Aberrant TGFβ1 Signalling and Cross-talk with Angiotensin II Signalling in COPD

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a place of mind

Abstract

Rationale: The airflow limitation in Chronic Obstructive Pulmonary Disease (COPD) is caused by either emphysematous destruction of parenchymal tissue, or small airways obstruction and/or obliteration. Our group previously characterized a gene expression signature for emphysema that included members of the TGFβ1 and Angiotensin (ANG) II signaling family. Further, we have shown that isolated parenchymal lung fibroblasts from severe COPD patients are unable to contract collagen 1α1 efficiently, but this impairment is reversed with the addition of TGFβ1. Thus, we hypothesize that dysregulated ANG II-TGFβ1 crosstalk within the lung fibroblasts of COPD patients disrupts normal wound repair processes leading to disease.

Methods: Parenchymal lung fibroblasts were isolated from the normal region of resected lung tissue from ex-smokers with healthy lung function, moderate COPD (GOLD 2) or severe COPD (GOLD 4) (n=5 each group). To evaluate changes in signalling induced by TGFβ1, cells were treated at passage 2 or 3 with TGFβ1 (10 ng/mL) or ANG II (50 nM), and supernatant and protein were collected at 48h for immunoblot analysis. Comparisons were analyzed using one-way ANOVA and student's t test.

Results: Our preliminary results indicate that there is a trend for decreased angiotensin II receptor type 1 (AT1R) expression at baseline between COPD and healthy control parenchymal fibroblasts. Specifically, this was observed in the moderate COPD (GOLD 2) derived fibroblasts. Treatment of parenchymal fibroblasts with TGFβ1 significantly further decreased AT1R protein expression ($p<0.05$), while angiotensin II receptor type 2 (AT2R) expression was not affected. Interestingly, fibroblasts derived from moderate COPD (GOLD 2) responded most strongly to TGFβ1 treatment in the reduction of AT1R expression compared to the control and severe COPD (GOLD 4) fibroblasts, indicating heterogeneity within disease severity. All fibroblasts demonstrated functional TGFβ1 canonical (SMAD2/3) signalling ($p<0.05$) but defective non-canonical (ERK/MAPK) signalling in response to TGFβ1 treatment. Collagen 1 production of parenchymal fibroblasts seemed to decrease with disease severity, but all increased production in response to TGFβ1 treatment, while angiotensin II increased collagen 1 production only in healthy control fibroblasts.

Conclusions: Our preliminary data indicate that the TGFβ1-ANG II signalling axis is dysfunctional in COPD parenchymal fibroblasts demonstrated by decreased AT1R expression via decreased ERK/MAPK signalling. This has important implications for understanding the disease biology of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide (1) and is the only disease with an increasing death rate, largely due to the ageing of the "Baby Boomer" population. The disease predominantly afflicts the elderly and is characterized by emphysematous destruction of the gas exchange surface and small airway obstruction and/or narrowing which lead to irreversible airflow limitation.

Previously, many groups have attempted to determine the molecular alterations that result in the development of emphysema. However, these studies have produced conflicting results due to the heterogeneous distribution of emphysematous lesions in diseased lungs and random sample acquisition. Therefore, our group used a combination of micro X-ray computed tomography to determine emphysema severity in lung tissue to correlate with the microarray gene expression profile of adjacent tissue. Our novel technique produced a molecular gene expression signature for emphysema, and we found that the top down-regulated genes in emphysema are involved in tissue repair and remodeling processes, including the Transforming growth factor (TGF)-β1, Angiotensin (ANG) and integrin signalling pathways.

The main cells involved in wound repair are fibroblasts due to their ability to produce extracellular matrix proteins such as collagen 1, and contract surrounding tissue to reduce wound size. The downregulation of many critical genes for fibroblast function suggests that these cells may have an aberrant phenotype in disease. TGFβ1 plays many roles in the wound repair process and is a potent activator of fibroblast function. Due to its pleiotropic effects, TGFβ1 is regulated by many factors including ANG II. Studies by other groups have shown a close relationship between the two signalling pathways that can form a positive feedback loop to perpetuate a profibrotic response in lung parenchymal fibroblasts (3).

Hypothesis

We hypothesize that dysregulation of ANG II-TGFβ1 crosstalk within the lung fibroblasts of COPD patients disrupts normal wound repair leading to disease.

Methods

Primary Fibroblast Isolation and Culture
Primary lung parenchymal fibroblasts from healthy control, moderate COPD (GOLD II) and very severe COPD (GOLD IV) ex-smokers were obtained through the kindness of our collaborators in Groningen, Netherlands and Hamilton, Ontario (Table 1). Lung tissue was obtained from patients undergoing surgery for lung resection, and the cells were isolated from the normal regions. Fibroblasts were harvested by seeding six-well tissue culture plates with tissue cut into 1 mm cubes and the growth medium Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1x antiobiotic-antimycotic, incubated at 37°C with 5% CO_2. After the fibroblasts had diffused out of the tissues, the tissues were removed, and cells were grown in monolayer cultures in the growth medium.

Table 1. Groningen and Hamilton primary ex-smoker lung fibroblast donor information

Donor	Age	Pack Years	M/F	FEV_1	FEV_1/FVC
Healthy (n=9)	68.7 ± 11.1	45.7 ± 21.8	6M/3F	91.5 ± 14.7	0.83 ± 0.10
COPD GOLD II (n=5)	72.0 ± 3.2	29.2 ± 12.5	5M	65.4 ± 12.3	0.54 ± 0.13
COPD GOLD IV (n=5)	58.8 ± 1.5	42.6 ± 15.7	2M/3F	18.5 ± 5.5	0.33 ± 0.19

TGFβ1 and ANG II Treatment of Lung Parenchymal Fibroblasts
Primary lung fibroblasts were grown to 70% confluence in 6-well tissue culture plates in 1 ml growth media then treated with or without recombinant human (rh) TGFβ1 (10 ng/ml) or ANG II (10 nM). Cells were incubated at 37°C with humidified air in 5% CO_2 and lysed after 24 h with buffer RLT containing β-mercaptoethanol to collect RNA, and with protein extraction buffer with protease and phosphatase inhibitor cocktails after 48 h to harvest protein.

Complementary DNA (cDNA) Synthesis and Real-time Polymerase Chain Reaction (RT-PCR)
mRNA was isolated using the Qiagen RNeasy Kit according to the manufacturer's protocols. mRNA concentration and purity was measured using NanoDrop 8000 UV-Vis Spectrophotometer. cDNA was created with 1 μg RNA using the SuperScript® II Reverse Transcriptase protocol. RT-PCR was performed according to the TaqMan® Gene Expression Assay protocol for both AT1R and GAPDH predesigned assays using a standard run on the Applied Biosystems ViiA7 Real-Time PCR Machine as follows: hold at 95°C for 10 min then 40 cycles between 15 sec at 95°C and 1 min at 60°C. Gene expression was calculated using the comparative CT method, and expression of AT1R was normalized to GAPDH to acquire the ΔΔCT values.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis and Immunoblot
Protein samples were loaded at equal concentrations in a SDS-polyacrylamide gel for electrophoretic resolution of the proteins of interest. Proteins in the gels were transferred to 0.45 μm nitrocellulose membranes, and probed with a cocktail of primary antibodies under denaturing conditions: the rabbit polyclonal antibody against AT1R, the rabbit polyclonal antibody against AT2R, the rabbit polyclonal antibody against SMAD2/3, the rabbit monoclonal antibody against phospho-SMAD2/3, the mouse monoclonal antibody against phospho-ERK1/2, the rabbit polyclonal antibody against ERK1/2, the rabbit polyclonal antibody against p38, the mouse monoclonal antibody against phospho-p38, the mouse monoclonal antibody against AKT and the rabbit polyclonal antibody against phospho-AKT. Supernatants were also loaded at equal concentrations into protein gels and after transfer onto 0.45 μm nitrocellulose membranes, was probed under native conditions with the rabbit polyclonal antibody against collagen 1, Alexa Fluor 680 goat anti-rabbit polyclonal antibody and the IRDye 800 conjugated goat anti-mouse polyclonal antibody were used to detect the corresponding primary antibodies. The membranes were imaged with the Odyssey Infrared Imaging System and protein band intensities were analyzed with the Odyssey software 1.1 (LI-COR Biotechnology). Results are expressed as protein of interest/β-tubulin ratios, and signaling activity is presented as ratios of phosphorylated form/total amount of the protein of interest, both of which are normalized to β-tubulin.

TGFβ1 Enzyme-Linked Immunosorbent Assay (ELISA)
Supernatants were diluted and acidified with 1N HCl to measure total TGFβ1 released by the parenchymal fibroblasts. The ELISA was performed according to the TGFβ1 E_{max}® Immunoassay protocol. The absorbance was measured at 450 nm using the Spectra Rainbow Microplate Reader (SLT Lab Instruments).

Whole Lung Sample Acquisition and Processing for NanoString
Lungs were obtained from COPD GOLD IV patients (n=3) who underwent lung transplantation at the University of Pennsylvania and from the Gift of Life Organ Procurement Organization in Philadelphia for donor lungs without COPD (n=3). The lungs were frozen in liquid nitrogen vapor after air inflation and cut into 2 cm thick slices. RNA was isolated using the Qiagen RNeasy Kit from a tissue cores extracted from each slice of lung according to the manufacturer's protocols. Isolated mRNA was sent to NanoString Technologies for gene expression analysis for AT1R, AT2R, COL1A1, TGFBR2, TGFBR1 and TGFB1. Gene count normalization was performed according to the nCounter™ Data Analysis Guidelines.

Statistics
Data are presented as mean ± SEM of at least 5 independent experiments. Paired comparisons were analyzed with the Student unpaired *t* test and one-way analysis of variance with Tukey post test was used for comparison of group data. All statistical analysis was done using Prism Version 5.04 (GraphPad Software Inc., San Diego, CA), with *P* values less than 0.05 considered statistically significant.

Results

Figure 1. AT1R protein expression is downregulated by TGFβ1 in parenchymal fibroblasts

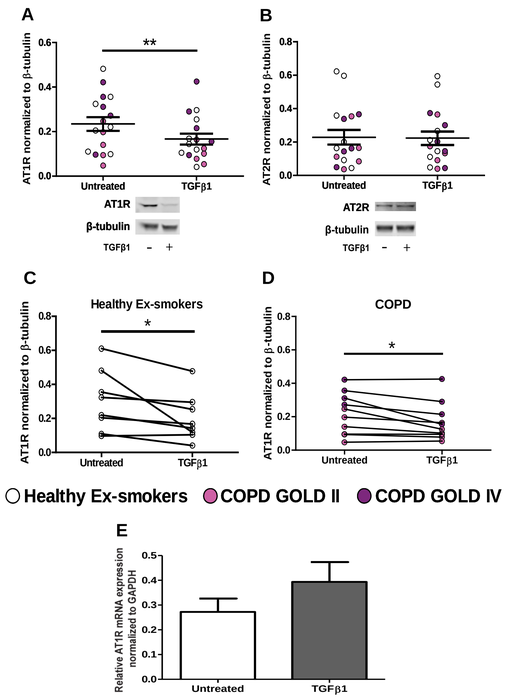

Figure 1. TGFβ1 down-regulates AT1R expression in lung parenchymal fibroblasts. Primary parenchymal fibroblasts from healthy ex-smokers (white circles, n=7), COPD GOLD II (pink circles, n=5) and COPD GOLD IV (purple circles, n=5) were treated with 10 ng/mL recombinant human TGFβ1 for 48 h to determine its effect on (A) AT1R and (B) AT2R protein expression. AT1R protein expression in response to TGFβ1 was plotted separately for (C) healthy ex-smokers, (D) COPD GOLD II and GOLD IV to determine differences in response. (E) AT1R mRNA expression was assessed by RT-PCR from healthy ex-smoker parenchymal fibroblasts (n=6) treated for 24 h with TGFβ1 (10 ng/mL) in 1% serum. * $p<0.05$ and ** $p<0.01$ by paired t-test.

Figure 2. TGFβ1 signalling is preserved in parenchymal fibroblasts derived from healthy ex-smokers and COPD patients

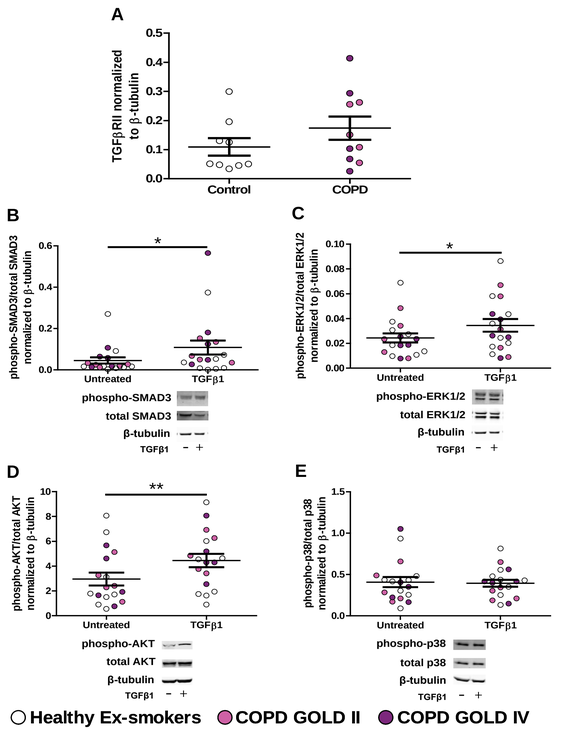

Figure 2. TGFβ1 signalling is intact in healthy and COPD ex-smoker parenchymal fibroblasts. Baseline protein expression of (A) TGFβRII was measured in healthy ex-smoker (white circles, n=9), COPD GOLD II (pink circles, n=5) and COPD GOLD IV (purple circles, n=5) parenchymal fibroblasts by immunoblot. To assess the functionality of TGFβ1 signalling, fibroblasts were treated with TGFβ1 (10 ng/mL) for 48 h, and activation of (B) SMAD, (C) ERK, (D) PI3K and (E) p38 pathways were assessed by measuring the phosphorylated to total protein ratios of the candidate signalling molecules for each pathway. * indicates $p<0.05$ and ** $p<0.01$ using paired t-test.

Figure 3. ANG II does not augment TGFβ1 production of ex-smoker parenchymal fibroblasts

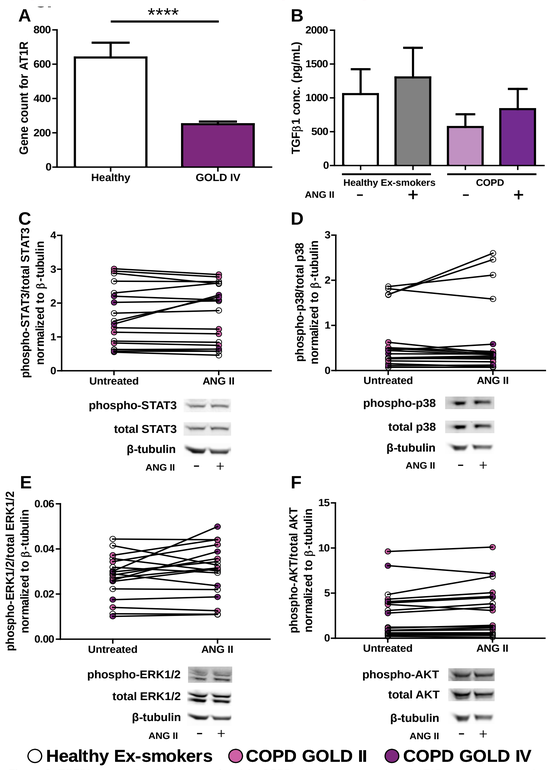

Figure 3. TGFβ1 expression is downregulated in COPD. (A) Gene expression of AT1R was measured using NanoString from healthy (n=3) and COPD GOLD IV (n=3) donor lungs. To evaluate the effect of ANG II on TGFβ1 production, the levels of TGFβ1 in parenchymal fibroblast culture supernatants measured by ELISA from healthy ex-smokers (gray bars, n=9) and COPD (purple bars, n=10) donors treated with ANG II for 48 h. Activation of (C) STAT3, (D) p38, (E) ERK and (F) PI3K activities were assessed in healthy ex-smoker (white circles, n=9), COPD GOLD II (pink circles, n=5) and GOLD IV (maroon circles, n=5) parenchymal fibroblasts after stimulation with 100 nM ANG II for 48 h by measuring the phosphorylated to total protein ratios of each candidate signalling molecule. **** signifies $p<0.0001$ using a student's t-test.

Figure 4. Collagen 1 production is augmented in COPD

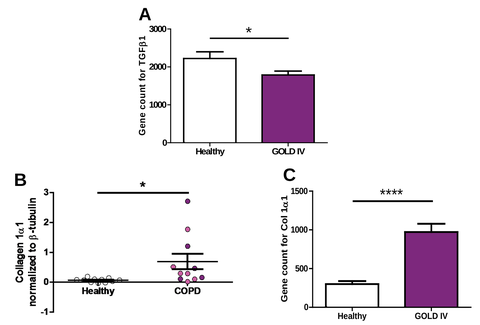

Figure 4. TGFβ1 augments collagen 1 production in primary parenchymal fibroblasts. Gene expression of (A) TGFβ1 was measured by NanoString in lung tissues extracted from healthy donors (n=3, open bar) and COPD GOLD IV patients (n=3, filled bar). To assess the effect of TGFβ1 on collagen 1 production, fibroblasts from donors with healthy lung function (white circles, n=11) or with COPD (GOLD II: pink circles, n=6, GOLD IV: purple circles, n=4) were treated with or without TGFβ1 (10 ng/mL) for 48 h. To examine differential responses to TGFβ1, (B) the level of TGFβ1-induced collagen 1α1 production was compared between the healthy ex-smokers and COPD donors, and validated with (C) gene expression analysis of collagen 1α1 in donor lungs. * is $p<0.05$ with a paired t-test while for gene expression analysis, * is $p<0.05$ and **** signifies $p<0.0001$ using a student's t-test.

Summary

- TGFβ1 downregulates AT1R protein expression in ex-smoker parenchymal-derived fibroblasts, but this response is blunted in COPD parenchymal fibroblasts.
- TGFβ1 signaling was shown to be intact in both healthy and COPD-derived parenchymal fibroblasts, thereby suggesting that TGFβ1 may mediate downregulation of AT1R by regulating receptor degradation.
- There is greater collagen 1 production in COPD lung tissues and parenchymal derived fibroblasts, which may be due to an over-exuberant response to TGFβ1.

Future Directions

- Examine the effect of TGFβ1 on AT1R receptor degradation.
- Evaluate the impact of the aberrant TGFβ1-ANG II crosstalk on COPD parenchymal fibroblast repair functions.

References
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