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Transforming growth factor-β_1-induced expression of connective tissue growth factor is enhanced in bronchial fibroblasts derived from asthmatic patients

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KEY WORDS

airway wall remodeling, asthma, connective tissue growth factor, transforming growth factor-β_1, Wnt signaling pathway

ABSTRACT

INTRODUCTION Bronchial asthma is accompanied by airway remodeling as well as increased secretion of cytokines, growth factors, and extracellular matrix proteins. Connective tissue growth factor (CTGF) has been suggested to contribute to many fibrotic disorders. However, the ability of human bronchial fibroblasts (HBFs) to express CTGF in response to transforming growth factor-β_1 (TGF-β_1) has not been studied so far.

OBJECTIVES The aim of the study was to investigate whether HBFs are able to express CTGF when stimulated with TGF-β_1.

PATIENTS AND METHODS All experiments were conducted on in vitro cultures of HBFs isolated from bronchial biopsies obtained from 8 patients with asthma and from 5 nonasthmatic individuals. We performed an analysis of changes in mRNA expression for CTGF and α-smooth muscle actin and in protein expression for CTGF.

RESULTS We have shown for the first time that HBFs derived from asthmatic patients are capable of higher CTGF expression when stimulated with TGF-β_1 compared with HBFs isolated from nonasthmatic individuals. Moreover, this effect is significantly reduced after the Wnt signaling pathway activation.

CONCLUSIONS Our results point to a pleiotropic effect of TGF-β_1, the elevated levels of which are observed in the bronchoalveolar lavage fluid obtained from asthmatic patients. The structural cells of the bronchi, fibroblasts, stimulated with TGF-β_1 begin to synthesize CTGF. Moreover, this process can be reversed by the GSK-3β inhibitor, which activates the Wnt signaling pathway. Our model, based on in vitro primary cell cultures, may be a valuable experimental approach to study the mechanisms underlying bronchial wall remodeling, and in the future it may lead to the development of new therapeutic strategies in asthma.

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INTRODUCTION Bronchial asthma is a chronic respiratory disease characterized by inflammation of the respiratory tract frequently leading to the airway wall remodeling. Bronchial hyperresponsiveness causes intermittent narrowing of the airways, wheezing, shortening of breath, coughing, and tightness in the chest. Although more and more people worldwide suffer from this disease, no treatment that would definitely prevent its long-term consequences has yet been developed.

Airway remodeling is a complex cascade of events, which involve immunocompetent cell infiltration, defective epithelium regeneration, smooth muscle cell proliferation and hyperplasia, increased mucus hypersecretion, and subepithelial fibrosis.[1] Subepithelial fibrosis results mainly from the transition of fibroblasts into myofibroblasts (FMT) and increased secretion of extracellular matrix proteins by both fibroblasts and myofibroblasts. Myofibroblasts are detectable as cells whose phenotype is an intermediate

between fibroblasts and smooth muscle cells.[2] In contrast to fibroblasts, myofibroblasts are characterized by the expression of α-actin isoform specific to smooth muscle cells (α-smooth muscle actin [α-SMA]) organized in stress fibers and, like fibroblasts, they have the ability to synthesize extracellular matrix components.[3] Moreover, they can play a role of inflammatory cells through the secretion of cytokines. One of the principal mediators of subepithelial fibrosis initiating FMT is transforming growth factor-β_1 (TGF-β_1).[4,5] Its elevated levels are detectable in the bronchoalveolar lavage fluid of asthmatics.[6] Recently, it has been demonstrated that mechanical tension[7] and cell-cell contacts[8] are also responsible for FMT.

Last-decade studies indicated that the secretion of connective tissue growth factor (CTGF) – one of the markers of fibrosis – is increased in numerous diseases.[9] CTGF is overexpressed in patients with systemic fibrotic disease, scleroderma.[10] Moreover, the secretion of CTGF can be induced by stimulation with TGF-β_1.[11]

Considering CTGF as one of the mediators of TGF-β_1 profibrotic action, we wondered whether human bronchial fibroblasts (HBFs) are capable of increased CTGF expression. Our previous studies demonstrated that HBFs isolated from the bronchial tissue of patients with asthma differentiate into myofibroblasts in vitro much more efficiently under the stimulation with TGF-β_1.[12] On the other hand, it has been reported that FMT can be inhibited by the activation of some signaling pathways within the cell, including the Wnt pathway (our unpublished data). Therefore, the main purpose of this study was to investigate whether bronchial fibroblasts from asthmatic patients can express CTGF after stimulation with TGF-β_1. We also tested whether the Wnt pathway activation can attenuate TGF-β_1-induced CTGF expression in these cells.

PATIENTS AND METHODS

Isolation of human bronchial fibroblasts and cell culture protocol

Bronchial biopsies were obtained from 8 patients with bronchial asthma and 5 nonasthmatic subjects during bronchoscopy. All patients were treated in the 2nd Department of Medicine of the Jagiellonian University, Kraków, Poland, and were in stable clinical condition. Patient characteristics are presented in the TABLE. The study was approved by the Jagiellonian University Ethics Committee (KBET/362/B/2003) and informed consent was obtained from all participants.

HBFs were isolated from bronchial biopsies as described previously.[12] Cells were cultured in DMEM with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2 and used between 5 to 15 passages. For experiments, cells were seeded at low density (5000 cells/cm^2) and cultured in serum-free DMEM supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Missouri, United States) with or without human recombinant TGF-β_1 (5 ng/ml; BD Biosciences, Franklin Lakes, New Jersey, United States) and GSK-3β inhibitor XII, TWS119 (5 μM; Callbiochem, La Jolla, California, United States). To inhibit the secretion of CTGF into the culture medium, brefeldin A was used (5 μg/ml; Sigma-Aldrich), which enhances accumulation of synthesized proteins in endoplasmatic reticulum of the cell (CTGF staining, western blot analysis).

Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was used to detect and quantify the expression of α-SMA and CTGF transcripts. The analysis was performed after 24-hour incubation with the stimuli. Total RNA was extracted by chaotropic lysis using the RNA extraction kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol, and cDNA was reversely transcribed using the High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Applied Biosystems, Foster City, California, United States) in the presence of RNase Inhibitor. Real-time PCR assays were done using iCycler Real Time PCR equipment (Biorad, Karlsbad, California, United States) and Sybr Green (Amresco, Solon, Ohio, United States) detection of amplification products. Specific α-SMA primers (5´-AATGATTCATAGGGCTTCAG-3´, 5´-ATTTGACCCAGAACTACTTT-3´), CTGF primers (5´-CCAACCGCAAGATCGGCGTG-3´, 5´-GTCTTCCAGTCGGTAAGCCGCG-3´), and housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (5´-AGAACATCATCCCTGCCTCTAC-3´, 5´-CTGTTGAAGTCAGAGGAGACCA-3´) were used to quantify the transcripts in each tested cell line. The relative abundance of specific mRNA transcripts was estimated on the basis of a cycle threshold (CT) value and recalculated against the housekeeping gene using the ΔCT method. ΔCT refers to $CT_{GAPDH} - CT_{\alpha\text{-}SMA}$ or CT_{CTGF}. All samples were run in duplicates. Primers design and control gel electrophoresis of amplification products ensured artifact-free results.

Protein assays

All protein assays were conducted after 48-hour incubation with the stimuli.

TABLE Characteristics of study participants

Characteristics	Nonasthmatics	Asthmatics
clinical diagnosis	chronic cough, chest X-ray abnormalities; excluded asthma, chronic inflammatory lung diseases, sarcoidosis, cancer	moderate-to-severe asthma according to GINA 2009 classification[13]
age, y (women, %)	56.7 ±7.3 (36)	44.1 ±9.2 (64)
duration of the disease	–	11.6 ±9.2
FEV_1 (% predicted)	97.8 ±12.2	77.4 ±21.6

Data are presented as mean ± standard deviation.

Abbreviations: FEV_1 – forced expiratory volume in 1 second, GINA – Global Initiative for Asthma

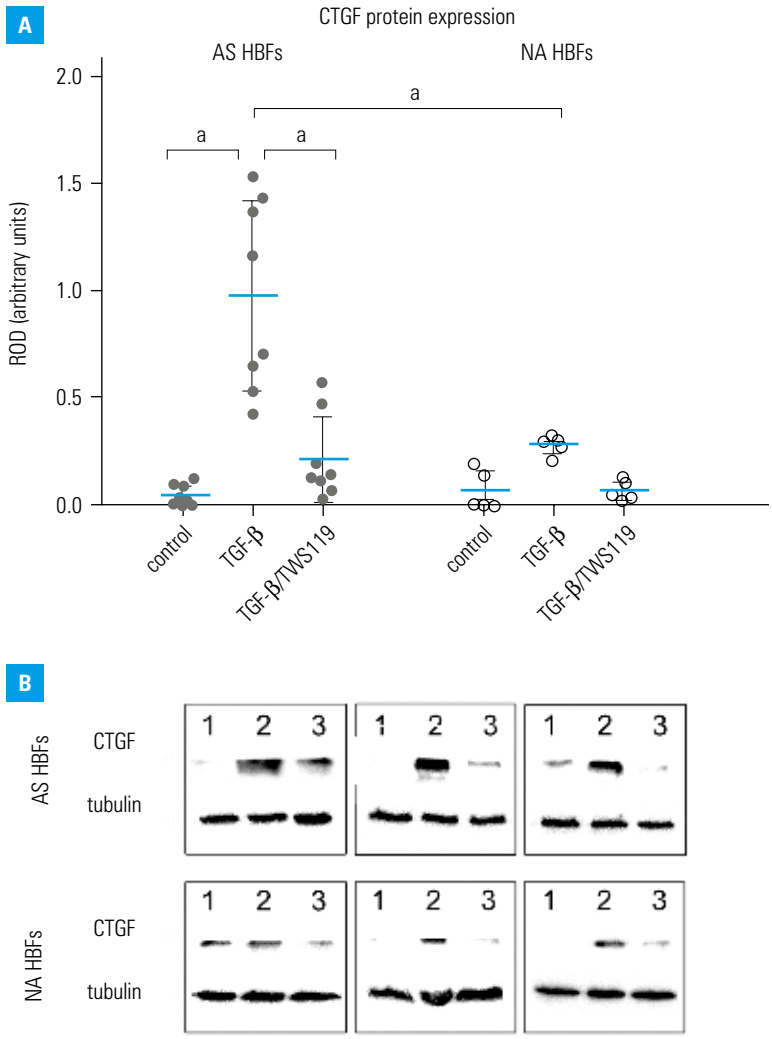

FIGURE 1 Expression of CTGF protein in comparison with tubulin levels in asthmatic and nonasthmatic HBFs

A – data presented as means ± standard deviation; each point represents the results obtained from a single HBF culture derived from AS and NA; **a** $P < 0.05$

B – representative western blots from AS (n = 3) and NA (n = 3) HBFs; 1 – control, 2 – TGF-β, 3 – TGF-β/TWS119

Abbreviations: AS – asthmatics, CTGF – connective tissue growth factor, HBFs – human bronchial fibroblasts, NA – nonasthmatics, ROD – relative optical density, TGF-β_1 – transforming growth factor-β_1

To visualize the intracellular localization of CTGF and α-SMA, immunocytochemical staining was performed. After 20-minute fixation in 3.7% formaldehyde, 10-minute permeabilization in 0.1% Triton X-100 and 45-minute blocking with 3% BSA, cells were stained with rabbit polyclonal anti-CTGF antibody (Sigma-Aldrich) and mouse monoclonal anti-α-SMA antibody (clone 1A4, Sigma-Aldrich) followed by anti-rabbit Alexa-546 antibody (Invitrogen, Carlsbad, California, United States) and anti-mouse Alexa-488 antibody (Sigma-Aldrich), respectively. Nuclei were stained with Hoechst 33 342 (Sigma-Aldrich). Visualization of specimens mounted in polyvinyl alcohol (Mowiol; Sigma-Aldrich) was performed with the Leica DM IRE2 microscope equipped with 40×, NA-1.25 HCX Plan Apo objective, Leica DC350FX camera, and Leica FW4000 software.

CTGF protein expression level was detected by the western blot analysis. Cells were lysed with the lysis buffer (0.1 mol/l Tris-HC1, 15% glycerol, 2 mM EDTA, 2% SDS, 10 mM phenylmethytsulfonyl fluoride, and 10 μg/ml aprotinin, pH 7.4). The protein concentration was determined using the Bradford method and the samples of supernatants containing 30 μg of protein from the whole cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and then transferred on the polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, England). After blocking in phosphate buffered saline (PBS) (0.1% Tween 80 in PBS) containing 5% skimmed milk at room temperature, the membrane was incubated for 2 hours with rabbit polyclonal anti-CTGF antibody (1:250) and mouse monoclonal antitubulin antibody (1:1000). After washing 3 times, the membrane was incubated for 1 hour with anti-rabbit and anti-mouse immunoglobulin G horseradish peroxidase-conjugated antibody (1:3000, Invitrogen), respectively. The extensively washed membranes were incubated with chemiluminescent reagent, Super Signal West Pico Substrate (Pierce, Rockford, Illinois, United States) for 10 minutes and the chemiluminescence signal was recorded using the MicroChemi imager (ALAB, Warsaw, Poland). The relative optical density of the signal bands was estimated using the ImageJ 1.45s software.

Statistical analysis Statistical analysis was performed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, California, United States). The comparison of parameters between the groups and experimental conditions was performed using a two-way analysis of variance for between-the-group comparisons and the Wilcoxon test for paired data. $P < 0.05$ was considered statistically significant.

RESULTS

Human bronchial fibroblasts from asthmatics are capable of connective tissue growth factor protein expression after stimulation with transforming growth factor β_1 TGF-β_1 was previously demonstrated to induce myofibroblast phenotype in asthmatic, in contrast to nonasthmatic, HBF cultures, as documented by α-SMA-positive cells and α-SMA protein expression.[8,12] Because CTGF expression is increased in many fibrotic diseases, we decided to investigate whether TGF-β_1 can induce CTGF protein level in asthmatic and nonasthmatic HBFs. As illustrated in FIGURE 1AB, CTGF protein expression is clearly enhanced following TGF-β_1 treatment in asthmatic HBFs in contrast to nonasthmatic HBFs ($P < 0.05$). Representative western blots (FIGURE 1B) show higher amounts of CTGF protein in asthmatic fibroblasts treated with TGF-β_1, and this distinguishes them from nonasthmatic controls. Our earlier studies (manuscript submitted) indicate that Wnt pathway activation can modulate TGF-β_1 signaling in bronchial fibroblasts and decrease their ability to differentiate into myofibroblasts. Following this rationale, we used GSK-3β specific inhibitor – TWS119 – to evaluate whether activated Wnt signaling may affect TGF-β_1-induced CTGF protein expression. Increased CTGF protein expression after TGF-β_1 treatment is attenuated by Wnt pathway activation (FIGURE 1AB). This effect is noticeably significant in asthmatic HBF cultures ($P < 0.05$).

Intracellular localization of connective tissue growth factor correlates with quantitative protein expression To demonstrate the intracellular

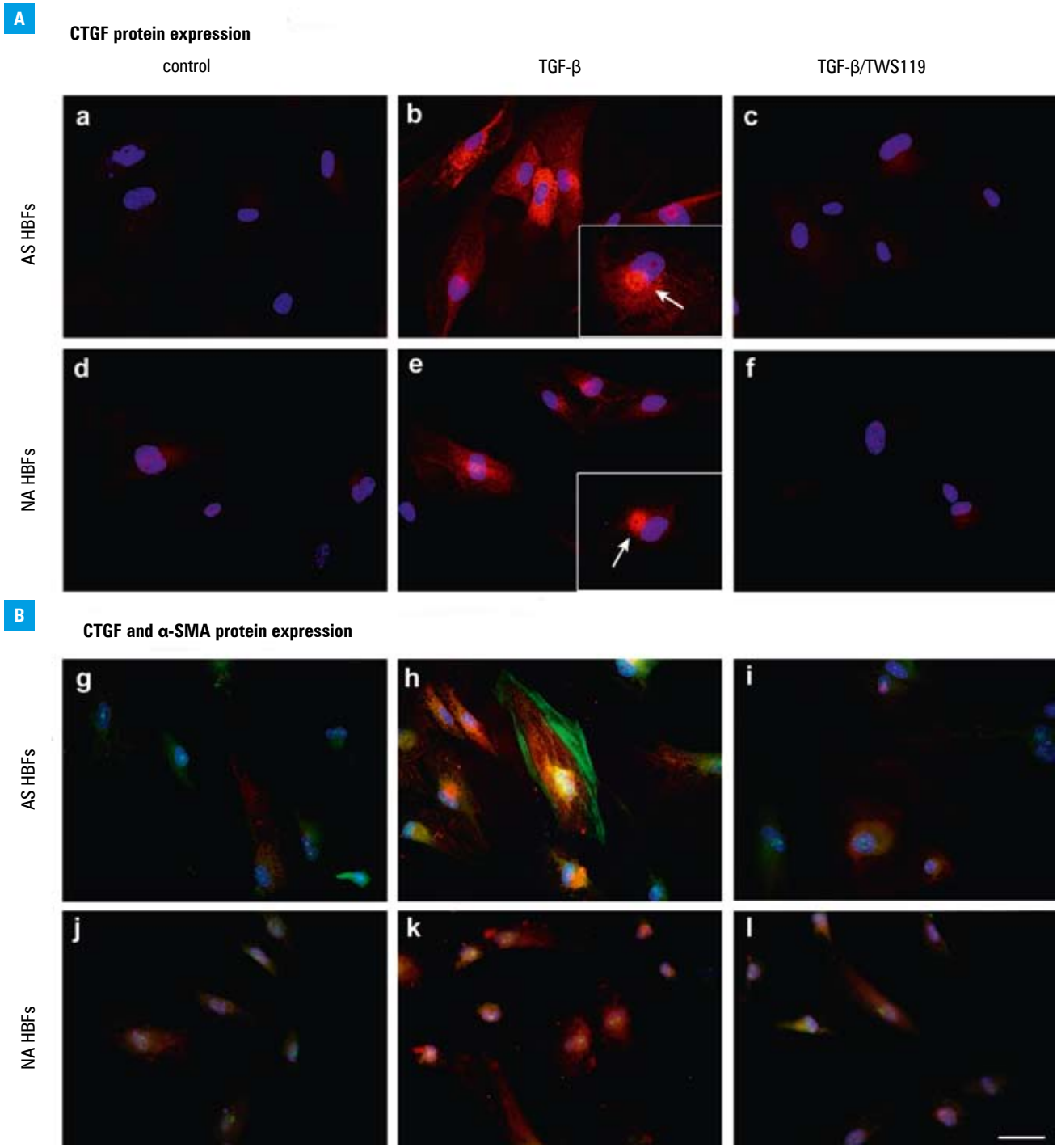

FIGURE 2 Intracellular localization of CTGF (**A**, **B**) and α-SMA (**B**) in asthmatic and nonasthmatic HBFs; cells were stained with anti-CTGF antibody (red, a–l) and anti-α-SMA antibody (green, g–l); nuclei were visualized with Hoechst dye (blue, a–l); bar represents 50 μm. Abbreviations: α-SMA – α-smooth muscle actin, others – see FIGURE 1

localization of CTGF protein, we stained HBFs with anti-CTGF antibody. We observed a higher number of CTGF positive cells in asthmatic HBFs after TGF-β_1 stimulation (FIGURE 2Ab) in comparison with nonasthmatic HBFs (FIGURE 2Ae). Likewise, the level of CTGF protein was greater in asthmatic HBFs treated with TGF-β_1. Furthermore, we did not observe any correlation between α-SMA protein incorporation into stress fibers and CTGF protein expression (FIGURE 2Bh). CTGF was present in almost every cell. We observed the prevalence of HBFs in which CTGF was localized near nuclei (FIGURE 2A – inserts).

At the mRNA level, transforming growth factor β_1 significantly increases transcripts for connective tissue growth factor in both asthmatic and nonasthmatic human bronchial fibroblasts Because many effects in cytokine signaling regulation are exerted at gene expression level, we further focused on the expression of CTGF transcripts after TGF-β_1 treatment in asthmatic and nonasthmatic HBFs. Surprisingly, we observed that the effect of TGF-β_1 in both asthmatic and nonasthmatic HBFs was the same (FIGURE 3A). The increased levels of CTGF mRNA were noticed after TGF-β_1 stimulation in asthmatic and nonasthmatic HBFs, and there was no statistically significant difference between the two groups. We also determined whether Wnt signaling could modulate TGF-β_1-induced

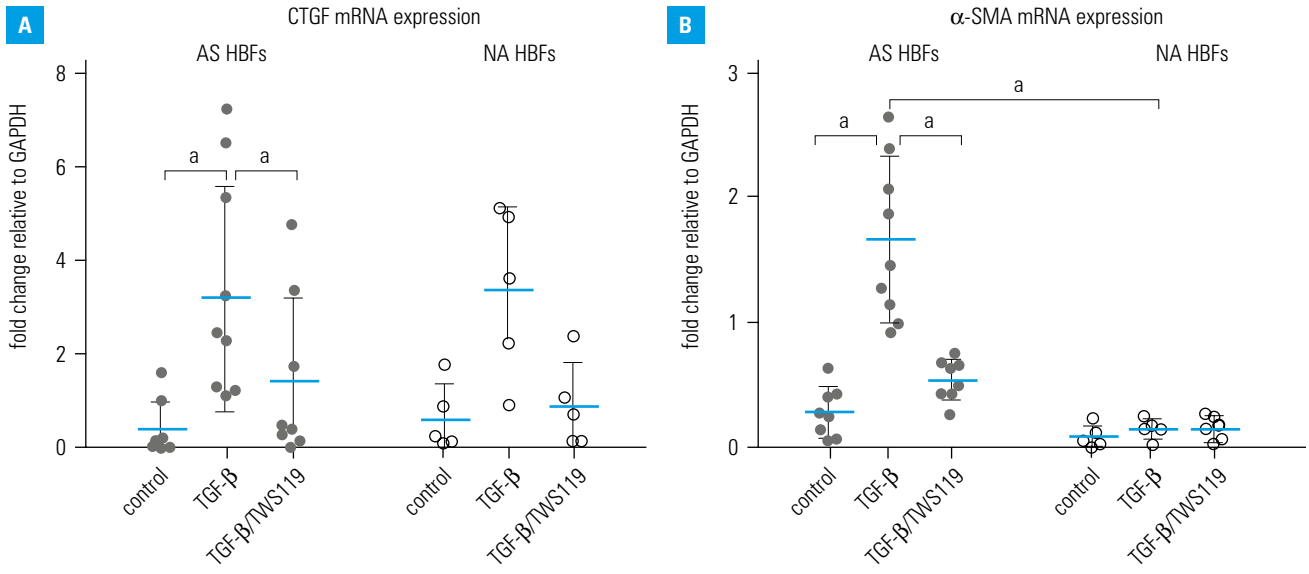

FIGURE 3 Expression of CTGF (**A**) and α-SMA (**B**) mRNA in comparison with GAPDH levels in asthmatic and nonasthmatic HBFs cultures; data presented as means ± standard deviation; each point represents the result obtained from a single HBF culture derived from NA and AS (the results presented are the means of at least 2 separated experiments for each culture); **a** $P < 0.05$ Abbreviations: GAPDH – glyceraldehyde 3-phosphate dehydrogenase, others – see **FIGURES 1** and **2**

CTGF mRNA expression. Specific activation of the Wnt pathway decreased the level of CTGF mRNA in both study groups (**FIGURE 3A**).

In line with our earlier results, showing that HBFs derived from asthmatic patients have higher capability of FMT,[12] we repeated the assays on α-SMA mRNA expression following TGF-β_1 stimulation (with or without TWS119) in both asthmatic and nonasthmatic HBFs. We found considerably higher effect of TGF-β_1 on α-SMA mRNA expression in asthmatic HBFs (**FIGURE 3B**; $P < 0.05$) compared with nonasthmatic HBFs, in which no FMT was detectable.

DISCUSSION Overexpression of CTGF is considered one of the key markers of fibrotic tissue pathologies.[9,14-16] Moreover, its level measured in biological fluids correlates with the degree of fibrosis.[17] A number of epidemiological studies revealed increased CTGF production in nephropathy, kidney and liver fibrosis, systemic scleroderma, and pulmonary disorders.[17,18] Significant amounts of CTGF were observed in the bronchoalveolar lavage fluid and plasma of patients with idiopathic pulmonary fibrosis.[9,19] Previous studies suggested that this cytokine might play an important role in airway remodeling during inflammation in asthma progression.[20,21] Furthermore, it has been demonstrated that CTGF can be produced in elevated amounts by asthmatic airway smooth muscle cells.[22,23] Considering that CTGF expression is potently induced by TGF-β[24] and that increased secretion of factors of the TGF-β family by fibroblasts and Th2 lymphocytes accompanies chronic inflammation in asthma, we stimulated HBFs with TGF-β_1 to determine whether these cells were capable of producing CTGF. For the experimental model, we used in vitro cultures of HBFs isolated from bronchial biopsies obtained from patients with and without asthma.

Our results have shown for the first time that TGF-β_1 may affect CTGF protein production in asthmatic HBFs. Intracellular CTGF protein levels are significantly higher in bronchial fibroblasts derived from patients with asthma compared with healthy controls. Our previous studies showed that enhanced capability for TGF-β_1-stimulated FMT in asthmatic HBFs may be attenuated by Wnt pathway activation. In the present study, we observed that the inhibition of GSK-3β kinase triggers a significant decrease in TGF-β_1-induced CTGF production, which seems independent of acquired myofibroblast phenotype.

Knowing that many effects of TGF-β are mediated by CTGF and that TGF-β can activate expression of CTGF gene,[17] we wanted to test whether this effect can also be measured on the mRNA level. The analysis of CTGF transcripts revealed that asthmatic and nonasthmatic HBFs do not differ in mRNA expression after stimulation with TGF-β_1, but in both cases elevated CTGF transcript level is attenuated by Wnt pathway activation. On the other hand, the α-SMA transcript level was significantly lower after TGF-β_1 stimulation in nonasthmatic HBFs, which is consistent with our previous results on α-SMA protein levels. Therefore, we postulate that TGF-β_1-induced CTGF expression in HBFs is independent of α-SMA expression.

Enhanced expression of CTGF protein in asthmatic HBFs may lead to increased secretion of this pleiotropic factor into extracellular space, but this hypothesis has not been studied so far. CTGF protein is mainly secreted from the cell via the Golgi apparatus,[25] and our fluorescent images suggest that HBFs from asthmatic patients may have greater ability to secrete CTGF. CTGF acts as a multifunctional growth factor regulating cell adhesion, migration, proliferation, survival, differentiation, and extracellular matrix deposition.[18,20] It has also been demonstrated to promote adhesion of fibroblasts to fibronectin by interaction with integrins.[26] On the other hand, fibroblast adhesion to extracellular matrix components promotes FMT.[7] Most of these phenomena are crucial in the development of inflammation in asthma and associated bronchial wall remodeling, but

none of the currently available therapies for asthma focuses on CTGF inhibition.

To fully understand the exact mechanisms of interaction between TGF-β and CTGF in promoting FMT, further studies are needed. The current results provide a solid hypothesis on the role of CTGF as an important player in bronchial wall remodeling. Further investigation of these effects may lead to the development of novel therapeutic targets for asthma.

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Indukowana transformującym czynnikiem wzrostu typu β_1 ekspresja czynnika wzrostowego tkanki łącznej jest zwiększona w fibroblastach oskrzelowych pochodzących od chorych na astmę

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SŁOWA KLUCZOWE

astma, czynnik wzrostowy tkanki łącznej, przebudowa ściany oskrzeli, ścieżka sygnalizacyjna Wnt, transformujący czynnik wzrostowy typu β_1

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** Katarzyna A. Wójcik jest studentką studiów doktoranckich na Uniwersytecie Jagiellońskim, Collegium Medicum.

STRESZCZENIE

WPROWADZENIE Przebiegowi astmy oskrzelowej towarzyszy przebudowa ściany oskrzela, a także zwiększone wydzielanie cytokin, czynników wzrostu i białek macierzy zewnątrzkomórkowej. Sugeruje się, że czynnik wzrostowy tkanki łącznej (*connective tissue growth factor* – CTGF) może mieć swój udział w zaburzeniach związanych z włóknieniem wielu narządów. Do tej pory nie badano jednak, czy ludzkie fibroblasty oskrzelowe (*human bronchial fibroblasts* – HBFs) pod wpływem transformującego czynnika wzrostu typu β_1 (*transforming growth factor-*β_1 – TGF-β_1) są zdolne do ekspresji CTGF.

CELE Celem pracy było zbadanie, czy ludzkie fibroblasty oskrzelowe (HBFs) mogą wykazywać ekspresję CTGF pod wpływem stymulacji TGF-β_1.

PACJENCI I METODY Wszystkie eksperymenty przeprowadzono w warunkach *in vitro*, na hodowlach HBFs wyizolowanych z bioptatów oskrzela, uzyskanych od 8 pacjentów z astmą oraz od 5 osób, u których nie stwierdzono astmy. Przeprowadzono analizę zmian w ekspresji mRNA dla CTGF oraz α-aktyny mięśni gładkich, a także białka CTGF.

WYNIKI Wykazano po raz pierwszy, że HBFs pochodzące od chorych na astmę mają zdolność do zwiększonej ekspresji CTGF pod wpływem TGF-β_1 w przeciwieństwie do HBFs wyizolowanych od osób niechorujących na astmę. Co więcej, efekt ten jest wyraźnie zmniejszony po aktywacji ścieżki sygnalizacyjnej Wnt.

WNIOSKI Uzyskane wyniki wskazują na plejotropowy efekt TGF-β_1, którego podwyższony poziom stwierdza się w popłuczynach oskrzelikowo-pęcherzykowych chorych na astmę. Strukturalne komórki oskrzeli, fibroblasty, pod wpływem TGF-β_1 podejmują syntezę CTGF. Co więcej, proces ten może zostać odwrócony przez zastosowanie inhibitora GSK3-β, aktywującego szlak sygnalizacyjny Wnt. Nasz model, oparty na pierwotnych hodowlach komórkowych in vitro, może stanowić cenny system eksperymentalny w badaniach mechanizmów leżących u podstaw przebudowy ściany oskrzeli, a w przyszłości doprowadzić do rozwoju nowych strategii terapeutycznych w astmie.