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Connective Tissue Growth Factor Expression in Human Bronchial Epithelial Cells

Amrita DOSANJH*

Department of Molecular and Experimental Medicine, the Scripps Research Institute, La Jolla 92122, USA

Abstract Connective tissue growth factor (CTGF) is a cysteine-rich protein that promotes extracellular matrix deposition. CTGF is selectively induced by transforming growth factor β and des-Arg kallidin in lung fibroblasts and increases steady-state mRNA levels of α type I collagen, 5α-integrin and fibronectin in fibroblasts. Bronchial epithelial cells have been proposed to functionally interact with lung fibroblasts. We therefore investigated if bronchial epithelial cells are able to synthesize CTGF. Human bronchial epithelial cells were grown to subconfluence in standard growth media. Proliferating cells grown in small airway growth media were harvested following starvation for up to 24 h. Expression of *CTGF* transcripts was measured by PCR. Immunocytochemistry was also completed using a commercially available antibody. The cells expressed readily detectable *CTGF* transcripts. Starvation of these cells resulted in a quantitative decline of *CTGF* transcripts. Direct sequencing of the PCR product identified human CTGF. Immunocytochemistry confirmed intracellular CTGF in the cells and none in negative control cells. We conclude that bronchial epithelial cells could be a novel source of CTGF. Bronchial epithelial cell-derived CTGF could thus directly influence the deposition of collagen in certain fibrotic lung diseases.

Key words bronchial epithelial cell; connective tissue growth factor; collagen deposition; remodeling

A wide variety of growth factors could be involved in the remodeling of airway tissue in diseases such as asthma. Collagen deposition and lung remodeling are now recognized features of a subset of asthmatic patients [1]. Connective tissue growth factor (CTGF) is an important regulator of collagen deposition that has not been extensively studied in the lung. CTGF was first isolated from human umbilical artery endothelial cells, and is a member of the *CCN* gene family, which contains insulin growth factor binding domains. CTGF is a cysteine-rich protein, with a molecular mass of approximately 38 kDa, that is transcriptionally regulated by transforming growth factor β (TGF-β) and other factors, such as des-Arg kallidin. CTGF promotes fibroblast proliferation, migration, adhesion and extracellular matrix (ECM) formation, and its overproduction might play a major role in pathways that lead to fibrosis [2,3].

In some asthmatics, the deposition of ECM and remodeling of the airways is observed, which leads to worsening clinical symptoms despite treatment. Epithelial activation and regulation of fibroblasts are proposed to be important in asthmatic remodeling. To better understand the process of remodeling in lung disease, it is important to identify the bronchial epithelial cell-derived factors that regulate the deposition of collagen and ECM in the lung. CTGF has been well studied in connective tissue and fibroblasts [4,5]. Although some insulin-like growth factors (IGF) have been identified in epithelial cells, the expression of CTGF, in particular, has not been described in bronchial epithelial tissue or cells.

We hypothesize that bronchial epithelial cells might produce CTGF, and thus can directly regulate the deposition of collagen and ECM by lung fibroblasts, which are in close proximity to bronchial epithelial cells.

Materials and Methods

Cell culture and RNA isolation

Human bronchial epithelial cells (HBEC) from two sepa-

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*Corresponding author: E-mail, dosamr@scripps.edu

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rate donors (Clonetics, San Diego, USA) were incubated and grown in small airway growth media (SAGM; Clonetics) in room air with 5% CO_2 at 37 ºC under sterile conditions. HBEC were grown to approximately 75% subconfluence in standard supplemented growth media SAGM. The cells were then washed and incubated at 37 ºC with 5% CO_2 in serum free/growth factor free basal medium for varying time periods. At the indicated time points, the cells were harvested, lysed and processed for isolation of RNA or protein, as described below. At confluence, the cells were harvested and total RNA extracted. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on total RNA from harvested cells. Experiments were performed in triplicate, and representative data are shown.

RT-PCR

Total RNA was isolated using RNA Stat60 (Tel-Test, Friendswood, USA) according to the manufacturer's instructions. RNA was quantified fluorimetrically using Sybr Green II (Molecular Probes, Eugene, USA).

The isolated RNA (1 μg) was then reversely transcribed using a 20 μl volume reaction, which consisted of 10 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Grand Island, USA), 4 μl of 5×RT buffer (Gibco BRL), 1 μl of 10 mM deoxyribonucleotide triphosphate (dNTP; Pharmacia, Uppsala, Sweden), 2 μl of 100 mM dithiothreitol, 0.5 μl random primer pd(N)6 (Pharmacia), 0.5 μl RNasin (Promega, Madison, USA), 1 μl DPEC H_2O, 10 μl total RNA. The reaction mixture was incubated for 1 h at 37 ºC. Expression of *CTGF* mRNA transcripts was semi-quantitatively measured by PCR at 59 ºC annealing temperature for 35 cycles, and compared to expression of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene transcripts. The primers used for these experiments were: 5′-AAGGTGTGGCTTTAGGAGCA-3′ (forward) and 5′-TTCACTTGCCAACCGCTGTC-3′ (reverse) (Genset, La Jolla, USA).

The PCR products were then separated on a 2% agarose gel. The isolated and anticipated product size was 300 bp. Sequencing analysis was completed using the ABI PRISM377 DNA sequencer (Applied Biosystems, Foster City, USA).

Quantitative real-time PCR

Total RNA was extracted from the bronchial epithelial cells, as described above, and digested DNA using a DNase-treatment kit (Qiagen, Valencia, USA). Total RNA (250–500 ng) was reversely transcribed using the Superscript RT kit (Qiagen), and one-twentieth of the cDNA was used for real-time quantitative PCR using the iCycler software package (Bio-Rad, Hercules, USA). The primers used were as follows: *CTGF* receptor forward primer 5′-CCCTCGCGGCTTACCG-3′; *CTGF* receptor reverse primer 5′-GGACCAGGCAGTTGGCTCT-3′; *GAPDH* forward primer 5′-GGGAAGGTGAAGGTCGGAGT-3′; *GAPDH* reverse primer 5′-TCCACTTTACCAGAGTTAAAAGCAG-3′. The following dual-labeled probes were obtained from BioSearch Technologies (Novato, USA): GAPD, 5′-6-carboxy-fluorescein (6-FAM)-ACCAGGCGCCCAATACGACCAA-6-carboxy-tetramethyl-rhodamine (6-TAMRA)-3′; CTGF; 5′-FAM-AAGACACGTTTGGCCCAGACCCAACT-black hole quencher 2 (BHQ-2)-3′. Standards, from 10 to 0.0001 amol of the PCR product cloned into pGEMTeasy, were run alongside the samples to generate a standard curve. All samples and standards were analysed in triplicate. The PCR reaction consisted of 1.5 mM Tris-HCl, 5 mM KCl, 2 mM dNTP, 200 ng of sense and antisense primers, either 5 pmol of CTGF dual-labeled probe or 12 pmol of GAPDH dual-labeled probe, 4 mM Mg^{2+} and 1 u of AmpliTaq Gold (Applied Biosystems) in a total volume of 50 μl. The reaction conditions were 95 ºC for 10 min followed by 50 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC. The starting amount of cDNA in each sample was calculated using the iCycler software package.

Antibody to CTGF protein

The commercially available CTGF polyclonal antibody developed by Torrey Pines Biomedical (La Jolla, USA) was used for immunocytochemistry. The primary antibody used was a rabbit antimouse CTGF polyclonal antibody which crossreacts with human CTGF (Torrey Pines Biomedical).

Immunocytochemistry

Bronchial epithelial cells were grown under standard conditions as described above. Cells were rinsed briefly with phosphate-buffered saline (PBS) and fixed in acetone-methanol (3:1) solution for 10 min at room temperature. The primary antibody was diluted 1:100, and a control/normal rabbit serum was also used, in a 1:100 ratio. The cells were incubated in primary antibody for 30 min, and secondary antibody fluorescein-isothiocyanate-goat F (ab) 2 antirabbit IgG (H+L; Caltag, Burlingame, USA) (1:100 dilution) for 30 min. The cells were washed between incubations with PBS. The slides were then viewed and photographed at final magnifications of 200× and 400×, using an Olympus BH2 fluorescence microscope [6].

Results

RT-PCR and quantitative detection of human *CTGF* mRNA in HBEC

The results of RT-PCR indicated cells expressed readily detectable CTGF transcripts. Direct sequencing of the PCR product confirmed that the generated product was human *CTGF*. Quantitative real-time PCR results indicated that, after extended starvation, bronchial epithelial cells showed a steady decline in *CTGF* transcripts compared to the non-starved state, adjusted for *GAPDH*. The quantitative data are expressed as the percentage or fold change in transcripts over time [**Fig. 1(A,B)**].

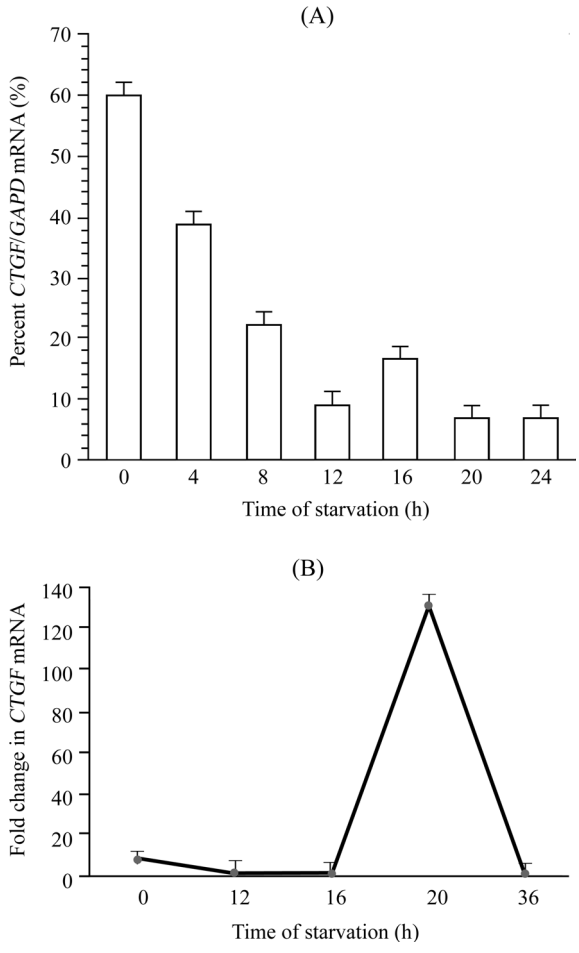

Fig. 1 Results of quantitative polymerase chain reaction analysis and changes in connective tissue growth factor (CTGF) transcript expression in human bronchial epithelial cells

(A) The expression of *CTGF* transcripts declined steadily in cells grown with growth factor deprivation from 0 to 24 h. (B) There was a significant change from 16 to 24 h.

Immunocytochemistry

The results of immunocytochemistry showed intracellular speckled cytoplasmic and perinuclear Golgi staining of the CTGF protein, as illustrated in **Fig. 2(A)**. **Fig. 2(B)** shows the control cells that were incubated with rabbit serum and secondary antibody only. There was no staining in these cells. These data lend further confirmation that in human airway epithelial cells, CTGF is present and readily detectable by a variety of techniques.

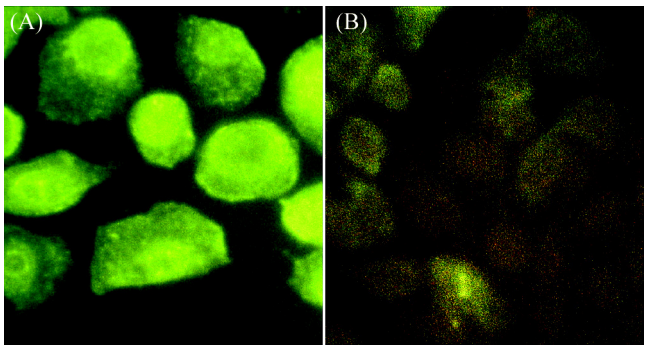

Fig. 2 Immunocytochemistry using rabbit anti-connective tissue growth factor (CTGF) antibody and human bronchial epithelial cells

The pattern of staining is cytoplasmic and Golgi (perinuclear). This confirms the presence of CTGF in these cells. (A) CTGF protein expression in human bronchial cells. (B) Control cells without staining.

Discussion

Our study was conducted to define the expression of CTGF in HBEC. These cells are metabolically active and key regulators of the inflammatory response in the lung. It is now established that in addition to lung fibroblasts, airway epithelial cells are important regulatory cells of the processing of collagen deposition and lung remodeling [7, 8]. Our study demonstrates that, *in vitro*, normal HBEC are capable of producing CTGF, a growth factor that in turn directly regulates the deposition of collagen in the lung. We found that both proliferating non-starved and starved HBEC are able to produce CTGF. Based on our analysis, epithelial cell starvation is followed by downregulation of CTGF transcripts. One possible explanation for this observation is that cells grown under conditions of growth factor deprivation, without adding exogenous growth factors, might in turn produce factors, such as tumor necrosis factor (TNF)-α, that lead to the

downregulation of *CTGF* expression. Another possibility is that the cells preferentially produce factors to maintain cellular proliferation, rather than CTGF, under these conditions.

The bronchial epithelial cells demonstrated both detectable *CTGF* transcripts and intracellular CTGF, under the conditions studied. Our findings have multiple implications for the ability of the bronchial epithelial cells to directly regulate deposition of collagen and ECM, which is important in the remodeling associated with asthma and other chronic lung diseases. Thus, intact undamaged airway epithelial cells might participate in remodeling, as a source of a growth factor known to directly regulate collagen deposition.

CTGF is a recently described cysteine-rich protein that regulates fibroblast proliferation and collagen deposition. CTGF is a member of a family of proteins called IGF binding proteins (IGFBPs), on the basis of its amino end protein sequence homology. CTGF was provisionally named IGFBP-8 and is now designated IGFB-rP in published reports, as it has IGF-independent and unique actions as well. The regulation of CTGF expression appears to be under the control of at least one potent pro-fibrogenic cytokine secreted by a wide variety of lung cells, including epithelial cells. The CTGF promoter contains TGF-β response elements. Prostaglandin E [2] attenuates the effect of TGF-β on CTGF production. TNF-α blocks the TGF-β upregulation induced by glucocorticoids [9,10].

In clinical, *CTGF* expression has been identified in a wide variety of diseases involving organ fibrosis, including sarcoidosis, scleroderma, systemic sclerosis and renal fibrosis [11,12]. There are studies demonstrating that in both pediatric and adult forms of pulmonary fibrosis, CTGF could play an important regulatory role. In asthma, there are many studies indicating that a subset of patients show signs of remodeling or pulmonary fibrosis. In the lung, the interaction between epithelial cells and fibroblasts might be important in the development of lung fibrosis [13]. Based on our findings, bronchial epithelial cells could potentially directly regulate lung fibroblast proliferation and collagen deposition by means of CTGF. Further *in vivo* investigation should be done to study the role of CTGF in diseases.

Based on published studies, CTGF has been thought to lack a role in regulating epithelial cell function, as it lacks TGF-β-like inhibitory effects on mink lung epithelial cells *in vitro*. In addition, the skin epidermis at sites of TGF-β administration *in vivo* does not demonstrate CTGF. Prior studies have demonstrated *CTGF* mRNA expression in the lung, although in lower abundance than the heart [14–16]. One study confirmed the presence of CTGF in alveolar type II epithelial cells from idiopathic lung fibrosis patients [15]. Our study, in contrast, demonstrates the presence of CTGF in HBEC which, unlike type II cells, are thought to be involved in the airway remodeling process.

Studies of epithelium of non-lung organs indicate there is intense CTGF staining of the epithelium within kidney collecting tubules and in olfactory, buccal, pharyngeal, esophageal and corneal organ tissue [17]. Postnatally, high levels of CTGF are also detectable in the kidney epithelium. In one study of Panc-1 epithelial pancreatic tumor cells, TGF-β-induced collagen I and CTGF production are observed [18,19]. In summary, our study demonstrates that lung epithelial cells produce CTGF. CTGF might represent a regulator of other target cells, such as lung fibroblasts and myofibroblasts.

We conclude that bronchial epithelial cells could be a novel source of CTGF, and that growth factors as well as stress might alter its expression. Bronchial epithelial cell-derived CTGF could thus directly influence the deposition of collagen in certain fibrotic lung diseases.

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References

1 Fahy JV, Corry DB, Boushey HA. Airway inflammation and remodeling in asthma. Curr Opin Pulm Med 2000, 6: 15–20
2 Duncan MR, Frazier KS, Abramson S, Williams S, Klapper H, Huang X, Grotendorst GR. Connective tissue growth factor mediates transforming growth factor β-induced collagen synthesis: Down-regulation by cAMP. FASEB J 1999, 13: 1774–1786
3 Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol Biol Cell 1993, 4: 637–645
4 Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ 1996, 7: 469–480
5 Mori T, Kawara S, Shinozaki M, Hayashi N, Kakinuma T, Igarashi A, Takigawa M *et al.* Role and interaction of connective tissue growth factor and transforming growth factor-β in persistent fibrosis: A mouse fibrosis model. J Cell Physiol 1999, 181: 153–159
6 Boes M, Dake BL, Booth BA, Erondu NE, Oh Y, Hwa V, Rosenfeld R *et al.* Connective tissue growth factor (IGFBP-rP2) expression and regulation in cultured bovine endothelial cells. Endocrinology 1999, 140: 1575–1780
7 Knight D. Epithelium-fibroblast interactions in response to airway

inflammation. Immunol Cell Bio 2001, 79: 160–164
8 Holgate ST. Epithelial damage and response. Clin Exp Allergy 2000, 30: 37–41
9 Ricupero DA, Rishikof DC, Kuang PP, Poliks CF, Goldstein RH. Regulation of connective tissue growth factor expression by prostaglandin E_2. Am J Physiol 1999, 277: 1165–1171
10 Abraham DJ, Shiwen X, Black CM, Sa S, Xu Y, Leask A. Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-β in normal and scleroderma fibroblasts. J Biol Chem 2000, 275: 15220–15225
11 Denton CP, Abraham DJ. Transforming growth factor-beta and connective tissue growth factor: Key cytokines in scleroderma pathogenesis. Curr Opin Rheumatol 2001, 13: 505–511
12 Allen JT, Knight RA, Bloor CA, Spiteri MA. Enhanced insulin-like growth factor binding protein-related protein 2 (connective tissue growth factor) expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Am J Respir Cell Mol Biol 1999, 21: 693–700
13 Zhang S, Smartt H, Holgate ST, Roche WR. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: An *in vitro* co-culture model of airway remodeling in asthma. Lab Invest 1999, 79: 395–405
14 Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF-β in cardiac fibroblasts and cardiac myocytes: A potential role in heart fibrosis. J Mol Cell Cardiol 2000, 32: 1805–1819
15 Pan LH, Yamauchi K, Uzuki M, Nakanishi T, Takigawa M, Inoue H, Sawai T. Type II alveolar epithelial cells and interstitial fibroblasts express connective tissue growth factor in IPF. Eur Respir J 2001, 17: 1220–1227
16 Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol 1996, 107: 404–411
17 Surveyor GA, Brigstock DR. Immunohistochemical localization of connective tissue growth factor (CTGF) in the mouse embryo between days 7.5 and 14.5 of gestation. Growth Factors 1999, 17: 115–124
18 Yokoi H, Sugawara A, MukoyamaM, Mori K, Makino H, Suganami T, Nagae T *et al*. Role of CTGF in profibrotic action of transforming growth factor-beta: A potential target for preventing renal fibosis. Am J Kidney Dis 2001, 38: 134–138
19 Wenger C, Ellenrieder V, Alber B, Lacher U, Menke A, Hameister H, Wilda M *et al*. Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. Oncogene 1999, 18: 1073–1080

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