Extracellular Matrix Proteins and Tissue Remodeling in Respiratory Diseases

Based on:
7.1 Summary

Remodelling of airways and blood vessels is an important feature of chronic obstructive pulmonary disease (COPD), however its molecular mechanisms are poorly understood. We examined the expression patterns of various extracellular matrix (ECM) components, including collagens (total collagen, subtypes collagen I, III and IV), fibronectin and laminin in bronchi from smokers with COPD (FEV₁ ≤75% pred.; n=15) and without COPD (FEV₁ ≥85% pred.; n=16). Immunohistochemical staining results were assessed by a validated visual scoring method (grade 0-4). Staining for ECM components was observed in the surface epithelial basement membrane (SEBM), and within the interstitium and vessels of the lamina propria and adventitia of airways. In COPD, total collagen was increased in the SEBM (p<0.01) at sites of intact bronchial epithelium, but was not changed in the interstitial space and vessels of the airway lamina propria and adventitia. Deposition of collagen I and III, however, was enhanced in the SEBM both at sites of damaged and of intact surface epithelium (p<0.05), lamina propria (p<0.02) and bronchial adventitia (p<0.05) in COPD. In COPD, fibronectin was increased in vessels of the lamina propria (p<0.05) and laminin in airway smooth muscle (p<0.01) and the microvasculature (p<0.05). FEV₁ values inversely correlated with collagens in the SEBM, fibronectin in bronchial vessels and laminin in the ASM. We conclude that smokers with COPD exhibit increased bronchial deposition of collagens I and III, fibronectin and laminin as part of the airway remodelling process in COPD.

7.3 Introduction

Chronic obstructive pulmonary disease (COPD) is a global health problem with increasing morbidity and mortality. One of the major causal factors is tobacco smoking, but of all smokers, only 10-20 percent develop COPD (1). Pathological features of COPD include thickening of airway walls, probably as a result of ongoing chronic inflammatory processes with an influx of neutrophils, macrophages and T-lymphocytes (2). The resultant changes in the airway wall in COPD include hyperplasia of subepithelial (myo-)fibroblasts and airway smooth muscle cells (3, 4).

Previous studies on the pathology of COPD have focused on alterations in small airways and parenchyma, where an infiltration of CD8⁺ T cells and macrophages, a loss in the number of alveolar-bronchiolar attachments and ECM (emphysema) with consequent loss of elastic recoil, and alveolar-peribronchial wall fibrosis with increased deposition of ECM proteins have been demonstrated (5-8). Thus far, few studies of COPD have focused on the larger airways (9-11). Bronchial epithelial loss and changes in large airway dimensions are found in COPD (3, 10, 12).
Tiddens and co-workers reported that the thickness of the wall area internal to the airway smooth muscle was increased in COPD, and that this increase correlated inversely with the FEV₁/FVC ratio, but these authors found no difference with respect to the airway smooth muscle mass (10).

Thickening of the surface epithelial basement membrane (SEBM), subepithelial fibrosis and the deposition of extracellular matrix proteins in the lamina propria are key features in asthma (13, 14). In COPD, however, changes in thickness of the SEBM and fibrosis of the mucosal lamina propria are less pronounced (15). Recent studies have indicated that the SEBM thickness in bronchial biopsies from smokers with chronic bronchitis was similar to that in normal subjects, unless features of asthma such as hyperresponsiveness or corticosteroid sensitivity were present as well (11, 15, 16). However, it has not been investigated in detail whether the composition of the SEBM is unchanged in COPD, and in addition, the lamina propria and adventitia may be altered.

We postulated that alterations in total or relative content of extracellular matrix proteins such as collagens, including subtypes I, III and IV, fibronectin, laminins and proteoglycans in the various compartments of the bronchial wall (SEBM, lamina propria, and bronchial adventitia and smooth muscle) are present in the airways of (ex-) smokers with COPD. In this study we investigated the localization and distribution pattern of various ECM markers in bronchial tissue from (ex-)smokers with or without COPD. Taken together, our results indicate that COPD is associated with increased deposition of ECM components in the bronchial airway wall. This may contribute to airway remodelling and airflow limitation.

7.4 Materials and methods

Bronchial tissue from lobectomy or pneumonectomy of current and ex-smokers, who underwent surgery for lung cancer, was obtained from the archive of the Pathology Departments of the Leiden University Medical Center (LUMC, Leiden, The Netherlands) and Southern Hospital (Rotterdam, The Netherlands), after approval of the study by the Medical Ethics committee of LUMC. All lung tissues were expanded by an injection syringe using 10 % phosphate-buffered formalin, and fixed for approximately 24 hours after which the tissues were further processed for embedding in paraffin and immunohistochemical staining. Samples of bronchial airways, located as far away as possible from the tumour were chosen for the study. Based on lung function outcome (see below), patients were assigned to the COPD and non-COPD groups (17-19). The patients in these two groups participated in a larger research project, part of which has been published previously (19, 20).

COPD group: Fifteen subjects were assigned to this group on the basis of the following parameters: forced expiratory volume in one second (FEV₁) <75% of predicted value before
bronchodilatation, FEV₁/FVC ratio <75%, a reversibility in FEV₁ ≤12% of predicted after 400 μg inhaled salbutamol, and a transfer factor for carbon monoxide (diffusion capacity) per litre alveolar volume (Kco) ≤80% of predicted value (21).

**Non-COPD group:** Sixteen subjects were assigned to this group based on the basis of the following data: FEV₁ >85% of predicted before bronchodilatation, FEV₁/FVC ratio >85%, and reversibility in FEV₁ ≤12% of predicted after 400 μg salbutamol inhalation. In order to exclude accompanying lung disease leading to a restrictive lung function, it was required that the total lung capacity (TLC) of each subject was over 80% of the predicted value (21).

Clinical data of all patients were examined for possible co-morbidity and medication use. All patients were free of symptoms of upper respiratory tract infection and none received antibiotics perioperatively. None of the patients received glucocorticosteroids in the three months prior to operation, but four patients received oral glucocorticosteroids perioperatively. After the selection based on lung function, all the lung tissues used for this study were checked histologically using the following exclusion criteria: (i) presence of tumour, (ii) presence of poststenotic pneumonia, (iii) fibrosis of lung parenchyma, and (iv) obstruction of the main bronchus (17, 18).

**Pulmonary Function Tests**

All pulmonary function tests were performed within 3 months prior to surgery. FEV₁ and forced vital capacity (FVC) were measured by spirometry, TLC and residual volume with the closed circuit helium dilution test and the Kco using the single breath-holding technique, as described by Quanjer et al. (21). Lung function data and other patient characteristics are shown in Table 5.1.

**Total collagen staining**

The total collagen fibers in bronchial tissue specimens were stained with Picro-sirius Red F3BA (22). Tissue sections of 4 μm thickness were treated with 0.2% aqueous phosphomolybdic acid and incubated in 0.1% Picro-sirius Red. Before dehydration, the slides were treated with 0.01N HCl and mounted. Slides were visualized under light microscope and collagen content was assessed using the same visual scoring method used for the analysis of the immunohistochemistry data (see below).

**Immunohistochemistry and quantification**

Sections of paraffin-embedded lung tissue were cut at 4 μm, mounted on Super Frost Plus® microscopic slides (Menzel-Gläser, Braunschweig, Germany) and processed for immunohistochemistry. Serial sections were used to detect the staining of collagen I, III, IV, fibronectin and laminin β2 employing immunohistochemistry. Sections were deparaffinized and rehydrated prior to incubation with specific purified mouse monoclonal antibodies. Anti-human
mouse monoclonal antibodies against collagen IV, fibronectin and laminin were purchased from NeoMarkers (Fremont, USA), collagen I from Sigma (St Louis, USA) and collagen III from Biogenex (San Ramon, USA), respectively. To block non-specific second antibody binding, sections were preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (5% BSA/PBS, pH = 7.4). Subsequently, sections were incubated overnight at 4 °C with primary antibodies against collagen I (1:150 v/v) or III (undiluted), fibronectin (1:500 v/v) and laminin β2 (1:150 v/v), or for 1 hour at room temperature in case of collagen IV (1:150 v/v). Immunostainings were performed after antigen retrieval by 0.1% protease treatment in PBS for 10 minutes at 37 °C or in case of collagen I by boiling in citrate buffer (10 mM citrate buffer, pH = 6.0) for 10 minutes in a microwave oven. Incubation for 30 minutes with secondary biotinylated anti-immunoglobulins (Multilink®, 1:75 dilution, Biogenex, San Ramon, USA) and tertiary complex of peroxidase-conjugated streptavidin at a dilution of 1:50 were used to enhance the detection sensitivity. Colour was developed using 0.025% of 3,3-diaminobenzidine (Sigma, St Louis, USA) in 0.01 mol/L PBS, containing 0.03% H₂O₂. Positive controls consisted of human breast carcinoma and placental tissue. Negative controls were not incubated with primary antibody. The optimal dilution for all antibodies was identified by examining the intensity of staining obtained with a series of dilutions: the optimum concentration resulted in specific and easily visible signal on paraffin sections of control specimens. Slides were counterstained with Mayer's hematoxylin, mounted and studied light-microscopically.

A visual scoring method was applied. For this purpose all tissues were analysed in a blinded fashion in random order by two independent observers, who were unaware of the clinical data of the case under study. Quantitative analysis was performed using a validated, arbitrary visual scale with grading scores of 0, 1, 2, 3 and 4 representing none, weak, moderate, intense and very intense staining, respectively (17, 18, 23, 24). We quantified the staining pattern of ECM proteins in the SEBM and subdivided the staining for sites where the bronchial epithelium was totally lost and the SEBM was denuded or not. Furthermore, the interstitial staining of the bronchial lamina propria and adventitia was assessed. Moreover, the staining pattern within either the microvasculature bronchial lamina propria or the adventitia was measured. The intensity of laminin expression in the ASM area was quantified. We also examined errors within and between observers by correlating the expression scores using Pearson’s analysis and found a very high correlation of 0.8 to 0.9.

**Statistical Analysis**

Data were analysed for statistical significance using the unpaired, two-tailed Students’ t-test as well as the non-parametric Mann-Whitney test, where appropriate. The expression data for ECM proteins were expressed as mean ± SEM. Furthermore, ECM proteins staining for different
compartments were correlated with FEV₁ using Pearson's correlation analysis. The individual collagen subtype values were correlated with the total collagen staining and with each other to evaluate co-localisation. Differences with \( p \leq 0.05 \) were considered to be statistically significant.

7.5 Results

Clinical Parameters
The clinical and lung function characteristics of all subjects included in the study are listed in Table 5.1. The COPD group demonstrated an elevated residual volume (RV), whereas the \( K_{co} \) was reduced (\( p<0.005 \)). The subjects in the two groups did not differ significantly in age, TLC, reversibility in FEV₁, smoking status (pack-years) or steroid use (Table 5.1).

Localization and quantification of extracellular matrix proteins
We investigated the localization of extracellular matrix proteins in the bronchial airways (Figures 5.1 and 5.2). ECM proteins were systematically assessed in the following sites: the surface epithelial basement membrane (SEBM), the connective tissue of the lamina propria and adventitia of the bronchial airway and in the bronchial blood vessels. We observed staining for collagen IV, fibronectin and laminin within the SEBM relatively more towards the apical side whereas collagen I and III were localized more towards the lamina propria in the reticular layer. Within vessel walls, staining for fibronectin was found in the (neo-)intima, for collagen IV and laminin in the medial and collagen I and III in the adventitial layer. In addition, laminin was immuno-localized at the apical side of the bronchial epithelium and in the airway smooth muscle (ASM) cell layer.

Representative examples of collagen staining in non-COPD (A, C, E and G) and COPD (B, D, F and H) samples are depicted in Figures 5.1 and 5.2. We quantified the staining pattern of ECM proteins in the SEBM and subdivided the staining for sites where the bronchial epithelium was damaged or not. All investigated ECM proteins were significantly increased at sites of epithelial denudation (Figure 5.3A-F, \( p<0.01 \)). We observed more intense staining for total collagen in the SEBM at sites of intact epithelium in subjects with COPD (1.5 fold increase, \( p < 0.05 \), Figure 5.3A). Figure 5.3B demonstrates that collagen I deposition is increased in COPD as compared to non-COPD patients in the SEBM at the areas of intact epithelium (2.3 fold increase, \( p < 0.001 \)) and damaged bronchial epithelium (1.6 fold increase, \( p < 0.01 \)), lamina propria and bronchial adventitia, (1.9 fold increase each, \( p < 0.001 \)).
Table 7.1 Subject Characteristics

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Abbreviations: Forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), total lung capacity (TLC), and residual volume (RV), are given as percentage of predicted. M = Male, F = Female, Pre-operative steroids use (y/n/u, yes/no/unknown) P values are given as COPD versus non-COPD. The patients in these two groups participated in a larger research project, part of which has been published previously (19, 20).

Figure 7.3C indicates that in COPD patients collagen III staining is elevated in the SEBM at sites of intact and damaged epithelium (1.5 and 1.4 fold increase, p < 0.01, respectively). Furthermore, at fibrotic sites of lamina propria (1.4 fold increase, p < 0.05) and adventitia (1.3 fold increase, p < 0.05) of the airway wall the collagen III staining is also increased (Figure 5.3C). Collagen IV protein, however, remained unaltered, irrespective of the presence of COPD (Figure 5.3D). Fibronectin deposition was higher in intima and (neo-)intima including endothelial cells of
bronchial blood vessels in COPD (Figure 5.3E). Laminin staining was more intense in the ASM layer (1.5 times, p < 0.01) and small vessels in the lamina propria (1.3 times, p < 0.01, Figure 5.3F). No other staining differences were observed between samples from subjects with and without COPD.

**Correlation of ECM proteins with clinical data**

Pearson’s correlation of ECM components with FEV₁ values (% predicted) in all COPD and non-COPD patients is summarized in Figure 5.4. We observed a significant inverse correlation with FEV₁ of the following parameters: total collagen staining in the SEBM underneath intact epithelium (r = -0.47, p < 0.01); collagen I staining in SEBM at sites with damaged epithelium (r = -0.61, p < 0.01); connective tissue of the bronchial adventitia (r = -0.67, p < 0.001, Figure 5.4A) and of the lamina propria (r = -0.53, p < 0.01). In the same regions similarly inverse correlation was found between collagen III and FEV₁ (r = -0.40, -0.42 and -0.48, p < 0.01, Figure 5.4B). Figure 5.4C illustrates that fibronectin is also inversely correlated with FEV₁ values in endothelium (r = -0.51, p < 0.01). Moreover, in ASM we found a significant inverse correlation between FEV₁ values and laminin (r = -0.61, p < 0.001, Figure 5.4D). When considering co-localization of total collagen with subtypes for collagen I, III and IV, we found a significant correlation between total collagen and collagen III in the SEBM at both damaged (r = 0.62, p < 0.001) and intact epithelium (r = 0.63, p < 0.001). No significant correlation was found between total collagen and collagen I and IV localization.

### 7.5 Discussion

In this study we showed that COPD is associated with an increased bronchial deposition of collagens I, III, IV, fibronectin and laminin. ECM proteins were observed in SEBM, lamina propria and adventitia of the bronchial walls and vasculature. We found that ECM protein deposition is increased in the SEBM at sites of damaged bronchial epithelium in all patients. In COPD patients, total collagen and predominantly collagens I and III subtype were further increased as compared to controls, while bronchial vessels showed increased deposition of fibronectin and laminin. FEV₁ values inversely correlated with collagens in the SEBM, fibronectin in bronchial vessels and laminin in the ASM. Taken together, these findings strongly suggest that deposition of ECM components contributes to the airway remodelling of COPD.
Figure 7.1 Photomicrographs of bronchial tissue sections from patients without COPD (A, C, E and G) and with COPD (B, D, F and H). Panels A and B show total collagen staining (Sirius-Red staining) in bronchial airway walls. Panels C and D show staining for collagen 1 in surface epithelial basement membrane (SEBM) and lamina propria. Panels E and F show collagen III protein staining in bronchial adventitial layer with bronchial vessels. Panels G and H show collagen IV staining in lamina propria. Arrows indicate sites of damaged bronchial epithelium. Counterstained with hematoxylin. Original magnification: x200. Scale bar = 50 μm.
Figure 7.2 Photomicrographs of bronchial tissue sections from patients without COPD (A, C, E and G) and with COPD (B, D, F and H). Panels A and B show fibronectin staining in bronchial lamina propria and panels C and D in vasculature. Panels E and F show laminin protein staining in the lamina propria and panels G and H in the adventitial layers with bronchial vessels. Arrows indicate sites of damaged bronchial epithelium: Counterstained with hematoxylin. Original magnification: x200. Scale bar = 50 μm.
**Figure 7.3** Graphic representations of extracellular matrix proteins (mean ± SEM) using visual scoring; (A) for total collagen, (B) for collagen I, (C) for collagen III, (D) for collagen IV, (E) for fibronectin and (F) for laminin. Abbreviations: surface epithelial basement membrane (SEBM), bronchial epithelium (B. E.), bronchial epithelium damaged or intact (D. E. or I. E.), lamina propria (L. P.), bronchial adventitia (B. A.) airway and vascular smooth muscle cells (ASM and VSM) and endothelial cells (EC). Staining score for non-COPD (white bars) and COPD groups (gray bars) are given. # P <0.05 SEBM scores of damaged versus undamaged bronchial epithelium. * P <0.05 versus the non-COPD group.
**Figure 7.4** Correlation with FEV₁ (% predicted) of total collagen in SEBM with undamaged bronchial epithelium (A), collagen III in the lamina propria (B), fibronectin in endothelial cells (EC, C) and laminin in VSM (D) of the combined patient groups (non-COPD and COPD). Correlation coefficient (r) was obtained using linear regression (Pearson's) analysis and significance level P value, P < 0.05.

An identical localization pattern of the various investigated ECM makers in the cartilaginous bronchial wall was present in our patients groups, which is in agreement with earlier reports describing their presence in the bronchial airways of asthmatics (13, 25-28). Several previous reports have demonstrated structural changes with fibrosis and deposition of ECM proteins as well as loss of elastic recoil in peripheral airways and lung parenchyma of COPD patients (29-31). Inflammation, with influx of CD8⁺ T-cells in peripheral airways and accumulation of macrophages has been reported (6, 32). Peribronchiolar and septal fibrosis are also found whereas alveolar extracellular matrix deposition is decreased in emphysema (5, 6, 32). Our
results demonstrate that COPD is also associated with changes in extracellular matrix protein deposition of larger airways. In asthma, SEBM thickening is prominent, as is the deposition in large airways of various ECM proteins, including collagens, fibronectin, laminins and proteoglycans in epithelial SEBM, subepithelial layers and bronchial vasculature (28, 33-35). In COPD, however, the few previous reports that are available have indicated that SEBM thickness remains unchanged, unless features of asthma such as hyperresponsiveness or corticosteroid sensitivity were present (3, 16, 28). However, we show here that the staining of total collagen, collagen I and III in SEBM is more intense in COPD as compared to controls. Furthermore, all investigated extracellular matrix proteins were upregulated at sites where the epithelial lining was damaged. These findings support the hypothesis of involvement of the bronchial epithelium and subepithelial (myo-)fibroblasts in damage and repair processes with tissue remodelling. Recent studies based on in vitro co-culture experiments indicate that effects of growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGF-1 and FGF-2) and transforming growth factor beta 1 (TGF-β1) on epithelial cells and (myo-)fibroblasts are necessary to mediate repair of epithelial injury by induction of cellular proliferation and collagen synthesis (36-39). The above mechanisms that were found in vitro could possibly also play a role in tissue remodelling and fibrosis during COPD.

We also investigated the deposition of ECM proteins in the bronchial vasculature of the bronchial lamina propria and adventitia. We show that COPD is associated with more deposition of collagen III and laminin in vascular media and adventitia, and with fibronectin in endothelial cells and also neo-intima of small muscular vessels. We and others have previously shown that structural changes to the pulmonary vasculature, including intimal and medial thickening with VSM hypertrophy and lumen narrowing, occur in COPD (19, 23, 40-42). We described that in the peripheral lung, vessel wall thickness was inversely correlated with FEV₁. Peinado et al. concluded that small pulmonary arteries of patients with mild COPD have endothelial dysfunction and intimal thickening (41, 42). In a recent paper, Santos et al. quantified the (immuno-)

histochemical staining pattern of various extracellular matrix components including elastin, total collagen and proteoglycans with the same visual scoring method employed by us and, previously, by several other authors (23, 24, 43, 44). Santos et al. reported no differences in small pulmonary arteries between COPD patients and smoking non-COPD controls. They did, however, find a positive correlation between the amount of collagen deposition and intimal thickening (23). In analogy to the ECM deposition in the bronchial wall, damage to the endothelial lining can induce vascular remodelling, vascular smooth muscle proliferation, metaplasia of VSM to (myo-)
fibroblasts, and increased synthesis and deposition of extracellular matrix proteins such as collagens and fibronectin (45). Our results support this hypothesis, which is likely also to contribute to vascular remodelling during the development of COPD.
Correlation analysis revealed a significant inverse correlation of FEV\textsubscript{1} values and total collagen and collagen I and III staining in the SEBM, fibronectin in intima of mucosal vessels and laminin expression in airway smooth muscle. These findings are consistent with the hypothesis of the development of structural abnormalities in the bronchial airway wall and in the vessel walls in patients with COPD causing airways obstruction. The exact mechanism remains unknown.

Taken together, our results indicate that COPD is associated with increased deposition of ECM components in the bronchial airway wall, as part of the airway remodelling and contributing to airflow limitation. Blockade of pathways that are likely to be involved in structural and functional abnormalities should be considered in the development of therapeutic interventions aimed to prevent chronic airflow limitation in COPD.

Acknowledgements

Financial support from The Netherlands Asthma Foundation (grant # NAF 32.97.73) is gratefully acknowledged.

7.6 References