

## CHAPTER 2

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# Scleroderma Lung Fibroblasts: Contractility and Connective Tissue Growth Factor

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and Richard M. Silver**

### Introduction

In the pathogenesis of pulmonary fibrosis in general and systemic sclerosis (SSc, scleroderma) in particular, lung fibroblasts undergo specific phenotypic modulation and develop cytoskeletal features similar to those of smooth muscle cells. These phenotypically altered, activated fibroblasts, or myofibroblasts, express a contractile isoform of actin ( $\alpha$ -smooth muscle actin) and promote contractility of lung parenchyma. Constitutively activated SSc fibroblasts produce an over abundance of collagen, fibronectin, and other extracellular matrix (ECM) proteins. They also overexpress several profibrotic receptors, including receptors for the dominant fibrogenic cytokine TGF- $\beta$ . Recently, we found that the main receptor for thrombin on lung fibroblasts, Protease-Activated Receptor (PAR)-1, is also abundantly expressed in scleroderma lung tissue and, furthermore, its expression is observed in association with myofibroblasts. Moreover, thrombin itself is capable of differentiating normal lung fibroblasts to a myofibroblast phenotype. Such differentiation occurs via protein kinase C and a RhoA-dependent pathway.

Additionally, thrombin induces several potent fibrogenic cytokines. CTGF, whose expression is constitutively upregulated in scleroderma fibroblasts and correlates well with the severity of lung fibrosis, is one such potent fibrogenic cytokine induced by thrombin. This study was undertaken to establish the link between the expression of CTGF and the contractile activity observed in scleroderma lung fibroblasts.

### Scleroderma Lung Fibrosis and Myofibroblasts

SSc is an autoimmune rheumatic disease that affects about 250,000 Americans, primarily females who are 30 to 50 years of age at disease onset. Currently, the leading cause of death in scleroderma patients is pulmonary dysfunction as a result of progressive interstitial lung fibrosis.<sup>1</sup> The conceptual process of lung fibrosis involves the presence of tissue injury, the release of fibrogenic factors, and the induction of myofibroblasts culminating in enhanced ECM deposition.<sup>2-4</sup> Myofibroblasts appear to be the principal mesenchymal cells responsible for tissue remodeling, collagen deposition, and the restrictive nature of the lung parenchyma associated with pulmonary fibrosis.<sup>5-8</sup>

We have demonstrated that myofibroblasts are present in the bronchoalveolar lavage fluid (BALF) of SSc patients and that myofibroblasts cultured from SSc BALF express more

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collagen I, III, and fibronectin than normal lung fibroblasts.<sup>9</sup> They also show a greater proliferative response upon exposure to TGF- $\beta$  and platelet-derived growth factor (PDGF) when compared to normal lung fibroblasts.<sup>9,10</sup> Recently we reported increased numbers of myofibroblasts in lung tissue from SSc where lesions with high ECM accumulation are present.<sup>11</sup> Several groups of investigators have demonstrated a correlation between fibrosis and  $\alpha$ -smooth muscle actin expressing myofibroblasts in a number of different tissues.<sup>5,8,12,13</sup> Myofibroblasts isolated from various fibrotic tissues, including lungs, are thought to be the primary source of collagen and other ECM proteins.<sup>5,9,11,12</sup> Studies in animals employing the bleomycin-induced model of pulmonary fibrosis have identified myofibroblasts to be the primary source of increased collagen expression and a major source of cytokines and chemokines as well.<sup>14,15</sup>

### TGF- $\beta$ , Thrombin and CTGF in SSc Lung Fibrosis

The presence of myofibroblasts has been extensively documented in active fibrotic lesions in many diseases, including SSc lung disease.<sup>6,8,9</sup> However, the precise sources of such myofibroblasts are still not well known. Relative contributions from circulating mesenchymal stem cells or from local trans-differentiation of epithelial cells to fibroblasts, demonstrated in other organ systems,<sup>16,17</sup> have not been observed in the lung. It seems as if lung fibroblasts may differentiate to a myofibroblast phenotype under the influence of local growth factors and cytokines.<sup>18</sup> One such growth factor is TGF- $\beta$ 1, the most potent profibrotic cytokine and a powerful stimulator of the production of extracellular matrix components, including collagen, fibronectin, fibrillin and proteoglycans.<sup>19</sup>

We recently reported that thrombin also mediates differentiation of lung fibroblasts to a myofibroblast phenotype, apparently at an even earlier stage than TGF- $\beta$ 1.<sup>11,20,21</sup> We demonstrated that the expression of the main receptor for thrombin in lung fibroblasts, PAR-1, is dramatically increased in lung tissue from scleroderma patients, mainly in lung tissue containing inflammatory and fibroproliferative foci.<sup>11</sup> PAR-1 expression decreases in the later stages of pulmonary fibrosis, suggesting that its role takes place early in the development of lung fibrosis. PAR-1, which is responsible for most cellular events induced by thrombin, colocalizes with myofibroblasts in scleroderma lung tissue, lending additional support for thrombin and PAR-1 in the process of lung fibroblast activation.<sup>11</sup>

Another protein essential for matrix synthesis and remodeling in scleroderma is CTGF. This growth factor is constitutively overexpressed by fibroblasts in fibrotic skin lesions as well as in lung fibroblasts from patients with systemic sclerosis.<sup>22-24</sup> The majority of the CTGF generated by scleroderma fibroblasts is associated with the cell layer, suggesting that CTGF acts in an autocrine fashion.<sup>22,24</sup> CTGF regulates deposition of ECM components in fibroblasts and is responsible for the persistent fibrotic reaction. Increased amounts of CTGF are found in scleroderma patients with more extensive skin involvement and a greater severity of fibrosis.<sup>23</sup> In addition to increased tissue expression, CTGF levels are increased in the serum of patients with scleroderma.<sup>23</sup> Higher levels of CTGF are seen in the diffuse form of scleroderma than in the limited form.<sup>23</sup> BALF from systemic sclerosis patients with active lung fibrosis contains much higher levels of CTGF compared to patients without fibrotic lung pathology.<sup>23</sup> We found that thrombin at physiological levels promotes overexpression of CTGF in normal lung fibroblasts approximately 8-fold, compared to only 3-fold induction by TGF- $\beta$  (Fig. 1). Similar results were reported from Laurent's laboratory.<sup>25</sup> Lung fibroblasts derived from scleroderma patients inherently contain approximately 5.5-fold protein level of CTGF compared to normal lung fibroblasts (Fig. 1).

Since thrombin and TGF- $\beta$  each induce CTGF production in fibroblasts, it has been suggested that CTGF may be a common downstream mediator for pathways associated with activation of both thrombin and TGF- $\beta$ .<sup>26</sup>

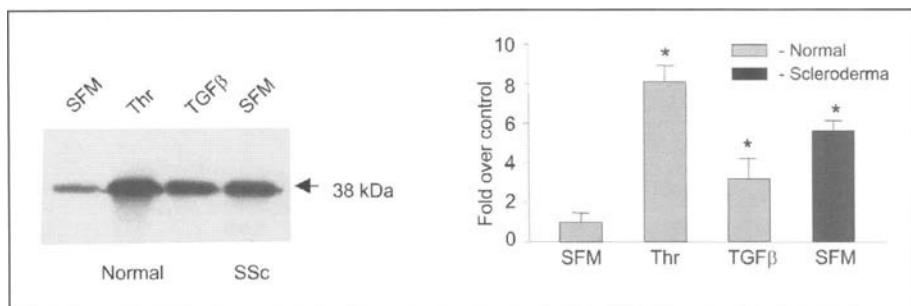


Figure 1. CTGF protein level in normal and SSc lung fibroblasts treated with thrombin or TGF- $\beta$ . Confluent lung fibroblasts were cultured in serum-free conditions for 24 hours followed by incubation for 24 additional hours in serum-free medium (SFM), thrombin (1 U/ml), or TGF- $\beta_1$  (5 ng/ml). The cells were harvested in PBS and solubilized in heparin-sepharose binding buffer containing 100 mM Tris, pH 7.4; 10 mM trisodium citrate; 150 mM NaCl; 1% Triton. CTGF protein was selected with heparin Sepharose (Amersham Biosciences), subjected to 10% polyacrylamide gel, and analyzed by Western blot with anti-CTGF antibody (R and D Systems, Inc). CTGF bands were quantified by densitometry using NIH Image software. The experiment was performed three times and mean values  $\pm$  SD is presented. The asterisk represents statistically significant differences ( $p < 0.05$ ) between normal lung fibroblasts stimulated with thrombin or TGF- $\beta$  versus nonstimulated cells and between SSc lung fibroblasts versus normal lung fibroblasts.

### Contractile Activity of CTGF

Contractility, a predominant feature of myofibroblasts, is equally promoted by either thrombin or TGF- $\beta$ .<sup>19-21</sup> Contractile forces of the myofibroblast are generated by  $\alpha$ -smooth muscle actin, which is extensively expressed in stress fibers and by large fibronexus adhesion complexes connecting intracellular actin with extracellular fibronectin fibrils.<sup>27</sup> Fibroblasts cultured in collagen matrices have been used to develop in vitro models of fibrocontractile diseases.<sup>28</sup> Studies in our laboratory have focused on stressed matrix contractions as an equivalent of myofibroblast contraction in vivo.<sup>20,21</sup> We observed that SSc lung fibroblasts contain an abundant amount of  $\alpha$ -smooth muscle actin and, accordingly, are capable of contracting collagen gels without exogenous treatment.<sup>20</sup> However, distinct SSc cell lines contract collagen gels to different degrees (Fig. 2A). To investigate the correlation between contractile activity and expression of CTGF by SSc lung fibroblasts, we measured the amount of CTGF using anti-CTGF antibody in collagen gels undergoing contraction by scleroderma lung fibroblasts. Collagen gels were digested with collagenase, and lung fibroblasts released from collagen were resolved on SDS-PAGE. CTGF bands were visualized by Western blot with anti-CTGF antibody and quantified by densitometry using NIH Image software. The association of the CTGF expression with ability of SSc lung fibroblasts to contract collagen gel was tested using Spearman rank correlation test. We found that cell lines containing more CTGF demonstrated greater contractile activity when compared to the cells containing less CTGF. For example, SSc cell line 3 having the highest CTGF level ( $895 \pm 62$  densitometric value) contracted collagen gels from ~15 mm in diameter to less than 6 mm in diameter (~9 mm contraction). In contrast, another SSc cell line expressing lower CTGF levels ( $412 \pm 97$  densitometric value) produced contraction of only about 5 mm (Fig. 2).

To investigate if CTGF can induce contractile activity by lung fibroblasts, we transfected normal lung fibroblasts with human CTGF in V5/His/pcDNA3.1 and then performed collagen gel contraction assays. We observed that lung fibroblasts transfected with CTGF contracted gels from 15 mm to ~7 mm diameter (~8 mm contraction), whereas nontransfected cells or cells transfected with vector as a control did not contract collagen gels (Fig. 3). To further confirm that CTGF can induce contractile activity by lung fibroblasts, we performed

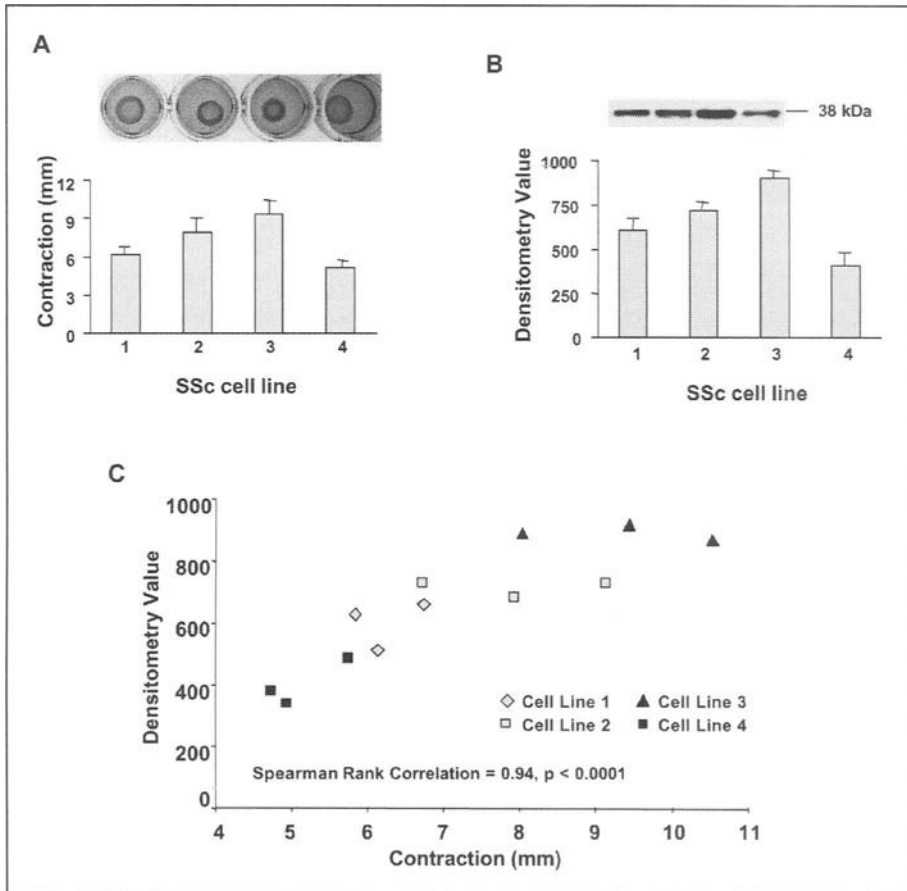


Figure 2. Contractile activity of SSc lung fibroblasts correlates with expression of CTGF. A) Collagen lattices were prepared using type I collagen from rat tail tendon as previously described.<sup>20,21</sup> SSc lung fibroblasts were suspended in collagen ( $2.5 \times 10^5$  cells/ml final concentration) and aliquoted into 24-well plates. Collagen lattices were polymerized for 45 min at 37°C followed by incubation with medium containing 10% fetal calf serum for 4 h, followed by overnight incubation in serum-free medium. To initiate collagen gel contraction, polymerized gels were gently released from underlying culture dish. The diameters of the gels were measured immediately and 24 h after release of gels. The degree of contraction was determined as difference between initial diameter of the gel and diameter of the gel in 24 h. B) Collagen gels were collected, digested with collagenase, and analyzed by Western blot using anti-CTGF antibody. CTGF bands were quantified by densitometry using NIH Image software. The experiment was performed three times and mean values  $\pm$  SD is presented. C) The correlation between the level of CTGF and ability of distinct SSc cell lines to contract collagen gel was analyzed by using the Spearman rank correlation.

collagen gel contraction assays utilizing normal lung fibroblasts treated with recombinant CTGF. Human CTGF in V5/His/pcDNA3.1 vector was purified from transfected He-La cells using ProBond nickel-chelating resin and heparin sepharose. We observed that CTGF induced collagen gel contraction in a dose-dependent manner with maximum effects at 100 ng/ml and 1  $\mu$ g/ml (Fig. 4A). Collagen gels contracted from 15 mm in diameter to ~9 mm (~6 mm contraction) within 24 hours of CTGF stimulation.

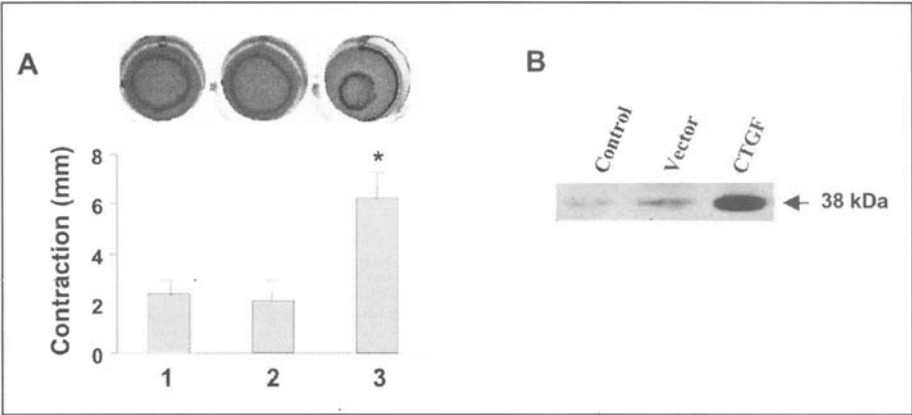


Figure 3. Overexpression of CTGF in normal lung fibroblasts induces collagen gel contraction. A) Normal lung fibroblasts were transiently transfected with CTGF DNA and cultured in collagen gel on 24-well plates. Polymerized gels were released from the underlying culture dish 24 h after transfection and incubated in serum-free medium for another 24 h. Column 1 represents nontransfected cells as a control, 2—cells transfected with vector, 3—cells transfected with CTGF. The experiment was performed three times and mean values  $\pm$  SD is presented. The asterisk represents statistically significant differences ( $p < 0.05$ ) between cells transfected with CTGF versus nontransfected cells. B) Collagen gels were collected, digested with collagenase, and analyzed by Western blot using anti-CTGF antibody.

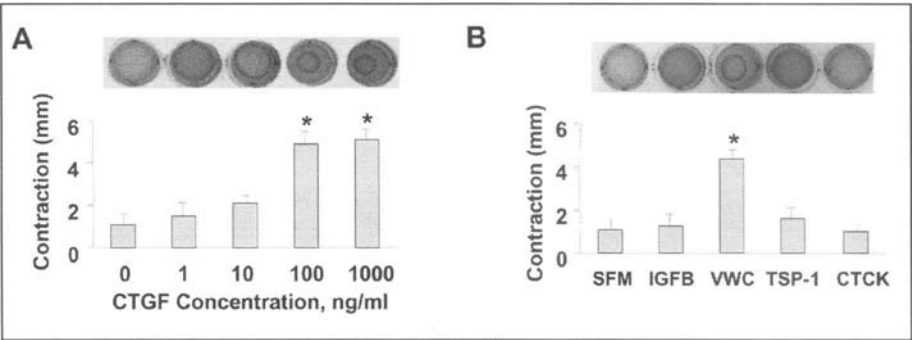


Figure 4. Recombinant CTGF induces collagen gel contraction by lung fibroblasts via VWC domain. Normal lung fibroblasts were cultured in collagen lattices as described earlier. A) CTGF (concentrations from 1 ng/ml to 1  $\mu$ g/ml) was added and collagen gel contraction was measured after 24 h of incubation. B) Normal lung fibroblasts were left in serum-free medium (SFM) or stimulated with 100 ng/ml of IGFB, VWC, TSP-I, and CTCK domains of CTGF for 24 h. Collagen gel contraction was measured as stated above. Data represent mean values  $\pm$  SD of three experiments, each performed in duplicate. The asterisk corresponds to statistically significant differences ( $p < 0.05$ ) between cells stimulated with whole CTGF (A) or CTGF domain (B) versus nonstimulated cells.

Next, we initiated a series of experiments to identify the structural module of CTGF responsible for contractile activity. Protein structural modules (also known as modular protein) are well demarcated and independently folded portions of proteins. Such domains are noncatalytic and bind specifically to short continuous peptide sequences in their binding partner(s) via one or more ligand-binding surfaces.<sup>29</sup> Modular protein domains are autonomous

in the sense that in most cases they may be removed from the original parent protein without compromising their ability to bind their cognate or target peptide ligands. Human CTGF protein contains 349 amino acids organized into four distinct structural modules following the signal peptide: insulin-like growth-factor-binding domain (IGFB) contains 63 amino acids (Gly31-Gly93); von Willebrand factor type C (VWC) domain or chordin-like domain<sup>30</sup> contains 60 amino acids (Cys103-Glu162); thrombospondin type I repeat (TSP1) contains 41 amino acids (Gln202-Cys242); and carboxyl-terminal cysteine knot-like domain (CTCK) contains 70 amino acids (Lys261-Pro330). The CTCK domain of CTGF is present in biological fluid and is sufficient for some biological activity.<sup>31</sup> Recently, Ball et al reported the functional activity of the CTCK domain produced as a maltose binding fusion protein in *E. coli*. After removal of the fusion part, recombinant CTCK promoted dose-dependent adhesion of fibroblasts, myofibroblasts, endothelial cells and epithelial cells.<sup>32</sup>

To determine whether any CTGF domains can autonomously induce contractile activity, they were cloned as glutathione S-transferase (GST)-fused proteins into pGEX2T vector and purified using Glutathione Sepharose. For collagen gel contraction assay by lung fibroblasts we used IGFB, VWC, TSP1, and CTCK domains of CTGF. We found that the VWC domain of CTGF in a concentration of 100 ng/ml demonstrated profound contractile activity, whereas IGFB, TSP1 and CTCK had no effect on collagen gel contraction (Fig. 4B). Collagen gels contracted from -15 mm in diameter to -10 mm (-5 mm contraction) within 24 hours after VWC stimulation, but remained unchanged after stimulation with IGFB, TSP1, or CTCK domains of CTGF.

## Conclusions

The role of myofibroblasts in various fibrotic disorders is currently well established. These smooth-muscle-like fibroblasts promote deposition of ECM proteins and contractility of lung parenchyma. The present studies were performed to characterize the contractile activity of SSc lung fibroblasts. Previously, we demonstrated that the early stages of interstitial lung disease of SSc are characterized by a prominence of cells possessing a myofibroblast phenotype. A major feature of such myofibroblasts is contractility, explained by an over-expression of  $\alpha$ -smooth muscle actin. Here, we demonstrate for the first time that the contractility of SSc lung fibroblasts depends on expression of CTGF as well, and that the VWC domain is primarily responsible for the contractile activity of CTGF in human lung fibroblasts. Future studies are required to identify the mechanisms by which CTGF stimulates collagen gel contraction.

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