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Basic fibroblast growth factor expression is increased in human renal fibrogenesis and may mediate autocrine fibroblast proliferation

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Background. Interstitial fibroblasts play a critical role in renal fibrogenesis, and autocrine proliferation of these cells may account for continuous matrix synthesis. Basic fibroblast growth factor (FGF-2) is mitogenic for most cells and exerts intracrine, autocrine, and paracrine effects on epithelial and mesenchymal cells. The aims of the present studies were to localize and quantitate the expression of FGF-2 in normal and pathologic human kidneys and to study the in vitro effects of FGF-2 on proliferation, differentiation, and matrix production of isolated cortical kidney fibroblasts.

Methods. FGF-2 protein expression was localized by immunofluoresence double labelings in normal and fibrotic human kidneys. Subsequently, interstitial FGF-2 labeling was determined semiquantitatively in 8 normal kidneys and 39 kidneys with variable degrees of interstitial fibrosis and was correlated with the morphometrically determined interstitial cortical volume. In addition, FGF-2 expression was quantitated by immunoblot analysis in three normal and six fibrotic kidneys. FGF-2 mRNA was localized by in situ hybridizations. Seven primary cortical fibroblast lines were established, and expression of FGF-2 and FGF receptor-1 (FGFR-1) were examined. The effects of FGF-2 on cell proliferation were determined by bromodeoxyuridine incorporation and cell counts, those on differentiation into myofibroblasts by staining for α-smooth muscle actin, and those on matrix synthesis by enzyme-linked immunosorbent assay for collagen type I and fibronectin. Finally, proliferative activity in vivo was evaluated by expression of MIB-1 (Ki-67 antigen).

Results. In normal kidneys, FGF-2 expression was confined to glomerular, vascular, and a few tubular as well as interstitial fibroblast-like cells. The expression of FGF-2 protein was increased in human kidneys, with tubulointerstitial scarring correlating with the degree of interstitial fibrosis ($r = 0.84$, $P < 0.01$). Immunoblot analyses confirmed a significant increase in FGF-2 protein expression in kidneys with interstitial scarring. In situ hybridization studies demonstrated low-level detection of FGF-2 mRNA in normal kidneys. However, FGF-2 mRNA expression was robustly up-regulated in interstitial and tubular cells in end-stage kidneys, indicating that these cells are the source of excess FGF-2 protein. Primary cortical fibroblasts express FGF-2 and FGFR-1 in vitro. FGF-2 induced a robust growth response in these cells that could be blocked specifically by a neutralizing FGF-2 antibody. Interestingly, the addition of the neutralizing antibody alone did reduce basal proliferation up to 31.5%. In addition, FGF-2 induced expression of α-smooth muscle actin up to 1.6-fold, but no significant effect was observed on the synthesis of collagen type I and fibronectin. Finally, staining for MIB-1 revealed a good correlation of interstitial FGF-2 positivity with interstitial and tubular proliferative activity ($r = 0.71$, $P < 0.01$ for interstitial proliferation, $N = 30$).

Conclusions. Interstitial FGF-2 protein and mRNA expression correlate with interstitial scarring. FGF-2 is a strong mitogen for cortical kidney fibroblasts and may promote autocrine fibroblast growth. Expression of FGF-2 correlates with interstitial and tubular proliferation in vivo.

Key words: FGF-2, fibrosis, kidney, interstitial fibroblast, cell proliferation.

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Fibrosis is a process that is characterized by excessive deposition of matrix components compromising organ function because of replacement of normal organ tissue. Tissue fibrosis can affect almost any organ but prefers tissues with common inflammations, such as the skin, the lung, the liver, and the kidney [1]. Inflammation is caused by the recruitment of infiltrating inflammatory cells such as lymphocytes and monocytes/macrophages. Inflammatory infiltration is followed by activation and proliferation of matrix-producing cells, mainly fibroblasts, which synthesize excessive amounts of extracellular matrix. Cytokines seem to play a decisive role in the pathogenesis of organ scarring. Human fetuses heal without scar early in gestation and begin to show scarring late in development [2]. The anatomical difference between the scarless fetal wound healing and the adult scar formation lies in the organization of matrix. Whereas

fibroblasts deposit matrix in an organized fashion, similar to normal skin in fetal wounds, adult wounds are characterized by the deposition of disorganized collagen fibers [3]. Studies by Whitby and Ferguson have demonstrated that the differential expression of certain cytokines is one of the key reasons for scar formation. Transforming growth factor-β (TGF-β) immunostaining, for example, was absent in fetal mouse wounds and was abundant in neonatal and adult mouse wounds [4]. Another cytokine that was markedly up-regulated in postnatal wounds with fibrosis was basic fibroblast growth factor (FGF-2). Whitby and Ferguson demonstrated that, similar to TGF-β, FGF-2 is present in neonatal and adult mouse lips but cannot be detected in fetal mouse lip wounds [4]. Thus, similar to TGF-β, FGF-2 may play an important role in the scarring process.

In the kidney, chronic tubulointerstitial fibrosis is the final common pathway of glomerular, vascular, or interstitial inflammations, often resulting in end-stage renal disease [5]. The severity of chronic tubulointerstitial disease is the single best histologic correlate of the decline in renal function and prognosis for organ function [1, 6]. As in other scarring processes, a number of cytokines have been implicated in renal fibrogenesis, including platelet-derived growth factor (PDGF) and TGF-β. PDGF is a strong mitogen for some but not all fibroblasts [7], and PDGF-BB has been shown to cause interstitial cell proliferation, the appearance of myofibroblasts, and kidney fibrosis in rats if given intravenously in high doses [8]. TGF-β affects all phases of wound healing, including the inflammatory response and matrix accumulation [9, 10]. Increased expression of TGF-β1 has been described in a variety of animal models and human diseases associated with renal fibrosis [reviewed in 1]. However, the role of other cytokines, such as FGF-2, is less well defined. FGF-2 belongs to the family of fibroblast growth and differentiation factors that currently contains nine members [11]. Up-regulated expression of FGF-2 has been linked to fibrogenesis in a number of animal models. Injection of FGF-2 into subcutaneous tissue of newborn mice caused slight fibrotic changes after seven days of injection, whereas simultaneous application with TGF-β resulted in persistent skin fibrosis [12]. Moreover, FGF-2 is a strong mitogen for pulmonary fibroblasts and hepatic stellate cells, and has thus been implicated in the pathogenesis of lung and liver fibrosis, although the exact role remains to be elucidated.

Regarding the kidney, FGF-2 protein was isolated from whole kidney homogenates as early as 1985 [13]. Floege et al, as well as Takeuchi et al, reported strong immunostaining in Bowman's capsule, the glomerular mesangium, blood vessels, and the interstitium, as well as within some tubules in rats [14, 15], although their results could not be confirmed by all investigators [16, 17] and the exact localization of FGF-2 protein in the kidney remains controversial. Recently, Floege et al analyzed FGF-2 expression in normal human kidneys using four different antibodies, and localized it most consistently to distal tubular epithelial and vascular smooth muscle cells [18]. FGF-2 is mitogenic for mesangial [19], proximal-tubular epithelial [20], glomerular endothelial [21], and glomerular epithelial cells [15]. Floege et al demonstrated that FGF-2 was synthesized by mesangial cells in vitro and in vivo [14]. Furthermore, FGF-2 infusions into rats injected with an antimesangial cell antibody (anti-Thy 1.1) resulted in a fourfold increase in glomerular cell proliferation [22] and led to increased podocyte injury and glomerulosclerosis in rats with membranous nephropathy [23]. Similarly, treatment of rats for 8 and 13 weeks with subcutaneous FGF-2 injections resulted in focal segmental glomerulosclerosis, with an increase of the peritubular interstitium [24]. Finally, Ray et al studied a transgenic mouse model of HIV-associated nephropathy and described increased interstitial staining for FGF-2 colocalized with extracellular matrix [25].

The aims of the present study were the evaluation of FGF-2 expression in normal and fibrotic human kidneys and its potential role in renal fibrogenesis.

METHODS

Materials

Rabbit polyclonal antibody to FGF-2 (Ab-2) and mouse monoclonal antibody to FGF receptor-1 (FGFR-1) were purchased from Calbiochem (La Jolla, CA, USA). Human recombinant FGF-1 and FGF-2 were from R&D Systems (Minneapolis, MN, USA), as was the neutralizing goat polyclonal antibody to FGF-2. Mouse monoclonal antibody to collagen type I was obtained from Southern Biotechnology (Birmingham, AL, USA). Polyclonal rabbit antibody to fibronectin antibody was purchased from Sigma (St. Louis, MO, USA), and mouse monoclonal antibody to heparan sulfate was from Boehringer Mannheim (Mannheim, Germany). For cell characterization, the following additional mouse monoclonal antibodies were used: anticytokeratin (Dako, Carpinteria, CA, USA), antivimentin (Boehringer Mannheim), anti-factor VIII (Dako), anti-human lymphocyte antigen-DR (HLA-DR) (Dako), anti–α-smooth muscle actin (Paesel+Lorei, Wiesbaden, Germany), anticollagen type III (Southern Biotechnology), anti-CD 44 (Pharmingen, San Diego, CA, USA), anti-CD 54, and anti-CD 68 (Dako). The mouse monoclonal antibody to collagen type IV was a generous gift from Steven Gay (University of Zürich, Zürich, Switzerland). Trypsin-ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium, and fetal calf serum (FCS) were obtained from GIBCO BRL Ltd. (Paisley, Scotland). Cell culture dishes were from Becton Dickinson (Franklin Lakes, NJ, USA).

Patient material

Kidney tissues from 39 patients with primary and secondary renal diseases and a variable degree of tubulointerstitial fibrosis were used in this study. Tissues included 32 biopsies and 7 nephrectomy specimens. The nephrectomies were performed in patients with end-stage renal disease caused by intractable hypertension or recurrent urinary tract infections. The use of parts of nephrectomy specimens or kidney biopsies for research purposes was approved by the ethics committee of the Georg-August-University, and written consent was obtained from all patients prior to nephrectomy or kidney biopsy. Normal-appearing kidney tissue from kidneys that proved either unsuitable for transplantation or were explanted because of tumor nephrectomies were used as controls ($N = 8$).

Cell culture

Human medullary fibroblast cell lines Tk 173 (obtained from a normal kidney) and Tk 188 (from a kidney with tubulointerstitial fibrosis) have been characterized previously [26] and were cultured in DMEM supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 U/mL). For the isolation of primary human renal fibroblasts, cortical parts of renal biopsy cylinders or nephrectomy specimens were cut and emerged in DMEM medium with 20% FCS and penicillin/streptomycin in the previously mentioned concentrations. When cells had grown to confluence, they were characterized by immunofluorescence for cytokeratin, vimentin, α-smooth muscle actin, collagen types I, III, IV, factor VIII, CD 68, and HLA-DR. Cells that were positive for vimentin and at least collagen types I and III, and negative for cytokeratin, factor VIII and HLA class II were considered to be fibroblasts and were used for induction assays in passages 2 through 8. For detection of heparan sulfate proteoglycane expression, fluorescence activated cell sorting (FACS) analysis was performed as described previously [26].

Specificity of the basic fibroblast growth factor antibody

To determine the specificity of the applied antibody, FGF-2 expression in Tk 173 and Tk 188 fibroblasts (which are easier to culture than primary fibroblasts) was first analyzed by immunocytochemistry as described previously, using the anti–FGF-2 and the secondary FITC-antirabbit antibodies at concentrations of 1:20 [27]. For Northern blot analyses, total cellular RNA was extracted from cultured cells using RNA-clean™ (AGS, Heidelberg, Germany) according to the manufacturer's instructions. Concentration of RNA was determined by absorbance at 260 nm, and samples were stored at −80°C prior to use. Northern blot analysis was performed as described [28]. Briefly, 40 μg of total RNA were electrophoresed on a 1.0% agarose gel containing 2.2 mol/L formaldehyde using 1 × MOPS, pH 7.0, as the running buffer. Photographs of ethidium-bromide–stained gels were taken under ultraviolet illumination. RNA was subsequently transferred to a nylon membrane (Hybond N; Amersham, Arlington Heights, IL, USA) by capillary transfer for 12 hours with 20 × standard saline citrate (SSC) as the transfer buffer. Blots were baked at 80°C and prehybridized for one hour at 68°C in prehybridization solution containing 0.5 mol/L Na_2HPO_4 buffer (pH 7.2), 0.5 mol/L EDTA (pH 8.0), 25% sodium dodecyl sulfate (SDS), and 1.5% blocking reagent. FGF-2 specific oligonucleotides (R&D Systems) were labeled with digoxigenin using a kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridizations were performed overnight at 58°C using the prehybridization solution. After washing, anti-DIG alkaline phosphate and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) substrate (both Boehringer Mannheim) were added, and autoradiograms were obtained.

Subsequently, immunoblots were performed as described previously [27]. Briefly, lysates from Tk 173 and Tk 188 fibroblasts were obtained by lysis with a detergent-based buffer containing 0.4% sodium deoxycholate, 1% NP-40, 1.9% EGTA, and 10 mmol/L Tris (pH 7.4). One hundred micrograms of total cellular protein and 50 ng FGF-2 control protein were run on an 18% SDS-polyacrylamide electrophoresis gel and transferred to a nitrocellulose membran (Hybond™ ECL™; Amersham) by electroblotting. Membranes were stained with Ponceau red to control for adequate transfer and equally loaded amounts. After blocking, the membrane was incubated with the respective antibody (anti–FGF-2 in a concentration of 1:40) followed by the secondary antibody (donkey-antirabbit, horseradish peroxidase-linked, concentration 1:3000; Amersham). Positive reaction products were identified by enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's protocol.

Immunhistochemical staining for basic fibroblast growth factor and morphometric evaluation of interstitial volume

Tissue from normal human kidneys, renal biopsies, and end-stage kidneys were snap frozen in liquid nitrogen. Sections of 4 to 6 μm were cut, mounted on microscope slides, and fixed in ice-cold acetone for 10 minutes. Sections were then allowed to dry, and unspecific binding was blocked by incubation with goat serum. Anti–FGF-2 antibody was added in a concentration of 1:20. After incubation for 60 minutes and several washes in phosphate-buffered saline (PBS), FITC-antirabbit was added in a concentration of 1:20. Positivity was evaluated with an Axiophot S100 microscope (Zeiss, Jena, Germany). Interstitial cortical staining for FGF-2 was rated by two

blinded investigators as in normal kidneys (+), increased (++), robustly increased (+++), or absent (−). Negative controls consisted in substitution of the primary antibody with an irrelevant rabbit polyclonal antibody or PBS.

In selected sections, double labelings were performed to characterize FGF-2–positive interstitial cells. In that case, an addition of the anti–FGF-2 antibody was followed by staining for α-smooth muscle actin, vimentin, cytokeratin, HLA-DR, CD 44, CD 54, and CD 68. FITC-anti-rabbit and the appropriate rhodamin-labeled antibody were added sequentially at a concentration of 1:20. Photographs were taken either by double or by sequential exposure using Zeiss camera and software (MC 200 Chip).

Selected sections from normal and fibrotic kidneys were also paraffin embedded after fixation in formaldehyde using a standard avidin-biotin complex (ABC) method, as described previously [27].

For morphometric evaluation, all tissue specimens were fixed in 4% buffered formalin solution (pH 7.4) and embedded in paraffin. Tissue sections were stained for trichrome or PAS, and point counting was performed as described by Bohle et al [29]. Only those specimens containing at least five glomeruli were evaluated. Five projection fields of the cortex were examined per kidney using a lattice of 1 cm at a magnification of ×250, discriminating between interstitium, tubular epithelia, glomeruli, and vasculature. Big blood vessels were ignored. The value for the interstitium was expressed as a percentage of the analyzed cortex.

Quantitation of basic fibroblast growth factor expression

Expression of FGF-2 protein was quantitated by immunoblot analysis. Briefly, lysates were obtained from six kidneys with considerable interstitial scarring and three control kidneys. Immunoblots were performed as described earlier in this article using 100 μg of protein and 50 ng of FGF-2 recombinant protein as positive control. After staining for Ponceau red, blots were analyzed densitometrically for later normalization. Positive reaction products were again identified by chemiluminescence using a Fluor-S™ Multiimager (Bio-Rad, Hercules, CA, USA), and densitometric analysis was performed with Multi-Analyst™ software (Bio-Rad).

Vector assembly and in situ hybridization for basic fibroblast growth factor

In situ hybridizations were performed on 6 μm sections of snap-frozen tissue from freshly explanted kidneys using a digoxigenin-labeled cRNA probe. FGF-2 cDNA was purchased from R&D Systems, digested with Hind III and BamHI, and cloned into pBS SK II (Stratagene, La Jolla, CA, USA) as described [30]. The new vector was named FGF-2-pBS. Correct ligation was verified by sequence analysis using Alf-Express (Pharmacia, Piscataway, NJ, USA). The vector was linearized with the appropriate enzyme, and FGF-2 sense/antisense riboprobes were synthesized and labeled with digoxigenin by in vitro transcription with the DIG RNA labeling kit (Boehringer Mannheim) as described [31]. Tissue sections were mounted on Sectionlock™ slides (Polysciences, Eppelheim, Germany), briefly heated to 50°C to ensure fixation, dried for 30 minutes, and fixed in 3% paraformaldehyde for 7 minutes. Washing in PBS and 2 × SSC was followed by prehybridization for 60 minutes at 43°C in hybridization buffer (Amersham) containing 50% formamide (Sigma). Hybridization was then performed in 40 to 60 μL hybridization buffer per section containing the cRNA probe at a concentration of 200 ng/mL, followed by washing in 2 × SSC for 5 minutes at 37°C and twice for 15 minutes in 0.1 × SSC, SDS at 68°C. After a final wash in 100 mmol/L Tris-HCl and 150 mmol/L NaCl for five minutes at room temperature, 1% blocking buffer and 10% goat serum (Dako) were added, and the sections were incubated with a 1:500 dilution of alkaline-phosphatase conjugated anti-DIG antibody for two hours at room temperature. Detection was performed after several washes in 100 mmol/L Tris-HCl, 150 mmol/L NaCl with detection buffer containing 0.18 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 0.34 mg/mL nitro blue tetrazolium (NBT), and 240 μg/mL levamisole. After 12 hours, color reaction was stopped by washing in 10 mmol/L Tris (pH 8) and 1 mmol/L EDTA for five minutes, and coverslips were mounted. Sections from human synovia, which is known to express FGF-2 abundantly, served as positive control.

Proliferation assay

Proliferation studies in primary cortical fibroblasts were performed using nonradioactive bromodeoxyuridine incorporation assays (Amersham) based on the method by Gratzner [32]. Four × 10^3 cells were plated per well in 96-well microtiter plates containing DMEM medium with the usual supplements and incubated overnight to allow cell attachment. Cells were then made quiescent by replacement of DMEM medium with Iscove's medium and subsequent incubation for 24 hours. In preliminary experiments, cytokines were added, and proliferation was measured after 24, 48, 72, and 96 hours. FGF-2 was used in concentrations of 0.1, 1, 10, and 100 ng/mL (equivalent to 5.5 × 10^{-12} mol/L, 5.5 × 10^{-11} mol/L, 5.5 × 10^{-10} mol/L, and 5.5 × 10^{-9} mol/L, respectively). In a second set of experiments, 1 ng/mL FGF-2 was added, and bromodeoxyuridine incorporation was measured after 72 hours. The specificity of the response to FGF-2 was determined by addition of a neutralizing FGF-2 antibody in a concentration of 10 μg/mL (which had been determined in titration experiments with Tk 173 fibroblasts) or a nonrelevant IgG. Both antibodies were also added without FGF-2 to study their

intrinsic effects on proliferation. Medium was changed to Iscove's containing BrdU/FdU in a dilution of 1:500 four hours prior to measurements. Cells were then washed three times in PBS and fixed in methanol containing 2% hydrogen peroxide followed by denaturation in 1N HCl for 10 minutes. After a further three times of washing in PBS containing 0.1% Tween and blocking for 30 minutes in PBS (0.1% Tween, 3% BSA), 50 μL of anti-BrdU antibody were added and incubated for 45 minutes. Cells were again washed three times in PBS/Tween, and peroxidase substrate was added (100 μL/well). Optical densities were subsequently determined photometrically at 405 nm (Dynatech MR 4000; Dynatech, Denkendorf, Germany). All experiments were performed in triplicate and repeated five times. Results are shown in percentage of negative control (Iscove's medium = 100%).

In a third set of experiments, confluent monolayers of fibroblasts were trypsinized, and cells were seeded at 4×10^3 cells per well in 96-well culture plates. After 12 hours, DMEM medium was replaced by Iscove's medium. After a further 24 hours, cells were incubated with various factors (FGF-2 at concentrations of 0.1, 1, 10, and 100 ng/mL, FGF-2 (1 ng/mL) + FGF-neutralizing antibody (10 μg/mL), FGF-2 (10 ng/mL) + irrelevant IgG, and FGF-2 (1 ng/mL) + heparin (10 μg/mL) for 72 hours before cell counts were performed. PDGF-BB at 10 ng/mL and heparin alone (10 μg/mL) served as controls. Again, all experiments were performed in triplicate and repeated five times.

Immunofluorescence staining of α-smooth muscle actin

The effects of FGF-2 on the differentiation of kidney fibroblasts into myofibroblasts were assessed by immunofluorescent staining of α-smooth muscle actin after continuous exposure to FGF-2 for 48 hours. Cells were seeded in microchamber slides and incubated in regular medium for 12 hours. After 24 hours of incubation in Iscove's medium for quiescence, FGF-2 was added in concentrations of 0.1, 1.0, and 10 ng/mL. At the end of incubation, cells were washed twice with PBS and fixed with ethanol/acetic acid (50:50, vol/vol) for 20 minutes at 4°C. The cells were subsequently washed again and incubated for two hours at room temperature with a 1:200 dilution of a monoclonal mouse antibody to human α-actin isoform of smooth muscle cells. Cells were then washed again with PBS and incubated for 60 minutes at room temperature with the FITC-conjugated rabbit-antimouse antibody. Control cultures were incubated without application of the primary antibody. Incubation with TGF-β1 (1 ng/mL) served as a positive control. Cells with the typical α-actin structure were considered to be α-actin positive. Additional staining with the Hoechst dye H33258 to identify nuclei was performed to facilitate cell counting. For each group, 500 cells were determined microscopically using an Axiophot S100 microscope (Zeiss) by a blinded investigator, and the percentage of α-smooth muscle actin-positive cells was calculated. Each experiment was repeated three times independently.

Matrix synthesis

Enzyme-linked immunosorbent assay (ELISA) of supernatants were performed as described [33], with some modifications, including the use of chemiluminescence to enhance sensitivity [34]. Eight $\times 10^3$ cells were plated per well, and cells were again made quiescent by incubation in serum-free medium for 24 hours. Next, FGF-2 was added in concentrations of 0.1, 1.0, and 10 ng/mL followed by incubation for 48 hours. Human recombinant TGF-β1 (R&D Systems) in a concentration of 1 ng/mL served as a positive control. Fifty μg/mL ascorbic acid and 50 μg/mL propionitrile (both from Sigma) were added in experiments evaluating collagen synthesis. Supernatants were transferred to a Maxisorp™ plate and incubated overnight at room temperature. Plates were subsequently dried for two hours and blocked with 3% dried milk. Incubation with 50 μL of the primary antibody (anticollagen type I was used in a concentration of 1:300 and antifibronectin at 1:5000) was followed by washing two times with PBS/0.1% Tween and incubation with the secondary antibody [antimouse-IgG-AP and antirabbit-IgG-AP (both Boehringer Mannheim, Germany), both used at a concentration of 1:1000]. After two additional wash steps, 100 μL of CSPD-Ready-to-Use substrate (Boehringer Mannheim) were added, and quantitation was performed in a luminometer (Mikrolumat CB 96P; Berthold, Bad Wildbad, Germany) using MikroWin™ software (Mikrotek, Overath, Germany). Nonspecific binding was determined by incubation with the secondary antibody only. The value was normally less than 5% of the total chemiluminescence and was subtracted from each assay. All assays were performed in triplicate and repeated five times. Standardization was obtained for the collagen type I ELISA using human type I collagen (Becton Dickinson, Bedford, MA, USA). The limit of detection was 1 ng/mL, with a working range of 10 to 1000 ng/mL. Human fibronectin (Sigma) served as standard for the fibronectin ELISA. The working range of that ELISA was 0.1 to 10 μg/mL, with a limit of detection of 10 ng/mL. All values were corrected for cell counts (10^3 cells), which were performed after transfer of supernatants.

Evaluation of cell proliferation in vivo

Immunostaining with MIB-1 (Dako) was used to evaluate cell proliferation in vivo. Five-micrometer thick sections were cut, dewaxed, and rehydrated according to routine procedures. Sections were pretreated in a microwave oven for 5 minutes and then incubated for 60 min-

utes at room temperature with the MIB-1 antibody, which was used in a concentration of 1:50. The streptavidin-biotin/alkaline phosphatase complex technique (Dako) was performed, and Fast Red™ was used as the chromogen. The sections were counterstained with hematoxylin. All stained nuclei were rated as positive. Proliferative activity within the interstitium and tubular epithelium was quantitated separately by counting MIB-1–positive cells in four fields at ×250 magnification using an integration grid.

Statistical analysis

All values are expressed as mean ± SEM unless specified. Analysis of variance (ANOVA) was used to determine statistical differences between growth factor-treated groups and controls using Sigma-Stat™ software (Jandel Scientific, San Rafael, CA, USA). Further analysis was carried out using Bonferroni's test to control for multiple testing. For nonnormally distributed data (MIB-1 staining), the Mann–Whitney rank sum test was applied. A correlation between semiquantitative interstitial FGF-2 staining, morphometric volume, and MIB-1 positivity was evaluated by Spearman's rank-order correlation coefficient. P values < 0.05 were considered significant.

RESULTS

Specificity of the anti-basic fibroblast growth factor antibody

Since the exact localization of FGF-2 in the kidney is still controversial, we first determined the specificity of the antibody used for our studies. First, immunofluorescence labeling demonstrated that the two fibroblast lines, Tk 173 and Tk 188, were positive for FGF-2 using the Ab-2 antibody. These results were corroborated by Northern and Western blot analyses (Fig. 1). Northern blot analysis confirmed that both fibroblast lines express FGF-2 mRNA, with a main band at 7.0 kb (Fig. 1A). In addition, the immunoblot demonstrated that the antibody detects a specific 18 kd band in the two fibroblast lines corresponding to the size of the recombinant protein (Fig. 1B). The two additional bands found mainly in Tk 173 fibroblasts probably correspond to multiple translation initiation sites [35]. There was no cross-reactivity of the antibody with FGF-1 (acidic fibroblast growth factor; data not shown).

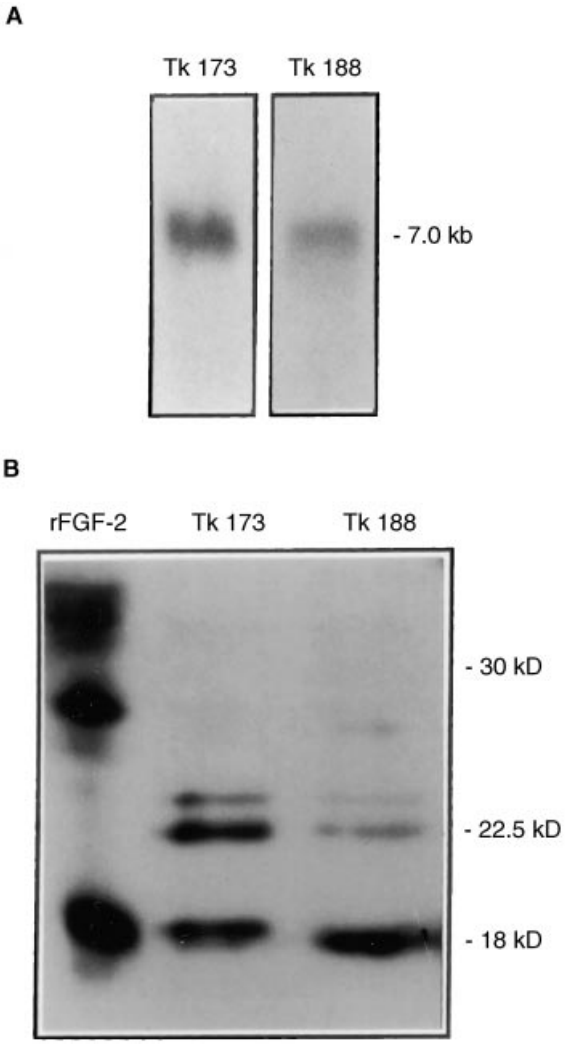

Fig. 1. Specificity of the anti–basic fibroblast growth factor (anti-FGF-2) antibody. The two kidney fibroblast cell lines Tk 173 and Tk 188 express FGF-2 as determined by Northern blot (*A*) and immunoblot analyses (*B*). Northern blot gave a main band at 7.0 kb, as expected for FGF-2 (A). Immunoblot analysis demonstrated a main band at 18 kD corresponding to the size of the recombinant protein. Additional bands at 22.5 and 24 kD correspond to multiple transplantation initiation sites (B). One hundred micrograms of total cellular protein were loaded onto each lane, and 50 ng of recombinant FGF-2 control protein (rFGF-2) served as positive control.

Basic fibroblast growth factor expression is expressed in interstitial fibroblast-like cells and increased in kidneys with tubulointerstitial fibrosis

Basic fibroblast growth factor expression was first analyzed in frozen sections of normal kidneys by indirect immunofluorescence. Immunolabeling of normal kidneys demonstrated FGF-2 protein within glomeruli, tunica media of blood vessels, few tubular (mainly distal by morphology), and many, albeit not all, interstitial cells (Fig. 2). The number of positive interstitial cells was considerably smaller in paraffin sections, probably because of differences in antigen stability (data not shown). To further characterize the FGF-2–positive cells within the interstitium, immunofluorescent double labelings were performed in normal kidneys and biopsies from kidneys with minimal interstitial involvement (interstitium morphometrically <25%). The results are depicted in Figure 2. Most of the FGF-2–positive cells within the interstitium stained also positive for CD 54 (intercellular

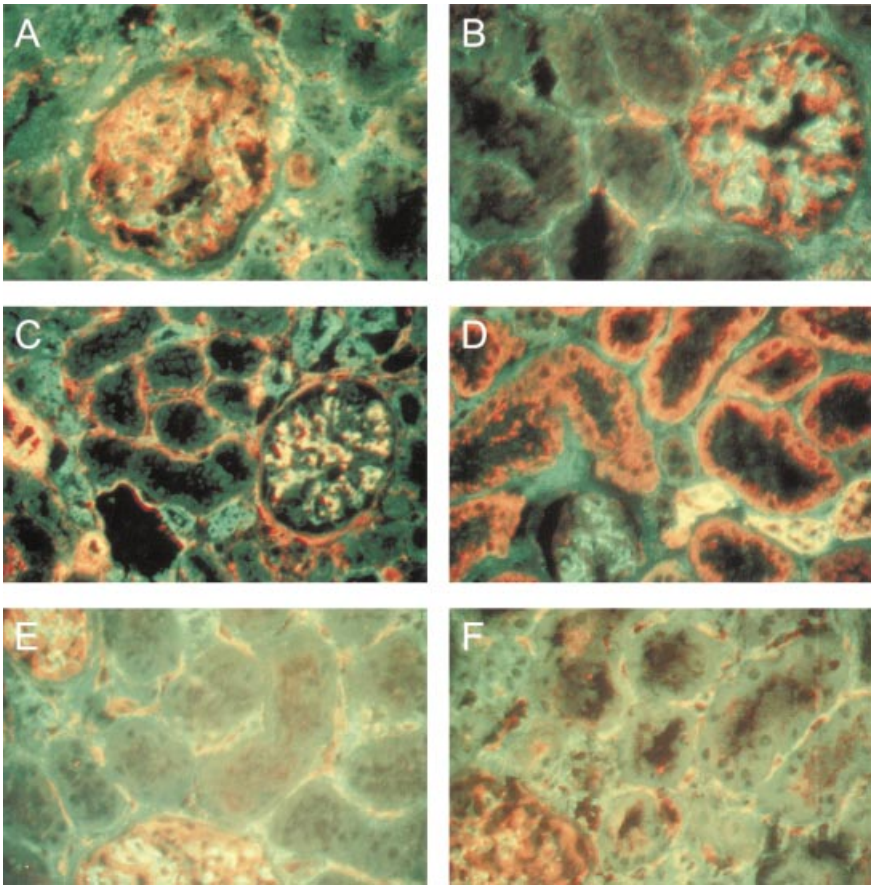

Fig. 2. Localization of FGF-2–positive cells in cortical tissue of a normal kidney by immunofluorescence. Indirect immunofluorescence double labelings for FGF-2 (FITC labeled) and HLA-DR (*A*), CD 68 (*B*), α-smooth muscle actin (*C*), cytokeratin (*D*), CD 54 (*E*), and CD 44 (*F*) (rhodamine labeled) were performed in a human kidney without interstitial scarring. There is a close correlation in staining within the interstitium for FGF-2 and α-smooth muscle actin, CD 54, and CD 44. However, some interstitial cells stain exclusively for α-smooth muscle actin (C), some exclusively for FGF-2 and not for CD 54 (E), or CD 44 (F). A number of cells coexpress FGF-2 and HLA-DR (A), although most interstitial cells stain only for FGF-2. There are very few coexpressions between FGF-2 and CD 68 (B). Interstitial cells do not express cytokeratin; however, some tubular cells display coexpression of FGF-2 and cytokeratin (D). Original magnification ×200.

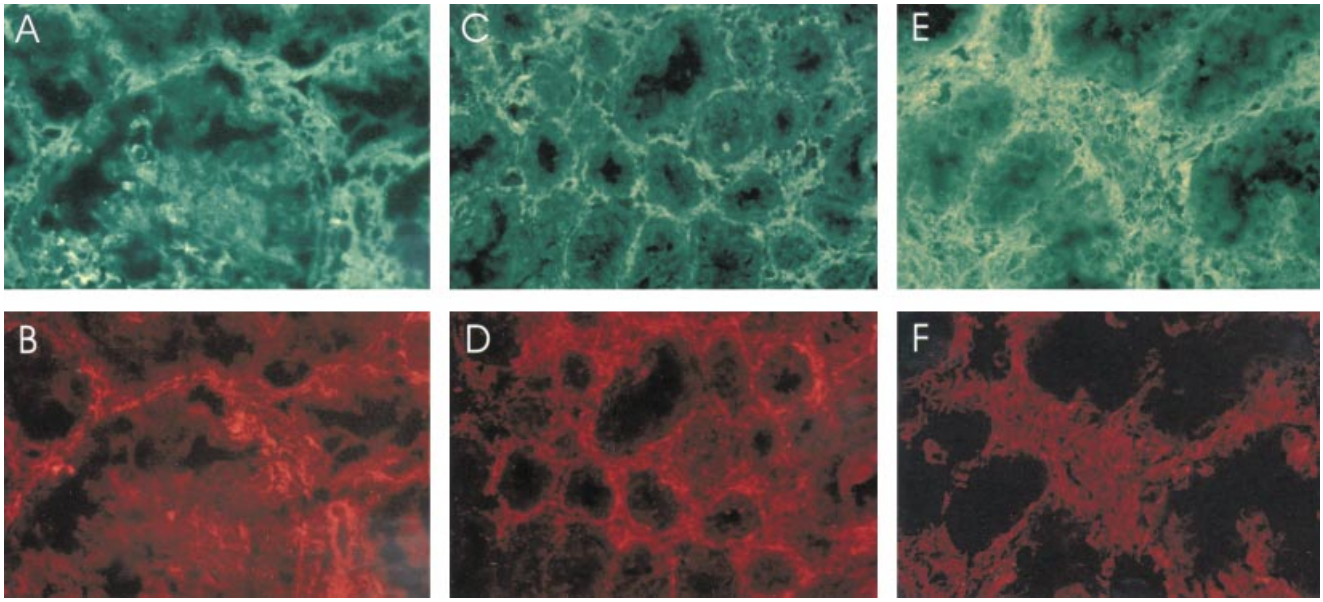

Fig. 3. Staining for FGF-2 in a fibrotic kidney. Indirect immunofluorescence for FGF-2 (*A, C, E*) and vimentin (*B*), α-smooth muscle actin (*D*), and CD 44 (*F*) in a human kidney with marked interstitial fibrosis. The staining for all four molecules is increased compared with a normal kidney. There is marked coexpression for FGF-2 and all three markers of interstitial fibroblasts; however, again, the overlap in staining is not complete. Some interstitial cells label exclusively for FGF-2 and some exclusively for α-smooth muscle actin (C and D). Original magnification ×400 (A and B) and ×200 (C, D, E and F).

adhesion molecule-1; Fig. 2E) and CD 44 (Fig. 2F), although many FGF-2–positive cells did not label for both. In addition, almost all FGF-2–positive cells did stain for vimentin as well (data not shown). There was a considerable overlap in interstitial staining for FGF-2 and α-smooth muscle actin (Fig. 2C); however, there were some cells that expressed α-smooth muscle actin but not FGF-2. There was some coexpression of FGF-2 and HLA-DR (Fig. 2A), but most FGF-2–positive interstitial cells were negative for the HLA class II molecule, which is expressed on dendritic cells and macrophages. Only occasionally was coexpression of FGF-2 and the macrophage marker CD 68 observed (Fig. 2B), indicating that only very few FGF-2–positive cells are macrophages. Judging by the difference in staining for CD 68 and HLA-DR, some of the cells that stained for FGF-2 may be dendritic cells, but the majority clearly are not. CD 44, CD 54, and α-smooth muscle actin have all been propagated as potential markers of fibroblasts [36, 37]. Thus, it seems reasonable to assume that most FGF-2–positive cells within the interstitium are probably fibroblasts or fibroblast-like cells.

We next compared the sections from eight normal renal tissues (from 3 female and 5 male patients, mean age 56.9 years) and 39 kidneys (from 12 female and 27 male patients, mean age 45.0 years), each with varying degrees of tubulointerstitial fibrosis. Since we were particularly interested in the correlation between FGF-2 expression and the degree of interstitial scarring, a variety of renal diseases was examined. Table 1 displays the characteristics of patients and tissue types. The relative percentage of the cortical interstitium was determined by morphometric analysis. In normal kidneys, the relative percentage of the interstitium varied between 15 and 25%. Conversely, in the nephrectomy specimens from patients with renal disease, the interstitium occupied between 49 and 72%. No change of FGF-2 expression was observed in normal human kidneys. However, robust up-regulation of FGF-2 expression was noted in kidneys with interstitial scarring. Table 1 summarizes the results. Figure 3 depicts the staining results in a kidney with marked interstitial fibrosis. FGF-2–positive cells (Fig. 3 A, C, and E) also stained positive for vimentin (Fig. 3B), α-smooth muscle actin (Fig. 3D), and CD 44 (Fig. 3F). Again, there was only little co-staining for the dendritic cell and macrophage marker HLA-DR and only occasional co-staining for the macrophage marker CD 68 (data not shown), excluding prominent expression in these cells. In paraffin-embedded sections, there was an accentuation of extracellular matrix staining, including tubular basement membrane, whereas fewer cellular elements were labeled.

When the expression of FGF-2 was correlated with the cortical interstitial volume, we found a good correlation ($r = 0.64$, $P < 0.01$). However, four of the biopsy specimens displayed an enlargement of the interstitial volume caused exclusively by interstitial infiltration without significant interstitial scarring (numbers 14, 15, 33, and 34). No increase in FGF-2 expression was observed in any of the four specimens. Thus, when FGF-2 protein expression was correlated with the interstitial volume only in those kidneys in which widening of the interstitial space was due to scarring, we found an even better correlation ($r = 0.84$, $P < 0.01$). Figure 4 illustrates the correlation graphically.

When the specimens from patients with glomerulonephritis ($N = 20$) were analyzed exclusively, the correlation was still significant ($r = 0.65$, $r < 0.01$). The number of individual diseases was too small to analyze them separately. However, looking at the specimens with IgA nephritis, there may be a biphasic expression pattern of FGF-2 with a +++ expression at interstitial volumes of 37 to 46% and a ++ expression at volumes of 55 to 71%. There was no correlation between interstitial FGF-2 staining and interstitial volume in the specimens from patients with tubulointerstitial nephritis.

FGF-2 protein expression is increased in cortical tissue from kidneys with interstitial fibrosis

Although our immunofluorescence labeling studies had shown a good correlation between interstitial volume and interstitial FGF-2 labeling, we wanted to confirm this finding by immunoblot analysis. Thus, protein was isolated from cortical tissue from three normal kidneys and six kidneys with marked interstitial scarring [from patients 10 (that is, FK1), 11 (FK2), 13 (FK3), 18 (FK4), 19 (FK5), and 24 (FK6)]. With the exception of FK1, all fibrotic kidneys contained more FGF-2 than their normal counterparts (Fig. 5A). Figure 5B summarizes the result after densitometric analysis ($P = 0.048$).

In situ hybridizations reveal increased FGF-2 mRNA expression within the tubulointerstitium in end-stage kidneys

In situ hybridizations were performed to localize FGF-2–synthesizing cells in three normal and three end-stage kidneys with interstitial fibrosis, tubular atrophy, and dilation. As expected, hybridization with the sense probe gave no signal (Fig. 6A). Conversely, hybridization with the antisense probe demonstrated weak staining within glomeruli and blood vessels, as well as occasional tubular and interstitial cells in normal kidneys (Fig. 6B), indicating that these cells synthesize FGF-2 themselves. Furthermore, similar to what was observed by immunofluorescent staining, in situ hybridizations of fibrotic end-stage kidneys revealed a robust increase in signal for FGF-2 mRNA within interstitial cells (Fig. 6 C, D). The majority of interstitial cells displayed FGF-2 mRNA positivity. There was also increased staining in tubular cells, even in some tubules that were dilated and atrophic. Conversely, there was no increased FGF-2 mRNA synthesis within the glomeruli of the analyzed kidneys, indicating that most FGF-2 is, in fact, synthesized in nonglo-

Table 1. Characteristics of the kidney specimens

Patient #	Sex	Age	Tissue	Diagnosis	FGF-2 staining	Interstitial volume
1	M	68	Biopsy	MGN	++	22%
2	M	31	Biopsy	MGN	+	25%
3	F	47	Biopsy	Chronic rejection	+++	55%
4	M	69	Biopsy	IgA nephropathy	+++	46%
5	M	54	Biopsy	Amyloidosis	++	48%
6	M	22	Biopsy	RPGN	++	38%
7	M	27	Biopsy	CRF	+++	65%
8	M	34	Biopsy	Chronic rejection	++	68%
9	M	60	Biopsy	IgA nephropathy	++	55%
10	F	41	Nephrectomy	ESRD	+++	68%
11	M	83	Nephrectomy	ESRD	++	61%
12	M	34	Biopsy	IgA nephropathy	+	36%
13	M	59	Nephrectomy	ESRD	+++	76%
14	M	53	Biopsy	TIN	+	78%
15	F	17	Biopsy	TIN	+	50%
16	M	49	Biopsy	Amyloidosis	++	40%
17	M	22	Biopsy	RPGN	+	22%
18	F	71	Nephrectomy	ESRD	+++	72%
19	M	27	Nephrectomy	ESRD	+++	70%
20	F	28	Biopsy	IgA nephropathy	++	37%
21	M	25	Biopsy	IgA nephropathy	+++	37%
22	M	25	Biopsy	Chronic rejection	+++	50%
23	M	48	Biopsy	Diabetic nephropathy	++	31%
24	F	57	Nephrectomy	ESRD	+++	49%
25	F	43	Biopsy	CRF	++	41%
26	M	27	Biopsy	IgA nephropathy	++	42%
27	M	30	Biopsy	FSGS	++	36%
28	M	42	Biopsy	FSGS	+	18%
29	F	22	Nephrectomy	Diabetic nephropathy	+	20%
30	F	58	Biopsy	RPGN	++	46%
31	M	49	Biopsy	FSGS	+++	74%
32	M	17	Biopsy	FSGS	+	27%
33	F	67	Biopsy	TIN	+	70%
34	M	78	Biopsy	TIN	+	46%
35	M	64	Biopsy	MGN	+	18%
36	F	29	Biopsy	MGN	++	42%
37	M	50	Biopsy	FSGS	+	22%
38	F	67	Biopsy	RPGN	++	25%
39	M	60	Biopsy	IgA nephropathy	++	71%
40	F	64	Nephrectomy	Normal kidney	+	23%
41	M	54	Nephrectomy	Normal kidney	+	19%
42	M	45	Nephrectomy	Normal kidney	+	25%
43	M	71	Nephrectomy	Normal kidney	+	21%
44	F	53	Nephrectomy	Normal kidney	+	15%
45	F	48	Nephrectomy	Normal kidney	+	16%
46	M	61	Nephrectomy	Normal kidney	+	24%
47	M	59	Nephrectomy	Normal kidney	+	20%

There were 6 cases of end-stage renal disease (ESRD), 4 cases of acute tubulointerstitial nephritis (TIN), 4 cases of rapidly progressive glomerulonephritis (RPGN), 7 cases of IgA nephropathy, 4 cases of membranous glomerulonephritis (MGN), 5 cases of focal segmental glomerulosclerosis (FSGS), 3 cases of chronic transplant rejection, 2 cases of amyloidosis, 2 cases of diabetic nephropathy, and 2 cases of chronic renal failure of unknown origin (CRF). Eight normal kidneys served as controls. Interstitial FGF-2 labeling was determined semiquantitatively, and interstitial volume determined morphometrically. Interstitial FGF-2 staining was rated as in normal kidneys (+), increased (++), or robustly increased (+++).

merular cells. Thus, we conclude that the results of the in situ hybridizations parallel the protein stainings, demonstrating that increased FGF-2 mRNA synthesis takes place in interstitial cells within the kidney and that increased protein detection is not due to mere accumulation of extrarenally synthesized FGF-2.

Detection of FGF-2 expression and its low- and high-affinity receptors in human primary cortical fibroblasts

To evaluate the possible role of FGF-2 in human renal fibrogenesis, the expression of FGF-2 and its high- and low-affinity receptors were examined in seven primary cortical fibroblast lines. All cell lines were characterized as fibroblasts and synthesized extracellular matrix components, including collagen types I and III, as well as fibronectin. Expression of FGFR-1, one of four high-affinity receptors, was analyzed by immunoblot analysis. The immunoblot analysis demonstrated a double band at approximately 120 kD in all seven fibroblast lines (Fig. 7A). The most robust expression of FGFR-1 was observed in fibroblasts isolated from kidneys 25, 31, and 39, with interstitial volumes of 41, 74, and 79%, respec-

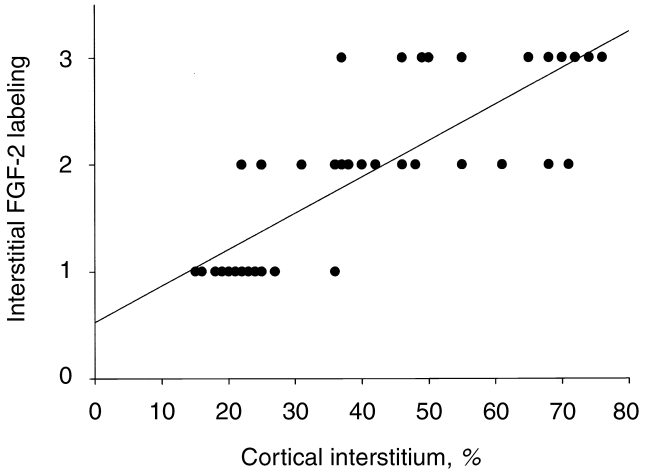

Fig. 4. Correlation of staining for FGF-2 and morphometric volume. There was a positive correlation between semiquantitative FGF-2 labeling and morphometrically determined volume. Four cases of acute tubulointerstitial nephritis have been omitted for this analysis (discussed in the **Results** section). $P < 0.01$; $r = 0.84$, by Spearman's rank order coefficient.

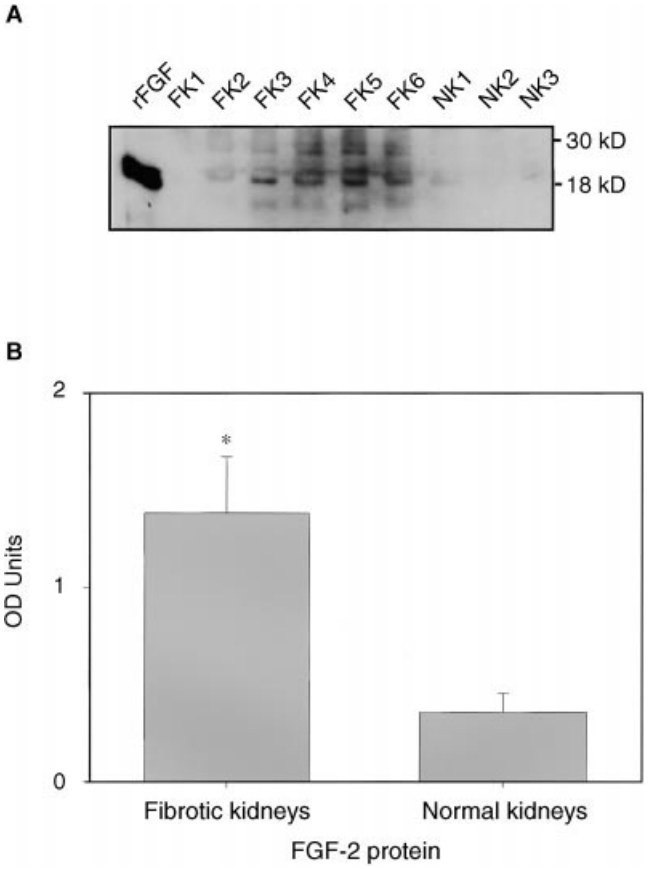

Fig. 5. Expression of FGF-2 protein in kidneys with and without interstitial scarring. FGF-2 protein expression was examined by immunoblot analysis in three normal (NK1 through NK3) and six kidneys with at least 10% interstitial fibrosis (FK1 through FK6). With the exception of FK1, all fibrotic kidneys express more FGF-2 than their nonfibrotic counterparts (*A*). (*B*) The densitometric analysis. *$P < 0.05$.

tively. In addition, FACS analyses confirmed the expression of heparan sulfate proteoglycane, which serves as a low-affinity receptor for FGF-2 in all seven cell lines. There was no difference in the level of expression (data not shown).

Basic fibroblast growth factor protein itself could also be detected in all seven fibroblast lines studied (Fig. 7B). Interestingly, the level of FGF-2 protein expression correlated with the degree of interstitial scarring of the kidney from which the cells were isolated. Whereas F33 and F34, isolated from patients 33 and 34 with tubulointerstitial nephritis and no interstitial scarring, displayed relatively moderate positivity for FGF-2, the level of expression was much higher in the fibroblasts isolated from kidneys with interstitial scarring. In addition, there was a good correlation between the relative level of FGF-2 protein expression in the seven fibroblast lines and the semiquantitative FGF-2 immunofluorescence labeling in the kidneys from which the cells were isolated ($r = 0.76$, $P = 0.038$).

FGF-2 is a strong mitogen for renal fibroblasts, induces differentiation into myofibroblasts, and has no major effect on matrix protein synthesis

To evaluate the function of FGF-2, proliferation, differentiation into myofibroblasts, and matrix protein synthesis of human renal fibroblasts were examined in five different primary cortical fibroblast lines. In a first set of experiments, performed in F25 fibroblasts, FGF-2 caused a robust induction of proliferation, which was time and dose dependent. The increase of bromodeoxyuridine incorporation was significant after 48 hours (187.8 ± 8.9%) at a concentration of 1 ng/mL. The maximum increase was seen with a concentration of 10 ng/mL after 72 hours (267.3 ± 24.9%). Figure 8 summarizes the findings obtained with bromodeoxyuridine incorporation after stimulation with FGF-2 at a concentration of 1 ng/mL for 72 hours in three of the five fibroblast lines. F25 and F26 were isolated from biopsies of patients 25 and 26, displaying increased interstitial volumes because of fibrosis of 41 and 42%, respectively. Conversely, F34 originated from patient 34 with tubulointerstitial nephritis and no interstitial scarring. Bromodeoxyuridine incorporation was increased between 166.3 ± 3.2% in F34 cells and 247.0 ± 15.4% in F26 fibroblasts. This increase could be specifically inhibited by addition of a neutralizing antibody to FGF-2, whereas a nonrelevant IgG did not have an effect on FGF-2–induced proliferation. Interestingly, the addition of the FGF-2 neutralizing antibody alone did decrease bromodeoxyuridine incorporation in all five fibroblast lines. However, because of the low level of basal proliferative activity, that decrease was

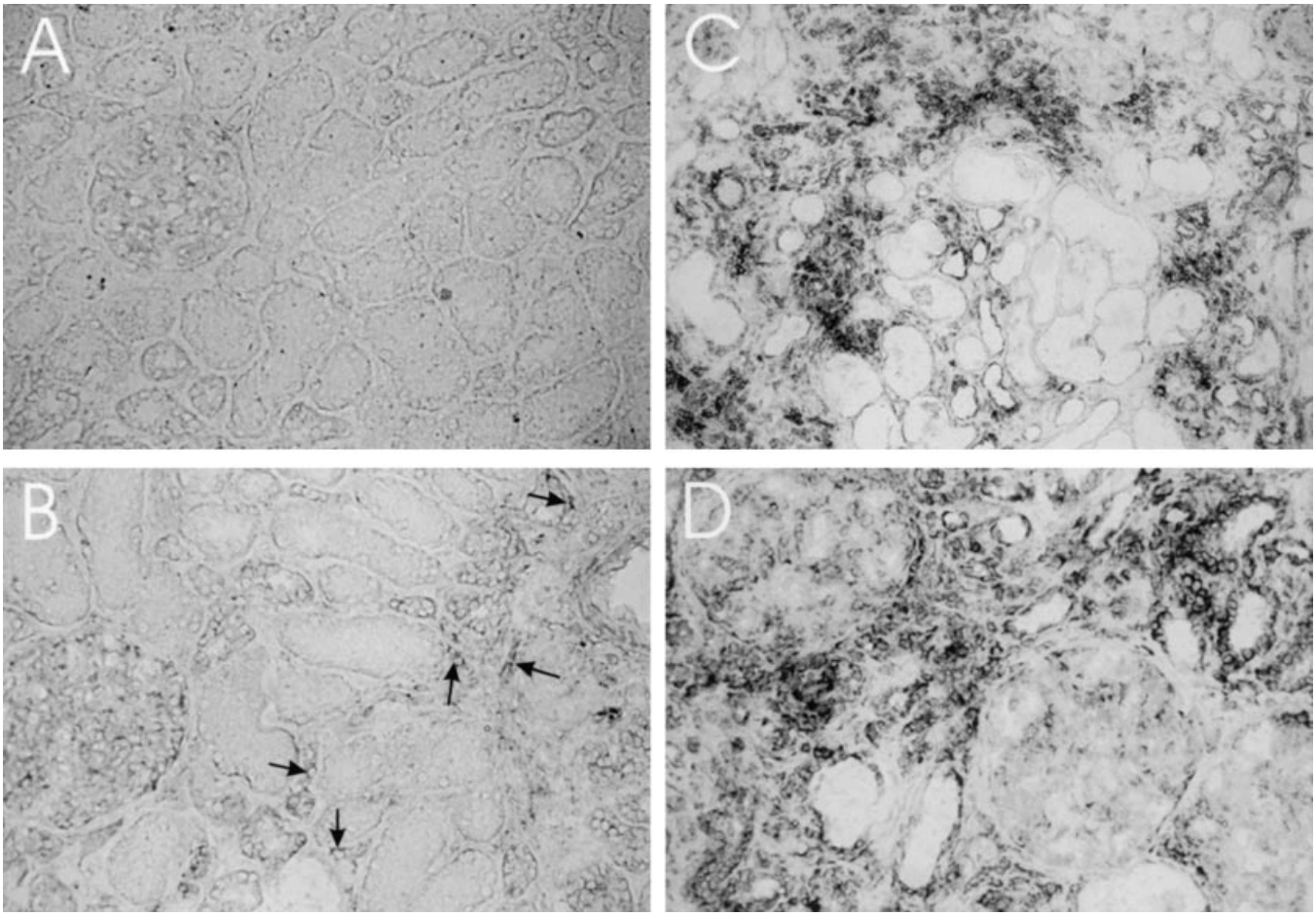

Fig. 6. In situ hybridizations for FGF-2 in normal control kidney and end-stage kidneys. There is no staining in a normal kidney hybridized with the FGF-2 sense riboprobe (*A*). Hybridization of a normal kidney with the FGF-2 antisense riboprobe revealed faint staining of some glomerular, tubular, vascular, and interstitial (arrows) cells. The hybridization signal was robustly up-regulated in kidneys from patients with end-stage renal disease (*C* and *D*). Staining for FGF-2 mRNA was concentrated within the interstitium (C) and in some tubular cells (D), even in some tubules that appear atrophic (C). Conversely, there was no increased staining for FGF-2 mRNA within glomeruli. Original magnification ×200 (A, B, and D) and ×100 (C).

statistically significant in only three cell lines (maximum inhibition 31.5% in F25 cells). Conversely, the addition of the nonrelevant IgG resulted in either no change or an increase in bromodeoxyuridine uptake. In a third set of proliferation experiments, bromodeoxyuridine incorporation essays were confirmed by cell counting. In these experiments, cell numbers increased dose dependently to a maximal 234.3 ± 15.8% ($P < 0.01$) in F25 and to 197.4 ± 18.6% ($P < 0.01$) in F34 fibroblasts after 96 hours of incubation at a concentration of 10 ng/mL. Again, the higher concentration (100 ng/mL) did not have any superior effect to the 10 ng/mL concentration. There was no additional effect of heparin, which by itself caused a slight reduction in proliferation (maximum inhibition of 18.9 ± 4.5%; NS).

Activation of fibroblasts into so-called myofibroblasts represents a key step in renal fibrogenesis. We evaluated the effects of FGF-2 on the expression of α-smooth muscle actin, which serves as a marker for myofibroblast formation. In two primary cortical fibroblast lines, F6 isolated from a kidney with mild interstitial scarring (interstitial volume 38%) and F39 from a kidney with an interstitial volume of 71%, FGF-2 resulted in a time- and dose-dependent induction of α-smooth muscle actin expression. Figure 9 summarizes the results observed after 72 hours. The increase was similar to that obtained with TGF-β1 (increase of α-smooth muscle actin-positive cells from 52.3 ± 3.3 to 82.3 ± 1.9% after 72 hours of incubation with 1 ng/mL FGF-2; $P < 0.01$). Of course, we do not know if these changes were due to de novo expression of α-smooth muscle actin or due to clonal selection of positive cells caused by cell population heterogeneity.

Finally, the influence of FGF-2 on extracellular matrix synthesis was evaluated by ELISAs for collagen type I and fibronectin in F6 and F39 fibroblasts. Whereas

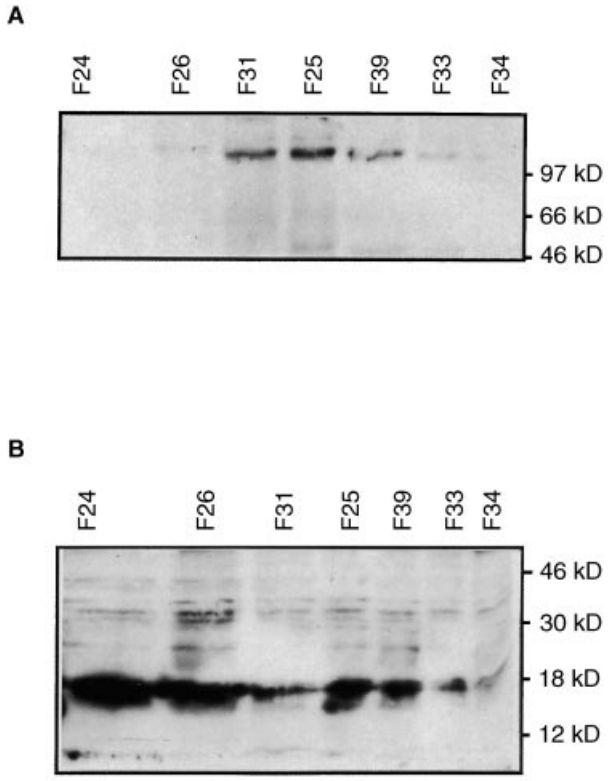

Fig. 7. Expression of FGF-2 and FGFR-1 in seven primary cortical fibroblast lines. (*A*) The expression of FGFR-1. (*B*) The expression of FGF-2 by immunoblot analyses. F33 and F34 were isolated from kidney biopsies without interstitial scarring, and the other five fibroblast lines were from kidneys with moderate (F25 and F26) and severe interstitial scarring (F24, F31, and F39). There was a good correlation between the degree of interstitial scarring and the expression of FGF-2, however, no such correlation could be found regarding the expression of FGFR-1 (discussed in the **Results** section).

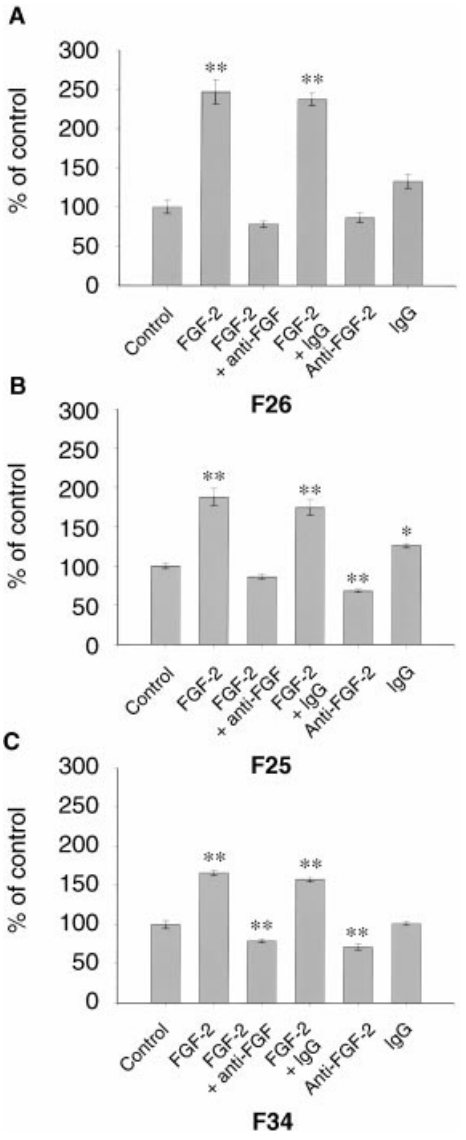

Fig. 8. Effects of FGF-2 on proliferation in primary cortical fibroblasts. FGF-2 was added in a concentration 1.0 ng/mL to F26 (*A*), F25 (*B*), and F34 (*C*) fibroblasts and proliferation was determined after 72 hours by bromodeoxyuridine incorporation. Specificity was determined by the addition of a neutralizing FGF-2 antibody and a nonspecific immunoglobulin (IgG). FGF-2 induced a robust proliferative response in all three fibroblast lines, which could be blocked specifically by the neutralizing FGF-2 antibody. The addition of the neutralizing FGF-2 antibody alone resulted in a significant inhibition of proliferation in two of the three tested cell lines, whereas the nonspecific IgG resulted rather in a promitogenic response. Values are given in percentage of negative control (Iscove's medium) and are a mean of five independent experiments. All experiments were conducted in triplicates. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control.

TGF-β1 (at a concentration of 1 ng/mL) increased secretion of collagen type I (from 101.0 ± 9.8 ng/10^3 cells to 1018.9 ± 98.5 ng/10^3 cells in F39 fibroblasts after stimulation for 48 h) and fibronectin (from 31.8 ± 4.5 μg/10^3 cells to 48.7 ± 3.5 μg/10^3 cells, again after 48 h of stimulation) compared with controls in both cell lines, no significant effect could be observed using three different concentrations of FGF-2, although there was a tendency for a decrease in secretion for collagen type I in both cell lines (data not shown).

Increased FGF-2 expression correlates with increased interstitial and tubular MIB-1 staining

After having established that FGF-2 is a mitogen for human cortical kidney fibroblasts in vitro, we tried to determine whether increased FGF-2 expression was correlated with increased proliferative activity in vivo. Thus, we stained for expression of the Ki-67 antigen, which is associated with cell proliferation using the MIB-1 antibody. We were able to perform stainings for MIB-1 in cortical tissue from 24 patients and 8 normal controls. Fifteen patient specimens could not be analyzed because of the lack of tissue that had been used up for other purposes (specimens from patients 5, 7, 14 through 17, 21, 23, 27, 29, 30, 32, and 35 through 37). In normal kidneys, only few interstitial and tubular cells displayed MIB-1 positivity (median 1.5 in both; Fig. 10A), whereas

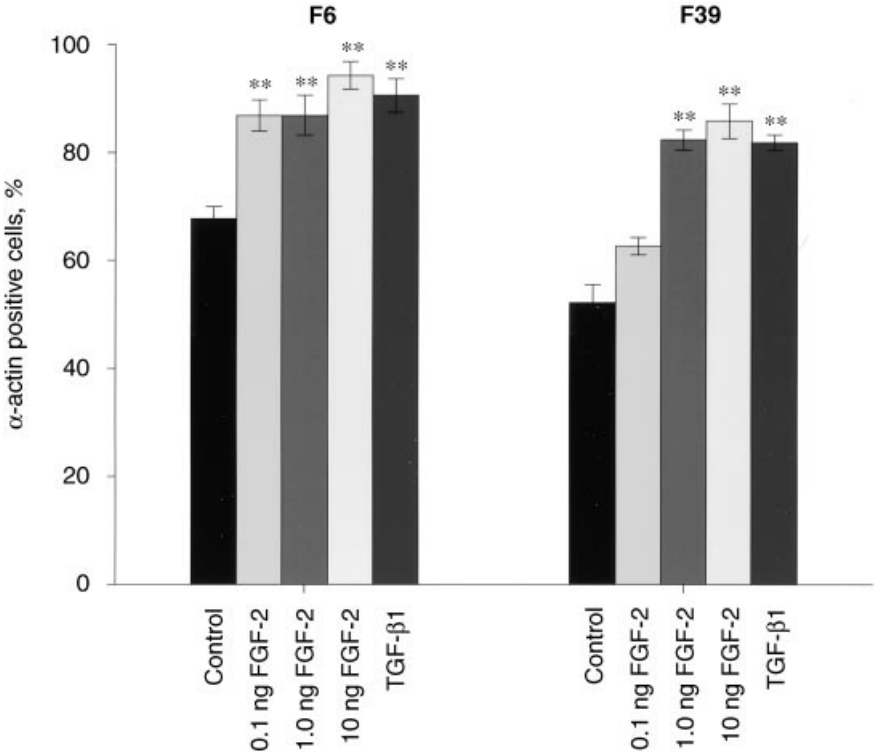

Fig. 9. Expression of α-smooth muscle actin in primary cortical fibroblasts. The number of α-smooth muscle actin-positive cells was determined after incubation with three different concentrations of FGF-2 (0.1, 1.0, or 10 ng/mL) for 48 hours. Iscove's medium served as a negative control, and TGF-β1 (1 ng/mL) served as a positive control. FGF-2 induced the expression of α-smooth muscle actin in both cell lines significantly. $^{**}P < 0.01$ vs. control.

the number of MIB-1–positive cells was considerably higher in patient specimens (median 13.5, $P = 0.001$ in interstitial, median 9.0, $P = 0.008$ in tubular cells; Fig. 10 B–D). The highest number of MIB-1–positive cells within the interstitium was observed in the two biopsies from patients with tubulointerstitial nephritis (numbers 33 and 34), which is not surprising given the high proliferative activity of infiltrating monocytes/macrophages and lymphocytes. When the number of MIB-1–positive cells was correlated with the semiquantitative FGF-2 staining, there was a positive correlation with the number of interstitial ($r = 0.48$, $P < 0.01$) and tubular ($r = 0.53$, $P < 0.01$) positive cells. However, the correlation was much more robust when we omitted the two patients with acute interstitial nephritis, who had a high proliferative activity of infiltrating mononuclear cells but a low level interstitial FGF-2 staining. In that case ($N = 30$), correlation of FGF-2 staining with MIB-1–positive interstitial cells ($r = 0.71$, $P < 0.01$) and tubular cells ($r = 0.66$, $P < 0.01$) was much better. The results are displayed in Figure 11. Thus, the results of the MIB-1 staining demonstrate that FGF-2 positivity is correlated positively with the number of MIB-1–positive cells within the interstitium and in tubular epithelia in scarring processes. This was even true when glomerular diseases were analyzed separately with a positive correlation between FGF-2 staining and interstitial MIB-1 positivity ($r = 0.64$, $P < 0.05$), although the number of analyzed tissues was small ($N = 11$). There does not seem to be a correlation of FGF-2 staining with acute inflammatory processes, although the number of tissues studied is too limited in that regard.

DISCUSSION

Virtually all forms of chronic progressive renal disease are accompanied by changes in the tubulointerstitium, irrespective of the primary renal disease [38]. This is not surprising since renal tubules, interstitium, and vasculature comprise more than 80% of the renal cortex [39]. Tubulointerstitial changes consist of interstitial inflammation followed by fibrosis, tubular atrophy, and dilation. Along with glomerular scarring, interstitial fibrosis is an accurate predictor of renal function, even for primary glomerular diseases [29]. One of the most perplexing features of renal fibrogenesis is the fact that fibrosis often progresses despite resolution of the primary inflammatory stimulus [40]. Thus, we and others have propagated the existence of autocrine cytokine loops, which may represent one of the key differences between regular wound healing and scarring [41–43].

The current studies that combined descriptional and functional analyses were undertaken to elucidate the role of FGF-2 in renal fibrogenesis. We have carefully analyzed the usefulness of the antibody used in our study given the controversial information regarding the detection of FGF-2. Different splice variants may be the reason that a variety of studies came to different conclusions

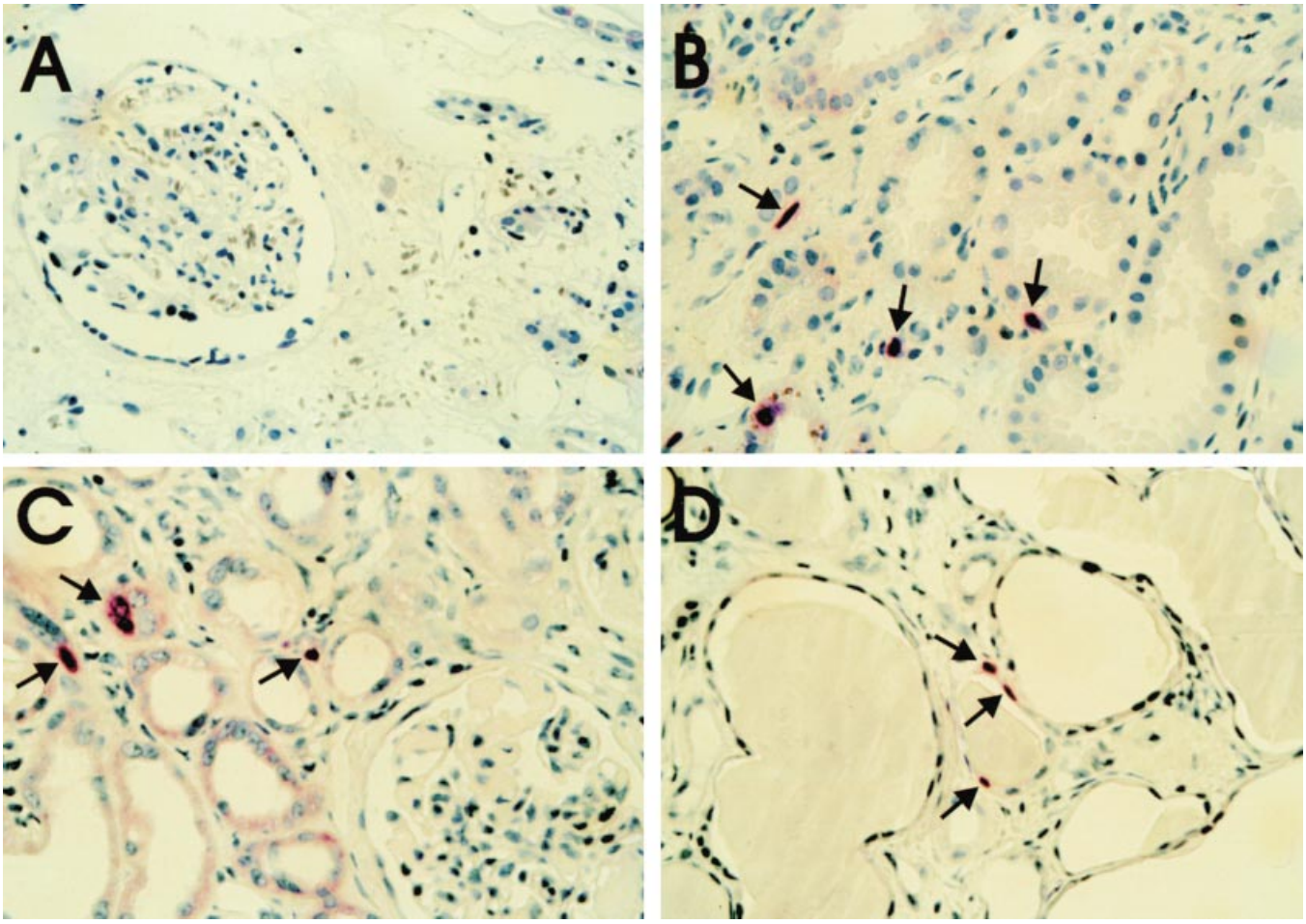

Fig. 10. Immunohistochemical staining for MIB-1–positive cells. Whereas normal kidneys displayed either no or only occasional MIB-1–positive cells (*A*), staining of kidneys with variable degrees of interstitial scarring demonstrate positive tubular and interstitial cells (highlighted by arrows). (*B*) Taken from patient 4 (IgA nephritis, interstitial space 46%, FGF-2 +++). (*C*) Taken from patient 36 (SLE, interstitial space 42%, FGF-2 ++). (*D*) Taken from patient 10 (end-stage renal disease caused by amyloidosis, interstitial space 68%, FGF-2 +++). Original magnification ×400.

regarding the localization of FGF-2. As was shown in the medullary Tk 173 and Tk 188 fibroblasts, the Ab-2 antibody mainly detects the 18 kD band, but also the 22.5 and 24 kD bands described previously [35]. These higher molecular weight forms may target intracellular FGF-2 to a nuclear localization [44]; however, their exact role is currently unclear. The higher molecular weight forms could not be detected in our primary cortical fibroblasts. Thus, the 18 kD band seems to be the dominating isoform in kidney fibroblasts.

Our study demonstrates that FGF-2 protein can be detected in glomerular, vascular, a few (mainly distal) tubular, as well as interstitial fibroblast-like cells in normal human kidneys. In cryosections, more interstitial cells were labeled than in paraffin-embedded tissue sections, which may explain the differences to the study by Floege et al, who described only occasional interstitial cell positivity [18]. The in situ hybridizations confirmed that the same cells that stain positive for FGF-2 in cryosections also synthesize FGF-2 mRNA. In addition, we have demonstrated for the first time that the expression of FGF-2 protein and mRNA is increased in kidneys with tubulointerstitial scarring, irrespective of the primary disease. Furthermore, our study has shown that primary cortical fibroblasts express FGF-2 and one of its high-affinity receptors. FGF-2 causes a robust induction in proliferation in these cells and induces α-smooth muscle actin expression as marker for myofibroblast formation but has no major effect on synthesis of collagen type I and fibronectin. Proliferative activity in tubular epithelial and interstitial cells is increased in diseased human kidneys and correlates with staining for FGF-2. Finally, basal proliferation in cortical fibroblasts can be reduced by the addition of a neutralizing antibody to FGF-2, pointing to a possible autocrine mechanism of fibroblast proliferation. Thus, our study provides evidence for the existence of an autocrine loop in human renal fibrogenesis, which is characterized by constitutive

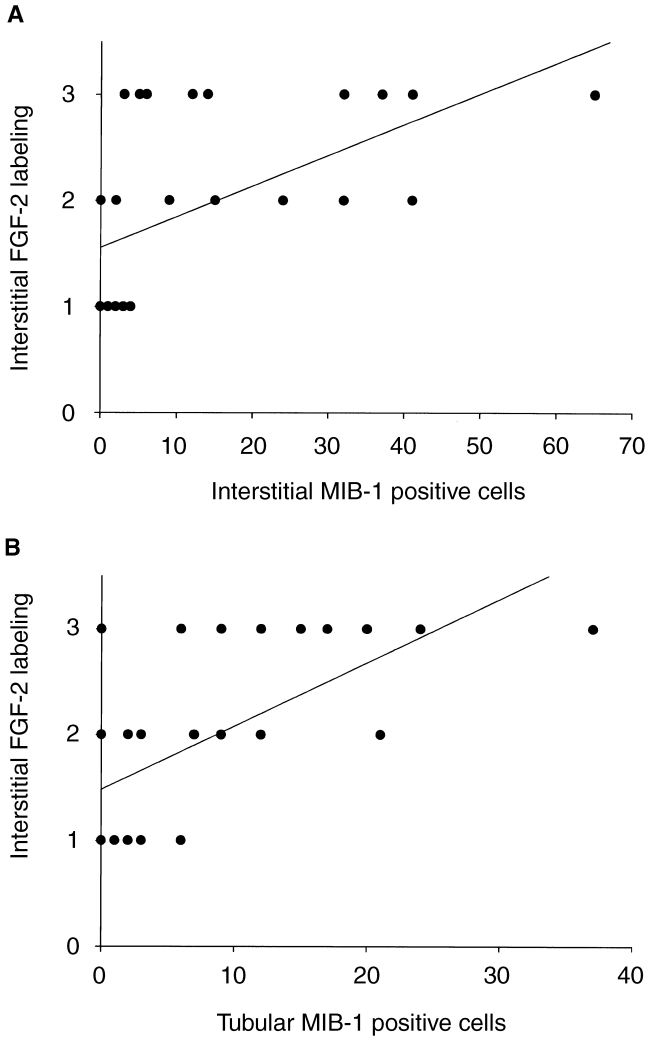

Fig. 11. Correlation of interstitial FGF-2 labeling with MIB-1–positive interstitial (*A*) and tubular epithelial (*B*) cells. There was a positive correlation between semiquantitative FGF-2 labeling and the number of proliferating interstitial (A; $P < 0.01$, $r = 0.71$), as well as tubular epithelial (B; $P < 0.01$, $r = 0.66$) cells. Two cases of acute tubulointerstitial nephritis have been omitted for this analysis (see results for details). The r depicts the value for Spearman's rank order coefficient.

expression of FGF-2 and its receptors, up-regulation in disease state, induction of proliferation, and decreased proliferation after neutralization of the cytokine.

The increased expression of FGF-2 in renal fibrogenesis, although demonstrated for the first time, is not surprising. The association of FGF-2 expression with proliferative fibrogenesis was first demonstrated by Gonzalez et al in patients with Dupuytren's contracture [45]. They noted already intense staining for FGF-2 associated with increased synthesis for FGF-2 and FGFR-1 mRNA in fibroblasts. Charlotte et al found increased expression of FGF-2 in the carbon tetrachloride model of rat liver fibrosis [46]. In that study, FGF-2 expression was localized to myofibroblastic liver cells. Interestingly, FGF-2 knockout mice display delayed wound healing [47], further pointing to a critical role for the cytokine in matrix formation. Unlike most other polypeptide growth factors, FGF-2 lacks a conventional leader sequence for secretion, and the mechanisms for its release are still unclear [11]. Chronically injured cells, however, release FGF-2 efficiently, and the mitogenic effect of the cytokine is potentiated by tissue injury [48]. In addition, as was originally shown by Mignatti, Morimoto, and Rifkin, FGF-2 may be secreted by viable cells [49].

What is the role of proliferation in renal fibrogenesis? Proliferation was thought to play only a little role, if at all, in the progression of renal disease. However, as was demonstrated by Nadasdy et al in a study of 28 human end-stage kidneys, even scarred kidneys display high proliferative activity in tubuli and interstitial cells [50]. Similar results were recently described by Thomas et al in the subtotal nephrectomy model of chronic renal failure [51]. These findings are corroborated by our study. It is intriguing to speculate that fibroblasts and tubular epithelial cells stimulate each other in the process of renal fibrogenesis, as indicated by the paralleled proliferation. We and others have recently demonstrated that tubular epithelial cells may acquire certain characteristics of fibroblasts [27, 52]. Although we did not examine this aspect in the current study, FGF-2 may be a candidate cytokine that could play a role in that process. Clearly, the interaction between tubular epithelial cells and fibroblasts plays a role not only in the initiation of interstitial inflammation, but also in progressive renal scarring. In the study by Ray et al in their transgenic mouse model of HIV-associated nephropathy, FGF-2 detection was associated with interstitial areas that contained both cell injury and proliferation [25]. Thus, proliferation does play a critical role in the progression of renal disease, and autocrine/paracrine secretion of FGF-2 may be important in the perpetuation of the process. Autocrine stimulation of cell proliferation caused by FGF-2 has been described in a number of cells, including tubular epithelial [43], endothelial [53], and vascular smooth muscle [54] cells. In these cell types, as in cortical fibroblasts, the efficacy of the neutralizing antibody indicates that the protein does get secreted before binding to its receptors.

Basic fibroblast growth factor exerts its effects via low- and four high-affinity receptors. The high-affinity receptors consist of four transmembrane tyrosine kinases (FGFR-1 to -4) [11]. Our study has demonstrated that primary cortical fibroblasts express FGFR-1, also known as *flg*, and heparan sulfate proteoglycan, which potentiates the binding to the high-affinity receptors [55]. Floege et al noted occasional interstitial staining for *flg* [18]. In our study, only three of the five examined fibroblast lines from kidneys with interstitial fibrosis displayed an up-regulated expression for FGFR-1. One possible explana-

tion is the redundancy of the fibroblast receptor system, including multiple splice variants. In a recent study by Ford et al, all seven receptor isoforms assayed by the group were expressed in whole cortex by reverse transcription-polymerase chain reaction assay [56]. Thus, increased expression of one of the other high-affinity receptors may explain the lack of up-regulation of FGFR-1 in two kidneys with interstitial scarring. Alternatively, down-regulation of FGFR-1 during a late stage of fibrogenesis may protect the kidney from uncontrolled proliferation. The mechanism of overexpression of FGF-2 and FGFR-1 has been described in certain tumor cells, including human hepatoma cells [57].

Heparan sulfate proteoglycan is a low-affinity receptor for FGF-2. Its expression in all seven primary cortical fibroblasts may explain why heparin (which otherwise is known to increase the proliferative effects of FGF-2) did not have any additional effects on proliferation. Conversely, the slight reduction in proliferation caused by heparin itself may be explained by competition with this low-affinity receptor [58].

Our findings of FGF-2 and FGFR-1 detection in human kidney fibroblasts or fibroblast-like cells are in concordance with observations by other groups in dermal [59], mammary gland [60], and human liver [61] fibroblasts. However, we extend those findings, demonstrating that renal fibroblasts (or fibroblast-like cells) do express FGF-2 in vivo as well. The interstitial cells that express FGF-2 are predominantly renal fibroblast-like cells as shown by double labelings. Vimentin, α-smooth muscle actin, CD 44, and CD 54 are all markers for fibroblasts in the kidney [36, 37]. However, though almost all CD 44- and CD 54-positive cells within the interstitium expressed FGF-2, some interstitial cells displayed exclusive FGF-2 positivity. In addition, a number of α-smooth muscle actin-positive cells were negative for FGF-2. Renal interstitial fibroblasts are a heterogeneous population [62], and studies on these cells are still hampered by the lack of a definite fibroblast marker [1]. Most of these cells seem to express FGF-2 in vivo, although some subpopulations may not. Conversely, some nonfibroblast cells such as monocytes/macrophages or dendritic cells express FGF-2, although the majority of these cells do not under normal conditions.

Previous studies suggested that extrarenal sources may contribute to FGF-2 deposition in the kidney [25]. Although we cannot completely exclude that possibility, it seems unlikely given the marked up-regulation of FGF-2 mRNA synthesis in fibrotic kidneys, as demonstrated by the in situ hybridization studies. Increased staining for FGF-2 in renal disease has been described before in various animal models [14, 25] and in patients with focal and segmental glomerulosclerosis and IgA nephropathy [63]. In that latter study, FGF-2 could be detected in glomeruli and in areas of tubulointerstitial damage, correlating with the degree of interstitial injury. Morita et al examined the expression of FGF-2 binding domains in human kidneys. FGF-2 binding heparan sulfate domains are present in the basement membrane proteoglycan perlecan, but are absent from other glycosaminoglycans such as fibroglycan and glypican [64]. Heparan sulfate was detected in sections with glomerular sclerosis and interstitial fibrosis; however, only the fibrotic sections contained FGF-2 binding [65]. Interestingly, a small population of macrophage-like parenchymal cells was identified in that study which expressed the FGF-2–binding domain on the cell surface, which is in concordance with the results of our study.

No significant effect of FGF-2 on synthesis of matrix components was observed in our study, although there was a tendency for decreased synthesis of collagen type I. Decreased synthesis of collagen type I in cells of mesenchymal origin after incubation with FGF-2 has been described before. Tan et al, for example, demonstrated a down-regulation of collagen gene expression in keloid fibroblasts [66]. Thus, unlike TGF-β1, a role of FGF-2 in the accumulation of extracellular matrix is not supported by our study nor by the literature. However, FGF-2 does induce expression of α-smooth muscle actin as an indicator of myofibroblast formation. In that regard, it is similar to TGF-β1, which is the prototype of a profibrogenic cytokine in the kidney. Expression of α-smooth muscle actin has been correlated with interstitial matrix formation and even with prognosis in a number of studies in the kidney [67] and other organs [68]. It certainly represents a critical step in fibrogenesis [69].

In summary, we have demonstrated that FGF-2 is expressed in interstitial fibroblast-like cells and that it is robustly up-regulated within these and tubular epithelial cells in human kidney fibrosis. Whereas FGF-2 does not seem to play a role in interstitial matrix production, it may have a critical role in fibroblast proliferation and myofibroblast formation. The continuity of fibroblast proliferation is probably one of the major differences between fibrosis and restitutional healing. Our study points to an important role of FGF-2 in that process. Clearly, further studies are necessary that delineate the differences between regular wound healing with no or minor scar formation and interstitial fibrosis resulting in progressive loss of organ function.

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APPENDIX

Abbreviations used in this article are: ABC, avidin-biotin complex; CRF, chronic renal failure; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESRD, end-stage renal disease; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; FGF-1, acidic fibroblast growth factor; FGF-2, basic fibroblast growth factor; FGFR-1, fibroblast growth factor receptor-1; FSGS, focal segmental glomerulosclerosis; MGN, membranous glomerulonephritis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; RPGN, rapidly progressive glomerulonephritis; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TGF-β, transforming growth factor-β; and TIN, acute tubulointerstitial nephritis.

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