Involvement of CTGF in TGF-\(\beta\)1–Stimulation of Myofibroblast Differentiation and Collagen Matrix Contraction in the Presence of Mechanical Stress

Qian Garrett,1 Peng T. Khaw,1 Timothy D. Blalock,2 Gregory S. Schultz,2 Gary R. Grotendorst,3 and Julie T. Daniels1

**PURPOSE.** This study was undertaken to investigate the role of connective tissue growth factor (CTGF) in fibroblast-to-myofibroblast differentiation and fibroblast-mediated collagen matrix contraction in the presence of mechanical stress.

**METHODS.** An in vitro three-dimensional contraction model of human corneal-fibroblast-seeded collagen lattices (FSCls) in the presence of mechanical stress generated by attaching the lattices to the culture well was used to measure FSC contraction. FSCls were treated with CTGF; TGF-\(\beta\)1; serum-free (SF) control medium; or TGF-\(\beta\)1 plus antisense oligodeoxynucleotide to CTGF; TGF-\(\beta\)1 plus scrambled-sequence oligodeoxynucleotide to CTGF; or TGF-\(\beta\)1 antibody. Expression of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) by fibroblasts in FSCls was detected by immunostaining and confocal microscopy, whereas ELISA was used for the fibroblasts cultured on plastic. The conditioned media were analyzed by ELISA for CTGF production.

**RESULTS.** Exogenous CTGF stimulated significantly less collagen matrix contraction and myofibroblast differentiation than TGF-\(\beta\)1, but similar to that stimulated by SF. TGF-\(\beta\)1 stimulated fibroblasts to express CTGF. CTGF antisense oligodeoxynucleotide inhibited TGF-\(\beta\)1-stimulated myofibroblast differentiation and FSC contraction. Exogenous CTGF circumvented the inhibitory effects of CTGF antisense on FSCl contraction. TGF-\(\beta\)1 antibody significantly inhibited FSCl contraction and myofibroblast differentiation under mechanical stress and SF control conditions.

**CONCLUSIONS.** In the presence of mechanical stress, CTGF is necessary for TGF-\(\beta\)1-stimulation of myofibroblast differentiation and subsequent collagen matrix contraction, but CTGF alone is not sufficient to induce myofibroblast differentiation and collagen matrix contraction. Thus, TGF-\(\beta\)1 appears to regulate multiple genes that are essential for fibroblast-mediated contraction of stressed matrix, one of which is CTGF.

**Investigative Ophthalmology & Visual Science, April 2004, Vol. 45, No. 4**

Copyright © Association for Research in Vision and Ophthalmology

1109
from fibroblasts at a later stage. Using free-floating collagen lattices, which are thought to represent the early stage of wound healing in vivo, we demonstrated the involvement of CTGF in mediating matrix contraction by fibroblasts involving matrix metalloproteinases (MMPs). However, the involvement of CTGF in mediating collagen matrix contraction in the presence of mechanical stress is not well known. The effect of mechanical stress is also important because, in a living organism, almost all tissues are constantly exposed to mechanical stresses, such as the forces originating from gravity and body movements.

To reflect the in vivo situation of mechanical tension, various models have been used to study cells and tissues in vitro. The model of fibroblasts grown in a three-dimensional collagen lattice has attracted considerable attention. Under these conditions, endogenous tension has been generated in the collagen matrix by tethering the lattice. This model provides an in vitro environment that approximates the wound-healing state found in vivo, thus offering the opportunity to investigate fibroblast-myofibroblast responses to growth factors during wound healing.

In this study we used the in vitro wound-contraction model of human corneal fibroblasts cultured within a three-dimensional network of collagen, in the presence of mechanical stress, to investigate whether CTGF is involved in myofibroblast differentiation and fibroblast collagen matrix contraction and whether stimulation of these events by TGF-β1 occurs through CTGF-dependent or -independent pathways. Our study demonstrates that exogenous CTGF is unlikely to be a direct mediator of myofibroblast differentiation and subsequent collagen matrix contraction in the presence of mechanical stress. TGF-β1 stimulation of these events could be CTGF dependent.

**METHODS**

**Growth Factors**

Recombinant human CTGF was prepared with a baculovirus expression system. Human recombinant TGF-β1 was obtained from R&D Systems (Oxford, UK).

**Antisense and Scrambled-Sequence Oligodeoxynucleotides to CTGF**

A 20-mer CTGF antisense oligodeoxynucleotide with the sequence GCC-AGA-AAG-CTC-AAA-CTT-GA was synthesized as previously described. A 20-mer oligodeoxynucleotide containing a scrambled nucleotide sequence with a random mix of all four bases was used as the negative control. Concentrated solutions of the CTGF antisense and scrambled oligodeoxynucleotides were made in the serum-free (SF) medium described in the next section and added to the cell cultures to produce final concentrations of 1, 10, and 100 μM.

**Cell Culture**

Human corneal fibroblasts were harvested and cultured, as previously described, from corneas donated, with informed consent, to Moorfields Eye Hospital Eye Bank. Briefly, fibroblasts were isolated from corneal tissue explants and cultured with fibroblast culture medium composed of Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Poole, UK) supplemented with 10% (wt/vol) fetal calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (all supplied by Invitrogen-Gibco Life Technologies, Paisley, Scotland, UK) at 37°C with 5% (vol/vol) CO₂ in air. Cultures were used for experimentation between passages 2 and 7. The tenets of the Declaration of Helsinki were followed and institutional ethics committee approval for the use of human cells was granted.

**ELISA for α-SMA**

To investigate the ability of TGF-β1 or CTGF to stimulate α-SMA expression of cultured monolayers, ELISA was used to quantify α-SMA expression as previously described. Briefly, human corneal fibroblasts were plated at 1×10⁴ cells/well in a 96-well tissue culture plate (Corning Costar, Corning, NY). Cells were serum starved overnight, after which growth factors were added in fresh SF medium. The cells were treated with concentrations of TGF-β1 or CTGF (0–10 ng/mL), and in SF DMEM supplemented with 1% (wt/vol) cell-culture-tested bovine serum albumin (BSA; Sigma-Aldrich), 100 IU/mL penicillin and 100 μg/mL streptomycin. SF DMEM supplemented with 1% BSA was used as the control treatment. After treatment of cells with growth factors for 3 days at 37°C with 5% (vol/vol) CO₂ in air, cells were fixed in methanol at −20°C for 30 minutes and nonspecific protein-binding sites were blocked for 1 hour by PBS containing 1% BSA (wt/vol) and 0.1% Tween-20 (vol/vol). Cells were incubated for 2 hours at 37°C with a monoclonal anti-α-SMA antibody (clone 1A4; Sigma-Aldrich) in PBS/1% BSA (1:2000 dilution). Cells were then incubated in horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Dako, High Wycombe, UK) in PBS/1% BSA (1:1000 dilution) for 1 hour at 37°C. Cells were washed with PBS and incubated with 100 μL TMB (3,3’,5,5’-tetramethyl-benzidine; One Solution Promega, Madison, WI) at RT for 15 minutes until the color developed. The color reaction was terminated by adding 1 N HCl to the wells (100 μL/well). Absorbance was read at 450 nm with a 630-nm reference filter, on a multifunction microplate reader (Safire; Tecan Austria GmbH, Grödig/Salzburg, Austria).

**Fibroblast-Seeded Collagen Lattice Preparation and Contraction Assay**

SF fibroblast-seeded collagen lattices (FSCLs) were prepared by mixing concentrated DMEM (Sigma-Aldrich) with type I rat tail collagen (5 mg/mL; Sigma-Aldrich), and the pH of the mixture was neutralized with 0.1 M NaOH before adding concentrated DMEM containing human corneal fibroblasts, to give a final cell density of 6.7 × 10³/mL of lattice mixture. To generate mechanical stress in the collagen matrix, after polymerization of the lattice mixture, FSCLs remained attached to the culture well and were cultured in the test medium for 48 hours, to allow the development of mechanical tension. To release, the lattice was completely detached with a pipette tip. FSCL contraction was measured over a period of 0 to 24 hours after release. At each time point the lattices were digitally photographed (Casio Computer Co. Ltd., Tokyo, Japan) from a fixed distance, and their areas were calculated in pixels with image analysis software (Image Tool; University of Texas Health Sciences Center San Antonio [UTHSCSA], San Antonio, TX). The lattices were rinsed with PBS (Invitrogen-Gibco) and fixed in 3.7% formaldehyde in PBS for 5 hours. Conditioned media from the FSCLs was collected from each treatment group. Triplicate samples per treatment were treated, unless otherwise indicated. Each experiment was repeated at least three times with cells from different donors.

**Growth Factor Stimulation of FSCL Contraction**

To assess fibroblast lattice contraction in response to growth factors, FSCLs were treated with concentrations of TGF-β1 or CTGF (0–10 ng/mL) in SF medium. The SF medium was used as a control.

**FSCL Contraction in Response to Inhibition of CTGF Gene Transcription**

To investigate whether FSCL contraction responds to inhibition of CTGF gene transcription in the presence of TGF-β1 and mechanical stress, FSCL was mechanically stressed and treated with TGF-β1 (10 ng/mL) plus antisense oligodeoxynucleotides to CTGF (1, 10, and 100 μM). TGF-β1 (10 ng/mL with a scrambled-sequence oligodeoxynucleotide (100 μM) was used as a negative control.
CTGF Protein Expression by Fibroblasts in FSCL

To investigate the ability of TGF-β1 to induce CTGF expression at the protein level, conditioned medium from FSCL was measured for CTGF protein expression using a capture sandwich ELISA with biotinylated and nonbiotinylated anti-CTGF antibodies and an streptavidin-biotinylated alkaline-phosphatase conjugate. Data are the mean ± SD of results in six samples. *Significant difference (P < 0.05) from the SF control.

FSCL Contraction in Response to Exogenous CTGF When CTGF Gene Transcription Is Inhibited

To test our postulation that CTGF may be essential in modulating FSCL contraction, human corneal fibroblasts were seeded on a culture plate, serum-starved overnight, and treated with CTGF (0.1, 1, 10 ng/mL) and TGF-β1 (0.1, 1, 10 ng/mL) for 3 days. SF medium was used as a control. Data are the mean ± SD of results in six samples. *Significant difference (P < 0.05) from the SF control.

Effect of TGF-β Antibody on FSCL Contraction and α-SMA Expression in the Presence of Mechanical Stress Only

To test whether endogenous TGF-β1 acts in an autocrine fashion to stimulate FSCL contraction and α-SMA expression under mechanical stress and SF conditions, FSCLs were mechanically stressed and treated with TGF-β1 (10 ng/mL) plus oligodeoxynucleotides to CTGF (100 μM) and exogenous CTGF (10 ng/mL). TGF-β1 (10 ng/mL), with a scrambled-sequence oligodeoxynucleotide (100 μM) and CTGF (10 ng/mL), was used as a negative control.

Immunodetection of α-SMA in FSCLs

The fixed collagen lattices were permeabilized for 15 minutes with 1% Triton X-100 (Sigma-Aldrich) at room temperature (RT). They were blocked for 1 hour with 10% FCS at RT. For immunostaining of α-SMA, the monoclonal anti-α-SMA antibody conjugated with Cy3 (Clone I44; Sigma-Aldrich) was added to the lattices (1:200 in blocking buffer) and incubated overnight at RT. After the lattices were rinsed with PBS and exposed for 1 minute to 4',6-diamidino-2-phenylindole (DAPI) dye (1:5000 in PBS; Sigma-Aldrich), they were rinsed and mounted on a glass slide with antifade agent. Cells were viewed with a laser scanning confocal microscope (LSM510; Carl Zeiss Meditec, Jena, Germany).

RESULTS

Induction of α-SMA Expression by TGF-β1 but Not by CTGF Alone

Myofibroblasts are central to wound contraction. Regulating the ability of fibroblasts to express α-SMA protein, a marker for myofibroblast differentiation, is crucial in controlling wound contraction. Therefore, we began by examining α-SMA protein expression by human corneal fibroblasts in monolayer in response to growth factor treatments. Figure 1 shows that compared with the SF control, TGF-β1, but not CTGF, significantly stimulated α-SMA expression with all the concentrations tested. The maximum effect occurred with 1 and 10 ng/mL. No significant difference in α-SMA expression was detected for CTGF at 0.1, 1, and 10 ng/mL. A concentration of 10 ng/mL for both TGF-β1 and CTGF was used for further experiments.

Stimulation of Mechanically Prestressed FSCL Contraction by TGF-β1 but Not by CTGF Alone

We next investigated the role of growth factors in fibroblast-mediated collagen matrix contraction in the presence of mechanical stress. Figure 2 shows that after release of prestressed FSCLs, all the treatment groups supported FSCL contraction. However, TGF-β1 stimulated significantly more contraction than SF medium or SF plus CTGF.
Effect of Inhibition of CTGF Gene Expression on TGF-β1-Stimulated FSCL Contraction

Because CTGF has been shown to mediate a number of TGF-β1-stimulated functions in fibroblasts, CTGF may present a downstream target for TGF-β1 regulation and play a role in mediating TGF-β1–stimulated matrix contraction under mechanical tension. To test this hypothesis, FSCLs were treated with TGF-β1, together with concentrations of antisense oligodeoxynucleotides that target CTGF mRNA or a scrambled CTGF oligodeoxynucleotide sequence control. As is shown in Figure 3, increasing the concentration of the CTGF antisense oligodeoxynucleotide from 1 to 100 μM inhibited TGF-β1–stimulated FSCL contraction in a dose-dependent manner, without toxicity to the cells (data not shown). The addition of a scrambled-sequence control did not show any significant difference in contraction compared with TGF-β1. These findings indicate that a level of CTGF protein may be synthesized by TGF-β1–treated fibroblasts in FSCLs.

Induction of CTGF Protein Synthesis by Mechanically Stressed Fibroblasts in FSCLs

To determine the effect of CTGF gene-downregulation, conditioned media from FSCLs treated with TGF-β1, TGF-β1 plus CTGF antisense oligodeoxynucleotides, or scrambled-sequence control or SF medium and SF plus CTGF antisense oligodeoxynucleotides were assayed for CTGF protein synthesis by ELISA. Figure 4 shows that a much higher level of CTGF protein was detected in the presence of TGF-β1 (~1.38 ng/mg; A) compared with the SF control (~0.15 ng/mg; B). Increasing concentrations of the CTGF antisense oligodeoxynucleotides from 1 to 100 μM inhibited TGF-β1–stimulated CTGF expression in a dose-dependent manner, and the scrambled control had little effect (Fig. 4A). Similarly, the level of CTGF protein expression decreased significantly when CTGF antisense oligodeoxynucleotide was added to the mechanically stressed lattices cultured with SF medium only (Fig. 4B).

Correlation of Increased Expression of α-SMA with Increased FSCL Contraction

To correlate the extent of fibroblast-to-myofibroblast differentiation during collagen matrix contraction, α-SMA assembly into stress fibers of cells in FSCLs in response to growth factors than CTGF and the SF control. No significant difference was found between CTGF- and SF-stimulated FSCL contraction.
and antisense oligodeoxynucleotide treatments was investigated. α-SMA expression was clearly shown, to various degrees, with all treatments (Fig. 5, histogram). The cell morphology also varied among the treatments (Figs. 5A–E). With TGF-β1, a known stimulator of myofibroblast differentiation, abundant formation of intracellular stress fibers, a characteristic of differentiated myofibroblast cells, was clearly seen (A, micrograph). However, the SF control (B), CTGF alone (C), and TGF-β1 plus antisense oligodeoxynucleotide to CTGF (D) shared similar cell morphologies, with much less stress fiber formation. The morphology of the cells treated with TGF-β1 plus scrambled oligodeoxynucleotides (E) was similar to those of the TGF-β1 treatment (A). In addition, the extent of myofibroblast differentiation also varied among the treatments. When the percentage of myofibroblast differentiation was quantified, TGF-β1 stimulated approximately 90% of the cells to differentiate into myofibroblasts (A, histogram). In contrast, α-SMA expression in exogenously added CTGF-treated FSCLs (C) did not significantly differ from control levels (~70%; B). Furthermore, with addition of CTGF antisense oligodeoxynucleotide to the TGF-β1 treatment, myofibroblast differentiation was significantly inhibited to approximately 40% (D), whereas addition of CTGF scrambled-sequence control (E) did not show significant inhibition compared with TGF-β1 treatment, suggesting that TGF-β1-induced myofibroblast differentiation may be mediated by a CTGF-dependent pathway.

**Induction of FSCL Contraction by Exogenous CTGF When CTGF Gene Transcription Is Inhibited**

To test whether exogenous CTGF can compensate for inhibition of CTGF gene transcription, exogenous CTGF was added to the treatment of TGF-β1, where the CTGF protein expression was inhibited by CTGF antisense oligodeoxynucleotides; and, as a result, the FSCL contraction occurred to a level similar to that with the treatment of TGF-β1 only (Fig. 6). Furthermore, addition of exogenous CTGF to the TGF-β1 treatment did not show any significant difference in stimulating FSCL contraction compared with the TGF-β1 treatment.

**Inhibitory Effect of TGF-β Antibody on FSCL Contraction and α-SMA Expression in the Presence of Mechanical Stress Only**

To test whether a high level of α-SMA expression by the mechanically stressed fibroblasts under the SF conditions was due to stimulation of the endogenous TGF-β, we investigated the effect of TGF-β function-blocking antibody on myofibroblast differentiation and collagen matrix contraction. Figure 7 (histogram) shows the contraction of mechanically stressed FSCL treated with monoclonal TGF-β1, β2, or β3 antibody was inhibited in a dose-dependent manner with maximum effect at the concentration of 3 μg/mL, at which contraction was inhibited from approximately 60% to 20%, compared with that of 

---

**Figure 5.** Expression of α-SMA in FSCL. Three-dimensional reconstruction of α-SMA in FSCLs by means of laser scanning confocal microscopy, after 2 days of culture, in the presence of mechanical stress, with the treatment of TGF-β1 (10 ng/mL) (A); SF (B); CTGF (10 ng/mL) (C); TGF-β1 (10 ng/mL) containing antisense oligodeoxynucleotides to CTGF (100 μM) (D); and TGF-β1 (10 ng/mL) containing CTGF scrambled-sequence control (100 μM) (E). α-SMA was visualized by means of immunofluorescence with a Cy3-conjugated monoclonal antibody against α-SMA. Histogram: the percentage of α-SMA-positive cells is expressed as a percentage of the total number of cells visualized by DAPI-stained nuclei, calculated from three randomly chosen microscopic fields. Inhibition was significant at *P* < 0.05 (*) when compared with TGF-β1 treatment. Scale bar, 20 μm. Final magnification, ×40.
the control. There was no difference in FSCL contraction among the SF controls with added mouse IgG at the different concentrations (data not shown). Furthermore, the monoclonal TGF-β1, -β2, -β3 antibody also inhibited α-SMA expression, reducing it from approximately 75% (Fig. 7A) to 45% (Fig. 7B), and little formation of intracellular stress fibers was observed in the cells treated with the TGF-β function-blocking antibody. These data support the hypothesis that endogenous TGF-β may act in an autocrine fashion to stimulate myofibroblast differentiation and subsequent collagen matrix contraction.

**DISCUSSION**

Corneal wound healing involves the activation of keratocytes to fibroblasts, migration of fibroblasts to the wound, fibroblast differentiation into myofibroblasts, and extracellular matrix deposition and remodeling. TGF-β plays a major role in initiating such complex cell-matrix-cytokine interactions. It stimulates both fibroblast proliferation and the synthesis and secretion of several extracellular matrix components. Consequently, much research has been performed to study TGF-β signaling pathways to identify agents that block the biological actions of TGF-β—such as blocking the wound-healing response and excessive matrix contraction that can induce pathologic scarring. One cytokine selectively induced in connective tissue cells by TGF-β is CTGF. CTGF mimics many actions of TGF-β in connective tissue cells. CTGF is known to have mitogenic effects on proliferation, migration, matrix production, angiogenesis, adherence, and granulation tissue formation. In addition, some of the effects of TGF-β, such as anchorage-independent growth of normal rat kidney (NRK) fibroblasts and collagen synthesis by these cells, have been demonstrated to be mediated through a CTGF-dependent pathway. Requirement of CTGF for TGF-β1-meditated matrix contraction by fibroblasts in the absence of mechanical tension, using an in vitro model representing the early stages of wound healing.

In the present study, we used an in vitro model representing the later stages of wound healing involving myofibroblasts and investigated whether the effect of TGF-β1 on differentiation of fibroblasts into myofibroblasts and subsequent matrix contraction under mechanical tension is mediated by CTGF-dependent pathways.

The myofibroblast is a central player in the wound repair and scarring process. The ability of cells to exert force on the collagen matrix has been shown to depend on the actin cytoskeleton, such as α-SMA. α-SMA is a critical component of the contractile apparatus of myofibroblasts with expression regulated by environmental factors specific to the wound tissue. The present study supports the previous findings that TGF-β1 is a potent stimulus of α-SMA expression in corneal fibroblasts. This effect was observed in monolayer cultures and in tethered three-dimensional collagen lattices. However, exogenous CTGF did not appear to stimulate α-SMA expression by human corneal fibroblasts at the levels that are significantly different from the SF control treatment. Scale bar, 20 μm.

**FIGURE 6.** Induction of FSCL contraction by exogenous CTGF when CTGF gene transcription was inhibited. Extent of FSCL contraction calculated by percentage reduction of the lattice areas measured in pixels, are shown after 2 days of culture, in the presence of mechanical stress, with the treatment of TGF-β1 (10 ng/mL); TGF-β1 (10 ng/mL) containing antisense oligodeoxynucleotides to CTGF (100 μM); TGF-β1 (10 ng/mL) containing CTGF antisense (100 μM) plus CTGF (10 ng/mL); TGF-β1 (10 ng/mL) containing scrambled-sequence control (100 μM) plus CTGF (10 ng/mL); or TGF-β1 (10 ng/mL) containing CTGF (10 ng/mL), and 24 hours after lattices were released from stress. Data are the mean ± SD of results in three samples. *Statistically significant differences (P < 0.05) from the TGF-β1 treatment.

**FIGURE 7.** Inhibitory effect of TGF-β1 on α-SMA expression in FSCL and FSCL contraction. Three-dimensional reconstruction of α-SMA that is present in FSCL by means of laser scanning confocal microscopy, after 2 days of culture, in the presence of mechanical stress, with treatment with SF (A) and SF containing the monoclonal TGF-β1, -β2, -β3 antibody at a concentration of 3 μg/mL (B). α-SMA was visualized by means of immunofluorescence with a Cy3-conjugated monoclonal antibody against α-SMA. The extent of FSCL contraction, calculated by percentage reduction of the lattice areas measured in pixels, is also shown after 2 days of culture, in the presence of mechanical stress, with the treatment of SF and SF containing monoclonal TGF-β1, -β2, -β3 antibody at concentrations of 0.03, 0.3, and 3 μg/mL, and 24 hours after lattices were released from stress. Data are the mean ± SD of results in three samples. *Statistically significant differences (P < 0.05) from the SF control treatment. Scale bar, 20 μm.
The study of CTGF, or connective tissue growth factor, has been further extended to understand its role in wound repair processes. Recent findings indicate that CTGF plays a critical role in myofibroblast differentiation and collagen matrix contraction. Our experiments demonstrated a correlation between CTGF levels and the degree of matrix contraction, confirming previous studies that show CTGF expression increases during the process of wound healing. However, understanding the precise mechanisms by which CTGF regulates these processes is still ongoing. This highlights the complexity of wound repair and the need for further research to elucidate the role of CTGF and its potential as a therapeutic target for various wound healing disorders.

**References**


