Both ICC and ISH
Mild-to-moderate rejection	7	7	4	4
Moderate-to-severe rejection (cellular)	3	3	2	2
Moderate-to-severe rejection (vascular/cellular)	6	5	4	3
Indeterminant infiltrate/NER	5	4	4	3
Allograft nephrectomy	12	12	8	8
Mature native kidney	19	13	16	10

[a] PDGF, platelet-derived growth factor; ICC, immunocytochemistry; ISH, *in situ* hybridization; NER, no evidence of rejection.

Washington). The antibody has previously been characterized in detail (30).

PDGF A-Chain. PDGF A-chain was detected using an affinity-purified rabbit polyclonal antibody raised against a 30-amino-acid peptide corresponding to the amino terminus of the human PDGF A-chain (Santa Cruz). Absorption studies and Western blots demonstrating the specificity of this antibody in mature kidney have been published previously (17).

Immunohistochemistry

Immunohistochemistry was performed on methyl Carnoy's-fixed, paraffin-embedded tissues following a standard avidin–biotin complex (ABC) method. Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. The sections were then incubated for 1 h with the primary antibody diluted in phosphate-buffered saline (PBS) plus 1% bovine serum albumin. After washes in PBS, the sections were sequentially incubated with biotinylated goat-anti-rabbit or horse anti-mouse antibody (Vector Laboratories), the ABC-Elite reagent (Vector), and finally 3,3′-diaminobenzidine (DAB; with nickel chloride enhancement) was used as the chromogen. Sections were counterstained with methyl green, dehydrated, and coverslipped.

Frozen sections of acetone or paraformaldehyde-fixed tissue were hydrated in PBS, blocked with 3% hydrogen peroxide, and washed in PBS. The sections were then incubated with the primary antibody and subsequently processed as above using a streptavidin-biotin immunoperoxidase method, counterstained with methyl green, dehydrated, and coverslipped.

For all samples, negative controls for the immunohistochemical procedures consisted of substitution of the primary antibody with irrelevant murine monoclonal antibodies or nonimmune rabbit antibody or PBS.

Double-Labeling Immunohistochemistry

Methyl Carnoy's-fixed, paraffin-embedded tissues were prepared for immunocytochemistry as described above. The slides were then sequentially incubated with the PDGF (R)-A 951 antibody against PDGF α-receptor, biotinylated goat anti-rabbit IgG (Vector), the ABC-Elite reagent (Vector), and finally DAB to give a brown reaction product. Then, the sections were incubated sequentially with murine monoclonal antibodies against α-smooth muscle actin, desmin, calponin, SM22α, CD 45, CD 68, or rabbit anti-PDGF-A. These were followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody or alkaline phosphatase-conjugated goat anti-rabbit antibody and the alkaline phosphatase blue substrate kit (Vector) plus 1 mM levamisole (Sigma), which yields a blue reaction product. After washing and counterstaining with nuclear fast red, the sections were dehydrated, coverslipped, and viewed.

In the case of double labeling for PDGF α-receptor and endothelial cells, the slides were first stained with PDGF (R)-A 951 as above to give a brown reaction product. Then, the sections were incubated with biotinylated *Ulex europaeus* agglutinin 1 (Vector), streptavidin-alkaline phosphatase, and the alkaline phosphatase blue substrate kit. The final steps were identical to those described above. Controls for the double labels were done by substitution of the first or second primary antibody with PBS, in which cases no double label was observed.

Molecular Probe

A 1718-bp fragment (nucleotides 76 to 1794 of the human PDGF α-receptor sequence) was cloned in both directions into pGEM3zf (+). The probe was a kind gift of R.A. Seifert and D. Bowen-Pope (University of Washington). The construct was linearized and transcribed into an antisense or sense riboprobe using reagents from Promega (Madison, WI), except [^{35}S]UTP, which was obtained from New England Nuclear (Boston, MA). The transcription reaction mixture contained 1 μg of PDGF α-receptor cDNA (sense or antisense), 250 μCi of [^{35}S]UTP, 500 μmol/L each of ATP, CTP, and GTP, 40 U of RNAsin, 10 mmol/L dithiothreitol (DTT), 40 mmol/L Tris, and 10 U of either SP6 or T7 polymerase. After 75 min at 37°C, the template DNA was digested by adding 1 U of DNAase (Promega) and incubating at 37°C for an additional 15 min. Free nucleotides were separated with a Sephadex G-50 column. The collected fraction containing labeled probe was then ethanol-precipitated and subjected to limited alkaline hydrolysis (31) to obtain fragments of approximately 200 bp. After hydrolysis, the probe was again precipitated, resuspended in nuclease-free water containing 10 mmol DTT, and stored at −20°C. Probes were used within 48 h.

In Situ Hybridization

Renal tissue that had been fixed in 10% formalin and embedded in paraffin was deparaffinized following the standard protocol. The sections were washed with 0.5× SSC (1× SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0) and digested with proteinase K (1 μg/ml) (Sigma) in 500 mM NaCl, 10 mM Tris digestion buffer for 40 min at 37°C. Several 0.5×-SSC washes were followed by prehybridization for 2 h in 50 μl of prehybridization buffer (0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM ethylenediamine tetra-acetic acid, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM DTT). The hybridizations were started by adding 500,000 cpm of ^{35}S-labeled riboprobe in 50 μl of prehybridization buffer and allowed to proceed overnight at 50°C. After hybridization, sections were washed with 0.5× SSC, treated with RNase A (20 μg/ml, 30 min room temperature), washed in 2× SSC (2 × 2 min), followed by three high-stringency washes in 0.1× SSC/Tween 20 (Sigma) at 37°C, and several 2× SSC washes. After the tissue was dehydrated and air-dried, it was dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 2 wk. After developing, the sections were counterstained with hematoxylin and eosin, dehydrated, mounted, and viewed. Positive cellular labeling was defined as five or more silver grains concentrated over a single cell.

Results

Morphologic Findings in Mature Adult Kidneys and Rejecting Transplants

By conventional light microscopy, the kidney specimens with arteriosclerosis obtained from tumor-related nephrectomies were also characterized by variable degrees of focal global glomerulosclerosis, tubular atrophy, and interstitial fibrosis. Arcuate and interlobular arteries demonstrated an expanded intima characterized by accumulation of spindled mesenchymal cells and matrix. Arteriolar vessels were occasionally narrowed by variable accumulations of subendothelial hyaline. Rejecting renal transplants also showed a wide variety of pathologic findings, including: (*1*) features of cellular (interstitial) rejection with mononuclear cell infiltrates and tubulitis; (*2*) features of both acute and chronic vascular rejection with subendothelial or intimal inflammation (acute rejection) involving muscular arteries and/or intimal accumulation of smooth muscle cells and matrix (chronic rejection); (*3*) occasional, focal ischemic and/or hemorrhagic infarctions of

variable size; and (*4*) extensive areas of interstitial fibrosis, associated with focal glomerulosclerosis.

PDGF α-Receptor Protein and mRNA Expression in Normal Parts of Mature Adult Kidneys and Technical Aspects

In preserved morphologically normal and near-normal portions of the renal parenchyma, immunocytochemical staining patterns for PDGF α-receptor and *in situ* hybridization patterns for PDGF α-receptor mRNA were identical to those described previously (19): PDGF α-receptor protein and mRNA expression was prominent in the renal interstitium, low grade in the mesangium, minimal in the vascular arterial media, and absent in all other renal structures of mature human kidneys. Peritubular staining (illustrated in Figures 1A and 5B) revealed PDGF α-receptor protein localization to elongated cell processes of interstitial fibroblasts, which may abut tubular basement membranes or peritubular capillaries, as described previously for this receptor (19) and for PDGF β-receptor (11). Also, similar to our previous report (19), usage of the four different antibodies against PDGF α-receptor generally yielded similar staining patterns (see below) under the various tissue storage and fixation conditions described in Materials and Methods. The polyclonal PDGF (R)-A 951 (Santa Cruz Biotechnology) antibody, which we have recently characterized in detail (19), was chosen for an extended study.

Vascular Expression of PDGF α-Receptor in Mature Adult Kidneys and Rejected Transplants

The morphologic findings by disease category by both immunocytochemistry and *in situ* hybridization are presented in tabular form in Table 2. By immunocytochemistry, PDGF α-receptor expression was normally absent or minimal in the arterial media and intima. Nine of 12 native kidneys (all those kidneys with portions fixed in methyl Carnoy's solution) examined in the current study contained arterial vessels in which foci of PDGF α-receptor expression were present in neointimal areas (Figure 1A). Occasional expression of PDGF α-receptor was also noted in cells of the media in these damaged vessels. Similar findings were obtained in rejected renal transplants, in which 10 of 13 specimens (all those fixed in methyl Carnoy's solution) showed positive staining of the neointima, ranging from small foci of receptor expression to massive, circumferential expression of PDGF α-receptor (Figure 1, B and C). Again, occasional cells of the arterial media stained positive for the receptor (Figure 1B). The pattern of vascular expression identified in the transplant nephrectomy specimens was also identified in acute and chronically injured muscular arteries present in transplant biopsies (Figure 2). In particular, as detailed below, the endothelial expression of PDGF A-chain identified previously in acute vascular rejection was present in conjunction with PDGF-α receptor expression by other cell types within the expanded intima. In arterial vessels of two rejected transplants but in none of the mature adult kidneys, we noted staining of the internal elastic membrane for PDGF α-receptor, similar to previous findings in fetal kidneys (19). PDGF α-receptor expression was always prominent in the

Table 2. Expression of PDGF α-receptor by diagnosis and by morphologic technique[a]

Diagnosis	Number Studied by ICC (No. by ISH)	Interstitium			Vessels				Glomeruli			Areas of Infiltrate
		Negative to Trace	++	++++	Endothelium + to +++	Adventitia + to +++	Media + to +++	Neointima +++ to +++	Negative to Trace +	Mesangium +	Visceral Epithelium +	++ Cells Present
Mild-to-moderate rejection	7 (4)	0 (0)	0 (4)	7 (0)	0 (0)	1 (1)	1 (0)	1 (0)	2 (3)	1 (1)	4 (0)	4 (2)
Moderate-to-severe rejection (cellular)	3 (2)	0 (0)	0 (1)	3 (1)	0 (0)	1 (1)	0 (0)	2 (0)	3 (1)	0 (1)	0 (0)	3 (1)
Moderate-to-severe rejection (vascular/cellular)	4 (4)	0 (0)	1 (1)	3 (3)	0 (0)	2 (1)	0 (0)	1 (0)	3 (2)	1 (1)	0 (0)	4 (3)
Indeterminant infiltrate	2 (2)	0 (0)	0 (0)	2 (2)	1 (0)	0 (0)	1 (0)	0 (0)	1 (2)	1 (0)	0 (0)	0 (1)
NER	2 (2)	0 (1)	0 (0)	2 (1)	0 (0)	1 (1)	0 (0)	2 (0)	2 (2)	0 (0)	0 (0)	2 (0)
Allograft nephrectomy	12 (8)	1 (7)	0 (0)	11 (1)	2 (0)	11 (2)	9 (0)	10 (4)	7 (6)	3 (2)	0 (0)	9 (3)
Mature native kidney	13 (16)	0 (1)	0 (9)	13 (6)	1 (0)	13 (14)	8 (3)	8 (3)	4 (4)	9 (12)	1 (0)	2 (4)

[a] For each category, the first number indicates the number of positive cases as detected by immunocytochemistry, and the adjacent number indicates the number of positive cases detected with *in situ* hybridization. Abbreviations as in Table 1.

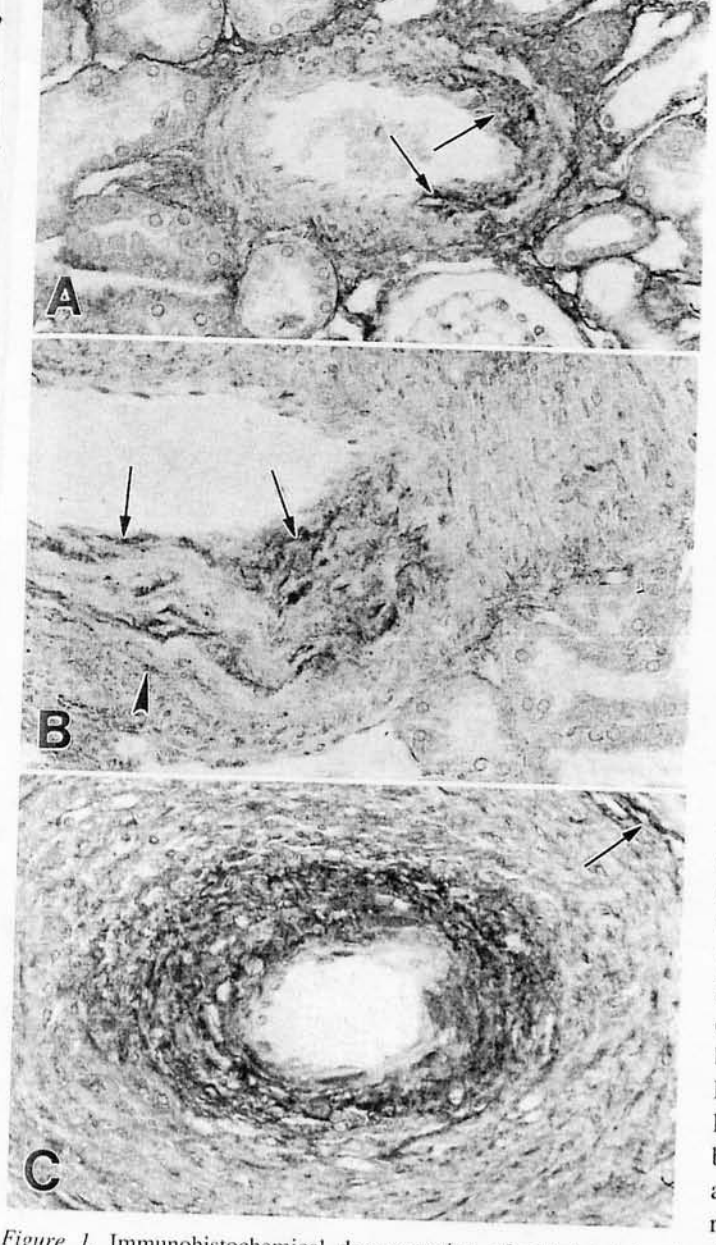

Figure 1. Immunohistochemical demonstration of platelet-derived growth factor (PDGF) α-receptor in renal vessels. (A) Small renal artery of mature adult individual with arteriosclerosis. Receptor expression is present in some intimal vascular cells (arrows), whereas endothelial cells and most medial smooth muscle cells fail to express PDGF α-receptor. There is expression of PDGF α-receptor in intertubular areas, in a distribution that at this magnification could correspond to either peritubular capillaries or interstitial fibroblasts. (B) Renal arterial vessel of a rejected kidney. Scattered intimal (arrows) and some medial (arrowhead) smooth muscle cells exhibit PDGF α-receptor expression. (C) Renal arterial vessel of a rejected kidney. Circumferential expression PDGF α-receptor in the inner layer of intimal cells. Outer intimal layers show low-grade or absent expression of PDGF α-receptor. Some receptor expression is also noted in the area of the internal elastic membrane (arrow). Magnification in A through C, ×400.

vascular adventitia of mature adult kidneys (Figure 1A). Adventitial staining in rejected kidneys was more variable and was completely lost in infarcted areas.

To further characterize the cells overexpressing PDGF α-receptor in damaged renal vessels of mature native kidneys with arteriosclerosis and rejected kidneys, double immunolabeling was performed. These data appeared to exclude the possibility that vascular endothelial cells (*Ulex*-positive) or infiltrating leukocytes (CD45-positive), in particular monocytes/macrophages (CD68-positive, data not shown), were responsible for the neointimal PDGF α-receptor expression (Figure 3, A through C). The PDGF α-receptor-positive cells frequently, but not uniformly, coexpressed several markers of differentiated smooth muscle cells, *i.e.*, α-smooth muscle actin (Figure 3) and the smooth muscle marker SM22α (32,33). Notably, SM22α was not expressed by the majority of medial smooth muscle cells (Figure 3F), but rather demonstrated a restriction to neointimal smooth muscle cells similar to the findings with PDGF α-receptor. Another smooth muscle maker, calponin, was found to have extensive expression by medial smooth muscle cells (Figure 3E), but not by intimal cells, and hence did not colocalize with PDGF α-receptor. Desmin was not expressed in the media or intima of renal vessels, and consequently no double labeling for desmin and PDGF α-receptor was observed in these areas (data not shown).

The above immunohistochemical findings were confirmed by *in situ* hybridization for PDGF α-receptor mRNA: In three of 11 specimens of mature adult kidneys with arteriosclerosis and in four of six rejected kidneys, mRNA expression was detected in the neointima, whereas medial smooth muscle cells were usually negative or showed low hybridization signal (Figure 4, A and B). Two of the eight allograft kidneys studied by *in situ* hybridization had uninterpretable results as a result of extensive infarction of the available tissue. Notably, in thrombosed vessels of the rejected kidneys, no intimal or medial PDGF α-receptor mRNA expression was detectable, but rare leukocytes within thrombi showed a hybridization signal. Hybridization signal intensity in the vascular adventitia was usually strong in mature native kidneys, but was variable in the rejected kidneys. In rejected kidneys with advanced ischemic damage, no mRNA expression of the adventitia was detectable. In leukocytic infiltrates surrounding some vessels, only rare cells contained PDGF α-receptor mRNA.

To assess the expression of the PDGF α-receptor ligand PDGF A-chain in the vasculature, serial sections were stained with antibodies to PDGF α-receptor and PDGF A-chain, and double immunolabeling of single sections also was performed. As described previously (17,18), PDGF A-chain is normally expressed in medial smooth muscle cells of renal vessels. Upregulated expression of A-chain, including expression in vascular endothelial cells and intimal cells, has been noted in vascular rejection (18). The present study confirms these observations and demonstrates a close spatial relationship between intimal, PDGF α-receptor-positive cells, and cells expressing PDGF A-chain (Figures 2 and 3B).

...each category, the first number indicates the number of positive cases as detected by immunocytochemistry, and the adjacent number indicates the number of positive cases detected with *in situ* hybridization. Abbreviations as in Table 1.

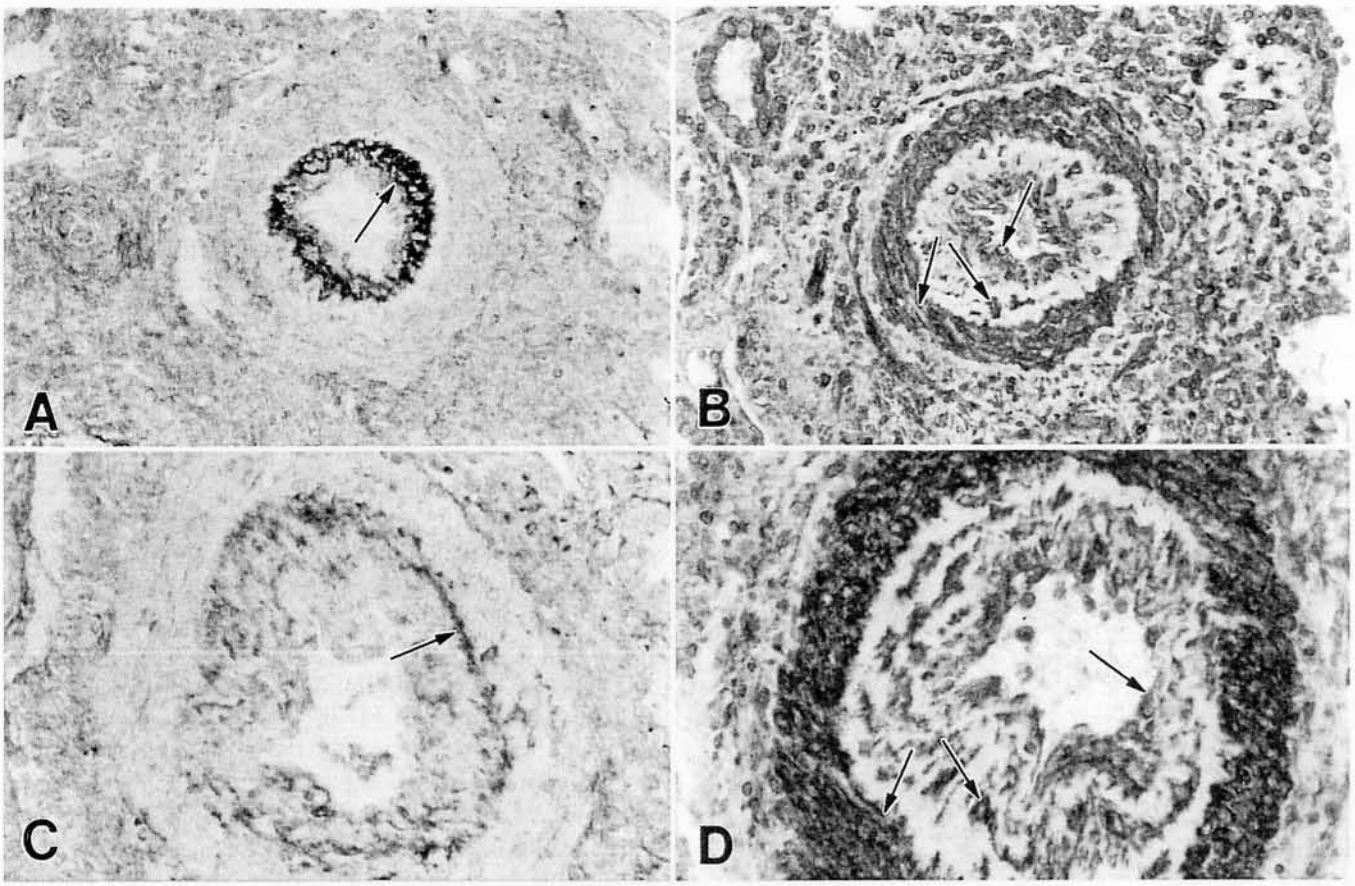

Figure 2. Immunostaining for PDGF α-receptor and PDGF A-chain in serial sections of rejecting renal arteries. (A and C) Renal arterial vessels from biopsies from different patients showing expression of PDGF α-receptor in intimal cells located beneath the endothelial surface (arrow). Magnification, ×400. (B and D) Same vessels as those shown in A and C stained with anti-PDGF A-chain antibody. PDGF A-chain is expressed in cells of the vascular media, intima, and some endothelial cells (arrows). Some mononuclear leukocytes infiltrating the intima and, most prominently within the interstitium, also express PDGF A-chain. Vessel pairs are Panels A and B and Panels C and D.

Glomerular Expression of PDGF α-Receptor in Mature Adult Kidneys and Rejected Transplants

As reported previously, PDGF α-receptor staining was usually undetectable in intact, undamaged glomeruli, but mild-to-moderate increases in mesangial PDGF α-receptor expression were occasionally detected both in mature native and rejected kidneys (data not shown). Prominent overexpression of glomerular PDGF α-receptor was noted in residual cellular elements within fibrosed parts of collapsed glomeruli and in adhesions (Figure 5A). In occasional adhesions, strands of PDGF α-receptor-positive cells could be observed, which extended from Bowman's capsule into the glomerular tuft (Figure 5B). Cellular glomerular crescents, which were present in some of the rejected kidneys, did not stain with the PDGF α-receptor antibody, but prominent receptor expression occurred in fibrotic crescents (similar to the image shown in Figure 5A). In many cases, these PDGF α-receptor-positive cells in sclerosed glomeruli also expressed α-smooth muscle actin, supporting their fibroblast origin (34,35).

Leukocyte infiltrates surrounding some glomeruli and vessels in mature adult and rejected kidneys were mostly PDGF α-receptor-negative, but occasional individual cells exhibited positive staining (Figure 5A). *In situ* hybridization for the expression of PDGF α-receptor mRNA in glomeruli of mature adult and rejected kidneys strictly mirrored the above expression pattern detected by immunocytochemistry (Figure 6, A through E).

Tubulointerstitial–Interstitial Expression of PDGF α-Receptor in Mature Adult Kidneys and Rejected Transplants

In areas with interstitial fibrosis both within mature adult and rejected kidneys, enhanced expression of both PDGF α-receptor protein and mRNA was noted (Figures 7 and 8, A and B). Double immunostaining for PDGF α-receptor and α-smooth muscle actin showed that many interstitial myofibroblasts, *i.e.*, α-smooth muscle actin-positive cells (34), expressed the receptor. However, not all myofibroblasts were double-labeled (data not shown). Most of the peritubular staining of PDGF α-receptor appears to be localized to the elongated cell processes of interstitial fibroblasts, similar to the pattern of localization of PDGF β-receptor shown in previous studies (11). Interstitial PDGF α-receptor protein and mRNA expression was completely lost in infarcted areas of rejected

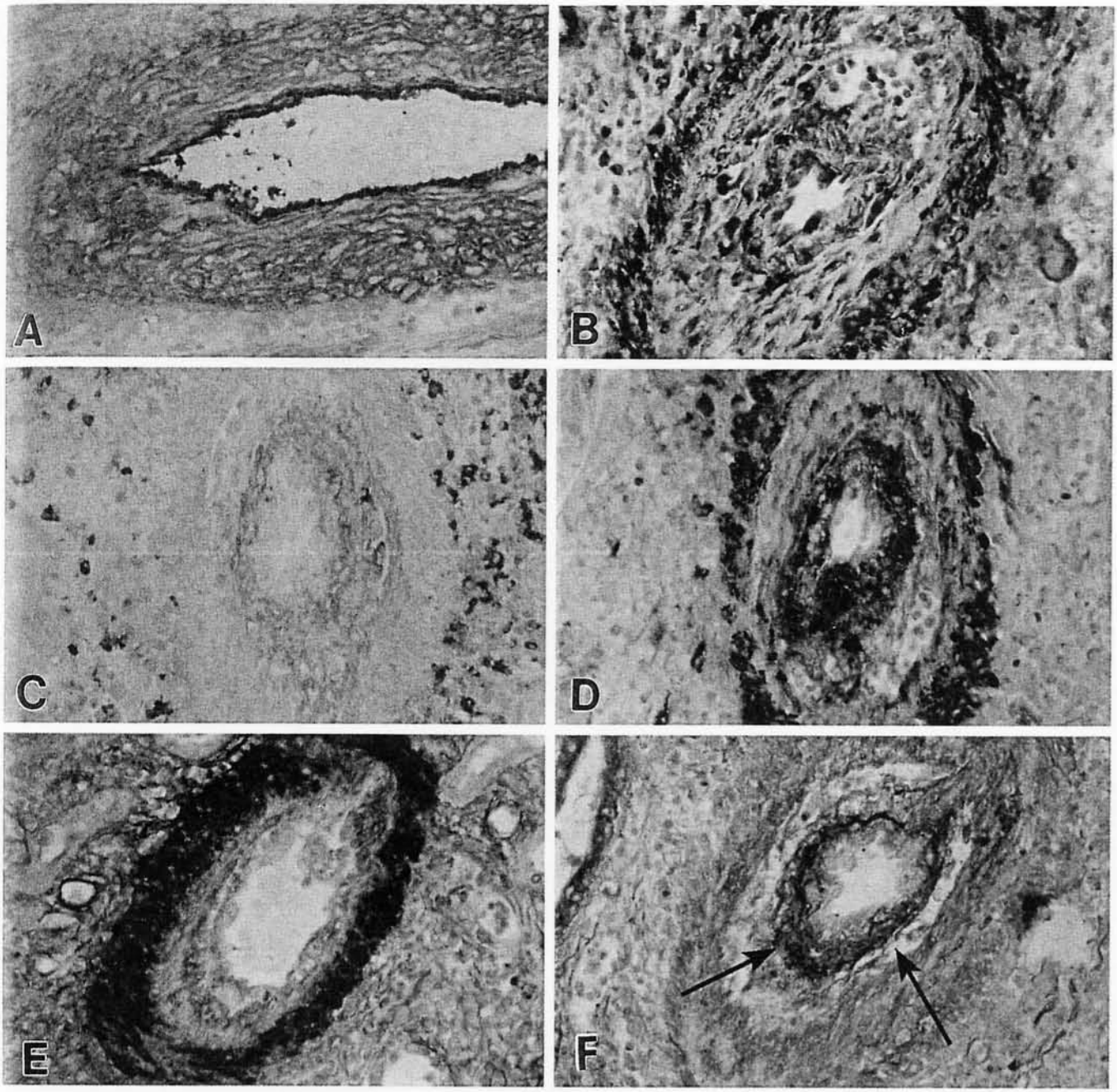

Figure 3. Double immunolabeling for PDGF α-receptor and cell-specific markers in vessels. (A) Renal arterial vessel without features of rejection in an allograft kidney. Expression of PDGF α-receptor (brown) and the endothelial marker *Ulex europaeus* lectin (blue), showing mutually exclusive staining. Magnification, ×400. (B) Rejecting arterial vessel in an allograft kidney. Expression of PDGF α-receptor (brown reaction product) is generally confined to intimal cells. PDGF A-chain (in blue) is expressed by medial smooth muscle cells and some intimal smooth muscle cells, a few of which show probable colocalization with PDGF α. Endothelial cells express PDGF A-chain exclusively. Magnification, ×400. (C) Same vessel as that shown in B, double-stained for PDGF α-receptor (brown) and the leukocyte common antigen CD45 (blue), showing mutually exclusive staining. (D) Same vessel as shown in B, double-stained for PDGF α-receptor (brown) and α-smooth muscle actin (blue), showing staining patterns that are predominantly mutually exclusive, although a few intimal cells may demonstrate double labeling. This cannot be ascertained further due to the spindled quality of such cells and the lack of identifiable discrete cell borders in this region. (E) Same vessel as shown in B, double-stained for PDGF α-receptor (brown) and calponin (blue), showing mutually exclusive staining. (F) Same vessel as shown in B, double-stained for PDGF α-receptor (brown) and SM22α (blue), showing double immunostaining of the majority of PDGF α-receptor-positive cells in the intima of this artery (arrow). In some cases, expression of SM22α in the intima was more restricted than detectable expression of PDGF α-receptor.

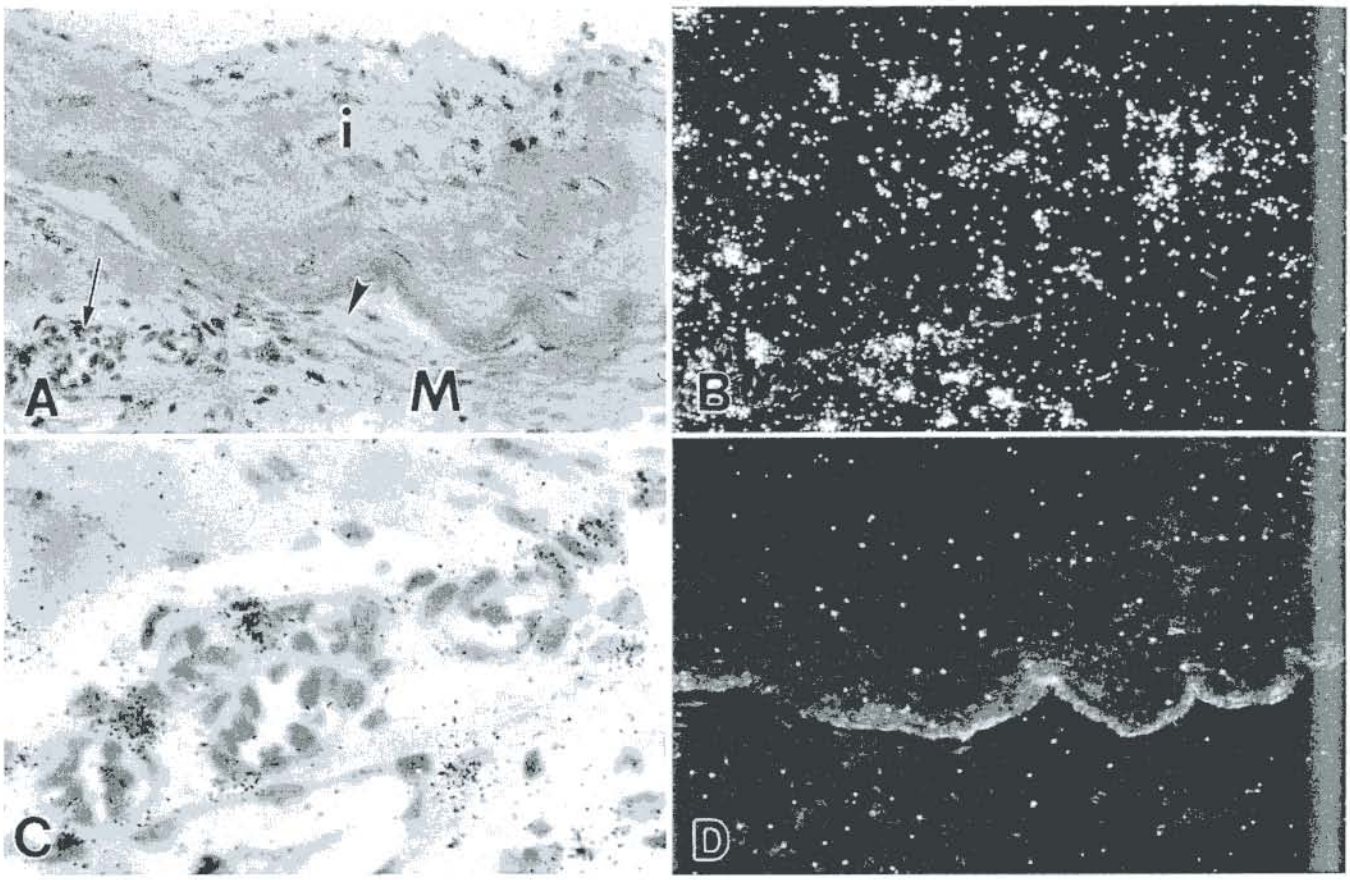

Figure 4. Demonstration of PDGF α-receptor in renal vessels by *in situ* hybridization. (A) Histologic appearance of a small renal artery of a mature adult. Some intimal thickening (i) is present. PDGF α-receptor is also expressed by a population of cells, most likely representing pericytes or adventitial fibroblasts, in the adventitial microvessels (arrow). M, media; arrowhead, internal elastic membrane. Magnification, ×400. (B) Dark field of the same area as shown in A, showing mRNA expression in the widened intima and the adventitial microvessel. (C) High-power photograph of the same area as shown in A, showing mRNA expression associated with the adventitial microvessels. Magnification, ×1000. (D) Dark field of the same area as shown in A and B, hybridized with the sense probe and showing no specific hybridization signal. Magnification, ×400.

kidneys. Tubular cells did not express PDGF α-receptor protein or mRNA in any of the specimens examined (Figure 1, A and B; Figure 5A; Figure 6, D and E; Figure 7; and Figure 8, A and B). A small subpopulation of mononuclear leukocytes present within inflammatory cell aggregates in both mature adult and rejecting kidneys showed expression of PDGF α-receptor (Figure 5). Double-immunolabeling studies demonstrate colabeling of PDGF α-receptor and CD-45 on only a few cells in areas of inflammatory cell infiltration. Most PDGF α-receptor-expressing cells in these areas fail to express the leukocyte antigen CD45, leading us to believe they are myofibroblast-like interstitial cells.

Discussion

The most intriguing finding of the current study in mature human kidneys with arteriosclerosis and rejecting renal transplants was the demonstration, for the first time, of expression of PDGF α-receptor protein and mRNA in intimal cells of the renal arterial vasculature. By double immunolabeling, we were able to exclude that these cells were infiltrating leukocytes, in particular monocytes/macrophages. Judging from the localization of the cells and the lack of *Ulex* lectin binding, these PDGF α-receptor-positive cells also did not represent endothelial cells. Thus, by means of exclusion, the intimal PDGF α-receptor-positive cells likely were smooth muscle cells that had migrated into the neointima or were the progeny of cells that previously had migrated into the neointima as a response to vascular degenerative and/or inflammatory changes. This hypothesis is substantiated by cell culture data showing that both arterial and venous smooth muscle cells can express PDGF α-receptor and/or respond to PDGF-AA (suggesting the presence of PDGF α-receptor) (36–39). Further immunocytochemical characterization of the PDGF α-receptor-positive neointimal cells indeed confirmed that many expressed one muscle-specific protein, SM22α, or a second muscle-specific protein, α-smooth muscle actin, as well. SM22α, a calponin-related protein whose function is unknown, is one of the earliest markers of differentiating smooth-muscle cells and is expressed, for example, as early as embryonic day 9.5 during mouse embryogenesis (32). In mature, large arteries, SM22α mRNA is constitutively expressed by medial smooth muscle cells, but has a much more restricted expression in atheroma-

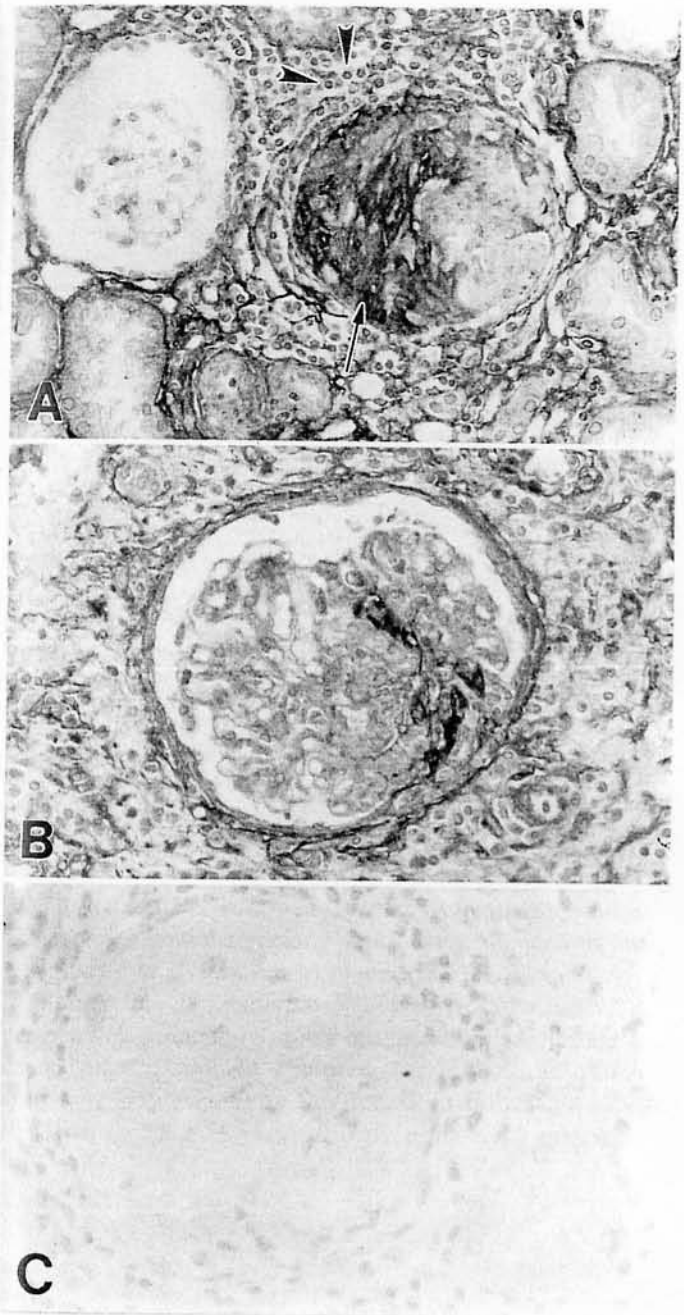

Figure 5. Immunohistochemical demonstration of PDGF α-receptor in glomeruli. (A) Kidney of a mature adult individual. PDGF α-receptor expression is markedly upregulated in the fibrotic area of a partially sclerosed glomerulus (arrow). A few of the surrounding leukocytes also express PDGF α-receptor (arrowheads). Magnification, ×400. (B) Rejected kidney. A strand of PDGF α-receptor-positive cells extends from Bowman's capsule into the glomerular tuft in an area of a synechial adhesion. Magnification, ×400. (C) Negative control for the same area shown in A. The tissue was stained with nonimmune rabbit IgG. Magnification, ×600.

tous intima. There, it is generally confined to fibrous cap regions and notably is absent in most α-actin-expressing smooth muscle cells of the intima (33). SM22α expression in the renal vasculature has not been described thus far, but it is interesting to note that in the small renal arteries, SM22α protein expression appeared to localize to intimal rather than medial smooth muscle cells. It is also noteworthy that the intimal PDGF α-receptor-positive cells usually failed to express some of the other smooth muscle/mesenchymal markers investigated, *i.e.*, calponin and desmin. Thus, intimal PDGF α-receptor expression appeared to identify a subset of phenotypically altered smooth muscle cells. The data of the current study thereby lend further support to the notion that phenotypic modulation of smooth muscle cells is an important feature of vascular damage (40).

The pathophysiologic role of PDGF α-receptor expression in intimal cells remains speculative. Two previous reports by our group have described the pathologic expression of PDGF A-chain protein and mRNA in a subpopulation of neointimal smooth muscle cells in mature human kidneys with arteriosclerosis (17) and rejecting renal allografts (18), and the expression of PDGF B-chain protein and mRNA in rejecting renal allografts (18), data that were confirmed in the current study. Furthermore, in acute renal allograft rejection, upregulated expression of PDGF A-chain by vascular endothelial cells has been detected as well (18). *In vitro* studies of human aortic endothelial cells exposed to allogeneic mononuclear cells from patients with cardiac allograft vasculopathy showed an increase in their synthesis of PDGF A- and B-chain (41,42). In conjunction with the data of the current study, these findings constitute the basis for an autocrine and/or paracrine system in which locally augmented production of PDGF A- and/or B-chain may contribute to the chemoattraction, hypertrophy, and/or proliferation of vascular smooth muscle cells in the intima. In support of this potential pathophysiologic role of vascular PDGF α-receptor, it has been demonstrated that vascular smooth muscle cells from spontaneously hypertensive rats, but not those from normotensive Wistar-Kyoto rats, expressed PDGF α-receptor in culture and responded to PDGF-AA with hypertrophy (43). This data should be interpreted in conjunction with data from other laboratories, which have demonstrated heterogeneity in the phenotype of cloned rat vascular smooth muscle cells from rat pups, including the presence or absence of detectable expression of PDGF α-receptor (44). These latter studies also found PDGF α-receptor in normal adult uninjured rat vascular smooth muscle cells. In experimental models of allograft arterial injury, expression of PDGF α-receptor by vascular smooth muscle cells, in conjunction with expression of its ligands PDGF A- and B-chains by various cell types present at sites of vasculopathy, has been implicated in the processes of smooth muscle cell migration and proliferation, which characterize chronic vascular rejection (45). However, it is recognized that the number of experimental studies that have sought to establish a role for PDGF A-chain or PDGF α-receptor in renal vasculopathies is quite limited at present, and more precise depictions of the mechanisms by which these molecules may exert an effect on renal injury are necessarily speculative.

Mild upregulation of PDGF α-receptor expression in both mature adult and rejecting kidneys was also observed in the mesangium of many glomeruli. The most likely cell type to

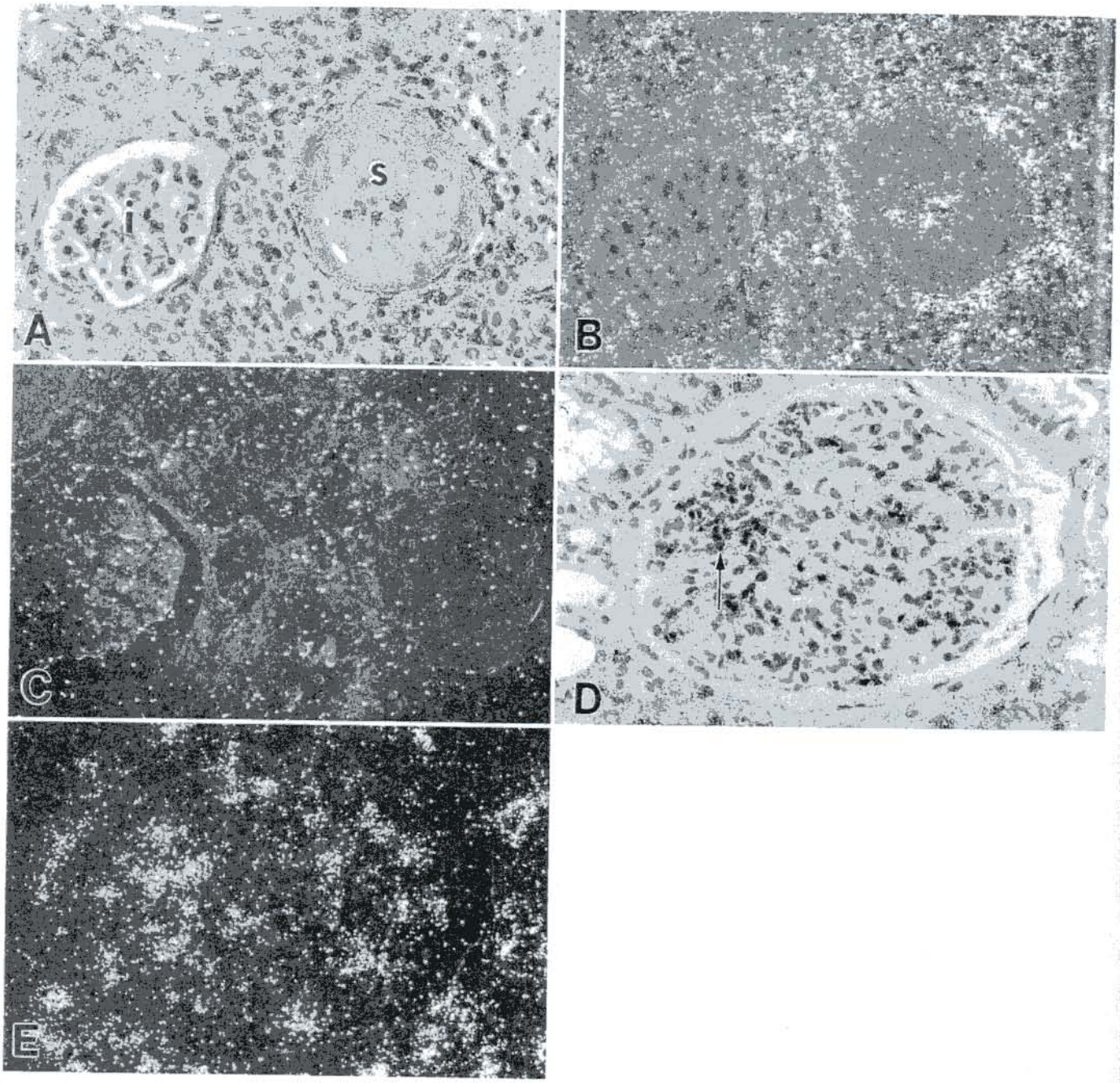

Figure 6. Demonstration of PDGF α-receptor in glomeruli by *in situ* hybridization. (A) Light microscopic image of an intact (i) and a globally sclerosed (s) glomerulus of a mature adult individual. The globally sclerosed glomerulus is surrounded by a mononuclear cell infiltrate. Magnification, ×400. (B) Dark field of the same area shown in A, showing mRNA expression in the residual cellular components in the sclerosed glomerulus but not the intact glomerulus, as well as in the area of the mononuclear cell infiltrate. Accumulation of grains in the intact glomerulus is not increased over background. (C) Dark field of the same area as shown in A, hybridized to a sense cRNA for PDGF α-receptor and showing no specific hybridization signal. (D) Light microscopic image of a glomerulus from an adult, with segmentally increased cellularity (arrow). Magnification, ×400. (E) Dark field of the same area shown in D, showing mRNA expression in many cells in mesangial areas, with the most prominent expression in the hypercellular area indicated in Panel D.

express PDGF α-receptor in the glomeruli is the mesangial cells, because mesangial cells have been shown to respond to PDGF-AA and express the receptor in culture (46–48). This assumption is strengthened by the observation that some of the glomerular PDGF α-receptor-positive cells also express α-smooth muscle actin, which specifically marks activated mesangial cells in glomeruli as long as Bowman's capsule is intact (27). Both Gesualdo's (49) and our own previous study (19) have demonstrated constitutive, albeit low-grade, expression of PDGF α-receptor mRNA and protein in mesangial areas. In addition, Stein-Oakley *et al.* (50) have recently reported that the glomerular PDGF α-receptor immunostaining

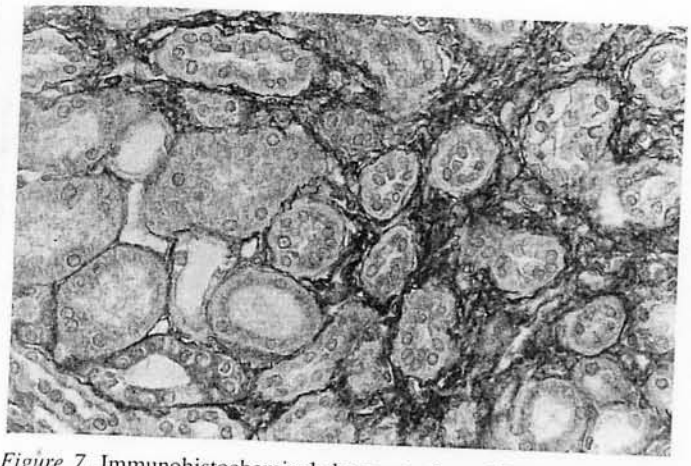

Figure 7. Immunohistochemical demonstration of PDGF α-receptor in the renal tubulointerstitium. Kidney of a mature individual. PDGF α-receptor expression is augmented in areas of interstitial fibrosis. No tubular expression of the receptor is present. Magnification, ×400.

was increased in patients with IgA nephropathy and focal segmental glomerulosclerosis, in particular in areas of mesangial hypercellularity. The findings of the present study therefore may relate to the fact that mesangial cell proliferation and activation are relatively common features in various types of glomerular diseases (35,51,52). The mild enhancement of glomerular PDGF α-receptor expression in mature adult and rejecting kidneys, as well as the glomerular overexpression of PDGF A- and B-chain in segmental glomerulosclerosis (50) and in experimental mesangial proliferative glomerulonephritis (12), may contribute to these processes.

Glomerular expression of PDGF α-receptor mRNA and protein was particularly prominent in adhesions, fibrotic crescents, and some globally sclerosed glomeruli. Because all three situations are characterized by breaks in Bowman's capsule, PDGF α-receptor expression likely reflects the migration of interstitial (myo-)fibroblasts into the capsular space. Renal interstitial fibroblasts already constitutively express moderate-to-high levels of PDGF α-receptor mRNA and protein (19), and the current study showed that this expression was enhanced even further in areas of myofibroblast accumulation, in which the fibroblasts express α-smooth muscle actin (34). An enhancement of PDGF α-receptor expression in renal interstitial fibrosis has also been noted in two previous studies (49,50). PDGF α-receptor expression by activated fibroblasts, *i.e.*, myofibroblasts, may play an important role in the pathogenesis of renal fibrosis, because studies in mice genetically deficient for the PDGF A-chain showed a defective pulmonary alveolar myofibroblast development (53). Furthermore, PDGF A-chain has been shown to mediate the proliferative response of fibroblasts to other cytokines, such as interleukin-1 (54).

In conclusion, in both mature adult human kidney and rejecting renal allografts, PDGF α-receptor expression was selectively upregulated in the three cell types that already constitutively exhibited some receptor synthesis, *i.e.*, interstitial fibroblasts, mesangial cells, and vascular smooth muscle cells. However, in these three cell types, PDGF α-receptor upregulation was not uniform, but mostly occurred in those cells that had undergone phenotypic modulation, *e.g.*, vascular smooth muscle cells that have migrated into the neointima or showed apparent enhanced expression of α-smooth muscle actin (fibroblasts and mesangial cells). In all three cases, the upregulated expression of PDGF α-receptor by the cells paralleled the occurrence of sclerosing changes, suggesting that PDGF α-receptor predominantly mediates fibrogenic responses in the kidney. Given the observed similarities of the findings in both mature kidneys with arteriosclerosis and rejecting allografts, our data support the notion that a variety of injurious conditions, including immunologic and nonimmune renal injury, can enter a final common pathway of tissue damage. Elucidation of the pathogenic mechanisms involved in this final pathway may

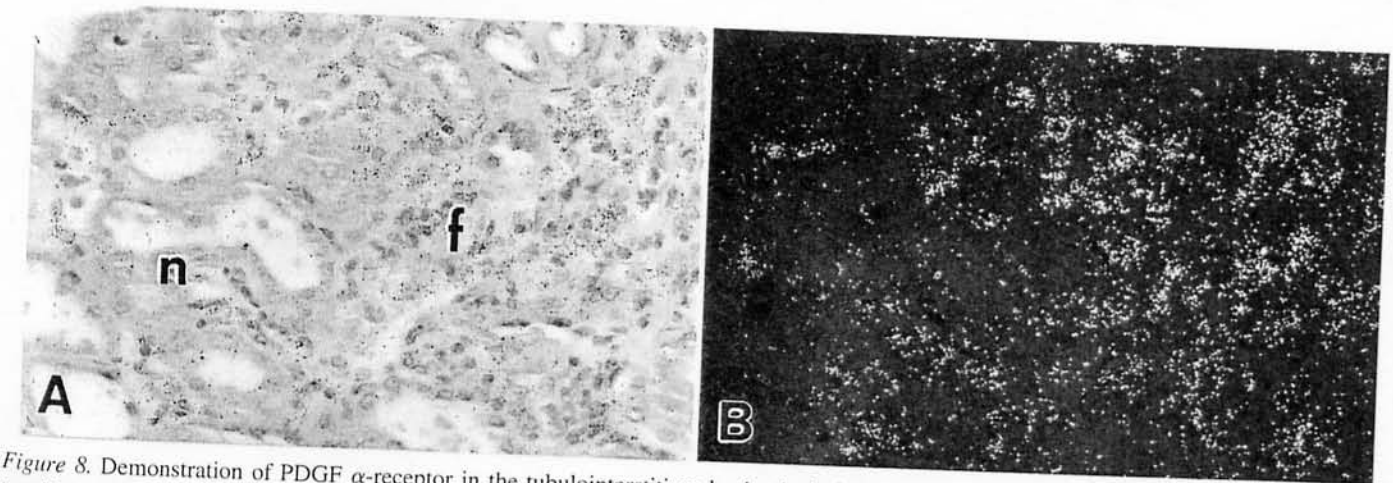

Figure 8. Demonstration of PDGF α-receptor in the tubulointerstitium by *in situ* hybridization. (A) Light microscopic image of a tubulointerstitial area with normal morphology (n) adjacent to a zone of interstitial fibrosis (f) with tubular atrophy, interstitial widening, and cellular infiltration. The infiltrating cells are mononuclear leukocytes (monocytes and lymphocytes) that are routinely found in areas of fibrosis. Widespread signal-localizing PDGF α-receptor mRNA is present in the intertubular areas, in concurrence with the immunohistochemical localization of PDGF α-receptor protein in interstitial areas. Magnification, ×400. (B) Dark field of the same area shown in A, showing some mRNA expression in the normal-appearing interstitium, but markedly enhanced mRNA expression in the fibrotic part.

ultimately result in novel therapeutic approaches to progressive renal disease.

Acknowledgments

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