

## Platelet-derived growth factor-D expression in developing and mature human kidneys

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### Platelet-derived growth factor-D expression in developing and mature human kidneys.

**Background.** Platelet-derived growth factor (PDGF) is a family of growth regulatory molecules composed of sulfide-bonded dimeric structures. Two well-studied PDGF peptides (PDGF-A and PDGF-B) have been shown to mediate a wide range of biological effects. PDGF-D is a newly recognized member of the PDGF family. Initial studies of the *PDGF-D* gene found its expression in cells of the vascular wall, suggesting that it could participate in vascular development and pathology. However, its localization in human kidney tissues has never been studied.

**Methods.** PDGF-D expression in fetal ( $N = 30$ ) and adult ( $N = 25$ ) human kidney tissues was examined by immunohistochemistry using an affinity-purified antibody raised to human PDGF-D. Antibody absorption with the immunizing peptide was employed to confirm the specificity of this antibody. PDGF-D protein and gene expression in human kidneys also were demonstrated by Western blotting and reverse transcription-polymerase chain reaction (RT-PCR).

**Results.** In the developing kidney, PDGF-D was first expressed by epithelial cells of comma- and S-shaped structures of the developing nephron, and most consistently in the visceral epithelial cells in the later stages of glomerular differentiation. In addition, PDGF-D could be found in mesenchymal, presumptively fibroblast cells in the interstitium of developing renal pelvis and in fetal smooth muscle cells in arterial vessels. In the adult normal kidney, PDGF-D was expressed by the visceral epithelial cells. There was persistent expression in arterial smooth muscle cells as well as in some neointimal smooth muscle cells of arteriosclerotic vessels, and expression in smooth muscle cells of vasa rectae in the medulla. PDGF-D could be identified at the basolateral membrane of some injured tubules in areas of chronic tubulointerstitial injury routinely encountered in aging kidneys. Western blotting of homogenates of adult kidneys demonstrated monospecific bands at 50 kD corresponding to previously established size parameter for this protein. RT-PCR of human kidney RNA resulted in a 918 basepair band, the

sequence of which corresponded to human *PDGF-D* (Genbank number AF336376).

**Conclusions.** To our knowledge, these are the first studies to localize PDGF-D in human kidneys and suggest that PDGF-D may have a role in kidney development. PDGF-D was shown to bind to PDGF  $\beta$  receptor, which localizes to mesangial cells, parietal epithelial cells, and interstitial fibroblasts, suggesting potential paracrine interactions between those cells and the visceral epithelium.

Platelet-derived growth factor (PDGF) is a family of growth regulatory molecules composed of sulfide-bonded dimeric structures. It was originally discovered as a factor promoting the proliferation of fibroblasts and smooth muscle cells [1, 2]. Until recently, two PDGF peptides, PDGF-A and PDGF-B, which are able to form three dimeric isoforms as PDGF-AA, PDGF-AB, and PDGF-BB, have been thought to comprise all of the members of the PDGF signaling system [3, 4]. These PDGF ligands exert their action on responsive cells by binding to the cell membrane tyrosine kinase receptors consisting of  $\alpha$ - and  $\beta$ -isoforms that dimerize upon ligand binding to form the PDGFR- $\alpha\alpha$ ,  $-\alpha\beta$ , or  $-\beta\beta$  isoforms [5–12]. The biological activity of PDGF-A and PDGF-B chains and PDGF  $\alpha$  and  $\beta$  receptors have been extensively studied in both experimental models and human settings [13–26].

As a result of analysis of the Biotechnology Information EST databases, two additional isoforms of PDGF termed PDGF-C and PDGF-D have been recently identified [27–29]. Human PDGF-D encodes a protein of 370 amino acids and has three domain structures: an amino-terminal CUB domain, a C-terminal PDGF/vascular endothelial growth factor (VEGF)-homology domain (growth factor domain or core domain), and an intermediary bridge region with no homology to other protein domains. There is also a single putative site for N-linked glycosylation in the core domain. The amino acid sequence of human PDGF-D is closely related to human PDGF-C, demonstrating 50% and 43% identity in the

**Key words:** growth regulatory molecule, platelet-derived growth factor, developing nephron, vascular development, protein PDGF-D, fibroblasts, glomerulogenesis arteriosclerosis.

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core domain and the full-length structure, respectively [38]. In addition, PDGF-D has approximately 25% overall amino acid identity with PDGF-A and PDGF-B [28]. A PDGF-D expression profile in different human organs including kidneys was reported by using real-time quantitative polymerase chain reaction (PCR) and Northern blot analysis [27, 28]. It also has been demonstrated that PDGF-D is able to bind with PDGF receptor  $\beta$  [27, 28]. PDGF-D could be expressed in human fibroblasts and cells of the vascular wall and could stimulate the proliferation of human smooth muscle cells, suggesting that it might play a role in vascular development and disease [29]. PDGF-D protein expression in mouse embryos using immunohistochemistry demonstrated its staining in developing heart, lung, kidneys and some muscle derivatives [27].

Although PDGF-D has been reported to be expressed in human kidney, its precise expression in specific cell types is controversial. Uutela et al could not find PDGF-D mRNA expression in a human kidney cell line [29]. To address this issue, our current study was designed to demonstrate PDGF-D expression in specific cell types by performing immunohistochemistry in developing and mature human kidneys. We also utilized Western blotting and reverse transcription (RT)-PCR in order to establish the existence of this molecule in human kidneys at protein and RNA levels.

## METHODS

### Tissue selection

Human fetal kidneys ( $N = 30$ ) with estimated gestational ages ranging from 54 to 122 days were obtained fresh from tissue examined after therapeutic abortions. These studies have received human subjects approval by an institutional IRB review panel (UW 96-1826-A-08). Tissues were fixed in methyl Carnoy's (methacarn) solution (60% methanol, 30% chloroform, 10% acetic acid) and processed and embedded in paraffin according to conventional techniques.

Normal-appearing human adult kidney tissues ( $N = 25$ ) were obtained fresh from uninvolved portions of kidneys surgically resected for localized renal cell carcinoma, or from cadaver donor kidneys unable to be utilized for transplantation, under an IRB approved protocol (M-1183). Tissues were fixed in methacarn solution and processed as stated earlier.

### Antibodies

**PDGF-D-chain.** Rabbit polyclonal antibody (lot no. E0259, provided by ZymoGenetics Inc., Seattle, WA, USA) is an affinity-purified antibody raised against the middle part of PDGF-D molecule. It recognizes the full-length human PDGF-D peptide but does not recognize the isolated growth factor domain (GFD) peptide. This

antibody is non-reactive with either full-length or growth factor domain of PDGF-C, or with PDGF-A and PDGF-B chains.

**PDGFR $\beta$ .** Rabbit polyclonal antibody (lot no. PR7649; provided by Dr. D.F. Bowen-Pope) recognizes PDGFR $\beta$  by Western blotting and stains mesangial cells by immunohistochemistry as previously described [14]. It detects patterns of PDGFR $\beta$  similar to those detected by antibody PR7212 as previously used and referenced by our group [16], but has the advantage of working well in methacarn fixed tissues.

**Muscle-specific actin.** Murine monoclonal antibody (clone 1A4; Dako Corp., Carpinteria, CA, USA) has been well characterized by Western blotting [30]. It has been previously demonstrated to recognize alpha-smooth muscle actin in methyl Carnoy's fixed tissues [16], and was used as a marker of mesangial cells as previously described [31].

**WT-1.** Rabbit polyclonal antibody (cat no. sc-192, lot no. D301; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was raised against a peptide of the carboxy terminus of Wilms' tumor (WT) protein of human origin. It has been characterized by tissue immunohistochemistry and Western blotting [32–35], and was used as a phenotypic marker of visceral epithelial cells (podocytes) as previously described [35, 36].

**Ulex europaeus agglutinin I.** A lectin glycoprotein (lot no. 91129; Vector Laboratories, Burlingame, CA, USA) established to be a marker of endothelial cells by lectin binding was used to identify glomerular and vascular endothelial cells as previously described [37, 38].

### Immunohistochemistry

Immunohistochemistry was performed on four-micron sections of methyl Carnoy's fixed, paraffin-embedded tissues following a standard avidin-biotin complex (ABC) method. Briefly, sections were deparaffinized in xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with 3% hydrogen peroxide and the samples were then rinsed in phosphate-buffered saline (PBS). For reduction of background labeling, the sections were blocked by using the avidin-biotin blocking kit (Vector Labs) per the manufacturer's instruction and Normal Goat Serum (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 30 minutes. The sections were incubated overnight at 4°C with rabbit anti-human PDGF-D polyclonal antibody diluted 1:100 in PBS containing 1% bovine serum albumin (BSA) and 5% non-fat dry milk. After washing in PBS, the sections were sequentially incubated with biotinylated goat anti-rabbit IgG (Vector Labs) and the ABC-Elite reagent (Vector Labs) Finally, 3,3'-diaminobenzidine (DAB; with nickel chloride enhancement) was used as the chromogen and sections were counterstained with methyl green, dehydrated and cover slipped.

For all samples, a negative antibody control consisted of substitution of the primary antibody with normal rabbit IgG. Transfected BHK-570 cells (expressing PDGF-C) or transfected Chinese hamster ovary (CHO) cells (expressing PDGF-D) as well as their parenteral BHK and CHO cells (kindly provided by ZymoGenetics) were used as positive and negative controls to establish the sensitivity and specificity of the antibody to PDGF-D for immunohistochemical procedures.

### Double labeling immunohistochemistry

Four-micron sections of methyl Carnoy's-fixed, paraffin embedded tissues were prepared for immunohistochemistry as described above. The slides were incubated with murine monoclonal antibody against  $\alpha$ -smooth muscle actin, rabbit polyclonal anti-PDGFR $\beta$  antibody, rabbit polyclonal WT-1 antibody, or Ulex europaeus agglutinin I. These were followed by biotinylated anti-mouse IgG, biotinylated goat anti-rabbit IgG, or biotinylated Ulex, and then the ABC-Elite reagent, and DAB to give a brown reaction. Peroxidase activity was blocked again with 3% hydrogen peroxide. Then, the sections were sequentially incubated with Normal Goat Serum to reduce the background labeling, rabbit polyclonal anti-human PDGF-D antibody with an overnight incubation, biotinylated goat anti-rabbit IgG, the ABC-alkaline phosphatase reagent (Vector Labs), and finally a blue substrate kit (Vector<sup>®</sup> Blue Alkaline Phosphatase Substrate Kit III; Vector Labs) to yield a blue reaction product. The slides were counterstained with Nuclear Fast Red (Vector Labs), dehydrated and cover slipped.

### Antibody absorption

The immunizing PDGF-D peptide used to generate anti-PDGF-D antibody was resuspended in PBS, incubated with rabbit polyclonal anti-PDGF-D antibody at 4°C overnight (the excess concentration of the peptide to antibody was 100:1), and then used as the absorbed primary antibody in a standard ABC immunohistochemistry procedure in kidney sections and BHK-570 cell lines expressing the full-length PDGF-D protein. Concurrent controls included repeating the procedure in an identical fashion but without the PDGF-D peptide absorption step.

### Protein preparation and Western blotting

Approximately 250 milligrams of frozen adult kidney were minced and washed thoroughly in PBS. After centrifugation and removing the tissue debris, the suspension buffer [0.1 mol/L NaCl, 0.01 mol/L Tris-HCl pH 7.6, 0.001 mol/L ethylenediaminetetraacetic acid (EDTA) pH 8.0, 1  $\mu$ g/mL aprotinin, and 100  $\mu$ g/mL phenylmethylsulfonyl fluoride (PMSF)] was added and the tissue was dispersed in Tissuemizer. For Western reducing conditions, 2 $\times$  sample buffer with sodium dodecyl sulfate

(SDS) and  $\beta$ -mercaptoethanol was added, and then the protein sample was boiled and the pellet discarded.

The protein samples were loaded on to 8 to 16% polyacrylamide gels, electrophoretically size-separated, and then transferred to nitrocellulose membranes. The membranes were rinsed with Tris-buffered saline (TBS; pH 7.6) and were blocked with 5% non-fat dry milk in TBS containing 1% BSA at 37°C for two hours. The membranes were then incubated overnight with a 1/1500 dilution of rabbit polyclonal anti-PDGF-D antibody 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 minutes, washed, developed with a chemiluminescent substrate (CSPD<sup>®</sup>; Applied Biosystems, Foster City, CA, USA) and exposed to film. The full-length PDGF-D protein (kindly provided by ZymoGenetics) was used as the positive protein control. As a negative antibody control, the primary antibody was replaced by normal rabbit IgG at an equivalent concentration. The full-length and growth factor domain PDGF-C protein also were used to confirm the non-reactive of anti-PDGF-D antibody to PDGF-C. In addition (not shown) this antibody does not cross-react with PDGF-A or PDGF-B protein.

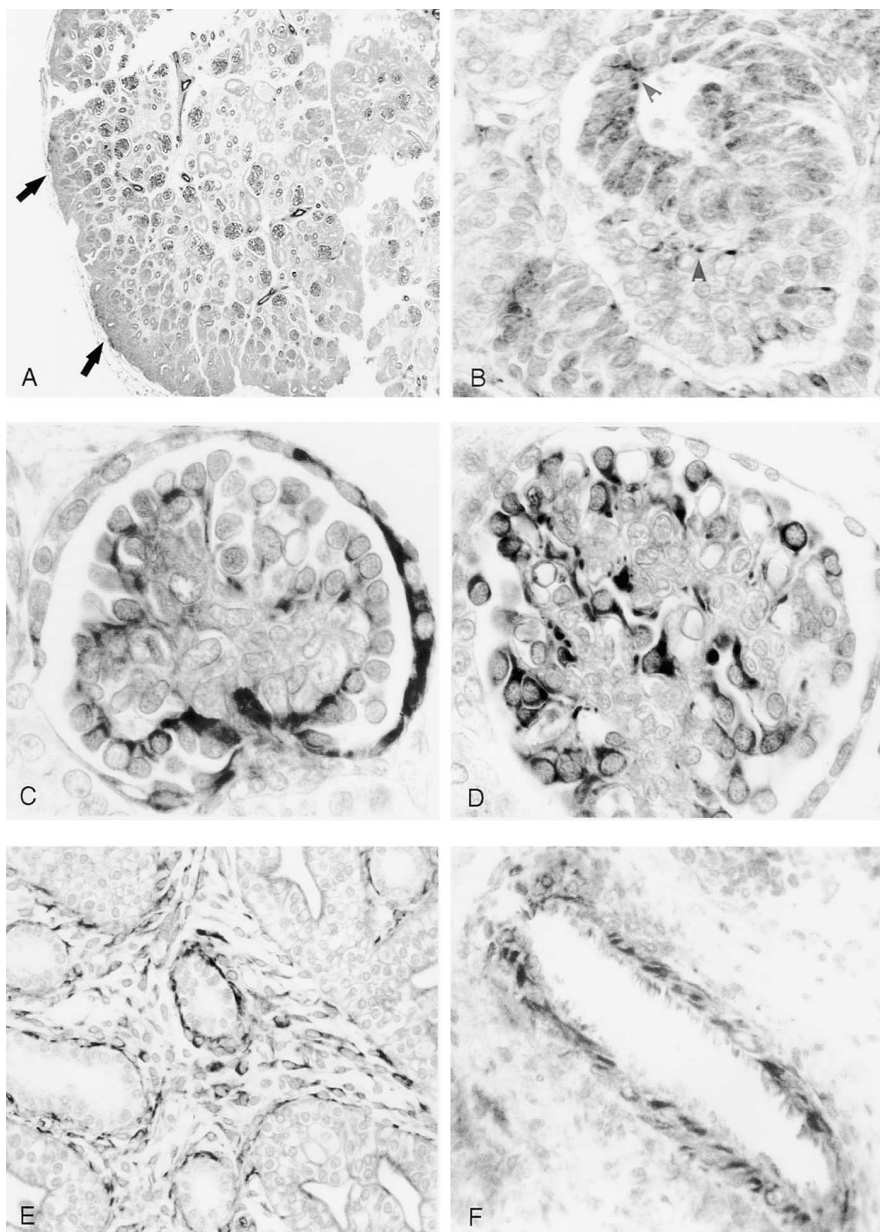
### RT-PCR

To demonstrate PDGF-D gene expression in kidney tissues, total cellular RNA from normal and transplant nephrectomies was extracted with RNAqueous-Midi kit (RNAqueous-Midi<sup>™</sup>; Ambion Inc., Austin, TX, USA) and stored at -70°C until use. Approximately two micrograms of RNA was mixed with the RT mixture per instructions in the OneStep RT-PCR kit (Qiagen Inc., Chatsworth, CA, USA) and then reverse-transcribed with PDGF-D primers (Life Technologies, Grand Island, NY, USA) for 30 minutes at 50°C. The sequences of the PDGF-D primers were 5'-GTGCAGAGTCCTAGATTCCC-3' for the forward primer and 5'-GAGGTGGTCTTGAGCTGCAG-3' for the reverse primer. After the hot start at 95°C for 15 minutes, 32 cycles of amplification were carried out with the following processes: denaturation at 94°C for one minute, annealing at 59°C for one minute, extension at 72°C for one minute in each cycle and 10 minutes at the last cycle. RT-PCR products were resolved on a 1% agarose gel containing ethidium bromide and were visualized under ultraviolet illumination. RNA was replaced by nuclease free water as the negative control for RT-PCR assays. The RT-PCR product was cloned and sequenced to confirm its identity with PDGF-D.

## RESULTS

### Immunohistochemistry

*Human fetal kidney.* Expression of PDGF-D was not identified in the metanephric blastema or the vesicle



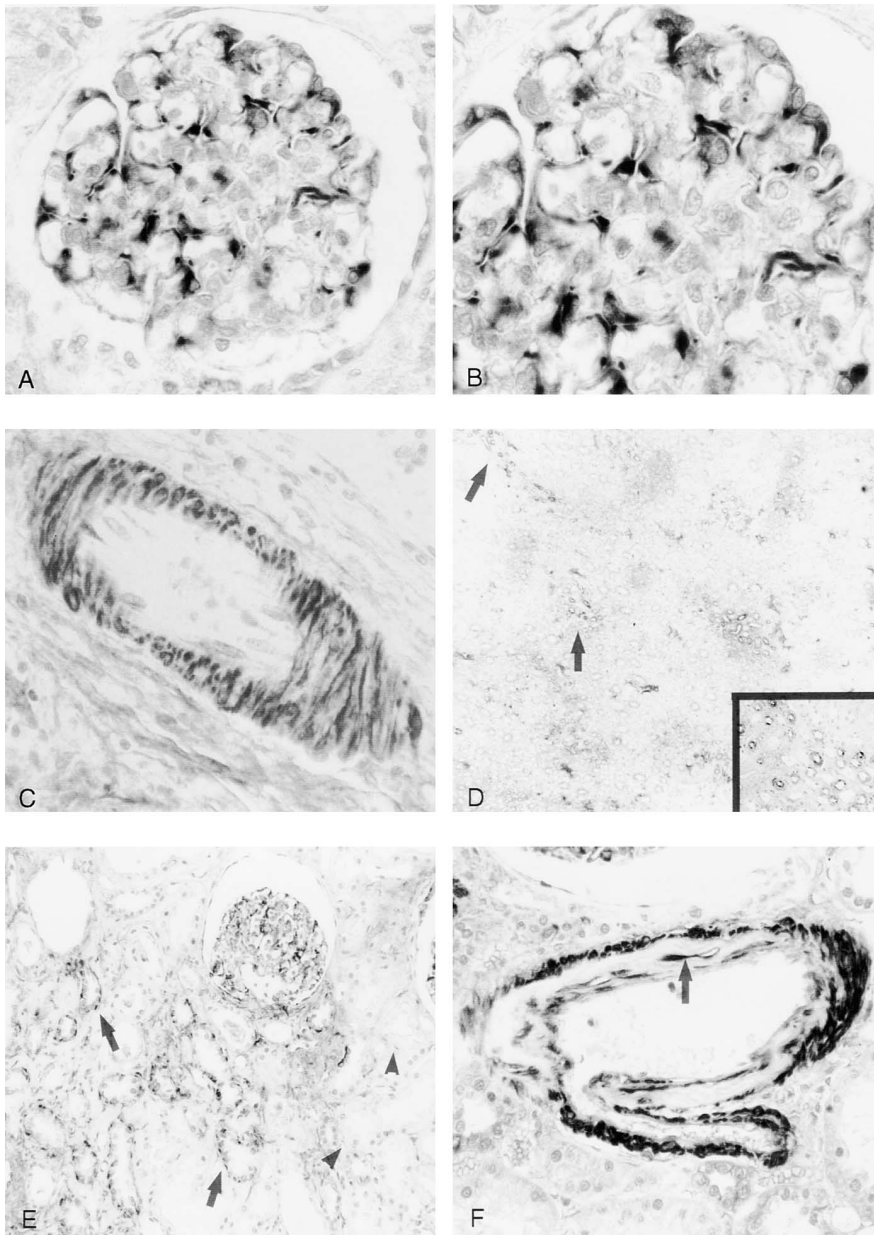
**Fig. 1. Immunolocalization of PDGF-D in developing human kidney.** (A) In a low power view of a fetal kidney of estimated 83 days of gestational age, PDGF-D is demonstrated in various stages of glomerulogenesis, but not in the metanephric blastema (arrows) or the vesicle developing as the earliest stage of glomerulogenesis. (B) PDGF-D is weakly expressed by epithelial cells (arrowheads) in the comma- and S-shaped structures. (C) PDGF-D is expressed by visceral epithelial cells and some parietal epithelial cells of the glomerulus at early stages of differentiation. (D) PDGF-D expression confined to visceral epithelial cells of the maturing glomerulus. (E, F) In extraglomerular structures, PDGF-D is expressed by fibroblast-like mesenchymal cells within the renal pelvis (E), and by medial smooth muscle cells of muscular arteries (F).

stage of early nephron formation (Fig. 1A). In the later stages of development, PDGF-D was weakly expressed by the epithelial cells of comma- and S-shaped structures (Fig. 1B) and strongly expressed by the visceral epithelial cells of more differentiated glomeruli (Fig. 1 C, D). PDGF-D was expressed by some parietal epithelial cells in differentiated glomeruli (Fig. 1C). Extraglomerular structures of developing kidney showing the expression of this protein included the basement membrane of immature collecting ducts, fibroblast-like mesenchymal cells in the interstitial area of the renal pelvis (Fig. 1E), and medial smooth muscle cells of arteries (Fig. 1F).

**Human adult kidney.** Most kidney specimens had normal histology by conventional light microscopy. Some

of them had foci of nonspecific chronic changes including global glomerulosclerosis, tubular atrophy, interstitial fibrosis and arteriosclerosis most likely related to aging [39].

In adult normal kidneys, PDGF-D was uniformly expressed by visceral epithelial cells but not by other glomerular structures (Fig. 2 A, B). There was widespread expression of this protein in the medial smooth muscle cells of arteries and arterioles that was similar to the expression pattern identified in developing kidney (Fig. 2C), as well as in smooth muscle cells of vasa rectae in the medullary area (Fig. 2D). PDGF-D expression was not detected in normal tubular and interstitial cells. There were focal changes in PDGF-D expression in areas of chronic renal damage, where it was identified in scler-



**Fig. 2. Immunolocalization of PDGF-D in mature human kidney.** (A, B show higher power view) PDGF-D is expressed by visceral epithelial cell of the glomerulus. (C) PDGF-D is expressed by medial smooth muscle cells of arteries, similar to that shown in Fig. 1F. (D) PDGF-D expression is present in the medullary area (arrows). PDGF-D staining structures in these areas are vasa rectae (inset). (E) PDGF-D is expressed at the basolateral membrane of some injured tubules (arrows), but not by normal tubules (arrowheads). (F) There is expression of PDGF-D by some neointimal cells (arrow) in arteriosclerotic vessels.

**Table 1.** Double labeling immunohistochemical staining pattern in developmental kidney

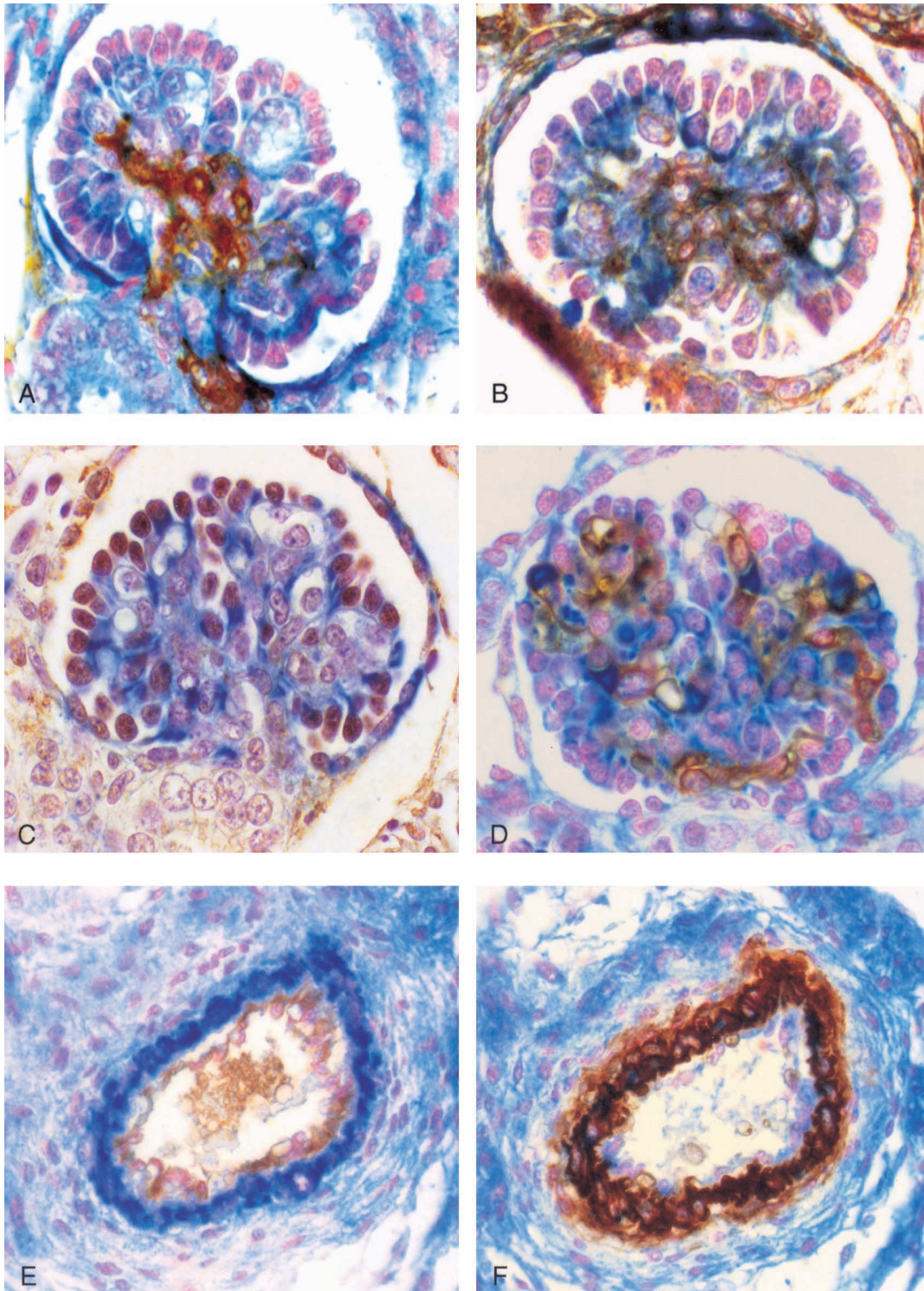
	Metanephric blastema	Metanephric vesicle	Comma-S-stage	Differentiated glomerulus	Mature glomerulus	Ureteric bud
PDGF-D	(-)	(-)	(+) faintly in EP	(+) VEP and PEP	(+) PEP	(-)
PDGFR $\beta$	(+)	(-)	(-)	(+) mesangium	(+) mesangium	(-)
$\alpha$ -smooth muscle actin	(-) <sup>a</sup>	(-) <sup>b</sup>	(-) <sup>c</sup>	(+) mesangium	(+) mesangium	(-)
WT-1	(-)	(+) focal	(+) EP	(+) nucleus of VEP	(+) nucleus of VEP	(-)
Ulex europaeus	(-) <sup>a</sup>	(-)	(+) EC	(+) EC	(+) EC	(-)

Abbreviations are: EP, epithelial cells; VEP, visceral epithelial cells; PEP, parietal epithelial cells; EC, endothelial cells; WT-1, Wilm's tumor. Symbols are: (-), negative; (+), positive.

<sup>a</sup>Components of vasculature in blastema and interstitium demonstrate the expression

<sup>b</sup>Perivascular mesenchymal cells are positive staining for alpha-smooth muscle actin

<sup>c</sup>Mesenchymal cells within the glomerular clefts demonstrate positive staining for alpha-smooth muscle actin



**Fig. 3. Double immunolabeling of PDGF-D with antibody markers of different cell types in developing human kidney.** (A) Alpha-smooth muscle actin (brown) is localized to mesangial cells, while PDGF-D (blue) is localized to visceral epithelial cells of the glomerulus. (B) PDGFR $\beta$  (brown) is expressed by mesangial cells, while PDGF-D (blue) is expressed by visceral epithelial cells of the glomerulus. (C) Co-localization of WT-1 (brown, nuclear stain), a phenotypic marker of visceral epithelial cells, and PDGF-D (blue, cytoplasmic stain) in the same cells in the differentiated glomerulus confirms that PDGF-D is expressed by visceral epithelial cells. PDGF-D also is expressed by some parietal epithelial cells. (D) Ulex europaeus agglutinin I (brown) stains endothelial cells, while PDGF-D (blue) is consistently expressed by visceral epithelial cells of the maturing glomeruli. (E) Ulex europaeus agglutinin I (brown) stains endothelial cells of muscular arteries, while PDGF-D (blue) stains medial smooth muscle cells. (F) Serial tissue section of that shown in E, demonstrating co-localization of alpha-smooth muscle actin (brown) and PDGF-D (blue) in the same cells confirms that PDGF-D positive cells are arterial smooth muscle cells. (Publication of this figure in color was made possible by a grant from ZymoGenetics, Seattle, Washington.)

**Table 2.** Double labeling immunohistochemical staining pattern in mature kidney

	Glomerular capillary EC	Glomerular mesangium	VEP	PEP	Tubule	Vessel	Interstitial cells
PDGF-D	(-)	(-)	(+)	(-)	(-) <sup>a</sup>	(+) <sup>b</sup> SMC	(-)
PDGFR $\beta$	(-)	(+)	(-)	(+)	(-)	(+) vascular adventitial cells	(+)
$\alpha$ -smooth muscle actin	(-)	(+)	(-)	(-)	(-)	(+) SMC	(+)
WT-1	(-)	(-)	(+)	(-)	(-)	(-)	(-)
Ulex europaeus agglutinin I	(+)	(-)	(-)	(-)	(-) <sup>c</sup>	(+) EC	(-)

Abbreviations are: EC, endothelial cells; VEP, visceral epithelial cells; PEP, parietal epithelial cells; SMC, smooth muscle cells. Symbols are: (-), negative; (+), positive.

<sup>a</sup>The staining is positive at the cellular basement membrane of the damaged tubules

<sup>b</sup>The staining also is positive in neointimal cells of arteriosclerotic vessels and vasa rectae in the medullary area

<sup>c</sup>The staining is positive in peritubular capillary endothelial cells

rotic glomeruli, in basolateral membranes of injured tubules (Fig. 2E), and in some neointimal cells of arteriosclerotic vessels (Fig. 2F).

### Double labeling immunohistochemistry

The localization of PDGF-D expression in developing and normal adult kidneys was confirmed by double-labeling immunohistochemical techniques using additional antibodies to identify phenotypic markers of different cell types. Ulex europaeus agglutinin I was used to identify endothelial cells. Expression of WT-1 identified visceral epithelial cells. While no specific markers of mesangial cells in tissue sections have been identified, PDGFR $\beta$  is expressed in the mesangium of differentiated glomeruli [16] and  $\alpha$ -smooth muscle actin, a marker for smooth muscle cells, has been identified in mesangial cells of differentiated glomeruli [16]. Furthermore, PDGFR $\beta$  and  $\alpha$ -smooth muscle actin have been shown to be expressed also in the activated mesangial cells in disease states of both experimental and human models [13, 14, 31, 40]. Therefore, PDGFR $\beta$  and  $\alpha$ -smooth muscle actin were used as markers of mesangial cells in this study.

In developing kidney, the results of double immunostaining for PDGFR $\beta$ ,  $\alpha$ -smooth muscle actin, WT-1, Ulex europaeus agglutinin I, and PDGF-D are summarized in Table 1 and shown in Figure 3. In mature normal kidney, the results of double immunostaining for these antibodies are summarized in Table 2 and shown in Figure 4. The double immunolabeling studies confirmed that PDGF-D was expressed by visceral epithelial cells of glomeruli in developing and mature kidneys. In addition, the co-expression of  $\alpha$ -smooth muscle actin and PDGF-D by neointimal cells of arteriosclerotic vessels indicates that PDGF-D expressing cells in this site are likely to be smooth muscle cells.

### Antibody specificity and Western blotting

Preincubation of anti-PDGF-D antibody with the immunizing peptide completely abolished the specific immunostaining of glomerular epithelial cells, and arterial smooth muscle cells of developing and mature kidneys

(Fig. 5 A-D). The immunostaining of the basement membrane of immature collecting ducts and that of fibroblast-like mesenchymal cells in the renal pelvis of developing kidney could be abolished also by the same procedure (Fig. 5 E and F). These findings helped establish the specificity of the antibody for the immunohistochemical procedures reported.

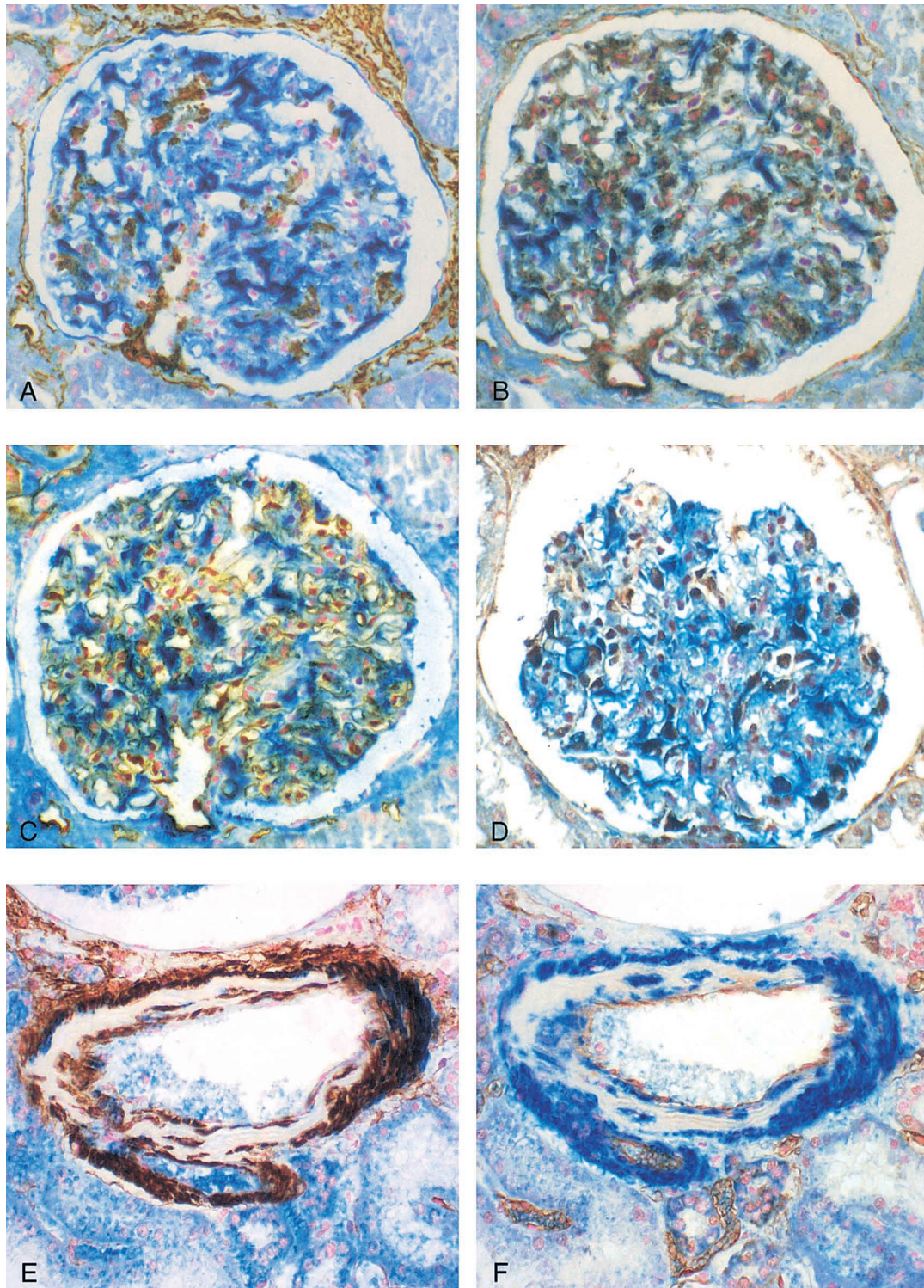
The presence of PDGF-D protein in human kidney was confirmed by Western blot analysis of tissue-extracted protein from normal and transplant kidneys using the polyclonal anti-PDGF-D antibody. The 50 kD monospecific bands corresponding to the size of the PDGF-D monomer as indicated by using a control preparation of PDGF-D full-length protein were identified in the kidney tissue extract running under reducing condition (Fig. 6). The anti-PDGF-D antibody recognized PDGF-D full-length protein at approximately 90 kD running under non-reducing condition (data not shown). These results were consistent with the biochemical properties of PDGF-D in other reports (90 kD unreduced and 49 to 55 kD reduced) [27, 28]. The specificity of this antibody also was confirmed by immunoblotting, which demonstrated that neither PDGF-C growth factor domain nor full-length protein reacted with anti-PDGF-D antibody.

### RT-PCR

Platelet-derived growth factor-D mRNA expression in the kidney was analyzed by RT-PCR. A PDGF-D nucleotide fragment of the expected length (918 bp) could be detected in kidney tissue extracts obtained from normal and transplant nephrectomies by using RT-PCR primers specific for PDGF-D (Fig. 7). The specificity of RT-PCR product was confirmed by DNA sequencing. The DNA sequence was matched with human PDGF-D nucleotides 365-1283 of Genbank number AF336376 (data not shown).

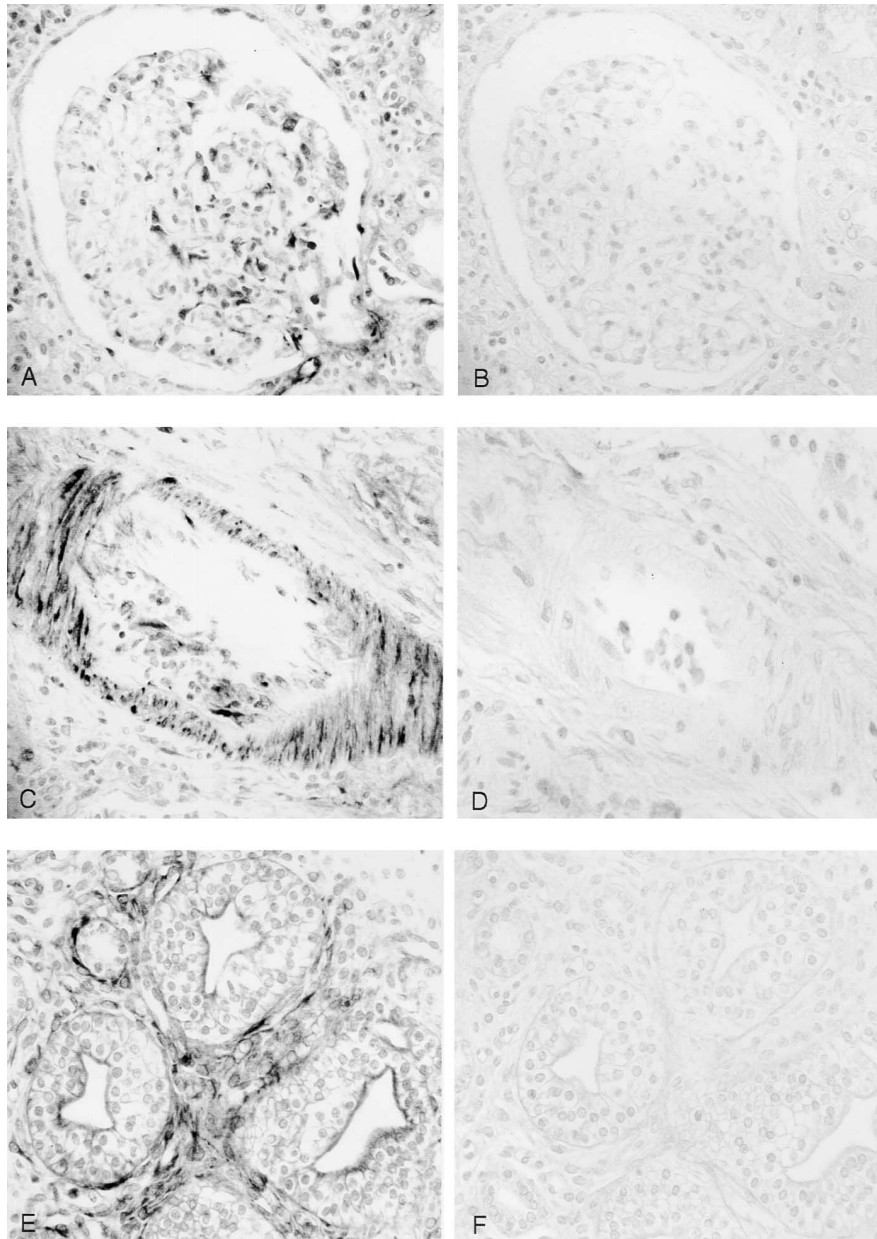
### DISCUSSION

This study demonstrates PDGF-D protein and gene expression in human kidneys by Western blotting and



**Fig. 4. Double immunolabeling of PDGF-D with antibody markers of different cell types in mature human kidney.** (A) Alpha-smooth muscle actin (brown) is expressed by mesangial cells and PDGF-D (blue) is expressed by visceral epithelial cells in the glomerulus. Alpha-smooth muscle actin also stains extraglomerular interstitial fibroblasts. (B) PDGFR $\beta$  (brown) stains mesangial cells, while PDGF-D (blue) stains visceral epithelial cells in the glomerulus. (C) Ulex europaeus agglutinin I (brown) binds endothelial cells, while PDGF-D (blue) stains visceral epithelial cells in the glomerulus. (D) Co-localization of PDGF-D (blue) with WT-1 (brown) in the glomerulus confirms that PDGF-D is expressed by visceral epithelial cells. (E, F) In arteriosclerotic vessels, PDGF-D (blue) is co-localized with alpha-smooth muscle actin (brown, in E) in medial smooth muscle cells and neointimal cells and is distinct from the double immunolabeling pattern with ulex europaeus agglutinin I (brown, in F) which binds endothelial cells. (Publication of this figure in color was made possible by a grant from ZymoGenetics, Seattle, Washington.)





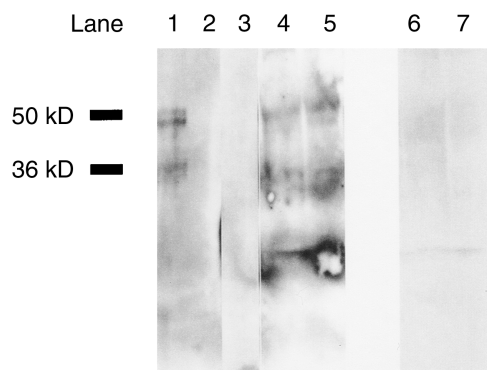
**Fig. 5. Immunostaining of mature (A–D) and developing (E, F) human kidneys with anti-PDGF-D antibody and with absorbed antiserum.** Tissue sections reacted with anti-PDGF-D antibody (A, C, and E). Adjacent sections to that shown in A, C, and E reacted with absorbed antiserum (B, D, and F). There is complete abolition of PDGF-D staining, confirming the specificity of this antibody.

RT-PCR in agreement with previous studies by others [27, 28]. We extend the information available on PDGF-D by localizing its expression in developing and mature human kidneys using immunohistochemical techniques.

Platelet-derived growth factor-D was not detectable in the earliest stages of glomerulogenesis, and it was not detected in the metanephric blastema or surrounding cortical interstitial cells. In contrast, PDGF  $\beta$  receptor was expressed by the primitive mesenchymal cells of the metanephric blastema and surrounding primordial interstitial cells. In later stages of glomerulogenesis,

PDGF-D was localized to epithelial cells transitioning from the early developing nephrons of the comma- and S-shaped stages to the visceral epithelial cells of differentiated glomeruli, concurrent with PDGF  $\beta$  receptor localization to the mesangial cells of differentiated glomeruli. In the developing pelvis, PDGF-D was expressed at the basement membrane of immature collecting ducts and by presumptive fibroblastic cells in the interstitium.

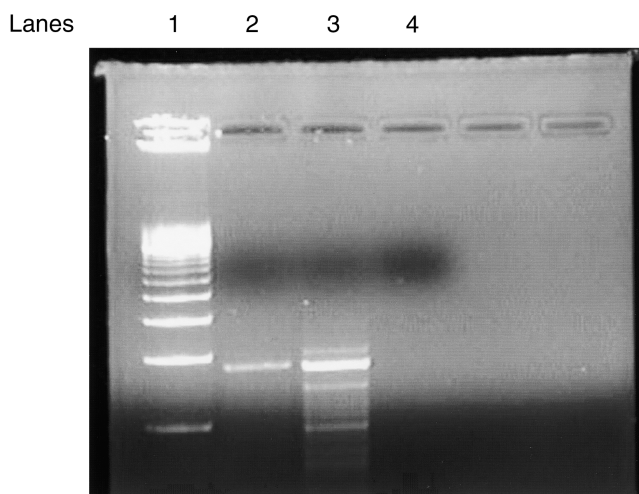
There are several growth factor-receptor interactions that are essential for epithelial-mesenchymal interactions in metanephric development. As recently reviewed



**Fig. 6. Western blot analysis utilizing anti-PDGF-D antibody reacts with the reduced form of PDGF-D full-length protein (lane 1) but does not react with PDGF-C growth factor domain protein (lane 2) or PDGF-C full-length protein (lane 3).** The antibody does react with normal kidney (lane 4) and transplant nephrectomy (lane 5). The immunoreactive protein in the 50 kD region corresponding to the PDGF-D full-length protein is identified in normal and transplant nephrectomies. The band in the 36 kD region corresponds to the N-terminally cleaved portion. No immunoreactive band is found in PDGF-C growth factor domain and full-length protein, confirming the specificity of antibody. Normal rabbit Ig G reacted with homogenates of normal kidney and transplant nephrectomy as a negative antibody control are demonstrated in lanes 6 and 7.

by Kanwar et al [41] and Bard [42], growth factors that have direct effects on individual compartments of kidney development include FGF-7, GDNF, VEGF, angiopoietin, and PDGF-B. FGF-7 knockout mice have small kidneys, while GDNF-deficient mice demonstrate kidney agenesis. VEGF and angiopoietin have been directly linked to the formation of the glomerular capillary network. PDGF-B and PDGF  $\beta$  receptor have been shown to be key regulatory molecules for mesangial development in the glomerulus, since mice in which PDGF-B or PDGF  $\beta$  receptor have been genetically deleted fail to develop mesangial cells [20, 21]. As PDGF-D is an additional ligand for PDGF  $\beta$  receptor and is expressed at early stages of glomerulogenesis, we speculated that PDGF-D may have roles in kidney development in a paracrine/juxtacrine manner, as PDGF-D might be secreted into the developing glomerulus and interact with the PDGF  $\beta$  receptor in regulating mesangial cell precursors or contributing to subsequent mesangial cell development.

In the fully differentiated glomeruli of fetal kidneys, and in glomeruli of mature human kidneys, there is persistent and uniform PDGF-D expression by visceral epithelial cells, again confirmed by double immunostaining. PDGF-D can be a phenotypic marker of visceral epithelial cells in both developing and mature kidneys and may be useful for studying the role of visceral epithelial cells in kidney diseases. The significance of widespread expression of PDGF-D by visceral epithelial cells is unknown. The principal receptor for PDGF-D, the PDGF  $\beta$  receptor, is constitutively expressed by mesangial cells



**Fig. 7. Reverse transcriptase-polymerase chain reaction analysis detected PDGF-D mRNA expression in human kidneys.** The DNA marker used was a 500-bp ladder (lane 1). A band corresponding to the expected length of PDGF-D PCR product (918 bp) is identified in normal and transplant nephrectomies (lanes 2 and 3, respectively). No expression is identified in the negative control sample (lane 4).

and parietal cells within the glomerulus, and by interstitial cells of fibroblast/myofibroblast type. PDGF-D may serve a local paracrine trophic function for glomerular cells bearing the PDGF  $\beta$  receptor. This might be similar to a role that has been hypothesized for vascular endothelial growth factor (VEGF), another growth factor constitutively expressed by visceral epithelial cells, with respect to the maintenance of fenestrations of glomerular capillary endothelial cells, which express a receptor protein for VEGF [43–47]. Alternatively, secreted PDGF-D conceivably could exert an effect on cells present in distal portions of the nephron and in the lower urinary tract, or perhaps systemically. Since so little is currently known about the regulation of PDGF-D release from various cell types, we are unable to offer a more specific schema for a functional role for constitutively expressed PDGF-D. Its ability to engage the PDGF  $\beta$  receptor strongly suggests a role for PDGF-D in promoting proliferation and migration of mesangial and interstitial cells in the kidney, based on numerous studies of injury accompanied by engagement of PDGF  $\beta$  receptor by its other principal ligand, PDGF-B chain [6, 9, 16, 20–22].

In renal arteries of developing and mature kidneys, PDGF-D is expressed by medial smooth muscle cells. It is additionally expressed by some neointimal cells of arteriosclerotic vessels. PDGF-D positive cells in the vascular intima are likely to be smooth muscle cells as demonstrated by double immunolabeling of these neointimal cells expressing PDGF-D with the phenotypic marker  $\alpha$ -smooth muscle actin. A previous study by Uutela et al demonstrated that PDGF-D stimulated proliferation and

survival of cultured serum-starved human coronary artery smooth muscle cells [29]. Taken together with our observations, these studies suggest that PDGF-D may have proliferative and chemotactic activity for smooth muscle cells that contributes to the development of vascular sclerosis, and that such PDGF-D activity is at least in part derived from neointimal cells present in such lesions where it may have local autocrine and paracrine effects.

In summary, we describe the localization of PDGF-D, a recently identified member of the PDGF family, in developing and mature kidneys. It is likely that this molecule has significant effects on renal development and on the renal response to injury in view of its ability to engage the PDGF  $\beta$  receptor and its expression in arteriosclerotic vessels.

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