ORIGINAL ARTICLE

VEGF synthesis is induced by prostacyclin and TGF-β in distal lung fibroblasts from COPD patients and control subjects: Implications for pulmonary vascular remodelling

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ABSTRACT

Background and objective: Involvement of pulmonary vascular remodelling is a characteristic sign in COPD. Vascular mediators such as vascular endothelial growth factor (VEGF) and prostacyclin may regulate fibroblast activity. The objective was to study the synthesis of VEGF and interactions with prostacyclin and transforming growth factor (TGF)-β1 in lung fibroblasts from patients with COPD and healthy control subjects. To further explore the autocrine role of synthesized VEGF on fibroblast activity, studies were performed in human lung fibroblasts (HFL-1).

Methods: Primary distal lung fibroblast cultures were established from healthy individuals and from COPD patients (GOLD stage IV). Lung fibroblasts were stimulated with the prostacyclin analogue iloprost and the profibrotic stimuli TGF-β1. VEGF synthesis was measured in the cell culture medium. Changes in proliferation rate, migration and synthesis of the extracellular matrix (ECM) proteins proteoglycans were analysed after stimulations with VEGF-A isoform 165 (VEGF165: 1–10 000 pg/mL) in HFL-1.

Results: Iloprost and TGF-β1 significantly increased VEGF synthesis in both fibroblasts from COPD patients and control subjects. TGF-β1-induced VEGF synthesis was significantly reduced by the cyclooxygenase inhibitor indomethacin in fibroblasts from COPD patients. VEGF significantly increased proliferation rate and migration capacity in HFL-1. VEGF also significantly increased synthesis of the ECM proteins biglycan and perlecan. The VEGF receptors (VEGFR), VEGFR1, VEGFR2 and VEGFR3, were all expressed in primary lung fibroblasts and HFL-1.

Conclusion: VEGF is synthesized in high amounts by distal lung fibroblasts and may have a crucial role in ongoing vascular remodelling processes in the distal lung compartments.

SUMMARY AT A GLANCE

Vascular endothelial growth factor (VEGF) is synthesized in high amounts by primary distally derived human lung fibroblasts. Our data indicate differences in cyclooxygenase (COX)-related responses in distal lung fibroblasts in COPD compared with healthy individuals, which may promote ongoing remodelling processes in the distal lung.

Key words: chronic obstructive pulmonary disease, extracellular matrix, fibroblasts, prostacyclin, vascular endothelial growth factor.

INTRODUCTION

Pulmonary vascular remodelling has an important role in the development of symptoms associated with reduced lung function in COPD.1,2 Vascular remodelling involves angiogenesis, bronchial vascularization and structural alterations of the vascular wall.1,3-6 Changes in vascular structures may impair gas exchange in alveoli resulting in hypoxic conditions with a number of systemic complications.7 Pulmonary hypertension with cardiovascular co-morbidity is common in COPD and associated with increased disease severity and mortality.8,9 Vascular endothelial growth factor (VEGF) is a pro-angiogenic growth factor that promotes proliferation and migration of endothelial cells. The most potent angiogenic member of the VEGF family is VEGF-A isoform 165 (VEGF165) that binds to VEGF receptor 2 (VEGFR2).10,11 VEGF is a key player in
pulmonary vascular remodelling and may therefore have a central role in COPD progression. VEGF is synthesized by epithelial cells, smooth muscle cells, mast cells, alveolar macrophages and fibroblasts. Fibroblasts are key regulators of extracellular matrix (ECM) proteins and constitute a rich source of growth factors. Interestingly, the vascular mediator prostacyclin may induce VEGF and prostacyclin is synthesized in high amounts by distal lung fibroblasts from severe COPD patients. Our recent data imply that severe COPD patients may have altered fibroblast function and defective repair mechanisms in the ECM structure in response to prostacyclin.

The aim of this study was therefore to study the synthesis of VEGF and its potential interactions with prostacyclin and the profibrotic mediator transforming growth factor (TGF)-β₁ in distal lung fibroblasts from COPD patients and control subjects. To further explore the possible role of VEGF on fibroblast activity, studies were performed in human lung fibroblasts (HFL-1) with the hypothesis that VEGF may affect ongoing remodelling processes in an autocrine fashion.

METHODS

Study subjects
Patient material from patients (n = 7) with severe COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV) undergoing lung transplantation at Lund University Hospital during 2006–2008, were included in this study. The patients were all ex-smokers, mean age 62 (53–66) and had stopped smoking at least 6 months before transplantation. Non-smokers (n = 5) with no history of smoking or other lung diseases, mean age 28 (19–29), were included as control subjects. Written consents were obtained from all participants. The study was approved by the Swedish Research Ethical Committee in Lund (FEK 213/2005, FEK 91/2006 and FEK 413/2008).

Primary distal lung fibroblast cultures and HFL-1
Primary distal lung fibroblast cultures were obtained from lung explants from COPD patients after lung transplantation and from transbronchial biopsy samples from control subjects as previously described. Primary lung fibroblasts and HFL-1 (CCL-153, ATCC, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% foetal clone serum (FCIII, Thermo Scientific, Waltham, MA, USA), 1% L-glutamine, 0.5% gentamicin and 5 μg/mL amphotericin B (all from Gibco BRL, Paisley, UK) at 37°C and 10% CO₂. Primary lung fibroblasts were used in passages 4–7 and HFL-1 in passages 16–21. DMEM supplemented with 0.4% FCIII was used throughout experimental conditions to avoid interactions with existing growth factors in the serum.

Analysis of VEGF₁₆₅ synthesis
VEGF synthesis was measured in cell culture medium by a commercially available ELISA kit for VEGF₁₆₅ (R&D Systems, Abingdon, England). To study the effects of prostacyclin and TGF-β₁ on VEGF synthesis, primary distal lung fibroblasts were pretreated with the unspecific cyclooxygenase (COX) inhibitor indomethacin (3 μM) to avoid interference with endogenous produced prostaglandins and stimulated with the prostacyclin analogue iloprost (1000 nM) (both from Cayman Chemicals, Linham, Sweden) and TGF-β₁ (10 ng/mL) (R&D Systems) in 0.4% serum for 24 h. The selective concentrations of indomethacin, iloprost and TGF-β₁ were chosen from previous stimulations in lung fibroblasts.

Immunocytochemistry
Fibroblasts (7000 cells/well) grown overnight on chamber slides were fixed in 4% paraformaldehyde. VEGFR1 antibody (Abcam, Cambridge, MA, USA), VEGFR2 antibody (AbDserotec, Oxford, UK) or VEGFR3 antibody (all diluted 1:200 in Tris Buffered Saline (TBS) with 1% BSA) were applied for 90 min in room temperature. Slides were rinsed in TBS and corresponding secondary antibody conjugated with Alexa fluorochrome 488 (diluted 1:200) (Molecular Probes Life Technologies, Carlsbad, CA, USA) applied together with DAPI (4’,6-diamidino-2-phenylindole) (300 nM) (Invitrogen Corp, Carlsbad, CA, USA) for nuclei staining. Slides were mounted in fluorescent mounting medium (Dako, Merck Life Science, Darmstadt, Germany).

Analysis of proteoglycan production
Proteoglycan production was determined as previously described. HFL-1 were cultured in 6-well plates and at confluence incubated in low sulphate DMEM (Gibco BRL) and stimulated with VEGF₁₆₅ (1–10 000 pg/mL) (R&D Systems) in [35S]-containing DMEM for 24 h. Proteoglycans in the cell medium were purified by ion exchanger DEAE52 and quantified by [35S]-sulphate incorporation on a scintillation counter (Wallac; Perkin Ellmer, Boston, MA, USA). The proteoglycans, biglycan, perlecan, versican and decorin, were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by densitometry.

Cell proliferation
Cell proliferation rate was determined as previously described. HFL-1 were plated in 96-well plates (Cellstar, Monroe, NC, USA) for 6 h and then stimulated with VEGF₁₆₅ (1–10 000 pg/mL) for 24 and 48 h. Cells were fixed in 1% glutaraldehyde (Sigma-Aldrich), stained with 0.1% crystal violet (Sigma-Aldrich), treated overnight with 1% Triton X (Merck, Darmstadt, Germany) and then quantified with a spectrophotometer plate reader at 595 nm.

Migration assay
Migration was analysed by seeding 130 000 cells/well in 6-well plates. At confluency, cells were incubated in 0.4% serum for 24 h. A scratch was made in the cell layer in each well with a 1-ml pipette tip and cells were stimulated with VEGF₁₆₅ (1–10 000 pg/mL) for 72 h. Changes in scratch area were imaged and...
analysed using the programme T-scratch (ETH, Zürich, Switzerland).

Statistical analysis
Data are shown for individual subjects as absolute values and presented as median. The non-parametric Mann–Whitney t-test was used to compare statistical differences between two groups. Two-ways repeated measurement analysis of variance (ANOVA) on ranks followed by the non-parametric Dunn’s post hoc test were used to compare differences in pharmacological treatments. Data for HFL-1 are presented as mean ± SEM and statistical analysis are performed with Student’s t-test and one-way repeated measurement ANOVA followed by the Holm-Sidak post hoc test.

Differences were considered to be statistical significant at $P < 0.05$. All analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Increased VEGF synthesis to iloprost and TGF-β1 in primary distal lung fibroblasts
VEGF synthesis was increased after stimulation with 1000 nM iloprost ($P < 0.05$; Fig. 1A,B) and 10 ng/mL TGF-β1 ($P < 0.01$; Fig. 1C,D) in fibroblasts from control subjects and COPD patients, but there were no significant differences in VEGF synthesis between fibroblasts from control subjects or COPD patients (Fig. 1).

![Diagram](image-url)

Figure 1  The prostacyclin analogue iloprost (1000 nM) significantly increased vascular endothelial growth factor (VEGF) synthesis in distal lung fibroblasts from control subjects ($n = 5$; A) and patients with COPD ($n = 7$; B). Transforming growth factor (TGF)-β1 (10 ng/mL) significantly enhanced VEGF synthesis in distal lung fibroblasts from control subjects ($n = 5$; C) and patients with COPD ($n = 7$; D) compared with unstimulated fibroblasts (A and B). TGF-β1-induced VEGF synthesis was significantly decreased by the unselective cyclooxygenase (COX) inhibitor indomethacin (3 µM) and in combination with iloprost in lung fibroblasts from COPD patients ($n = 7$; D) but not in fibroblasts from control subjects ($n = 5$; C). Iloprost had no further effect on VEGF synthesis after TGF-β1 stimulation on fibroblasts from control subjects (C). Other prostaglandins than prostacyclin appear to be involved in TGF-β1-induced VEGF synthesis in fibroblasts from COPD patients (D). Data are presented as median with individual values (the different symbols represent the individual subjects (control subjects 1-5 and COPD subjects 1-7)) and median values are represented as a line. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical analyses are performed with analysis of variance (ANOVA) on ranks followed by Dunn’s post hoc test to compare differences between control and COPD fibroblasts and within the control and COPD fibroblasts after iloprost and TGF-β1 stimulation.
Treatments with indomethacin and iloprost had no further effect on VEGF synthesis compared with TGF-β1 alone in fibroblasts from control subjects (Fig. 1C). However, other prostaglandins appear to be involved in VEGF-mediated responses to TGF-β1 in COPD as treatment with indomethacin and iloprost in combination with TGF-β1 significantly reduced VEGF synthesis ($P < 0.05$; Fig. 1D) and iloprost treatment had no further inhibitory effects compared with indomethacin in fibroblasts from COPD patients.

**Expression of VEGFR in lung fibroblasts**

Primary lung fibroblasts from COPD patients and control subjects and HFL-1 all expressed VEGFR1, VEGFR2 and VEGFR3 (Fig. 2).

**Effect of VEGF165 stimulation on proliferation rate and migration capacity**

VEGF synthesis was significantly increased by TGF-β1 (10 ng/mL) in HFL-1 ($P < 0.01$; Fig. 3A). VEGF significantly increased proliferation rate after 48 h (Fig. 3B) and migratory capacity at 100 pg/mL of VEGF after 24 h ($P < 0.05$), 48 h ($P < 0.01$) and 72 h ($P < 0.05$; Fig. 4) in HFL-1.

**Effect of VEGF165 stimulation on proteoglycan synthesis in HFL-1**

Interestingly, VEGF significantly increased synthesis of the individual proteoglycans perlecan at 10 pg/mL of VEGF ($P < 0.05$; Fig. 5A) and biglycan at 10–10 000 pg/mL of VEGF ($P < 0.05$; Fig. 5B). Versican (Fig. 5C) and decorin (Fig. 5D) synthesis were not significantly altered by VEGF stimulation in HFL-1.

**DISCUSSION**

In this study, we show that VEGF synthesis is induced by the prostacyclin analogue iloprost and TGF-β1 in distal lung fibroblasts from control subjects and patients with severe COPD, and that VEGF promotes proliferation, migration and proteoglycan synthesis in HFL-1. Other studies have shown that VEGF synthesis

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![Figure 2](image-url)  
**Figure 2** Representative stainings are shown in green for vascular endothelial growth factor receptor 1 (VEGFR1) (A, D, G), VEGFR2 (B, E, H) and VEGFR3 (C, F, I), and nuclei staining with DAPI (4',6-diamidino-2-phenylindole) in blue in lung fibroblasts from control subjects (A–C), patients with COPD (D–F) and in human lung fibroblast (HFL-1) (G–I). Scale bars are indicated as 50 μm in (A–I). Image analyses for fibroblasts from COPD patients or control subjects were scanned and analysed using the ScanScope slide scanner (Aperio, Vista, CA, USA) and image analyses for HFL-1 were performed with Nikon Eclipse microscope, camera Nikon DS-Qi1Mc and software programme NIS-Elements AR 3.0 (Nikon, Tokyo, Japan).
is increased by prostacyclin analogues in other cell types\(^{15,32,33}\).

The crosstalk between prostacyclin and VEGF may be important during inflammation in distal lung by promoting angiogenesis in hypoxic areas with poor circulation and ventilation. Altered VEGF expression in combination with hypoxia may thereby be crucial in the onset of vascular remodelling\(^ {34}\). Expression of

![Figure 3](image1)

**Figure 3** Vascular endothelial growth factor (VEGF) synthesis and effect of VEGF on proliferation in human lung fibroblast (HFL-1). VEGF synthesis was significantly increased after stimulation with transforming growth factor (TGF)-\( \beta_1 \) (10 ng/mL) compared with medium alone in HFL-1 \((n = 5, A)\). VEGF significantly enhanced proliferative rate after 48 h at the concentrations 10–10 000 pg/mL \((n = 5, B)\), data are presented as mean ± SEM and statistical analysis are performed with Student’s t-test and one-way repeated measurement analysis of variance (ANOVA) followed by the Holm–Sidak post hoc test. **\(P < 0.01\); ***\(P < 0.001\).

![Figure 4](image2)

**Figure 4** Effect of vascular endothelial growth factor (VEGF) on migratory capacity in human lung fibroblast (HFL-1). VEGF 100 pg/mL increased migratory capacity measured by closure of scratch after 24, 48 and 72 h \((n = 5\) individual experiments, A) \(\square\) control; \(\square\) VEGF 1 pg/mL; \(\square\) VEGF 10 pg/mL; \(\square\) VEGF 100 pg/mL; \(\square\) VEGF 1000 pg/mL; \(\square\) VEGF 10 000 pg/mL). Representative images of scratch from starting point \((0\) h) and after 72 h in control well \((\text{only} \ 0.4\% \ \text{medium}, B)\) and stimulation with VEGF 100 pg/mL \((C)\). The red points are markers for imaging at the different time points. Data are expressed as % of scratched area from day 0 presented as mean ± SEM. Statistical analysis is performed with Student’s t-test and one-way repeated measurement analysis of variance (ANOVA) followed by the Holm–Sidak post hoc test. *\(P < 0.05\); **\(P < 0.01\).
prostacyclin synthase was upregulated in HFL-1 promoting VEGF synthesis in tumours during hypoxic conditions. Importantly, in our previous study, distal lung fibroblasts from severe COPD patients had an altered response to prostacyclin that resulted in a defect repair of the collagen network that could affect emphysema progression. A noteworthy finding in this study was that fibroblasts from COPD patients, in contrast to control subjects, responded to COX inhibition after TGF-β1 stimuli with decreased VEGF synthesis independent of iloprost treatment. These data imply that other prostaglandins, such as PGE2, are involved in VEGF-mediated responses in COPD. PGE2 is released in high amounts by COPD fibroblasts and has been shown to induce VEGF via PGE2 receptor EP2 (E-prostanoid 2) and cAMP activation.

In a small study, we showed that TGF-β1 induced high amounts of both prostacyclin and PGE2 in distal lung fibroblasts from severe COPD patients compared with control subjects. Interestingly, activation of the COX-2 pathway in endothelial cells may promote VEGF-induced angiogenesis through p38 and c-Jun N-terminal kinase (JNK). However, except for the COX-mediated response, we could not detect any significant differences in VEGF synthesis between fibroblasts from patients with severe COPD and control subjects. In line with these findings, expression of VEGF in pulmonary arteries did not differ between patients with severe COPD with emphysema and non-smoking control subjects, whereas patients with mild to moderate COPD showed increased expression of VEGF.

Increased VEGF expression was associated with bronchial angiogenesis that inversely correlated with lung function in COPD patients. COPD patients with chronic bronchitis had increased VEGF levels in sputum in contrast to COPD patients with emphysema who had decreased VEGF levels. Patients with acute exacerbations presented high levels of VEGF in the circulation compared with stable COPD patients and healthy subjects, and increasing circulating VEGF longitudinal course appears to be related to poorer COPD prognosis. In this study, all the VEGFR were expressed by the lung fibroblasts, and especially VEGFR3, which has not previously been shown. A decreased expression of VEGFR2 in parenchymal

Figure 5 Effect of vascular endothelial growth factor (VEGF) on synthesis of the extracellular matrix (ECM) proteins proteoglycans. VEGF (10–10 000 pg/mL) significantly increased synthesis of the individual measured proteoglycans perlecan (A) and biglycan (B), whereas there were no significant effects on versican (C) or decorin (D). Proteoglycan synthesis was related to the total amount of proteins in the corresponding cell layer that were analysed by a commercially available protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Data are presented as mean ± SEM and statistical analysis is performed with one-way repeated measurement analysis of variance (ANOVA) followed by the Holm–Sidak post hoc test. n = 6 with duplicates in each experiment. *P < 0.05.

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regions in severe COPD patients did correlate with increased endothelial cell death\(^5\) and inhibition of VEGFR2 in an animal model resulted in emphysematous lung structure and cell apoptosis.\(^{20}\) Interestingly, VEGF may act both as a promoter of endothelial cell function and a negative regulator of vascular smooth muscle cells and vessel maturation in combination with platelet-derived growth factor,\(^{35}\) highlighting the complex role of VEGF in vascular remodelling. VEGF has the ability to bind to multiple proteins, such as proteoglycans, present in the ECM.\(^{36,38}\)

In this study, we showed for the first time that VEGF significantly increased synthesis of the proteoglycans biglycan and perlecan in HFL-1. Biglycan is important for migration of cells\(^39\) and may upregulate VEGF expression.\(^{40}\) VEGF-A gene expression was significantly decreased in biglycan-deficient mice.\(^38\) Perlecan is a major ECM protein, essential for the structure of vascular basement membranes\(^30,36\) and a crucial cofactor for VEGF binding and storage of VEGF.\(^36\) The interaction between perlecan and VEGF-A induced VEGFR2 signalling in endothelial cells\(^41\) and downregulation of perlecan resulted in reduced angiogenesis in vivo.\(^42\)

Although the ECM changes are minor, our data imply that biglycan and perlecan may have unique roles in regulating cell migration and angiogenesis, related to VEGF storage or stabilization of the ECM. Interestingly, our previous data showed that perlecan and biglycan synthesis are reduced in fibroblasts from severe COPD patients.\(^30\)

Recently, promising results have been obtained with kinase inhibitor in patients with idiopathic pulmonary fibrosis that acts partly on targets downstream of VEGFR (Hostettler et al., 2014).\(^{43}\) However, the role of a kinase inhibitor on different stages of COPD is unknown and VEGF treatment may have different outcomes depending on disease progression and severity. COPD patients with milder stages of COPD and with peribronchial fibrosis may respond more favourably to anti-VEGF treatment, whereas severe COPD patients with emphysema may benefit for therapies that restore the VEGF/VEGFR2 axis. In this study, we did not have access to distal lung fibroblasts from earlier stages of COPD or smokers and therefore used HFL-1 for exploring the role of VEGF on fibroblast function. Our results are probably linked to disease stage since we could not detect any differences related to age or different sampling techniques, that is, transbronchial biopsies versus lung explants, in our previous studies.\(^{18,30}\)

Overall, VEGF generates small effects on lung fibroblasts such as migration, proliferation and ECM synthesis. In conclusion, as proof of concept, our in vitro data indicate that VEGF is induced by prostacyclin and TGF-β and synthesized in high amount by distally derived lung fibroblasts, and VEGF acts in an autocrine fashion by increasing ECM synthesis, migration and proliferation of HFL-1. Our data also indicate differences in COX-related responses in distal lung fibroblasts in COPD compared with healthy individuals, which may promote ongoing remodelling processes in the distal lung.

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12. Santos S, Peinado VI, Ramirez J, Morales-Blanhir J, Bastos R, Roca J, Rodriguez-Roisin R, Barbera JA. Enhanced expression of vascular endothelial growth factor in pulmonary arteries of smokers and therefore used HFL-1 for exploring the role of VEGF on fibroblast function. Our results are probably linked to disease stage since we could not detect any differences related to age or different sampling techniques, that is, transbronchial biopsies versus lung explants, in our previous studies.\(^{18,30}\) Overall, VEGF generates small effects on lung fibroblasts in this study but may nevertheless have an important modulatory role in autocrine and paracrine fashions in distal lung. Importantly, the synthesized levels of VEGF by the fibroblasts are in line with the concentration of VEGF that shows most prominent effects on the fibroblasts such as migration, proliferation and ECM synthesis. In conclusion, as proof of concept, our in vitro data indicate that VEGF is induced by prostacyclin and TGF-β and synthesized in high amount by distally derived lung fibroblasts, and VEGF acts in an autocrine fashion by increasing ECM synthesis, migration and proliferation of HFL-1. Our data also indicate differences in COX-related responses in distal lung fibroblasts in COPD compared with healthy individuals, which may promote ongoing remodelling processes in the distal lung.


