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Localization of fibroblast growth factor-2 (basic FGF) and FGF receptor-1 in adult human kidney[1]

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Background. The expression pattern of fibroblast growth factor-2 (FGF-2; basic FGF), a pleiotrophic growth factor, as well as one of its receptors (FGFR1), in the kidney is highly controversial.

Methods. Using an approach that combines multiple antibodies for immunohistochemistry and correlative *in situ* hybridization, we assessed the intrarenal expression of both FGF-2 and FGFR1 in 13 specimens of adult kidney removed during tumor nephrectomy.

Results. The FGF-2 expression pattern in the kidneys as detected by immunohistochemistry was variable and depended on the antibody used. The most consistent expression of FGF-2 protein was demonstrated in glomerular parietal epithelial cells, tubular cells (mainly of the distal nephron), as well as arterial endothelial cells. These locations also corresponded to areas of FGF-2 mRNA expression. Additionally, by immunohistochemistry, FGF-2 protein was detected in arterial smooth muscle cells and occasional podocytes. The expression of FGFR1 protein and mRNA was most consistently present in tubular cells of the distal nephron and in vascular smooth muscle cells. *In situ* hybridization, but not immunohistochemistry, also suggested FGFR1 expression in cells that could not be precisely identified within the glomerular tuft as well as some interstitial cells.

Conclusion. These data suggest potential autocrine and paracrine pathways within the FGF-2 system, particularly within the vascular walls and in the distal nephron, and thereby provide information for further mechanistic understanding of the role of the FGF-2 system in human renal disease.

Fibroblast growth factor-2 (FGF-2; also known as basic FGF) is a pleiotrophic growth factor with a wide distribution throughout the body. It is presumed to play important roles in embryogenesis, vascular homeostasis, atherosclerosis, wound healing, angiogenesis, and cancer [reviewed in 1–6]. In the kidney, experimental evidence suggests that FGF-2 may serve as an endogenous amplifier of cytotoxic damage following immune-mediated injury to glomerular cells [7], and that it may contribute to the development of focal segmental glomerulosclerosis [8, 9]. Furthermore, FGF-2 exhibits mitogenic actions on various renal cells, such as mesangial cells [10–12], glomerular endothelial and epithelial cells [13, 14], tubular epithelial cells [15], and vascular smooth muscle cells [5]. Through its mitogenicity and chemotactic action on endothelial and smooth muscle cells, FGF-2 also acts as an angiogenic factor [3, 5].

Fibroblast growth factor-2 exists in multiple isoforms in the kidney [11], which arise from alternative splicing. This alternative splicing determines whether the isoforms localize to the cytoplasm or nucleus [16]. Following cellular release, FGF-2 may be sequestered in the extracellular matrix by binding to heparan sulfate proteoglycans (HSPGs) [5]. HSPGs in the cell membrane also act as low-affinity receptors for FGF-2 and are necessary for signaling by this peptide [17]. In addition to HSPG low-affinity receptors, four high-affinity FGF receptors have been identified (FGFR1 to FGFR4), of which FGFR1, FGFR2, and FGFR3 bind FGF-2 [3, 18]. It is controversial as to whether FGFR4 can also bind FGF-2 [19, 20]. Further complexity is added by the fact that FGFR1, FGFR2, and FGFR3 each exist as different splice variants (for example, IIIb and IIIc variants), which influence binding affinities for FGF-2 [3, 18]. Currently, the best characterized FGF-2 receptor that exhibits the highest binding affinity (K_d 50 to 150 pM) is FGFR1, also known as *flg* [18].

Data about renal sites of FGF-2 and FGFR1 expression are necessary to achieve a comprehensive understanding of how FGF-2 may function in renal pathophysiology. In

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this respect previous studies have yielded controversial data. FGF-2 expression in adult human kidney has been described as almost absent [21], largely confined to mid-size blood vessels [22] or to tubular cells [23], present in vessels and cells of Bowman's capsule but not tubules [24], or widely expressed in endothelial, glomerular, tubular and medullary interstitial cells [25]. In the case of FGFR1, its expression within the human kidney has also been reported variably, ranging from absent [23] to expression in tubular cells with minimal or negative immunostaining of vessels and glomeruli [26–28].

In our current study, we reassessed the previously mentioned issues using a combined approach of immunohistochemistry with multiple antibody preparations, as well as correlative *in situ* hybridization to describe the localization of FGF-2 as well as FGFR1 in nondiseased adult human kidney. Our data provide a basis for further mechanistic understanding of the role of the FGF-2 system in human renal disease.

METHODS

Tissue selection

Macroscopically normal-appearing human kidney tissue ($N = 13$) obtained fresh from uninvolved portions of kidneys surgically resected for localized renal cell carcinoma was fixed in 10% neutral-buffered formalin, and was processed and embedded in paraffin according to conventional techniques. Portions of these kidneys were also fixed in methyl Carnoy's solution and were paraffin embedded as previously described [29]. Four micrometer sections of all specimens were stained with the periodic acid-Schiff reagent, silver methenamine, and hematoxylin and eosin using conventional techniques for light microscopy and evaluation of the overall morphology.

Antibodies

FGF-2. Basic fibroblast growth factor/147 (bFGF/147; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and bFGF/Ab2 (Oncogene Science, Cambridge, MA, USA) are affinity-purified rabbit polyclonal antibodies raised against a peptide corresponding to amino acids 40 to 63 mapping within the amino terminal region of human FGF-2. Both antibodies recognized FGF-2 in methyl Carnoy's fixed, paraffin-embedded sections.

Clone 3H3/Ab3 is a murine monoclonal IgG_1 antibody to human recombinant FGF-2 (Oncogene Science). The specific FGF-2 epitope(s) recognized by the antibody is unknown. This antibody recognized FGF-2 in steam-heated, formalin-fixed sections and in methyl Carnoy's fixed, paraffin-embedded sections.

The fourth antibody, clone DE6, is a murine monoclonal IgG_1 antibody to human recombinant FGF-2 (kindly provided by Synergen, Boulder, CO, USA). The specific FGF-2 epitope(s) recognized by the antibody is unknown. This antibody recognized FGF-2 in steam-heated, formalin-fixed sections and in methyl Carnoy's fixed, paraffin-embedded sections.

FGFR1. Flg/C-15 (Santa Cruz Biotechnology) is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 808 to 822 mapping within the intracellular carboxy terminal region of human FGFR1. The antibody recognizes FGFR1 in methyl Carnoy's fixed, paraffin-embedded sections. The antibody is nonreactive with FGFR2 (bek), FGFR3, or FGFR4, as determined by Western blotting against the purified proteins corresponding to these receptors.

Clone VBS1 is a murine monoclonal IgM antibody raised against the extracellular ligand-binding domain of FGFR1 isolated from bovine coronary venular endothelial cells, as described [30]. The antibody was extensively characterized previously and shown to specifically immunoprecipitate FGFR1 at 120 kDa [30, 31].

Tubular markers. EMA, clone E29 (Dako, Carpenteria, CA, USA), is a mouse monoclonal IgG2a that is specific for epithelial membrane antigen [38]. It reacts with normal epithelium in a variety of tissues and has been characterized by comparative immunocytochemistry with several other polyclonal and monoclonal anti-EMA antibodies [38].

Rabbit anti–Na,K-ATPase (Upstate Biotechnology Inc., Lake Placid, NY, USA) is a whole rabbit antiserum created against the β-1 subunit of rat Na,K-ATPase [39]. It was characterized with Western blotting.

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 one hour with the primary antibody diluted in phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA). Following washes in PBS, the sections were sequentially incubated with biotinylated goat-anti-rabbit or horse-antimouse antibody (Vector Labs, Burlingame, CA, USA), the ABC-Elite reagent (Vector Labs), and finally 3,3′-diaminobenzidine (DAB; with nickel chloride enhancement) was used as the chromogen. Sections were counterstained with methyl green and were dehydrated and cover slipped. Sections of all 13 kidneys were stained with all of the antibodies. To confirm the staining patterns, all antibodies were also used for immunohistochemistry on acetone-fixed frozen tissue sections.

Double immunocytochemistry was performed to detect FGF-2 expression in combination with the tubular markers, EMA and Na,K-ATPase. Briefly, slides were incubated with either AB3 or bFGF/147, peroxidase-

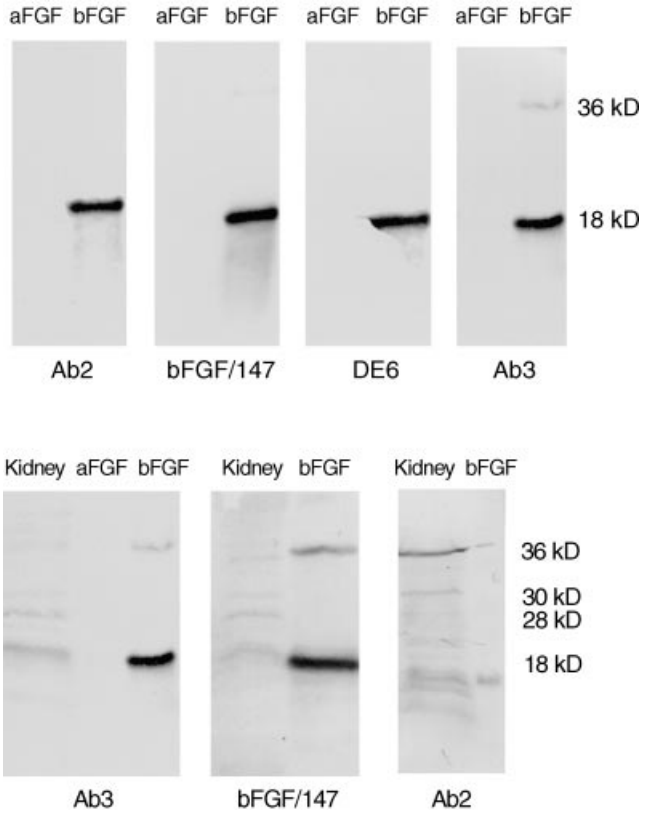

Fig. 1. Western blots show the specificity of each antiserum/antibody system employed in this study for fibroblast growth factor-2 (FGF-2; bFGF) with adjacent control lane showing absence of binding to FGF-1 (aFGF; top). The bottom three Western blots show the ability of these antibodies to recognize the appropriate FGF-2 substrates in extract of whole human kidney.

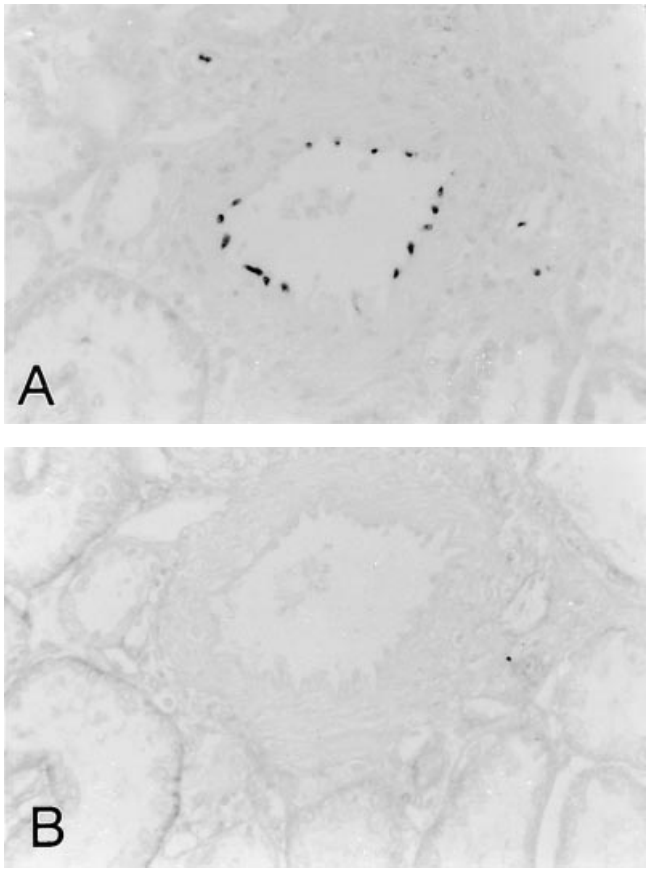

Fig. 2. Solid-phase preabsorption of anti–fibroblast growth factor-2 (FGF-2) antibody. (*A*) Section of human kidney stained with antibody Ab3, which had been preabsorbed with buffer only (**Methods** section). Positive immunostaining of arterial endothelium is evident. (*B*) The same section stained with Ab3, which had been preabsorbed with FGF-2 (**Methods** section). The immunostaining pattern present in (A) is abolished and now is confined to tubular basement membranes.

conjugated secondary antibody and were reacted with DAB to give a brown colored product. After washing, residual peroxidase blocking with hydrogen peroxide and normal serum blocking, the slides were then stained with the second primary antibody (EMA or Na,K-ATPase), biotinylated secondary antibody, ABC-HRP (Vector) and were reacted with Vector VIP peroxidase substrate kit (Vector) to give a purple reaction product.

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

In further control experiments, solid-phase preabsorption of the anti–FGF-2 antibodies was carried out by coating recombinant FGF-2 or FGF-1 (both from Oncogene Research Products) onto 96-well polystyrene plates overnight at 4°C in 0.2 M carbonate buffer (pH 9.6) at a concentration of 2.5 μg/ml. Control wells contained buffer only. Plates were then washed with PBS and blocked with 1% BSA for one hour at room temperature. Following this, one of the four anti–FGF-2 antibodies was added at a concentration of 1 μg/ml bFGF/147, 4 μg/ml (Ab2), 0.5 μg/ml (Ab3), or a 1:1000 dilution (DE6), incubated overnight at 4°C, and then employed for immunostaining. Further controls in the case of the bFGF/147 antibody included wells coated with the peptide used for immunization (amino acids 40 to 63 mapping within the amino terminal region of human FGF-2; Santa Cruz) at a concentration of 10 or 50 μg/ml. Absorbed antisera were then used for repeat immunohistochemical procedures as otherwise detailed earlier in this article.

Western blotting

To confirm the specificity of the four antibodies against FGF-2 employed for immunohistochemistry, Western blotting was performed using recombinant human FGF-2 and FGF-1 (Santa Cruz Biotechnology) and homogenates of adult human kidney. Kidney samples were prepared as previously described [8, 12]. Samples were electrophoresed on a 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and were then blotted onto 0.2 μm

Table 1. Summary of immunohistochemistry and *in situ* hybridization data on the expression of FGF-2 in mature human kidney

Cell-type or intrarenal structure	Immunohistochemistry with anti-FGF-2 antibody				*In situ* hybridization
	bFGF/147	Ab2	Ab3	DE6	
Arterial endothelial cells	+	±	+	+	+
Vascular smooth muscle cells	+	±	±	+	−
Neointimal smooth muscle cells	+	±	±	+	−
Vascular adventitial cells	−	−	−	+	−
Venous endothelium	−	−	±	+	−
Peritubular endothelium	±	−	±	+	−
Cells within glomerular tuft	+	±	±	+	−
Glomerular basement membrane	−	−	−	−	NA
Podocytes	±	−	−	±	−
Parietal glomerular epithelial cells	+	+	+	+	±
Tubules	+[a]	+[a]	+[a]	+[a]	+[a]
Interstitial cells	±	±	±	+	±
Tubular basement membrane, interstitial matrix	±	−	−	±	NA
Interstitial infiltrates	−	−	−	±	−

Symbols are: (+) positive immunostaining or expression of mRNA; (±) positive immunostaining or expression of mRNA under some circumstances or staining conditions only (see text); (−) absent immunostaining or mRNA expression. NA is not applicable.

[a] See text for further details

nitrocellulose membranes. The blots were blocked with 5% BSA in PBS for one hour and then incubated with primary antibody or an equal concentration of normal rabbit IgG or monoclonal mouse IgG_1 (Dako) diluted in Tris-buffered saline containing 1% BSA for two hours at room temperature. After washing, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG or goat antimouse antibody (Dako) for one hour. The blots were then visualized with 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium (BCIP-NBT).

Molecular probes

The following probes were used: (*a*) a 1300 bp cDNA corresponding to nucleotides 435 to 1735 of human FGF-2 cloned into pKS+ Bluescript (kindly provided by I. Drummond, Charlestown, MA, USA); (*b*) pCD115, a 1200 bp *flg* cDNA corresponding to the region encoding the entire extracellular domain, cloned into the pGEM 1 plasmid (kindly provided by Volkard Lindner, Department of Pathology, University of Washington, Seattle, WA, USA); and (*c*) a 4 kb cDNA encoding the full-length sequence of human FGFR1β ligated into the PCR-2 plasmid. All constructs were linearized and transcribed into an antisense or sense riboprobe, as described previously [34]. Briefly, the transcription reaction mixture contained 1 μg cDNA (sense or antisense), [^{35}S]-uridine triphosphate ([^{35}S]-UTP), adenosine triphosphate (ATP), cytidine 5′-triphosphate (CTP) and guanosine 5′-triphosphate (GTP), RNAsin, dithiothreitol, Tris, and either SP6 or T7 polymerase. After 75 minutes, the template DNA was digested by adding DNAse and incubating at 37°C for an additional 15 minutes. Free nucleotides were separated with a Sephadex G-50 column. The collected fraction containing labeled probe was then ethanol precipitated, resuspended in nuclease-free water containing 10 mmol dithiothreitol, and stored at −20°C. Probes were used within 48 hours.

Pilot studies were performed with the *flg* and FGFR1β probes in which the ^{35}S-UTP–labeled probes were treated to limited alkaline hydrolysis in order to generate fragments of approximately 200 bp. The hydrolyzed and full-length transcripts were used on replicate sections. The *in situ* hybridization signal was equivalent with both probes, and the full-length probes were used in subsequent experiments to maintain high hybridization stringency.

In situ hybridization

Kidney tissues, which were fixed in 10% formalin and embedded in paraffin, were deparaffinized following standard protocol. *In situ* hybridization was then performed as described previously [32, 33]. Briefly, the sections were washed with 0.5 × standard saline citrate (SSC) and digested with proteinase K (1 μg/ml; Sigma Chemical Co., St. Louis, MO, USA) in Tris buffer. Several 0.5 × SSC washes were followed by prehybridization for two hours. 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, washed in 2 × SSC, followed by three high-stringency washes in 0.1 × SSC/Tween 20 (Sigma) at 50°C, and several 2 × SSC washes. After the tissue was dehydrated and air dried, it was dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA) and exposed in the dark at 4°C for two weeks. 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.

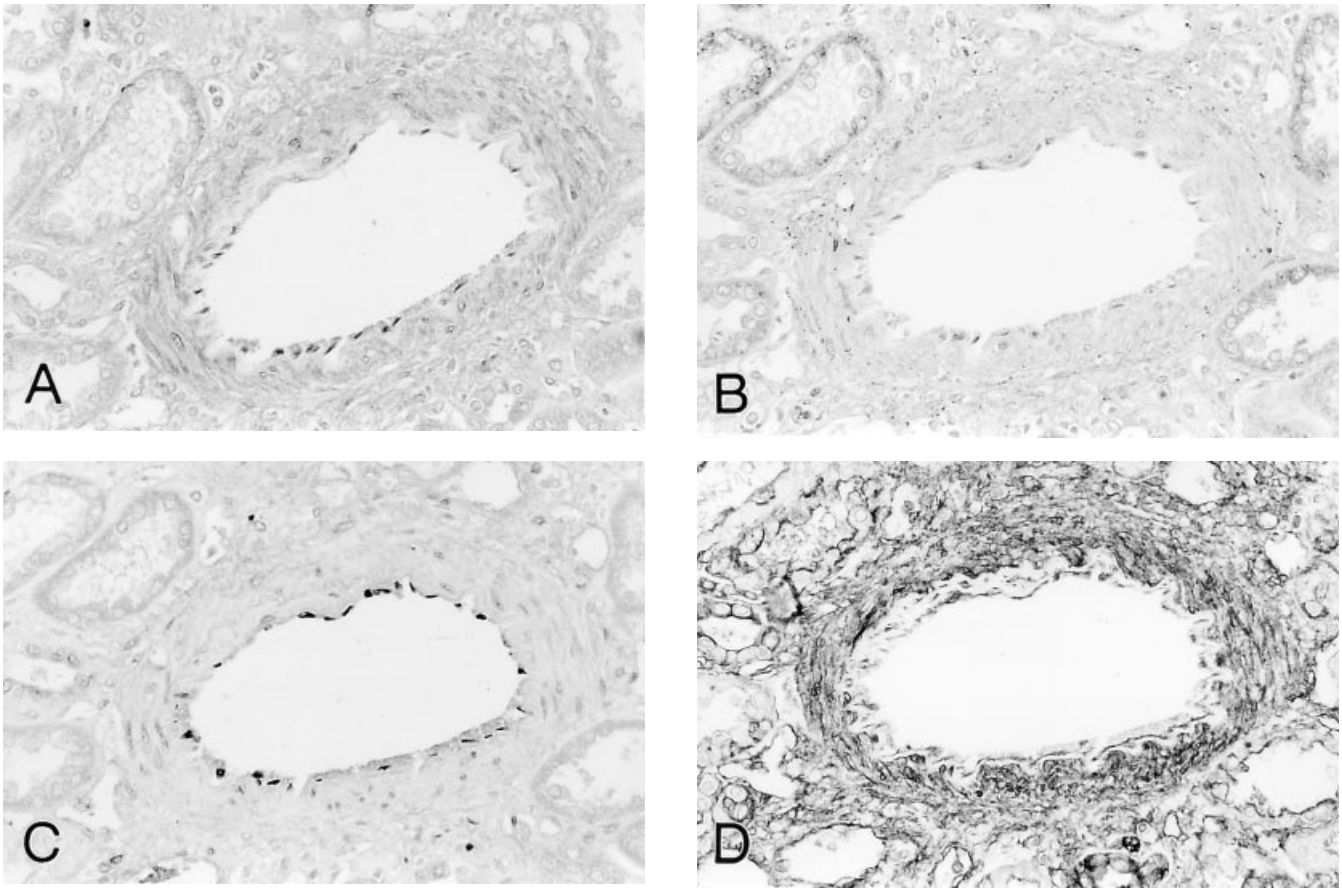

Fig. 3. Vascular immunostaining for fibroblast growth factor-2 (FGF-2). Serial sections of a small cortical artery stained with the four anti–FGF-2 antibodies. (*A*) Polyclonal antibody bFGF/147. Positive immunostaining of endothelial cells in a nuclear pattern, as well as weak staining of medial smooth muscle cells, is present. (*B*) Polyclonal antibody Ab2. Weak staining of endothelial cells in a nuclear pattern as well as staining of tubular cells is noted. (*C*) Monoclonal antibody Ab3. Strong nuclear staining of endothelial cells is present. (*D*) Monoclonal antibody DE6. Positive immunostaining of endothelial cells, medial smooth muscle cells, adventitial cells, interstitial cells, and tubular cells is present in a cytoplasmic pattern.

RESULTS

Morphological findings in adult human kidneys

By conventional light microscopy, the adult kidney specimens were, for the most part, morphologically normal, although focal areas demonstrating variable degrees of focal and segmental glomerulosclerosis, tubular atrophy, interstitial fibrosis, and arteriosclerotic changes were present. These changes were completely consistent with nephrosclerotic changes of aging and were frequently concentrated in the immediate subcapsular cortex. None of these changes were extensive or indicative of a superimposed primary disease process.

Characterization of anti–FGF-2 antibodies

All four antibodies were used in Western blotting experiments to detect recombinant FGF-1 and recombinant FGF-2 (Fig. 1). Additionally, the Ab2, Ab3, and bFGF/147 antibodies were used in Western blotting experiments using lysates derived from adult kidney cortex (the DE6 antibody has previously been characterized extensively in kidney homogenates; Fig. 1) [8]. Each of the antibodies recognized recombinant FGF-2, with a major band detected at approximately 18 kDa and a second weaker band at approximately 36 kDa. None of the antibodies showed cross-reactivity with FGF-1. In addition, the three commercially available antibodies recognized several bands in the tissue lysates with approximate molecular weights of 18, 28, 30, and 36 kDa. The appearance of multiple FGF-2 variants at these approximate molecular weights has been previously reported in both human tissue [34] and cultured cells [12, 35], and is consistent with the presence of several FGF-2 isoforms.

In further studies, we investigated the effect of an incubation of the bFGF/147 or Ab2 antibody with an excess of recombinant human FGF-2 prior to immunohistochemistry. Similar to previous reports [36, 37], this did not abolish the positive immunostaining with the antibodies but rather resulted in prominent staining of tubular basement membranes and adjacent interstitial matrix (data not shown).

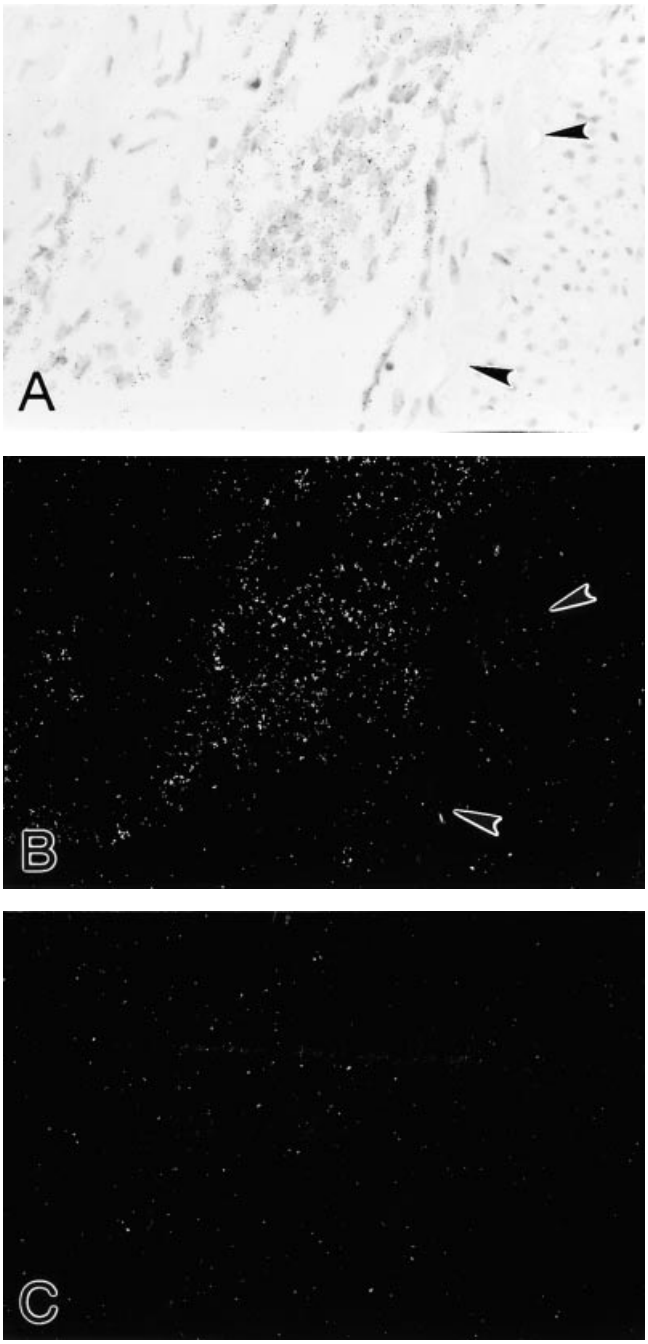

Fig. 4. Vascular expression of fibroblast growth factor-2 (FGF-2) mRNA. Tangential section of an arcuate artery probed with antisense probe (bright field: *A*; dark field: *B*), as well as with a sense probe (*C*). The hybridization signal is confined to the endothelial layer, whereas the signal over medial smooth muscle cells is indistinguishable from background. Arrowheads indicate internal elastic membrane.

Given this observation, we also carried out solid-phase preabsorption experiments with the four anti–FGF-2 antibodies. In these experiments, endothelial, tubular, and glomerular cell staining was completely abolished when the Ab3 monoclonal anti–FGF-2 antibody was incubated with immobilized complete FGF-2 (Fig. 2). Immunostaining with the Ab2 and DE6 antibodies was moderately reduced but not abolished following incubation with immobilized complete FGF-2 (data not shown). Immunostaining with the bFGF/147 was reduced in samples that were incubated with the immobilized immunizing peptide (partial FGF-2; data not shown). For all antibodies, a control incubation with immobilized FGF-1 instead of FGF-2 yielded a staining pattern that was similar to that observed with nonabsorbed antibody to FGF-2 (data not shown). Furthermore, in most instances, some enhancement of tubular basement membrane staining was noted in those samples that had been incubated with immobilized FGF-2 (Fig. 2). This suggests that, analogous to the experiments in which antibody was incubated with excess FGF-2 (discussed earlier in this article), some leakage of the immobilized antigen may have occurred, which resulted in subsequent binding of the FGF-2/anti–FGF-2 antibody complex to the sections.

FGF-2 expression in adult human kidneys

Immunohistochemical findings and the findings of *in situ* hybridization in the kidneys are summarized in Table 1. The usage of the four different antibodies against FGF-2 yielded staining patterns that were relatively similar, but some important differences evolved with respect to tissue structures exhibiting positivity, as well as to the intracellular localization of immunoreactive FGF-2 (that is, cytoplasmic stain vs. nuclear stain; Table 1). No qualitative differences in localization patterns were obtained when frozen, cryostat tissue sections were used in place of fixed tissue sections for the immunohistochemical procedures.

Vasculature. Arterial endothelial cells consistently expressed FGF-2 protein with all four antibodies used (Fig. 3). Except for the DE6 antibody, which yielded a cytoplasmic signal, all other antibodies localized detectable FGF-2 to the nuclei of the endothelial cells (Fig. 3). Compared with endothelial cells, the staining intensity in vascular smooth muscle cells of the vascular media was weaker, but in many instances, positive immunostaining of variable degrees was observed (Fig. 3). Adventitial cells only exhibited immunoreactivity with the DE6 antibody (Fig. 3D). In atherosclerotic vessels, FGF-2 immunoreactivity of variable degrees was noted in vascular smooth muscle cells of the neointima with all four antibodies. By *in situ* hybridization, FGF-2 mRNA was detectable in endothelial cells but not in medial or neointimal smooth muscle cells nor adventitial cells of renal arteries (Fig. 4).

Compared with arterial endothelial cells, the degree of detectable immunostaining was lower in endothelial cells of peritubular capillaries and veins, where it ranged from absent to focally detectable with the four antibodies employed (Figs. 5 and 7). *In situ* hybridization also failed

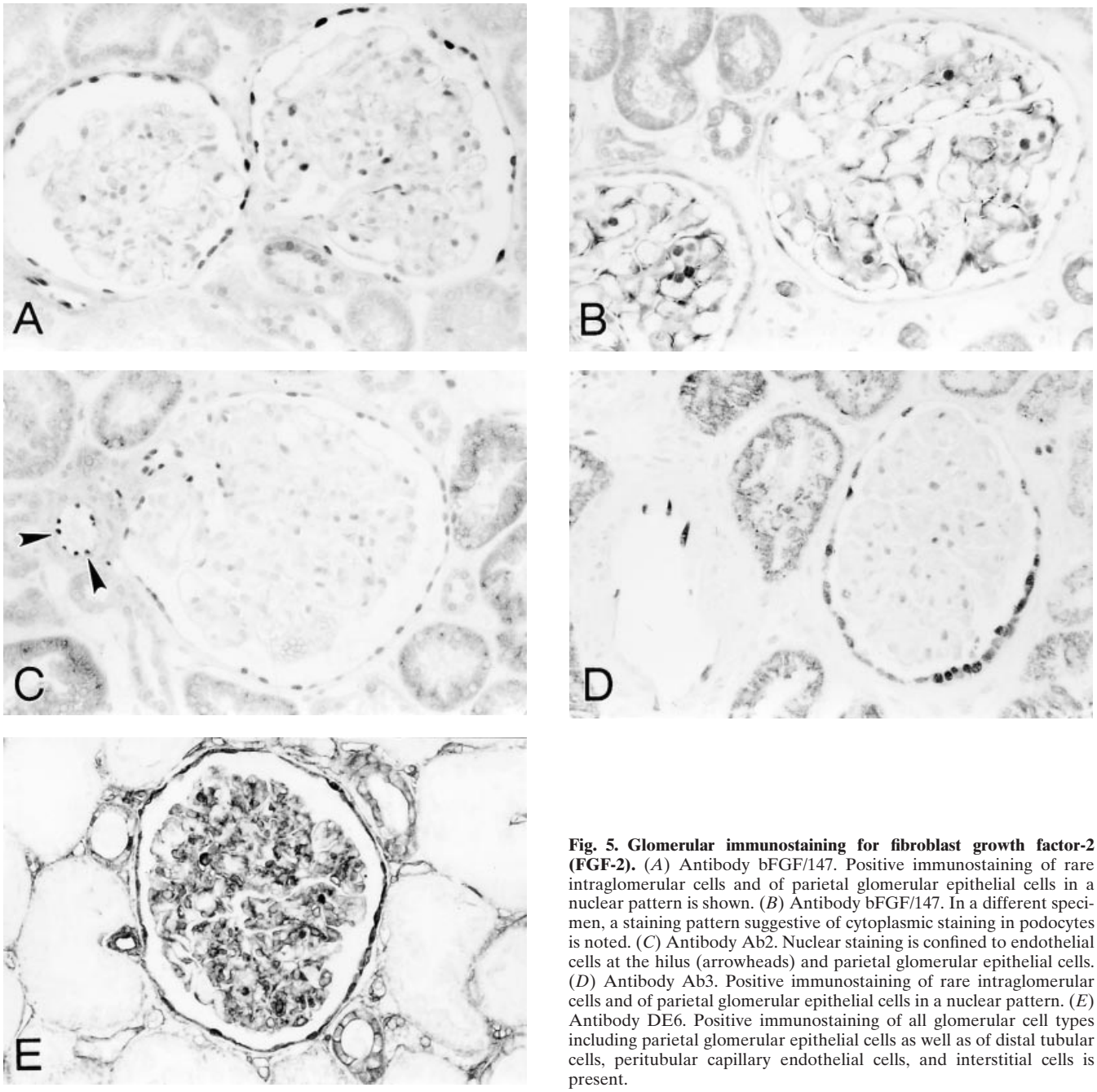

Fig. 5. Glomerular immunostaining for fibroblast growth factor-2 (FGF-2). (*A*) Antibody bFGF/147. Positive immunostaining of rare intraglomerular cells and of parietal glomerular epithelial cells in a nuclear pattern is shown. (*B*) Antibody bFGF/147. In a different specimen, a staining pattern suggestive of cytoplasmic staining in podocytes is noted. (*C*) Antibody Ab2. Nuclear staining is confined to endothelial cells at the hilus (arrowheads) and parietal glomerular epithelial cells. (*D*) Antibody Ab3. Positive immunostaining of rare intraglomerular cells and of parietal glomerular epithelial cells in a nuclear pattern. (*E*) Antibody DE6. Positive immunostaining of all glomerular cell types including parietal glomerular epithelial cells as well as of distal tubular cells, peritubular capillary endothelial cells, and interstitial cells is present.

to detect FGF-2 mRNA expression in capillary or venous endothelium.

Glomeruli. Within the glomerular tuft, that is, within mesangial and endothelial cells as well as leukocytes of adult kidneys, a variable number of cells displayed immunoreactivity with all four antibodies tested (Fig. 5). Whereas in some cases, the infrequency of the cells suggested labeling of circulating or infiltrating leukocytes (Fig. 5A), it was more widespread with the DE6 antibody and consistent with FGF-2 expression in mesangial and endothelial cells (Fig. 5E). No staining of the glomerular basement membrane was noted. Staining of podocytes was inconsistently present with the polyclonal bFGF/147 and the monoclonal DE6 antibody, but rare to absent with the two other antibodies (Fig. 5). In contrast, parietal glomerular epithelial cells stained positively using all four antibodies, albeit with patterns that ranged from cytoplasmic to nuclear localization (Fig. 5). By *in situ* hybridization, FGF-2 mRNA was detectable in occasional parietal glomerular epithelial cells only (Fig. 6).

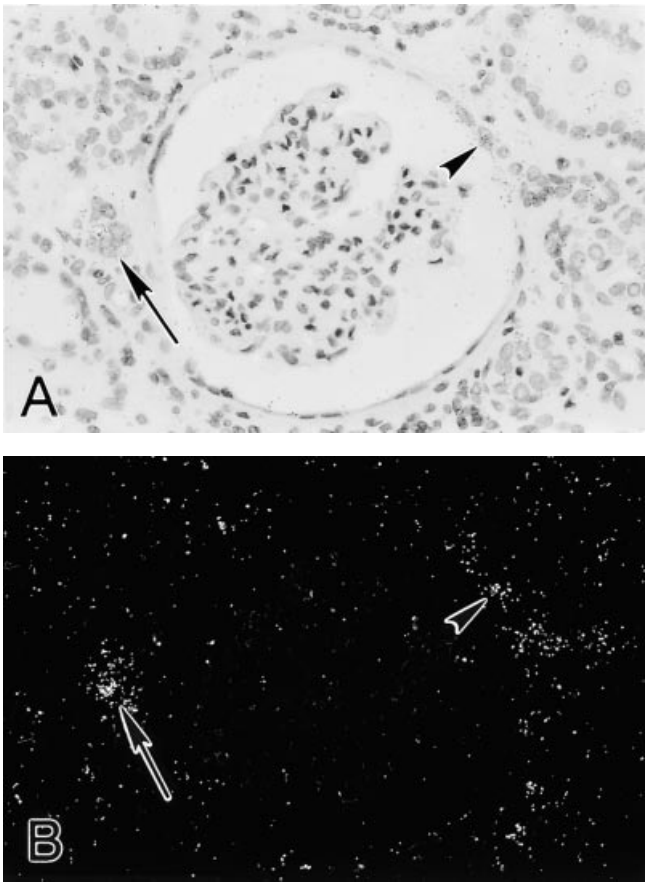

Fig. 6. Glomerular *in situ* hybridization for fibroblast growth factor-2 (FGF-2) mRNA. (*A*) Light and (*B*) dark field image. Hybridization signal is indistinguishable from background within the glomerular tuft but is present in rare glomerular parietal epithelial cells (arrowheads), as well as distal tubular cells (arrows).

FGF-2 protein and mRNA expression was markedly reduced in sclerotic glomeruli (data not shown).

Tubules. By immunohistochemistry using the four antibodies, tubules consistently expressed FGF-2 in adult kidneys (Figs. 5 and 7). *In situ* hybridization also confirmed the presence of FGF-2 synthesis in tubular cells (Fig. 8). A more detailed analysis of the tubular segments was based on morphological criteria to identify tubular segments. Proximal tubules were identified in the cortex by their prominent brush border membrane, distal tubules by their more cuboidal and smaller cells, as well as a less prominent brush border membrane and collecting ducts by the absence of a brush border membrane and columnar cells. Based on these criteria, the detailed analysis revealed that particularly the epithelium of distal tubules, including the macula densa, as well as collecting ducts, exhibited positive immunolocalization (Fig. 7). Staining patterns within individual cells again were variable and ranged from cytoplasmic staining (Fig. 7C) to exclusively nuclear staining, especially in the case of staining with the AB3 monoclonal antibody (Fig. 7 A, B).

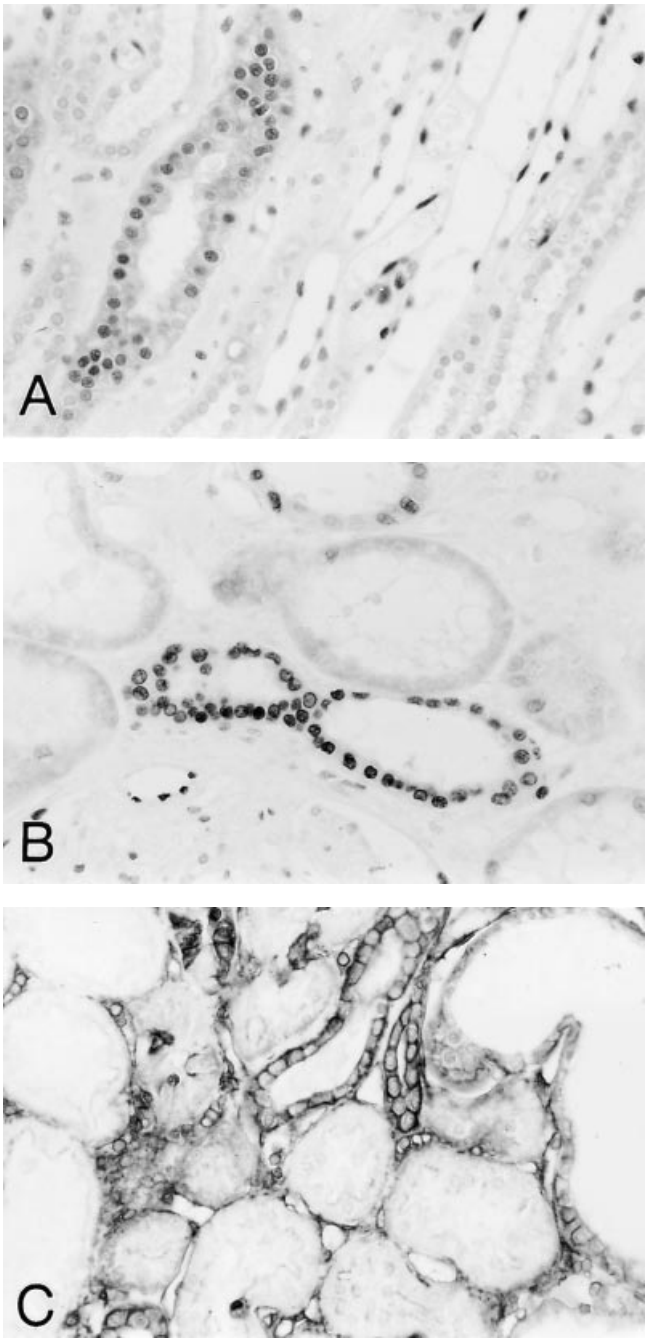

Fig. 7. Tubular immunostaining for fibroblast growth factor-2 (FGF-2). (*A*) Antibody bFGF/147. Medulla. Positive immunostaining of collecting duct cells as well as of peritubular capillary endothelial cells in a nuclear pattern is present. (*B*) Antibody Ab3. Cortex. Positive immunostaining of cells of the macula densa in a nuclear pattern. (*C*) Antibody DE6. Cortex. Staining is present in cells of distal but not proximal tubules, a dilated distal tubule as well as in interstitial cells and peritubular capillary endothelial cells.

To confirm the localization of FGF-2 in specific tubular segments, double immunohistochemistry was performed with Ab3 and bFGF/147 in combination with markers of distal tubules such as EMA and Na,K-ATPase (Fig. 9).

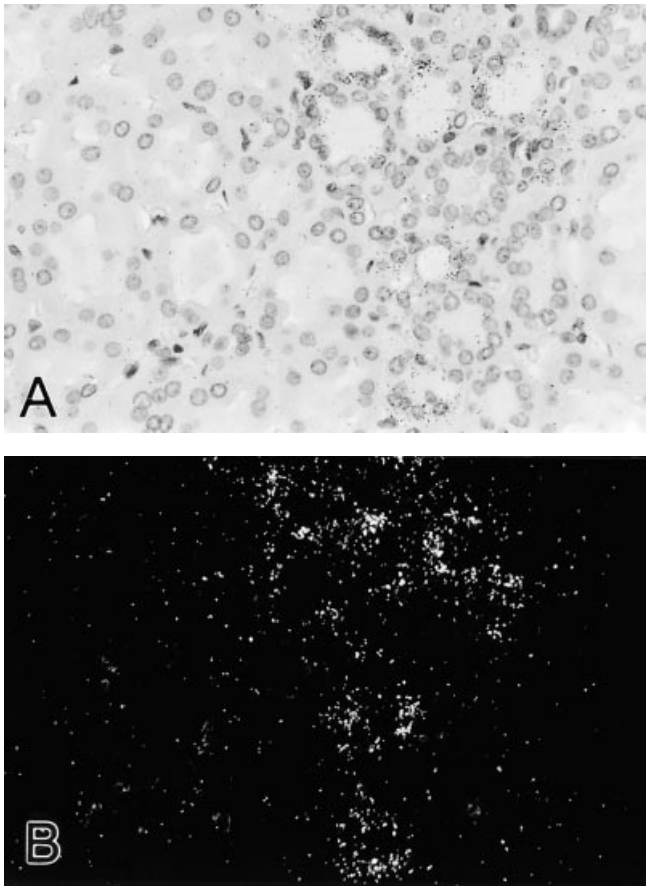

Fig. 8. Tubular *in situ* hybridization for fibroblast growth factor-2 (FGF-2) mRNA. (*A*) Light and (*B*) dark field image. Corticomedullary junction. Hybridization signal is present in distal tubules and collecting ducts but not in proximal tubular cells or interstitial cells.

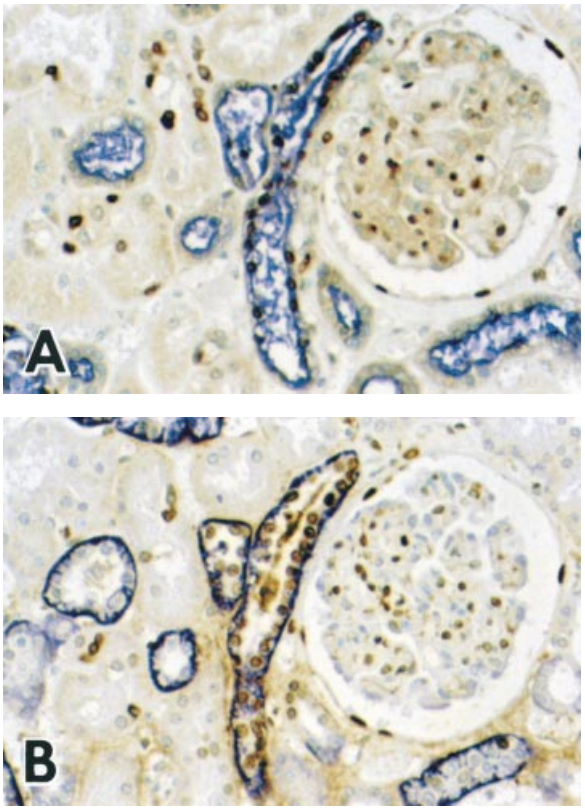

Fig. 9. Double immunohistochemistry. (*A*) bFGF-147 (brown) and EMA (purple) colocalize in distal tubules. (*B*) In a replicate tissue section, Ab3 (brown) and Na,K-ATPase (purple) also colocalize in distal tubules.

These experiments demonstrated colocalization of the markers with FGF-2 and thus confirm that FGF-2 is primarily expressed in cells of the distal tubule.

Interstitium. Using the DE6 monoclonal antibody against FGF-2, positive immunostaining was detectable in the interstitium of adult kidneys (Figs. 3D, 5E, and 7C.). Reactivity was weak to absent when the other antibodies to FGF-2 were employed, and only rare cells, which often appeared to represent peritubular capillary endothelial cells, exhibited signal. Staining of the extracellular matrix and/or the tubular basement membrane was occasionally observed, particularly in areas with interstitial fibrosis, using the bFGF/147 and DE6 antibodies (Fig. 10). In focal interstitial infiltrates, the majority of mononuclear cells failed to exhibit positive immunostaining with three of the four anti–FGF-2 antibodies, whereas the DE6 antibody occasionally yielded positive staining in a high percentage of infiltrating cells (data not shown). By *in situ* hybridization, only very rare interstitial cells contained FGF-2 mRNA (data not shown). Because of the rarity of such cells and technical problems, these cells could not be phenotyped. Their rarity, however, would suggest that they did not represent intrinsic renal cells, but rather infiltrating or circulating leukocytes. As in the case of immunohistochemistry, the

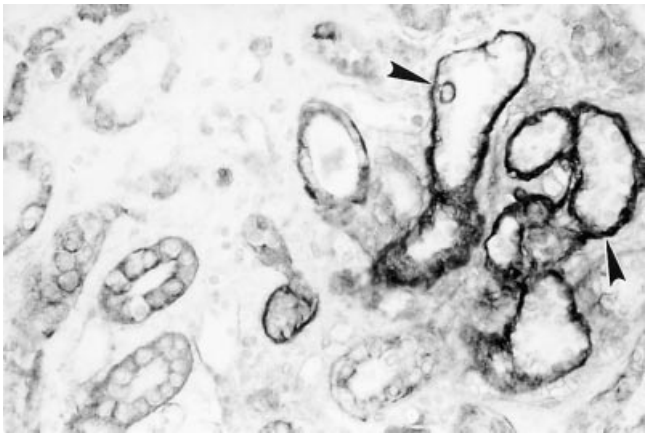

Fig. 10. Interstitial immunostaining for fibroblast growth factor-2 (FGF-2) in renal interstitial fibrosis. Antibody bFGF/147. In an area of cortical fibrosis, immunoreactivity is present in distal tubular cells and also in the extracellular matrix, particularly the tubular basement membrane of some tubules (arrowheads).

Table 2. Summary of immunohistochemistry and *in situ* hybridization data on the expression of FGFR1 in mature human kidney

Cell-type or intrarenal structure	Immunohistochemistry with anti-FGFR1 antibody		*In situ* hybridization with probe	
	VBS1	Flg/C-15	FGFR1β	*flg*
Arterial endothelium	−	−	−	−
Vascular smooth muscle cells	±	±	+	+
Neointimal smooth muscle cells	±	±	+	+
Vascular adventitial cells	−	−	−	−
Venous endothelium	−	−	−	−
Peritubular endothelium	−	−	−	−
Cells within glomerular tuft	±	±	+	+
Glomerular basement membrane	−	−	NA	NA
Podocytes	−	−	−	−
Parietal glomerular epithelial cells	−	±	±	±
Tubules	+[a]	+[a]	+[a]	+[a]
Interstitial cells	±	±	±	±
Tubular basement membrane, interstitial matrix	−	−	NA	NA
Interstitial infiltrates	±	±	±	±

Symbols are: (+) positive immunostaining or expression of mRNA; (±) positive immunostaining or expression of mRNA under some circumstances or staining conditions only (see text); (−) absent immunostaining or mRNA expression. NA is not applicable.

[a] See text for further details

majority of mononuclear cells in interstitial infiltrates did not exhibit detectable amounts of FGF-2 mRNA.

FGFR1 expression in adult kidneys

Immunohistochemical findings and the findings of *in situ* hybridization in the kidneys are summarized in Table 2.

Vasculature. Within the arterial vasculature, endothelial cells failed to express FGFR1 in adult kidneys with both the polyclonal Flg/C-15 and monoclonal VBS1 antibody (Fig. 11 A, B). By *in situ* hybridization with either of the two FGFR1 probes, most arterial endothelial cells also did not appear to express mRNA (Fig. 11 C, D), but the expression of FGFR1 mRNA in adjacent smooth muscle cells at times rendered the definitive localization of grains to specific cell types difficult. Smooth muscle cells stained positively to a variable degree for FGFR1 protein using the two anti-FGFR1 antibodies. Furthermore, both FGFR1 mRNA and protein were detectable in neointimal lesions in atherosclerotic vessels (Fig. 11 B–D). Vascular adventitia failed to express FGFR1 protein or mRNA.

Capillary and venous endothelium did not express FGFR1 mRNA or protein in normal kidneys.

Glomeruli. By immunohistochemistry, most glomerular cells were unreactive with both of the anti-FGFR1 antibodies (Figs. 11A and 12A). Frequently, however, a few individual cells within the glomerular tuft stained positively for FGFR1 (Figs. 11A and 12A). These latter cells often localized to capillary lumina, suggesting that they represented circulating leukocytes. In contrast to the immunohistochemical findings, *in situ* hybridization with both cDNAs for FGFR1 demonstrated mRNA expression in cells of the glomerular tuft (Fig. 12 B, C). The signal was usually not detectable at the edge of the glomerular tuft (Fig. 12 B, C), suggesting that the mRNA expression was mostly derived from mesangial and/or glomerular endothelial cells and/or infiltrating/circulating leukocytes. Parietal glomerular epithelial cells occasionally stained positively with the Flg/C15 antibody (Fig. 12D) and expressed FGFR1 mRNA (Fig. 12 B, C), whereas no signal was obtained over Bowman's capsule with the VBS1 antibody.

Tubules. Immunohistochemistry with the two anti-FGFR1 antibodies as well as *in situ* hybridization with the two cDNAs consistently demonstrated FGFR1 protein and mRNA expression in tubular cells. Most of the tubular segments were identified as loops of Henle, distal tubules, and collecting ducts (Figs. 11A, 12 A–C, and 13). The immunohistochemical staining pattern within the positive tubular segments was not uniform, and staining of individual cells ranged from negative to staining of the cell membrane to diffuse cytoplasmic stain (Fig. 13). In the absence of specific immunohistochemical markers of the collecting duct cells, it was not possible to unequivocally assign FGF-1 expression to either intercalated or principal cells.

Interstitium. By immunohistochemistry, rare cells in the interstitium of normal kidneys stained positively for FGFR1 using either antibody (Fig. 12D). No staining of the extracellular matrix or tubular basement membrane was noted. No enhanced staining was noted in areas of interstitial fibrosis. Most leukocytes in focal infiltrates also failed to express FGFR1 protein, but in some cells, strong staining was noted. *In situ* hybridization in normal kidneys demonstrated strong FGFR1 mRNA expression in some cells of the interstitium, but no enhanced mRNA expression in areas of interstitial fibrosis (Fig. 12 B–C). As in the case of immunohistochemistry, however, most cells in mononuclear infiltrates failed to exhibit FGFR1 mRNA expression, again suggesting that either a subfraction of leukocytes and/or rare intrinsic interstitial cells express FGFR1 mRNA.

DISCUSSION

Using an approach that combines multiple antibodies for immunohistochemistry and *in situ* hybridization, we demonstrate intrarenal expression of both FGF-2 and FGFR1 under all conditions employed. This study is noteworthy for the heterogeneity of some of the findings. This contrasts with previous studies on other cytokines and their receptors in human kidney, in which we have used the same approach and obtained consistent protein and mRNA data [29, 32, 33, 38, 39]. In the case of FGF-2,

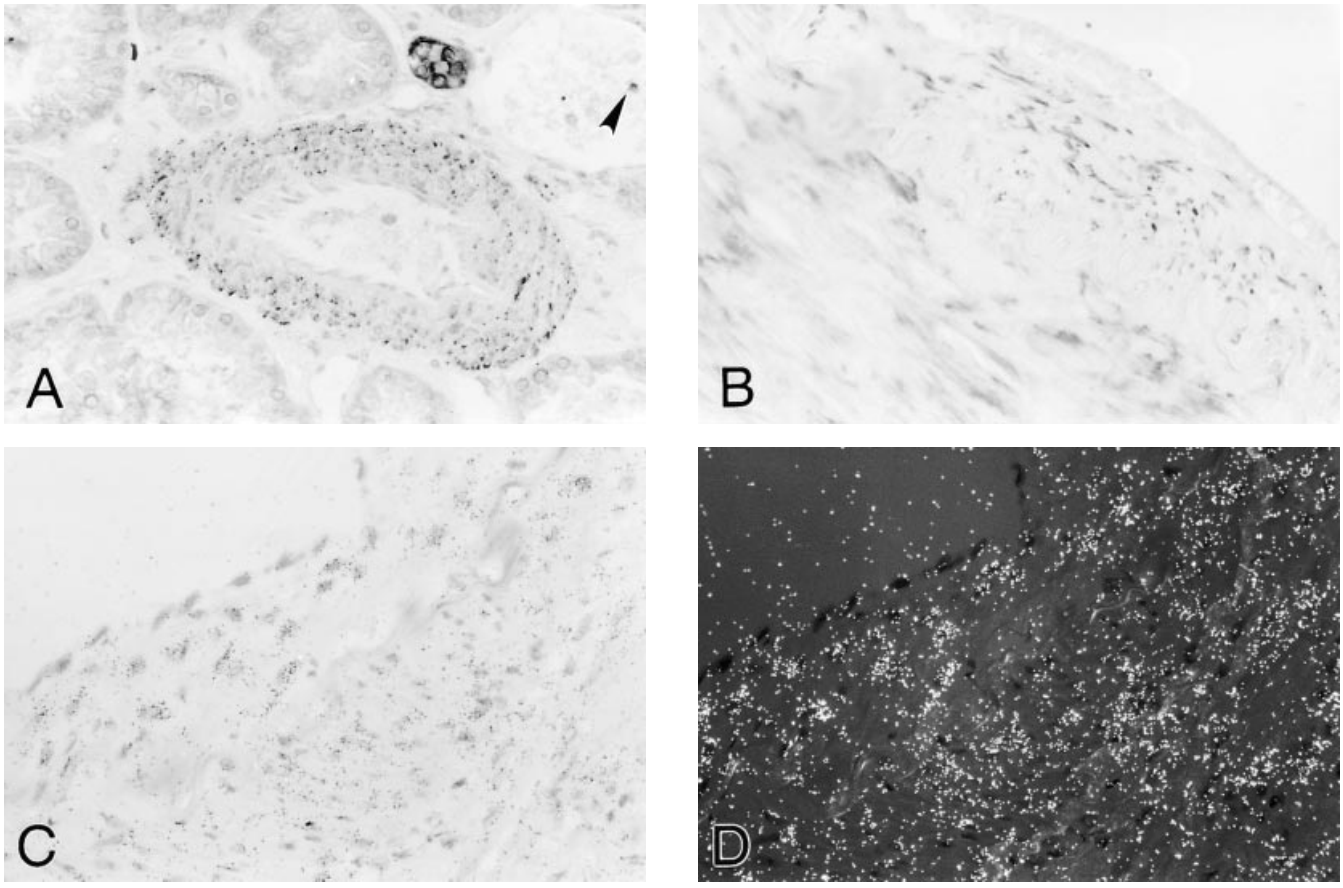

Fig. 11. Renal vascular expression of fibroblast growth factor receptor-1 (FGFR1) protein and mRNA. (*A*) Adult cortex, polyclonal antibody Flg/C-15. FGFR1 is expressed in smooth muscle cells of the vascular media as well as in cells of a distal tubule and a single cell within the glomerular tuft (arrowhead). (*B*) Artherosclerotic vascular damage with neointima formation, stained with monoclonal antibody VBS1. FGFR1 is expressed in vascular smooth muscle cells of both media and neointima but not in endothelial cells. (*C and D*) Arcuate artery with moderate neointima formation; *in situ* hybridization using the FGFR1β cDNA, bright and dark field image. FGFR1 mRNA localizes to smooth muscle cells of the media and neointima, but not to endothelial cells.

it has been reported that fixation conditions have a major influence on the apparent localization of the cytokine within rat kidney [35] and also on the apparent intracellular localization of FGF-2, that is, localization to nuclear versus cytoplasmic compartments [40, 41]. Although differences in fixatives may account for some of the discrepancies between our *in situ* hybridization data and immunostaining results, all material for immunohistology was fixed and processed in the same manner. Consequently, the apparent variability, particularly in the case of FGF-2 expression, must be attributed to differences among the antibodies employed for immunostaining.

To demonstrate that the different staining patterns with the FGF-2 antibodies were specific, several control experiments were performed. Similar to previous reports [36, 37], we noted that it was not possible to demonstrate specificity of the immunostaining pattern by preabsorption of the antibodies with excess antigen. Rather, in this case, there is binding of some of the exogenous FGF-2 used in the absorption procedures to HSPGs within basement membranes and the extracellular matrix [37]. This bound exogenous FGF-2 is then labeled in the subsequent tissue immunohistochemical procedures. However, using Western blots, as well as absorption of the antibodies to a solid phase, we demonstrate that all four antibodies react specifically with recombinant FGF-2 and do not cross-react with FGF-1. We therefore conclude that the different expression patterns of FGF-2 may result from the complexity of the FGF-2/FGFR1 system, particularly its multiple isoforms, and the resulting differences in antibody specificities [42]. Furthermore, in the case of FGF-2, it has been described that mRNA expression is much less widespread in comparison to protein expression, which reflects the fact that FGF-2 is mostly stored in the cytoplasm and/or extracellular matrix and synthesized only on demand [43]. Similar considerations also apply to FGFR1, where again, the occurrence of different splice variants may result in apparently different staining patterns.

Within glomeruli, the demonstration of FGF-2 protein

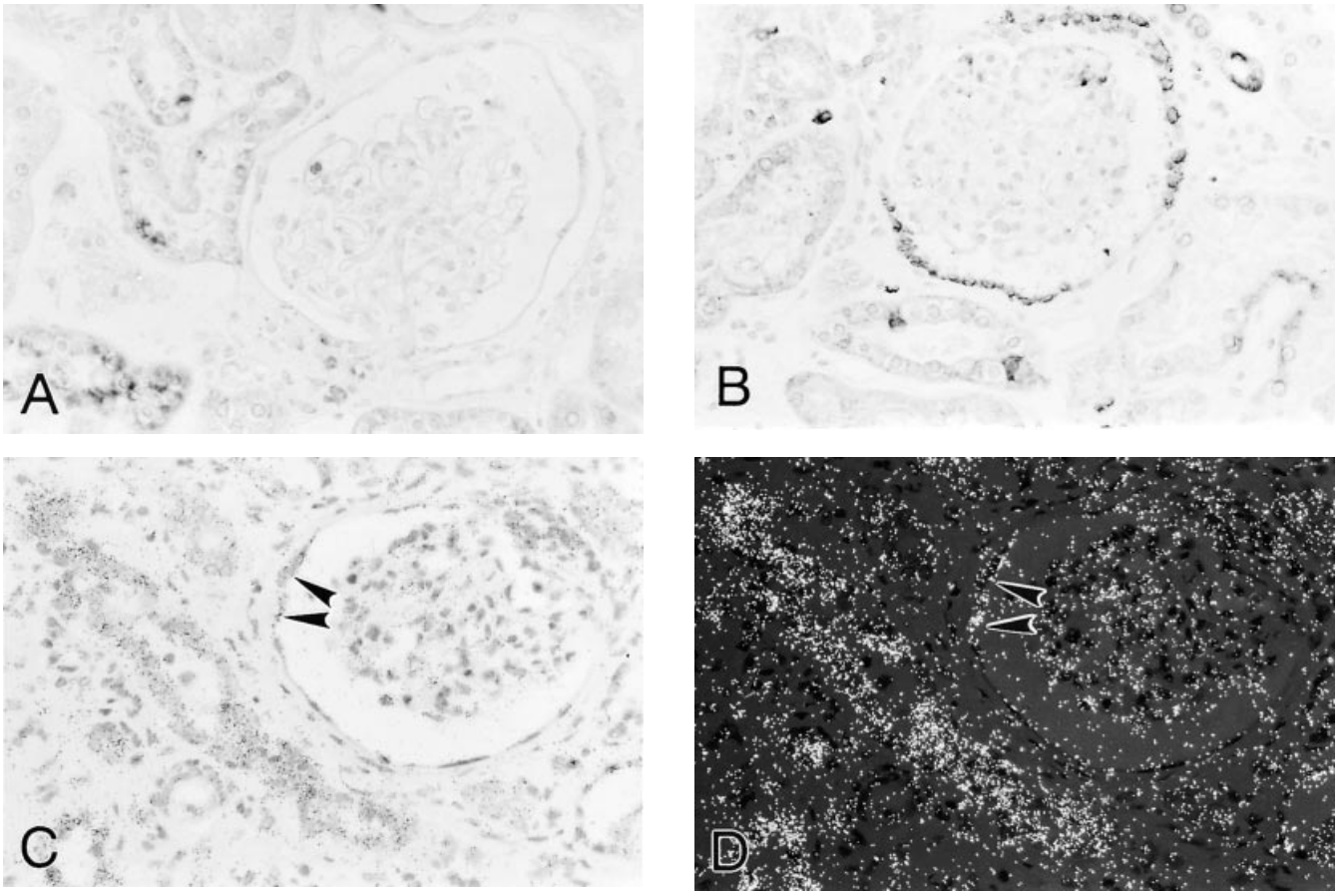

Fig. 12. Glomerular expression of fibroblast growth factor receptor-1 (FGFR1) protein and mRNA. (*A*) Adult cortex, monoclonal antibody VBS1. FGFR1 is expressed in a single cell within a lumen of the glomerular tuft as well as in some cells of distal tubules. (*B*) Renal cortex, polyclonal antibody Flg/C-15. FGFR1 is expressed in parietal glomerular epithelial cells, rare cells within the glomerular tuft, some distal tubular cells, as well as in rare interstitial cells. (*C*) Light and (*D*) dark field image. Renal cortex with moderate interstitial fibrosis; *in situ* hybridization with the FGFR1β cDNA. FGFR1 mRNA is present within cells of the glomerular tuft, rare parietal glomerular epithelial cells (arrowheads), and cells of distal tubules.

in a typical mesangial pattern was exceptional in this study. This contrasts with findings in rats, where, using comparable conditions (methyl Carnoy's fixed, paraffin-embedded tissue, DE6 monoclonal antibody), we noted prominent expression of FGF-2 in mesangial cells *in vivo* [11]. Rather, in the present study, occasional visceral and in particular parietal glomerular epithelial cells were most consistently positive for FGF-2. Similarly, Stein-Oakley et al, using a different anti–FGF-2 monoclonal antibody on cryostat, paraformaldehyde-fixed sections, described FGF-2 expression in nuclei of parietal glomerular epithelial cells in normal human kidney [25]. In contrast to FGF-2, the expression of FGFR1 in glomeruli in this study was notable for the relative abundance of mRNA transcripts but low intraglomerular expression of FGFR1 protein. Because our anti-FGFR1 antibodies were directed against different portions of the FGFR1, the predominance of a particular splice variant is unlikely to underlie this observation. Rather, our data suggest low translational efficacy in the case of FGFR1. This assumption is consistent with the literature, in which Ford et al describe prominent expression of FGFR1 mRNA (with low to absent expression of FGFR2, FGFR3, and FGFR4 mRNA) in normal rat glomeruli [44], whereas we and others have reported absent or only minimal FGFR1 protein expression in both normal rat and human glomeruli [8, 23, 28].

Tubular cells, mainly of the distal nephron (distal tubules, collecting ducts), were one of the two sites in which we consistently detected FGF-2 mRNA and protein under all experimental conditions. Previously, Hughes and Hall, using a polyclonal anti–FGF-2 antibody (Oncogene Science, NY, USA) in formalin-fixed tissues, as well as Stein-Oakley et al also described tubular FGF-2 protein expression without commenting on the specific localization [23, 25]. However, in the rat, a detailed study by Cauchi et al using various fixation conditions and two polyclonal anti–FGF-2 antibodies is consistent with our

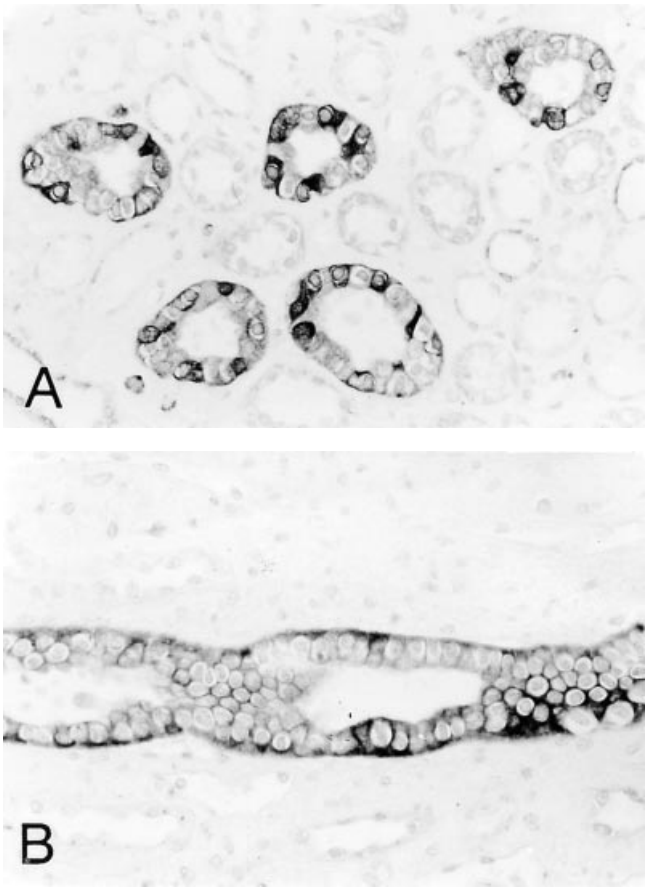

Fig. 13. Tubular expression of fibroblast growth factor receptor-1 (FGFR1) protein. (*A*) Adult medulla, polyclonal antibody Flg/C-15. FGFR1 is expressed in some cells of collecting ducts and some cells of loops of Henle. (*B*) Normal medulla, monoclonal antibody VBS. FGFR1 is expressed to a variable degree in cells of a collecting duct.

observations in humans, in that FGF-2 immunoreactivity was described in the S3 segments of proximal tubules, thick ascending limbs of the loop of Henle, distal tubules, and collecting ducts [36]. Our parallel demonstration of high-level FGFR1 protein and mRNA expression in some, but not all, cells of the distal nephron suggests that FGF-2 may have an important physiological autocrine or paracrine role in the maintenance of tubular and/or peritubular functions. The pathophysiological relevance of FGFR1 expression in these sites is indicated by studies in which chronic, high-dose administration of FGF-2 to rats or cynomolgus monkeys induced tubular dilation and cast formation [45]. It is also of interest in this context that Kanda et al have shown that antagonism of FGF-2 in rats with a neutralizing antibody reduced peritubular endothelial but not tubular cell proliferation during the compensatory growth that follows uninephrectomy [46]. Because compensatory growth of tubules is mostly derived from proximal tubular cells [47], these data appear compatible with our findings, which showed that these cells fail to express FGF-2 and FGFR1.

In the renal interstitium of adult human kidneys, only rare cells expressed FGF-2 or FGFR1 under most conditions of our study. In contrast to Stein-Oakley et al, we failed to observe increased cell-associated FGF-2 expression in fibrotic areas [25]. Rather, FGF-2 protein in these instances localized to the extracellular matrix and tubular basement membrane, that is, the known extracellular binding sites for FGF-2 [37]. Another interesting observation of our current study was that FGFR1 appeared to label some circulating leukocytes in both the renal interstitium and glomerular capillaries, supporting observations of Kerby et al, who noted FGFR1 protein expression in some interstitial inflammatory cells of rejecting transplants [27].

Apart from tubular cells, the second intrarenal site at which we most consistently detected intense constitutive expression of FGF-2 mRNA and protein was the arterial endothelium. Less consistently, FGF-2 expression was identified in capillary and venous endothelium, as well as in vascular smooth muscle cells. Using frozen sections, others have detected FGF-2 in the basement membrane of normal human blood vessels and/or occasional capillary endothelial cells [21, 22]. Similar to this study, Stein-Oakley et al described prominent endothelial expression of FGF-2 in normal human kidney using formalin-fixed, paraffin-embedded material [25]. Combined with our demonstration of FGFR1 expression in vascular smooth muscle cells, where FGFR1 is the predominantly expressed FGFR type [48], the intense endothelial FGF-2 expression provides the basis for a paracrine pathway of FGF-2 signaling. The functional relevance of this pathway is supported by several experiments in rats, in which arteries were mechanically wounded and which demonstrate that FGF-2 released from injured cells of the vascular wall mediates smooth muscle proliferation as well as angiogenesis [49–51]. The exogenous administration of FGF-2 has also been shown to promote endothelial regeneration after injury in rats [52]. This suggests that, at least in the rat, endothelial cells also express FGFR. Our failure to detect FGFR1 on endothelial cells may therefore relate to low-grade expression in humans or suggest that FGFRs other than FGFR1 are predominantly expressed on these cells.

In summary, we describe the renal expression of FGF-2 and FGFR1 in mature human kidney. One of the important findings of this study is that despite considerable effort, it was not always possible to clarify definitively whether these proteins, especially FGF-2, are expressed at particular sites. Given the complexity of the FGF system, the clarification of these open questions may be a difficult task. On the other hand, we provide evidence for definitive expression of FGF-2 and FGFR1 in the vascular wall and cells of the distal nephron. These data will serve to explain the pathophysiological role of FGF-2 in health and disease.

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