TGF-β and CTGF have overlapping and distinct fibrogenic effects on human renal cells

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Am J Physiol Renal Physiol 283: F707–F716, 2002. First published May 14, 2002; 10.1152/ajprenal.00007.2002.—Transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) are ubiquitously expressed in various forms of tissue fibrosis, including fibrotic diseases of the kidney. To clarify the common and divergent roles of these growth factors in the cells responsible for pathological extracellular matrix (ECM) deposition in renal fibrosis, the effects of TGF-β and CTGF on ECM expression in primary human mesangial (HMCs) and human proximal tubule epithelial cells (HTECs) were studied. Both TGF-β and CTGF significantly induced collagen protein expression with similar potency in HMCs. Additionally, αs(I)-collagen promoter activity and mRNA levels were similarly induced by TGF-β and CTGF in HMCs. However, only TGF-β stimulated collag enous protein synthesis in HTECs. HTEC expression of tenascin-C (TN-C) was increased by TGF-β and CTGF, although TGF-β was the more potent inducer. Thus both growth factors elicit similar profibrogenic effects on ECM production in HMCs, while promoting divergent effects in HTECs. CTGF induction of TN-C, a marker of epithelial-mesenchymal transdifferentiation (EMT), with no significant induction of collag enous protein synthesis in HTECs, may suggest a more predominant role for CTGF in EMT rather than induction of excessive collagen deposition by HTECs during renal fibrosis.

mesangial cells; tubule epithelial cells; collagen; tenascin; epithelial-mesenchymal transdifferentiation; connective tissue growth factor; transforming growth factor-β

PROGRESSIVE RENAL DISEASE can be caused by multiple pathogenetic mechanisms, leading to end-stage renal failure. The progressive renal diseases predominantly originate in the glomerulus, causing glomerular injury and subsequent sclerosis characterized by prominent intraglomerular accumulation of extracellular matrix (ECM) components. Among the resident cells of the glomerulus, mesangial cells are primarily responsible for excessive ECM deposition (55). In recent years, tubulointerstitial damage has been recognized as an equally important component of progressive renal disease (57). It is now believed that glomerular injury triggers tubular cell activation, leading to tubulointerstitial inflammation and fibrosis. Activated tubular cells produce a number of cytokines, chemokines, and profibrogenic growth factors, which act in an autocrine and paracrine fashion (60). Increasing evidence also suggests that tubular epithelial cells have the capacity to undergo epithelial-mesenchymal transdifferentiation (EMT), thereby becoming interstitial fibroblasts (43), a major cell type responsible for interstitial fibrosis (1).

Transforming growth factor-β (TGF-β) is implicated as a key mediator in the development of kidney fibrosis. Elevated expression of TGF-β isoforms has been demonstrated in the glomeruli and tubulointerstitium of patients with renal diseases characterized by excessive ECM accumulation (63). Elevated TGF-β has also been observed in various animal models of kidney fibrosis (11, 21, 27, 46, 53). Moreover, blocking TGF-β via neutralizing antibodies resulted in reduction of glomerulosclerosis in experimental models (6, 54, 69). More recent studies have shown that connective tissue growth factor (CTGF) expression is strongly upregulated in diabetic nephropathy and other progressive renal diseases (33). A strong correlation between the number of CTGF-positive cells and degree of injury at sites of chronic tubulointerstitial damage was also observed (33). However, in contrast to TGF-β, the significance of CTGF in development of renal fibrosis is not fully defined.

The relationship between TGF-β and CTGF in the stimulation of ECM synthesis has been characterized recently. TGF-β-induced collagen synthesis is blocked...
with specific anti-CTGF antibodies or antisense CTGF oligonucleotides in NRK-fibroblasts in vitro and in wound healing in vivo (15). Based on these observations, it has been proposed that CTGF is a downstream mediator of the fibrogenic effect of TGF-β. In support of these studies, NRK-49F cells treated with CTGF antisense oligonucleotides showed significant inhibition of fibronectin and α1(I)-collagen mRNA upon TGF-β stimulation (66). However, additional studies using rat mesangial cells demonstrated that neutralizing antibodies to CTGF inhibit TGF-β-mediated fibronectin synthesis but not TGF-β induction of α-smooth muscle actin (4), indicating differential effects of TGF-β and CTGF on the mesangial cell response to injury in vitro. Furthermore, recent studies performed in human dermal fibroblasts suggest an additional divergent role for CTGF and the closely related CCN family member Cyr61 as matrix remodeling factors (7, 8). Thus CTGF may be involved in matrix deposition or matrix degradation, depending on either the cell type or the specific experimental conditions.

Given the fact that CTGF is prominently expressed in both glomerular and tubular compartments in progressive human renal diseases, the goal of this study was to systematically compare the profibrogenic effects of CTGF via a vis TGF-β. Because of the possible cell-type-specific effects of CTGF, we used human renal cells for our studies, including human mesangial cells (HMC) and human proximal tubular epithelial cells (HTEC).

MATERIALS AND METHODS

Cell Culture

Human kidney tissues were obtained from surgical nephrectomy specimens and donor transplant kidneys, which were determined unsuitable for transplant in accordance with Institutional Review Board guidelines. HMCs were isolated from glomeruli by collagenase treatment and differential sieving of minced cortical areas of the renal tissue (34). Cells were confirmed to be mesangial by morphological criteria and by the presence of staining to actin, Thy-1, and myosin, and by the absence of staining for cytokeratin and factor VIII. For some experiments, HMCs were purchased from Clonetics (Rockville, MD). HMCs were grown in medium containing a 1:1 mixture of DMEM and Ham’s F-12 supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and 20% FBS. HTECs were isolated by collagenase treatment and fabric filtering of minced tissue (25). Cells were confirmed to be epithelial by morphological and positive staining for cytokeratin and negative staining for factor VIII. HTECs were grown in medium containing a 1:1 mixture of DMEM and Ham’s F-12 supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), L-glutamine (2 mM), and epidermal growth factor (10 ng/ml). Serum-free medium (SFM) contained only the 1:1 mixture of DMEM and Ham’s F-12 and 0.1% BSA. All culture media was purchased from Gibco-BRL (Grand Island, NY). Cells were grown at 37°C in 5% CO₂.

Recombinant CTGF/CTGF Antibody

Human recombinant CTGF (rCTGF) was generated using a baculovirus system to infect Drosophila cells (19). rCTGF was purified using a heparin-Sepharose affinity column, and rCTGF fractions were analyzed via Western blot analysis for the absence of contamination by other growth factors. Goat anti-human CTGF antibody was generated as previously described by Duncan et al. (15).

Adenoviral Constructs

Adenoviral vectors expressing CTGF and green fluorescent protein (GFP) were generated using the method described by He et al. (26). Briefly, the cDNA encoding full-length human CTGF (provided by Gary Grotendorst) was cloned in the shuttle vector pAdTRACK-CMV, which contains a GFP expression cassette driven by a separate CMV promoter. The shuttle vector containing CTGF was cotransformed into Escherichia coli BJ5183 cells with the AdEasy-1 adenoviral backbone plasmid, which lacks the E1 and E3 regions of the adenoviral genome. Linearized recombinant plasmid DNA was then transfected into 293 cells, an adenoviral packaging cell line, using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) to generate the recombinant adenovirus expressing CTGF and GFP from separate CMV promoters (AdCTGF). An adenovirus expressing GFP alone (AdGFP) was generated via the same method for use as a control vector.

3[H]Proline Incorporation Assay

Cells were plated in 12-well plates and grown to visual confluence, followed by incubation in SFM containing 0.1% BSA supplemented with ascorbic acid (50 μg/ml) and β-aminopropionitrile (64 μg/ml) for 24 h. Cells were then treated with indicated concentrations of TGF-β (R&D Systems, Minneapolis, MN) or human rCTGF for 48 h. To inhibit the CTGF response and demonstrate specificity of the rCTGF, human fibroblasts were pretreated with goat polyclonal CTGF antibody or normal goat IgG (50 μg/ml) for 30 min before addition of rCTGF (5 ng/ml). Alternatively, cells were transduced with AdCTGF or AdGFP as a control at a multiplicity of infection (MOI) of 25 for 48 h. The [3H]proline incorporation assay was performed as previously described (30). Briefly, 20 μCi/ml L-[2,3,4,5-3H]proline were added during the last 24 h of incubation. Aliquots of culture medium normalized for cell number were concentrated in a SpeedVac, denatured by boiling in SDS sample buffer, and separated by 6% SDS-PAGE. After electrophoresis, gels were enhanced by fluorography (Fluoro-Hance; Research Products International, Mount Prospect, IL) and visualized by autoradiography. Protein levels were quantitated using NIH Image densitometry software.

RNA Isolation and Northern Blot Analysis

Confluent cultures of HMCs and HTECs were serum starved for 24 h, followed by stimulation with TGF-β or rCTGF at the indicated concentrations for 24 h. Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method (10) and electrophoresed on a 1% agarose gel. Northern blot analysis was performed as previously described (30). Briefly, after RNA transfer to a nylon membrane, hybridization was performed overnight using [32P]cDNA probes for α1(I)-collagen (COL1A2), tenasin, or 18S rRNA. Membranes were washed and exposed in a cassette for quantitation using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
**Measurement of Spliced and Unspliced COL1A2 by RT-PCR**

HMCs were grown to confluence in 10-cm² dishes and serum starved for 24 h, followed by incubation with TGF-β or rCTGF for 24 h. HMCs serum starved in the absence of growth factor stimulation served as the control. Alternatively, cells were transfected with AdCTGF or AdGFP as a control for 48 h at an MOI of 50. Total RNA was isolated and DNase treated, and 1 μg RNA was converted to cDNA using a random hexamer primer. Spliced collagen was amplified using an upper primer to exon 1 (5’-CGC GGA CTT TGT TGC TGC TTG-3’) and a lower primer spanning exons 7 and 8 (5’-GGA AAC CCT GAT GGC CTG GG-3’) and yielded a 321-bp product. Unspliced collagen was amplified using the same upper primer and lower primer to intron 1 (5’-GGA CCT TGA CTT CCA CCA C-3’) and yielded a 393-bp product. 18S rRNA was amplified using the QuantumRNA Classic 18S Standards (Ambion, Austin, TX) to yield a 488-bp product. cDNA for unspliced collagen (1 μl) and cDNA for spliced and 18S rRNA (0.5 μl) were used for amplification using 0.6 μM of each primer and 1 mM MgCl₂ in a total volume of 50 μl. Spliced and unspliced collagen were amplified in the presence of 5 and 6% formamide, respectively. Amplification was for 95°C for 5 min and then cycles of 95°C for 1 min, 59°C for 30 s, and 72°C for 45 s, followed by a 5-min extension at 72°C. Spliced and unspliced collagen and 18S rRNA were amplified for 24, 32, and 23 cycles, respectively. Product sizes were measured with a 100-bp DNA ladder.

**Plasmid Constructs, Transient Transfection, and Luciferase Assay**

The COL1A2-luciferase construct contains sequences from −353 to +58 bp of the human COL1A2 promoter fused to the luciferase reporter gene, as previously described (14). For transient transfections, HMCs were seeded in six-well plates (80,000 cells/well) in medium containing 10% FCS. The next day, cells were transfected with 1 μg plasmid DNA using FuGENE6 transfection reagent (Roche) following the manufacturer’s recommendations. Each condition was performed in triplicate. Later (24 h), medium was changed to 2% FCS with or without growth factors. After transfection (48 h) and after addition of growth factors (24 h), cells were harvested in 300 μl lysis buffer/well (Promega, Madison, WI), and protein concentration of pooled triplicate samples was determined using the Bio-Rad (Hercules, CA) protein assay based on the Bradford method. Luciferase activity was measured in cell lysates normalized for protein concentration. These transfection conditions consistently gave reproducible results. In some experiments, a construct containing the Renilla luciferase (pRL-TK; Promega) was cotransfected with the collagen promoter and used as an additional control for transfection efficiency. Luciferase activities in cell lysates were measured with a dual luciferase reporter assay system (Promega).

**Western Blot Analysis**

**TGFβRI/TGFβRII.** Confluent cultures of HMCs were serum starved for 24 h and then stimulated with growth factors [TGF-β, rCTGF, platelet-derived growth factor (PDGF)-AB] at the indicated concentrations for an additional 24 h. Cells were collected and lysed in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride followed by bicinechonic acid protein assay (Pierce, Rockford, IL) to determine total protein concentration. Equal amounts (50 μg) of total proteins per sample were separated via 8% SDS-PAGE (TβRII) or 10% SDS-PAGE (TβRI) and transferred to nitrocellulose membranes for Western blotting. Polyclonal rabbit antibodies against TβRI (R-20; Santa Cruz Biotechnology, Santa Cruz, CA) and TβRII (Medical University of South Carolina Antibody Facility) were incubated at dilutions of 1:500 in 3% milk/Tris-buffered saline (TBS) overnight at 4°C. After washes in Tween-TBS (TTBS), membranes were probed with anti-rabbit-horseradish peroxidase (HRP) secondary antibodies (Amersham Pharmacia, Piscataway, NJ) in 3% milk/TBS at a dilution of 1:3,000 for 1.5 h at room temperature. After washes in TTBS, proteins were visualized using enhanced chemiluminescence (ECL) reagents (Amersham). Protein levels were quantitated using NIH Image densitometry software.

**Tenasin-C.** Confluent cultures of HMC or HTEC in 12-well plates were serum starved for 24 h, followed by stimulation with TGF-β or rCTGF (2.5, 5, and 10 ng/ml) for 72 h. To inhibit rCTGF stimulation, cells were pretreated with neutralizing CTGF antibody (50 μg/ml) or normal goat IgG as a control for 30 min, followed by addition of rCTGF (5 ng/ml) for 72 h. Aliquots of conditioned media normalized for cell number were analyzed for tenasin protein levels via 6% SDS-PAGE followed by transfer to a nitrocellulose membrane. The membrane was probed with a mouse monoclonal antibody against human tenasin-C (TN-C; 1:1,000, kindly provided by Dr. Wolfgang Rettig, Boehringer Ingelheim Pharma) used in a previously published study by our laboratory (36). After washes in TTBS, the blot was incubated with anti-mouse-HRP secondary antibody (1:3,000). After final washes in TTBS, tenasin protein levels were visualized with ECL and quantitated as above.

**Statistical Analysis**

Student’s t-test analysis using GraphPad InStat Statistics Software (version 1.12) was performed to determine statistical significance. Values of ≤0.05 were considered statistically significant.

**RESULTS**

**TGF-β and CTGF are Inducers of ECM Proteins in HMC**

Previous studies have shown that TGF-β is a potent inducer of collagen gene expression in HMCs, including collagen types I, III, and IV (47). The effects of TGF-β and CTGF on production of collagenous proteins were examined in confluent HMCs using the [³H]proline incorporation assay followed by SDS-PAGE. This assay allows for assessment of newly synthesized total collagenous proteins (Fig. 1, A and B). Incubation of cells with low concentrations of either TGF-β or CTGF consistently led to a similar increase in collagenous protein synthesis, suggesting that both factors could independently contribute to elevated ECM deposition in HMCs. Collagens I, III, and IV were induced to a similar extent by TGF-β and CTGF. As shown in Fig. 1C, the stimulation of collagenous protein synthesis by CTGF is abrogated in the presence of neutralizing antibody against CTGF, which confirms the specificity and purity of the rCTGF used for these experiments. To provide additional support for the ability of CTGF to induce collagenous proteins in HMCs, cells were transfected with an adenoviral vector expressing CTGF (AdCTGF) or green fluorescent protein
CTGF endogenously expressed via the adenoviral vector was an equally potent inducer of collagenous proteins compared with the rCTGF added exogenously. Because induction of collagen type I is specifically associated with renal fibrosis, we next investigated the effects of TGF-β and CTGF on COL1A2 promoter activity using a COL1A2 promoter/reporter construct as described in MATERIALS AND METHODS. Recent studies have determined that TGF-β stimulates COL1A2 gene transcription via the Smad3 and Sp1 response elements located in the proximal region of the COL1A2 promoter (9, 47, 68). Furthermore, it has been reported that CTGF stimulates the COL1A2 promoter in human fibroblasts (56). We observed that both TGF-β and CTGF significantly stimulate the COL1A2 promoter activity in HMCs with similar potency, suggesting that the CTGF response element is also located in the proximal promoter region (Fig. 2A). The specific cis elements and cognate transcription factors mediating CTGF stimulation of the COL1A2 promoter remain to be characterized.

To further confirm the effects of CTGF on COL1A2 transcription, we used a previously described method based on the PCR (51). RT-PCR using primers designed for spliced and unspliced (newly transcribed) collagen type I RNA was performed to analyze the effect of CTGF on COL1A2 mRNA expression. HMCs were stimulated with CTGF for 24 h or left unstimulated as a control. Figure 2B, left, demonstrates that CTGF induced COL1A2 steady-state mRNA levels (spliced) with a 1.33-fold increase in COL1A2 spliced mRNA when normalized for 18S RNA levels (compare lanes 1 and 3). Furthermore, CTGF increased COL1A2 heterogeneous nuclear RNA (unspliced RNA) levels (Fig. 2B, right, compare lanes 2 and 4) consistent with CTGF stimulation of COL1A2 promoter activity (Fig. 2A), which collectively suggests induction of collagen gene transcription by CTGF. Additionally, endogenously expressed CTGF in HMCs transduced with AdCTGF also induced COL1A2 transcription compared with HMCs transduced with AdGFP as a control (Fig. 2B, right). The average degree of increase in unspliced/newly transcribed COL1A2 by rCTGF and endogenously expressed CTGF (AdCTGF) was 1.72 when normalized for 18S RNA levels. Taken together, these observations (Figs. 1 and 2) suggest that TGF-β and CTGF have similar effects on collagen expression in HMCs.

**TGF-β Receptor Expression Is Not Modulated by Either TGF-β or CTGF in HMCs**

TGF-β receptors are upregulated in various models of renal fibrosis (27, 29, 58); however, very little is
known about regulation of their expression. To determine whether stimulation of collagen by CTGF is mediated via upregulation of TGF-β receptors, TGF-β receptor levels were analyzed in HMCs. We examined the effects of TGF-β, CTGF, and PDGF-AB on the type I and type II receptor levels in HMCs by Western blot and RT-PCR. Neither TGF-β nor CTGF had appreciable effects on TGF-β receptor expression at the protein (Fig. 3A) or mRNA (data not shown) levels. Interestingly, however, PDGF-AB significantly decreased receptor type II levels by 35% (Fig. 3B), without affecting the levels of type I receptor. The functional significance of this observation is not clear at the present time.

**TGF-β and CTGF Have Divergent Effects on ECM Production by HTECs**

We next examined the effects of TGF-β and CTGF on collagenous protein synthesis in HTECs. HTECs predominantly produce collagen type IV, which was significantly increased in response to TGF-β (Fig. 4A). CTGF treatment reduced synthesis of collagenous proteins in HTECs. Furthermore, the lowest concentration of CTGF used in our study (1 ng/ml) was most inhibitory (Fig. 4B). However, this decrease did not reach statistical significance.

To determine if the differential effects of TGF-β and CTGF in HTECs were specific for collagen type IV or whether other ECM proteins were also differentially regulated by these growth factors, we analyzed TN-C expression. In healthy kidney, TN-C is diffusely expressed in the medulla, but it is ubiquitously overexpressed in areas of tubulointerstitial damage, regardless of diagnosis (59). Therefore, TN-C expression may serve as an early marker of fibrosis. The effects of TGF-β and CTGF on TN-C steady-state mRNA and protein expression in HTECs were determined by Northern blot and Western blot analysis, respectively. TGF-β significantly induced TN-C protein levels (Fig. 5A and B), which correlated with induction of TN-C mRNA levels (Fig. 5D). CTGF also significantly stimulated production of TN-C protein, although with lesser potency than TGF-β (Fig. 5A and B). The increase in TN-C protein was abolished in HTECs treated with neutralizing CTGF antibody before addition of rCTGF, demonstrating the specificity of the stimulatory effect of CTGF (Fig. 5C). A more modest stimulation of TN-C mRNA levels was observed after addition of various doses of CTGF compared with TGF-β (Fig. 5D). Collectively, these data suggest that under the experimental conditions used in our study, TGF-β and CTGF have opposing effects on collagen IV synthesis in HTECs but similar effects on TN-C synthesis in HTECs. We also analyzed the effects of TGF-β and CTGF on TN-C production in HMCs. Cultured
A: \[ ^{3}H \] proline incorporation assay was performed after stimulation of HTEC with the indicated concentrations of TGF-β or CTGF for 48 h as described in MATERIALS AND METHODS. A representative gel is shown. B: graphical representation of relative collagenous protein levels after addition of TGF-β or CTGF for 48 h compared with control levels arbitrarily set to 1. Values are the averages from 3 independent experiments performed in duplicate: *P = 0.05 and ** P = 0.004.

HMCs synthesized TN-C, but TN-C levels were not modulated by either TGF-β or CTGF (data not shown).

DISCUSSION

The profibrogenic role of TGF-β in various fibrotic diseases, including renal fibrosis, is well established. More recently, CTGF has also been proposed as a key cytokine responsible for elevated deposition of ECM proteins in vivo. Both TGF-β and CTGF are universally overexpressed in various diseases characterized by tissue fibrosis (5, 22, 31, 42). However, although the role of TGF-β as a potent inducer of ECM synthesis in a variety of cells, including renal cells, is well documented, little is known regarding the mechanisms by which CTGF affects ECM deposition. Original studies by Duncan et al. (15) using embryonic rat kidney cells (NRK fibroblasts) have demonstrated that CTGF is a mediator of the fibrogenic effects of TGF-β, including proliferation and ECM production. Subsequent studies have shown induction of collagen type I and fibronectin by CTGF in rat mesangial cells (48, 49). Additionally, CTGF has been shown to induce expression of fibronectin in mouse tubule epithelial cells (61). On the other hand, Chen et al. (7) have demonstrated that adhesion of human fibroblasts to CTGF resulted in the formation of focal adhesion complexes and activation of FAK, paxillin, and Rac kinases, which correlated with prolonged mitogen-activated protein kinase activation and induction of metalloproteinases MMP-1 and MMP-3 (7). The latter study suggests a role for CTGF as a matrix-remodeling factor. Finally, there is increasing evidence that CTGF and a closely related factor, Cyr61, have proangiogenic functions (2, 3). These studies underscore the cell-type-specific effects of this pleiotropic growth factor. Our study was undertaken to characterize the possible profibrogenic functions of CTGF compared with TGF-β in human renal cells.

HMCs in culture constitutively produce ECM proteins, mainly collagen types I, III, and IV (47). ECM synthesis can be further upregulated by low concentrations (2.5–5 ng/ml) of TGF-β. Under our experimental conditions, similar upregulation of ECM was also observed with low doses of CTGF (2.5–5 ng/ml). In addition, the pattern of stimulated ECM proteins was indistinguishable between TGF-β and CTGF, suggesting that both factors induce a similar profibrogenic program in HMCs. In previous studies using rat mesangial cells, stimulation of collagen type I and fibronectin was also observed after addition of either TGF-β or CTGF (49). However, in contrast to our study, stimulation of matrix proteins was achieved with much higher doses of CTGF (20 ng/ml). It is possible that HMCs are more responsive than rat cells to CTGF. However, we cannot exclude the possibility that the difference in CTGF potency was the result of the different sources of CTGF, which is not commercially available. To further compare the effects of TGF-β and CTGF on ECM synthesis, we focused on collagen type I, which is highly upregulated in fibrotic conditions. Furthermore, TGF-β regulation of collagen type I is relatively well defined. In agreement with previous studies, CTGF and TGF-β stimulated COL1A2 mRNA levels in HMCs (40, 47). In addition, our studies demonstrate that similar to TGF-β, CTGF expressed either exogenously or endogenously (via adenoviral vector) upregulates the collagen type I gene at the level of transcription. It is well documented that TGF-β upregulation of the COL1A2 promoter in HMCs and fibroblasts occurs via Smad3 and Sp1. Although TGF-β and CTGF response elements map to the proximal promoter region, it is unlikely that Smad3 is directly involved in the CTGF response. However, TGF-β stimulation of the CTGF promoter is Smad3 dependent (28). In the future, we may characterize the specific transcription factors that mediate CTGF stimulation of the collagen type I gene. Together, these studies demonstrate that TGF-β and CTGF can similarly stimulate ECM production by HMCs in vitro, suggesting that both growth factors may contribute to glomerulosclerosis.

Expression of TGF-β receptors is elevated in human renal diseases (64) and in various animal models of renal fibrosis (27, 29, 53, 58). However, relatively little is known about the factors that upregulate TGF-β receptor expression in general and in renal cells in particular. Some of the factors that have been previously implicated in modulating TGF-β receptor levels in renal cells include ANG II (62), high glucose (32), and leptin (23). On the other hand, TGF-β has been shown to downregulate its receptor expression in rat kidney fibroblasts (20). In this study, there was no effect of either CTGF or TGF-β on type I and type II
TGF-β receptor protein and mRNA levels in HMCs, suggesting that these profibrotic growth factors do not enhance ECM production via upregulation of the classic transmembrane TGF-β signaling receptors. In contrast to our recent studies using dermal fibroblasts, which demonstrated selective upregulation of TGF-β receptor type II by PDGF-AB (13), PDGF-AB selectively reduced TGF-β type II receptor levels in normal adult HMCs. The relevance of this observation is presently unknown. However, differential regulation of TGF-β receptor subunits has been noted in other experimental models. For example, TGF-β type II receptor is exclusively induced by glucose treatment in mouse mesangial cells (32), whereas both receptor mRNA and protein levels were elevated by either high glucose or mechanical stretch in rat mesangial cells (50). We have also observed discordant expression of TGF-β receptor subunits in vivo in the remnant model of chronic renal failure (Gore-Hyer E and Trojanowska M, unpublished observations). In addition, altered ratios of TGF-β receptors have been reported in atherosclerotic lesions (37) and liver cirrhosis (52). These observations may suggest that alterations in the TGF-β signaling pathway via modulation of receptor levels are common features of various fibrotic diseases. Further studies are necessary to understand the implications of altered TGF-β receptor ratios in the context of fibrosis.

Tubulointerstitial injury correlates with the decline in renal function and progression of renal disease (57). Furthermore, elevated levels of TGF-β and CTGF correlate with tubulointerstitial fibrosis (21, 33, 61). Therefore, we analyzed whether HTECs may directly contribute to tubulointerstitial fibrosis by responding to profibrogenic growth factors such as TGF-β and CTGF. Our data demonstrate that ECM protein synthesis by HTECs is significantly increased in response to TGF-β. Elevated collagen synthesis in response to TGF-β was previously observed in rat tubular cells (12). In contrast to HMCs, HTECs do not express increased collagenous proteins in response to CTGF. In fact, the lowest doses of CTGF used in our study decreased the levels of secreted collagenous proteins. This decrease may be either due to inhibition of ECM synthesis or due to stimulation of MMPs, as previously shown in dermal fibroblasts (7). These data also suggest that unlike NRK cells, in which CTGF was shown to mediate the profibrogenic effects of TGF-β, the profibrogenic effects of TGF-β may be CTGF independent in HTECs. Further studies are needed to resolve these issues. Although our studies suggest that CTGF is not a potent stimulator of collagenous protein synthesis in HTECs, it has been demonstrated that CTGF can induce fibronectin expression in mouse proximal tubule epithelial cells (61). Thus CTGF may act as a fibrogenic stimulus in HTECs to induce fibronectin accumulation in renal fibrosis.

There is increasing evidence that EMT is one of the mechanisms contributing to the pathogenesis of tubulointerstitial fibrosis (41). EMT, the process whereby...
epithelial cells transform into mesenchymal cells, occurs during development and in pathological processes such as tumorigenesis (24). Recent studies suggest that the increased numbers of interstitial fibroblasts, which are the primary cell type responsible for ECM deposition in tubulointerstitial fibrosis (1), may be derived in part by the transdifferentiation of tubular epithelial cells into fibroblasts/myofibroblasts (39, 41, 44). During renal fibrosis, EMT is thought to occur when tubular epithelial cells acquire mesenchymal/fibroblast characteristics, including expression of vimentin (38), α-smooth muscle actin (17), and fibroblast specific protein-1 (36), which allow the cells to migrate through their basement membrane into the interstitium where the cells are then identified as fibroblasts/myofibroblasts. It has been demonstrated that HTECs undergoing EMT acquire a migratory phenotype in vitro, which likely facilitates their translocation from the tubular basement membrane to the renal interstitium in vivo (65, 67). Furthermore, alterations in the basement membrane composition, including downregulation of collagen type IV, are important for epithelial cell migration during EMT (67). Interestingly, this study demonstrates the novel finding that CTGF induces TN-C in HTECs, albeit with lesser potency than TGF-β. The matricellular protein TN-C is significantly elevated in tubulointerstitial lesions of various kidney diseases (45) and is expressed during development at the sites of EMT (16). TN-C possesses both adhesive and anti-adhesive properties, which may promote cell migration in tumor metastasis, embryonic development, and wound healing (35). Thus it is possible that TN-C is one of the factors facilitating EMT in vitro and in vivo during renal fibrosis. Our data demonstrating the ability of CTGF to induce TN-C and also diminish collagen IV protein synthesis in HTECs may indicate a predominant role for CTGF in the induction of EMT during the progression of tubulointerstitial fibrosis.

Several studies have demonstrated a pivotal role for TGF-β in EMT in various physiological processes and in kidney fibrosis (17, 43, 67). The divergent effects of TGF-β and CTGF on collagen IV expression in HTECs suggest that these growth factors may direct different HTEC functions in the progression of EMT and tubulointerstitial fibrosis. Although TGF-β significantly induced collagenous proteins in HTECs, CTGF diminished collagen protein expression, which may elicit the changes in basement membrane composition necessary to facilitate EMT. Thus, in addition to TGF-β, CTGF may be an important and distinct mediator of HTEC transdifferentiation in interstitial fibroblasts. In support of the potential role of CTGF in EMT in vivo, Frazier et al. (18) observed CTGF, TGF-β, and PDGF expression in close proximity to epithelial cells in transition to the myofibroblast phenotype in the remnant model of renal fibrosis. It is tempting to speculate that CTGF may contribute to EMT during renal fibrosis by stimulating the migratory phenotype of HTECs via alterations in ECM composition (decreasing collagen type IV expression) and via its induction of TN-C, which may further promote cell migration. Further studies are required to test the specific mechanisms of CTGF in EMT.

In conclusion, our study demonstrates that the profibrogenic potential of CTGF is equal to that of TGF-β in HMCs, with both growth factors exhibiting similar stimulation of collagen gene expression at the promoter, mRNA, and protein levels. To date, various profibrotic effects of CTGF have been demonstrated predominantly using rat or mouse cell lines. However, given the cell type- and/or cell line-dependent properties of in vitro cultures, it is necessary to fully characterize the effects of CTGF in primary human cells. These findings are also important to clarify the profibrotic actions of CTGF in human renal cells in view of the emerging pleiotropic nature of CTGF as a matrix-remodeling factor capable of inducing matrix degradation in human dermal fibroblasts (7). Furthermore, this study reveals distinct effects of TGF-β and CTGF on ECM expression by HTECs. Both growth factors induce expression of TN-C, a marker of EMT, which is overexpressed in renal tubulointerstitial disease (45, 59). However, opposite to TGF-β, CTGF did not significantly induce collagen protein synthesis but slightly decreased collagen type IV protein levels in HTECs, which may indicate a distinct role for CTGF in EMT. TGF-β has recently been shown to have both CTGF-dependent and independent effects in various cell types and experimental systems. Therefore, it will be of great interest to determine the overlapping and distinct roles of TGF-β and CTGF in human tubular epithelial cells undergoing EMT, a potential mechanism contributing to renal tubulointerstitial fibrosis.

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