Reduction in Connective Tissue Growth Factor by Antisense Treatment Ameliorates Renal Tubulointerstitial Fibrosis

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Abstract. Connective tissue growth factor (CTGF/CCN2) is one of the candidate factors mediating fibrogenic activity of TGF-β. It was shown previously that the blockade of CTGF by antisense oligonucleotide (ODN) inhibits TGF-β-induced production of fibronectin and type I collagen in cultured renal fibroblasts. The in vivo contribution of CTGF in renal interstitial fibrosis, however, remains to be clarified. With the use of a hydrodynamics-based gene transfer technique, the effects of CTGF antisense ODN are investigated in rat kidneys with unilateral ureteral obstruction (UUO). FITC-labeled ODN injection via the renal vein showed that the ODN was specifically introduced into the interstitium. At day 7 after UUO, the gene expression of CTGF, fibronectin, fibronectin ED-A, and α1(I) collagen in untreated or control ODN-treated obstructed kidneys was prominently upregulated. CTGF antisense ODN treatment, by contrast, markedly attenuated the induction of CTGF, fibronectin, fibronectin ED-A, and α1(I) collagen genes, whereas TGF-β gene upregulation was not affected. The antisense treatment also reduced interstitial deposition of CTGF, fibronectin ED-A, and type I collagen and the interstitial fibrotic areas. The number of myofibroblasts determined by the expression of α-smooth muscle actin was significantly decreased as well. Proliferation of tubular and interstitial cells was not altered with the treatment. These findings indicate that CTGF expression in the interstitium plays a crucial role in the progression of interstitial fibrosis but not in the proliferation of tubular and interstitial cells during UUO. CTGF might be a potential therapeutic target against tubulointerstitial fibrosis.

Tubulointerstitial fibrosis is a common feature of progressive renal injury in almost all forms of renal diseases. It has been shown that tubulointerstitial injury is a more consistent predictor of functional impairment than glomerular damage (1,2). Among proposed mechanisms by which the renal injury progresses, TGF-β is postulated to play a central role in the development of tubulointerstitial fibrosis as well as glomerulosclerosis (3–5). TGF-β promotes the accumulation of extracellular matrix (ECM), through the enhanced synthesis of ECM proteins and inhibition of their degradation (3–5). Elevated renal expression of TGF-β has been shown in nearly every experimental model of renal injury characterized by fibrosis (5); transgenic overproduction of TGF-β1 into the circulation in mice resulted in marked tubulointerstitial fibrosis with severe glomerulonephritis (6). These studies have provided plausible evidence for TGF-β and its downstream pathway as a potential therapeutic target against renal fibrosis (5,6).

Long-term suppression of TGF-β, however, is still controversial because of its antiproliferative and anti-inflammatory effects (6). These notions have therefore prompted us to investigate the mechanisms specific to the profibrotic action of TGF-β.

Connective tissue growth factor (CTGF/CCN2) (7) is thought to be an important factor that mediates some of downstream events of fibrogenic properties of TGF-β (8–11). In cultured fibroblasts, CTGF gene expression is strongly induced by TGF-β (10–12), in a smad 3/smad 4-dependent manner (13). Addition of CTGF in turn potently stimulates fibroblast proliferation and ECM protein synthesis (14). In the kidney, enhanced expression of CTGF has been shown in proliferative and fibrotic lesions of various human and experimental renal diseases, including glomerulonephritis and diabetic nephropathy (15–18). We have reported that CTGF mRNA is markedly upregulated, subsequent to TGF-β1, in the interstitial fibrotic areas and tubular epithelial cells as well as in parietal glomerular epithelial cells in a rat model of tubulointerstitial fibrosis (19). These observations led to the hypothesis that CTGF could serve as a downstream effector of TGF-β in the kidney. Indeed, we reveal that the blockade of CTGF using antisense oligonucleotide (ODN) efficiently inhibits TGF-β-induced fibronectin production in cultured renal interstitial fibroblasts (11,19). Moreover, it has already been shown that TGF-β–induced...
collagen synthesis is CTGF dependent, using the cells treated with neutralizing CTGF antibody or those stably transfected with an antisense CTGF gene (20). Although these observations strongly suggest that CTGF is a key mediator of ECM production, the role of CTGF in renal fibrogenesis in vivo still remains to be elucidated.

Previous reports have shown that proliferation of both tubular and interstitial cells was observed after unilateral ureteral obstruction (UUO) (21,22). Inhibition of the renin-angiotensin system attenuates cell proliferation in the obstructed kidney (21). However, the blockade of TGF-β, which has an antiproliferative action, increases tubular and interstitial cell proliferation (22). CTGF stimulates both cell proliferation and ECM accumulation in cultured fibroblasts (10), but its action in interstitial fibroblasts in vivo remains unclear. In the present study, we investigated the effects of CTGF antisense ODN, with a kidney-targeted gene transfer technique using hydrodynamic pressure (23), on ECM production and interstitial fibrosis during the progression of obstructive nephropathy. Furthermore, we evaluated the proliferation of tubular and interstitial cells in obstructive nephropathy with CTGF antisense treatment.

Materials and Methods

In Vivo Gene Transfer Using Hydrodynamics-Based Technique

All animal experiments were conducted in accordance with our institutional guidelines for animal research. Naked plasmid or ODN was introduced into rat kidney by means of a recently developed hydrodynamics-based gene transfer technique (23). In brief, male Wistar rats that weighed ~200 g were anesthetized with pentobarbital, and the left kidney was exposed by midline incision. To first examine functional expression of the transgene introduced, 1 mg of pCAGGS-LacZ (a gift from Dr. J. Miyazaki, Osaka University) diluted in 750 μl of Ringer solution was injected into the left kidney via the renal vein using a 24-gauge SURFLO intravenous catheter (Terumo, Tokyo, Japan), after clamping the left renal vein. Blood flow was reestablished immediately after injection. Two days after injection, frozen kidney sections (4 μm thick) were embedded in OCT compound fixed in cold acetone, incubated with rabbit anti-collagen IV (Chemicon International, Temecula, CA) for 1 h, followed by incubation with rhodamine-conjugated anti-rabbit IgG for 1 h. The specimens were also stained with the nuclear acid dye 4',6-diamidino-2-phenylindole (DAPI; Vector laboratories, Burlingame, CA). The slides were developed with confocal laser scanning microscopy (LSM 5 Pascal; Carl Zeiss Vision, Munich, Germany).

UUO with CTGF Antisense ODN Transfer

After rats were anesthetized, the left ureter was ligated with 4-0 silk at two points (19). Antisense or control ODN (200 μg) in 750 μl of Ringer solution was then injected into the left kidney via the renal vein. The sequences of phosphorothioate ODN (Bex) for rat CTGF used were as follows: antisense ODN, 5'-GAG GGA GGC GAG CAT GGT-3'; and control reverse ODN, 5'-TGG TAC GAG CAG AAG CAG-3'. The antisense sequence is complementary to rat CTGF cDNA around the translation initiation codon (underlined in sequences); the sequence was effective in blocking CTGF expression in vitro (11,19). For sham operation, vehicle was injected into the kidney without ureteral ligation. Rats were killed 7 d after UUO or sham operation (n = 7 each), and the kidneys were harvested.

Northern Blot Analysis

Total RNA from the whole kidney was extracted by the acid guanidinium thiocyanate-phenol-chloroform method. Northern blot analysis was performed using 30 μg of total RNA as described (19). Briefly, hybridization was performed at 42°C overnight with [32P]-labeled probes for rat CTGF, TGF-β1, fibronectin (19), fibronectin extra domain (ED)-A (nucleotides 5370 to 5632) (24), or human pro-α1(1) collagen cDNA (11). The membranes were washed at 55°C in 1× SSC/0.1% SDS, and autoradiography was performed for 24 h with BAS-2500 system (Fuji Photo Film, Tokyo, Japan). The amount of RNA loaded was normalized with human glyceraldehyde-3-phosphate dehydrogenase (Clontech, Palo Alto, CA).

Histology and Immunohistochemistry

For histologic analysis, sagittal kidney sections fixed with 4% buffered paraformaldehyde were embedded in paraffin, and 2-μm-thick sections were stained with Masson’s trichrome. The fibrotic area was measured quantitatively using a computer-aided manipulator (KS400, Carl Zeiss Vision) with slight modifications (25,26). Thirty randomly selected fields were examined by two investigators without knowledge of the origin of the slides. Then the results were expressed as the percentage area of interstitial fibrosis (27).

For immunohistochemical analyses of CTGF, type I collagen, α-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA), and Ki-67, the sections were deparaffinized, washed with PBS, and treated with 3% H2O2 in methanol for 10 min (19). The samples were incubated with rabbit polyclonal anti-CTGF (Abcam, Cambridge, UK), goat polyclonal anti-type I collagen (Southern Biotechnology, Birmingham, AL), mouse monoclonal anti-α-SMA (DAKO Japan, Kyoto, Japan), mouse monoclonal anti-PCNA (DAKO), or mouse monoclonal anti–Ki-67 (DAKO) antibody for 1 h. After incubation with biotin-conjugated secondary antibodies, the specimens were processed using LSAB+ kit (DAKO) and developed with 3,3′-diaminobenzidine tetrahydrochloride. PCNA-positive cells of renal tubules and the interstitium were separately counted in 20 high-power fields (21). For immunofluorescence study of fibronectin ED-A, cryostat sections (4 μm thick) fixed in cold acetone were incubated with mouse anti-fibronectin ED-A (Abcam) for 1 h, followed by incubation with FITC-labeled anti-mouse IgG (Biosource International, Camarillo, CA). The slides were developed by confocal laser scanning microscopy. Ratio of positively stained areas was computer analyzed using NIH Image and expressed as percentages (27).

Western Blot Analysis

Western blot analysis was performed as described (19) with some modifications. The whole kidneys were homogenized in ice-cold...
solution that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 10 mM Na₂HPO₄, 10 mM NaF, 10 mg/ml aprotinin, 2 mM dithiothreitol, 2 mM sodium orthovanadate, and 1 mM PMSF. The homogenates were centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatants were treated with Laemmli’s sample buffer. Samples (30 μg protein/ lane) were separated by 12.5% SDS-PAGE and electrophoretically transferred onto Immobilon polyvinylidene difluoride filter (Millipore, Bedford, MA). Filters were incubated with anti–α-SMA or anti-PCNA for 1 h, and immunoblots were then developed using horse-radish peroxidase–linked donkey anti-mouse Ig (BioRad, Richmond, CA) and a chemiluminescence kit (Amersham, Arlington Heights, IL). β-Actin (antibody from Sigma) was used as an internal control.

Statistical Analyses

Data are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA or the Kruskal-Wallis test as appropriate. P < 0.05 was considered statistically significant.

Results

Localization of FITC-Labeled CTGF Antisense ODN

For investigating the validity of the gene transfer method, pCAGGS-LacZ plasmid was injected into the kidney. Positive X-gal staining was observed most prominently in the interstitial cells 2 d after transfection (Figure 1A). Similar staining was observed at day 7 (not shown). Then we transferred FITC-labeled ODN into the kidney. Green fluorescence was seen in the interstitial areas throughout the left kidney (Figure 1B) but not in the contralateral kidney (not shown). Using double staining with DAPI, a substantial portion of FITC-labeled ODN was introduced into the nuclei (Figure 1C). When the specimens were stained with anti-collagen type IV, ODN was observed outside the basement membrane of the tubular epithelium (Figure 1D). Thus, most of the transgene introduced was present in the interstitial areas and active in the interstitial cells, consistent with a previous report (23).

Effect of CTGF Antisense ODN on CTGF and TGF-β1 Gene Expression

To explore the role of CTGF in tubulointerstitial fibrosis, we examined the effect of CTGF antisense ODN transfer in rat kidney with obstructive nephropathy. Figure 2 illustrates the expression of CTGF mRNA in the whole kidney on day 7 after ureteral ligation, the time point when the induction of CTGF mRNA becomes pronounced subsequent to TGF-β1 mRNA upregulation (19). CTGF mRNA expression was significantly elevated in the obstructed kidneys by 2.1-fold as compared with the sham-operated kidneys. Introduction of CTGF antisense ODN into the kidney markedly inhibited CTGF mRNA expression by 86 ± 18% (n = 7) compared with the vehicle-treated obstructed kidney (Figure 2B), indicating efficient transfer of ODN into the kidney. Immunohistochemical analyses revealed that CTGF was significantly reduced in the interstitial cells but not in the arterioles or glomerular epithelial cells with antisense treatment (Figure 3).

Interestingly, TGF-β1 mRNA expression was not altered with CTGF antisense ODN treatment compared with vehicle (2.1- versus 2.2-fold of sham operation; Figure 2B). There were no significant changes with control ODN treatment. These findings indicate that the transfer of CTGF antisense ODN efficiently reduced CTGF upregulation, without affecting TGF-β1 mRNA expression.

Effect of CTGF Antisense ODN on ECM Accumulation and Interstitial Fibrosis

To evaluate the role of CTGF in promoting ECM accumulation, we examined the expression of fibronectin, fibronectin ED-A, and α1(I) collagen genes after CTGF antisense ODN treatment by Northern blotting (Figure 2). Fibronectin mRNA expression was upregulated in the obstructed kidneys (2.0-fold of sham operation) and significantly attenuated (by 55 ± 8%; P < 0.05, n = 7) with the transfer of CTGF antisense ODN. Expression of fibronectin ED-A and α1(I) collagen genes was markedly upregulated after UUO, and such induction was also significantly suppressed in CTGF antisense ODN treatment (by 45 ± 8% and 66 ± 10% of vehicle, respectively; Figure 2B).

Immunohistochemical analyses revealed that the expression of fibronectin ED-A was markedly increased after UUO (Figure 4A). Fibronectin ED-A was deposited around the Bowman’s capsules (arrows) and interstitial fibrotic areas (arrowheads). Treatment with CTGF antisense ODN reduced the amount of fibronectin ED-A deposition in the interstitial areas but not around the Bowman’s capsules (Figure 4A). Similar
Results were observed for the expression of type I collagen (Figure 4B). Type I collagen deposition was markedly enhanced in the interstitial fibrotic areas of the untreated group and significantly reduced with the introduction of CTGF antisense ODN (Figure 4B).

Histologically, the untreated and control ODN-treated obstructed kidneys developed tubular atrophy and epithelial flattening with tubulointerstitial fibrosis in Masson's trichrome staining (Figure 5A). The fibrotic areas determined in blue staining were significantly attenuated in the obstructed kidneys that were treated with CTGF antisense ODN as compared with control ODN (6.8 ± 0.8% versus 12.3 ± 0.8%; P < 0.01; n = 7; Figure 5B).

Effect of CTGF Antisense ODN on α-SMA Expression

Phenotypic alterations of interstitial fibroblasts and tubular epithelial cells may be one of the most crucial events during the development of tubulointerstitial fibrosis (28). Expression of α-SMA in the interstitial areas becomes upregulated along with ECM accumulation and is thought to indicate the alteration to myofibroblasts (28,29). To explore the mechanisms of amelioration of renal fibrosis, we next examined the effect of the blockade of CTGF on α-SMA expression. Figure 6 shows that α-SMA expression was markedly increased after UUO by Western blot analysis of the whole kidney. Uptregulation of α-SMA expression was significantly decreased in CTGF antisense ODN-treated kidneys as compared with control ODN-treated kidneys (2.8- versus 6.5-fold of sham operation, respectively; P < 0.05; n = 6). Immunohistochemical analysis also revealed that the enhanced expression of α-SMA was observed mainly in the interstitial areas of the obstructed kidney, and the staining was significantly attenuated with CTGF antisense ODN treatment (Figure 6C). These findings indicate that the blockade of CTGF may inhibit transition from fibroblasts to myofibroblasts.

Effect of CTGF Antisense ODN on Cell Proliferation

An increase in proliferation of tubular and interstitial cells occurs during obstructive nephropathy, which might represent a certain phase of repair process (22). CTGF exerts a potential proliferation activity on fibroblasts in vitro (10). To explore the effect of CTGF on renal cell proliferation during obstructive nephropathy, we investigated PCNA and Ki-67 expression by Western blot and immunohistochemical analyses. Ki-67 antigen is a marker of active phases of the cell cycle. As shown in Figure 7, Western blot analysis revealed that PCNA expression in the whole kidney was significantly increased after ureteral ligation. When CTGF antisense ODN was transferred into the obstructed kidney, such treatment did not alter PCNA expression as a whole (Figure 7, A and B). Immunohistochemical study also indicated that enhanced proliferation of both tubular and interstitial cells in the obstructed kidney determined by PCNA and Ki-67 expression was unaffected with antisense ODN treatment (Figure 7, C through F). These results suggest that CTGF does not play a crucial role in cell proliferation during the development of interstitial fibrosis in obstructive nephropathy.

Discussion

In the present study, we investigate the effects of CTGF antisense ODN transfer on ECM production and histologic changes in a rat model of obstructive nephropathy, using a kidney-targeted gene transfer technique. We reveal for the first time that the blockade of CTGF in vivo significantly ameliorates the development of renal interstitial fibrosis. Accumulating evidence has indicated have been developed, which CTGF has a multifunctional property, including fibroblast proliferation, ECM protein synthesis, endothelial cell migration and smooth muscle cell differentiation.
angiogenesis, and chondrocyte proliferation and differentiation (8–12,30–32). Recently, CTGF-deficient mice have been developed, which exhibit skeletal dysmorphism with the decreased expression of specific ECM components in the cartilage (33). Among a number of physiologic and pathophysiologic roles of CTGF proposed, we particularly focused on its role in renal fibrogenesis activated by TGF-

We demonstrate in this study that the reduction in CTGF effectively attenuates the upregulation of ECM proteins in a rat model of obstructive nephropathy. Northern blot analysis exhibited that the transfer of CTGF antisense ODN greatly reduces fibronectin, fibronectin ED-A, and α1(I) collagen mRNA expression (Figure 2). Fibronectin ED-A, a splice variant of fibronectin, is strongly induced by TGF-β and is crucial for the alteration to the myofibroblastic phenotype (24). In UUO rats, fibronectin ED-A is mostly deposited around the Bowman’s capsules and fibrotic areas (34). Immunohistochemical analysis revealed that the treatment with CTGF antisense ODN reduced the amount of fibronectin ED-A deposition in the interstitial fibrotic areas but not around the Bowman’s capsules (Figure 4A). CTGF antisense ODN also significantly reduced type I collagen deposition in the interstitial areas, leading to the amelioration of tubulointerstitial fibrosis (Figure 5). It has been reported that, by this hydrodynamics-based gene transfer technique, the transgene is introduced mainly into renal interstitial fibroblasts (14,15). Together with the present data, these observations suggest that this strategy could provide specific knockdown of CTGF in the interstitial areas of the targeted kidney.

Interestingly, our results show that CTGF antisense treatment does not affect TGF-β1 mRNA expression in the kidney (Figure 2). A number of reports so far have shown that the treatment of renal fibrosis in obstructive nephropathy is generally associated with the inhibited expression of TGF-β, such as upon blocking of the renin-angiotensin system (21), in PAI-1–deficient mice (35), and by directly inhibiting TGF-β with antisense ODN (36) or neutralizing antibodies (22). Hepatocyte growth factor also ameliorates renal fibrosis with the inhibition of TGF-β (37). These studies have suggested that TGF-β is a final common pathway leading to renal fibrosis. However, a recent report has shown that the treatment of hepatocyte growth factor ameliorates renal fibrosis through the attenuation of CTGF induction in 5/6 nephrectomized TGF-β transgenic mice, without reducing TGF-β1, β2, or β3 (38). It has been shown that the addition of recombinant CTGF does not stimulate TGF-β1 mRNA expression in renal interstitial fibroblasts (14) or in mesangial cells (17). Thus, a decrease in CTGF may lessen renal fibrosis without affecting TGF-β expression. In the present study, >80% reduction of CTGF mRNA resulted in ~50 to 60%, but not complete, inhibition of ECM deposition after UUO (Figures 2 through 5). This may be due to the incomplete efficacy of this type of treatment or to the presence of other major pathways to interstitial fibrosis. Nevertheless, these findings strongly suggest that CTGF should serve as one of the bone fide downstream mediators of TGF-β’s profibrotic action in vivo and could become a more specific target against renal fibrosis.

Fibronectin ED-A is thought to be a key player in the...
differentiation into myofibroblasts, which are characterized by α-SMA expression (28,29). Anti-fibronectin ED-A antibody specifically blocks TGF-β-stimulated α-SMA induction (24). Our results reveal that α-SMA expression is potently inhibited by blocking CTGF expression in the obstructed kidneys. Therefore, CTGF may function to induce myofibroblast differentiation in vivo, by activating fibronectin ED-A or by acting synergistically with it.

Proliferation of both tubular and interstitial cells is documented during obstructive nephropathy (21,22). This process might involve some repair mechanisms, because blocking of TGF-β in the obstructed kidney results in significantly increased tubular proliferation along with the protection against interstitial fibrosis (22). CTGF stimulates cell proliferation in some cells (10,30–32), and CTGF-null mice exhibit decreased PCNA-positive cells in the chondrocytes (33). CTGF therefore may be related to cell proliferation after UUO. It is interesting however, that the proliferation of the tubular and interstitial cells in the obstructed kidney was not inhibited by the treatment with CTGF antisense ODN (Figure 7). These findings suggest that CTGF does not play a crucial role in proliferation in the obstructed kidney but is an important mediator specific to fibrogenesis. The reason that the blockade of TGF-β and CTGF causes different effects on proliferation remains to be elucidated and requires further clarification.

CTGF is potently activated by TGF-β but may also be induced through TGF-β-independent pathways, e.g., by glucocorticoids (39), angiotensin II/calcineurin (40), and ad-
vanced glycation end products (41). This notion further supports that CTGF can become a promising therapeutic target as a final common pathway of renal fibrosis. Recently, CTGF has been shown to bind to TGF-β1 to activate phosphorylation of smad 2 (42). These findings suggest that CTGF may transduce its signals in part through binding to TGF-β1.

Downstream pathways in the CTGF signaling still remain unclear. CTGF binds directly to integrin αvβ3 and αIIbβ3, which are candidate receptors for CTGF to promote cell adhesion, migration, and proliferation (30,43). CTGF also binds to LDL receptor-related protein (44), which serves as a functional receptor for MMP-9 (45). We and others have already shown that Cyr61, another member of the CCN family, may exert distinct actions from CTGF in terms of ECM production and mesangial activation, while potentially sharing the same receptors (8,9,46). Further clarifications therefore are essential to

Figure 5. Treatment with CTGF antisense ODN suppresses interstitial fibrosis. (A) Representative Masson’s trichrome–stained sections. S, sham operation; U, UUO with vehicle injection; R, UUO with CTGF reverse ODN (200 μg) introduction; AS, UUO with CTGF antisense ODN (200 μg) introduction. (B) Semiquantitative score of tubulointerstitial fibrosis in the cortex from the kidneys. Mean ± SE; n = 7; *P < 0.05; **P < 0.01; #P < 0.01 between AS and S. Magnification, ×200.
elucidate the signaling pathways and mechanisms by which CTGF induces ECM production in vitro and renal fibrosis in vivo.

In summary, the present study reveals that the treatment with CTGF antisense ODN by a kidney-specific gene transfer reduces ECM production in experimental obstructive nephropathy, ameliorating the development of interstitial fibrosis. Our study demonstrates the potential role of CTGF in renal fibrosis in vivo and opens up the possibility of a novel therapeutic approach for a variety of renal diseases leading to tubulointerstitial fibrosis.

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References
33. Ivkovic S, Yoon BS, Popoff SN, Saffadi FF, Libuda DE, Stephenson RC, Daluiski A, Lyons KM: Connective tissue growth...


