TGF-β1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2)

Frank Strutz, Michael Zeisberg, Anja Renziehausen, Birgit Raschke, Volker Becker, Cees van Kooten, and Gerhard A. Müller

Department of Nephrology and Rheumatology, Georg-August-University Göttingen, Göttingen, Germany, and Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands

TGF-β1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2).

Background. The prognosis of primary renal disease is often dependent on the degree of tubulointerstitial scarring. Scarring is caused by proliferation and excessive matrix production of renal fibroblasts and possibly other cellular elements. Transforming growth factor-β (TGF-β) is the most important cytokine for the induction of matrix synthesis in the kidney. However, its effects on renal fibroblast proliferation have not been determined. We have recently demonstrated that the expression of basic fibroblast growth factor (FGF-2) is robustly up-regulated in human kidneys with tubulointerstitial fibrosis and that FGF-2 is a potent inducer of fibroblast proliferation. The present study examined the interaction between TGF-β1 and FGF-2 in human renal fibroblasts.

Methods. Experiments were performed on a transformed medullary fibroblast line and on primary cortical kidney and skin fibroblasts isolated from human biopsies. mRNA levels of FGF-2 and TGF-β1 were analyzed by Northern blot analyses. Changes in protein expression were examined by immunoblots and enzyme-linked immunosorbent assay (ELISA). Bromodeoxyuridine incorporation assays and cell counts were used to analyze cell proliferation. The expression of cell cycle-regulatory proteins cyclin-dependent kinase (cdk) 2 and the cdk inhibitor p27^{kip1} were determined by immunoblots.

Results. Stimulation of renal fibroblasts with FGF-2 resulted in no change of TGF-β1 mRNA expression, whereas incubation of the cells with TGF-β1 induced FGF-2 mRNA up to 3.51 ± 0.21-fold after six hours. This increase could be blocked almost completely by the addition of cyclohexamide, indicating that the process is in large part dependent on protein synthesis. The up-regulation in FGF-2 mRNA expression was paralleled by de novo detection of FGF-2 protein in the supernatant, peaking after 12 to 24 hours, as determined by Western blot and ELISA, whereas cellular protein was only increased up to 2.1-fold. Interestingly, both methods detected release of FGF-2 protein to the supernatant already at three hours, indicating a role for TGF-β1 in directly releasing preformed FGF-2. Since

TGF-β1 induced FGF-2, which results in fibroblast proliferation, we hypothesized that TGF-β1 may cause fibroblast proliferation mediated by FGF-2. This hypothesis was verified by cell proliferation assays demonstrating that stimulation of renal fibroblasts with TGF-β1 resulted in an up to 3.21 ± 0.28-fold increase in bromodeoxyuridine incorporation and a 1.95 ± 0.16-fold increase in cell number after 72 hours. This mitogenic effect of TGF-β1 could be blocked completely by the addition of a neutralizing antibody to FGF-2 or the tyrosine kinase inhibitor tyrphostin AG1296, which blocks FGF receptor (FGFR) tyrosine kinase activity. Conversely, a neutralizing antibody to epidermal growth factor (EGF) or the tyrphostin B42, which inhibits EGF receptor signal transduction, had no effect. Interestingly, a neutralizing antibody to PDGF had only minor effects in primary kidney fibroblasts but reduced TGF-β1–induced proliferation considerably in primary skin fibroblasts. Finally, TGF-β1–induced proliferation in kidney fibroblasts was paralleled by a robust increase in cdk 2 protein expression up to 72 hours, whereas p27^{kip1}, whose activity is maintained by TGF-β in epithelial cells, was down-regulated up to 48 hours.

Conclusions. Our studies demonstrate, to our knowlege for the first time, that TGF-β1 induces proliferation in human renal fibroblasts and that this process is mediated largely by FGF-2. The induction of proliferation by TGF-β1 via induction of FGF-2 may play an important role in the autonomy of renal fibroblast growth and thus in the pathogenesis of human fibrogenesis.

Interstitial fibrosis invariably accompanies the course of chronic renal failure toward end-stage renal disease [1]. A great number of studies have demonstrated that interstitial scarring is an accurate predictor of renal function in primary glomerular diseases, diabetic nephropathy, hypertensive nephropathy, polycystic kidney disease, and chronic pyelonephritis [reviewed in 2]. Cytokines play an important role in the pathogenesis of renal fibrogenesis, including platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). PDGF is a strong inducer of proliferation in some but not all fibroblasts [3]. TGF-β is probably the most important cytokine for the accumulation of matrix in renal fibrogenesis [4]. It induces the formation of interstitial matrix

proteins, including collagen types I and III, as well as fibronectin. Furthermore, it induces the expression of the plasmin activator inhibitor-1, thus delaying matrix degradation [5]. TGF-β1 expression was found to be elevated in several animal models of chronic progressive renal disease associated with interstitial fibrosis, including Heymann nephritis [6], antiglomerular basement membrane disease [7], purine aminonucleoside nephrosis [6], hypertensive [8] and obstructive nephropathy [9], chronic rejection [10], as well as most recently in a model of fibrosis following papillary necrosis [11]. Furthermore, mice expressing the TGF-β1 transgene in the liver under control of the murine albumin promoter developed glomerulosclerosis and tubulointerstitial fibrosis [12]. Thus, TGF-β has been proposed as a potential target for gene therapy to prevent the progression of chronic renal disease [13].

The role of other cytokines in renal fibrogenesis is less well defined. One potential candidate for an important role in the pathogenesis of kidney fibrosis is basic fibroblast growth factor (FGF-2). FGF-2 is a single-chain polypeptide with a molecular weight of 18 kD, which binds to four high-affinity receptors [FGF receptor-1 (FGFR-1) to FGFR-4]. These FGFRs function as protein tyrosine kinases and regulate a wide variety of cellular processes [reviewed in 14]. FGF-2 has been implicated in the pathogenesis of skin, liver, and lung fibrosis. Charlotte et al, for example, found increased expression of FGF-2 in carbon tetrachloride-induced liver fibrosis [15]. In the kidney, FGF-2 is a potent mitogen for proximal tubular epithelial and mesangial cells [16, 17]. Furthermore, subcutaneous injections into rats for 8 and 13 weeks caused focal segmental glomerulosclerosis and widening of the peritubular interstitium [18]. Finally, in a transgenic mouse model of HIV nephropathy, interstitial FGF-2 staining was increased and colocalized with extracellular matrix [19]. Fibroblasts are the main effector cells in human renal fibrogenesis, although other cells may play a role as well as was demonstrated by us and other groups [20, 21]. Recently, we have shown that FGF-2 protein and mRNA expression are increased in human kidneys with tubulointerstitial fibrosis and that the degree of fibrosis correlates well with staining for FGF-2 [22]. Moreover, we have shown that FGF-2 is a potent mitogen for human cortical fibroblasts and that these cells express the FGFR-1 as well as low-affinity receptors (heparan sulfate proteoglycans). Since a neutralizing antibody to FGF-2 was capable of inhibiting basal fibroblast proliferation, we concluded that FGF-2 may mediate autocrine fibroblast proliferation.

Thus, the aim of the present study was to analyze the interaction between FGF-2 as a mediator of fibroblast proliferation and TGF-β1 as an inducer of extracellular matrix synthesis in human renal fibroblasts in vitro. We demonstrate that TGF-β1 induces FGF-2 synthesis on the mRNA and protein levels robustly, which may result in the release of preformed FGF-2. Furthermore, we show that TGF-β1 promotes proliferation in medullary and cortical fibroblasts and that this effect is mediated mainly by induction of FGF-2. Finally, we demonstrate that TGF-β1–induced proliferation is characterized by increased synthesis of cyclin-dependent kinase (cdk) 2 and a decrease in p27^{kip1} synthesis up to 48 hours.

METHODS

Materials

Human recombinant FGF-2 and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA), as were the neutralizing antibodies to epidermal growth factor (EGF; goat polyclonal), FGF-2 (goat polyclonal), PDGF (rabbit polyclonal), and TGF-β (chicken polyclonal). Rabbit polyclonal antibody to FGF-2 (Ab-2) was obtained from Calbiochem (La Jolla, CA, USA). The following mouse monoclonal antibodies were used for cell characterization: anticollagen type I, anticollagen type III (both from Southern Biothechnology, Birmingham, AL, USA), anticytokeratin (Dako, Carpinteria, CA, USA), antivimentin (Boehringer Mannheim, Mannheim, Germany), antifactor VIII (Dako), anti–HLA-DR (Dako), anti–α-smooth muscle actin (Paesel+Lorei, Wiesbaden, Germany), anti-CD 44 (Pharmingen, San Diego, CA, USA), and 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). FGF-2 and TGF-β1–specific oligonucleotides were purchased from R&D Systems, and tyrphostines AG1296 and B42 were from Calbiochem-Novabiochem (Nottingham, UK). Rabbit polyclonal anti-cdk 2 antibody was also obtained from Calbiochem-Novabiochem. The anti-p27^{kip1} antibody was from Transduction Laboratories (Lexington, KY, USA). Ham's F-12 medium was from Seromed Biochem. KD, Berlin, Germany. Bovine collagen, insulin, transferrin, selenium, hydrocortisone, and tri-iodothyronine were purchased from Sigma (St. Louis, MO, USA). Trypsin-ethylenediaminetetraacetic acid [0.05/0.02% (wt/vol)], 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).

Cell culture

Cortical fibroblasts and tubular epithelial cells were cultured from biopsies after patients had given their consent to a second biopsy for research purposes. Skin fibroblasts were also established from skin biopsies at the time of kidney biopsy as controls. The use of parts of kidney and skin biopsies for research purposes was approved by the Ethics Committee of the Georg-August-

University. Written consent was obtained from all patients prior to kidney and skin biopsy. Biopsies were performed according to standard procedures using the Biopty gun with a 18-gauge needle under real-time ultrasound guidance. For isolation of primary human kidney fibroblasts, cortical parts of renal biopsy cylinders were cut and emerged in DMEM medium supplemented with 20% FCS, penicillin (100 U/mL), and streptomycin (100 U/mL). When cells had grown to confluency, cells were split 1:1 and were characterized by immunofluorescence for cytokeratin, vimentin, α-smooth muscle actin, collagen types I, III, and IV, factor VIII, CD 44, CD 54, CD 68, and HLA-DR. Cells that were positive for vimentin, CD 44, CD 54, α-smooth muscle actin, and the collagen types I and III and negative for cytokeratin, factor VIII, and HLA-class II were considered to be fibroblasts and used for induction assays in passages 2 through 8. In selected stainings, additional staining with the Hoechst dye H33258 was performed to facilitate cell counting. For quantitation of expression of α-smooth muscle actin, cytokeratin, and collagen types I and III, 400 cells were counted, and the relative percentage of positive cells was determined.

Primary human proximal tubular epithelial cells (PTECs) were cultured according to the method by Detrisac et al and characterized as described previously [23, 24]. PTECs were grown on a matrix of bovine collagen and heat-inactivated FCS and cultured in serum-free DMEM and Ham's F-12 medium in a 1:1 ratio supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), selenium (5 μg/mL), hydrocortisone (36 ng/mL), triiodothyronine (40 pg/mL), and EGF (10 ng/mL). Cells were used in passages 3 through 6.

Epithelial skin biopsies, obtained at the time of renal biopsy from the puncture site, were treated as described previously for cortical fibroblast culture. Skin fibroblasts were characterized as described for kidney fibroblasts and used in passages 2 through 8. Human renal medullary fibroblast cell lines Tk 173 (obtained from a normal kidney) and Tk 188 (from a kidney with tubulointerstitial fibrosis) have been characterized previously [25] and were cultured in DMEM supplemented with 10% FCS, penicillin, and streptomycin (100 U/mL each).

mRNA analyses

To analyze possible interactions between FGF-2 and TGF-β1, cells were rested for 24 hours in Iscove's medium prior to stimulation with FGF-2 (1 ng/mL) or TGF-β1 (1 ng/mL). After 0, 3, 6, 9, 12, and 24 hours, total cellular RNA was extracted using RNA-clean™ (AGS, Heidelberg, Germany) according to instructions available from the manufacturer with minor modifications. RNA concentrations were determined at 260 nm, and samples were stored at −80°C prior to use. Northern blot analysis was performed as described previously with

modifications [20]. Briefly, 40 μg of total RNA were electrophoresed on a 1.0% agarose gel containing 2.2 mol/L formaldehyde. One × MOPS, pH 7.0, was used as the running buffer. Ethidium bromide-stained gels were subsequently photographed under ultraviolet illumination, and RNA was transferred to a nylon membrane (Hybond N; Amersham, Arlington Heights, IL, USA) by capillary transfer overnight. Blots were than baked at 80°C for two hours 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 ethylenediaminetetraacetic acid (EDTA; pH 8), 25% sodium dodecyl sulfate (SDS), and 1.5% blocking reagent. RNA from TGF-β1–stimulated cells was then hybridized with FGF-2–specific oligonucleotides, RNA from FGF-2–stimulated cells with TGF-β1 specific oligonucleotides. Oligonucleotides had been labeled with digoxigenin according to instructions from Boehringer Mannheim. Hybridizations were performed for 12 hours at 58°C. After washing, anti-DIG alkaline phosphatase and CSPD substrate (both Boehringer Mannheim) were added. Positive reaction products were identified by chemiluminescence using a Fluor-S™ Multiimager (Bio-Rad, Hercules, CA, USA), and densitometric analysis was performed using Multi-Analyst™ software (Bio-Rad). All blots were stripped and hybridized with digoxigenin labeled 18S-RNA (Ambion, Austin, TX, USA) as control. Data from five different blots with RNA from independent experiments were summarized and are given after correction for 18S-RNA density.

For further investigation of the mechanisms of TGF-β1–induced FGF-2 mRNA synthesis, experiments were repeated in the presence of cyclohexamide (1 μg/mL) to inhibit translation. These experiments were repeated three times, and results are given corrected for 18S-RNA density. Toxicity of cyclohexamide was evaluated by lactic acid dehydrogenase (LDH) measurements in supernatants by routine procedures.

Protein measurements

Protein synthesis of FGF-2 after induction by TGF-β1 was analyzed by immunoblot analysis and enzyme-linked immunosorbent assay (ELISA). Tk 173 cells were plated at a density of 6000 per well and were serum deprived over 24 hours by culture in Iscove's medium. Subsequently, cells were stimulated with 1 ng/mL TGF-β for 0, 3, 6, 12, 24, and 48 hours without additional additives. Lysates were obtained with a detergent-based buffer containing 0.4% sodium deoxycholate, 1% NP-40, 1.9% EGTA, and 10 mmol/L Tris (pH 7.4); 100 μg of total cellular protein were run on an 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane (Hybond™ ECL™; Amersham). Supernatants were concentrated ×10 and loaded after correction for cell number. Membranes

were subsequently stained with Ponceau red to control for adequate transfer and equally loaded amounts, blocked with a phosphate-buffered saline (PBS) solution containing 0.2% Tween 20 and 5% dry milk and incubated with an anti–FGF-2 antibody at a concentration of 1:40. After several washes, and incubation with the secondary antibody (donkey-anti-rabbit, horseradish peroxidase linked; Amersham) ECL-Plus (Amersham) was added according to the manufacturer's protocol to identify positive reaction products by chemiluminescence using the Fluor-S™ Multiimager. Western blots were independently performed three times from independent experimental series with qualitatively similar results. Densitometric analyses were carried out using Multi-Analyst™ software, and data of all series were summarized.

Fibroblast growth factor-2 protein secretion was quantitated by commercial ELISA (Qantikine immunoassay human bFGF; R&D Systems, DFB 50) in parallel experiments according to instructions available from the manufacturer. This assay has a sensitivity of 3 pg/mL with a working range of 10 to 640 pg/mL. There is no known cross-reactivity to other cytokines and growth factors. Cells were plated and prepared as detailed. Subsequently, cells were stimulated with 1 ng/mL TGF-β for 0, 3, 6, 12, 24, and 48 hours. Cells were counted after supernatants had been removed to account for TGF-β1–induced proliferation, and FGF-2 measurements were corrected for cell number. Results are given in pg per mL per 1000 cells.

Proliferation assays

Proliferation studies were performed using bromodeoxyuridine incorporation assays and cell counts. In a first set of experiments, Tk 173 and Tk 455 were plated at 4×10^3 cells per well in 96-well microtiter plates containing DMEM medium with FCS and incubated overnight. Cells were then made quiescent by replacement of DMEM with Iscove's medium and subsequent incubation for 24 hours. TGF-β1 was added thereafter in a concentration of 1.0 ng/mL, and proliferation was measured after 24, 48, 72, and 96 hours by nonradioactive bromodeoxyuridine incorporation assays (Amersham) based on the method by Gratzner et al [26]. Assays were performed according to the manufacturer's instructions with some modifications. Iscove's medium without additives served as the negative, and FGF-2 in a concentration of 10 ng/mL served as the positive control. Neutralizing antibodies to TGF-β1, FGF-2, or EGF were added to selected cells stimulated with TGF-β1. The appropriate concentrations of the neutralizing antibodies (10 μg/mL for anti-FGF-2, 10 μg/mL for anti-EGF, 10 μg/mL for anti-PDGF, and 1 μg/mL for anti–TGF-β1) were identified in control experiments neutralizing the proliferative effects of EGF (1 ng/mL) on PTECs, FGF-2 (1 ng/mL) and PDGF (1 ng/mL) on Tk 173 fibroblasts, or neutral-

ization of the stimulation of collagen type I secretion by TGF-β1 (10 ng/mL) in Tk 188 cells. Medium was changed to Iscove's containing bromodeoxyuridine/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 further washing for three times in PBS containing 0.1% Tween and blocking for 30 minutes in PBS (0.1% Tween, 3% bovine serum albumin), 50 μL of anti-bromodeoxyuridine 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, Denkendorf, Germany).

In a second set of experiments, confluent monolayers of Tk 173 and Tk 455 kidney fibroblasts were trypsinized, and cells were seeded at 4×10^3 cells/well in 96-well culture plates. After 12 hours of incubation, DMEM medium was replaced by Iscove's medium. After an additional 24 hours, cells were stimulated with three different concentrations of TGF-β1 (0.1, 1.0, and 10 ng/mL). FGF-2 at a concentration of 10 ng/mL served as positive control, and Iscove's medium alone served as the negative control. Anti–TGF-β1 and anti–FGF-2 were added to selected wells in the previously mentioned concentrations. In addition, the effect of an irrelevant IgG (anti–HLA-DR) was examined in a concentration of 10 μg/mL. Cells were counted after 72 hours using a Neubauer chamber.

The effect of the tyrosine kinase inhibitors tyrphostin AG1296, which inhibits the FGF-receptor tyrosine kinase, and tyrphostin B42 (AG 490), which blocks the EGF receptor kinase, was then analyzed in a similar experimental setting. The dose of the tyrphostines was determined in experiments neutralizing the proliferative effects of FGF-2 (1 ng/mL) on Tk 173 fibroblasts and of EGF (1 ng/mL) in PTECs. Tk 173 and Tk 455 kidney fibroblasts were again plated at 4×10^3 cells per well. TGF-β1 was added at a concentration of 1 ng/mL after 24 hours of incubation in Iscove's medium. In addition to TGF-β, neutralizing antibodies to TGF-β or FGF-2 were added (both at concentrations of 10 μg/mL) as well as the tyrphostines AG 1296 (at a concentration of 5 μmol/L) and B42 (concentration 500 nmol/L). Iscove's alone served as negative control. Cells were counted after 24, 48, 72, and 96 hours. In control experiments, the effects of both tyrphostines on FGF-2–induced proliferation of both fibroblast lines after 72 hours were assayed. Serial LDH measurements were obtained by standard procedures from supernatants containing the tyrphostines to exclude toxic effects.

In a final set of proliferation experiments, primary cortical fibroblasts Tk 458, Tk 469, and primary skin fibroblasts Sk 448 and Sk 449 were plated at a density

of 4×10^3 cells per well. All cells were made quiescent by incubation in Iscove's medium without additives for 24 hours and subsequently stimulated with TGF-β1 at 1 ng/mL. In selected wells, neutralizing antibodies to TGF-β1 (10 μg/mL), FGF-2 (10 μg/mL), PDGF (10 μg/mL), or EGF (5 μg/mL) were added, and bromodeoxyuridine incorporation was determined after 72 hours. All proliferation assays were performed in triplicate and repeated four times.

Immunoblot analyses for cell cycle proteins

Western blot analyses were performed as described previously in this article with minor modifications. Tk 173 and Tk 455 fibroblasts were incubated with 1 ng/mL TGF-β1, and lysates were obtained after 0, 24, 48, and 72 hours. Again, 100 μg of protein were run on an 18% SDS-PAGE gel, and membranes were stained with Ponceau red after transfer. Anti-cdk 2 antibody was used in a concentration of 1:500, and anti-p27^{kip1} antibody was used in a concentration of 1:2500. Donkey-anti-rabbit and goat-anti-mouse horseradish peroxidase-linked antibodies (Amersham) were used as the secondary antibodies. Positive reaction products were identified by chemiluminescence as described previously in this article. Quantitative analyses were again obtained using Multi-Analyst™ software. In control experiments, cells were stimulated for 0, 24, 48, and 72 hours with TGF-β1 (1 ng/mL) in the presence of the FGF-2–neutralizing antibody (10 μg/mL). Immunoblot analyses were performed twice with qualitatively similar results.

Statistical analyses

All values are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to determine statistical differences between growth factor treated groups and controls using Sigma-Stat™ software 2.03 (Jandel Scientific, San Rafael, CA, USA). Further analysis was carried out using Bonferroni's method to control for multiple testing. *P* values ≤0.05 were considered significant.

RESULTS

Characterization of primary cortical fibroblasts

Primary fibroblast line Tk 455 was established from the biopsy of a 29-year-old female (C.M.) with IgA nephropathy and tubulointerstitial fibrosis involving 25% of the interstitium. Tk 458 originated from the biopsy of a 48-year-old male (B.G.) with diabetic nephropathy and minimal (<10%) interstitial involvement, and Tk 469 was cultured from the biopsy of a 39-year-old female (S.M.) with marked interstitial fibrosis. After the second passage, the percentage of cytokeratin positive cells was less than 5% in each culture. All cells were positive for vimentin, CD44, and CD54 by indirect immunofluorescence. The percentage of positive cells for collagen type
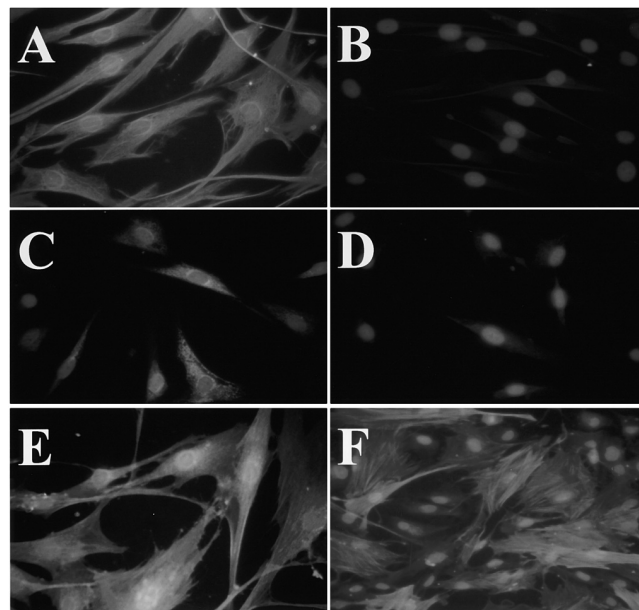

Fig. 1. Characterization of primary cortical fibroblasts by immunofluorescence. Primary cortical fibroblast line Tk 455 stained positively for vimentin (*A*), collagen type I (*C*), collagen type III (*E*), and α-smooth muscle actin (*F*). No staining was observed for cytokeratin (*B*) and factor VIII (*D*). Costainings with the Hoechst dye H33258 identifying cell nuclei were performed.

I was 96% in Tk 455 cells, 95% in Tk 458, and 96% in Tk 469 fibroblasts. Regarding collagen type III, the percentages were 94, 92, and 95%, respectively. The percentage of α-smooth muscle actin–positive cells was 88% in Tk 455, 78% in Tk 458, and 83% in Tk 469 fibroblasts. All three cell lines were negative for HLA-DR, factor VIII, and CD68. Figure 1 demonstrates the staining patterns observed by indirect immunofluorescence labeling for vimentin, cytokeratin, factor VIII, collagen types I and III, and α-smooth muscle actin in Tk 455 fibroblasts. Primary skin fibroblast lines Sk 448 and Sk 449 were obtained from a 57-year-old male with IgA nephropathy and a 30-year-old male with membranous glomerulonephritis, respectively. All cells were negative for cytokeratin and positive for vimentin and collagens type I and III.

TGF-β1 induces FGF-2 mRNA and protein synthesis

To study the interactions between FGF-2 and TGF-β1 in human kidney fibroblasts, possible changes in cytokine expression were first studied by Northern blot analyses. The basal level of TGF-β1 mRNA expression in Tk 173 and Tk 455 fibroblasts was very low, and stimulation with 0.1, 1.0, or 10 ng/mL FGF-2 did not result in any change in expression level for TGF-β1 mRNA (data not shown). Conversely, stimulation of both fibroblast lines with 1.0 ng TGF-β/mL caused an increase in FGF-2 mRNA as early as three hours (175.6 ± 23.7) after initia-
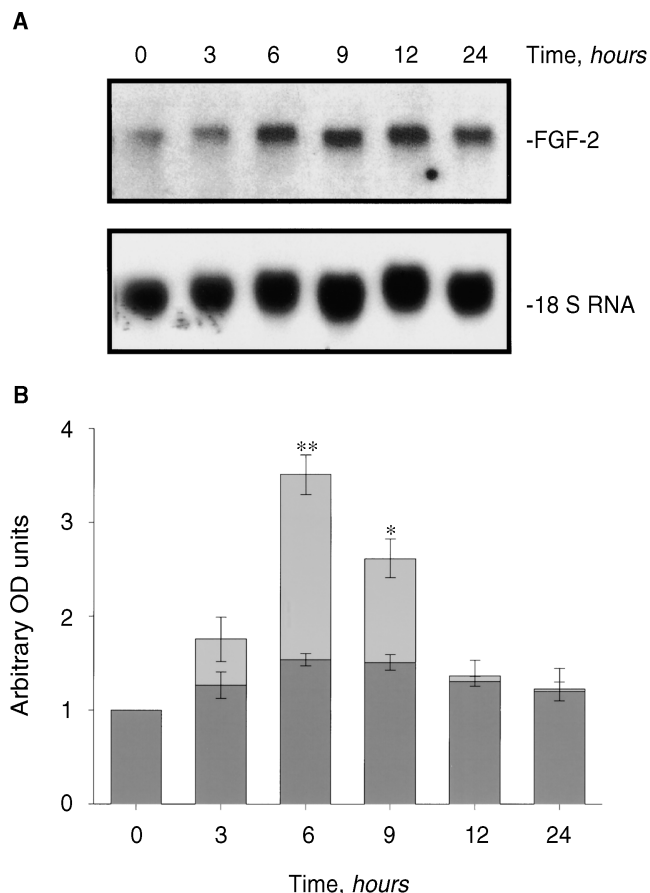
A

B

Fig. 2. FGF-2 mRNA levels in Tk 173 fibroblasts after stimulation with TGF-β1 (1 ng/mL) for 0, 3, 6, 9, 12, and 24 hours. (*A*) One representative Northern blot with a main band at 7.0 kb 18S-RNA was used as internal control. (*B*) The densitometric analyses after five different stimulations (■) and mRNA changes after the addition of cyclohexamide (■). *P < 0.05; **P < 0.01 vs. control (= 0 hour).

A

B

Fig. 3. Detection of FGF-2 protein in the supernatant by immunoblot (*A*) and ELISA (*B*). Cells were stimulated with 1 ng/mL TGF-β1 for 0, 3, 6, 12, 24, and 48 hours. Protein of supernatant was corrected for cell number. (A) A characteristic immunoblot illustrating the robust increase in FGF-2 protein in the supernatant peaking after 24 hours. The de novo detection of the protein after only three hours of stimulation points to a role of TGF-β1 in release of preformed FGF-2. (B) A summary of ELISAs for FGF-2 in the supernatant confirming the immunoblot data. Protein values were corrected for cell number. *P < 0.05; **P < 0.01 vs. control (= 0 hour).

tion of stimulation (Fig. 2). In Tk 173 fibroblasts, induction peaked after six hours (350.9 ± 21.2% compared with basal level, $P < 0.001$) and was still robustly elevated after 9 hours (261.6 ± 20.7%, $P = 0.048$) before returning to its basal level after 12 and 24 hours. Figure 2B summarizes the findings of five independent experiments in Tk 173 fibroblasts. This increase could be almost completely blocked by addition of cyclohexamide in a concentration of 1 μg/mL (maximum increase to 153.7 ± 6.3% after 9 h, $P = 0.096$), indicating that protein synthesis is required for induction of FGF-2. In these experiments, toxic effects were excluded by serial measurements of LDH, which did not change. Similar increases in FGF-2 mRNA were obtained in Tk 455 fibroblasts, although absolute values were somewhat lower (maximum increase to 245 ± 18.9% after 9 h, $P = 0.035$). Again, induction of FGF-2 was almost completely prevented by cyclohexamide.

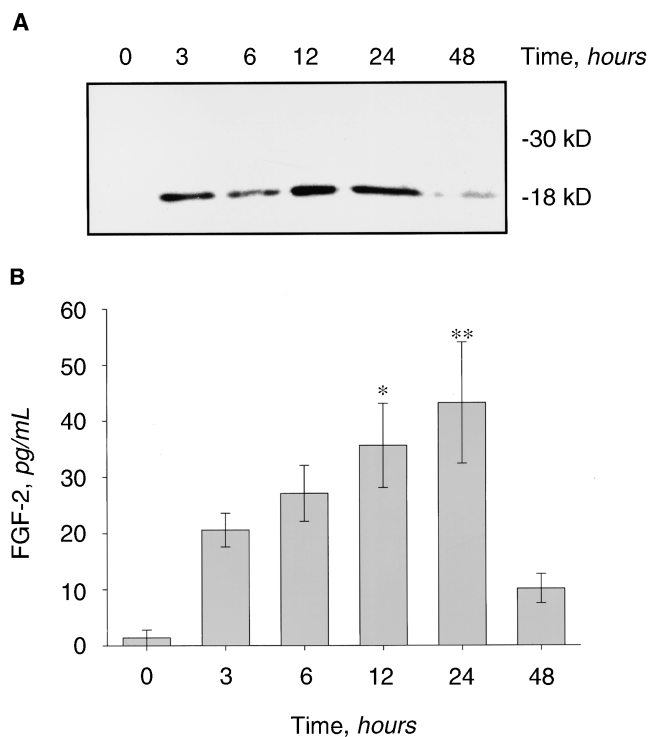To assess whether this increase in FGF-2 mRNA synthesis was paralleled by a rise in FGF-2 protein production, immunoblots for intracellular and secreted FGF-2 as well as ELISAs for FGF-2 in the supernatant were performed in Tk 173 fibroblasts. Immunoblot analysis demonstrated one main band at 18 kD. The relative amount of intracellular FGF-2 protein increased at maximum by a factor of 2.1 ± 0.23 ($P < 0.05$) after 24 hours (data not shown). Conversely, there was a robust increase in FGF-2 protein in the supernatant. This increase peaked after 12 to 24 hours of incubation with TGF-β1 (Fig. 3A). Furthermore, the increase in FGF-2 protein in the supernatant was confirmed by ELISA. There was an increase from 1.4 ± 1.4 ng/mL/4000 cells at 0 hours to a maximum 43.3 ± 10.8 ng/mL/4000 cells at 24 hours ($P = 0.003$; Fig. 3B). Thus, increased mRNA values are paralleled by increased values for intracellular and particularly secreted FGF-2 protein. Additionally, the detection of FGF-2 protein in the supernatant after only three hours of incubation with TGF-β1 implies that TGF-β1 may result in the release of preformed FGF-2. A similar mechanism has recently been proposed for the release of TGF-β1 from tubular epithelial cells after stimulation with FGF-2 [27].
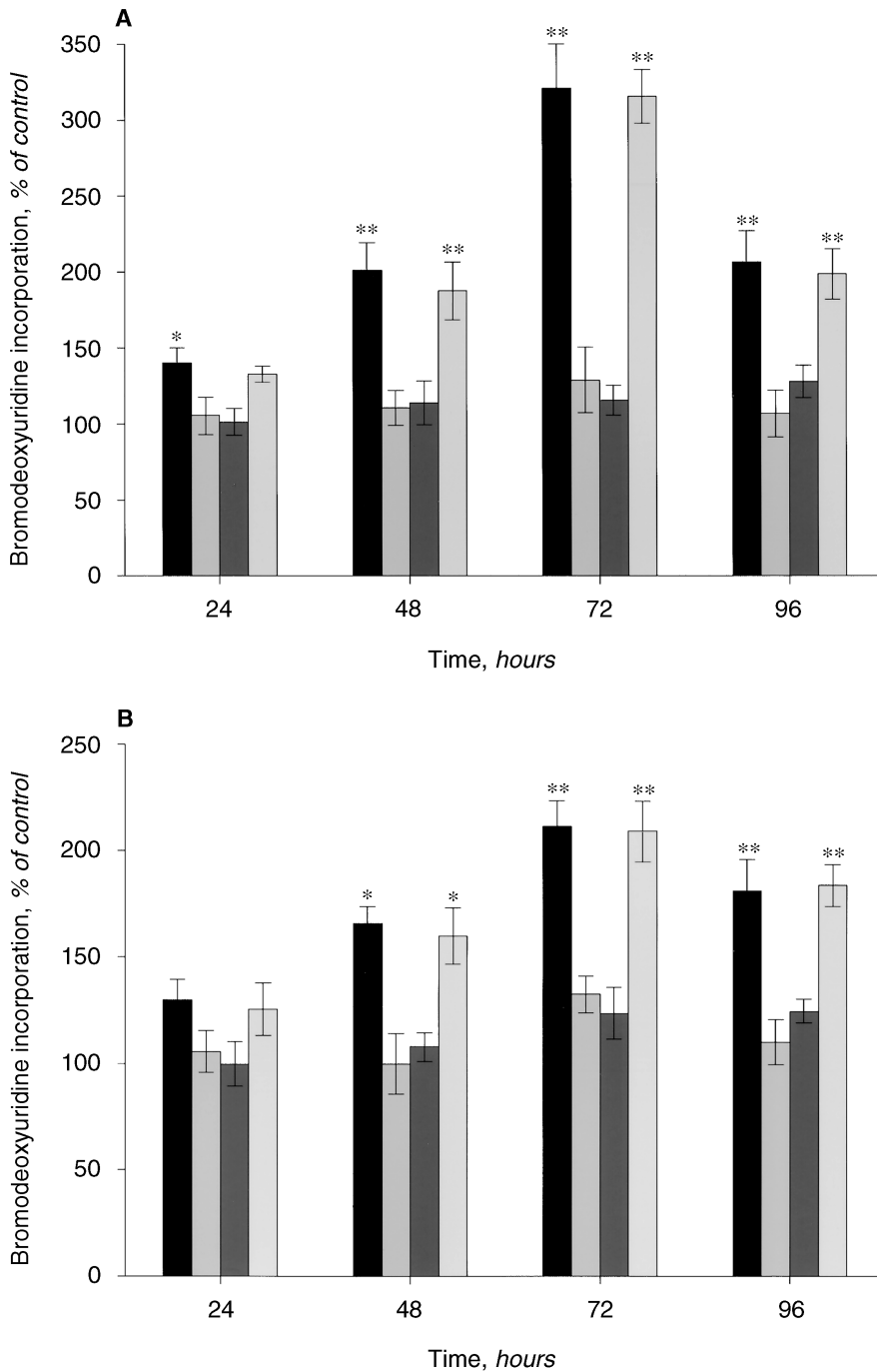
Fig. 4. **TGF-β1–induced proliferation as determined by bromodeoxyuridine incorporation in human kidney fibroblasts.** (*A*) The time dependency of TGF-β1–induced proliferation in Tk 173 fibroblasts after stimulation with TGF-β1 at 1 ng/mL. Cells grown in Iscove's medium without additives were used as controls (= 100%). (*B*) The time dependency in Tk 455 cells. The addition of a neutralizing antibody to TGF-β (gray columns) and to FGF-2 (dark gray columns) prevented the TGF-β1–induced proliferation, whereas a neutralizing antibody to EGF (light gray columns) had no effect. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

TGF-β1 induces proliferation in human kidney fibroblasts in time- and dose-dependent fashion

Since TGF-β1–induced FGF-2 and FGF-2 is a strong mitogen for renal fibroblasts, we speculated that TGF-β1 may induce proliferation in these cells. Figure 4 demonstrates the results obtained in Tk 173 (Fig. 4A) and Tk 455 (Fig. 4B) fibroblasts after stimulation with TGF-β1 (1 ng/mL) for 24, 48, 72, and 96 hours. TGF-β1 induced a time-dependent increase in bromodeoxyuridine incor-

poration, which peaked in both cell lines after 72 hours (bromodeoxyuridine incorporation $321 \pm 28.9\%$ in Tk 173 fibroblasts and $210.9 \pm 12.3\%$ in Tk 455 fibroblasts). This increase could be inhibited by addition of neutralizing antibodies to TGF-β1 and to FGF-2. Conversely, the addition of a neutralizing antibody to EGF had no significant effect. FGF-2 (10 ng/mL) alone was used as the positive control and increased bromodeoxyuridine uptake to $375.9 \pm 28.7\%$ of controls in Tk 173 and to

285.3 ± 24.1% in Tk 455 cells (data not shown). Next, we analyzed the dose dependency of TGF-β1–induced stimulation. Although all three doses tested induced cell proliferation in both fibroblast lines, 1.0 ng/mL had the most robust effect (increases to 195.5 ± 16.6% of controls in Tk 173 and by 161.8 ± 17.2% in Tk 455 cells after 72 h), as shown in Figure 5, indicating that higher doses may have additional antiproliferative effects. Again, neutralizing antibodies to TGF-β1 and FGF-2 prevented the increase in cell number, whereas an irrelevant IgG had no effect (Fig. 5). Cell counts for stimulation with FGF-2 were increased to 278.3 ± 25.6% and to 214.5 ± 17.8%, respectively (data not shown). At the same time these experiments demonstrated that bromodeoxyuridine incorporation was paralleled by increases in absolute cell number. However, examining the time course of cell counts determined that the peak in proliferation after induction by TGF-β1 (1 ng/mL) was after 96 hours (increases in cell number by 205.9 ± 23.4% in Tk 173 and by 171.5 ± 17.4% in Tk 455 fibroblasts), although the additional increase after 72 hours was only minimal. Thus, as expected, the peak in cell counts was later than the peak in bromodeoxyuridine incorporation. Absolute values were much higher in transformed Tk 173 fibroblasts than in primary Tk 455 fibroblasts because of a higher rate of basal proliferation.

Neutralization of FGF receptor kinase inhibits TGF-β1–induced proliferation in renal fibroblasts

To analyze further the role of FGF-2 in TGF-β1–induced fibroblast proliferation, we used two different tyrphostines (AG compounds). Tyrphostines inhibit tyrosine kinases by binding to the substrate binding site. Tyrphostines AG1296, which blocks FGF-2 receptor tyrosine kinase activity, and B42 (AG 490), which inhibits specifically EGF receptor kinase autophosphorylation, were used. The results are depicted in Figure 6. The addition of tyrphostin AG1296 blocked TGF-β1–induced proliferation completely in both fibroblast lines. Conversely, tyrphostin B42 had no significant effect, indicating that EGF does not play a role in the process of TGF-β1–induced fibroblast proliferation. At the concentration used, AG1296 completely prevented the induction of proliferation by FGF-2 alone in Tk 173 and Tk 455 fibroblasts, whereas again, B42 had no significant effects (data not shown). Measurements of LDH demonstrated that there was no difference between supernatants from cells with and without tyrphostines excluding toxic effects at the applied doses (data not shown).

Neutralization of PDGF inhibits TGF-β1–mediated cell proliferation in skin but not in kidney fibroblasts

We next analyzed the effects of TGF-β1 on two additional cortical primary fibroblasts (Tk 458 and 469). Furthermore, since fibroblasts are known to be heterogeneous, we determined the effects of TGF-β1 on skin fibroblasts. As depicted in Figure 7, TGF-β1 (1 ng/mL) induced a robust increase in bromodeoxyuridine incorporation in the two primary kidney [217.2 ± 22.2% in Tk 458 and 295.0 ± 17.5% in Tk 469 fibroblasts compared with controls (= 100%)] and in the two skin (234.5 ± 12.6% in Sk 448 and 275.6 ± 8.9% in Sk 449 cells) fibroblast lines after 72 hours. Thus, the mitogenic response was even more pronounced in the fibroblasts derived from a kidney with little interstitial involvement. Again, specificity of the effect was determined by addition of a neutralizing antibody to TGF-β1, and a neutralizing antibody to EGF had no effect on TGF-β1–induced increase in uptake of bromodeoxyuridine. However, whereas the neutralizing antibody to FGF-2 almost completely abolished the response to TGF-β1 in the two cortical fibroblast lines (reduction to 93.2 ± 12.3 and to 112.0 ± 15.6%), there was only partial inhibition in the two skin fibroblast lines (reduction to 156.7 ± 8.9 and 164.5 ± 15.1%). Interestingly, the addition of a neutralizing antibody to PDGF caused only slight decreases in TGF-β1–induced proliferation in the kidney fibroblast lines but reduced bromodeoxyuridine uptake considerably in Sk 448 and 449 (to 130.9 ± 14.3 and 143.6 ± 12.7% of controls; Fig. 7). Thus, whereas PDGF may play only a minor role in TGF-β1–induced proliferation in kidney fibroblasts, it may have a more important function in skin fibroblasts.

TGF-β1 stimulation of kidney fibroblasts results in up-regulation of cdk 2 and down-regulation of p27[kip1]

To elucidate further the effects of TGF-β1 on cell proliferation in human kidney fibroblasts, we analyzed the effects on expression of cdk 2 and p27[kip1] by immunoblot analyses. The results are shown in Figure 8. Expression of cdk 2 was robustly up-regulated by TGF-β1 (1 ng/mL) in a time-dependent fashion in both cell lines (Fig. 8A). As expected, basal cdk 2 expression was much higher in transformed Tk 173 cells than in primary Tk 455 fibroblasts. Still, a similar increase could be observed in both cells up to 72 hours. Conversely, the expression of p27[kip1], which inhibits proliferation, was down-regulated up to 48 hours time dependently (Fig. 8B). However, after 72 hours of incubation with TGF-β1, levels of p27[kip1] returned back to basal levels, possibly explaining why TGF-β1 induced proliferation decreases thereafter. Interestingly, in contrast to cdk 2, basal levels of p27[kip1] were not significantly different in the two cell lines. In control experiments in the presence of a neutralizing antibody to FGF-2, no significant changes in cdk 2 or p27[kip1] were observed (data not shown), adding additional proof that the mitogenic effects of TGF-β1 are mediated via FGF-2.
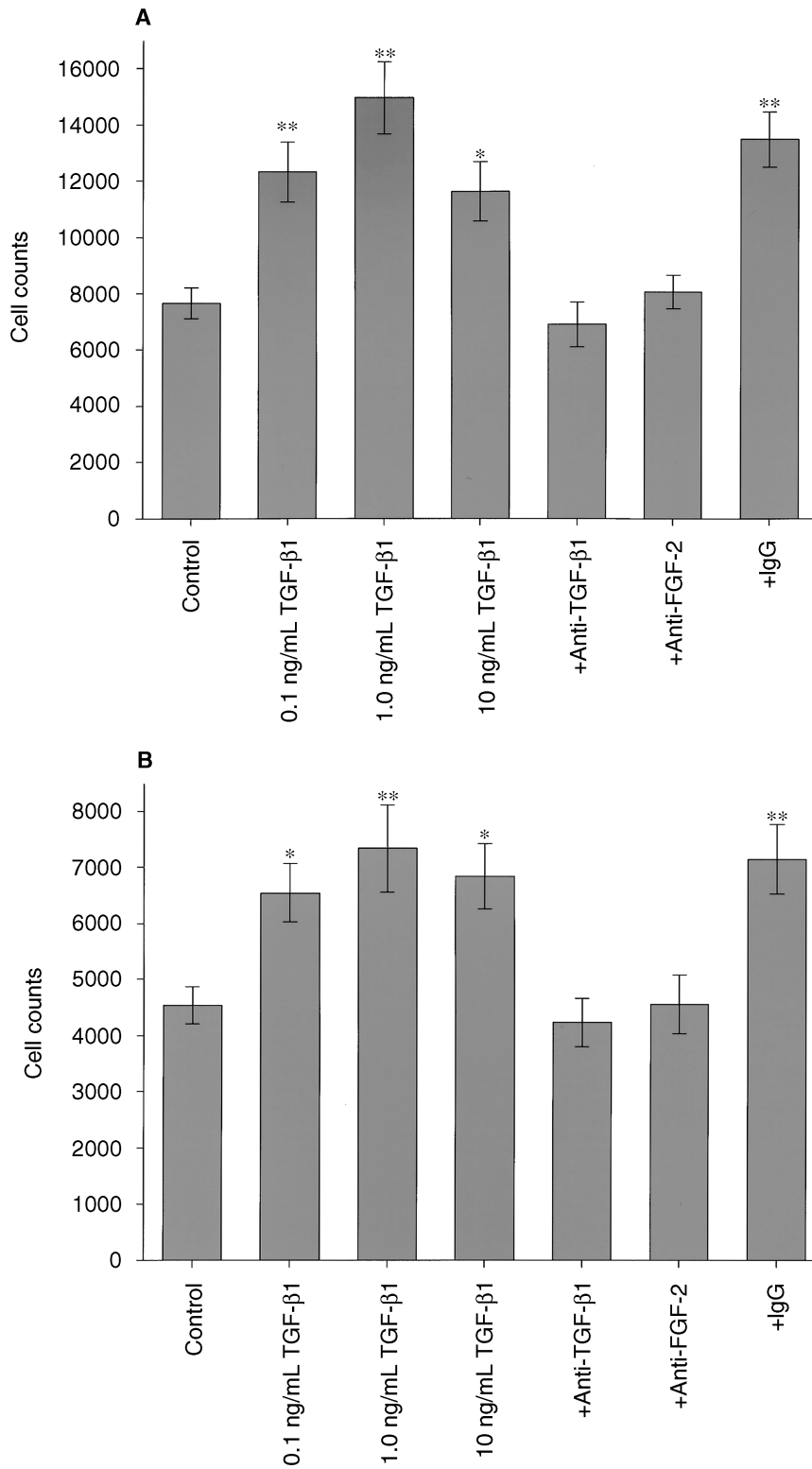
Fig. 5. Partial dose dependency of TGF-β1–induced proliferation in Tk 173 (*A*) and Tk 455 (*B*) fibroblasts, indicating a maximal pro-mitogenic effect of 1 ng/mL. Cells were originally plated at a density of 4000 per well and stimulated with 0.1, 1.0 or 10 ng/mL TGF-β1 and counted after 72 hours. Again, neutralizing antibodies to TGF-β and FGF-2 inhibited TGF-β1–induced proliferation, whereas an irrelevant IgG had no effect. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control (Iscove's medium = 100%).
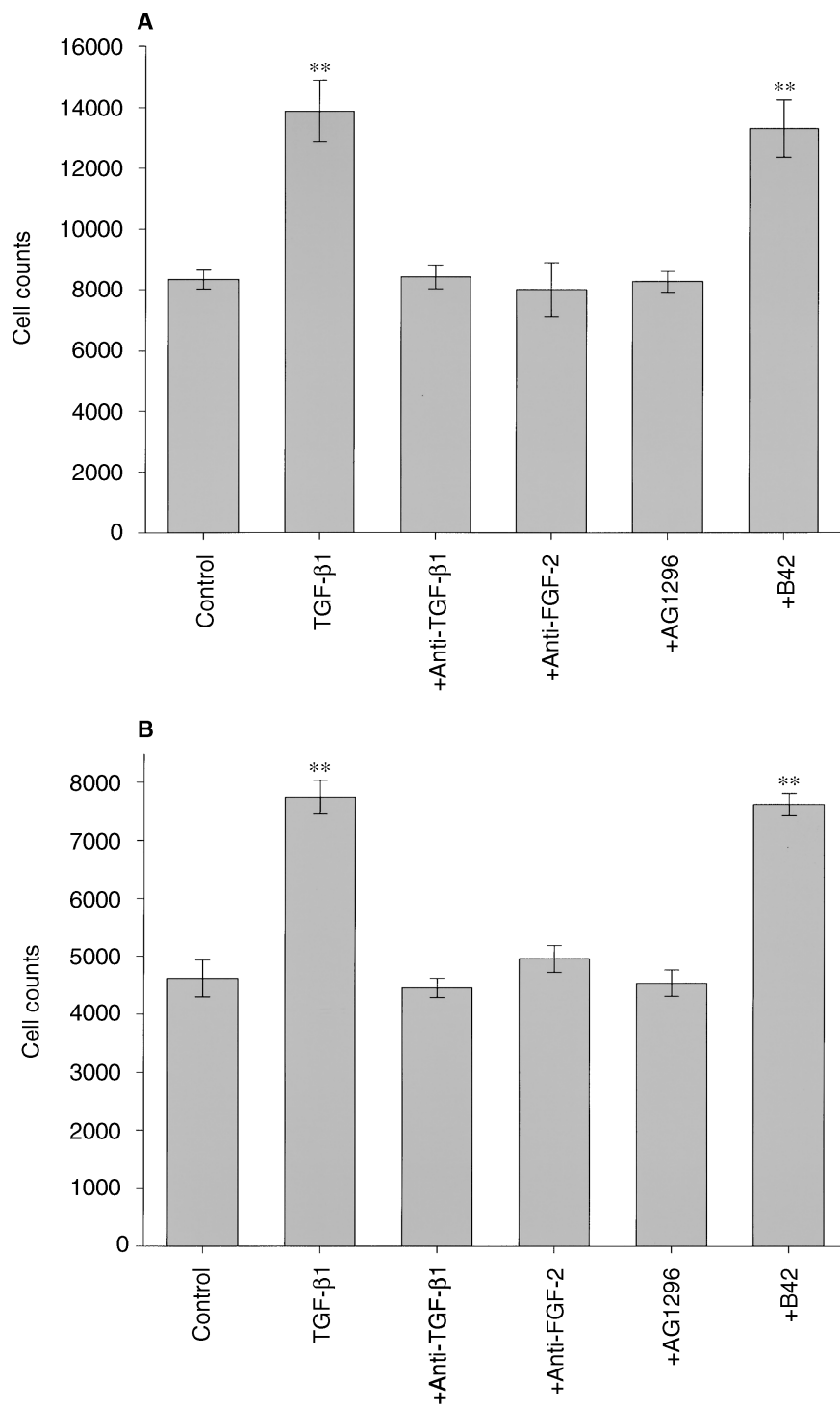
Fig. 6. **Effects of two tyrphostines on TGF-β–induced proliferation in kidney fibroblasts.** Cells were plated at a density of 10^3 per well and counted after 72 hours of stimulation with TGF-β1 (1 ng/mL). Whereas the FGF-receptor tyrosine kinase inhibitor tyrphostin AG1296 inhibited TGF-β1–induced proliferation in Tk 173 (*A*) and Tk 455 (*B*) kidney fibroblasts, no effect was seen with tyrphostin B42, which blocks EGF receptor signal transduction. Cells grown in Iscove's medium alone served again as controls. Neutralizing antibodies to TGF-β1 and FGF-2 demonstrated the specificity of induced proliferation. **$P < 0.01$ vs. control.

DISCUSSION

Transforming growth factor-β1 plays an important role in matrix accumulation in renal fibrogenesis [4]. The cytokine exists in three isoforms, of which TGF-β1 is the best studied [28]. TGF-β1 inhibits proliferation in most cells, including glomerular epithelial and endothe-

lial, as well as tubular epithelial cells [4]. However, its effects on fibroblast proliferation are largely unknown. Our study demonstrates that TGF-β1 induces a robust delayed mitogenic response in medullary and cortical fibroblasts. Our results indicate that this effect is largely mediated via induction of FGF-2 synthesis and secretion,
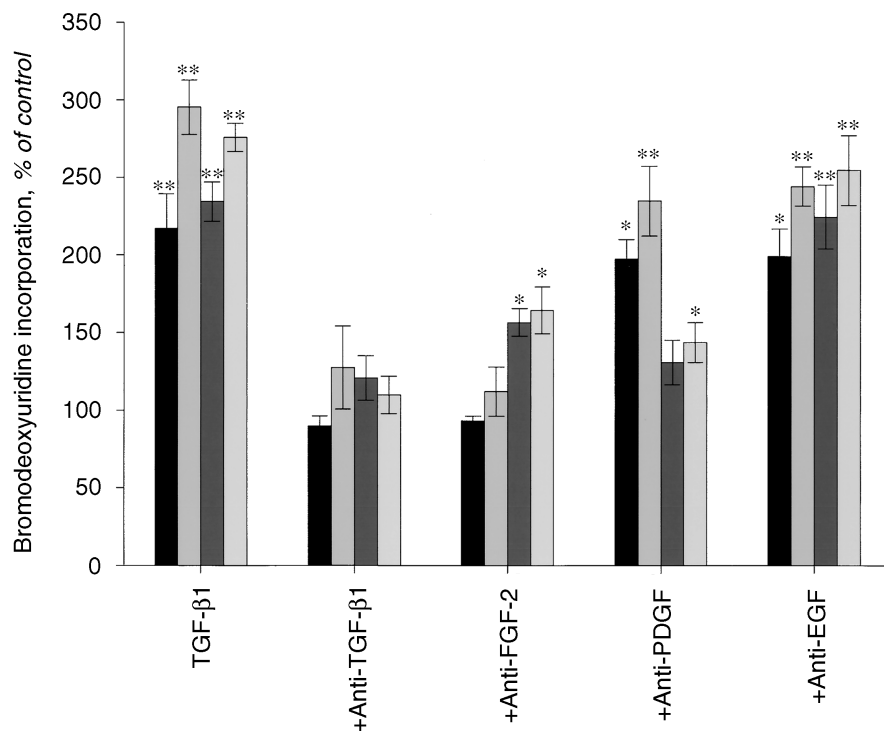
Fig. 7. Effects of a neutralizing antibody to PDGF on TGF-β1–induced proliferation in primary kidney and skin fibroblasts. Primary cortical fibroblast lines Tk 458 (■) and Tk 467 (gray boxes) as well as primary skin fibroblast lines Sk 448 (■) and Sk 449 (□) were subjected to stimulation with TGF-β1 (1 ng/mL). In addition, neutralizing antibodies to TGF-β1, FGF-2, PDGF, or EGF were added. After 72 hours, bromodeoxyuridine incorporation was determined. Unstimulated cells served as controls (= 100%). Whereas the neutralizing antibody to FGF-2 inhibited TGF-β1–induced proliferation in cortical fibroblasts, only partial inhibition was observed in skin fibroblasts. Conversely, the neutralizing antibody to PDGF prevented TGF-β1–induced proliferation in skin fibroblasts but had only minor effects in kidney fibroblasts. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control.

although additional effects may play a role as well. In addition, we have shown that stimulation with TGF-β1 results in increased expression of cdk 2 and decreased synthesis of p27[kip1]. Finally, we demonstrate that the promitogenic effect of TGF-β1 is not confined to renal fibroblasts but can be observed in skin fibroblasts as well. However, PDGF may play a more important role in the mediation of the mitogenic effects of TGF-β1 in skin fibroblasts.

Despite its name, TGF-β1 leads to a growth arrest in the G_1 phase of the cell cycle in most cells. A special family of protein kinases called the cdks regulate the cell cycle [29]. These cdks associate with a group of proteins called cyclins, and only these dimers have the necessary kinase activity to induce cell cycle progression. During the G_1 phase, at least two cyclins form dimers with their associated cdks: cyclin D/cdk 4 (or its homologue cdk 6) and cyclin E/cdk 2 [29]. The kinase activity of these cyclin/cdks complexes is inhibited by cdk inhibitors. p27[kip1] is one of those inhibitors and is capable of inhibiting cyclin D and cyclin E complexes, possibly by interaction with the catalytic cleft for ATP. Antimitogenic effects are mediated through an increase in cdk inhibitors such as p27[kip1]. For example, the growth-inhibitory effects of high glucose on mesangial cells are mediated by TGF-β1 [30]. The effects of TGF-β1 in these mesangial cells are characterized by increased expression of the cdk inhibitor p27[kip1] [31]. p27[kip1] has been recently proposed as a safeguard against inflammatory injury [32].

Shankland et al, in mesangial cells, found that the proliferative response to FGF-2 and PDGF was determined mainly by down-regulated p27[kip1] levels, although the authors observed an up-regulation of cdk 2 as well [33]. In our study, p27[kip1] expression was decreased by TGF-β1 up to 48 hours and returned to almost baseline after 72 hours, which may explain why DNA synthesis (as evaluated by bromodeoxyuridine incorporation) decreases thereafter. Conversely, expression of cdk 2 was robustly increased up to 72 hours, demonstrating that TGF-β1–induced proliferation is caused by an up-regulation of cdk 2 and a down-regulation of one of its inhibitors on the level of cell cycle-regulating proteins. Furthermore, our results demonstrate that transformed and nontransformed human kidney fibroblasts respond similarly to TGF-β1, although the level of basal proliferation (and cdk 2 expression) was higher in transformed Tk 173 cells. This is of interest, since culture of primary renal fibroblasts is cumbersome and loss of response to TGF-β1 has been described after immortalization of rodent fibroblasts [34]. In addition, although we tested only a limited number of cell lines, it shows that medullary and cortical fibroblasts may react in a similar manner to TGF-β1, and that there does not seem to be any major difference in response if fibroblasts are derived from a fibrotic or nonfibrotic kidney. However, we cannot exclude the possibility that the in vitro conditions favor a mitogenic response to TGF-β1.
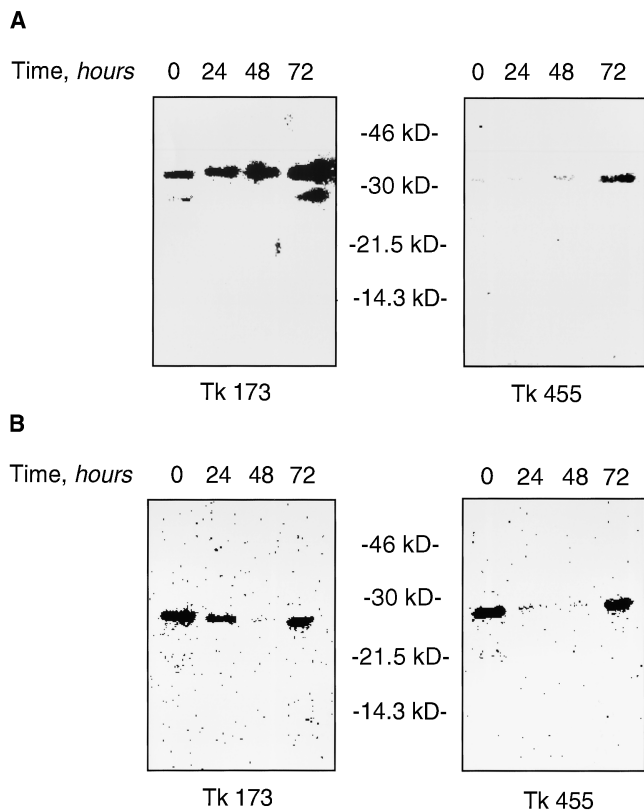
Our article is not the first to describe promitogenic ef-

A

Time, *hours*   0   24   48   72   0   24   48   72

-46 kD-

-30 kD-

-21.5 kD-

-14.3 kD-

Tk 173   Tk 455

B

Time, *hours*   0   24   48   72   0   24   48   72

-46 kD-

-30 kD-

-21.5 kD-

-14.3 kD-

Tk 173   Tk 455

Fig. 8. Immunoblots for cdk 2 (*A*) and p27^{kip1} (*B*) in Tk 173 and Tk 455 human kidney fibroblasts. Cells were stimulated with 1 ng/mL TGF-β1 for 0, 24, 48, and 72 hours. Both cell lines displayed robust up-regulation of cdk 2 (33 kD) after 48 and 72 hours. Tk 173 cells had a much higher basal level of cdk 2 protein expression compared with primary Tk 455 fibroblasts. Conversely, levels of the cyclin-dependent kinase inhibitor p27^{kip1} (27 kD) were similar at base line in both cell lines and were decreased after 24 and 48 hours before returning to basal levels after 72 hours.

fects of TGF-β1 in cells of mesenchymal origin. As a matter of fact, TGF-β was originally described as promoting anchorage independent growth in normal rat kidney fibroblasts in the presence of EGF [35]. Rosenbaum et al reported that TGF-β1 caused a dose- and time-dependent induction of proliferation in human myofibroblastic liver cells [36]. Similar to the results in our study, that analysis described inhibition of this effect by addition of a neutralizing antibody to FGF-2. Although protein levels were not studied in that article, the group was able to demonstrate an up-regulation in mRNA levels for FGFR-1 and -2. Similar results were recently reported for pulmonary fibroblasts in which TGF-β1 markedly induced FGFR-1 and FGFR-2 [37]. Although we did not study the expression of FGFRs in our study, we were able to demonstrate that an inhibitor of the FGFR tyrosine kinase prevented the mitogenic response to TGF-β1, whereas an inhibitor of the EGF receptor tyrosine kinase did not. However, the tyrphostin AG1296 not only blocks FGFR tyrosine kinase but additionally

inhibits PDGF receptor tyrosine kinase activity. We therefore tested the effects of a neutralizing antibody to PDGF and found only a modest effect in kidney fibroblasts. However, a considerable reduction of TGF-β1–induced proliferation was notable in skin fibroblasts, further corroborating the concept of fibroblast heterogeneity [38]. In that regard, it will be interesting to study the interactions between FGF-2 and connective tissue growth factor (CTGF), which seems to mediate part of the effects of TGF-β1 on matrix synthesis [39].

Interactions between TGF-β1 and FGF-2 have been described in the kidney before. Recently, Phillips et al demonstrated that exposure of human proximal tubule cells to TGF-β1 in a concentration of 10 ng/mL resulted in an up to 1.5-fold increase in FGF-2 mRNA, which was maximal after 24 hours and was paralleled by a time-dependent increase in FGF-2 protein secretion [27]. Moreover, a recent study by Johnson et al demonstrated that tubular epithelial cells secreted TGF-β1, which in turn stimulated cell growth in human cortical fibroblasts [40].

Thus, our data corroborates data from the literature that TGF-β1 induces a proliferative effect in organ fibroblasts and that this effect may be mediated by FGF-2. However, our study is, to our knowledge, the first to study the effects of TGF-β1 on medullary and cortical fibroblasts and the first to neutralize systematically a variety of potentially involved cytokines. A simplified concept of the effects of TGF-β1 and FGF-2 on fibroblast proliferation is depicted in Figure 9. What is the role of proliferation in the process of renal scarring? Cell proliferation in general is believed to be central to the renal response to injury resulting in renal fibrosis [41]. Recently, Shankland et al demonstrated that two animal models of chronic progressive renal disease had a more progressive course in p27^{kip1} knockout mice with increased glomerular and tubular cell proliferation [32]. Interstitial cell proliferation in human end-stage kidneys was analyzed by Nadasdy et al. To their surprise, they found that even scarred kidneys displayed high proliferative activity in tubuli and interstitial (noninflammatory) cells [42]. Interstitial cell proliferation was also described in models of interstitial fibrosis due to 5/6 nephrectomy [43, 44]. Finally, we described recently a close correlation between interstitial proliferation [determined by MIB-1 (Ki-67) labeling] and interstitial FGF-2 staining [22].

In summary, our study has demonstrated that TGF-β1 induces FGF-2 mRNA and protein synthesis in medullary and cortical renal fibroblasts. This effect results in a delayed time- and partly dose-dependent mitogenic response to TGF-β1 in kidney fibroblasts. Induction of proliferation by TGF-β1 can be blocked almost completely by the addition of a neutralizing antibody to FGF-2 in kidney but not in skin fibroblasts. The mitogenic effects are paralleled by an increase in cdk 2 and a decrease in p27^{kip1} syntheses. We conclude that the
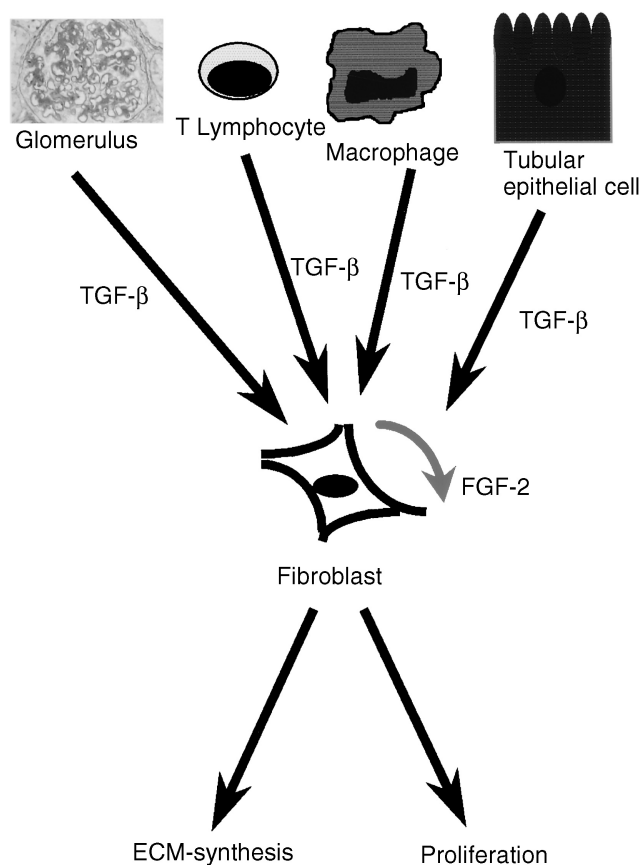
Fig. 9. Proposed mechanism of FGF-2 in renal fibrogenesis. Release of TGF-β1 by glomerular, tubular epithelial, and infiltrating cells results in an increased synthesis of FGF-2 in fibroblasts possibly mediating autocrine proliferation in these cells.

induction of FGF-2 by TGF-β1 may play an important role in the mediation of autocrine and paracrine fibroblast proliferation, and thus in progressive renal disease.

APPENDIX

Abbreviations used in this article are: ATP, adenosine 5′-triphosphate; cdk, cyclin-dependent kinase; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethyleneglycol tetraacid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FGF-2, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; Ig, immunoglobulin; kD, kilodalton; LDH, lactic acid dehydrogenase; PAS, periodic acid Schiff; PDGF, platelet-derived growth factor; PTEC, proximal tubular epithelial cell; SEM, standard error of the mean; TGF-β, transforming growth factor-β.

REFERENCES

1. NATH KA: Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 20:1–17, 1992
2. STRUTZ F, MÜLLER GA: Renal fibrogenesis and progression, in *Immunologic Renal Diseases*, edited by NEILSON EG, COUSER WC, Philadelphia, New York, Lipincott-Raven, 1997, pp 705–726
3. KNECHT A, FINE LG, KLEINMAN KS, et al: Fibroblasts of rabbit kidney in culture. II. Paracrine stimulation of papillary fibroblasts by PDGF. *Am J Physiol* 261:F292–F299, 1991
4. BORDER WA, NOBLE NA: Mechanisms of disease: Transforming growth factor β in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
5. MOLL S, MENOUD P-A, FULPIUS T, et al: Induction of plasminogen activation inhibitor type 1 in murine lupus-like glomerulonephritis. *Kidney Int* 48:1459–1468, 1995
6. EDDY A: Protein restriction reduces transforming growth factor β and interstitial fibrosis in chronic purine aminonucleoside nephrosis. *Am J Physiol* 266:F884–F893, 1994
7. DOWNER G, PHAN SH, WIGGINS RC: Analysis of renal fibrosis in a rabbit model of crescentic nephritis. *J Clin Invest* 82:998–1006, 1988
8. HAMAGUCHI A, KIM S, OHTA K, et al: Transforming growth factor-β1 expression and phenotypic modulation in the kidney of hypertensive rats. *Hypertension* 26:199–207, 1995
9. KANETO H, MORRISSEY J, KLAHR S: Increased expression of TGF-β1 mRNA in the obstructed kidney of rats with unilateral ureteral ligation. *Kidney Int* 44:313–321, 1993
10. SHIHAB FS, YAMAMOTO T, NAST CC, et al: Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. *J Am Soc Nephrol* 6:286–294, 1995
11. MO W, BRECKLIN C, GARBER SL, et al: Changes in collagenases and TGF-β precede structural changes in a model of chronic renal fibrosis. *Kidney Int* 56:145–153, 1999
12. KOPP JB, FACTOR VM, MOZES M, et al: Transgenic mice with increased plasma levels of TGF-beta 1 develop progressive renal disease. *Lab Invest* 74:991–1003, 1996
13. BORDER WA, NOBLE NA: TGF-beta in kidney fibrosis: A target for gene therapy. *Kidney Int* 51:1388–1396, 1997
14. SLAVIN J: Fibroblast growth factors: At the heart of angiogenesis. *Cell Biol Int* 19:431–444, 1995
15. CHARLOTTE F, WIN KM, PRÉAUX AM, et al: Immunolocalization of heparin-binding growth factors (HBFG) types 1 and 2 in rat liver: Selective hyperexpression of HBGF-2 in carbon tetrachloride-induced fibrosis. *J Pathol* 169:471–476, 1993
16. ZHANG GH, ICHIMURA T, WALLIN A, et al: Regulation of rat proximal tubule epithelial cell growth by fibroblast growth factors, insulin-like growth factor-1 and transforming growth factor-beta, and analysis of fibroblast growth factors in rat kidney. *J Cell Physiol* 148:295–305, 1991
17. FLOEGE J, ENG E, LINDNER V, et al: Rat glomerular mesangial cells synthesize basic fibroblast growth factor: Release, upregulated synthesis, and mitogenicity in mesangial proliferative glomerulonephritis. *J Clin Invest* 90:2362–2369, 1992
18. KRIZ W, HÄHNEL B, RÖSENER S, et al: Long-term treatment of rats with FGF-2 results in focal segmental glomerulosclerosis. *Kidney Int* 48:1435–1450, 1995
19. RAY PE, BRUGGEMAN LA, WEEKS BS, et al: bFGF and its low affinity receptors in the pathogenesis of HIV-associated nephropathy in transgenic mice. *Kidney Int* 46:759–772, 1994
20. STRUTZ F, OKADA H, LO CW, et al: Identification and characterization of fibroblast-specific protein 1 (FSP1). *J Cell Biol* 130:393–405, 1995
21. NG YY, HUANG TP, YANG WC, et al: Tubular epithelial-myofibro-

blast transdifferentiation in progressive tubulointerstitial fibrosis in 5/6 nephrectomized rats. *Kidney Int* 54:864–876, 1998

22. STRUTZ F, ZEISBERG M, HEMMERLEIN B, *et al*: Basic fibroblast growth factor (FGF-2) expression is increased in human renal fibrogenesis and may mediate autocrine fibroblast proliferation. *Kidney Int* 57: 1521–1538, 2000

23. DETRISAC CJ, SENS MA, GARVIN AJ, *et al*: Tissue culture of human kidney epithelial cells of proximal tubule origin. *Kidney Int* 25:383–390, 1984

24. GERRITSMA JS, HIEMSTRA PS, GERRITSEN AF, *et al*: Regulation and production of IL-8 by human proximal tubular epithelial cells in vitro. *Clin Exp Immunol* 103:289–294, 1996

25. MÜLLER GA, FRANK J, RODEMANN HP, *et al*: Human renal fibroblast cell lines (tFKIF and tNKF) are new tools to investigate pathophysiologic mechanisms of renal interstitial fibrosis. *Exp Nephrol* 3:127–133, 1995

26. GRATZNER HG: Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218:474–475, 1982

27. PHILLIPS AO, TOPLEY N, MORRISEY K, *et al*: Basic fibroblast growth factor stimulates the release of preformed transforming growth factor β1 from human proximal tubular cells in the absence of de novo gene transcription or mRNA translation. *Lab Invest* 76:591–600, 1997

28. O'KANE S, FERGUSON MWJ: Transforming growth factor βs and wound healing. *Int J Biochem Cell Biol* 29:63–78, 1997

29. BOTTAZZI ME, ASSOIAN RA: The extracellular matrix and mitogenic growth factors control G1 phase cyclins and cyclin-dependent kinase inhibitors. *Trends Cell Biol* 7:348–352, 1997

30. WOLF G, SHARMA K, CHEN Y, *et al*: High-glucose induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int* 42:647–656, 1992

31. WOLF G, SCHROEDER R, ZIYADEH FN, *et al*: High glucose stimulates expression of p27[Kip1] in cultured mouse mesangial cells: Relationship to hypertrophy. *Am J Physiol* 273:F348–F356, 1997

32. OPHASCHAROENSUK V, FERO ML, HUGHES J, *et al*: The cyclin-dependent kinase inhibitor p27[kip1] safeguards against inflammatory injury. *Nat Med* 4:575–580, 1998

33. SHANKLAND SJ, PIPPIN J, FLANAGAN M, *et al*: Mesangial cell proliferation mediated by PDGF and bFGF is determined by levels of the cyclin kinase inhibitor p27[kip1]. *Kidney Int* 51:1088–1099, 1997

34. SORRENTINO V, BANDYOPADHYAY S: TGF beta inhibits Go/S-phase transition in primary fibroblasts: Loss of response to the antigrowth effect of TGF beta is observed after immortalization. *Oncogene* 4:569–574, 1989

35. ROBERTS AB, ANZANO MA, LAMB LC, *et al*: New class of transforming growth factors potentiated by epidermal growth factor: Isolation from non-neoplastic tissues. *Proc Natl Acad Sci USA* 78:5339–5343, 1981

36. ROSENBAUM J, BLAZEJEWSKI S, PREAUX A-M, *et al*: Fibroblast growth factor 2 and transforming growth factor β1 interactions in human liver fibroblasts. *Gastroenterology* 109:1986–1996, 1995

37. THANNICKAL VJ, ALDWEIB KD, RAJAN T, *et al*: Upregulated expression of fibroblast growth factor (FGF) receptors by transforming growth factor-beta1 (TGF-beta1) mediates enhanced mitogenic responses to FGFs in cultured human lung fibroblasts. *Biochem Biophys Res Commun* 251:437–441, 1998

38. MÜLLER GA, STRUTZ F: Renal fibroblast heterogeneity. *Kidney Int* 48(Suppl 50):S33–S36, 1995

39. DUNCAN MR, FRAZIER KS, ABRAMSON S, *et al*: Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: Down-regulation by cAMP. *FASEB J* 13:1774–1786, 1999

40. JOHNSON DW, SAUNDERS HJ, BAXTER RC, *et al*: Paracrine stimulation of human renal fibroblasts by proximal tubule cells. *Kidney Int* 54:747–757, 1998

41. COUSER WG, JOHNSON RJ: Mechanisms of progressive renal disease in glomerulonephritis. *Am J Kidney Dis* 23:193–198, 1994

42. NADASDY T, LASZIK Z, BLICK KE, *et al*: Tubular atrophy in the end-stage kidney: A lectin and immunohistochemical study. *Hum Pathol* 25:22–28, 1994

43. KLIEM V, JOHNSON RJ, ALPERS CE, *et al*: Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. *Kidney Int* 49:666–678, 1996

44. THOMAS GL, YANG B, WAGNER BE, *et al*: Cellular apoptosis and proliferation in experimental renal fibrosis. *Nephrol Dial Transplant* 13:2216–2226, 1998