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Short communication

# Connective tissue growth factor regulates transition of primary bronchial fibroblasts to myofibroblasts in asthmatic subjects

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ABSTRACT

Fibroblast to myofibroblast transition (FMT) contributes to bronchial wall remodelling in persistent asthma. Among other numerous factors involved, transforming growth factor type β (TGF-β) plays a pivotal role. Recently it has been demonstrated that connective tissue growth factor (CTGF), a matricellular protein, combines with TGF-β in the pathomechanism of many fibrotic disorders. However, it is not clear whether this interaction takes place in asthma as well.

Primary cultures of human bronchial fibroblasts from asthmatic and non-asthmatic subjects were used to investigate the impact of CTGF and TGF-$\beta_1$ on the fibroblast to myofibroblast transition. The combined activity of TGF-$\beta_1$ and CTGF resulted in an average of 90% of FMT accomplished in cell lines derived from asthmatics. In this group FMT was highly dependent on the presence of CTGF produced by the cells, as shown by gene silencing experiments with the specific siRNA.

Results support the important role of CTGF biosynthesis in the asthmatic bronchi amplifying FMT. This is evidenced by inhibition of TGF-$\beta_1$-induced FMT following CTGF silencing in asthmatic bronchial fibroblasts. CTGF is produced by fibroblasts and contributes to the FMT phenomenon in positive loop-back, inducing and boosting TGF-$\beta_1$ triggered FMT. Thus, CTGF is a promising target for pharmacological intervention in secondary prevention of bronchial remodelling in asthma.

## 1. Introduction

Remodelling of asthmatic airways is frequently observed in patients with moderate to severe asthma [1]. This complex process follows chronic inflammation and leads to irreversible narrowing of the bronchial tree. Remodelling of airways depends on numerous cell types. Histological examination of affected subjects reveals structural abnormalities of epithelium with subepithelial fibrosis, increased angiogenesis, and smooth muscle cells proliferation, as well as accumulation of extracellular matrix, suggesting the involvement of fibroblasts and other mesenchymal cells [2].

Increased airway smooth muscle (ASM) mass in asthmatic bronchi may result from the cell proliferation and hypertrophy or influx of blood-derived mesenchymal progenitors, as well as transdifferentiation of epithelial cells or fibroblasts into a contractile phenotype. Therefore, it is plausible that fibroblasts to myofibroblasts transition (FMT) can contribute both to overproduction of extracellular matrix and smooth muscle hyperplasia in asthmatic bronchi [3]. Various stimuli, such as growth factors, pro-inflammatory cytokines, mechanical tension and mesenchymal-epithelial interactions may induce a phenotypic switch of fibroblasts, resulting in a gradual increase of α-smooth muscle actin (α-SMA) expression. α-SMA is a protein of contractile apparatus and the lineage marker for ASM [4].

Myofibroblasts can be experimentally induced from fibroblasts by transforming growth factor type β (TGF-β), a cytokine readily produced by many cell types. Connective tissue growth factor (CTGF) is another profibrotic protein involved in wound healing and numerous other pathologies. CTGF is a matricellular protein which cooperates with TGF- β in the progression of kidney, pancreas, retina, and skin fibrosis [5]. Increased levels of this growth factor have also been found in the lung tissue and plasma of asthmatics [6,7]. Our study was designed to investigate if CTGF can participate in FMT of airways. For this purpose, we used in vitro models of primary fibroblast cultures taken from asthmatic and non-asthmatic subjects.

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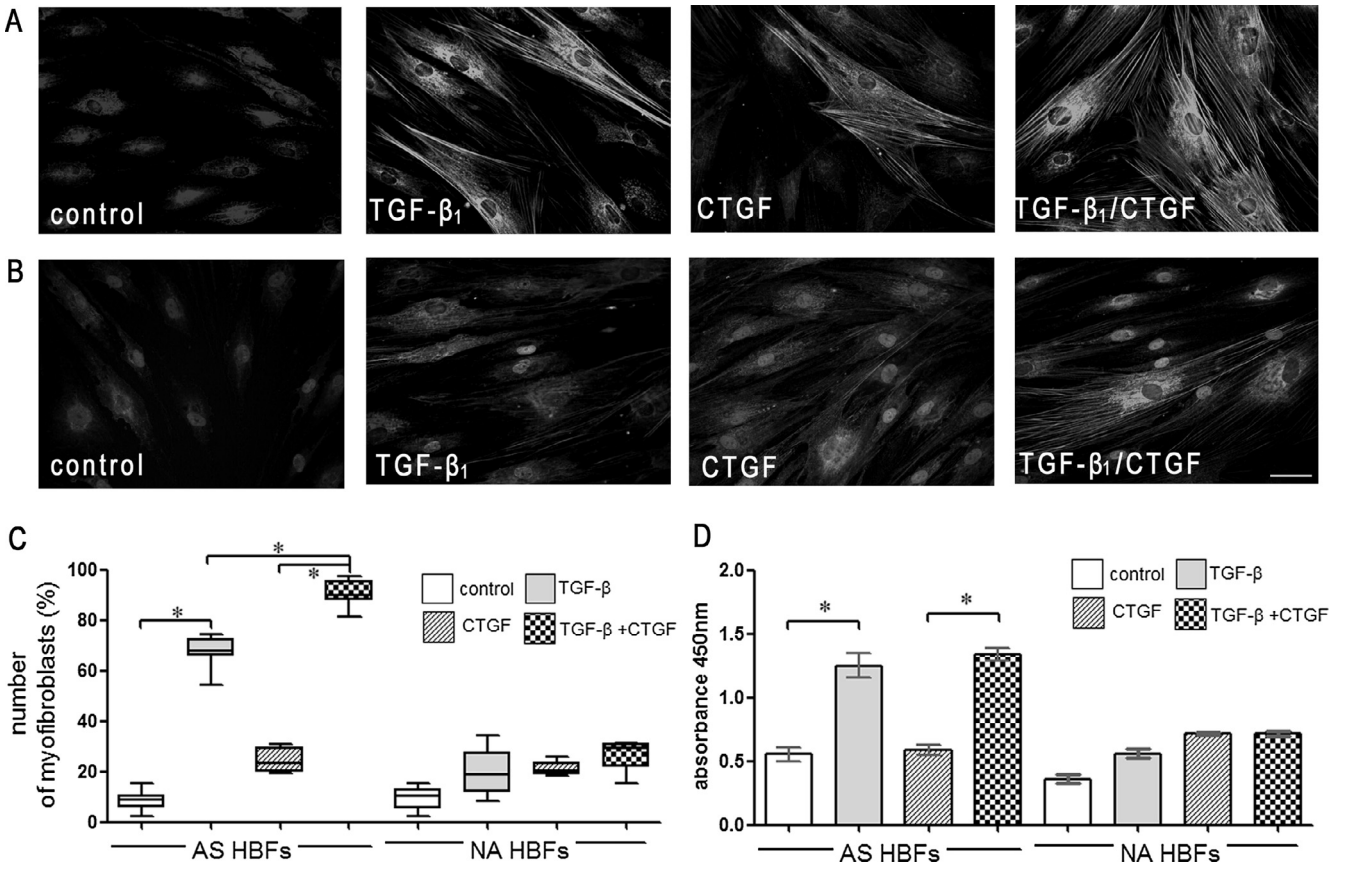


**Fig. 1.** CTGF promotes TGF-$\beta_1$-induced FMT. (A,B) Representative immunofluorescence microphotography showing cells with α-SMA positive stress fibers derived from AS and NA HBFs. (C) Fraction of myofibroblasts following TGF-$\beta_1$ and/or CTGF treatment in AS and NA HBFs cultures. (D) ELISA measurements of α-SMA protein in AS and NA HBFs cultures after TGF-$\beta_1$ and/or CTGF treatment. Values represented as means with SD, $^{*}$ $p < 0.05$.

## 2. Material and methods

Bronchial biopsies were obtained from 8 asthmatic (AS) and 5 non-asthmatic (NA) subjects. Details of the fibroblast cultures used to establish primary cell lines, characterized by CTGF expression, are described elsewhere [8]. The patients' characteristics are presented in supplementary material. Approval for the study was given by the Jagiellonian University Ethics Committee (KBET/211/B/2013 and KBET 122.6120.69.2015) and informed consent was obtained from all fibroblast donors.

The human bronchial fibroblasts (HBFs) were cultured in DMEM (Sigma Aldrich) and supplemented with 10% foetal bovine serum (FBS; Gibco) at standard tissue culture conditions (37 °C, 5% $CO_2$, 95% humidity). The experimental cultures' HBFs were seeded at low density (5000 cells/cm$^2$) in serum-free DMEM and supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich) both with, and without human recombinant TGF-$\beta_1$ (5 ng/ml; BD Biosciences) and human recombinant CTGF (20 ng/ml.; Sigma-Aldrich). Inhibition of CTGF gene expression in HBFs was achieved using CTGF-specific siRNA complementary to nucleotides 1272–1290 of CTGF*(CCN2)* mRNA (NM_001901.2; 150 nM, Sigma Aldrich). As a control, non-targeting siRNA (60 nM, Santa Cruz Biotechnology) was used. For the transfection, each oligonucleotide was encapsulated in Lipofectamine2000 reagent (0.3% final concentration, Invitrogen). After 24 h of exposure to liposomes, cells were washed with DMEM and cultured in the supplemented DMEM medium for the following 24 h.

FMT was ascertained by immunocytochemical analysis using mouse anti-α-SMA Mab to visualize α-SMA positive stress fibers in HBFs. The number of myofibroblasts were counted for the entire culture surface. Whole cells lysate was used to quantify α-SMA protein by ELISA and Western blot. Simultaneously, the mRNA abundance of *ACTA2* in total cellular RNA was measured by Real-time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR) using *GAPDH* as the internal housekeeping transcript. Detailed online descriptions of molecular and statistical methods are presented as further supporting information.

## 3. Results and discussion

Our results support the enhanced FMT of bronchial fibroblasts derived from asthmatics, a phenomenon which seems to be maintained for at least several passages of primary cells *in vitro*. Following co-stimulation with TGF- $\beta_1$ and CTGF, this transition is extremely efficient and renders a contractile phenotype of 90% of cells on average. However, in response to TGF- $\beta_1$ stimulation, CTGF is produced by HBFs as autocrine and paracrine growth factor [8].

Therefore, we evaluated HBFs response to the recombinant CTGF. An increase of α-SMA positive myofibroblasts was observed, however, the magnitude of this response differed between AS and NA cell lines. This was also noticeable after concurrent stimulation with CTGF and TGF-$\beta_1$, causing the highest percentage of FMT in AS HBFs, while the effect was fourfold less in NA cells (91 ± 5% stimulated cells from AS vs. 23 ± 7% from NA; Fig. 1A and C). Quantification of α-SMA using ELISA fluorescence did not reveal any differences between AS cells stimulated with TGF-$\beta_1$ alone or in combination with CTGF, although CTGF alone did not induce α-SMA (Fig. 1D). This was in contrast with results of NA cells stimulation responding similarly to CTGF + TGF-$\beta_1$ than to CTGF alone (Fig. 1D). Thus, AS HBFs showed an inherent alteration of sensitivity to CTGF induced FMT in contrast to NA.

Since we previously observed that HBFs can produce [8] and release CTGF following TGF-$\beta_1$ stimulation (Supplementary Fig. 1), the