Chapter 5

Structure-function relationships between extracellular matrix in the airway smooth muscle and airway function in asthma

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Abstract

Introduction:
Altered deposition of extracellular matrix (ECM) in the airway smooth muscle (ASM) layer as observed in asthma may influence ASM mechanical properties. We hypothesized that ECM in ASM is associated with airway function in asthma. First, we investigated the difference in ECM expression in ASM between asthma and controls. Second, we examined whether ECM expression is associated with bronchoconstriction and bronchodilation in vivo.

Methods:
Our cross-sectional study comprised 19 atopic mild asthma patients, 15 atopic and 12 non-atopic healthy subjects. Spirometry, methacholine responsiveness, deep breath-induced bronchodilation ($\Delta R_{ns}$), and bronchoscopy with endobronchial biopsies were performed. Positive staining of elastin, collagen I, III, and IV, decorin, versican, fibronectin, laminin, and tenasin in ASM was quantified as fractional area and mean density. Data were analyzed using Pearson’s or Spearman’s correlation coefficient.

Results:
ECM expression in ASM was not different between asthma and controls. In asthmatics, fractional area and mean density of collagen I and III were correlated with methacholine dose response slope and $\Delta R_{ns}$, respectively ($r = 0.71$, $p < 0.01$; $r = 0.60$, $p = 0.02$). Furthermore, ASM collagen III and laminin in asthma were correlated with FEV$_1$ reversibility ($r = -0.65$, $p = 0.01$; $r = -0.54$, $p = 0.04$).

Conclusion:
In asthma, ECM in ASM is related to the dynamics of airway function in absence of differences in ECM expression between asthma and controls. This indicates that the ASM layer in its full composition is a major structural component in determining variable airways obstruction in asthma.
Introduction

Airway remodeling in asthma includes alteration in extracellular matrix (ECM) deposition and increase in airway smooth muscle (ASM) mass [1,2]. It is difficult to associate aspects of remodeling with disease severity or degree of airways obstruction and hyperresponsiveness [3]. However, recent studies suggest that remodeling of ASM is an exception to this [4]. First, it has been shown that the clinical expression of asthma [5], airway hyperresponsiveness [6] and impaired airway relaxation [7] are associated with mast cell counts in the ASM layer in asthma. Second, the deposition of ECM in- and outside the ASM layer in asthma seems to be related to its clinical severity and is altered as compared to healthy controls [8,9].

ECM affects both the synthetic-proliferative [10-12] and mechanical properties of ASM, which may be reflected by the altered airway mechanics observed in asthma [13]. However, contradictory results have been reported in vitro as to whether the ECM in the asthmatic ASM layer leads to enhanced or rather constrained shortening and force generation of the ASM cells. Elastic and collagen fibers in the airway wall may diminish bronchoconstriction by giving radial constraint to ASM or rather enhance it when peribronchial pressure becomes less negative due to airway remodeling [14,15]. Proteoglycans and glycoproteins, for instance versican and laminin respectively, may play an important role as well in determining the resiliency of ASM [16,17].

In a previous study performed in asthma patients only, we have demonstrated that FEV₁, airway hyperresponsiveness, and deep-breath induced bronchodilation were associated with the expression of selective ASM proteins and ECM components in endobronchial biopsies [18]. It is unknown whether such structure-function relationship exists for major ECM components in ASM such as proteoglycans and glycoproteins. In particular, human in vivo studies regarding this structure-function relationship are lacking.

In the present study, we hypothesized that ECM in the ASM layer is associated with airway function in asthma. The first aim was to investigate whether there are differences in ECM in the ASM layer between atopic asthma and healthy atopic and non-atopic controls. The second aim was to investigate whether there are significant associations between ECM in the ASM layer (elastin,
collagen I, III, and IV, the proteoglycans decorin and versican, and the glycoproteins fibronectin, laminin, and tenasin) and the dynamics of airway function in vivo (induced bronchoconstriction and bronchodilation).

Methods

Design and subjects

This cross-sectional study consisted of 2 visits. At visit 1, subjects were screened for eligibility to participate according to the in- and exclusion criteria (see below). Spirometry and methacholine bronchoprovocation test were performed. At visit 2, the respiratory system resistance ($R_{rs}$) and reactance ($X_{rs}$) were measured followed by FEV$_1$ reversibility testing and bronchoscopy with endobronchial biopsies.

The study population consisted of three groups: 1. atopic mild asthma (n = 19); 2. healthy atopic control (n = 15); 3. healthy non-atopic control (n = 12). Subjects were recruited by the Department of Respiratory Medicine of the Academic Medical Center Amsterdam.

Asthma patients had controlled disease according to GINA guidelines [19]. The inclusion criteria were: aged 18-50 years; non-smoking or stopped > 12 months with ≤ 5 pack years; no exacerbations within 6 weeks prior to participation; steroid-naïve or stopped using steroids by any dosing route ≥ 8 weeks prior to participation; no pulmonary diseases other than asthma; no lung medication except inhaled short-acting β2-agonists as rescue therapy; airway hyperresponsiveness defined by a methacholine PC$_{20}$ ≤ 8 mg/mL; post-bronchodilator FEV$_1$ > 70% of predicted; atopy defined by a positive skin prick test. The inclusion criteria for healthy control subjects were similar to those of the asthma patients, except that they had no pulmonary diseases and no airway hyperresponsiveness (methacholine PC$_{20}$ > 8 mg/mL). The atopic status of healthy controls was determined by skin prick test, in order to delineate the contribution of atopy as such to the ECM composition in the ASM layer.
All subjects gave written informed consent prior to enrolment. This study was approved by the Medical Ethics Committee of the Academic Medical Center Amsterdam and is registered at the Netherlands Trial Register (NTR1306).

**Airway function and skin prick test**

Spirometry was performed according to European Respiratory Society (ERS) recommendations [20]. Reversibility was determined by measuring FEV\(_1\) pre- and post-inhalation of 400\(\mu\)g Salbutamol. PC\(_{20}\) was measured by methacholine bronchoprovocation test using the standardized tidal volume method with a maximum methacholine dose of 16 mg/mL [21]. Because 24 out of the total 27 healthy control subjects did not reach a PC\(_{20}\) at the highest methacholine concentration, we used the validated dose response slope to analyze airway responsiveness [22]. \(R_s\) and \(X_s\) were measured with a FOT device (Woolcock Institute, Sydney, Australia) at 8Hz during 60 seconds of tidal breathing according to ERS recommendations [23]. Additionally, deep breath-induced bronchodilation was assessed by measuring the change in resistance (\(\Delta R_s\)) and reactance (\(\Delta X_s\)) after a deep inspiration (7). The skin prick test included 12 common aeroallergen extracts.

**Bronchoscopy**

Fiberoptic bronchoscopy was performed according to international recommendations [24]. Participants received local anaesthetic by Lignocaine spray 1% in the nose, and 1% and 10% in the throat. Additional Lignocaine 1% solution was instilled into lung segments to dampen the cough reflex. The bronchial tree was inspected by autofluorescence bronchoscopy (SAFE 3000, Pentax, Japan). Two endobronchial biopsies per patient were taken at B4-6 of the right lung with a cup forceps (Pentax KW2411S). Special care was taken in positioning the forceps laterally to the bronchial carina in order to minimize the amount of connective tissue and in the same time maximize the yield of ASM in the biopsies. After collection, the biopsy specimens were fixed in 4% buffered formaldehyde and embedded in paraffin.

**Histochemistry and immunohistochemistry**

Biopsy specimens were cut into 4\(\mu\)m sections and stained with H&E for initial analysis as described previously [7,9]. Next, sections were stained with Elastica-van Gieson for analysis of elastin in the
ASM layer. Antigen retrieval and the primary antibodies used for labeling of ECM (collagen I, III, and IV, decorin, versican, fibronectin, laminin, and tenascin) are shown in Table 1. Briefly, the paraffin sections were dewaxed and rehydrated. Prior to overnight incubation with the primary antibody, a 3% H$_2$O$_2$ solution was applied for 40 minutes to inhibit endogenous peroxidase activity. A streptavidin-biotin (LSAB kit, DAKO, Glostrup, Denmark) or a non-biotin (Novocastra Novolink, Leica Biosystems, Newcastle Upon Tyne, UK) detection method was applied as secondary antibody staining. All sections were incubated with antibodies from the same batch within one session and counterstained with Harris haematoxylin. Negative control staining was obtained by replacing the primary antibodies by PBS or isotype-matched control antibodies.
### Table 1 - Antibodies used for immunohistochemical analyses

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pre-treatment</th>
<th>Species</th>
<th>Dilution</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>Citrate</td>
<td>Goat</td>
<td>1:2500</td>
<td>Polyclonal</td>
<td>US Biological, Swampscott, MA, USA</td>
</tr>
<tr>
<td>Collagen III</td>
<td>Trypsin</td>
<td>Mouse</td>
<td>1:400</td>
<td>III-53</td>
<td>Oncogene &amp; Calbiochem, Darmstadt, Germany</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Citrate</td>
<td>Mouse</td>
<td>1:300</td>
<td>CIV-22</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Decorin</td>
<td>Chondroitinase</td>
<td>Rabbit</td>
<td>1:400</td>
<td>Polyclonal</td>
<td>Sigma-Aldrich, Saint Louis, MO, USA</td>
</tr>
<tr>
<td>Versican</td>
<td>Trypsin</td>
<td>Mouse</td>
<td>1:600</td>
<td>2-b-1</td>
<td>Seikagaku Co., Tokyo, Japan</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Citrate</td>
<td>Rabbit</td>
<td>1:15000</td>
<td>Polyclonal</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Laminin</td>
<td>Proteinase K</td>
<td>Mouse</td>
<td>1:500</td>
<td>Monoclonal</td>
<td>Sigma-Aldrich, Saint Louis, MO, USA</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Pepsin</td>
<td>Mouse</td>
<td>1:12000</td>
<td>BC-24</td>
<td>Sigma-Aldrich, Saint Louis, MO, USA</td>
</tr>
</tbody>
</table>
Morphometry

Images of the microscopic slides were captured digitally (Leica DMR, Leica Microsystems, Wetzlar, Germany). Selection of the ASM area for analysis was based on alpha-SMA stain and morphology of the ASM cells, which were elongated and disposed in bundles [18]. The area of positive staining for each antibody within the selected ASM was determined by color threshold. To define this threshold, sections of 6-8 subjects per study group stained with each antibody were analyzed to achieve the best range of positivity. Afterwards, the color data file created for each antibody was applied to all cases stained with the same antibody using Image-Pro Plus 4.1 (Media Cybernetics, Bethesda, USA) at 200x magnification as described previously [9]. Fractional area was expressed as percentage of the total selected ASM area. Mean density of the immunohistochemical staining was measured by the image analysis software.

Data analysis

Fractional area and mean density were compared between study groups using unpaired t-test or Mann-Whitney U test. Correlations between ECM and airway function parameters were examined using Pearson’s or Spearman’s correlation coefficient. Statistical analyses were performed using SPSS 18 (IBM Corporation, New York, USA) with a p-value of < 0.05 considered statistically significant.

Statistical power was calculated from our previous data on smooth muscle elastin staining [8]. This showed that 13 subjects per group allowed detecting 12% between-group difference in fractional area of elastin in ASM with at least an 80% power at the 5% level of significance.

Results

Subjects

The subject characteristics of the study groups were largely comparable (Table 2). As expected, post-bronchodilator FEV1 %predicted was significantly lower, whereas the dose response slope and R² were significantly higher in asthma as compared to healthy controls.
Fractional area and mean density of ECM in ASM

Biopsy specimens of 5 out of 19 asthma patients, 3 out of 15 healthy atopic, and 2 out of 12 healthy non-atopic subjects contained no ASM and therefore could not be analyzed.

Figure 1 shows representative images of paraffin sections stained for elastin, collagen I, III, and IV, decorin, versican, fibronectin, laminin, and tenascin. The fractional area of elastin in ASM was not different between asthma and controls with or without atopy (p > 0.05, Figure 1a). Similarly, there was no difference in fractional area or mean density of all other stained ECM components in ASM between the study groups (p > 0.05, Figure 1b-i).

ECM in ASM and dynamics of airway function

There were highly significant correlations between fractional area and mean density of ECM in ASM and parameters reflecting bronchoconstriction or bronchodilation. Notably, this was observed in the asthma patients only. First, fractional area of collagen I was positively correlated with the methacholine dose response slope (r = 0.71, p < 0.01, Figure 2a). Second, there was a positive correlation between fractional area of collagen III and \( \Delta R_n \) (r = 0.60, p = 0.02, Figure 2b). Finally, fractional area of collagen III and laminin were inversely correlated with FEV\(_1\) reversibility (r = -0.65, p = 0.01, Figure 2c; r = -0.54, p = 0.04, Figure 2d). Similar significant correlations were observed when fractional area was replaced with mean density (Table 3). In contrast, there were no significant correlations between ECM in ASM and FEV\(_1\) or other spirometric values representing the level of airway function (p > 0.05).
Table 2 - Characteristics of asthmatic and healthy subjects

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subjects (n)</strong></td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td><strong>Male/Female (n)</strong></td>
<td>4/15</td>
<td>5/10</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>25 (21-47)</td>
<td>27 (20-49)</td>
</tr>
<tr>
<td><strong>FEV\textsubscript{1}, post-beronchodilator (%pred.)</strong></td>
<td>96 (13)</td>
<td>109 (13)</td>
</tr>
<tr>
<td><strong>FEV\textsubscript{1}/FVC (%pred.)</strong></td>
<td>0.81 (0.08)</td>
<td>0.89 (0.06)</td>
</tr>
<tr>
<td><strong>PC\textsubscript{20} (mg/mL)</strong></td>
<td>1.81 (0.95-3.46)</td>
<td>&gt; 8</td>
</tr>
<tr>
<td><strong>Dose response slope (% decline FEV\textsubscript{1} / \mu mol methacholine)</strong></td>
<td>3.05 (1.29-8.13)</td>
<td>0.32 (0.17-0.44)</td>
</tr>
<tr>
<td><strong>R\textsubscript{rs} 8Hz FOT (cmH\textsubscript{2}O/L/s)</strong></td>
<td>3.68 (0.82)</td>
<td>2.99 (0.66)</td>
</tr>
<tr>
<td><strong>X\textsubscript{rs} 8Hz FOT (cmH\textsubscript{2}O/L/s)</strong></td>
<td>0.35 (0.35)</td>
<td>0.35 (0.16)</td>
</tr>
</tbody>
</table>

\( a \) Mean (min-max)
\( b \) Mean (SD)
\( c \) Geometric mean (95% CI)
\( d \) Median (P25-P75)
\( * \) Asthma vs. Healthy, atopic p = 0.008; Asthma vs. Healthy, non-atopic p = 0.008
\( ** \) Asthma vs. Healthy, atopic p < 0.001; Asthma vs. Healthy, non-atopic p < 0.001
\( *** \) Asthma vs. Healthy, atopic p = 0.012; Asthma vs. Healthy, non-atopic p = 0.037

FEV\textsubscript{1}: forced expiratory volume in 1 second; FVC: forced vital capacity; PC\textsubscript{20}: provocative concentration of methacholine causing a 20 % drop in forced expiratory volume in 1 second; R\textsubscript{rs}: respiratory system resistance; X\textsubscript{rs}: respiratory system reactance; %pred. = % predicted.

Table 3 - Structure-function relationship in patients with asthma: Fractional area and mean density

<table>
<thead>
<tr>
<th></th>
<th>Fractional area</th>
<th>Mean density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( p\text{-value} )</td>
</tr>
<tr>
<td>Collagen I - Dose response slope</td>
<td>0.71</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Collagen III - ( \Delta R\textsubscript{rs} )</td>
<td>0.60</td>
<td>0.02</td>
</tr>
<tr>
<td>Collagen III - FEV\textsubscript{1} reversibility</td>
<td>-0.65</td>
<td>0.01</td>
</tr>
<tr>
<td>Laminin - FEV\textsubscript{1} reversibility</td>
<td>-0.54</td>
<td>0.04</td>
</tr>
</tbody>
</table>

FEV\textsubscript{1}: forced expiratory volume in 1 second; \( \Delta R\textsubscript{rs} \) = change in respiratory system resistance after deep inspiration.
Figure 1 - Extracellular matrix inside the airway smooth muscle layer
Representative images of biopsies stained for elastin (a), collagen I (b), III (c), and IV (d), decorin (e), versican (f), fibronectin (g), laminin (h), and tenascin (i). Fractional area of each ECM component is presented in a box-plot with the whiskers representing minimum and maximum values. Scale bar = 50μm; FA = fractional area expressed as percentage of the total selected ASM area per ECM component staining; A = asthma; HA = healthy atopic; HNA = healthy non-atopic.
Figure 2 - Correlations between extracellular matrix inside the airway smooth muscle layer and dynamics of airway function in patients with asthma

Significant correlations were found between fractional area of collagen I and dose response slope (a), collagen III and ΔRrs, collagen III and FEV₁ reversibility (c), and laminin and FEV₁ reversibility (d). FEV₁ = forced expiratory volume in 1 second; ΔRrs after DI = change in respiratory system resistance after deep inspiration.
Discussion

The present study demonstrates structure-function relationships between extracellular matrix within the airway smooth muscle layer and indices of bronchoconstriction and bronchodilation in asthma. We observed that fractional area of collagen I and III were positively correlated with the methacholine dose response slope and change in $R_{ns}$ after deep inspiration, respectively. Additionally, fractional area of collagen III and laminin were inversely correlated with FEV$_1$ reversibility. These structure-function relationships existed in asthmatics only, even though there was no difference in fractional area and mean density of ECM between asthma and healthy controls. ECM in the ASM layer was not associated with FEV$_1$ or other spirometric values representing the level of airway function. Our results indicate that the ECM in the ASM layer is related to the dynamics of airway function in asthma. This suggests that the ASM layer contributes to the physiological phenotype of asthma.

To our knowledge this is the first in vivo human study examining the structure-function relationship between ECM in ASM and airway function using endobronchial biopsies in mild asthma patients and healthy controls. The association between ECM components and the dynamics rather than level of airway function is a novel finding and contributes to the current evidence found in literature pointing towards the ASM layer as a key player in determining airway mechanics [13].

We did not observe differences in fractional area or mean density of ECM in ASM between asthma patients and healthy controls with or without atopy. Previous studies have shown that the extent of various aspects of airway remodeling is different between asthma with varying severity [25,26]. Additionally, the elastic fiber content in the superficial elastic fiber network attached to the basement membrane is different between fatal asthma and healthy controls [27]. When considering ECM in the ASM layer, fatal asthmatics showed increased fractional area of elastic fiber and fibronectin as compared to non-fatal asthmatics [9]. These data suggest that ECM remodeling in ASM is dependent on disease severity. Therefore, it may not be surprising that the mild asthmatics in the current study did not show significantly different fractional area or mean density of ECM as compared to healthy controls. This is in keeping with an earlier observation showing that the
percentage positive staining of decorin and versican inside the ASM layer of moderate asthma was not different from healthy controls [28].

We have analyzed both the fractional area and mean density of several ECM components in ASM by immunohistochemistry. The quantitative results not only present a detailed analysis of the distribution of ECM components in ASM, but also give an estimate of the amount of ECM. Other morphometric analysis methods e.g. immunoblotting may have provided complementary data. However, previous studies have shown that immunohistochemistry data have a good correlation with results obtained by those analysis methods [29,30].

Collagen I, III, and laminin were significantly correlated with the dynamics of airway function. This was observed in asthma patients only, which fits in with the results of a previous study showing that airway wall mechanics are different in asthma as compared to controls [31]. Yet it is unclear whether ECM in ASM enhances or rather constrains shortening and force generation of the ASM cells. The results of our study indicate that collagen I, III, and laminin in the asthmatic ASM layer lead to a more reactive, stiff and less distensible airway as is shown by positive correlations with the dose response slope and $\Delta R_{rs}$, and inverse association with $FEV_1$ reversibility. These results suggest that ECM in the ASM layer of asthma patients leads to a deterioration in airway function and enhanced airway narrowing. This is in keeping with the results from our previous study showing that airway hyperresponsiveness was inversely correlated with the ASM contractile proteins desmin and MLCK [18]. Additionally, the results from our previous and current studies suggest that a decrease in ASM contractile proteins and an increase in ECM proteins lead to less change in $\Delta R_{rs}$. This supports the hypothesis that in atopic mild asthma, allergen exposure may lead to a change in ASM phenotype from contractile to proliferative [18].

ECM produced by ASM cells may not be the only component inside the ASM layer that determines airway function [32]. It has been shown recently that ASM-derived ECM proteins stimulate mast cells to differentiate into a fibroblastoid phenotype, which may play an important role in causing airway dysfunction [33]. Additionally, ASM phenotype itself may be influenced by ECM [16,34] as well as
intramuscular mast cells [5,6,35]. Apparently, ASM cell phenotype and ASM microenvironment determines airway function in asthma, most likely in very close interaction.

The three groups of well-characterized subjects and the extensive physiology together with the quantitative immunohistochemistry seem to represent the strengths of our study. In addition, our study featured sufficient statistical power. However, there are some limitations. First, each study group had to consist of 13 subjects according to the *a priori* sample size calculation. However, only 12 subjects in the healthy atopic and 10 in the healthy non-atopic study group were available for statistical analysis. We renewed the power calculations by a new analysis based on the current numbers of subjects and found that a between-group difference of 13% in fractional area of elastin in ASM could be detected with a power of 80% at the 5% level of significance. We therefore believe that this study was sufficiently powered. Furthermore, as mentioned above, the absence of differences in fractional area of ECM components in ASM is in keeping with results from previous studies. Second, we have included mild asthma patients only, because patients had to have controlled disease without steroid therapy. The benefit is that this avoids any bias potentially induced by steroids. Third, biopsies were taken from subsegmental levels of the right lung. As the degree of airway remodeling in asthma may vary with asthma severity and location in the bronchial tree [9], subsequent studies are needed to allow a more detailed delineation of the structure-function relationship throughout the bronchial tree. Fourth, structural components of the airway wall other than the ASM layer, for instance the submucosa, may be important as well in determining airway function [36]. Therefore, airway structural components and the inflammatory cell profile in-and outside the ASM layer need to be analyzed in conjunction with airway physiology. In the future this may result in the identification of novel therapeutic targets leading to a more efficient, effective, and personalized treatment, which not only relieves symptoms, but also tackles the pathologic basis of asthma at an early stage and thereby prevents deterioration of airway function.
Conclusion

The current study shows structure-function relationships between extracellular matrix within the airway smooth muscle layer and dynamics of airway function in patients with asthma. These findings were observed even in the absence of significant differences in fractional area or mean density of elastin, collagen I, III and IV, decorin, versican, fibronectin, laminin, and tenascin in the airway smooth muscle layer between asthma and healthy controls. Our study confirms and extends existing evidence showing that the airway smooth muscle layer in its full composition is a major structural component of the airways in determining airway function in asthma.
References


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