Respiratory Physiology & Neurobiology 177 (2011) 236–240

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Pulmonary fibroblasts from COPD patients show an impaired response of elastin synthesis to TGF-β1

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ARTICLE INFO

Article history:
Accepted 17 April 2011

Keywords:
Chronic obstructive pulmonary disease
Human lung fibroblasts
Transforming growth factor-β1
Proliferation
Elastin

ABSTRACT

Insufficiency of tissue repair by pulmonary fibroblasts may contribute to the decrease in elastic fibres in chronic obstructive pulmonary disease (COPD). In this study, the repair function of COPD fibroblasts was assessed by examining the response to transforming growth factor (TGF)-β1. Primary pulmonary fibroblasts were cultured from lung tissue of COPD patients and smoking control subjects. Cellular proliferation was measured with Alamar Blue reduction method. Levels of tropoelastin mRNA and soluble elastin was measured using real-time RT-PCR and Fastin elastin assay respectively. The percentage of increase in proliferation and elastin production after TGF-β1 (1 ng/ml) treatment was calculated for fibroblasts from each subject. COPD fibroblasts showed slower proliferation than control fibroblasts, and a reduced response to TGF-β1 stimulation. The promotive effect of TGF-β1 on elastin synthesis in control fibroblasts was significantly diminished in fibroblasts from COPD patients. Our findings indicate that COPD lung fibroblasts have a significantly decreased response to TGF-β1 in terms of proliferation and elastin production.

1. Introduction

Chronic obstructive pulmonary disease (COPD), characterised by irreversible airflow obstruction, is an important medical problem globally (GOLD, 2009; Hogg and Timens, 2009). The main site of airflow obstruction in COPD is the small airways (Hogg and Timens, 2009). This is assumed to be due primarily to emphysematous changes where there is loss of elastic fibres in the alveolar walls and subsequent destruction of the alveoli (Black et al., 2008; Merrilees et al., 2008). This destruction results in the loss of alveolar attachments to the small airways which in turn is thought to lead to the collapse of the small airways in expiration which results in airflow obstruction (Jeffery, 2001).

The development of emphysema is currently regarded to be due to the imbalance of tissue injury and repair (Chung and Adcock, 2008; Sharafkhaneh et al., 2008). The chronic and abnormal inflammatory process induced by tobacco smoking contributes to increased extracellular matrix degradation and promotes the structural changes in lung parenchyma (GOLD, 2009). On the other hand, studies suggest that the tissue repair capacity of lung fibroblasts, which are responsible for the extracellular matrix regeneration and maintenance, appears to be decreased in COPD. One piece of evidence supporting the impaired repair theory is the reduced proliferation of fibroblasts seen in patients with COPD compared to patients with normal lung function, despite a comparable smoking history (Holz et al., 2004; Hostettler et al., 2008; Muller et al., 2006).

Transforming growth factor (TGF)-β1 is a cytokine with many different effects on cell proliferation and differentiation and on inflammation. TGF-β1 can mediate fibroblast activation and proliferation (Rahimi and Leof, 2007). It can also promote the formation of elastin (Kuang et al., 2007; Kucich et al., 2002; McGowan et al., 1997) and this could help repair damage to the lungs. A recent study by Togo et al. (2008) showed that lung fibroblasts from subjects with moderate to severe COPD produce higher levels of TGF-β1 compared to fibroblasts from non-COPD subjects. However, while exogenous TGF-β stimulated collagen gel contraction and chemotaxis as well as the release of fibronectin in normal fibroblasts, a similar effect was not observed in fibroblasts from COPD patients, indicating a reduced response to TGF-β1 in tissue repair by lung fibroblasts from COPD patients.

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doi:10.1016/j.resp.2011.04.019

Taking these facts together, we put forward the hypothesis that TGF-β1-stimulated proliferation and elastin production might be decreased in lung fibroblasts from COPD patients. We cultured lung fibroblasts from patients with COPD and from individuals with a comparable smoking history but normal lung function to determine if COPD lung fibroblasts treated with TGF-β1 display impaired repair mechanism.

2. Methods

2.1. Subjects

Primary human fibroblasts were cultured from lung tissue obtained at surgery for bronchial carcinoma. Patients had their lung function measured preoperatively. Subjects who had a clearly documented history of asthma or bronchodilator reversibility (increase in FEV1 > 10% of predicted normal values) were excluded. Subjects with other lung diseases such as bronchiectasis and interstitial lung disease were also excluded. The study was approved by Auckland Ethics Committee and Ethics Committee of Zhongshan Hospital of Fudan University where samples were collected, and written informed consent for the acquisition of material for research was obtained preoperatively.

2.2. Cultures of human lung fibroblasts

Distal pulmonary fibroblasts were cultured from peripheral pleura-free parenchymal specimens of the resected lobe remote from the tumor. Tissues specimens collected at surgery were immediately transferred into explant culture medium [Dulbecco's modified Eagle medium (DMEM), 10% fetal calf serum (FCS), penicillin (100 ng/ml), and streptomycin (100 ng/ml)] (Invitrogen), minced with a scalpel (1–2 mm^2), and the pieces transferred into 25 cm^2 culture dishes for primary culture at 37 °C in 5% CO_2. In addition to assessment of the morphology of the fibroblasts, the purity of the cultured primary lung fibroblasts was confirmed by staining with vimentin (fibroblast marker), cytokeratin (epithelial marker), von Willebrand factor (endothelial markers) and with desmin (smooth muscle marker).

2.3. The measurement of cellular proliferation

At passage 2, fibroblast proliferation was measured using the Alamar Blue (AB) assay (Invitrogen) as described previously (Al-Nasiry et al., 2007). Briefly, fibroblasts were seeded at 5000/well into 96 well plates in serum-free culture medium and AB added at a final concentration of 10%. After a 6-h incubation in a humidified atmosphere containing 5% CO_2, the %AB reduction, which proved linear to cell number, was measured and recorded as baseline reading. After an additional culture period of 24 h in complete culture medium (5% CO_2 at 37 °C), %AB reduction was measured again. A proliferation index was calculated as 24 h reading divided by the baseline reading. To evaluate the impact of TGF-β1 on the cell proliferation, cells were cultured with or without 1 ng/ml recombinant human TGF-β1 (R&D system) for 48 h, and the percentage of increase in proliferation index after treatment was calculated.

2.4. Real-time PCR

Total RNA was extracted from 48-h subconfluent passage 3 cultures using Trizol Reagent (Invitrogen Life Technologies), and 1 μg of total RNA transcribed using SuperScript® ViLO™ cDNA synthesis kit (Invitrogen Life Technologies) according to the manufacturer's instructions.

Real-time PCR was performed using Express SyBR® GreenERTM SuperMix with Premixed ROX (Invitrogen Life Technologies) following the manufacturer's instructions. The following primers were used: elastin forward, 5′-TCT GAG GTT CCC ATA GGT TAG GG-3′; elastin reverse, 5′-CTA AGC CTG CAG CAG CTC CT-3′; GAPDH forward, 5′-TGA GCA CCA GAT TGT CTC CT-3′; and GAPDH reverse, 5′-GCA TCA AAG GTG GAA GAC TG-3′. PCR assays were performed in triplicate on the 7900HT real-time PCR machine (Applied Biosystems) with cycler conditions as follows: incubation for 2 min at 50 °C followed by another incubation step of 95 °C for 10 min, then 15 s at 95 °C and 1 min at 60 °C for 40 cycles. Reaction specificity was evaluated by melting curve analysis which was performed by heating the plate from 55 to 95 °C and measuring SYBR Green I dissociation from the amplicons. The calculation of threshold cycles (Ct values) and further analysis of these data were performed by the Sequence Detector software. The relative mRNA expression of target gene in each sample were quantified and normalized to the GAPDH mRNA levels by the 2^{-ddCt} method (Fink et al., 1998).

2.5. Quantification for elastin

Passage 3 cells were seeded at 50,000 cells/well in 6-well plates and cultured for one week in the presence or absence of 1 ng/ml recombinant human TGF-β1 (R&D system). Cell-free supernatant was collected for measurement of soluble elastin using the Fastin elastin assay kit from Biocolor according to the manufacturer's instruction.

2.6. Statistics

All statistical analyses were performed using GraphPadPrism 5.0 for Windows. D'Agostino and Pearson omnibus normality test was performed initially. If data were normally distributed, they were expressed as mean ± SD, and unpaired *t*-tests were performed to calculate statistical differences between groups. Otherwise, data were expressed as median (range), and comparisons were made by the Mann–Whitney *U*-test. Correlation analyses were carried out using Pearson or Spearman methods depending on the normality of the data distribution. Differences were considered significant at the level of $p < 0.05$.

3. Results

3.1. Clinical and demographic features of the subjects

A total of 18 subjects were enrolled into the study and divided into control group ($n = 8$) and COPD group ($n = 10$) according to post-bronchodilator spirometry. The control subjects had forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ≥ 0.7. The patients with FEV1/FVC < 0.7 were classified as COPD. The two groups were similar in age, gender and smoking status (Table 1). The two groups differed significantly in lung function. As expected, the subjects in COPD group had significantly lower FEV_1% pred and FEV_1/FVC% than control subjects.

3.2. COPD fibroblasts showed lower cellular proliferation and decreased response to TGF-β1

Due to the fact that AB is a stable water-soluble dye and is non-toxic to the cells, continuous monitoring of cultures overtime is possible. Therefore, the AB assay was used to quantifying numbers of the viable cells in the fibroblasts cultures. A proliferative index was calculated to represent the proliferative capacity. As shown in the left part of Fig. 1A, in the absence of TGF-β1, the cellular proliferation was slower in the fibroblasts from subjects with COPD (1.27 ± 0.23) than fibroblasts from control subjects (1.64 ± 0.28) ($p = 0.007$).

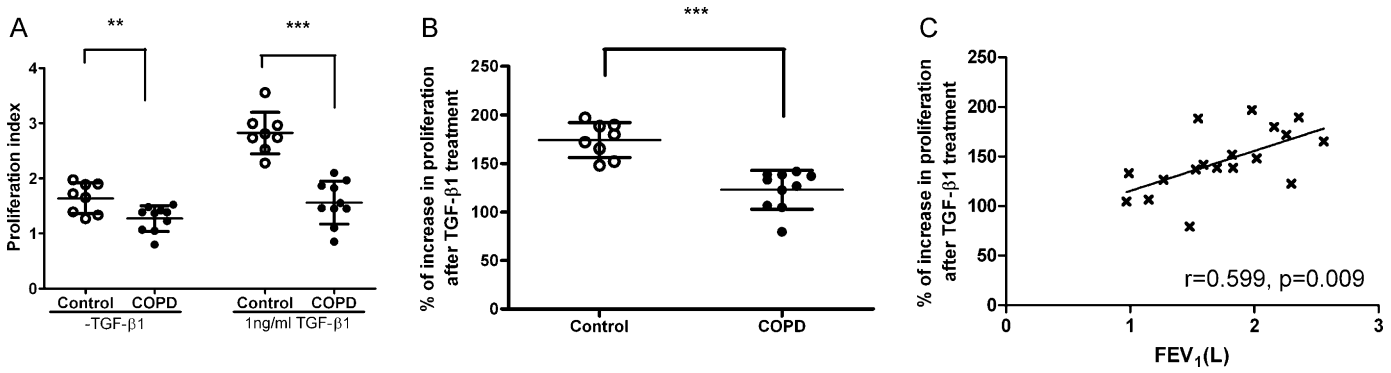

Fig. 1. Reduced proliferation and poor response to TGF-β1 in primary lung fibroblasts from patients with COPD. (A) Proliferation index (Alamar Blue assay) showing significantly decreased proliferative capacity of fibroblasts cultured from COPD subjects compared with control subjects both with and without TGF-β1 treatment. (B) The percentage of increase in proliferation index after 48-h incubation with 1 ng/ml TGF-β1 was significantly lower for lung fibroblasts from COPD patients than for cells from control subjects. (C) The percentage of increase in proliferation index after TGF-β1 treatment was positively correlated with the value of FEV_1. Control indicates subjects with normal lung spirometry ($n = 8$); COPD indicates subjects with COPD ($n = 10$). Data are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p \leq 0.001$.

1 ng/ml of TGF-β1 stimulated the proliferation of lung fibroblasts from both COPD and control subjects. However, the proliferative effect on the cells from COPD patients was significantly reduced compared to control cells. As shown in the right half of Fig. 1A, the proliferation index for COPD fibroblasts was significantly much lower than that for control cells (1.56 ± 0.39 vs 2.82 ± 0.38, $p < 0.0001$). The difference (percentage of increase) in the presence and absence of TGF-β1 for the COPD group and control group was 123 ± 20% and 174 ± 18% respectively ($p = 0.0001$) (Fig. 1B). Furthermore, the percentage of increase in the proliferation index after TGF-β1 stimulation was positively correlated with the values of FEV_1 ($r = 0.599$, $p = 0.009$) (Fig. 1C).

3.3. The stimulative effect on elastin synthesis by TGF-β1 was decreased in lung fibroblasts from COPD patients

The basal expression of elastin mRNA was not statistically different between the two groups. The relative expression for COPD group and control group was 7.27 (1.23, 15.00) and 1.88 (0.14, 4.84) respectively ($p = 0.169$). However, the protein level of soluble elastin in cell-free supernatants was higher for the COPD group compared to the control group (54.49 ± 14.28 ng/ml vs 33.33 ± 17.51 ng/ml, $p = 0.0065$).

Fibroblasts from the COPD patients responded poorly to TGF-β1 with respect to elastin transcription and production of soluble elastin. As shown in Fig. 2, TGF-β1 led to a 6-fold increase in mRNA transcription and a 1.5-fold increase in protein secretion of elastin in control fibroblasts. In contrast, TGF-β1 had little effect on elastin synthesis and secretion in COPD fibroblasts.

Table 1
Clinical and demographic features of the subjects.

	COPD[a]	Control[b]	*p*-Value
Gender (M/F)	5/5	5/3	NS
Age (years)	65.3 ± 9.8	69.5 ± 3.1	NS
Smoking history (pack-years)	43.0 ± 17.7	30.0 ± 10.0	NS
FEV_1 (*L*)	1.56 ± 0.38	2.25 ± 0.39	0.0012
FEV_1% pred	68.69 ± 18.70	90.17 ± 5.38	0.0128
FVC (*L*)	2.75 ± 0.76	2.97 ± 0.45	NS
FVC % pred	86.2 ± 20.5	89.0 ± 10.2	NS
FEV_1/FVC%	57.4 ± 8.8	79.3 ± 9.4	<0.0001

Data are presented as mean ± SD. COPD, chronic obstructive pulmonary disease; M, male; F, female; FEV_1, forced expiratory volume in one second; % pred, % predicted; FVC, forced vital capacity; NS, non-significant.

[a] Subjects had an FEV_1/FVC < 70%.

[b] Subjects had an FEV_1/FVC ≥ 70%.

4. Discussion

These results demonstrate that lung fibroblasts from subjects with COPD have a significantly reduced response to TGF-β1 with respect to cellular proliferation and elastin production, supporting the idea that impairment of repair mechanisms may be important in the development of COPD.

The cellular response to TGF-β1 can be extremely variable, promoting as well as antagonizing proliferation, apoptosis, and differentiation depending on cell type and stimulation context (Rahimi and Leof, 2007). It can mediate fibroblast activation and proliferation yet induce apoptosis and cell arrest in epithelial and endothelial cells (Rahimi and Leof, 2007). Our data showed that the proliferation of pulmonary fibroblasts from COPD subjects was significantly reduced compared to control fibroblasts. These results are consistent with the findings of other two groups (Holz et al., 2004; Muller et al., 2006). Furthermore, the proliferative effect of TGF-β1, clearly evident for control fibroblasts, was significantly diminished in COPD fibroblasts. As reviewed by Klass et al. (2009), TGF-β1 exerts potent stimulatory effects on fibroblast proliferation *in vivo* and *in vitro* and is implicated in normal wound healing. Our findings that lung fibroblasts from COPD patients have an impaired proliferative function and, more importantly, a blunted response to TGF-β1 indicate that the normal tissue repair mechanisms may be compromised in COPD lung.

Loss of elastic fibres is an established feature of COPD and emphysema (Black et al., 2008; Hogg and Timens, 2009; Merrilees et al., 2008). A previous immunohistochemistry study showed that the content of elastin fibres is decreased in the lungs of the patients with COPD (Merrilees et al., 2008). Elastin is a very long-lived molecule with most elastin deposition occurring early in life and with little turnover in adult life (Shapiro et al., 1991), but following injury there is marked increase in the synthesis of elastin (Lucey et al., 1998; Massaro and Massaro, 1997). Several experimental studies have reported that elastin synthesis is increased in emphysematous lungs in animal models (Chambers and Laurent, 1996). Similarly, investigations on human emphysematous lungs report increased synthesis of elastin (Fukuda et al., 1989). In a recent study, Deslee et al. (2009) reported that elastin mRNA expression was significantly increased in the alveolar walls in very severe COPD compared to donors, non-COPD and stages 2–3 COPD), but elastic fibre content was not increased per lung volume. Consistently, we found an increase in the basal soluble elastin production by fibroblasts from COPD subjects. The loss of elastic fibres, therefore, might be result from the assumption that enhanced elastin synthesis is not sufficient to restore elastic fibres with insoluble crosslinked elastin. Previous studies indicate that enhanced synthesis of versi-

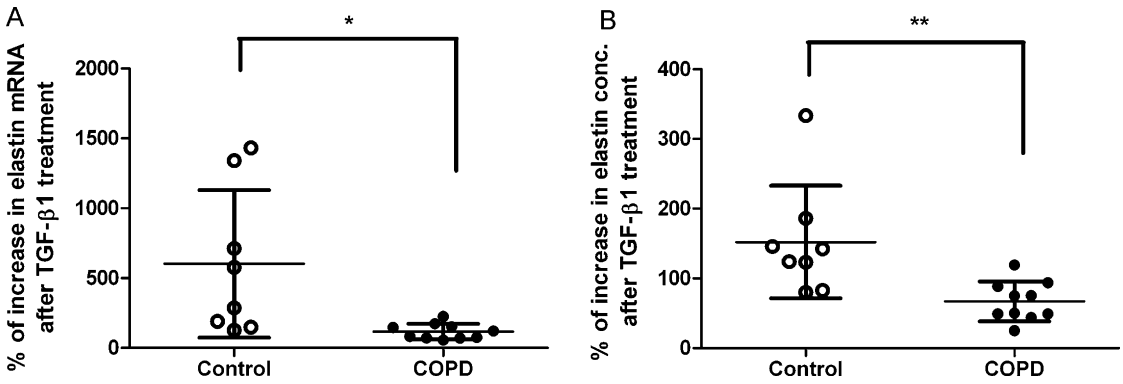

Fig. 2. TGF-β1 had little stimulative effect on the synthesis and secretion of elastin by primary lung fibroblasts from subjects with COPD. Cells were cultured in the presence or absence of 1 ng/ml TGF-β1 and the percentage of increase in elastin mRNA (A) and protein production (B) were measured using real-time PCR and Fastin elastin assay respectively. Control indicates subjects with normal lung spirometry ($n = 8$); COPD indicates subjects with COPD ($n = 10$). Data are expressed as mean ± SD. $^{*}p < 0.05$, and $^{**}p < 0.01$.

can likely explains the inability of COPD fibroblasts to repair elastic fibres as this proteoglycan inhibits assembly of tropoelastin into fibres (Huang et al., 2006). Another explanation is that the enhanced tropoelastin production after injury is not sufficient to compensate for the proteolytic degradation and destruction.

TGF-β1 is an important cytokine for matrix formation and tissue repair, and has been shown to promote the synthesis of elastin (Kuang et al., 2007; Kucich et al., 2002; McGowan et al., 1997). Thus it would be expected to have a role in repair of elastic fibres lost as a result of smoking or other inflammatory insults. The stimulative effect of TGF-β1 on elastin mRNA transcription and protein production observed in lung fibroblasts from subjects of normal lung function in our study is consistent with the repair function of TGF-β1. However, such enhanced synthesis response was not present in the fibroblasts from COPD patients. This lack of response to TGF-β1 indicates that fibroblasts undergo a significant phenotypic change with the development of COPD. Together with the slower proliferation of pulmonary fibroblasts, these findings provide a new explanation for the fact that patients with COPD have an impaired capacity to form enough new elastic fibres to compensate for the degradation and destruction.

Although the interaction of TGF-β1 with its cell surface receptors has been extensively investigated, the precise molecular events down-stream from the activated receptors that mediate responses elicited by TGF-β1 remains to be fully characterized. One study showed that reduced responsiveness to the gel contraction and chemotaxis effect of TGF-β observed in lung fibroblasts from COPD patients is associated with increased levels of the inhibitory protein Smad7 and not attributable to the alterations of TGF-β receptors (Togo et al., 2008). Apart from the Smad pathway, molecules in other signal transduction pathways, including phosphatidylinositol 3-kinase (PI_3K)-Akt and MAPKs, are also involved in intracellular TGF-β1 signalling (Chen et al., 2006). An *in vitro* study in human embryonic lung fibroblasts showed that it is PI_3K-Akt activation, but not p38 or p42/44 phosphorylation, that participates in the TGF-β induced elastin transcription (Kuang et al., 2007). Another potential mechanism may be differential release of PGE_2 which inhibits fibroblast proliferation. Togo et al. (2008) have reported that lung fibroblasts from COPD patients release more PGE_2 than fibroblasts from non-COPD patients.

These findings indicate that complex modulation and cross-talk between different signalling pathways might underlie the reduced response to TGF-β1 in COPD fibroblasts. Further studies will be needed to clarify precise mechanisms. It will also be important to demonstrate similar responses on fibroblasts taken from COPD patients without lung cancer, although in this study all patients had cancer, with the only difference being the COPD status. Thus any modulation of fibroblasts phenotype and subsequent differential responses to TGF-β1 by tumor derived cytokines would likely be similar in COPD and non-COPD fibroblasts.

In summary, primary lung fibroblasts from patients with COPD have a reduced responsiveness to TGF-β1 with respect of cellular proliferation and elastin synthesis. Our data suggest that restoration of the response to TGF-b1 may be an effective strategy to overcome the impaired repair capacity and alter the progression of COPD.

Acknowledgements

This paper is dedicated to our close friend and mentor Professor Peter Black who died suddenly in January 2010. Professor Black was instrumental in the conception and design of this study. His death is a huge loss to us personally and to the COPD research community. This work was financially supported by Auckland Medical Research Foundation, National Natural Science Foundation of China (No. 81000013),Shanghai Leading Talent Projects (2010No.036 to JMQ), and Shanghai Leading Academic Discipline Project (Project Number: B115).

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