Enhanced Bronchial Expression of Extracellular Matrix Proteins in Chronic Obstructive Pulmonary Disease

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Abstract

Remodeling of airways and blood vessels is an important feature in chronic obstructive pulmonary disease (COPD). By using immunohistochemical analysis, we examined bronchial expression patterns of various extracellular matrix (ECM) components such as collagens (subtypes I, III, and IV), fibronectin, and laminin β2 in patients with COPD (forced expiratory volume in 1 second [FEV1] ≤ 75%; n = 15) and without COPD (FEV1 ≥ 85%; n = 16) and correlated expression data with lung function. Quantitative analysis revealed enhanced levels (P < .01) of total collagens I, III, and IV in surface epithelial basement membrane (SEBM) and collagens I and III in bronchial lamina propria (P < .02) and adventitia (P < .05) in COPD. Distinct and increased (P < .05) vascular expression of fibronectin accounts for intimal vascular fibrosis, whereas laminin β2 (P < .05) was elevated in airway smooth muscle (ASM). FEV1 values inversely correlated with collagens in the SEBM, fibronectin in bronchial vessels, and laminin in the ASM. Our data suggest that COPD exhibits increased bronchial deposition of ECM proteins that contribute to deteriorated lung function and airway remodeling.

With increasing morbidity and mortality, chronic obstructive pulmonary disease (COPD) remains a global health problem. One of the major causal factors is tobacco smoking, but of all smokers, COPD develops in only 10% to 20%.1 Pathologic 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.2,3 The resultant changes in the airway wall in COPD include hyperplasia of subepithelial myofibroblasts and airway smooth muscle (ASM) cells.2,3

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 emphysema with consequent loss of elastic recoil, and alveolar-peribronchial wall fibrosis with increased deposition of extracellular matrix (ECM) proteins have been demonstrated.3,5 In a recent study, Hogg et al6 have shown that the progression of COPD is associated with the accumulation of inflammatory mucous exudates in the small airways. Thus far, only a few studies on COPD have focused on the larger airways where bronchial epithelial loss and changes in large airway dimensions have been reported in COPD.2,7-10 Tiddens and coworkers7 reported that the thickness of the wall area internal to the ASM was increased in COPD and that this increase correlated inversely with the forced expiratory volume in 1 second (FEV1)/forced vital capacity (FVC) ratio, but these authors found no significant increase in the ASM mass.

Thickening of the surface epithelial basement membrane (SEBM), subepithelial fibrosis, and the deposition of ECM proteins in the lamina propria are key features in asthma.11,12 In COPD, however, changes in thickness of the SEBM and
fibrosis of the mucosal lamina propria are less pronounced.\textsuperscript{12} Studies have indicated that the SEBM thickness in bronchial biopsy specimens from smokers with chronic bronchitis was similar to that in healthy subjects, unless features of asthma such as hyperresponsiveness or corticosteroid sensitivity were present as well.\textsuperscript{9,12,13} However, it has not been studied in detail whether the composition of the SEBM is unchanged in COPD, and, in addition, the lamina propria and adventitia may be altered.

We hypothesized that alteration in total or relative content of ECM 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, bronchial adventitia, and smooth muscle) are present in the airways of smokers and ex-smokers with COPD. We studied the quantitative distribution patterns of various ECM markers in bronchial tissue samples from smokers and ex-smokers with or without COPD.

**Materials and Methods**

**Selection of Specimens**

Bronchial tissue samples from current and/or ex-smokers, who underwent surgery for lung cancer, were obtained at Leiden University Medical Centre, Leiden, the Netherlands, and Southern Hospital, Rotterdam, the Netherlands, after approval of the study by the Leiden University Medical Centre Medical Ethics Committee. Lung tissue samples were fixed for 24 hours by perfusion fixation using an injection syringe filled with 10% phosphate-buffered formalin, embedded in paraffin, and processed for immunohistochemical analysis. Samples of bronchial airways, located as far away as possible from the tumor, were chosen for the study. Based on lung function outcome, patients were assigned to COPD and non-COPD groups.\textsuperscript{14-16} The patients in these 2 groups participated in a larger research project, part of which has been published.\textsuperscript{10,16}

**COPD Group**

Fifteen subjects were assigned to this group on the basis of the following parameters: an FEV\textsubscript{1} of 75% or less of predicted value before bronchodilatation, an FEV\textsubscript{1}/FVC ratio less than 75%, a reversibility in FEV\textsubscript{1} of 12% or less of the predicted value after 400 µg of albuterol inhalation. To exclude accompanying lung disease leading to a restrictive lung function, it was required that the total lung capacity (TLC) of each subject be more than 80% of the predicted value.\textsuperscript{17}

Clinical data for all patients were examined for possible comorbidity 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 during the 3 months before operation, but 4 patients received oral glucocorticosteroids perioperatively. After selection based on lung function, all lung tissue samples used for the study were checked histologically by using the following exclusion criteria: (1) presence of tumor, (2) presence of poststenotic pneumonia, (3) fibrosis of lung parenchyma, and (4) obstruction of the main bronchus.\textsuperscript{14,15}

**Pulmonary Function Tests**

All pulmonary function tests were performed within 3 months before surgery. The FEV\textsubscript{1} and FVC were measured by spirometry, TLC and residual volume with the closed circuit helium dilution test, and the K\textsubscript{CO} using the single breath-holding technique, as described by Quanjer et al.\textsuperscript{17} Lung function data and other patient characteristics are shown in Table II.

**Staining of Total Collagen Fibers**

The total collagen fibers in bronchial tissue specimens were stained with Picro-sirius red F3BA (Sigma, St Louis, MO).\textsuperscript{18} Tissue sections, 4 µm thick, 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 hydrochloric acid and mounted. Slides were visualized under a light microscope, and collagen content was assessed using a visual scoring method (see “Quantitative Analysis of Staining”).

**Immunohistochemical Analysis**

Sections of paraffin-embedded lung tissue were cut at 4 µm and mounted on Super Frost Plus microscopic slides (Meinzl-Gläser, Braunschweig, Germany), and serial sections were used to detect the staining of collagen I, III, and IV, fibronectin, and laminin β2 using immunohistochemical analysis. Anti-human mouse monoclonal antibodies against collagen IV, fibronectin, and laminin were purchased from NeoMarkers, Fremont, CA; collagen I from Sigma; and collagen III from BioGenex, San Ramon, CA.

Sections were deparaffinized, rehydrated, and preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.4. Subsequently, sections were incubated overnight at 4°C with primary antibodies against collagen I (1:150 vol/vol) or III (undiluted), fibronectin (1:500 vol/vol), and laminin β2 (1:150 vol/vol) or for 1 hour at room temperature for collagen.
IV (1:150 vol/vol). Immunostaining was performed after antigen retrieval by 0.1% protease treatment in PBS for 10 minutes at 37°C or, for collagen I, by boiling in citrate buffer (10 mmol/L of citrate buffer, pH 6.0) for 10 minutes in a microwave oven. Incubation for 30 minutes with secondary biotinylated anti-immunoglobulins (Multilink, dilution 1:75; BioGenex) and a tertiary complex of peroxidase-conjugated streptavidin at a dilution of 1:50 were used to enhance the detection sensitivity. Color was developed using 0.025% of 3,3-diaminobenzidine (Sigma, St Louis) in 0.01 mol/L of PBS, containing 0.03% hydrogen peroxide.

Positive control samples consisted of human breast carcinoma and placental tissue. 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 hematoxylin, mounted, and studied light microscopically.

**Quantitative Analysis of Staining**

A visual scoring method was applied. For this purpose, all tissue samples were analyzed in a blinded manner in random order by 2 independent observers (A.R.K. and A.W.W.) who were unaware of the clinical data for 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 no, weak, moderate, intense, and very intense staining, respectively.10,14-16,19 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 the microvasculature bronchial lamina propria or the adventitia was measured. We also examined errors within and between observers by correlating the

<table>
<thead>
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<th>Table 1</th>
<th>Subject Characteristics and Clinical Parameters*</th>
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<tr>
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<td>Mean ± SEM</td>
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<td>.239</td>
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*FEV<sub>1</sub>, FVC, TLC, and RV are given as percentage of predicted value. P values are given as COPD vs non-COPD.
expression scores using Pearson analysis and found a very high correlation of 0.8 to 0.9.

Statistical Analysis

Data were analyzed for statistical significance using the unpaired, 2-tailed Student t test and the nonparametric Mann-Whitney test, as appropriate. The expression data for ECM proteins were expressed as mean ± SEM. Furthermore, ECM proteins staining for different compartments were correlated with FEV₁ by using Pearson correlation analysis. The individual collagen subtype values were correlated with total collagen staining and with each other to evaluate colocalization. Differences with a P value of .05 or less were considered statistically significant.

Results

Clinical Parameters

The clinical and lung function characteristics of all subjects included in the study are listed in Table 1. The COPD group demonstrated an elevated residual volume, whereas the Kco was reduced (P < .005). Subjects in the 2 groups did not differ significantly in age, TLC, reversibility in FEV₁, smoking status (pack-years), or steroid use (Table 1).

Localization and Quantification of Extracellular Matrix Proteins

We studied the localization of ECM proteins in the bronchial airways Image 1 and Image 2. ECM proteins were assessed systematically in the following sites: the SEBM,
connective tissue samples 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 toward the apical side, whereas collagen I and III were localized toward 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 layer, and for collagen I and III in the adventitial layer. In addition, laminin was immunolocalized at the apical side of the bronchial epithelium and in the 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 Images 1 and 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 studied ECM proteins were increased significantly at sites of epithelial denudation (Figure II) \( P < .01 \). We observed more intense staining for total collagen in the SEBM at sites of intact epithelium in subjects with COPD (1.5-fold increase; \( P < .05 \); Figure 1A). Figure 1B demonstrates that collagen I deposition was increased in COPD compared with that in non-COPD patients in the SEBM at the areas of intact epithelium (2.3-fold increase; \( P < .001 \)) and damaged bronchial epithelium (1.6-fold increase; \( P < .01 \)), and lamina propria and bronchial adventitia (1.9-fold increase each; \( P < .001 \)). Figure 1C indicates that in patients with COPD, collagen III staining is elevated in the SEBM at sites of intact and damaged epithelium (1.5- and 1.4-fold increases, respectively, \( P < .01 \)). Furthermore, at fibrotic sites of lamina propria (1.4-fold

**E** and **F**, Collagen III protein staining in bronchial adventitial layer with bronchial vessels. **G** and **H**, Collagen IV staining in lamina propria. Arrows indicate sites of damaged bronchial epithelium (**A-H**. hematoxylin counterstain, original magnification \( \times 200 \)).
increase; \( P < .05 \) and adventitia (1.3-fold increase; \( P < .05 \)) of the airway wall, collagen III staining also is increased (Figure 1C). Collagen IV protein, however, remained unaltered, irrespective of the presence of COPD (Figure 1D). Fibronectin deposition was higher in the intima and (neo-)intima, including endothelial cells of bronchial blood vessels in COPD (Figure 1E). Laminin staining was more intense in the ASM layer (1.5-fold; \( P < .01 \)) and small vessels in the lamina propria (1.3-fold; \( P < .01 \); Figure 1F). No other staining differences were observed between samples from subjects with or without COPD.

Correlation of ECM Proteins With Clinical Data

Pearson correlations of ECM components with FEV\(_1\) values (percentage predicted) in all COPD and non-COPD patients are summarized in Figure 2. We observed a significant inverse correlation with FEV\(_1\) of the following parameters: total collagen staining in the SEBM under intact epithelium (\( r = -0.47; P < .01 \)); collagen I staining in SEBM at sites with damaged epithelium (\( r = -0.61; P < .01 \)); connective tissue of the bronchial adventitia (\( r = -0.57; P < .001 \); Figure 2A) and of the lamina propria (\( r = -0.43; P < .01 \). In the same regions, inverse correlations also were found between collagen III and FEV\(_1\) (\( r = -0.40, r = -0.42, \) and \( r = -0.54; P < .01 \); Figure 2B). Figure 2C illustrates that fibronectin also was correlated inversely with FEV\(_1\) values in endothelium (\( r = -0.51; P < .01 \)). Moreover, in ASM, we found a significant inverse correlation between FEV\(_1\) values and laminin (\( r = -0.61; P < .001 \); Figure 2D). When considering colocalization of total collagen with subtypes for collagen I, III, and IV,

![Image 2](https://example.com/image2.png)

**Image 2** Bronchial tissue sections from patients without chronic obstructive pulmonary disease (COPD) (A, C, E, and G) and with COPD (B, D, F, and H). Fibronectin staining in bronchial lamina propria (A and B) and vasculature (C and D).
we found a significant correlation between total collagen and collagen III in the SEBM at damaged \((r = 0.62; P < .001)\) and intact epithelium \((r = 0.63; P < .001)\). No significant correlation was observed between total collagen and collagen I and IV localization.

### Discussion

In this study, we showed that COPD is associated with an increased bronchial deposition of collagens I, III, and 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 patients with COPD, total collagen and predominantly collagens I and III were increased further compared with levels in control subjects, whereas bronchial vessels showed increased deposition of fibronectin and laminin. FEV\(_1\) values inversely correlated with collagens in the SEBM, fibronectin in bronchial vessels, and laminin in the ASM. Taken together, these findings suggest that deposition of ECM components contributes to the airway remodeling in COPD.

An identical localization pattern of the various studied ECM markers in the cartilaginous bronchial wall was present in our patient groups, which is in agreement with earlier reports describing their presence in the bronchial airways of patients with asthma and patients with bronchopulmonary dysplasia.\(^{11,20-22}\) Several reports have demonstrated structural changes with fibrosis and deposition of ECM proteins and loss
Figure 1 Graphic representations of extracellular matrix proteins (mean ± SEM) using visual scoring for total collagen (A), for collagen I (B), for collagen III (C), for collagen IV (D), for fibronectin (E), and for laminin (F). Staining score for non–chronic obstructive pulmonary disease (COPD) (white bars) and COPD groups (black bars) are given. * P < .05 vs the non-COPD group.
† P < .05 SEBM scores of damaged vs undamaged bronchial epithelium. ASM, airway smooth muscle cells; BA, bronchial adventitia; BE, bronchial epithelium; DE, damaged bronchial epithelium; EC, endothelial cells; IE, intact bronchial epithelium; LP, lamina propria; SEBM, surface epithelial basement membrane; VSM, vascular smooth muscle cells.
of elastic recoil in the peripheral airways and lung parenchyma of patients with COPD.\textsuperscript{6,23} Inflammation, with influx of CD8\textsuperscript{T} cells in peripheral airways and accumulation of macrophages has been reported.\textsuperscript{5,24} Peribronchiolar and septal fibrosis also are found, whereas alveolar ECM deposition is decreased in emphysema.\textsuperscript{5,24}

Our results demonstrate that COPD also is associated with changes in ECM 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.\textsuperscript{25-27} In COPD, however, the few reports that are available have indicated that SEBM thickness remains unchanged, unless features of asthma such as hyperresponsiveness or corticosteroid sensitivity were present.\textsuperscript{5,13} However, we show here that the staining of total collagen, collagen I, and collagen III in SEBM is more intense in COPD than in control subjects. Furthermore, all studied ECM proteins were up-regulated at sites where the epithelial lining was damaged. These findings support the hypothesis of involvement of the bronchial epithelium and subepithelial myofibroblasts in damage and repair processes with tissue remodeling.

Studies based on in vitro coculture experiments indicate that effects of growth factors such as epidermal growth factor, fibroblast growth factors 1 and 2, and transforming growth factor β1 on epithelial cells and myofibroblasts are necessary to mediate repair of epithelial injury by induction of cellular proliferation and collagen synthesis.\textsuperscript{28-31} The aforementioned mechanisms that were found in vitro possibly could also have a role in tissue remodeling and fibrosis during COPD. We also studied the deposition of ECM proteins in the bronchial vasculature of the bronchial lamina propria and adventitia. We found 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 the neointima of small muscular vessels. We and others have previously shown that structural changes to the pulmonary vasculature, including intimal and medial thickening with vascular smooth muscle (VSM) hypertrophy and lumen narrowing, occur in COPD.\textsuperscript{16,19,32} We described that in the peripheral lung, vessel wall thickness was correlated inversely with FEV\textsubscript{1}. Peinado et al\textsuperscript{33,34} concluded that small pulmonary arteries of patients with mild COPD have endothelial dysfunction and intimal thickening.

In the present study, we used the method of visual scoring on immunohistochemically stained bronchial tissue sections, which also was used by Santos et al\textsuperscript{19} and others\textsuperscript{10,35} for the staining pattern of various ECM components, including elastin, total collagen, and proteoglycans. Santos and colleagues\textsuperscript{19} reported no differences in small pulmonary arteries between COPD patients and smoking non-COPD control subjects, but a positive correlation between the amount of collagen deposition and intimal thickening was found. Analogous to the ECM deposition in the bronchial wall, damage to the endothelial lining can induce vascular remodeling, VSM proliferation, metaplasia of VSM to myofibroblasts, and increased synthesis and deposition of ECM proteins such as collagens and fibronectin.\textsuperscript{36} Our results support this hypothesis, which likely also contributes to vascular remodeling during the development of COPD.

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Correlation with FEV\textsubscript{1} (% predicted) of total collagen in surface epithelial basement membrane with undamaged bronchial epithelium (A, \(r = -0.67; P < .001\)), collagen III in the lamina propria (B, \(r = -0.48; P < .01\)), fibronectin in EC (C, \(r = -0.51; P < .01\)) and laminin in VSM (D, \(r = -0.61; P < .001\)) of the combined patient groups (non–chronic obstructive pulmonary disease [COPD] and COPD). The correlation coefficient (r) was obtained using linear regression (Pearson) analysis and significance level (\(P < .05\)) was considered at absolute values of \(r > 0.37\). Staining scores of 0, 1, 2, 3, and 4 represent no, weak, moderate, intense, and very intense staining, respectively. ASM, airway smooth muscle cells; BA, bronchial adventitia; EC, endothelial cells; FEV\textsubscript{1}, forced expiratory volume in 1 second; LP, lamina propria.}
\end{figure}
Correlation analysis revealed a significant inverse correlation of FEV₁ values and total collagen and collagen I and III staining in the SEBM, fibronectin in the intima of mucosal vessels, and laminin expression in ASM. Focus is now on the bidirectional relationship between ASM cells and the ECM. In addition to increased synthesis of ECM proteins, ASM cells can be involved in the down-regulation of matrix metalloproteinases and the up-regulation of tissue inhibitors of metalloproteinases. 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 airway obstruction. Taken together, our results indicate that COPD is associated with increased deposition of ECM components in the bronchial airway wall as part of airway remodeling 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.

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References


