Role of connective tissue growth factor in fibronectin expression and tubulointerstitial fibrosis

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Yokoi, Hideki, Masashi Mukoyama, Akira Sugawara, Kiyoshi Mori, Tetsuya Nagae, Hisashi Makino, Taka- yoshi Suganami, Kensei Yahata, Yuriko Fujinaga, Issei Tanaka, and Kazuwa Nakao. Role of connective tissue growth factor in fibronectin expression and tubulointerstitial fibrosis. Am J Physiol Renal Physiol 282: F933–F942, 2002; 10.1152/ajprenal.00122.2001.—Connective tissue growth factor (CTGF) is one of the candidate factors mediating downstream events of transforming growth factor-β (TGF-β), but its role in fibrogenic properties of TGF-β and in tubulointerstitial fibrosis has not yet been clarified. Using unilateral ureteral obstruction (UUO) in rats, we analyzed gene expression of TGF-β1, CTGF, and fibronectin. We further investigated the effect of blockade of endogenous CTGF on TGF-β-induced fibronectin expression in cultured rat renal fibroblasts by antisense oligodeoxynucleotide (ODN) treatment. After UUO, CTGF mRNA expression in the obstructed kidney was significantly upregulated subsequent to TGF-β1, followed by marked induction of fibronectin mRNA. By in situ hybridization, CTGF mRNA was detected mainly in the interstitial fibrotic areas and tubular epithelial cells as well as in parietal glomerular epithelial cells in the obstructed kidney. The interstitial cells expressing CTGF mRNA were also positive for α-smooth muscle actin. CTGF antisense ODN transfection into cultured renal fibroblasts significantly attenuated TGF-β-stimulated upregulation of fibronectin mRNA and protein compared with control ODN transfection, together with inhibited synthesis of type I collagen. With the use of a reporter assay, rat fibronectin promoter activity was increased by 2.5-fold with stimulation by TGF-β1, and this increase was abolished with antisense CTGF treatment. Thus CTGF plays a crucial role in fibroblast synthesis induced by TGF-β, suggesting that CTGF blockade could be a possible therapeutic target against tubulointerstitial fibrosis.

TUBULOINTERSTITIAL FIBROSIS is a common feature of progressive renal diseases regardless of the initiating insult (4, 41). It has been shown in a number of clinical as well as experimental studies that tubulointerstitial injury is a more consistent predictor of functional impairment than glomerular damage (41, 44). Mechanisms by which the interstitial fibrosis progresses are not well understood, but various cytokines are thought to be involved in fibrogenic and inflammatory processes by stimulating fibroblast proliferation, macrophage infiltration, and extracellular matrix (ECM) accumulation (18).

Among them, multiple lines of evidence have indicated transforming growth factor-β (TGF-β) as a key cytokine underlying the development of tissue fibrosis, including tubulointerstitial fibrosis as well as glomerulosclerosis (5, 18). TGF-β enhances the synthesis of ECM proteins such as collagen types I, III, and IV, fibronectin, and laminin (5, 18, 46). TGF-β also promotes ECM accumulation by increasing the production of protease inhibitors such as plasminogen activator inhibitor-1 and by decreasing the activity of proteases such as matrix metalloproteinases (5, 18, 46). Furthermore, TGF-β stimulates fibroblast migration and proliferation (5) and also is chemotactic for monocytes and macrophages (5, 18). Transgenic mice overexpressing TGF-β1 in the liver with high plasma levels of active TGF-β1 develop marked tubulointerstitial fibrosis with severe glomerulonephritis (48). In accordance with these observations, elevated renal expression of TGF-β mRNA or protein has been reported in nearly every experimental model of renal failure characterized by fibrosis (3). In an obstructive nephropathy model, augmented expression of TGF-β in fibrotic tissue, produced mainly in the interstitial fibroblasts and macrophages, greatly paralleled the increased interstitial expression of fibronectin and collagen types I, III, and IV (4, 14, 18, 33, 34). On the basis of these findings, it has been suggested that blocking of TGF-β or its downstream pathway becomes a potential antifibrotic strategy for chronic renal diseases (6). However, the molecular mechanisms for profibrotic effects of TGF-β have not yet been fully elucidated.

Connective tissue growth factor (CTGF), originally isolated from conditioned media of human umbilical vein endothelial cells (10), belongs to a new family of...
cysteine-rich growth factors (the CCN family) that consists of CTGF/fisp-12, celf10/cyr61, and nov (8, 47). In cultured fibroblasts, CTGF gene expression is strongly induced by TGF-β but not by other growth factors, such as epidermal growth factor, platelet-derived growth factor, or basic fibroblast growth factor (26). Addition of CTGF, in turn, potently stimulates fibroblast proliferation and ECM protein synthesis (19). In human diseases, CTGF gene expression was detected in fibroblasts of sclerotic lesions from patients with systemic sclerosis (25) and in fibrotic areas of atherosclerotic plaques (42). Recently, it has been shown that CTGF expression is upregulated in proliferative and fibrotic glomerular lesions of various human renal diseases, including glomerulonephritis and diabetic nephropathy (29). Subsequent reports using in vitro and animal models revealed that CTGF mRNA and protein are increased in cultured mesangial cells as well as in the renal cortex in a diabetic milieu, suggesting the involvement of CTGF in the pathogenesis of diabetic glomerulosclerosis (39, 45). Although these observations have led to the hypothesis that CTGF is a candidate factor mediating fibrogenic properties of TGF-β (22), the role of CTGF in tubulointerstitial fibrosis still remains unclarified.

In the present study, to explore the implication of CTGF in tubulointerstitial fibrosis, we investigated the time course of TGF-β, CTGF, and fibronectin gene expression in an obstructive nephropathy model in rats by Northern blot analysis. Localization of CTGF mRNA expression was also investigated by in situ hybridization. Furthermore, to evaluate the contribution of CTGF to fibronectin and collagen expression induced by TGF-β, we inhibited endogenous CTGF by antisense oligodeoxynucleotide (ODN) in cultured rat renal fibroblasts and analyzed the effect of its blockade by Northern blot analysis, immunoblotting, and luciferase reporter assay.

METHODS

Unilateral ureteral obstruction. All animal experiments were conducted in accordance with our institutional guidelines for animal research. Male Wistar rats weighing 200–250 g were subjected to either unilateral ureteral obstruction (UUO) or sham operation (14, 33, 40). In UUO rats under pentobarbital anesthesia, the right ureter was ligated with 4–0 silk at two points through a midline abdominal incision and cut between the ligatures to prevent retrograde infection. Rats were killed at 12 h and 3, 6, or 14 days after UUO or sham operation (n = 4 at each time point), and both the obstructed kidney and the contralateral kidney were harvested. Northern blot analysis was performed using 40 μg of total RNA prepared from each kidney.

In situ hybridization. In situ hybridization was performed as described, with some modifications (11, 38). A cDNA fragment of rat CTGF (nucleotides 1221–1803) (57) subcloned into pGEM-T-Easy vector (Promega, Madison, WI) was used to produce antisense and sense riboprobes. After digestion with a restriction enzyme SpeI or Eco47III, antisense and sense cRNA riboprobes were transcribed in vitro from the linearized plasmids using digoxigenin (DIG)-labeled UTP and an RNA labeling kit (DIG RNA Labeling Kit SP6/T7; Roche Diagnostics, Mannheim, Germany). Seven-micrometer-thick sections of paraffin-embedded renal tissues were placed on silanized slides (DAKO Japan, Kyoto, Japan). Sections were deparaffinized, treated with 0.2 M HCl for 20 min, digested with 10 μg/ml proteinase K for 10 min at 37°C, fixed with 4% paraformaldehyde for 5 min, and treated with 2 mg/ml glycine for 30 min. The specimens were incubated with prehybridization buffer (50% denitized formamide/× standard saline citrate (SSC)) for 30 min in a humidified chamber at 45°C. Then, DIG-labeled riboprobes (final concentration, 1 μg/ml) were added to hybridization solution containing 50% denitized formamide, 10% dextran sulfate, 1× Denhardt’s solution, 10 mM Tris–HCl, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 250 μg/ml denatured salmon testis DNA, and 250 μg/ml yeast tRNA. Hybridization was performed in a humidified chamber for 16 h at 45°C. Thereafter, the slides were washed once with 5× SSC and once with 2× SSC containing 50% formamide at 45°C. Then, they were treated with 20 μg/ml RNase A for 30 min at 37°C. Washing was continued once with 2× SSC and twice with 0.1× SSC. The sections were blocked with 1× blocking reagent (Roche), washed with 100 mM maleic acid buffer (pH 7.5) containing 150 mM NaCl, and then incubated with alkaline phosphatase-conjugated Fab fragments of sheep anti-DIG antibody (Roche) at a dilution of 1:500 for 30 min at room temperature. They were visualized on reaction with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate for 16 h at room temperature according to the DIG Nucleic Acid Detection Kit protocol (Roche). The slides were counterstained with hematoxylin.

Histology and immunohistochemistry. For histological analysis, sagittal kidney sections were fixed with 4% buffered formaldehyde and embedded in paraffin. Two-micrometer-thick sections were stained with Masson’s trichrome.

For immunohistochemical analysis of α-smooth muscle actin (α-SMA), serial paraffin-embedded fixed paraffin sections were deparaffinized, washed with PBS, and treated with 3% H2O2 in methanol for 10 min to quench endogenous peroxidase activity. The specimens were incubated with murine anti-α-SMA antibody (DAKO) for 1 h at room temperature, processed using LSAB+ kit (DAKO), and developed with 3,3′-diaminobenzidine tetrahydrochloride.

Cell culture. Normal rat kidney fibroblasts (NRK-49F cells) and normal rat kidney epithelial cells (NRK-52E cells) were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM (GIBCO BRL, Rockville, MD) containing 10% fetal calf serum (FCS; Sanko Junyaku, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin. For TGF-β stimulation, cells at ~90% confluence were made quiescent in serum-free DMEM supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, and 10 ng/ml selenium (ITS; Sigma, St. Louis, MO). After 24 h of serum starvation, cells were stimulated with 1–10 ng/ml of recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) and further incubated for 1–48 h. Northern blot analysis was performed using 20 μg of total RNA prepared from each culture.

Northern blot analysis. Total RNA from the whole kidney or cultured cells was extracted by the acid guanidinium thiocyanate-phenol-chloroform method. Northern blot analysis was performed as described previously (38, 40). Briefly, total RNA was electrophoresed on a 1.4% agarose gel and transferred to a nylon membrane filter (Bionyne; Pall BioSupport, East Hills, NY). Hybridization was performed at 42°C overnight with 32P-labeled cDNA probes for rat CTGF (nucleotides 1221–1803) (57), TGF-β1 (1142–1546) (52), and fibronectin (619–1082) (50), which were prepared by standard reverse-transcription PCR method. The membranes
were washed at 55°C in 1× SSC/0.1% SDS, and autoradiography was performed for 12 h with the BAS-2500 system (Fuji Photo Film, Tokyo, Japan). The amount of RNA loaded in each lane was normalized with 28S or 18S rRNA.

CTGF antisense oligonucleotide transfection. Transfection of antisense ODN into cultured cells was performed as described (31) with some modifications. The sequences of phosphorothioate oligonucleotides (Kurabo, Osaka, Japan) for rat CTGF used in this study were as follows: antisense ODN, 5'-GAC GGA GCC GAG CAT GGT-3'; and control reverse ODN, 5'-TGG TAC GAG CGG AGG CAG-3'. The antisense sequence is complementary to rat CTGF cDNA (57) around the translation initiation codon (underlined in sequences). Transfection into NRK-49F cells was carried out by cationic lipofection with TransFast Reagent (Promega) according to the manufacturer’s instructions. Cells (1 × 10^6/dish) were plated into 10-cm dishes and serum-starved in DMEM with ITS for 24 h. Oligonucleotide and the reagent in a charge ratio of 1:1 were allowed to aggregate for 10 min at room temperature, and cells were transfected with 0.5 μM of ODN in serum-free DMEM. After 2 h of incubation, the cells were overlaid with growth medium containing FCS to achieve the final concentration of 5% and then stimulated with 3 ng/ml TGF-β1 for 12–48 h. Northern blot analysis was performed using 15 μg of total RNA prepared from each culture.

Western blot analysis. Western blot analysis was performed as described previously (55). Cells were lysed on ice in a solution containing 1 M Tris-HCl (pH 7.5), 12 mM β-glycerophosphate, 0.1 M EGTA, 1 mM pyrophosphate, 5 mM NaF, 10 mg/ml aprotinin, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cell lysates were centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatants were treated with Laemmli’s sample buffer. Equal amounts of samples (40 μg/lane) were separated by 12.5% SDS-PAGE and electrophoretically transferred onto Immobilon polyvinylidene difluoride filters (Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 5% methanol at 100 V for 1 h. Filters were incubated with antibodies against fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA) or type I collagen (Calbiochem, San Diego, CA) overnight at 4°C. Immunoblots were then developed by an enhanced chemiluminescence protocol with horseradish peroxidase-linked donkey anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) and a chemiluminescence kit (Amersham, Arlington Heights, IL). α-Tubulin (antibody from Sigma) was used as an internal control.

Plasmid construction and luciferase reporter assay. The promoter region (nucleotide −531/+1) of the rat fibronectin gene (13) was PCR amplified from Wistar rat genomic DNA with the following primers: forward, 5'-GGA CAA GGT AGT GCC GAC TTA ACG-3' and antisense, 5'-GGC CAC TTA ACG-3'. The PCR product of the expected size was subcloned into pGEM-T-Easy vector (Promega) to construct pGEM-T-rFN531. pGL3-Basic vector (Promega) was digested with HindIII, then blunted with Klenow fragment, and ligated with EcoRI-Linker (Takara, Tokyo, Japan). The rat fibronectin promoter region was cleaved with EcoRI from pGEM-T-rFN531 and inserted into the EcoRI sites of pGL3-Basic vector (rFN531-Luc). The sequence and direction of the inserted fragment were confirmed by the dyeodeoxy chain-termination method using a Dye Terminator cycle sequencing kit FS and 373B DNA sequence (Applied Biosystems, Foster City, CA).

A luciferase reporter assay was performed as described elsewhere (17). In brief, NRK-49F cells were plated into six-well plates at 5 × 10^4/well and serum-starved in DMEM with ITS. After 24 h of incubation, cells were transfected in serum-free DMEM by cationic lipofection with 2 μg/well of rFN531-Luc and 50 nM of phosphorothioate ODN (antisense or control ODN for rat CTGF), along with 0.1 μg/well of pRL-SV40 (Promega) as an internal control for transfection efficiency. After 6 h of incubation, the medium was changed to DMEM with ITS, and the cells were stimulated with 3 ng/ml TGF-β1 for an additional 24 h. Cells were then lysed, and the luciferase activity was determined using a Dual Luciferase Reporter Assay Kit (Toyo Ink, Tokyo, Japan).

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using analysis of variance followed by Scheffe’s test. P < 0.05 was considered statistically significant.

RESULTS

CTGF expression in obstructive nephropathy. To investigate the involvement of CTGF in tubulointerstitial fibrosis, we examined changes in CTGF gene expression together with those in TGF-β1 and fibronectin messages in rat kidney after obstructive nephropathy. Figure 1 illustrates the expression of TGF-β1, CTGF, and fibronectin mRNA at 12 h and 3, 6, and 14 days after ureteral obstruction. The staining intensity of 28S rRNA verified equivalent loading of RNA samples in each lane (Fig. 1A). TGF-β1 mRNA expression was increased in the right obstructed kidneys as early as
12 h compared with control sham-operated or contralateral kidneys and remained high at day 14 (Fig. 1B), as reported in previous studies (14, 33, 34, 40). CTGF mRNA expression was significantly upregulated subsequent to TGF-β1 in the obstructed kidneys at day 3 (1.7-fold of control) (Fig. 1, A and B). The upregulation became more pronounced at days 6 and 14 (4.1- and 6.9-fold of control, respectively). This increase was

![Image of in situ hybridization for CTGF mRNA in the kidney after UUO in rats.](http://ajprenal.physiology.org/)

Representative results from control sham-operated rats (A and B; ×400) and UUO rats at day 14 (C and D; ×400) after surgery are shown. In the control kidney (cortex (A) and medulla (B)), CTGF mRNA expression was confined only in the glomerular tuft, presumably in podocytes (arrows). At day 14 after UUO (cortex (C) and medulla (D)), CTGF mRNA was upregulated in glomerular epithelial cells (arrows) and in mesangial areas (asterisk) as well as in the interstitial fibrotic areas (arrowheads) and tubular epithelial cells (double arrow). The contralateral kidney at day 14 (cortex (E) and medulla (F)) showed increased CTGF mRNA expression in the glomerulus (asterisk) but not in the tubulointerstitial areas. No significant signal was detected from the same sample as in C with the sense probe (G; ×400). Masson’s trichrome-stained sections from the sham-operated kidney showed normal appearance (H; ×400); after UUO, the obstructed kidney at day 14 exhibited increased extracellular matrix deposition with markedly atrophic tubules (I; ×400).
followed by marked augmentation of fibronectin mRNA induction in the obstructed kidney, which appeared progressively throughout the time course and was more prominent than that of CTGF (17-fold of control at day 14) (Fig. 1B). These findings indicate that the upregulation of TGF-β1 and CTGF gene expression precedes that of fibronectin expression after obstructive nephropathy, suggesting that CTGF may mediate the fibronectin upregulation induced by TGF-β1 during the course of tubulointerstitial fibrosis. The contralateral kidney showed a slight but significant increase in CTGF and fibronectin gene expression at day 14, without a significant change in TGF-β1 expression (Fig. 1B).

In situ hybridization and histology. In situ hybridization for CTGF mRNA gave signals only in the glomerular tuft and no apparent signal in the tubulointerstitial area in the sham-operated kidney (Fig. 2, A and B). In the obstructed kidney, on the other hand, CTGF mRNA expression was increased in fibrotic areas and tubular epithelial cells as well as parietal glomerular epithelial and mesangial cells at day 14 after UUO (Fig. 2, C and D). The contralateral kidney showed a slight increase in CTGF expression in the glomerulus, presumably in the mesangial area, but signals were not apparent in the tubulointerstitial area (Fig. 2, E and F). No significant signal was detected when serial sections from the same sample were hybridized with the sense probe (Fig. 2G). Histologically, the sham-operated kidney had a normal appearance (Fig. 2H). In the obstructed kidney at day 14, collecting ducts and distal tubules displayed tubular atrophy and epithelial flattening with marked tubulointerstitial fibrosis (Fig. 2I).

By immunohistochemistry, interstitial expression of α-SMA, a marker for myofibroblasts (15), was markedly increased after UUO (Fig. 3B), as reported previously (27). The interstitial cells expressing CTGF mRNA were also positive for α-SMA staining (Fig. 3, A and B, arrows).

TGF-β-stimulated expression of CTGF and fibronectin mRNA in cultured cells. In models of tubulointerstitial fibrosis, interstitial fibroblasts are thought to be the major cell type of TGF-β production and action to stimulate ECM accumulation (4, 18), whereas macrophages may be another important source of TGF-β production (14, 18). In situ hybridization has revealed that CTGF is induced in the tubulointerstitium mainly in the interstitial fibroblasts and tubular epithelial cells. Although TGF-β is already shown to stimulate the expression of both CTGF and fibronectin in cultured fibroblasts (22, 26), their relationship has not been evaluated. To investigate this, we first examined the dose- and time-dependent induction by TGF-β of CTGF and fibronectin gene expression using cultured rat renal fibroblasts and renal epithelial cells (Fig. 4).

After confirming that TGF-β1 showed a well-paralleled CTGF and fibronectin gene upregulation in NRK-49F cells in a dose-dependent manner (Fig. 4A), we examined the time course of CTGF and fibronectin mRNA induction by using 3 ng/ml of TGF-β1 (Fig. 4B). CTGF gene expression was significantly upregulated by TGF-β1 stimulation in 3 h, showed a peak ~12 h after exposure (3.9-fold above baseline), and then declined at 48 h. In contrast, fibronectin mRNA expression was gradually increased from 6 h and showed a significant upregulation at 24 and 48 h after stimulation. Thus, as observed in the obstructive nephropathy model, the upregulation of fibronectin expression by TGF-β1 was rather delayed compared with that of CTGF. TGF-β1 also stimulated CTGF and fibronectin expression in NRK-52E cells, but the extent of induction was less remarkable than in NRK-49F cells (Fig. 4C).

Effects of CTGF antisense transfection on fibronectin and type I collagen synthesis. The findings that TGF-β induces CTGF expression in an earlier time course than fibronectin and that TGF-β and CTGF share a number of effects including fibronectin induction have led to the hypothesis that CTGF may serve as a downstream mediator of TGF-β action (22). To explore the role of CTGF in TGF-β-induced fibronectin expression, we examined the effect of CTGF antisense ODN transiently transfected into NRK-49F cells by the cationic lipofection method. As shown in Fig. 5, CTGF antisense ODN markedly inhibited TGF-β1-induced CTGF mRNA expression at 12 and 48 h after stimulation compared with control reverse ODN, indicating efficient transfection of ODN into the cells. Under this condition, TGF-β1-induced fibronectin mRNA expression was significantly (by ~70%) attenuated at 48 h.

Fig. 3. Interstitial fibrotic area in the obstructed kidney from UUO rats. A: in situ hybridization of CTGF mRNA reveals increased expression in the interstitial cells (arrows). B: immunohistochemical staining for α-smooth muscle actin (α-SMA) of the same section shows that CTGF-positive cells are also positive for α-SMA.
after transfection in antisense ODN-treated fibroblasts compared with control ODN-treated cells (+19 vs. +60% of vehicle-treated cells, \( P < 0.05 \)) (Fig. 5B). Western blot analysis confirmed the inhibited synthesis of fibronectin protein by CTGF antisense treatment (Fig. 6). Furthermore, CTGF antisense ODN also inhibited TGF-\( \beta \)-stimulated production of type I collagen at 48 h after transfection (Fig. 6). These findings strongly suggest that the increased production of fibronectin and type I collagen by TGF-\( \beta \) is mediated by, for the most part, the induction of CTGF expression in cultured renal fibroblasts.

Effects of CTGF antisense transfection on fibronectin promoter activity. We next examined the effect of the blockade of CTGF expression on fibronectin promoter activity. For this purpose, we constructed a luciferase reporter plasmid carrying the promoter region (−531 to +1) of the rat fibronectin gene (13). This fibronectin promoter construct rFN531-Luc showed a 2.5-fold increase in luciferase activity on stimulation by 3 ng/ml TGF-\( \beta \) (Fig. 7). Treatment with control ODN had no significant effect on luciferase activity, showing that a nonspecific effect by ODN transfection was negligible. In this condition, treatment with CTGF antisense ODN almost completely abolished TGF-\( \beta \)-stimulated
induction of fibronectin promoter activity (Fig. 7). These results indicate that CTGF plays a critical role in TGF-β1-induced transcriptional activation of the fibronectin gene in cultured renal fibroblasts.

**DISCUSSION**

Previous studies have shown the close relationship between the increased expression of TGF-β and the progression of glomerulosclerosis and tubulointerstitial fibrosis, suggesting a role of this cytokine in the pathogenesis of fibrotic renal diseases (3, 5, 18, 46). Indeed, blocking of TGF-β1 with neutralizing antisera or antisense oligonucleotide effectively suppresses matrix protein accumulation and mesangial expansion in experimental glomerulonephritis (1, 7). Similarly, the beneficial effects on renal histology and function by treatment with anti-TGF-β antibody have been reported in experimental diabetic nephropathy models (53, 58).

Upregulation of TGF-β is consistently documented in experimental obstructive nephropathy (3, 14, 33, 34), but the pathogenic role of TGF-β in this particular fibrosis model is less defined. Studies so far have indicated the importance of the activated renin-angiotensin system in stimulating TGF-β after UUO, and the inhibition of angiotensin II generation or its receptor signaling has been shown to successfully prevent the progression of fibrosis concomitantly with reduced TGF-β expression (3, 28, 34, 43). Recently, blocking of TGF-β in obstructive nephropathy using TGF-β1 antisense ODN (27) or anti-TGF-β antibody (37) has been reported, resulting in significant amelioration of tubulointerstitial fibrosis. These studies have provided plausible evidence for TGF-β as a potential therapeutic target against renal fibrosis (6). An important caveat for this strategy, however, is that long-term suppression of TGF-β, a multifunctional cytokine, might be detrimental. TGF-β has a modulatory role in the immune system, mainly suppressing the inflammatory response (9). In fact, TGF-β1-null mice exhibit excessive inflammation with tissue necrosis in specific organs, leading finally to organ failure and death (49). Moreover, TGF-β1 may also function as an endogenous antiangiogenic and antitumor factor for certain malignancies (21), thus rendering this methodology less feasible in humans. Therefore, to design antifibrotic strategies, it is important to elucidate the mechanisms and downstream pathways specific to the profibrotic action of TGF-β.

In the present study, we reveal that CTGF is a likely mediator of TGF-β-stimulated fibronectin induction in a rat model of interstitial fibrosis and in cultured renal interstitial fibroblasts on the basis of the following findings. CTGF expression was markedly upregulated subsequent to TGF-β from an early stage of tubulointerstitial fibrosis after ureteral obstruction, followed by a marked increase in fibronectin mRNA (Fig. 1). This observation is compatible with different time courses for TGF-β activation between CTGF and fibronectin noted in renal fibroblasts in culture (Fig. 4B).Interstitial fibroblasts have been shown to be the major site of TGF-β upregulation in obstructive nephropathy (27). Using in situ hybridization, we for the first time demonstrated the upregulation of CTGF mRNA in the cells of interstitial fibrotic areas as well as in tubular epithelial cells in the obstructive nephropathy model (Fig. 2). In the sham-operated kidney, we detected CTGF mRNA expression only in cells of the glomerular tuft (Fig. 2A), presumably podocytes (29, 30). It has been reported that myofibroblasts and fibroblasts may be the main source of CTGF expression in tubulointerstitial fibrosis in a human renal biopsy specimen (29). Consistent with this observation, the immunohistochemical staining for α-SMA, a myofibroblast marker,
revealed that most of the CTGF-positive cells were also positive for α-SMA in the obstructed kidney (Fig. 3). Together with the potent profibrotic property of CTGF (19, 22), these findings suggest CTGF mediation of TGF-β-dependent fibroblast induction. One interesting finding in the UUO model is a significant upregulation of CTGF mRNA in the glomeruli of the contralateral kidney without a change of TGF-β1 expression (Figs. 1B and 2E), suggesting a possible TGF-β-independent stimulation. A previous report showed a similar increase in collagen IV mRNA in the contralateral kidney but along with TGF-β1 upregulation, which was abolished by angiotensin-converting enzyme inhibition (28). Although TGF-β dependence is not clear at present, this might reflect mechanical or humoral signals to the contralateral kidney for increased matrix production in this model.

To clarify CTGF dependence of TGF-β-stimulated fibroblast induction, we employed antisense strategy in cultured rat renal fibroblasts. Northern blot analysis indicated that the introduction of CTGF antisense ODN abolished TGF-β-induced CTGF expression at 12 h and thereafter significantly attenuated fibronectin mRNA and protein synthesis (Figs. 5 and 6). Furthermore, the antisense CTGF treatment allowed almost completely abolished the increased fibronectin promoter activity stimulated by TGF-β (Fig. 7). These results strongly suggest that TGF-β-induced fibroblast expression is mostly dependent on CTGF in cultured renal fibroblasts. The difference in inhibition magnitude among these experiments may be due to the difference in transfection efficiency. CTGF antisense ODN also inhibited TGF-β-stimulated type I collagen synthesis (Fig. 6). Recently, CTGF has been reported to mediate TGF-β-induced collagen synthesis in NRK fibroblasts using cells treated with anti-CTGF antibodies or those stably transfected with an antisense CTGF gene (16). Such CTGF dependence in this cell line has also been demonstrated in anchorage-independent growth induced by TGF-β (35). Taken together, these findings indicate that CTGF plays a crucial role in mediating various important actions of TGF-β.

Increased CTGF expression has been shown in a variety of human and experimental diseases characterized by fibrosis, including studies in the kidney (29, 30, 39, 45), skin (19, 25, 26), blood vessels (42), lung (36), and liver (22). Whether CTGF plays a role in vivo in fibrosis progression of these disease states still remains to be elucidated and obviously requires further clarification. Whether CTGF upregulation in those conditions is TGF-β dependent is another issue to be clarified. Of note, it has been reported that dexamethasone potently induces CTGF while suppressing TGF-β (12), suggesting the presence of a TGF-β-independent pathway of CTGF activation and also the possible involvement of CTGF in profibrotic actions of glucocorticoids. Besides stimulating fibrogenesis, CTGF exerts various biological actions, including endothelial cell migration and proliferation (2, 54), angiogenesis (2, 54), and vascular smooth muscle cell apoptosis (23), thereby potentially participating in tissue remodeling in various disease states.

Fibronectin is a major ECM protein serving as a scaffold for the deposition of other proteins; it also functions as a fibroblast chemoattractant (20). Furthermore, fibronectin promotes differentiation of fibroblasts to myofibroblasts (51), which may be a crucial phenomenon in the pathogenesis of tubulointerstitial fibrosis (15, 32). We demonstrate that CTGF plays a critical role in fibroblast gene induction activated by TGF-β, but the signaling mechanisms of this cascade activation are yet to be determined. TGF-β-induced fibronectin expression has been shown to be dependent on the activation of c-Jun N-terminal kinase in human fibrosarcoma cells (24). It has also been shown that the activator protein-1 element in the rat fibroblast gene is important in angiotensin II-stimulated fibronectin induction in vascular smooth muscle cells (56). TGF-β and CTGF share multiple biological actions including fibrogenesis but, at the same time, have other actions that may not overlap one another (22). Therefore, it is important to elucidate the signaling pathway and mechanisms by which CTGF induces fibronectin expression.

In summary, the present study demonstrates that CTGF expression is upregulated in obstructive nephropathy, followed by a marked induction of fibronectin expression. CTGF blockade resulted in a marked inhibition of TGF-β-induced fibronectin expression and its promoter activity in cultured renal fibroblasts, suggesting that CTGF is crucial in mediating fibronectin induction in the TGF-β-stimulated pathway. Our study opens the possibility that blockade of CTGF should provide a novel therapeutic strategy for treating various renal diseases leading to fibrosis.

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