TGF-β1 induces IL-8 and MCP-1 through a connective tissue growth factor-independent pathway

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Submitted 20 June 2005; accepted in final form 28 September 2005

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TRANSFORMING GROWTH factor-β (TGF-β) and more recently connective tissue growth factor (CTGF) are recognized as important fibrogenic cytokines. Accumulating evidence has demonstrated that both TGF-β1 and CTGF induce matrix production in the kidney (2, 5, 9, 16, 19, 36). We recently demonstrated that CTGF facilitates TGF-β1 to induce fibrogenesis in human primary proximal tubule cells and cortical fibroblasts and that blockade of the type II TGF-β receptor abrogates CTGF-induced production of fibronectin and collagen IV (22). Fibronectin is a key interstitial matrix protein that serves as a scaffold for the deposition of other proteins. Furthermore, it functions as a fibroblast chemotactant, promoting their differentiation, which may be a crucial phenomenon in the pathogenesis of tubulointerstitial fibrosis (37). Collagen IV is predominantly a basement membrane protein expressed in the proximal tubular cells. Both fibronectin and collagen IV are known to be key matrix proteins whose expression is dysregulated as part of the renal fibrotic response. The integral relationship between TGF-β and CTGF is clear in renal fibrosis, and indeed antifibrotic therapies targeting CTGF are under development. However, whether CTGF is also involved in the immunomodulatory effects of TGF-β remains unclear.

TGF-β is known to have pleotropic immunomodulatory effects, functioning as an anti-inflammatory cytokine when secreted from certain classes of T cells as well as many other types of nonlymphoid cells, but as a proinflammatory cytokine in many epithelial cell types including the proximal tubule cells of the kidney (17, 33, 34). TGF-β controls the differentiation, proliferation, and activation of immune cells and it is implicated in immune abnormalities linked to cancer, autoimmune, opportunistic infections, and fibrotic complications. In contrast, in the proximal tubule cells of the kidney it is known to stimulate the production of proinflammatory cytokines including interleukin-8 (IL-8), macrophage chemotactic protein-1 protein (MCP-1), MCP-4, and RANTES (8, 17, 33, 38). Although production of these cytokines is traditionally thought to promote renal damage (18), more recently it has been recognized that these cytokines may participate in resolution of renal injury (26). Hence, it is important to clearly understand the factors controlling the production of these cytokines in the kidney.

There are three isoforms of TGF-β in the mammalian cells: TGF-β1, TGF-β2, and TGF-β3. Among these three isoforms, TGF-β1 is the only one that circulates in the bloodstream, is the most highly expressed isoform in the kidney, and has been most closely linked to the pathophysiology of progressive nephropathy (4). IL-8 is a prototype chemokine of the C-X-C family of structurally related small cytokines that have potent chemotactic activity for neutrophils and lymphocytes in inflammatory and immune responses (15) promoting their migration to the site of inflammation (7). MCP-1 is the most potent known chemotactic factor for monocytes, subsequently inducing monocyte migration and differentiation to macrophages (1). Studies suggest that upregulation of MCP-1 is a common regulatory pathway involved in the progressive dia-

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betic nephropathy as well as inflammatory renal diseases (30). Chemokines (IL-8 and MCP-1) are closely related to the urinary excretion of protein in experimental models (28, 29) and are known to be involved in the pathogenesis of the early phase of renal fibrosis.

Clearly, emerging evidence suggests that CTGF is an important downstream mediator of the profibrotic effects of TGF-β1 (23, 31, 37). However, its role in the regulation of TGF-β1-induced chemokine production is still unknown. The present study was to determine the role of CTGF in mediating TGF-β1-induced chemokine production.

MATERIALS AND METHODS

Human kidney-2 cells. The immortalized proximal tubular cell line human kidney-2 (HK-2) derived from normal kidney (ATCC) was grown in Keratinocyte-SFM media (Invitrogen) under conditions as previously described (20).

Materials. TGF-β1 was purchased from Sigma, and recombinant human CTGF was a generous gift from FibroGen. CTGF and non-specific small interference RNA (siRNA; 21-mer RNA molecules) were chemically synthesized (Ambion). The human IL-8 ELISA kit was purchased from R&D Systems, and the human MCP-1 immunoassay kit was from BioSource International. Mouse monoclonal fibronectin antibody was from Neomarkers, and rabbit polyclonal collagen IV antibody was from Abcam. The CTGF polyclonal antibody used was raised in a New Zealand White rabbit, against full-length purified recombinant human CTGF protein as previously described (25).

Experimental protocol. Three-hundred picomoles of CTGF siRNA per well on a six-well plate were introduced into HK-2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In parallel wells, to act as negative controls, cells were transfected with a nonspecific siRNA. Twenty-four hours after transfection, cells were exposed to 2 ng/ml TGF-β1 for 48 h. Conditioned media was collected and centrifuged to remove cell debris for measurement of chemokine secretion (IL-8 and MCP-1) and matrix production (fibronectin and collagen IV). RNA was also extracted from all cells under all experimental conditions (control, TGF-β1 treated, nonspecific siRNA plus TGF-β1, CTGF siRNA plus TGF-β1, and CTGF mRNA levels were determined by RT-PCR and further confirmed by quantitative real-time PCR). CTGF protein levels were determined by immunocytochemistry staining.

To determine whether CTGF induced IL-8 and MCP-1, cells were exposed to 20, 200, and 400 ng/ml CTGF for 24, 48, and 72 h, respectively. Conditioned media were collected and centrifuged to remove cell debris and then stored at −80°C for subsequent IL-8 and MCP-1 quantification using ELISA. Conditioned media from 200 ng/ml treatment with CTGF were used to measure fibronectin secretion to serve as a positive control and confirm that the CTGF used in the experiments was active.

The basal expression levels of chemokines (IL-8 and MCP-1) and matrix proteins (fibronectin and collagen IV) in the CTGF gene-silenced cells were determined. As detailed above, 300 pmol of
nonspecific or CTGF-specific siRNAs were introduced into HK-2 cells. Media were changed at the final 24 h of the 48-h period after transfection. Conditioned media were collected for IL-8, MCP-1, fibronectin, and collagen IV. RNA was extracted for verification of gene silencing in the cells. siRNA. 21-mer RNA molecules were chemically synthesized (Ambion). The complementary oligonucleotides were 2’-deprotected, annealed, and purified by the manufacturer. The sequence targeting CTGF (accession no. NM_001901) was 5’-AAGAUGAUGGAGAGAUGGCA-3’. Experimental protocols were described as above.

**RT-PCR and real-time PCR.** RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA was treated with DNASE I (Invitrogen) and then reverse transcribed using Superscript II RT (Invitrogen). Semiquantitative RT-PCR and quantitative real-time PCR were used to assess transcript levels of CTGF. For semiquantitative RT-PCR, sequence-specific primers for human CTGF (accession no. NM_001901) and β-actin (accession no. NM_001101) were as follows: CTGF: forward 5’-CGAGCTAAATCTCGTGAGAGT-3’, reverse: 5’-CCATGTCTCCGTACATCTTC-3’; and β-actin: forward 5’-GCTGTCGTGCAACCGCTC-3’, reverse 5’-CAAACATGATCTGGGTATCCTTC-3’. The sizes of the PCR products for CTGF and β-actin are 208 and 353 bp, respectively. Amplification products were electrophoresed through 1.5% (wt/vol) agarose gels and visualized by ethidium bromide staining. Bands were scanned and quantitated by densitometry using NIH Image software v1.60. β-Actin was used as an internal control for sample normalization. To further confirm the RT-PCR data, real-time PCR was used to measure CTGF mRNA. Specific primers for sample normalization. To further confirm the RT-PCR data, real-time PCR was used to measure CTGF mRNA. Specific primers were designed as follows: CTGF: forward 5’-GGCTTACCGGCTGAGAGAG-3’, reverse 5’-AGGAGGCGGTGTTGATCATTGG-3’; 18S served as an internal control: forward 5’-GGCTTACCGGCTGAGAGAG-3’, reverse 5’-AGGAGGCGGTGTTGATCATTGG-3’.

**ELISA.** Conditioned media samples were subjected to IL-8 and MCP-1 immunoassays. Conditioned media were collected and centrifuged at 3,000 rpm and 4°C for 10 min to remove cell debris and then stored at −80°C. IL-8 and MCP-1 were quantified using an ELISA according to the manufacturer’s instructions. Western blotting. Conditioned media samples were subjected to SDS-PAGE under reducing conditions. Proteins were then transferred to nitrocellulose membranes and probed with antibodies specific for CTGF. The membranes were then incubated with HRP-conjugated secondary antibodies and developed with ECL. The intensity of the bands was quantitated by densitometry using NIH Image software v1.60. At least three independent experiments were performed, and the results were consistent.

**Results.** Exposure to TGF-β1 led to a significant increase in IL-8 and MCP-1 production in both nonspecific siRNA and CTGF siRNA-treated cells. The increase in IL-8 production was more pronounced in CTGF siRNA-treated cells, whereas MCP-1 production was similar in both groups. These results suggest that TGF-β1 regulates IL-8 production independently of CTGF expression, whereas MCP-1 production may be modulated by CTGF.

**Discussion.** The results of this study suggest that TGF-β1 induces IL-8 and MCP-1 production in HK-2 cells, which may contribute to the progression of kidney fibrosis. The mechanism by which TGF-β1 regulates IL-8 production is independent of CTGF expression, whereas MCP-1 production may be modulated by CTGF. These findings provide insight into the role of TGF-β1 in kidney fibrosis and suggest potential therapeutic targets for the treatment of kidney disease.
to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). Nonspecific binding sites were blocked overnight (5% nonfat milk and 0.1% Tween-20 in PBS), after which the membranes were incubated in primary fibronectin or collagen IV antibody for 2 h at room temperature, followed by being washed four times after which they were incubated with peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech) for 1 h and again washed four times. The blots were then detected using ECL (Amersham Pharmacia Biotech). The bands corresponding to fibronectin (220 kDa) or collagen IV (180 kDa) were quantitated using NIH Image software v1.60. Coomassie brilliant blue staining was used to confirm that an equal amount of protein was loaded in each lane.

Statistical analysis. All results are expressed as a percentage of the control values (100%) with the exception of real-time PCR results, which are expressed as a fold-change compared with the control value. In each experiment, at least two data points were collected and each experiment was repeated three times; hence n = 6 unless otherwise stated. Results are expressed as means ± SE. Statistical comparisons between groups were made by ANOVA, with pairwise multiple comparisons made by Fisher’s protected least-significant difference test. Analyses were performed using the software package Statview version 4.5 (Abacus Concepts, Berkely, CA). P values <0.05 were considered significant.

RESULTS

CTGF siRNA mediated gene silencing. Three hundred picomoles of CTGF siRNA per well on a six-well plate significantly decreased CTGF mRNA expression by ∼94%. A RT-PCR representative gel is shown in Fig. 1A and further confirmed by real-time PCR (Fig. 1B). CTGF protein level was determined by immunocytochemistry staining (Fig. 1C).

TGF-β1 induces IL-8 and MCP-1 is independent of CTGF. Cells exposed to 2 ng/ml TGF-β1 for 48 h significantly upregulated IL-8 production to 356 ± 88% (P < 0.05) compared with control values (Fig. 2A). However, IL-8 production did not change in the presence of 2 ng/ml TGF-β1 in the cells in which the CTGF gene was silenced, compared with the cells exposed to nonspecific siRNA [P = not significant (NS); Fig. 2A]. Similar results were seen in MCP-1. Cells exposed to 2 ng/ml TGF-β1 for 48 h significantly upregulated MCP-1 to 2,344 ± 583% (P < 0.05) compared with control values (Fig. 2B). MCP-1 expression did not change in the cells in which the CTGF gene was silenced in the presence of 2 ng/ml TGF-β1 compared with the cells transfected with nonspecific siRNA (P = NS; Fig. 2B). These data suggest that TGF-β1-induced upregulation of IL-8 and MCP-1 is independent of CTGF.

Basal expression of IL-8 and MCP-1 was not altered in CTGF gene-silenced cells. Cells in which the CTGF gene was silenced had no change in the basal expression of IL-8 (Fig. 2C) or MCP-1 (Fig. 2D).

CTGF does not induce IL-8 or MCP-1 in a dose- and time-dependent manner. We further determined whether CTGF induced IL-8 and MCP-1 expression in a dose- and time-dependent manner. Cells were exposed to different doses of CTGF (20, 200, and 400 ng/ml) for 24, 48, and 72 h, respectively. Conditioned media samples were then collected for IL-8 and MCP-1 quantification. No stimulation of IL-8 (A) or MCP-1 (B) production was observed. To serve as a positive control and confirm that the CTGF used in the experiments was active, fibronectin secretion was measured on conditioned media collected from cells treated with 200 ng/ml CTGF for 48 h (C). Results are means ± SE and are standardized as a percentage of the control. *P < 0.05 vs. control; n = 6.

Fig. 3. CTGF does not induce IL-8 or MCP-1 in a dose- and time-dependent manner. Cells were exposed to different doses of CTGF (20, 200, and 400 ng/ml) for 24, 48, and 72 h, respectively. Conditioned media samples were then collected for IL-8 and MCP-1 quantification. No stimulation of IL-8 (A) or MCP-1 (B) production was observed. To serve as a positive control and confirm that the CTGF used in the experiments was active, fibronectin secretion was measured on conditioned media collected from cells treated with 200 ng/ml CTGF for 48 h (C). Results are means ± SE and are standardized as a percentage of the control. *P < 0.05 vs. control; n = 6.
dependent manner. Cells were exposed to 20, 200, and 400 ng/ml CTGF for 24, 48, and 72 h, respectively, and IL-8 and MCP-1 expression levels did not change compared with control values at each time point (P = NS in all cases; Fig. 3, A and B).

However, cells exposed to 200 ng/ml CTGF for 48 h increased fibronectin secretion to 176 ± 12% (P < 0.05) compared with control values. This confirmed that the CTGF used in the experiments was active and served as a positive control (Fig. 3C).

TGF-β1-induced fibronectin and collagen IV production is differentially mediated by CTGF. Cells exposed to 2 ng/ml TGF-β1 for 48 h significantly induced fibronectin and collagen IV to 252 ± 2% (P < 0.05; Fig. 4A) and 248 ± 37% (P < 0.05; Fig. 4B), respectively, compared with control values. Basal expression of fibronectin was significantly reduced in CTGF-silenced cells to 64 ± 10.6% compared with cells transfected with nonspecific siRNA (P < 0.05; Fig. 4C), although basal collagen type IV secretion was not substantially altered in the absence of autocrine CTGF production (P = NS; Fig. 4D). The TGF-β1-induced increases in fibronectin were attenuated in the cells in which CTGF was silenced (P < 0.05; Fig. 4A) and to a lesser extent for collagen IV (P = 0.06; Fig. 4B) compared with nonspecific siRNA suggesting that TGF-β1-induced fibronectin and collagen IV secretion is differentially regulated.

**DISCUSSION**

This study, to our knowledge, is the first to report that TGF-β1 induces IL-8 and MCP-1 through a CTGF-independent pathway. Increasing evidence supports the hypothesis that CTGF is an important downstream mediator of the profibrotic effects of TGF-β1 and the present studies reinforce this view. However, the role of CTGF in TGF-β1-induced immunomodulation has to date not been investigated. Our studies demon-

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**Fig. 4.** A and B: TGF-β1-induced fibronectin and collagen IV are differentially mediated by CTGF. Conditioned media samples collected in Fig. 2, A and B, were used to measure fibronectin and collagen IV secretion using Western blotting as described in MATERIALS AND METHODS. A and B: fibronectin and collagen IV secretion, respectively. Coomassie brilliant blue staining served as a loading control. Results are means ± SE and are standardized to a percentage of the control. *P < 0.05 vs. control; #P < 0.05 vs. nonspecific siRNA; n = 6. C and D: basal secretion levels of fibronectin and collagen IV in cells in which the CTGF gene is selectively silenced. Conditioned media samples collected in Fig. 2, C and D, were used to measure fibronectin and collagen IV secretion using Western blotting. Fibronectin (C) and collagen IV (D) expression were measured by Western blotting. Coomassie Brilliant Blue staining served as a loading control. Results are means ± SE and are standardized to a percentage of the control. *P < 0.05 vs. control; n = 6.
strate that CTGF does not mediate TGF-β1-induced alterations in chemokine production in proximal tubular cells. Clearly, targeting TGF-β1 to yield antifibrotic effects has not been successfully largely due to the adverse systemic effects on the inflammatory and immunomodulatory responses. The present results lend support to the view that targeting CTGF is likely to be an effective antifibrotic strategy, but without modification of proinflammatory pathways in the kidney. As modulation of CTGF has not been demonstrated to result in significant alterations in the systemic or local inflammatory responses in organs other than the kidney (14), it is likely that strategies to reduce CTGF will allow specific targeting of the fibrotic response without deleterious effects on inflammatory and immunomodulatory pathways. Although combined anti-inflammatory and antifibrotic effects may be desirable in many forms of progressive kidney disease, the phases of inflammation and fibrosis are likely to be separated in time and targeting of treatment to different phases of diseases is clearly preferred.

IL-8 and MCP-1 are differentially regulated by TGF-β1 in different organs, with several studies in nonrenal cells suggesting that IL-8 and other chemokines are either inhibited or not modified in the presence of TGF-β1 (11, 13). In the kidney, both IL-8 and MCP-1, the cytokines chosen for examination in these studies, are synthesized by proximal tubular cells (8) and are closely related to the function of epithelial foot processes (38). Our findings that TGF-β1 upregulates IL-8 and MCP-1 are consistent with previous reports that TGF-β1 induces IL-8 and MCP-1 in proximal tubular epithelial cells (34). In contrast, others have reported that TGF-β1 downregulates MCP-1 in human proximal tubular epithelial cells (8). Clearly, this is at odds with our own observations and others suggesting that an elevation in MCP-1 occurs in association with an increase in TGF-β1 in models of progressive renal disease (3, 27). The present studies clearly demonstrate that TGF-β1 stimulates the production of these chemokines which is independent of the downstream production of CTGF as effective silencing of the CTGF gene did not modify TGF-β1-induced chemokine production. Furthermore, direct exposure of the HK-2 cells to CTGF did not induce any alteration in the basal production of these chemokines.

Conversely, the profibrotic actions of TGF-β1 have been demonstrated to be largely dependent on intact CTGF mRNA expression (10, 32), although both overlapping and distinct fibrogenic effects in human renal cells have been observed (9). This is consistent with reports demonstrating that fibrogenic responses in the tubulointerstitium can be attenuated when CTGF is reduced using either specific anti-CTGF antibodies or antisense CTGF oligodeoxynucleotides (6). More recently, it has been demonstrated using a transgenic model with overexpression of TGF-β1 that modification of CTGF, again using antisense CTGF oligodeoxynucleotides (19), attenuates renal fibrogenesis even in the presence of ongoing high levels of TGF-β1. Several reports indicate that CTGF can be expressed independently of the expression of TGF-β1 (40), resulting in a fibrogenic response. Interestingly, in this model modification of the expression of CTGF did not reduce basal collagen IV production and only reduced TGF-β1-stimulated collagen IV production by a small but statistically insignificant amount. Conversely, in which cells the CTGF gene was effectively silenced had a significant reduction in both basal and TGF-β1-stimulated fibronectin production (35, 39). This is in contrast to mesangial cells, where neutralization of CTGF using anti-CTGF antibody reduced both fibronectin and collagen IV production induced by advanced glycation end products (40). The reason for this discrepancy is not clear, although it is appreciated that a complex interplay between cytokines and matrix proteins exists, which may be differentially regulated by TGF-β1-dependent and -independent factors, resulting in unique downstream signaling cascades and specific induction of responsive genes in different cell types (16). Additionally, studies in a human fibrosarcoma cell line have demonstrated that TGF-β1 induces fibronectin through a c-Jun NH2-terminal kinase-dependent, Smad4-independent pathway (12), whereas studies using mouse embryonic fibroblasts null for Smad3 demonstrate that Smad3 is integral to the synthesis of collagen (24). Our studies would suggest that while both matrix proteins are stimulated by TGF-β1, CTGF is more important in the basal and TGF-β1-mediated increase in fibronectin in HK-2 cells.

In summary, we demonstrated using gene-silencing techniques in the proximal tubular model of HK-2 cells that TGF-β1-induced chemokine production occurs via a CTGF-independent pathway. Conversely, TGF-β1 plays important roles in both fibrosis and immunomodulation, whereas CTGF has a more specific role as a downstream mediator of TGF-β1 in inducing fibrosis in the absence of modifying inflammatory responses.

ACKNOWLEDGMENTS

CTGF was a generous gift from FibroGen (San Francisco, CA).

GRANTS

These studies were supported by the National Health and Medical Research Council of Australia and the Juvenile Diabetes Research Foundation. W. Qi is supported by a National Health Medical Research Council Scholarship.

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