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High ICAM-1 gene expression in pulmonary fibroblasts of COPD patients: a reflection of an enhanced immunological function

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Summary

Chronic obstructive pulmonary disease (COPD) is characterized by destruction of extracellular matrix (ECM) in parenchymal areas, whereas the bronchial walls can show fibrosis. In addition, an extensive inflammatory process is observed. CD8+ T cells, located throughout the lung, and epithelial cells in centrally located airways produce cytokines involved in the inflammatory process. These cytokines may influence the present fibroblasts, the key effectors in the defective ECM repair and maintenance in COPD.

We explored the effects of the cytokine microenvironment on cell-cell interaction gene expression in pulmonary fibroblasts of controls (n=6), GOLD stage II (n=7) and stage IV (n=7) COPD patients. We simulated the *in vivo* microenvironment using supernatants of CD3/CD28 stimulated CD8+ T cells isolated from peripheral blood of COPD patients, supernatant of a bronchial epithelial cell line, or a combination of both.

Our data shows that fibroblasts of COPD patients display an altered response to the cytokine microenvironment, depending on both the disease stage and the central or peripheral location in the lung. Especially adhesion related genes are upregulated in fibroblasts of COPD patients, which can indicate a more pronounced role of fibroblasts in the inflammatory process in COPD, possibly resulting in reduced function as effectors of ECM repair.

Keywords: COPD, lung fibroblasts, ICAM-1, chronic inflammation, CD8+ T cells.

Abbreviations

COPD = Chronic obstructive pulmonary disease

ECM = Extracellular matrix

GOLD = Global initiative for chronic obstructive lung disease

FEV_1 = Forced expiratory volume in 1 second

FVC = Forced vital capacity

ICAM-1 = Intracellular adhesion molecule -1

Introduction

About 15-20% of cigarette smokers develop chronic obstructive pulmonary disease (COPD), which comprises a heterogeneous combination of chronic bronchitis and emphysema (1;2). Emphysema is characterized by chronic inflammation, alveolar destruction and loss of alveolar attachments, causing a permanent enlargement of airspaces and loss of elastic recoil of peribronchiolar attachments, which results in airflow limitation (1;3;4). To date, the underlying mechanisms of the vulnerability to cigarette smoke have not been elucidated (1;2;5;6).

We have proposed that besides the generally accepted imbalance between oxidants and anti-oxidants and between proteases and their inhibitors resulting in excess tissue destruction, a hampered tissue repair process is involved in the pathogenesis of emphysema (7;8). Fibroblasts are important in tissue repair and modulation by producing extracellular matrix (ECM) proteins, ECM-degrading enzymes and their inhibitors (9). We have previously observed that lung fibroblasts of patients with severe COPD exhibit an altered ECM production profile compared to fibroblasts of controls pointing to an important role of fibroblasts in the defective ECM modulation observed in COPD (8;10-12).

Besides tissue degradation and destruction, a chronic inflammatory process underlies COPD, with infiltrating inflammatory cells producing a large variety of cytokines (3;6;13-16). In addition, cigarette smoke damages epithelial cells, cells that are an important source of growth factors like TGFβ (3). This complex cytokine microenvironment most likely contributes to the altered behavior of pulmonary fibroblasts of COPD patients. In addition to ECM production, fibroblasts can actively be involved in the inflammatory processes via cytokine and chemokine production, and by direct interaction with inflammatory cells (7;17;18). We hypothesize that the local cytokine microenvironment determines the balance between ECM production and pro-inflammatory functions of fibroblasts.

The current study was conducted to further explore the role of fibroblasts in COPD, and especially to investigate the influence of the complex cytokine microenvironment of the diseased lung on cell-to-cell interaction and matrix production. Because *in vivo* analysis of human pulmonary fibroblasts is not possible, we designed an *in vitro* model of the local cytokine microenvironment of the diseased lung, using supernatants of stimulated epithelial and CD8+ T cells. The cytokine microenvironment is complex and consists of many cytokines and chemokines. By using supernatants we generated a model as close as possible to the *in vivo* situation. Epithelial and CD8+ T cells are known as key sources of the cytokines in the fibroblast microenvironment of the diseased lung (19). Therefore, we treated fibroblasts either with a supernatant of CD3/CD28 stimulated CD8 positive T cells isolated from peripheral blood of COPD patients, or a supernatant of a bronchial epithelial cell line, or a combination of both supernatants. We compared the effects of the supernatants on the expression of cell-interaction and matrix related genes in fibroblasts by real time PCR, using cDNA- macro-filter array as initial gene expression exploration. Protein expression analysis was used to confirm mRNA expression. Fibroblasts from both COPD patients (GOLD stage II/III and stage IV) and control patients were studied.

Materials and Methods

Subjects

To investigate mRNA expression, lung tissue was obtained from 20 individuals. The clinical characteristics of the groups are presented in table 1. Informed consent was given by all patients. Classification of severity of COPD was based on the 2003 Global initiative for Chronic Obstructive Lung Disease (GOLD) criteria (4;20). Three groups were studied: Individuals with moderate (GOLD stage II, n=5) and severe COPD (GOLD stage III, n=2) were combined to one group (stage II/III), individuals with very severe COPD (stage IV, n=7), and individuals with histologically normal lungs (n=6). Patients had no α_1-antitrypsin deficiency. Emphysema was defined as histologically abnormal enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of alveolar walls and without obvious fibrosis (1;2;4), and was assessed by routine histological examination of lung tissue by an experienced pulmonary pathologist (WT).

GOLD stage II/III tissue was obtained from non-involved lung tissue from patients undergoing resective surgery for pulmonary carcinoma. Tissue was always taken as far away as possible from the tumor, or from a non-involved lobe. Histopathologically emphysematous lesions were present, yet lesions were of limited but varying severity. Moderate emphysema was noted histopathologically as isolated or unattached segments of viable alveolar septal tissue or isolated cross sections of pulmonary vessels.

Tissue from the very severe COPD group (GOLD stage IV) as obtained from patients with COPD undergoing surgery for lung transplantation or lung volume reduction. The resected tissue showed both macroscopically and microscopically severe emphysematous lesions, often accompanied by bullae.

The control group consisted of donor lung tissue that had not been used for transplantation because of technical or logistical (unilateral transplantation) reasons. Technical reasons were related to doubt as to technical transplantability in relation to available recipients or doubts about the condition of the lung. In case of the latter, lung tissue was included only after additional investigations (including histopathology), showed no signs of COPD (1), nor of any other significant pathology.

Isolation and culture of lung fibroblasts

Pulmonary fibroblast cultures were established from parenchymal lung tissue by means of an explant technique as described previously (11). Absence of mycoplasma contamination in the fibroblast cultures was confirmed with a mycoplasma detection kit (Roche Diagnostics, Almere, The Netherlands). Isolated cells were characterized as fibroblasts by morphological appearance and expression pattern of specific proteins (11). All cells exhibited a characteristic staining pattern for vimentin, fibronectin, and the fibroblast marker prolyl-4-hydroxylase and lacked immunoreactivity for keratin. Less than 5% of cells were positive for desmin and α-smooth muscle cell actin.

Supernatants

Epithelial supernatant was obtained from a bronchial epithelial cell line, 16HBE (21) (kind gift from Dr. D.C. Gruenert, University of Vermont, California Pacific Medical Center Research Institute, San Francisco, CA). 16HBE cells were grown to confluence in 'Complete' medium (Ham's F12 medium (BioWhittaker Cambrex Verviers, Belgium) supplemented with 10% FCS (PAA laboratories, Linz, Austria), 2

mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (BioWhittaker Cambrex)). After confluence, culture medium was replaced with medium containing 0.5% FCS for 24 hours. This supernatant was harvested, and stored at -80°C.

To obtain culture supernatant of CD8+ T cells, peripheral blood was collected from seven patients with COPD. Informed consent was given by all patients. Heparinized blood was obtained and layered on Ficoll-paque (Amersham Biosciences, Uppsala, Sweden). The interface containing peripheral blood mononuclear cells (PBMCs) was collected. PBMCs were resuspended at 1 x 10^7 cells/ml in HBSS/2% FCS for purification of CD8 cells. CD8 cells were isolated by positive selection using anti-CD8 magnetic beads and Detach-a-bead (Dynal, Wirral, UK). The purity of the isolated CD8 T cells was determined by FACS analysis on a coulter Epics ELITE (Beckman Coulter, Hialeah, Florida, USA) with CyQ-labeled anti-CD8, PE-labeled anti-CD4 and FITC-labeled anti-CD3 antibodies (BD Bioscience, Oxford, UK). The CD8 cells were 87% pure containing 7% CD4 T cells. Isolated CD8 cells were resuspended in culture medium containing 0.5% FCS and incubated for 24 hours. Isolated CD8 cells were stimulated with anti-CD3 and anti-CD28 antibodies by replacing the culture medium with 0.5% FCS medium containing 5% anti-CD3 (clone WT-32) and anti-CD28 antibodies (clone 20-4996, CLB Sanquin, Amsterdam, The Netherlands) for 24 hours. Supernatant was then harvested, centrifuged and stored at -80°C.

Fibroblast culture for cDNA arrays

Fibroblasts from confluent cultures were seeded in culture plates (Corning BV, Amsterdam, The Netherlands) in 'complete medium' and grown to confluence (passage 5). Culture medium was replaced with medium containing 0.5% FCS for 24

hours. Medium was replaced with new 0.5% FCS medium, either with or without supernatant of the 16HBE cell line (1/25 diluted), supernatant of CD8 cells stimulated with anti-CD3 and anti-CD28 (1/10 diluted) or a combination of supernatant of 16HBE cells and CD8 cells (1/25 and 1/10 diluted final concentration respectively). Optimal stimulation concentration and duration was determined in pilot experiments (data not shown). After 6 hours, cells were harvested for RNA isolation.

Preparation of RNA and hybridization of cDNA arrays

Fibroblast total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA quantity was determined by optical density measurements at 260 nm. Three μg RNA per patient was pooled per group. Total RNA was treated with DNase I and run over a column to remove genomic DNA (Qiagen).

The Atlas Cell Interaction expression array (BD Biosciences Clontech, Palo Alto, CA, USA) was used to identify differences in the expression pattern of 265 known genes in the fibroblasts from the three patient groups treated with supernatants of 16HBE cells, CD8 cells, or their combination. The Atlas expression arrays are based on a filter array technique (macro-array) that uses a nylon filter spotted with cDNA's of the genes of interest that can be hybridized with cDNA of the samples to be analyzed. Two to five μg total RNA was used for cDNA synthesis using a commercial kit (BD Biosciences). The cDNA was labeled by incorporation of [α-^{32}P] dCTP (10 μCi/μl). The probes were hybridized when the counts per minute were between 2-8 x 10^6, with the ExpressHyb hybridization solutions applied to the cDNA arrays following the user manual (BD Biosciences). The membranes were exposed to a phosphorimaging screen at room temperature for 3 days. Quantification

of bound cDNA was performed using Phosphorimaging system (Molecular Dynamics, Sunnyvale, CA).

Analysis of cDNA array data

We compared gene expression patterns of fibroblasts from the three groups after incubation with supernatant of 16HBE cells, CD8 cells, or the combination and at basal culture conditions, using AtlasImage 2.0 software (BD Biosciences Clontech). In order to subtract the common variance and find the differentially expressed genes, a new method was designed based on the data reduction tool “principal component analysis” (22-24).

In brief, a matrix was prepared with all filter data placed in columns. To each value, 1 was added to avoid values of zero. The natural logarithm was taken to improve normality according to the following formula: $A' = 1.442695 * \ln(A + 1)$. All columns were standardized to mean = 0 and standard deviation = 1. Subsequently, the principal components were calculated and the first principal component was deducted from the data matrix. Of the remaining data, the inverse of the natural logarithm was taken. Ratios were determined and a gene expression ratio of at least a 2 fold increase compared to basal culture conditions was considered relevant.

Real time PCR

To obtain a more uniform patient group we excluded the only patient who had longstanding bronchitis (COPD stage IV) for PCR and subsequently for the s-ICAM ELISA and immunohistochemistry. To validate the results of the array data, we performed quantitative real time PCR analysis for ICAM-1 mRNA expression on individual patient fibroblast samples used in the array study. Three ng mRNA was

transcribed into cDNA by reverse transcriptase II (Invitrogen, Breda, the Netherlands). Real time PCR was performed on an ABI7900HT sequencer with "Assay on Demands" from Applied Biosystems (Foster City, CA, USA), according to their instructions. Data were analyzed by the ∆∆Ct method (25). In brief, Ct values of the genes of interest were corrected for Ct values from a household gene (Ribosomal protein S9), resulting in a ∆Ct value. This ∆Ct was normalized to the average ∆Ct value of the basal control group. The log value was taken and ratios between stimulated and basal condition were calculated. Medians were taken from the individual patients per category.

s-ICAM protein expression

Of all fibroblasts samples, supernatants were harvested to determine s-ICAM levels produced by fibroblasts. s-ICAM levels were determined by a commercial s-ICAM ELISA kit (CLB/Sanquin, Amsterdam, The Netherlands)). In brief, wells are coated with a capturing s-ICAM antibody en subsequently incubated with the supernatant samples. After incubation, wells are washed and incubated with a peroxidase labeled anti-s-ICAM antibody. Staining was visualized using TMB (3,3',5,5'-tetramethylbenzidine) . Absorption was read at 450nm on a standard photospectrometer.

Immunohistochemistry

Immunohistochemistry was performed on 3µm formalin fixed, paraffin embedded lung tissue using a standard two-step immunoperoxidase protocol. Sections were incubated with a primary monocolonal antibody recognizing ICAM-1 (kind gift from Dr. Boyd (26)). Envision (Dakopatts, Glostrup, Denmark) in combination with DAB (Sigma, St. Louis, Missouri, USA) was used to visualize

antibody binding. Slides were semi-quantitatively scored for the number of ICAM-1 positive cells in the submucosa. (1 = few ICAM-1 positive cells, 2 = moderate number of ICAM-1 positive cells, 3 = large number of ICAM-1 positive cells).

Statistical analysis

Differences in subject characteristics, real time data and immunohistochemistry scores between the 3 study groups were analyzed using Kruskall-Wallis test followed by the Mann-Whitney U test. Differences in smoking habits and gender between the three study groups were analyzed using the Fisher's exact test. The level of significance used was 0.05. All reported P-values are two-sided.

Results

Patient groups

Pack-years smoking histories were similar in subjects with moderate/severe and very severe COPD (table 1). Patients with very severe COPD were significantly younger than patients with moderate COPD. Material of our stage II group mainly consists of males, material of the stage IV group mainly consists of females. From our archives, it was not possible to obtain material, which was equally divided in males and females per COPD stage. The control group did not significantly differ in age or gender distribution from the COPD groups. As control group we used lung material from disapproved donor lungs. It is not possible to obtain all clinical characteristics of the donors, as could be obtained form our patient group. However, this material is analysed by an experienced pathologist and classified as normal tissue.

Array results in general

A total of 51 out of 265 genes tested were differentially expressed on the cell interaction arrays. Genes were categorized in three groups: genes involved in adhesion and migration, genes involved in extracellular matrix production or modulation, and genes involved in cellular regulation. Overall, cell-interaction genes were especially differentially expressed in the control and stage IV group. The stage II/III group showed only minor changes in gene expression profiles.

Gene expression with epithelium supernatant stimulation

After epithelial supernatant stimulation, 22 out of 265 genes were differentially expressed when compared to fibroblasts under basal culture conditions (table 2A, B, C): in particular genes involved in adhesion and regulation (table 2A and C). Epithelial supernatant influenced predominantly gene expression in control fibroblasts, but had hardly an effect on COPD fibroblasts. One gene was in particular differentially expressed: ICAM-1 expression was down regulated (-4.4 times) in the control group and, in contrast, up regulated in both COPD groups (4.5 and 5.7 fold in stage II/III and stage IV respectively).

Gene expression after CD8 supernatant stimulation

Twenty-nine genes were differentially expressed upon CD8 supernatant stimulation (table 2A, B, C). Twenty-three genes were differentially expressed in the stage IV COPD patient group and only 9 and 3 genes in stage II/III and control group, respectively. Most regulated genes reflected adhesion processes and extracellular matrix substances. Notably ICAM-1 expression was highly up regulated in both COPD groups (10.2 and 9.5 fold in stage II/III and stage IV respectively) whereas the control group showed no regulation of ICAM-1 mRNA expression.

Gene expression after combined CD8 and epithelial supernatant

After stimulation with a combination of CD8 and epithelial supernatants, 24 genes were differentially regulated (table 2A, B, C). Fibroblasts of controls treated with the combined supernatants showed primarily alteration in the expression of genes involved in cell regulatory processes. Fibroblasts of stage II/III COPD patients showed minimal alteration under these conditions whereas in the stage IV COPD group, ICAM-1 showed a surprisingly high increase in mRNA expression (25.5 fold).

Real time PCR

Arrays were used to find indications which genes would be most fit for further analysis of genes of interest by real-time PCR. ICAM-1 expression was by far the most prominent change found in the arrays to explore and confirm by RT-PCR. We found the highest ICAM-1 expression in stage IV fibroblasts under influence of the epithelial and CD8 supernatant combination (figure 1). Epithelial supernatant alone also significantly elevated the ICAM-1 gene expression in stage IV compared to stage II and controls. CD8 supernatants also caused the highest level of ICAM-1

mRNA expression in the stage IV group, but this was not significant due to large patient variation in combination with the relative small number of subjects.

s-ICAM protein expression

To verify the mRNA expression, we also determined s-ICAM levels in the supernatants of all fibroblast samples, using an s-ICAM ELISA. Hardly any s-ICAM was detected under basal conditions. Stimulation with the combination of CD8 and epithelial supernatant showed a trend towards an increase in s-ICAM levels in stage IV fibroblasts (p=0.055) (figure 2). Epithelial and CD8 supernatant stimulation alone did not affect the protein levels of s-ICAM.

Immunohistochemistry

To further verify the highly elevated mRNA expression of stage IV fibroblasts, we also analyzed ICAM-1 protein expression in tissue sections of the same control and COPD patients from whom fibroblasts were derived, as used in the array study. In the submucosa, where a large number of fibroblasts is located and which is likely to be influenced by local epithelial and CD8 derived factors, we found a significantly larger number of ICAM-1 positive cells in stage IV COPD compared to controls and stage II COPD (figure 3). Most positive cells represented elongated fibroblast-like cells (figure 4). This supports our observation of the high increase in ICAM-1 mRNA expression in stage IV COPD fibroblasts. **.**

Discussion

Smoking is known to induce CD8+ T cell influx and epithelial activation in the airway wall in COPD patients. We explored the modulating effects of CD8+ T cell and epithelial cell derived mediators on gene expression profiles of fibroblasts obtained from GOLD stages II/III, and IV, as well as controls. We clearly established that the cytokine microenvironment differentially modulates pulmonary fibroblast gene expression in COPD patients compared to controls. In particular adhesion related genes were differentially expressed, and an interaction was apparent when the fibroblasts were exposed to the combination of the CD8 and epithelial supernatants. Thus, factors in the microenvironment of the fibroblast may contribute to the altered fibroblast behavior observed in COPD. Since fibroblasts are essential cells in the modulation of the ECM, an alteration in their behavior could reflect the underlying cause of the defective ECM repair observed in COPD.

Our experiments were performed as closely related to the actual lung microenvironment as possible but still represent an *in vitro* model for the *in vivo* situation. The use of supernatants represent a source of uncertainty and variation but the alternative, to use combinations of individual cytokines, would have resulted in a selection bias. We used CD8 positive T cells from peripheral blood of COPD patients as a representative for the CD8 T cells in the diseased lung. A primary epithelial cell line was used because it is not possible to obtain sufficient cultures of primary epithelial cells and fibroblasts of the same patient. Our model is an appropriate model reflecting the microenvironment in COPD in order to study the role of mediators derived from CD8 T cells en epithelial cells on fibroblasts.

ICAM-1 showed a striking differential gene expression pattern between COPD and controls, which was supported by elevated production of s-ICAM in combination

with elevated expression in lung tissue of COPD patients. In the arrays, the combination of epithelial and CD8 supernatant showed the largest differential expression in stage IV fibroblasts. This observation was confirmed by results of the real-time PCR and was also supported by finding a near-significant trend ($p=0.055$) towards a higher production of s-ICAM-1 by stage IV fibroblasts as compared to controls and stage II/III fibroblasts. In addition, stage IV COPD patients showed the largest number of ICAM-1 positive cells ($p=0.0079$), mostly fibroblasts, in the submucosa of the small airways where an influence of CD8 cells as well as epithelial cells is to be expected. Although an effect of supernatant of epithelial cells and of CD8 cells on ICAM gene expression seemed to be present in the macro-array, this could not be confirmed using RT-PCR.

ICAM-1 is an important adhesion molecule involved in a number of inflammatory processes such as neutrophil and lymphocyte trafficking and it acts as accessory molecule in antigen presentation (32). ICAM-1 expression is regulated by a variety of cytokines like IL-1, IL-4, IFNγ, TGFβ and TNFα (32;33). The CD8+ T cells known to be elevated in number in COPD patients are an important source of IL-4 and TNFα, while epithelial cells are an important source of TGFβ. Thus these cytokines may be the key element of the supernatants explaining the elevated ICAM-1 expression.

Several studies have pointed to a role of ICAM-1 expression in the pathogenesis of COPD. ICAM-1 concentrations in BAL of COPD patients are increased and cigarette smoke extract increases ICAM-1 production by macrophages (34;35). In addition, an impressive decline in plasma ICAM-1 concentration after smoking cessation (36). Finally, fibroblasts are able to interact with inflammatory cells via adhesion molecules like ICAM-1 and VCAM-1 (33), and increased ICAM-1

expression is associated with leukocyte transmigration along fibroblasts (37). Therefore, it is likely that an increased ICAM-1 expression on fibroblasts of COPD patients points to important interactions with inflammatory cells. It is known that increased numbers of CD8+ T lymphocytes are present throughout the lung in smoking subjects with COPD (13;16;27-29), whereas epithelial cells cover more centrally located airways. The cytokines produced by both CD8+ T cells and epithelial cells are known modulators of fibroblast activity in COPD (2;13;30;31). We used supernatant of stimulated CD8+ T cells and epithelial cells to create an *in vitro* model reflecting the *in vivo* situation of pulmonary fibroblasts. An important aspect of our study is that we used a supernatant of CD8+ T cells isolated from peripheral blood of COPD patients without chronic bronchitis because CD8+ T cells of COPD patients have been shown to possess an altered cytokine production profile (15;29). Thus, more centrally (bronchially) located fibroblasts are modulated by both epithelial and CD8 derived factors, whereas more distally (parenchymal) located fibroblasts will be less regulated by bronchial epithelial cell-derived factors. In the specific microenvironments of the bronchial area, where both sources of mediators are present, the balance between mediators of epithelial and CD8+ T cells may ultimately determine the effect on fibroblast gene expression in COPD patients.

Fibroblasts are multipotent cells, their activity and function can be modulated based on local environmental factors. A major feature of fibroblasts is the production of ECM components, initiated in part by TGFβ stimulation. Fibroblasts are also involved in immunological processes, playing an important role in the switch from an acute, resolving inflammation to a chronic persistent inflammation (17;18). Buckley et al. suggested that this chronic inflammation occurs because of disordered fibroblast behavior, which leads to inappropriate survival and retention of leukocytes within

inflamed tissue (18;38). Thus it is not surprising that fibroblasts in COPD tissue show a functionally altered behavior under the influence of the local cytokine microenvironment (11;18). The differentially expressed adhesion-related genes we find in stage IV COPD fibroblasts when stimulated with CD8 supernatant supports the theory of a prominent role of fibroblasts in the chronic inflammatory process observed in COPD.

Besides the strong up regulation of ICAM-1, several other adhesion related genes were shown to be up regulated. It seems likely therefore that COPD fibroblasts, especially in stage IV COPD, are easily induced by the local cytokine microenvironment to interact with inflammatory cells and thus play a supportive role in the ongoing inflammation. Fibroblasts differentiated to a more immunological orientated function may not be fully capable of maintaining the ECM or may exhibit altered ECM repair mechanisms. These functional changes could contribute to destruction or inadequate maintenance of the ECM, causing some of the specific features of COPD. We have previously demonstrated a decreased decorin and biglycan immunohistological staining in lung tissue of stage IV COPD patients, signifying the altered ECM modulation (10).

In conclusion, fibroblasts of COPD patients display an altered response to the cytokine microenvironment in the COPD lung, depending on both the disease stage and the central or peripheral location in the lung. The local cytokine microenvironment appears to skew the COPD fibroblasts to interact with inflammatory cells instead of a focus on ECM maintenance, which may be pivotal in the pathogenesis of COPD. Furthermore our findings suggest that tissue repair mechanisms will differ in the parenchyma versus the bronchial wall in COPD.

Acknowledgments

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Table 1: Characteristics of the patient and control groups.

	Control	**GOLD stage II/III**	**GOLD stage IV**
Number of subjects	6	7	7
Age (years)	44.5 (25-71)	69 (44-81)	55 (44-61)
Male / Female	3 / 3	7 / 0	1 / 6
Smoking (Ex / C)	ND	2 / 5	7 / 0
Pack-years	ND	40 (14-58)	30 (12-54)
FEV_1 % predicted	ND	73 (40-76)	18 (13-29)
FEV_1 / FVC %	ND	55 (44-60)	28 (24-66)

All values are presented as median values with ranges in parentheses. Ex = ex smokers, not smoking for at least one year, C = current smoker; FEV_1 % predicted = Forced Expiratory Volume in 1 second expressed as percentage of predicted value; FVC = forced vital capacity; ND = not determined.

Table 2: Gene expression of fibroblasts stimulated with supernatants of epithelial, CD8 cells, or the combination compared to basal culture conditions

A: Adhesion related genes

Epithelial sup			CD8 sup			Epithelial and CD8 sup			
C	**StII/III**	**StIV**	**C**	**StII/III**	**StIV**	**C**	**StII/III**	**StIV**	**Gene**
					2.1				LFA-1
			4.5		8.1				CD27L
-4.4	**4.5**	**5.7**		**10.2**	**9.5**	**3.9**		**25.5**	**ICAM1**
		2.2	3.4		3.3			2.1	envoplakin
2.1			3.0		4.9				CD47
	2.5				2.2	-2.0		3.2	ninjurin-1
			3.2		8.0	2.4			ezrin
					-2.1				cadherin 5
					-3.7			-2.0	cadherin 6
					-2.0				cadherin 8
			-2.5			-2.5			desmocollin
2.4									beta catenin
2.2			2.4						desmoplakin III
					2.9	2.0			integrin alpha 8
2.3									vitronectin receptor alpha
-2.2									CD36 antigen
-2.2									semaphorin; CD100
	2.0								semaphorin E
				-2.1					CD9 antigen, MRP-1
-2.4								2.0	E-selectin
-2.2									CD153 antigen

B: Matrix related genes

Epithelial sup			CD8 sup			Epithelial and CD8 sup			
C	**StII/III**	**StIV**	**C**	**StII/III**	**StIV**	**C**	**StII/III**	**StIV**	**Gene**
						2.5			TIMP1
					2.8				tissue-type plasminogen activator
									procollagen 1 alpha 2 subunit
	2.1				2.7				procollagen 2 alpha 1 subunit
2.2						-2.1			procollagen 3 alpha 1 subunit
					-2.0				collagen 6 alpha 1
					2.6				collagen 11 alpha 2 subunit
					2.4			2.3	fibronectin precursor (FN)
					-2.7				SPARC
					2.3				decorin

C: Regulation related genes

Epithelial sup			CD8 sup			Epithelial and Cd8 sup			
C	**StII/III**	**StIV**	**C**	**StII/III**	**StIV**	**C**	**StII/III**	**StIV**	**Gene**
-3.6			-2.3						FASL
						-2.2			ILGF factor binding protein 6
2.5			2.0		2.3	2.0			MARCKS-related protein
		2.1							ras-like protein TC10
2.2					3.0	-2.0		2.5	ras-related C3 botulinum toxin
				-2.2					transforming protein rhoB
		3.5					2.9		TIAM1
			2.2			2.0			thrombospondin 1
					2.3				alpha-2-macroglobulin
					-2.3	2.1			CD91
						2.1		2.3	CD40L
						2.1			CD59
-2.9									lymphotoxin-alpha
								2.1	tumor necrosis factor receptor
						-2.1			neurogenic locus notch protein
-2.2									delta-like protein precursor (DLK)
							2.1		Wnt-5A
						2.7			Wnt-8B
2.9	2.2					2.7			neurotrophic tyrosine kinase R3
					2.0				caveolin-2

Ratios were determined between stimulated fibroblasts and fibroblasts under basal culture conditions. A minimum of two-fold higher or lower expression is considered relevant. Sup, supernatant; C, control; StII/III, Stage II and Stage III; StIV, Stage IV according to GOLD criteria.

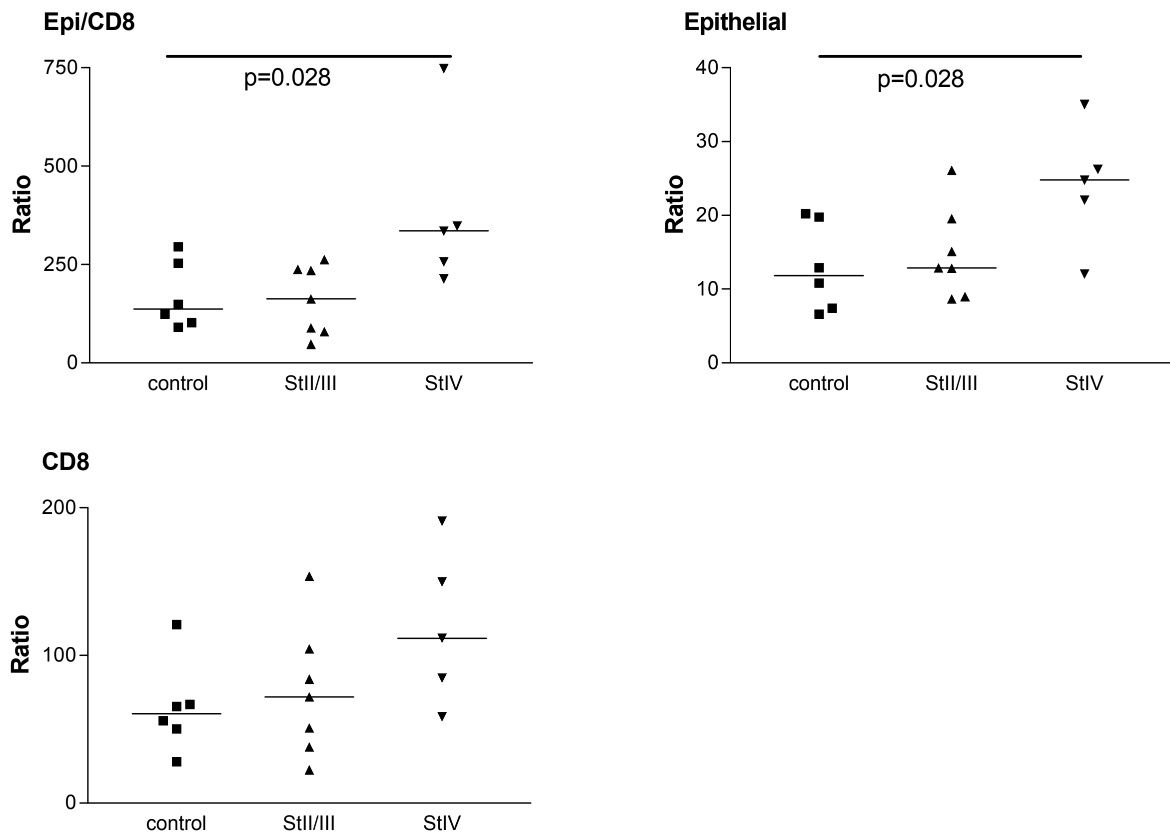


Figure 1: Real time ICAM-1 mRNA expression of fibroblasts stimulated with supernatants of epithelial and/or CD8 cells. Ratio compared to basal culture conditions. One stage IV sample was lost due to technical reasons.

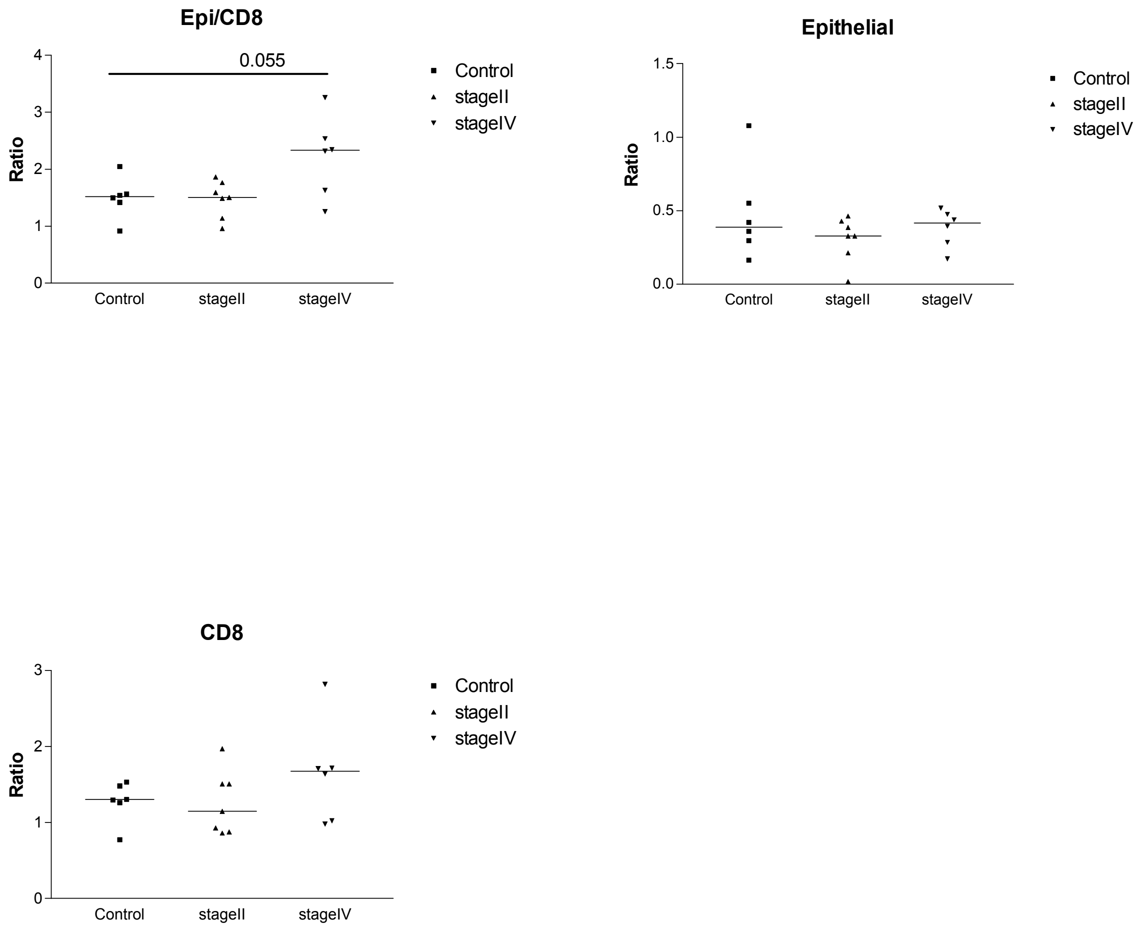


Figure 2: s-ICAM-1 protein expression of fibroblasts stimulated with supernatants of epithelial and/or CD8 cells. Ratio compared to basal culture conditions.

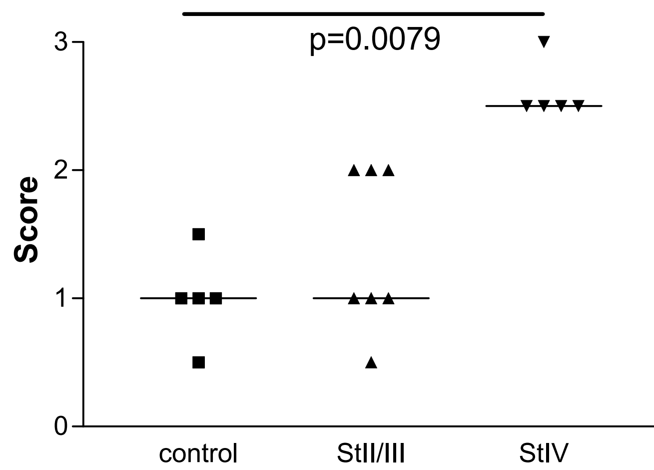
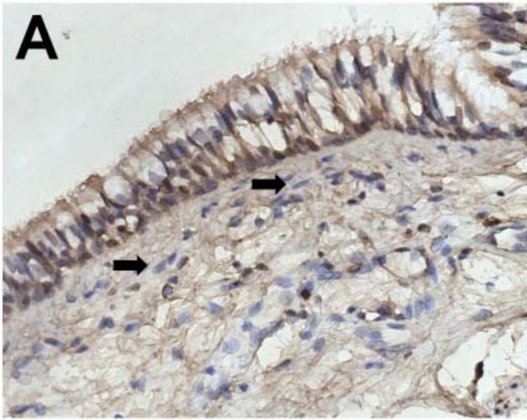
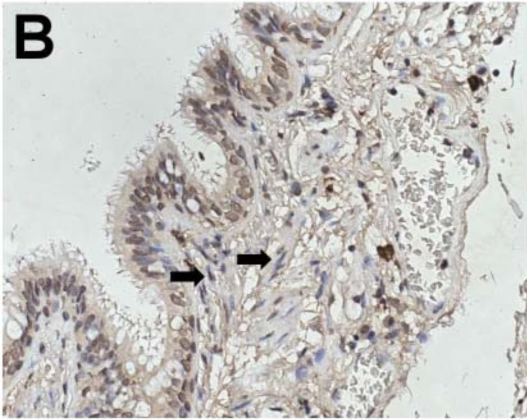


Figure 3: Semi-quantitative score of number of ICAM-1 positive cells in the submucosa. Relative frequency of ICAM-1 positive cells is significantly increased in stage IV COPD.


A


B

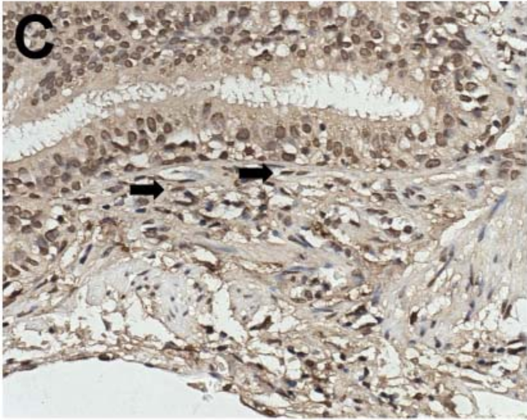

C

Figure 4: ICAM-1 staining (brown) in lung tissue of control (A), stage II COPD (B), and stage IV COPD (C). In control as well as stage II COPD, hardly any ICAM-1 positive cells are observed in the submucosa. Note the ICAM-1 negative, elongated fibroblasts (arrow) in the submucosa. In stage IV COPD, numerous ICAM-1 positive cells are observed. Note the ICAM-1 positive elongated fibroblasts (arrow). Counterstained with hematoxylin, magnification 400x.

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