American Journal of Pathology, Vol. 163, No. 5, November 2003

Mediation of Transforming Growth Factor-β_1-Stimulated Matrix Contraction by Fibroblasts

A Role for Connective Tissue Growth Factor in Contractile Scarring

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Excessive cell-mediated tissue contraction after injury can lead to morbid contractile scarring in the body. In the eye this can cause blindness because of posterior capsule opacification, proliferative vitroretinopathy, failure of glaucoma filtration surgery, and corneal haze. During repair, transforming growth factor (TGF)-β and connective tissue growth factor (CTGF) genes are co-ordinately expressed. Although TGF-β and CTGF stimulate new matrix deposition, their role and regulation during contractile scarring is unknown. In this study, an *in vitro* model of collagen matrix contraction culminating from tractional forces generated by fibroblasts showed that both TGF-β_1 and CTGF-stimulated contraction. Using a specific anti-sense oligodeoxynucleotide to CTGF the pro-contractile activity of TGF-β_1 was found to be mediated by CTGF. During contraction fibroblasts produced similar levels of matrix metalloproteinases (MMPs)-2 and -9 with TGF-β_1 or CTGF and a modest increase in MMP-1 with CTGF only (indicated by zymography and enzyme-linked immunosorbent assay). The requirement of MMPs for contraction was demonstrated using a broad-spectrum synthetic inhibitor. This study demonstrates a new function for CTGF in mediating matrix contraction by fibroblasts involving MMPs and suggests a novel regulatory mechanism for TGF-β-stimulated contraction. Inhibition of CTGF activity or gene transcription could be a suitable target for anti-scarring therapies. *(Am J Pathol 2003, 163:2043–2052)*

Co-ordinated phenotypic changes, extracellular matrix deposition, and remodeling by a variety of cells are central to tissue formation during embryogenesis, development, and repair. Perturbations in control of the cascade of tissue generation are likely to result in embryonic tissue malformation, fibrotic disorders, and abnormal wound healing.

The tissue repair process is regulated by a number of peptides including cytokines and growth factors. Transforming growth factor (TGF)-β mRNA and protein is increased in sites of tissue repair[1,2] and is a potent stimulator of connective tissue formation.[3–5] TGF-β up-regulates fibroblast proliferation[6] and extracellular matrix synthesis[7,8] and reduces matrix degradation after injury.[9]

During tissue repair and early development, TGF-β gene expression is co-ordinately regulated with that of connective tissue growth factor (CTGF).[1,10] CTGF is a cysteine-rich, heparin-binding protein[11] whose gene expression is strongly induced by TGF-β in fibroblasts.[1,12] A 1-hour exposure to TGF-β is sufficient to induce CTGF gene transcription for up to 36 hours in fibroblasts.[13] A novel TGF-β response element controls transcription in both human and murine CTGF promoters.[13] These observations suggest that CTGF is a downstream mediator of TGF-β activity in fibroblasts.[13] Indeed, CTGF mediates a number of TGF-β-stimulated wound healing functions,[14] including collagen synthesis during granulation tissue formation.[15]

Contraction and remodeling of matrix are important elements of the tissue repair process,[16] however excessive contraction induces pathological scarring in a variety

Supported by the Royal National Institute for the Blind, UK (to J. T. D., P. T. K.); the National Institutes of Health (grant number NEI EYO 5587 to G. S. S., T. D. B.); the Eranda Foundation (to J. T. D.); the Hayman Trust (to J. T. D.); the Moorfields Eye Hospital Special Trustees (to J. T. D.); and the Moorfields Eye Hospital Executive Trust (to J. T. D., P. T. K.).

The views presented represent those of the authors and not necessarily those of the funding bodies.

Accepted for publication July 31, 2003.

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of tissues including parts of the eye, skin, liver, and kidney.[17–21] For example, CTGF mRNA has been found in plaques of human cataracts, the major blinding disease, and the highly contractile membranes of posterior capsule opacification.[22] Wound contraction is governed by a combination of two fibroblast-driven mechanisms; tractional forces generated by fibroblasts migrating into a wound,[23–25] and differentiation of fibroblasts into a highly contractile myofibroblast phenotype.[26,27]

Using an *in vitro* model representing matrix contraction culminating from generation of tractional forces by fibroblasts,[24,28] we have recently shown that matrix metalloproteinases (MMPs) are produced by Tenon's capsule fibroblasts during this process in response to serum and that they are essential for contraction.[29] Using a similar model we have previously shown that in response to serum retinal pigment epithelial cells can also contract collagen matrix in an MMP-dependent manner.[30] This has important implications for our understanding of the mechanism of proliferative vitreoretinopathy.

The MMPs are essential for turnover of matrix during homeostasis and pathology.[31] Increasing evidence suggests that members of the MMP family are also involved in many processes besides matrix degradation, including recruitment of stem and progenitor cells from the bone marrow niche[32] and cleavage of growth factors including CTGF.[33] Furthermore, using an *in vivo* model of subconjunctival wound healing we found that application of a broad spectrum MMP inhibitor significantly reduced matrix deposition, contraction, and ultimately scarring in the rabbit.[34]

TGF-β strongly induces matrix contraction involved in scarring.[35–37] However, involvement of CTGF in the regulation of matrix contraction either independently or via control of TGF-β activity has not been addressed despite the co-expression of these growth factors during wound healing. Our study demonstrates a new function for CTGF in mediating matrix contraction by fibroblasts via utilization of MMPs and also indicates a novel regulatory mechanism for TGF-β-mediated contraction. This data suggests that CTGF could be a suitable target for anti-scarring therapies in a number of pathologies throughout the body using agents that inhibit CTGF activity or CTGF gene expression.

Materials and Methods

Growth Factors

Recombinant human CTGF was prepared as previously described using a baculovirus expression system.[14] Human recombinant TGF-β_1 was purchased from R&D Systems (Oxford, UK).

Neutralizing Antibody to CTGF

Anti-human CTGF IgG-neutralizing antibodies were prepared as described previously by injecting recombinant human CTGF into goats, isolating total IgG fraction with protein G Sepharose chromatography, followed by CTGF affinity column chromatography.[15]

Anti-Sense Oligodeoxynucleotides to CTGF

The human, mouse, and rat CTGF mRNA genes were analyzed for unique, nonrepetitive, 20 mer nucleotide sequences with high GC contents that would minimize self-hybridization and provide stability of the oligodeoxynucleotide mRNA complex. A total of 81 nucleotide sequences were selected, and 20 mer anti-sense oligodeoxynucleotides were synthesized with thioate ester linkages replacing the phosphate ester linkages, 2′-*O*-methoxyethyl ribose groups coupled at base positions 1 to 5 and 16 to 20, and 5-methylcytosine substituted for all cytosines. The anti-sense oligodeoxynucleotides were tested for the ability to reduce CTGF mRNA levels using a cell culture-screening assay.[38,39] Briefly, mouse endothelial cells (bEND.3; American Type Culture Collection, Manassas, VA) were grown to confluence and then treated with 150 nmol/L of oligodeoxynucleotide and 10 μg/ml of lipofectin (Life Technologies, Inc, Rockville, MD) for 4 hours. The cells were washed and grown for 24 hours then total RNA was isolated from the cells by guanidine isothiocyanate and phenol-chloroform extraction. CTGF mRNA expression was measured by Northern blotting. The gel blots were stripped and reprobed for expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase to confirm equal RNA loading. The CTGF mRNA levels from the Northern blot analysis were expressed as a percentage of the levels of CTGF mRNA in control cells, which were treated with lipofectin only, after normalization to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The oligodeoxynucleotide with the sequence, GCC-AGA-AAG-CTC-AAA-CTT-GA, corresponds to nucleotides 987 to 1007 of the human CTGF mRNA, reduced the level of mouse CTGF mRNA in bEND cells by 86%, and was selected as the CTGF anti-sense oligodeoxynucleotide. A scrambled mismatch control 20 mer that contained a random mix of all four bases was used as a control to assess specificity and toxicity of the phosphorothioate oligodeoxynucleotide.

MMP Inhibitor

The broad-spectrum MMP inhibitor GM 6001[40,41] (a generous gift from Glycomed, CA) was dissolved in dimethyl sulfoxide to give a stock concentration of 10 mmol/L. The stock was diluted in culture medium such that 1% (v/v) dimethyl sulfoxide was the maximum concentration cells were exposed to during experiments. A structural analog of GM 6001 (GM 6001-negative control; CN Biosciences UK, Nottingham, UK) with no MMP inhibitory activity was treated similarly and used as a negative control.

Cell Culture

Human fibroblasts were harvested and cultured from corneas donated, with informed consent, from Moorfields

Eye Hospital Eye Bank as previously described.[42] Briefly, fibroblasts were isolated from corneal tissue explants and cultured with Dulbecco's modified Eagle's medium (Sigma Chemical Company, Poole, Dorset, UK) supplemented with 10% w/v newborn calf serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all supplied by Gibco Life Technologies, Paisley, Scotland, UK). For experimentation fibroblasts were used at passage two or three.

Contraction Model

Free-floating relaxed fibroblast-populated collagen gels were prepared with modifications of the method of Bell and colleagues,[28] as previously described.[42] These gels are an *in vitro* representation of contraction resulting from tractional forces generated by fibroblasts migrating through matrix such as a wound.[24,28] Triplicate serum-free fibroblast-populated collagen gels were prepared in 48-well tissue culture plates. The gels contained 2.5 mg/ml of type I rat-tail collagen (Sigma Chemical Company, Poole, Dorset, UK) and 1×10^5 fibroblasts per ml of gel mixture. After gelation of the collagen solution, each gel was fed with 500 μl of test medium and detached from the well. Contracting gels were digitally photographed (Casio Computer Co. Ltd., Japan) and the gel areas were calculated in pixels using image analysis software (Image Tool; The University of Texas Health Science Center in San Antonio (UTHSCSA), San Antonio, TX). To achieve reproducible values with respect to magnification and camera resolution, the camera was mounted on a retort stand and every photograph was taken from the same specified distance. The tissue-culture plate, containing the gels to be photographed, was mounted on a light box in the same position on each occasion. Conditioned medium from contracting fibroblast-populated collagen gels was collected from each treatment group and stored at −20°C in siliconized Eppendorfs until analyzed.

Growth Factor Stimulation of Contraction

To assess matrix contraction, gels were fed with concentrations of TGF-β_1 or CTGF (0 to 250 ng/ml) in serum-free Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin before release.

CTGF Inhibition during Contraction

To inhibit CTGF gene transcription in the presence of TGF-β_1, fibroblast-populated collagen gels were fed with serum-free Dulbecco's modified Eagle's medium containing 1% (w/v) bovine serum albumin and 25 ng/ml recombinant human TGF-β_1 together with concentrations of anti-sense oligodeoxynucleotides to CTGF or a scrambled sequence control (0 to 100 μmol/L) before release. After 24 hours of contraction, media was removed and replenished with fresh test media. To assess the effect of inhibition of CTGF gene transcription on basal levels of contraction gels fed with serum-free medium only plus concentrations of oligodeoxynucleotides to CTGF and the scrambled control were also included. To inhibit CTGF protein activity in the presence of TGF-β_1, gels were treated as above, and CTGF-neutralizing antibody (10 μg/ml) or an IgG control were added.

CTGF Enzyme-Linked Immunosorbent Assay (ELISA)

CTGF was measured in cultured cells using a capture sandwich ELISA with biotinylated and nonbiotinylated affinity-purified goat polyclonal antibodies to human CTGF. Briefly, a flat-bottom ELISA plate (96-well; Costar, Cambridge, MA) was coated with 50 μl of goat anti-human CTGF antibody (which recognizes predominantly epitopes in the N-terminal half of the CTGF molecule) at a concentration of 10 μg/ml in phosphate-buffered saline (PBS)/0.02% sodium azide for 1 hour at 37°C. The wells were washed four times and incubated with 300 μl of blocking buffer (PBS/0.02% sodium azide/1% bovine serum albumin) for 1 hour at room temperature. The wells were washed again four times and 50 μl of recombinant human CTGF standard or sample was added and incubated at 37°C for 1 hour. After washing, 50 μl of biotinylated goat anti-human CTGF was added at a concentration of 2 μg/ml and incubated at room temperature in the dark then 50 μl of alkaline phosphatase-conjugated streptavidin (Zymed, South San Francisco, CA) was added at a 1:1000 dilution and incubated at room temperature for 1 hour after washing. The wells were washed again and incubated with 100 μl of alkaline-phosphatase substrate solution (1 mg/ml *p*-nitrophenyl phosphate; Sigma, St. Louis, MO) in sodium carbonate/bicarbonate buffer/0.02% sodium azide, pH 9.6) until the reaction developed. Absorbance readings were taken at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The values for CTGF concentration were normalized for total protein content in the sample using bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL).

Zymography

MMP activity in conditioned medium was demonstrated by gelatin zymography (10% zymogram gelatin gels), using the manufacturer's buffers and instructions (Mini Cell; Invitrogen, Groningen, The Netherlands). Briefly samples were diluted in sample buffer (1:1) and electrophoresed through gelatin-impregnated zymogram gels at 150 V for 90 minutes. Kaleidoscope molecular weight markers (BioRad, Hemel Hempstead, UK) were also included. The gels were incubated at room temperature in renaturing buffer for 30 minutes then washed in developing buffer for a further 30 minutes. Fresh developing buffer was added and the gels were incubated for 16 hours at 37°C. Zymograms were stained with 0.5% Coomassie blue (BioRad) for 90 minutes before destaining until clear bands of MMP activity appeared against a blue background. The sum intensity of the bands was calculated using a Kodak Digital Science electrophoresis doc-

umentation and analysis system 120 (Eastman Kodak Co., Rochester, NY).

MMP-1 ELISA

Total MMP-1 was measured in undiluted conditioned media using a Biotrak human two-site sandwich ELISA (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK) in accordance with the manufacturer's instructions. Briefly, samples and standards were adsorbed onto duplicate wells of a microtiter plate precoated with anti-MMP-1 antibody. A secondary polyclonal antibody to MMP-1 was added and subsequently quantified using the horseradish-peroxidase conjugate/tetramethylbenzidine (TMB) substrate detection system.

MMP Inhibition during Contraction

To assess the involvement of MMPs in fibroblast-mediated matrix contraction, collagen gels were fed with 25 ng/ml of TGF-β_1 or CTGF plus 1% bovine serum albumin together with concentrations of the broad-spectrum MMP inhibitor GM 6001 or the control molecule (0 to 100 μmol/L) before release.

Cell Viability

Fibroblast viability in the presence of each test medium was monitored using a kit incorporating the reagent WST-1 according to the manufacturer's instructions (Boehringer Mannheim Diagnostics and Biochemicals, Lewes, UK). Briefly, fibroblasts were seeded into triplicate wells of 96-well tissue-culture plates, at a density of 2.5×10^3 per well in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, and cultured for 4 hours. The medium was aspirated and replaced with 100 μl of test medium. At time 0 and 4 days 10 μl of WST-1 reagent was added to each well and the plate was incubated at 37°C for 2 hours. The absorbance, related to the number of viable cells converting the reagent to colored formazan crystals, was read at 450 nm.

Statistical Analysis

Experiments were repeated at least three times. One-way analysis of variance was performed using computer software (SPSS for Windows; SPSS Inc., Chicago, IL). The observed significance levels were adjusted with the Bonferroni test for multiple comparisons. A P value of <0.05 was considered significant.

Results

TGF-β_1 and CTGF Stimulate Fibroblast-Mediated Matrix Contraction

Remodeling and contraction of newly deposited extracellular matrix is an integral part of wound healing. To determine whether CTGF could stimulate fibroblast-mediated matrix contraction, fibroblast-populated collagen gels were used as an *in vitro* model of wound matrix contraction. TGF-β_1 stimulated matrix contraction by fibroblasts in a dose-dependent manner, as did CTGF ($P < 0.001$; Figure 1). This indicated a new function for CTGF in regulating fibroblast activity.

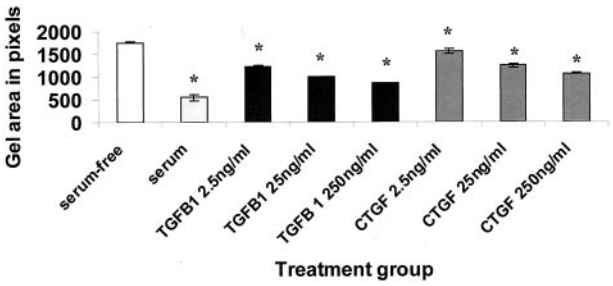

Figure 1. TGF-β_1 and CTGF stimulated fibroblast-mediated collagen matrix contraction. Fibroblast-populated collagen gel areas, measured in pixels, are shown after 3 days of culture in the presence of concentrations of TGF-β_1 or CTGF. Serum-free medium and medium containing 10% (v/v) serum were used as negative and positive controls, respectively. Both TGF-β_1 and CTGF stimulated contraction in a concentration-dependent manner. The error bars represent the SEM. The **asterisk** indicates a significant difference ($P < 0.05$) from the serum-free control.

Inhibition of CTGF Gene Transcription Reduces TGF-β_1-Stimulated Contraction

Because CTGF can mediate a number of TGF-β-stimulated functions in fibroblasts,[15,43,44] we hypothesized that CTGF may also mediate TGF-β_1-stimulated matrix contraction. To test this, the fibroblast-populated collagen gels were cultured with TGF-β_1 together with concentrations of anti-sense oligodeoxynucleotides to CTGF or with a scrambled CTGF oligodeoxynucleotide sequence control. As shown in Figure 2, increasing the concentration of the CTGF anti-sense oligodeoxynucleotide from 1 to 100 μmol/L progressively reduced the percentage inhibition of contraction relative to the degree of contraction stimulated by TGF-β_1 ($P < 0.001$). Furthermore, the highest dose of anti-sense oligodeoxynucleotide (100 μmol/L)

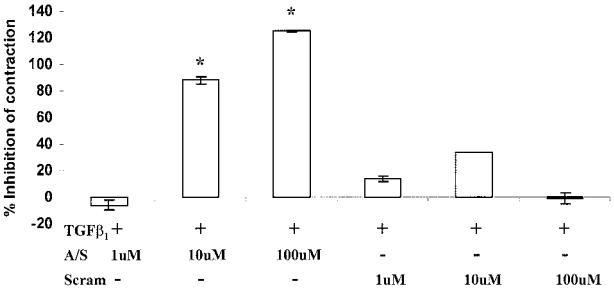

Figure 2. The addition of anti-sense oligodeoxynucleotides to CTGF significantly inhibited fibroblast-mediated collagen contraction in the presence of 25 ng/ml of TGF-β_1 after 2 days of culture. The data are plotted as the percentage inhibition of contraction relative to the degree of contraction stimulated by TGF-β_1 only. Statistical analysis of the raw data (actual gel areas) indicated that concentrations of 10 and 100 μmol/L of anti-sense oligodeoxynucleotide to CTGF significantly inhibited matrix contraction compared to gels treated with TGF-β_1 only. Although the scrambled sequence control seemed to have some inhibitory effect, it was not found to be statistically significant. The error bars represent the SEM. The **asterisks** indicate statistically significant differences ($P < 0.05$).

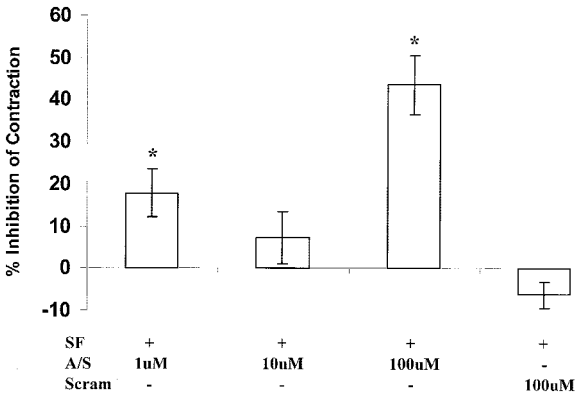

Figure 3. The addition of anti-sense oligodeoxynucleotides to CTGF significantly inhibited basal levels of fibroblast-mediated collagen contraction under serum-free conditions after 2 days of culture. The data are plotted as the percentage inhibition of contraction relative to the degree of contraction stimulated under serum-free conditions alone. Statistical analysis of the raw data (actual gel areas) indicated that concentrations of 1 and 100 μmol/L of anti-sense oligodeoxynucleotide to CTGF significantly inhibited matrix contraction compared to serum-free cultured gels. The scrambled sequence control had no significant effect. The error bars represent the SEM. The **asterisks** indicate statistically significant differences ($P < 0.05$).

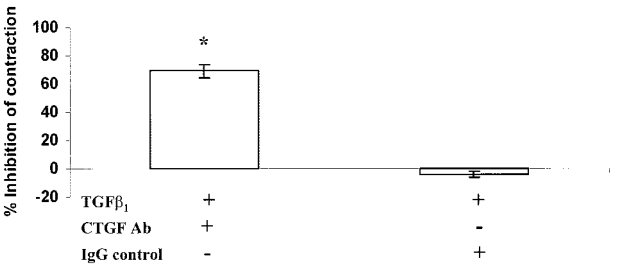

Figure 4. The addition of CTGF-neutralizing antibody significantly inhibited fibroblast-mediated collagen contraction in the presence of 25 ng/ml of TGF-β_1 after 7 days of culture. Again the data are plotted as the percentage inhibition of contraction relative to the degree of contraction stimulated by TGF-β_1 only. The IgG control had no significant effect on contraction. The error bars represent the SEM. The **asterisk** indicates a statistically significant difference ($P < 0.05$).

reduced gel contraction to a level less than that of serum-free medium, suggesting that there is a basal level of expression of CTGF by fibroblasts in the absence of exogenously added TGF-β_1. Addition of a scrambled 20 mer did not significantly reduce gel contraction stimulated by TGF-β_1. These results suggest that CTGF mediates gel contraction stimulated by TGF-β_1 in fibroblast-populated collagen matrix, and also suggests that there is a low, basal level of CTGF synthesis by fibroblasts even in the absence of exogenously added TGF-β_1.

Inhibition of CTGF Gene Transcription Reduces Basal Levels of Contraction

Under serum-free conditions, anti-sense oligodeoxynucleotides to CTGF also significantly inhibited fibroblast-mediated matrix contraction by day 3 ($P < 0.05$; Figure 3). However, unlike the complete inhibition of contraction seen with the highest dose of anti-sense oligodeoxynucleotides to CTGF in cells primed with TGF-β_1, less than half (44%) of contraction was inhibited under serum-free conditions.

Neutralization of CTGF Protein TGF-β_1-Stimulated Matrix Contraction

Addition of affinity-purified IgG antibodies that neutralize CTGF also significantly reduced TGF-β_1-stimulated matrix contraction ($P < 0.001$; Figure 4), again supporting the premise that CTGF mediates promotion of matrix contraction by fibroblasts in response to TGF-β_1.

Anti-Sense Oligodeoxynucleotide-Inhibited CTGF mRNA Expression in bEND.3 Cells

The anti-sense oligodeoxynucleotides were tested for the ability to reduce CTGF mRNA levels using the bEND cell culture-screening assay (Figure 5). The oligodeoxynucleotide with the sequence, GCC-AGA-AAG-CTC-AAA-CTT-GA, corresponding to nucleotides 987 to 1007 of the human CTGF mRNA, reduced the level of mouse CTGF mRNA in bEND cells by 86%, and was selected as the CTGF anti-sense oligodeoxynucleotide.

Anti-Sense Oligodeoxynucleotide Inhibited CTGF Protein Release in Human Fibroblasts

To assess if anti-sense oligodeoxynucleotide reduced CTGF gene transcription in human corneal fibroblasts, levels of CTGF protein in conditioned media was measured by ELISA. Increasing concentrations of the CTGF anti-sense 20 mer (1, 10, and 100 μmol/L) progressively decreased CTFG protein levels in conditioned media samples collected 24 hours (Figure 6, open boxes) or 48 hours (Figure 6, shaded boxes) after addition of TGF-β_1 and the 20 mer. Addition of the scrambled sequence oligodeoxynucleotide (100 μmol/L) did not reduce the level of CTGF protein induced by TGF-β_1 at 24 hours (threefold increase) and 48 hours (fivefold increase), indicating the effect of the anti-sense oligodeoxynucleotide was not because of nonspecific inhibition of protein synthesis.

Gelatinases Are Produced during Fibroblast-Mediated Matrix Contraction

Members of the MMP family are up-regulated during a number of tissue-remodeling events such as embryogenesis and development, metastasis, and wound healing.[31,45,46] To determine whether MMPs were produced during *in vitro* contraction of collagen by fibroblasts, conditioned medium collected from contracting gels was evaluated by gelatin zymography (Figure 7). Bands of proteolytic activity corresponded to the molecular weights of pro-MMP-9 (92 kd) and pro- and active MMP-2 (72 and 66 kd) were detected in all samples of conditioned media from gels that were incubated in serum-free medium (Figure 7A, lane 1) or medium supplemented with increasing concentrations of TGF-β_1 (Figure 7A, lanes 2 to 5) or CTGF (Figure 7A, lanes 6 to 9). However, the levels of pro- or active MMP-2 in conditioned media

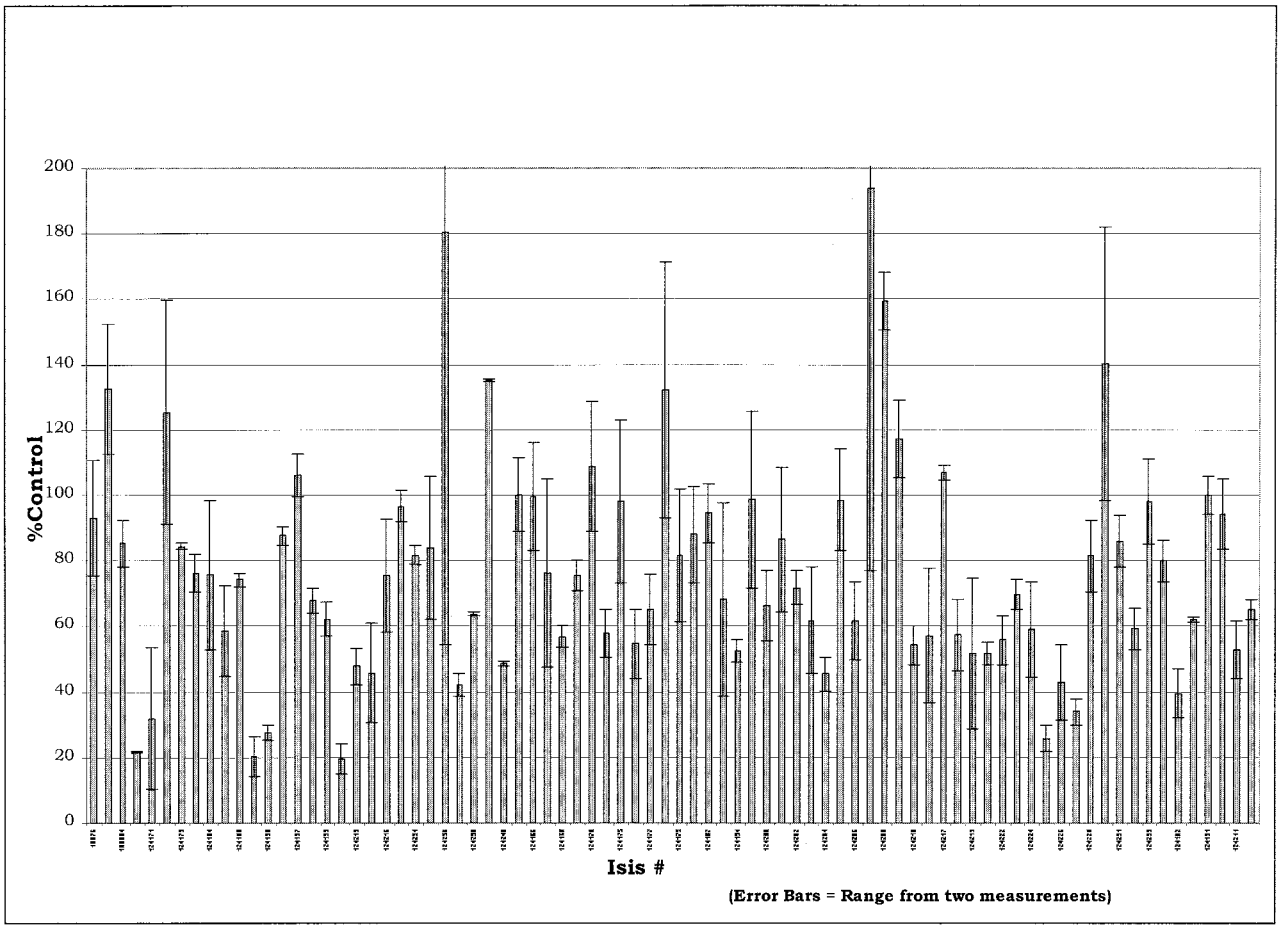

Figure 5. Anti-sense oligodeoxynucleotides (MOE gapmers at 150 nmol/L) were tested for the ability to reduce CTGF mRNA levels using the bEND cell culture-screening assay. The oligodeoxynucleotide, with the sequence GCC-AGA-AAG-CTC-AAA-CTT-GA, corresponds to nucleotides 987 to 1007 of the human CTGF mRNA, reduced the level of mouse CTGF mRNA in bEND cells by 86%, and was selected as the CTGF anti-sense oligodeoxynucleotide. This is represented by the 12th bar from the **left** (the oligo with the lowest percent control value). The error bars represent the range from two experiments.

did not change substantially, even though gel contraction increased with increasing concentrations of TGF-β_1 (Figure 7B) or CTGF (Figure 7C). The only exception was a decrease in active MMP-2 at the highest concentration of TGF-β_1. The faint band corresponding to the molecular

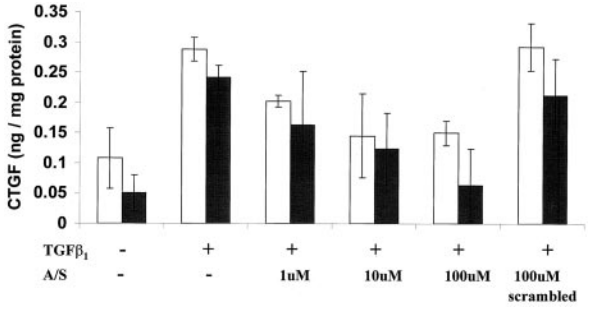

Figure 6. CTGF anti-sense oligodeoxynucleotide reduced CTGF protein produced by fibroblasts in the presence of TGF-β_1. To determine the specificity of CTGF gene down-regulation, conditioned medium collected from contracting gels fed with 25 ng/ml of TGF-β_1 or TGF-β_1 plus concentrations of anti-sense oligodeoxynucleotide to CTGF or scrambled sequence control were assayed by ELISA at 24 and 48 hours to determine the levels of CTGF protein. At 24 hours all concentrations of the CTGF anti-sense oligodeoxynucleotide reduced CTGF protein in the presence of TGF-β_1 (**open boxes**). By 48 hours the 1 μmol/L concentration was no longer potent (**shaded boxes**). The scrambled control sequence had no effect. The error bars represent SEM.

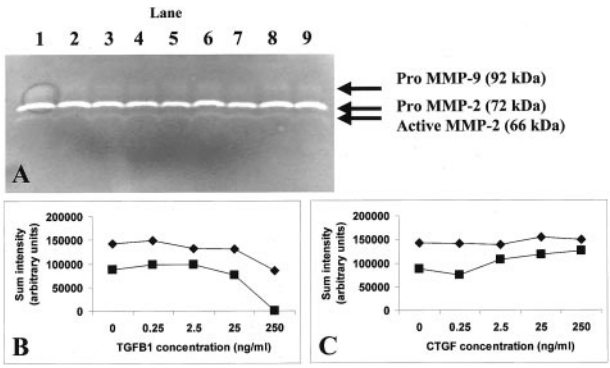

Figure 7. MMPs were produced during fibroblast-mediated collagen matrix contraction. Conditioned media collected from gels contracting in the presence of concentrations of TGF-β_1 or CTGF for 3 days were analyzed by zymography for the presence of MMPs (**A**). The **lanes** represent MMP activity in medium collected from gels treated with: serum-free medium (**lane 1**), TGF-β_1 at 0.25 ng/ml (**lane 2**), 2.5 ng/ml (**lane 3**), 25 ng/ml (**lane 4**), and 250 ng/ml (**lane 5**) and CTGF at 0.25 ng/ml (**lane 6**), 2.5 ng/ml (**lane 7**), 25 ng/ml (**lane 8**), and 250 ng/ml (**lane 9**). The **top faint band** corresponds to the molecular weight of pro-MMP-9, the **middle band** to pro-MMP-2, and the **bottom band** to active MMP-2. No stimulation above basal levels (**lane 1**) with either growth factor was detected. After semiquantitative image analysis of the bands, TGF-β_1 appeared to have an inhibitory effect on active MMP-2 activity (**B**) while CTGF had little effect (**C**).

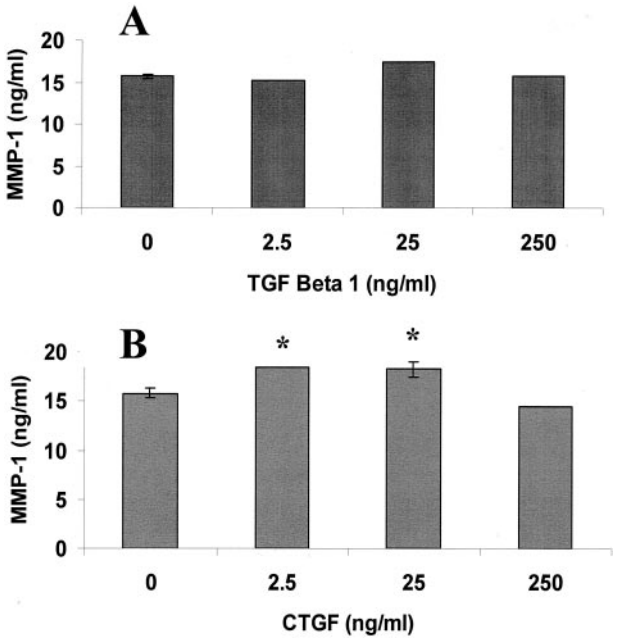

Figure 8. Using an ELISA measuring total MMP-1 protein in conditioned medium collected on day 3, it was found that TGF-β_1 had no significant effect on MMP-1 protein production during collagen gel contraction (**A**). CTGF induced a modest, yet statistically significant increase in MMP-1 protein ($P < 0.05$) at the concentrations indicated by the **asterisks** (**B**). The error bars represent SEM.

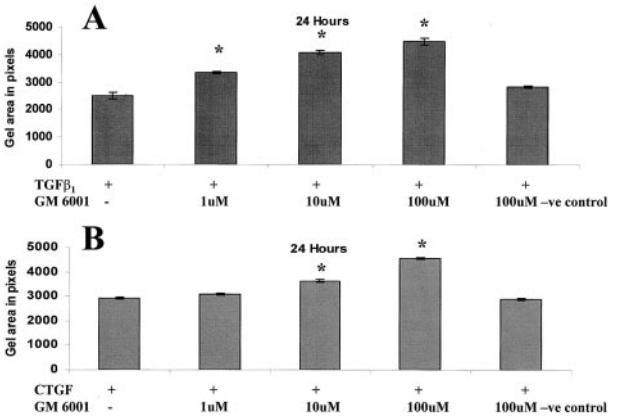

Figure 9. Addition of the broad-spectrum MMP inhibitor, GM 6001, significantly ($P < 0.05$) reduced collagen contraction in a dose-dependent manner in gels cultured with 25 ng/ml of TGF-β_1 (**A**) or 25 ng/ml of CTGF (**B**). The negative control analog of GM 6001 had no effect. The **asterisks** indicate significant differences in gel areas between growth factor-treated and growth factor plus MMP inhibitor-treated groups. The error bars represent the SEM.

weight of pro-MMP-9 was not strong enough to accurately measure using the image analysis software.

MMP-1 Is Produced during Fibroblast-Mediated Matrix Contraction

MMP-1 (collagenase) is capable of cleaving the triple helix structure of type I collagen[47] making it thermally unstable and susceptible to further proteolysis by gelatinases. Because MMP-1 may facilitate fibroblast-mediated matrix contraction by cleavage of collagen, the total amount of MMP-1 released into conditioned media during 48 hours of contraction was measured by ELISA. As shown in Figure 8A, addition of TGF-β_1 (2.5, 25, and 250 ng/ml) did not alter levels of MMP-1 compared to the average level present in serum-free conditioned medium. In contrast, CTGF at 2.5 and 25 ng/ml induced a modest, yet statistically significant increase ($P < 0.05$), in MMP-1 levels.

Broad Spectrum MMP Inhibition Reduces TGF-β_1 and CTGF-Stimulated Matrix Contraction by Fibroblasts

To determine whether MMPs were required for CTGF and/or TGF-β_1-stimulated matrix contraction by fibroblasts, the broad-spectrum MMP inhibitor GM 6001 was added to the culture medium. MMP inhibition was found to significantly reduce both TGF-β_1 and CTGF stimulated matrix contraction in a dose-dependent manner ($P < 0.001$; Figure 9). Addition of the inactive structural analogue of GM 6001 did not reduce contraction, and evaluation of metabolic activity using the WST-1 indicator of cell viability, showed that GM 6001 was not toxic to fibroblasts.

Discussion

A series of highly orchestrated events lead to the deposition, remodeling, and contraction of matrix that occurs during the normal process of tissue repair after injury. Previous reports demonstrate that TGF-β plays a significant role in this process.[16] However, excessive matrix deposition and contraction induced by elevated levels of TGF-β can lead to pathological scarring.[48–53] As a result the TGF-β family has become a target for modulating the wound-healing response. This strategy has been shown to work clinically,[54] however, the control of scarring is still incomplete. Understanding the control mechanisms involved in TGF-β-mediated events should facilitate the development of new therapeutic strategies to control aberrant wound healing such as scarring.

Like TGF-β, CTGF acts as a fibroblast chemoattractant and mitogen and also stimulates production of extracellular matrix components.[11,14,43] The potential role of CTGF in fibroblast-mediated matrix contraction is currently unknown. Our data demonstrate CTGF, like TGF-β_1, stimulates collagen matrix contraction in a dose-dependent manner in the absence of other exogenous growth factors. This indicates a potential new regulatory role for CTGF in the process of wound healing. After injury, resting fibroblasts become activated and start to migrate into the wound, generating tractional forces leading to matrix contraction. Fibroblasts become orientated parallel to the wound bed and along expected lines of stress.[26,27,55] The cells acquire focal adhesions, stress fibers, and extracellular fibronectin fibrils[55] and acquire a proto-myofibroblast phenotype.[27] The model used in our experiments is thought to mimic this process *in vitro*.[56] These new data expands the known activities of CTGF by

demonstrating that CTGF is sufficient to induce fibroblast-mediated collagen matrix contraction.

The CTGF gene is transcriptionally activated by TGF-β,[10] and is known to mediate a variety of TGF-β-stimulated fibroblast activities.[15,43,44] However, no studies have previously looked at CTGF in relation to TGF-β-mediated wound contraction. This study shows for the first time that the potent stimulatory effects of TGF-β on fibroblast matrix contraction can be regulated by another growth factor, ie, CTGF. Our anti-sense oligodeoxynucleotide study clearly demonstrated that contraction of relaxed gels by TGF-β_1-stimulation could be negated by inhibiting CTGF gene transcription and that this occurred without toxicity to the cells. Inhibition of CTGF protein activity using a neutralizing antibody corroborated these findings.

TGF-β_1 stimulates the differentiation of fibroblasts (or protomyofibroblasts) into myofibroblasts that are characterized by *de novo* expression of α-smooth muscle actin, increased expression of ED-A fibronectin, increased expression of stress fibers, and increasingly complex formation of focal adhesions.[27] However, for TGF-β_1 to achieve the transition from fibroblast to myofibroblast via the Smad signaling pathway,[57] mechanical tension must also be applied to the cells.[55,58] Our contraction model confirmed these findings because very few cells with assembled α-smooth muscle actin stress fibers, ie, myofibroblasts were observed (unpublished data). This indicates a role for TGF-β_1 in the generation of tractional forces by fibroblasts in a collagen matrix under the control of CTGF. In fact, recent data have shown that CTGF activates TGF-β signals by direct binding in the extracellular space.[59] Perhaps this mechanism is involved in complex process of TGF-β-mediated matrix contraction. Our data has very important implications for TGF-β biology as it demonstrates a new and alternative control mechanism for this procontractile influence of TGF-β on fibroblasts. Interestingly, in the absence of any exogenous stimulation by growth factors, inhibition of CTGF gene expression was not as potent. This suggests that although CTGF still seems to be involved it is likely that alternative signaling pathways are being used by the cells in the absence of exogenous or in the presence of autocrine levels of TGF-β_1. This data further supports the hypothesis that CTGF is an important mediator of TGF-β_1-stimulated collagen matrix contraction by fibroblasts.

The requirement of MMP activity for the penetration and movement of a number of cell types through ECM has been identified.[30,60–62] These reports suggest that movement of cells through ECM and subsequent matrix contraction (processes involved in scarring) may involve MMPs. We recently demonstrated that broad-spectrum MMP inhibition reduced matrix deposition, contraction, and scarring in a rabbit model of subconjunctival wound healing.[34] Furthermore, we recently reported that fibroblast-mediated matrix contraction induced by cellular tractional forces is dependent on MMP activity.[29] In those *in vitro* experiments the fibroblasts were responding to serum. In the current study we investigated the potential involvement of MMPs in TGF-β_1 and CTGF-mediated collagen matrix contraction. During contraction TGF-β_1 did not up-regulate the levels of the gelatinases MMP-2 and MMP-9 or the collagenase MMP-1. Yet basal levels of MMPs were required for contraction because the presence of TGF-β_1 was not sufficient to stimulate contraction in the face of broad-spectrum MMP inhibition. In contrast CTGF induced a modest increase in MMP-1 protein production during contraction, which was reduced by MMP inhibition. Although the increase in MMP-1 by CTGF was found to be statistically significant, the potential biological significance of this finding is yet to be established. CTGF has recently been shown to increase MMPs -2, -3, -9, and -14 in vascular endothelial cells possibly for initiation of angiogenesis during early hypoxia.[63] The interactions between these growth factors and MMPs during fibroblast-mediated matrix contraction are clearly complex and the subject of further investigation. These data do however indicate a novel feature of both TGF-β_1 and CTGF function in the utilization of MMPs for fibroblast-mediated matrix contraction.

Using gene array technology, CTGF gene expression was found to be stress-responsive.[64] A fourfold to sixfold increase was found in the mRNA levels for CTGF from fibroblasts cultured in tethered collagen gels, which induce mechanical stress on the cells, compared to fibroblasts cultured in free-floating relaxed gels as in our experiments. The authors also suggested that TGF-β alone is not sufficient to induce full induction of CTGF expression as the application of neutralizing antibodies to TGF-β could not negate CTGF expression induced by mechanical stress.[64] Excessive contraction of extracellular matrix by myofibroblasts is thought to play a key role in fibrotic pathologies. It has previously been shown that CTGF did not mediate TGF-β induction of the myofibroblast phenotype when fibroblasts were cultured in the presence of the growth factor on plastic.[65] However, the cells were not studied within a matrix under mechanical stress. To further dissect the mechanisms by which CTGF may contribute to matrix contraction, which is thought to occur via a combination of fibroblast migration into a wound and myofibroblast contraction within the wound,[18] the ability of CTGF to induce the myofibroblast phenotype under mechanical stress is currently being investigated in our laboratory.

Our present data has highlighted several novel functions of CTGF; stimulation of fibroblast-mediated collagen matrix contraction, utilization of MMPs for this contraction and the regulation of TGF-β_1-mediated matrix contraction. These findings help to further our understanding of CTGF and its important interactions with TGF-β during events crucial to wound healing. The data are also important clinically as they suggest CTGF may be a suitable therapeutic target for the control of contractile scarring.

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