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Connective tissue growth factor regulates the key events in tubular epithelial to myofibroblast transition in vitro

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Abstract

Connective tissue growth factor (CTGF) has been reported to play an important role in mediating the profibrotic effects of transforming growth factor-β (TGF-β) in various renal diseases. To elucidate the role of CTGF in renal tubular epithelial-myofibroblast transdifferentiation, we examined the expression of α-smooth muscle actin (α-SMA), vimentin, tenascin-C, and collagen IV expression upon the stimulation of CTGF in cultured human proximal tubular epithelial cell line (HKC), and further investigated the effects of endogenous CTGF blockade on the transdifferentiation process induced by TGF-β. It is revealed that upon the stimulation of recombinant human CTGF (rhCTGF, 2.5 or 5.0 μg/L), the expression of α-SMA and tenascin-C mRNA increased significantly ($p < 0.01$), while collagen IV gene expression decreased significantly ($p < 0.01$), all in a dose-dependent manner. The percentage of α-SMA-positive cells was significantly larger in the rhCTGF-stimulated groups than that in negative control (38.9%, 65.5% vs. 2.4%, respectively, $p < 0.01$) as confirmed by flow cytometry. Both cytoplasmic and secretory tenascin-C expression was upregulated by the stimulation of rhCTGF ($p < 0.01$). Under this condition, collagen IV secreted into the culture media was lowered markedly ($p < 0.01$). On RT-PCR analysis, TGF-β1 upregulated CTGF gene expression, preceding that of α-SMA. The α-SMA mRNA expression induced by TGF-β1 was significantly inhibited by CTGF antisense oligodeoxynucleotide (ODN) transfection ($p < 0.01$). With prolonged incubation time, CTGF antisense ODN also inhibited intracellular α-SMA protein synthesis, as demonstrated by indirect immuno-fluorescence. So it is concluded that CTGF could promote the transdifferentiation of human renal tubular epithelial cells towards myofibroblasts in vitro, both directly and as a downstream mediator of TGF-β, and CTGF blockade would be a possible therapeutic target against tubulointerstitial fibrosis.

Keywords: Connective tissue growth factor; Transforming growth factor-β; Human renal tubular epithelial cell; Transdifferentiation; α-Smooth muscle actin; Antisense oligodeoxynucleotide

1. Introduction

Renal tubulointerstitial fibrosis is considered to be the common final pathway leading to end-stage renal failure, irrespective of the initial renal injury (Eddy, 2000; Becker and Hewitson, 2000). It has also been shown that tubulointerstitial fibrosis is a more consistent predictor of functional impairment than glomerular damage (Nath, 1992). The process of tubulointerstitial fibrosis involves the loss of renal tubular epithelial cells and the accumulation of extracellular matrix (ECM) proteins, such as collagen (type I, III), fibronectin, and laminin. Although tubular epithelial cells can synthesize a variety of ECM proteins (Creely et al., 1992; Tang et al., 1997),

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it is thought that myofibroblasts are the main source of the increased ECM deposition seen in renal fibrosis and, indeed, in other types of tissue fibrosis (Zhang and Phan, 1999; Hogemann et al., 1993). The number of myofibroblasts identified by the expression of α-smooth muscle actin (α-SMA) is the best prognostic indicator of functional impairment of the kidney in both human and experimental glomerulonephritis (Roberts et al., 1997; Fan et al., 1999; Essawy et al., 1997). Therefore, it is crucial to clarify the origin of myofibroblasts and how myofibroblasts production of ECM is regulated.

Currently, little is known about the origin of myofibroblasts within the injured kidney. It has been suggested that interstitial myofibroblasts may derive from the differentiation of fibroblasts, the migrating of perivascular smooth muscle cells or local proliferation (Tang et al., 1996; Zhang et al., 1995). Emerging evidence also suggests that tubular epithelial cell has the capacity to undergo epithelial–mesenchymal transdifferentiation (EMT), thereby becoming interstitial myofibroblast (Strutz et al., 1995). However, the mechanism(s) regulating tubular EMT process remains largely unknown.

Cytokines and growth factors play an important role in the EMT process. Among them, transforming tissue growth factor-β1 (TGF-β1), a multiple functional cytokine with profibrotic properties, has been implicated in the pathogenesis of renal fibrosis in both experimental and human glomerulonephritis (Hill et al., 2000; Shankland et al., 1996; Böttinger and Bitzer, 2002). In vitro, TGF-β1 has been shown to induce EMT of mammary epithelial cells and many kinds of embryonic cells (Miettinen et al., 1994; Potts et al., 1991). Furthermore, TGF-β1 has been shown to stimulate expression of α-SMA by cultured human proximal tubular epithelial cells (Fan et al., 1999). These results suggest that TGF-β may be an important inducer of EMT.

Connective tissue growth factor (CTGF) is a newly identified growth factor, which belongs to the family of cysteine-rich growth factors that consists of CTGF/fisp-12, cef/cyr61, and nov (Yokoi et al., 2002). In cultured fibroblasts, CTGF gene expression is strongly induced by TGF-β and not by other growth factors, such as epidermal growth factor, platelet-derived growth factor, or basic fibroblast growth factor (Igarashi et al., 1993). Addition of CTGF, in turn, potently stimulates fibroblast proliferation and ECM synthesis (Frazier et al., 1996). Recently, it has been shown that CTGF expression is upregulated in the proliferative and fibrotic interstitial lesions of various human renal diseases (Ito et al., 1998). Although all these observations have led to the hypothesis that CTGF is a candidate factor mediating fibrogenic properties of TGF-β, the role of CTGF in renal tubular EMT still remains unclarified.

In the present study, to explore the implication of CTGF in renal tubular EMT, we examined the direct effect of CTGF on the expression of α-SMA, vimentin, tenascin-C, and collagen IV in cultured human renal proximal tubular epithelial cell line (HKC). Furthermore, to evaluate the contribution of CTGF to the EMT process induced by TGF-β1, we inhibited endogenous CTGF by antisense oligodeoxynucleotide (ODN) and analyzed the effects of its blockade on the transdifferentiation process induced by TGF-β.

2. Materials and methods

2.1. Cell culture

HKC cells (kindly provided by Professor Zhihong Liu, Division of Nephrology, Jinling Hospital, Nanjing, China) were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2 in RPMI 1640, containing 10% fetal calf serum (Invitrogen, USA), 2.50 g/L HEPES (Invitrogen, USA), 0.30 g/L L-glutamine, 1.80 g/L sodium bicarbonate, 100 kU/L penicillin and 100 mg/L streptomycin. After adding 0.25% trypsin (Invitrogen, USA) for digestion, 2×10^6 cells were grown in 50 mL plastic culture bottles (for indirect immuno-fluorescence and immunocytochemistry examination, cells were cultured in four-chamber glass slides). Prior to use, subconfluent cells were incubated in serum-free medium supplemented with 1.0% bovine serum albumin (BSA, Roche, Switzerland) for 24 h.

To observe the effects of CTGF on tubular EMT, serum-starved HKC cells were incubated with different concentrations of recombinant human CTGF (rhCTGF, 2.5 or 5.0 μg/L, FibroGen, USA) for 24 or 48 h. Then the cells or the culture media were collected to examine the expression of α-SMA, vimentin, tenascin-C, and collagen IV using RT-PCR, indirect immuno-fluorescence, immunocytochemistry, Western blot analysis, and enzyme-linked immunosorbent assay (ELISA) methods, respectively. To investigate the effects of TGF-β on the expression of CTGF and α-SMA, serum-deprived HKC cells were incubated for 0–24 h in the presence of 10.0 μg/L recombinant human TGF-β1 (rhTGF-β1, R&D System, USA, No: AV332072), then the cells were collected and total RNA was extracted for RT-PCR. To examine the effect of CTGF antisense ODN on tubular expression of α-SMA, serum-starved cells were transfected with CTGF sense or antisense ODN prior to the stimulation of TGF-β1, then cell lysate and slides were harvested for RT-PCR and indirect immuno-fluorescence examinations.

2.2. Morphological observation

After rhCTGF (2.5 or 5.0 μg/L) was added to the medium, the cells were observed every 6 h. After stimulation with rhCTGF for 48 h, HKC cells were observed with phase contrast microscopy and photos were taken.

2.3. Semi-quantitative RT-PCR

Total RNA was extracted from HKC cells with TRIzol Reagent (Invitrogen, USA) according to the instructions of the manufacturer. RNA samples were quantified by measurement of optic absorbance at 260 nm and 280 nm in a spectrophotometer, with the A_{260}/A_{280} ratio ranging from 1.8 to 2.0, which indicated a high purity of the extracted RNA. The concentration of total RNA was calculated according to A_{260}. Aliquots of total RNA (2.0 μg) from each sample were reverse-transcribed into cDNA according to the instructions of the First Strand cDNA Synthesis Kit manufacturer (MBI, Lithuania). Equal amounts of the reverse transcriptional products were subjected to PCR amplification. We co-amplified the housekeeping gene β-actin to allow semi-quantitative comparison of PCR products. Amplification was started with 5 min of denaturation at 94 °C followed by 30 cycles (for β-actin, 27 cycles). Each cycle consists of 45 s at 94 °C, 45 s at 55 °C, 60 s at 72 °C. The final extension was for 10 min at 72 °C. After amplification, 5.0 μl of each PCR reaction product was electrophoresed through a 1.5% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL). Gels were scanned using an EPSON GT-800 Scanner (EPSON, Japan) and photos were taken. The mRNA levels of CTGF, α-SMA, tenascin-C, and collagen IV were normalized with β-actin mRNA levels. The mean mRNA level of the untreated HKC cells was regarded as 1.0 arbitrary unit. At least three independent PCR procedures were performed to allow statistical analysis.

All PCR primers were synthesized by Sangon Biotech, China. The sequences and the amplified lengths are seen in Table 1. DNA marker (DL2000) was a product of Takara, China.

2.4. Indirect immuno-fluorescence

After HKC cells were stimulated with rhCTGF (2.5 or 5.0 μg/L) or transfected with CTGF sense or antisense ODN and stimulated with TGF-β1 (10.0 μg/L) for 48 h, HKC cells were fixed in ethanol–acetone (1:1 mixed) for 15 min at 4 °C. After rinsing with phosphate-buffer saline, cells were incubated with mouse anti-human α-SMA or vimentin antibody (Sigma, USA) at 4 °C for 12 h. Then FITC-labeled goat anti-mouse antibody (Zhongshan Biotech, China) was added and further incubated for 45 min at 37 °C. Negative control was made by replacing the first antibody with phosphate-buffer saline. After the slides were covered with glycerol buffer, the expression of α-SMA and vimentin were observed under fluorescence microscopy and photos were taken. The number of HKC cells stained with α-SMA or vimentin antibody was determined by counting the number of positive-stained cells in a total of at least 600 cells under magnification of 400× in each group. Data from five experiments were expressed as the mean percentage ± SE.

2.5. Flow cytometry

The intracellular α-SMA protein expression was also measured by flow cytometric analysis. After stimulation with CTGF (2.5 or 5.0 μg/L) for 48 h, HKC cells were harvested and washed in phosphate-buffer saline. Then the cells were fixed in 1.0% paraformaldehyde for 15 min and permeated by 0.2% Triton X-100 (Sigma, USA). After washing, aliquots of 1×10^6 cells/mL were incubated for 30 min at 37 °C with mouse anti-human α-SMA (Sigma, USA, 1:1000 diluted). To correct for nonspecific binding, phosphate-buffer saline solution instead of the first antibody was added to the blank control tube. After washing with phosphate-buffer saline, 1:50 diluted FITC-labeled goat anti-mouse IgG antibody (Zhongshan Biotech, China) was added and incubated for 45 min. After washing, 1×10^6 cells in each group were subjected to flow cytometric analysis, and the percentage of positive cells was measured.

2.6. Immunocytochemistry

HKC cells were cultured in four-chamber glass slides in the absence or presence of rhCTGF (2.5 or 5.0 μg/L),

Table 1
Primers used in the RT-PCR analysis

Templates	Primer sequences	Product lengths (bp)
α-SMA	Sense 5′-GCTCACGGAGGCACCCCTGAA-3′ Antisense 5′-CTGATAGGACATTGTTAGCAT-3′	589
Tenascin-C	Sense 5′-GGGTCCTCAAGAAAGTCAT-3′ Antisense 5′-ACTCCATTCACGCACTTGC-3′	636
Collagen IV	Sense 5′-CGGGGTTACAAGGTGTCATTGG-3′ Antisense 5′-GCCAAGTATCTCACCTGG-3′	332
CTGF	Sense 5′-AACTATGATTAGAGCCAACTGCCTG-3′ Antisense 5′-TCATGCCATGTCTCCGTACATCTTC-3′	477
β-actin	Sense 5′-CCTTCCTGGGCATGGAGTCCTG-3′ Antisense 5′-GGAGCAATGATCTTGATCTTC-3′	208

and were stained with monoclonal antibody of tenascin-C (Sigma, USA) using immunocytochemistry according to the instructions of Immunocytochemical Detection Kit (Zhongshan Biotech, China). Briefly, HKC cells were rinsed in phosphate-buffer saline, fixed in 2% paraformaldehyde, preincubated with 10% fetal calf serum to block nonspecific binding, and then incubated with tenascin-C antibody or irrelevant isotype control antibody for 60 min. After washing with phosphate-buffer saline, endogenous peroxidase was inactivated by incubation in 0.3% H_2O_2 in methanol for 15 min, incubated with peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated anti-peroxidase complexes, followed by development with diaminobenzidine to produce a brown color. All procedures were performed at room temperature. The number of HKC cells stained with tenascin-C antibody was determined by counting the number of positive-stained cells in a total of at least 600 cells under magnification of 400× in each group. Data from five experiments were expressed as the mean percentage ± SE.

2.7. Western blot

After HKC cells were stimulated with rhCTGF (2.5 or 5.0 μg/L) for 48 h, the media were cleared by centrifugation. Aliquots of cleared media normalized for cell number were concentrated in a spin concentrator (Universal, USA), taken up in 4× reducing sample buffer, boiled for 5 min, electrophoresed in 10% SDS-PAGE gels, and then transferred to nitrocellulose. Blocked membranes were incubated with monoclonal anti-tenascin-C antibody (1:1000, Sigma, USA) in Tris-buffered saline containing 0.05% Tween-20 (TTBS)/4% BSA overnight at 4 °C, washed, and then incubated with horseradish peroxidase-conjugated second antibody (1:50 diluted, Zhongshan Biotech, China) for 1 h. Bands were visualized by enhanced Chemiluminescence kit (Zhongshan Biotech, China) and captured on an X-ray film. Protein levels were quantitated using Chemiluminescence Detection System (Biorad, USA).

2.8. ELISA

Collagen IV in conditioned media was quantitatively measured by ELISA method according to the manufacturer's instructions to the collagen IV-ELISA Detection Kit (Jingmei Biotech, China).

2.9. CTGF antisense ODN transfection

The sequences of phosphorothioate oligonucleotides (Sangon Biotech, China) for human CTGF used in this study are as follows: antisense ODN, 5′-TACTGGC GGCGGT*CAT*-3′; sense ODN, 5′-ATGACCGCCGC CAGTA-3′. The antisense sequence is complementary to human CTGF cDNA around the translation initiation codon (italicized in sequence). Transfection into HKC cells was carried out by cationic lipofection with Lipofectamine Reagent according to the manufacturer's instructions. Briefly, HKC cells (2×10^6) were serum-starved for 24 h before transfection. Then antisense or sense ODN and Lipofectamine Reagent (Invitrogen, USA) in a charge ratio of 1:1 were aggregated for 45 min at room temperature, and cells were transfected with 3.0 μM of ODN in serum free RPMI 1640. After 18 h of incubation, the cells were overlaid with growth medium containing fetal calf serum to achieve the final concentration of 10% and stimulated with TGF-β1 (10.0 μg/L) for 6 or 48 h. Then the cells and medium were collected, respectively for the detection of CTGF and α-SMA.

2.10. Statistical analysis

All data were presented as mean ± SE. One-way analysis of variance followed by Newman–Keuls method of Q test using Statistical Package for the Social Sciences Software (version 11.5) was performed to determine statistical significance. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of rhCTGF on the morphology of HKC cells

Under phase contrast microscopy, HKC cells cultured in six-well plates produced a confluent monolayer with cobblestone morphology. Culture of HKC cells in 2.5 μg/L of rhCTGF induced profound morphological changes, with cells developing marked hypertrophy, becoming elongated, and losing the cobblestone growth pattern. The number of cells undergoing morphological transformation was further increased when rhCTGF was added at a dose of 5.0 μg/L (Fig. 1).

3.2. CTGF-stimulated expression of α-SMA, tenascin-C, and collagen IV mRNA in cultured HKC cells

After being deprived of serum for 24 h, HKC cells were incubated with rhCTGF (2.5 or 5.0 μg/L) for 24 h. Then total RNA was extracted from cell lysate and subjected to RT-PCR analysis. It was demonstrated that the addition of low dose rhCTGF (2.5 μg/L) for 24 h induced a marked increase in α-SMA and tenascin-C mRNA expression ($p < 0.01$). In the high dose rhCTGF (5.0 μg/L)-treated cells, the mRNA levels of α-SMA and tenascin-C were even higher ($p < 0.01$, Fig. 2). Interestingly, collagen IV mRNA expression was suppressed by rhCTGF, and this suppression effect was more

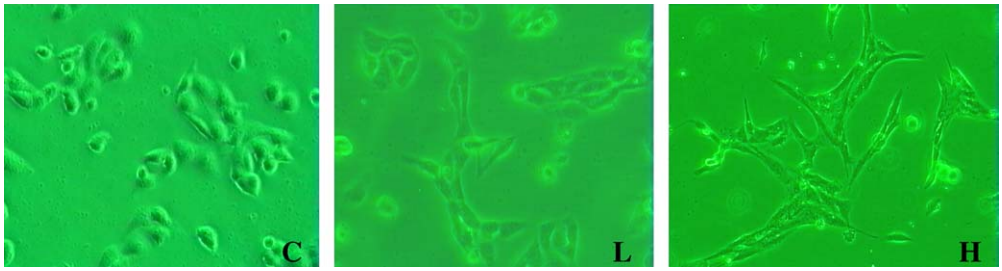

Fig. 1. Effect of CTGF on the morphology of HKC cells (phase contrast microscopy, ×200). C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h.

dramatic under a high concentration (5.0 μg/L) of rhCTGF.

3.3. Effect of rhCTGF on intracellular α-SMA expression

Under fluorescence microscopy, almost no cell was stained with α-SMA under normal culture condition. After HKC cells were cultured in a medium containing 2.5 μg/L of rhCTGF for 48 h, the percentage of α-SMA positive-stained cells was upregulated markedly. In the high dose rhCTGF (5.0 μg/L)-treated cells, the ratio of α-SMA positive-stained cells was even larger (Fig. 3a).

3.4. Flow cytometric analysis of α-SMA expression by HKC cells

Flow cytometry of Triton X-100-permeabilized HKC cells was used to quantitate rhCTGF-induced α-SMA protein expression. As shown in Fig. 4, few HKC cells expressed α-SMA in the cytoplasm under normal culture condition. Addition of rhCTGF caused a dose-dependent increase in the percentage of HKC cells expressing α-SMA protein, with almost 2/3 (65.5 ± 7.8%) of the cells being positive when cultured in 5.0 μg/L rhCTGF (Fig. 4).

3.5. Effect of rhCTGF on intracellular vimentin expression in HKC cells

As a second phenotype marker of rhCTGF-induced transdifferentiation of HKC cells, intracellular vimentin expression was examined by indirect immuno-fluorescence method. It was showed that few HKC cells expressed vimentin under normal culture condition. After HKC cells were stimulated with 2.5 μg/L of rhCTGF for 48 h, the expression of vimentin increased significantly. HKC cells cultured in a high concentration of rhCTGF (5.0 μg/L) showed a further increase in the percentage of vimentin positive-stained cells (Fig. 3b).

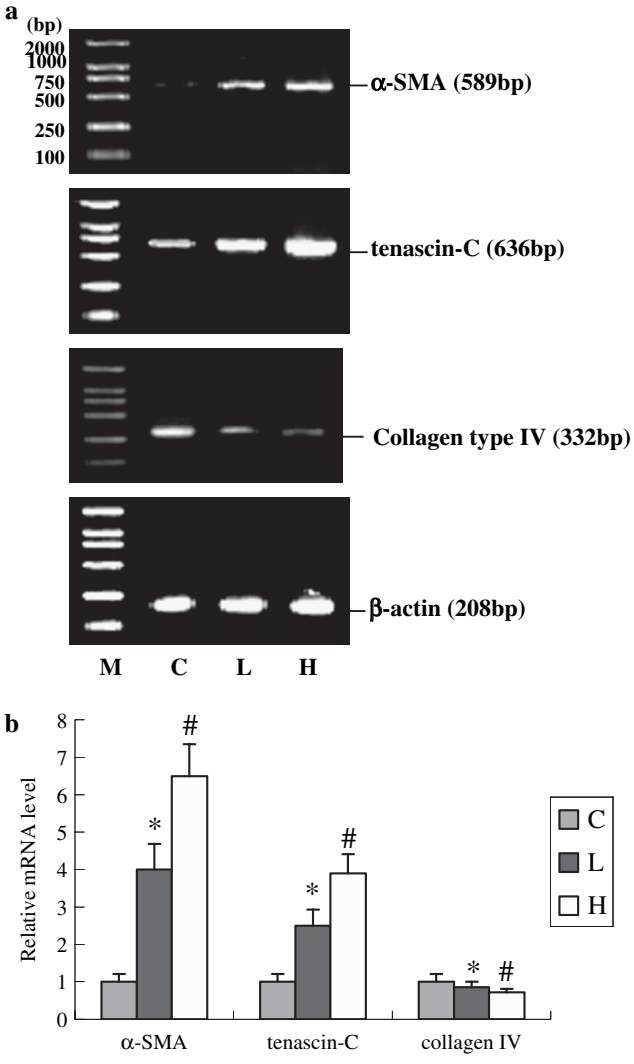

Fig. 2. RT-PCR analysis for CTGF-induced expression of α-SMA, tenascin-C, and collagen IV mRNA in HKC cells. a: Representative RT-PCR results. M, marker; C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 24 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 24 h. b: Relative mRNA levels of α-SMA, tenascin-C, and collagen IV, which are normalized with β-actin levels. $n = 3$. $^{*}p < 0.01$, vs. C; $^{\#}p < 0.01$, vs. L.

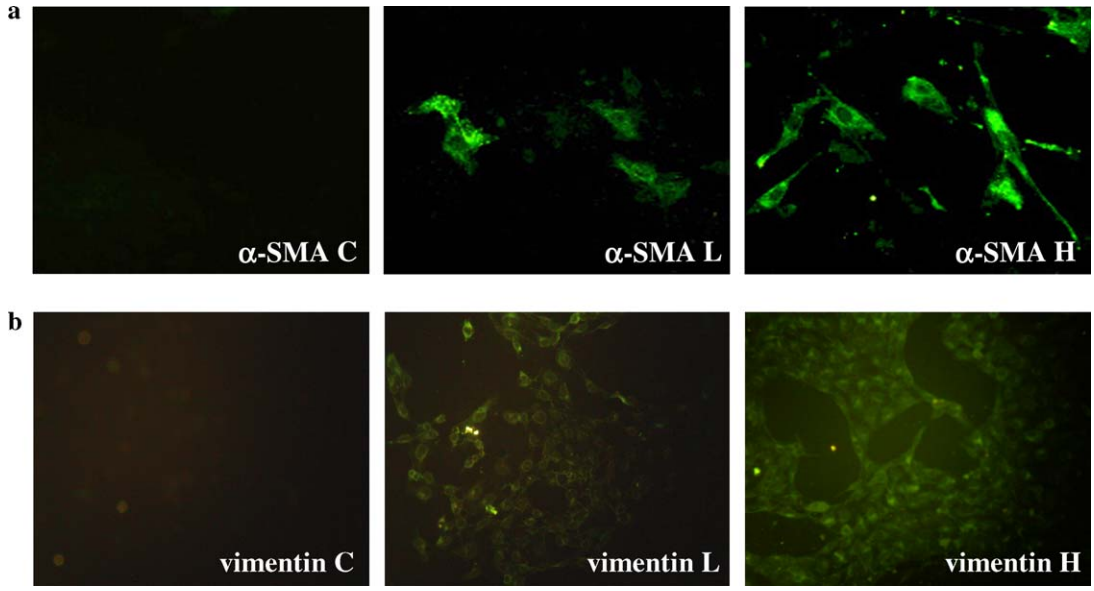

Fig. 3. rhCTGF-induced expression of α-SMA and vimentin in the cytoplasm of HKC cells (indirect immuno-fluorescence). a: Expression of α-SMA (×400). C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h. b: Expression of vimentin (×200). C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h.

3.6. Effects of rhCTGF on ECM production by HKC cells

To identify whether tubular ECM proteins were also regulated by rhCTGF, the intracellular and secreted tenascin-C protein expression was analyzed. Immunocytochemical result showed that after HKC cells were stimulated with 2.5 μg/L of rhCTGF for 48 h, the expression of tenascin-C increased moderately. HKC cells cultured in a high concentration of rhCTGF (5.0 μg/L) showed a more significant increase in the percentage of cells expressing tenascin-C in the cytoplasm (Fig. 5). Western blot analysis showed that rhCTGF also significantly upregulated tenascin-C protein levels in the culture media in a dose-dependent manner ($p < 0.01$, Fig. 6).

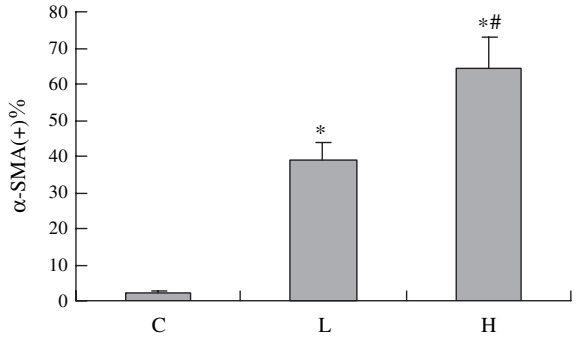

Fig. 4. Flow cytometric analysis α-SMA expression by HKC cells upon the stimulation of rhCTGF. C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h. $n = 3$. *$p < 0.01$, vs. C; $^{\#}p < 0.01$, vs. L.

As is well known, HKC cells predominantly produce collagen IV under normal culture condition. In this study, low concentration of rhCTGF (2.5 μg/L) inhibited collagen IV production significantly ($p < 0.01$), while higher concentration of rhCTGF (5.0 μg/L) exhibit more inhibitory effect ($p < 0.01$, as compared with low dose rhCTGF-treated, Fig. 7), as showed by ELISA.

3.7. TGF-β1-stimulated expression of CTGF and α-SMA mRNA in HKC cells

After deprived of serum for 24 h, HKC cells were incubated with TGF-β1 (10.0 μg/L) for 0, 3, 6, 12, and 24 h, respectively. As demonstrated by RT-PCR analysis, CTGF mRNA expression was significantly upregulated by TGF-β1 stimulation, showed a peak at 3 h after exposure and then declined but still kept at a relatively high level ($p < 0.01$ vs. C). In contrast, α-SMA mRNA expression increased from 6 h and showed a more significant upregulation at 12–24 h after stimulation ($p < 0.01$ vs. C, Fig. 8). Thus, the upregulation of α-SMA expression was delayed compared with that of CTGF.

3.8. Effects of CTGF antisense ODN transfection on the expression of CTGF and α-SMA mRNA

To further explore the role of CTGF in TGF-β1-induced tubular EMT, we examined the effect of CTGF antisense ODN transfection on the α-SMA expression induced by TGF-β1. As shown in Fig. 9, CTGF

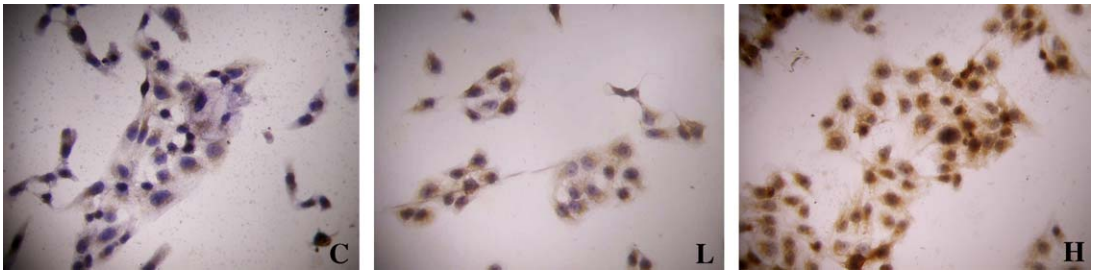

Fig. 5. Immunohistochemistry showing the expression of tenascin-C in HKC cells (×400). C, untreated control; L: treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h.

antisense ODN markedly inhibited TGF-β1 (10.0 μg/L)-induced CTGF mRNA expression at 6 h after stimulation compared with sense ODN ($p < 0.01$), verifying efficient transfection into HKC cells. Under this condition, TGF-β1-induced α-SMA mRNA expression was also significantly suppressed ($p < 0.01$, as compared with sense ODN-transfected, Fig. 9).

3.9. Effect of CTGF antisense ODN transfection on α-SMA protein expression

Indirect immuno-fluorescence staining showed that cytoplasmic α-SMA protein expression increased significantly after HKC cells were incubated with 10.0 μg/L TGF-β1 for 48 h ($p < 0.05$ vs. C). Transfection of CTGF antisense ODN attenuated TGF-β1-stimulated increase in α-SMA expression ($p < 0.05$). When HKC cells were treated with CTGF sense ODN, cytoplasmic α-SMA protein expression kept almost unchanged ($p > 0.05$, as compared with TGF-β1-stimulated, Fig. 10).

4. Discussion

EMT, the process whereby epithelial cells transform into mesenchymal cells, often occurs during development and in pathological processes such as tumorigenesis (Hay and Zuk, 1995). Recent studies suggest that the increased number of interstitial myofibroblasts, which are the primary cell type responsible for ECM deposition in tubulointerstitial fibrosis (Strutz and Muller, 1999; Wang et al., 2001; Blom et al., 2002), may be derived in part by the transdifferentiation of tubular epithelial cells (Ng et al., 1998; Muller et al., 2000; Okada et al., 2000a). During renal fibrosis, EMT is thought to occur when tubular epithelial cells acquire mesenchymal/fibroblast characteristics, including expression of vimentin (Muchaneta-Kubara and el Nahas, 1997), α-smooth muscle actin (Fan et al., 1999), and fibroblast specific protein-1 (Makhluf et al., 1996), which allow the cells to migrate through their basement membrane into the interstitium where the cells are then identified as myofibroblasts. Therefore, we explored whether human renal tubular epithelial cells directly contribute to tubulointerstitial fibrosis by responding to profibrogenic growth factors such as CTGF and TGF-β in this study.

In the present study, it is revealed that HKC cells became elongated upon the stimulation of rhCTGF, which may be due to the cytoskeleton rearrangement and polarity disruption caused by CTGF. Using RT-PCR analysis, indirect immuno-fluorescence, and flow cytometry methods, we observed high levels of α-SMA mRNA and protein, as well as elevated vimentin expression in rhCTGF-treated HKC cells after 24 or 48 h stimulation. Given the fact that α-SMA is the specific marker of myofibroblast, these results strongly suggested that renal

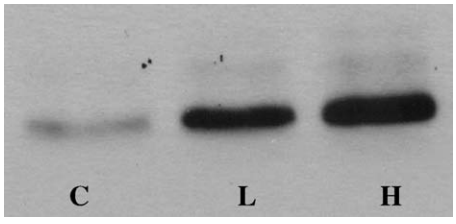

Fig. 6. Western blot analysis of tenascin-C in the cultured media upon the stimulation of rhCTGF. C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h.

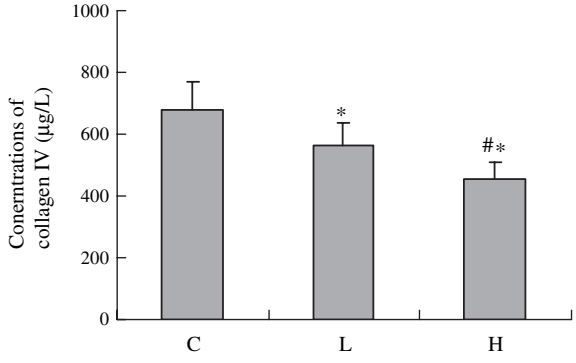

Fig. 7. Effect of rhCTGF on the collagen IV production by HKC cells (ELISA). C, untreated control; L, treatment with low dose rhCTGF (2.5 μg/L) for 48 h; H, treatment with high dose rhCTGF (5.0 μg/L) for 48 h. $n = 3$. $^{*}p < 0.01$, vs. C; $^{\#}p < 0.01$, vs. L.

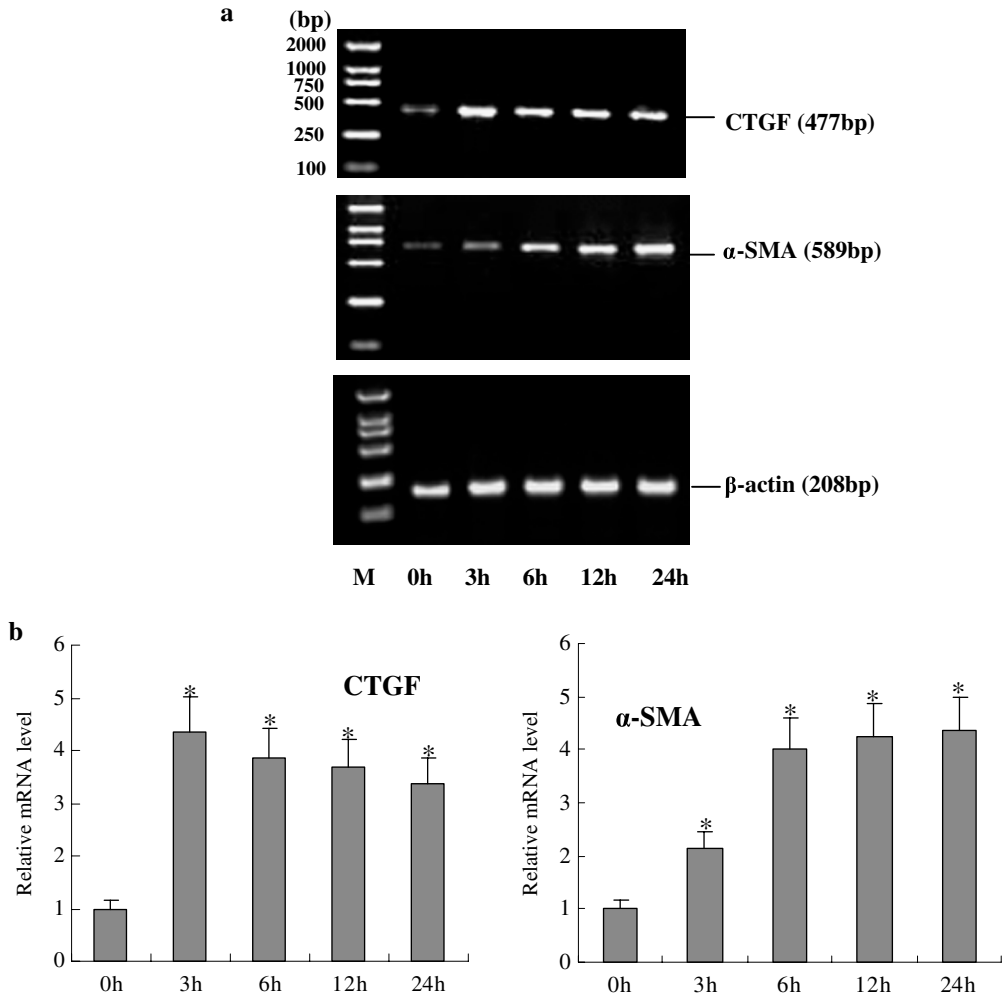

Fig. 8. RT-PCR analysis for TGF-β1-induced expression of CTGF and α-SMA mRNA in HKC cells. a: Representative RT-PCR results. M, marker; lane 0h, 3h, 6h, 12h, 24h showed the results after treatment with 10.0 μg/L TGF-β1, for 0, 3, 6, 12 and 24 h, respectively. b: Relative mRNA levels of CTGF and α-SMA at 0, 3, 6, 12, and 24 h after TGF-β1 (10.0 μg/L) stimulation, which are normalized with β-actin levels. $n = 3$. *$p < 0.01$, vs. C (0 h).

tubular cells could transdifferentiate into myofibroblasts on the stimulation of CTGF.

It is reported that the matricellular protein tenascin-C is significantly elevated in tubulointerstitial lesions of various kidney diseases (Okada et al., 1996) and is expressed during development at the sites of EMT (Erickson and Bourdon, 1989). Tenascin-C possesses both adhesive and anti-adhesive properties, which may promote cell migration in tumor metastasis, embryonic development, and wound healing. It has also been demonstrated that HKC cells undergoing EMT acquire a migratory phenotype in vitro, which likely facilitates their translocation from the tubular basement membrane to the renal interstitium in vivo (Yang and Liu, 2001; Zeisberg et al., 2001). Thus it is possible that tenascin-C is one of the factors facilitating EMT in vitro and in vivo during renal fibrosis. Furthermore, alterations in the basement membrane composition, including downregulation of collagen IV, are important for epithelial cell migration during EMT (Zeisberg et al., 2001; Gore-Hyer et al., 2002). This study demonstrated that CTGF induced tenascin-C expression and simultaneously diminished collagen IV protein synthesis in HKC cells, so it is tempting to speculate that CTGF may contribute to EMT process by stimulating the migratory phenotype of tubular epithelial cells via the induction of phenotype changes (expression of α-SMA), alterations in ECM composition (decreasing collagen IV expression), and its induction of tenascin-C. All of the above actions may lead to the complete transformation and translocation of tubular epithelial cells into interstitial myofibroblasts. In support of the potential role of CTGF in EMT in vivo, Frazier et al. (2000) 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.

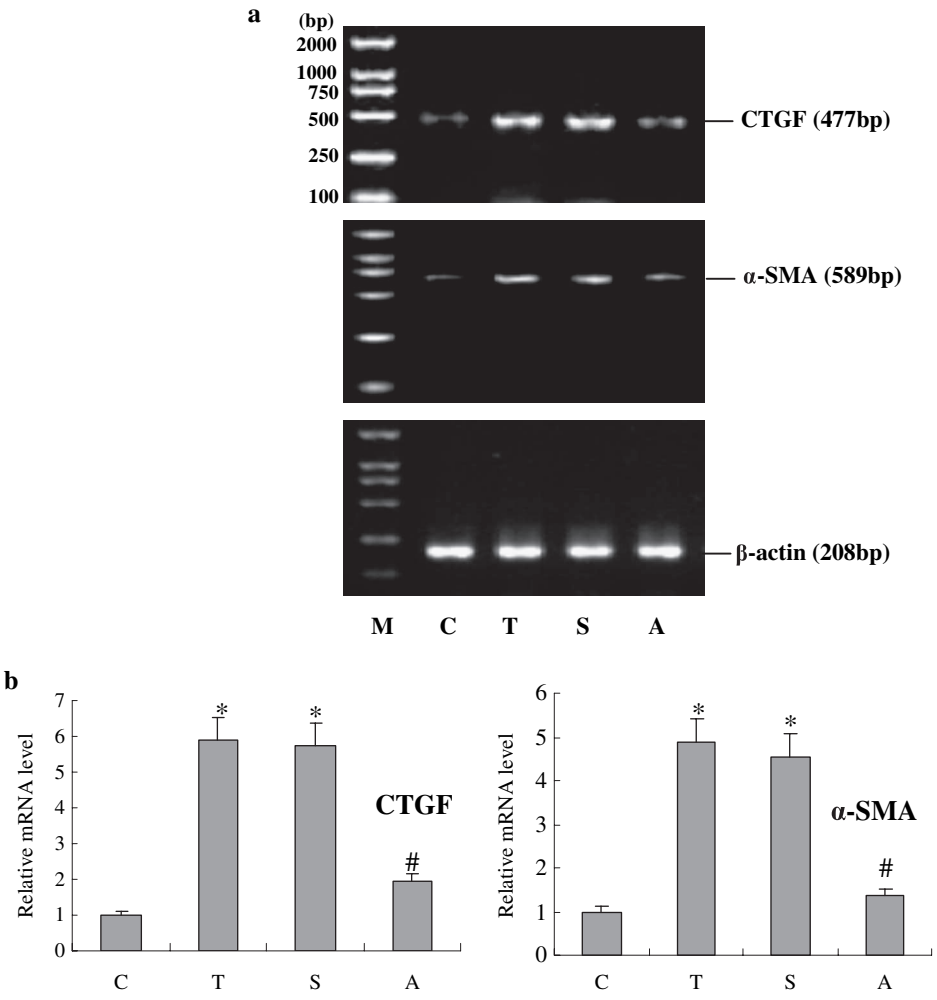

Fig. 9. Inhibitory effects of CTGF antisense ODN on TGF-β1-induced expression of CTGF and α-SMA mRNA in HKC cells. a: Representative RT-PCR results. M, marker; C, untreated control; T, treatment with TGF-β1 (10.0 μg/L) for 6 h; S, transfection with CTGF sense ODN + treatment with TGF-β1 (10.0 μg/L) for 6 h; A, transfection with CTGF antisense ODN + treatment with TGF-β1 (10.0 μg/L) for 6 h. b: Relative mRNA levels of CTGF and α-SMA, which are normalized with β-actin levels. $n = 3$. $^{*}p < 0.01$ vs. C; $^{\#}p < 0.01$, vs. sense-transfected.

Another important observation in this study was that CTGF-induced transdifferentiation of HKC cells was dose-dependent. This was not just a matter of adding more rhCTGF to get a larger number of cells to trans-differentiate, but there was a threshold concentration of CTGF that had to be reached in order to achieve complete transdifferentiation. This was well illustrated by a culture of HKC cells in 1.0 μg/L rhCTGF that induced a minor increase in α-SMA mRNA and protein expression but failed to drive a complete morphological transformation (data not shown). Indeed, phase contrast microscopy of these cells showed little morphological change. The development of complete morphological transformation of HKC cells required culture in 2.5 μg/L

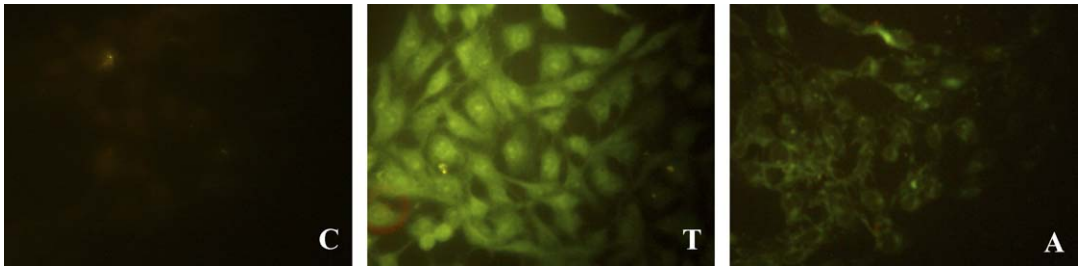

Fig. 10. Effect of CTGF antisense ODN transfection on TGF-β1-induced α-SMA protein expression in the cytoplasm of HKC cells (indirect immuno-fluorescence, ×400). C, untreated control; T, stimulated by TGF-β1 (10.0 μg/L) for 48 h; A, antisense ODN transfection + TGF-β1 (10.0 μg/L) stimulation for 48 h.

rhCTGF, and increasing the concentration of rhCTGF from 2.5 to 5.0 μg/L increased the number of cells undergoing transdifferentiation. This dose-dependent pro-transdifferentiation effect of CTGF was very similar to that of TGF-β1 as demonstrated previously (Okada et al., 2000b; Fan et al., 1999).

The findings that TGF-β1 induces CTGF expression in an early time course than α-SMA and that TGF-β1 and CTGF share a number of effects including α-SMA induction have led to the hypothesis that CTGF may serve as a mediator of TGF-β action. To further clarify CTGF dependence of TGF-β1-stimualted tubular EMT, we employed antisense strategy in cultured HKC cells. RT-PCR analysis indicated that the introduction of CTGF antisense ODN abolished TGF-β1-induced CTGF mRNA expression at 6 h and thereafter attenuated α-SMA mRNA and protein expression. These results strongly suggest that TGF-β1-induced tubular EMT is mostly dependent on CTGF. Recently, CTGF has been reported to mediate TGF-β1-induced collagen type synthesis in NRK-fibroblasts using cells treated with anti-CTGF antibody or antisense CTGF gene (Duncan et al., 1999). Such CTGF dependence in ECM accumulation induced by TGF-β has also been demonstrated in renal tubular epithelial cells (Yokoi et al., 2002). Taken together, these findings indicate that CTGF plays a crucial role in mediating various profibrotic actions of TGF-β.

In summary, this study provided evidence that CTGF can induce complete transdifferentiation of the normal human tubular epithelial cells into myofibroblasts on the basis of cell morphology, expression of the mesenchymal marker (α-SMA and vimentin), the upregulation of tenascin-C expression, and the downregulation of collagen IV. CTGF blockade resulted in a marked inhibition of TGF-β-induced tubular transdifferentiation process, suggesting that CTGF is crucial in mediating the EMT induction in the TGF-β-stimulated pathway. Hence, blockade of endogenous CTGF could potentially offer unique opportunities to attenuate the tubular EMT process and, thereby, to inhibit the myofibroblast activation and further prevent renal interstitial fibrogenesis.

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