

Differences in airway remodeling between subjects with severe and moderate asthma

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Background: Airway remodeling in asthma comprises a range of structural changes. Several studies have suggested an association between these changes and disease severity. The relationship between the extent of remodeling and lung function is not well defined.

Objective: We sought to contrast the structural changes in the airways of well-defined groups of subjects with severe and moderate asthma and to correlate the extent of remodeling with disease severity.

Methods: Endobronchial biopsy specimens were obtained from 15 subjects with severe and 13 subjects with moderate asthma. Epithelial integrity, cell-layer areas, subepithelial fibrosis, and the distance between epithelial and airway smooth muscle (ASM) layers were measured by means of image analysis. Collagen was identified by using Van Gieson stain, and ASM was defined by using smooth muscle α -actin immunostaining. Specific immunostains were performed for the evaluation of RANTES, IL-8, and eotaxin expression as markers of ASM phenotype.

Results: ASM area was greater in subjects with severe ($0.24 \pm 0.03 \text{ mm}^2$) than in subjects with moderate ($0.05 \pm 0.01 \text{ mm}^2$) asthma ($P < .001$). The distance between the epithelial and ASM layers was less in the severe group ($0.12 \pm 0.01 \text{ mm}$) than in the moderate group (0.24 ± 0.02 , $P < .001$). A trend toward greater subepithelial fibrosis in subjects with severe asthma did not reach statistical significance. IL-8 and eotaxin expression, but not RANTES expression, were increased in the ASM of subjects with severe asthma compared with in subjects with moderate asthma.

Conclusion: Smooth muscle alteration is the key structural change that distinguishes severe from moderate asthma, and phenotypic change in ASM might contribute to the difficulty

in obtaining adequate control in some subjects with severe asthma. (J Allergy Clin Immunol 2005;116:544-9.)

Key words: Asthma, airway remodeling, structural changes, subepithelial fibrosis, airway smooth muscle, human bronchial biopsies, tissue morphometry

Asthma is a chronic inflammatory disease characterized by bronchoconstriction, pulmonary inflammation, and airway remodeling.¹ It has been suggested that reversibility in asthma is associated with mild disease but that with disease progression, varying degrees of remodeling of the airways might lead to irreversibility.² Subjects with mild and moderate asthma have pulmonary function that is relatively well preserved. These subjects respond appropriately and quickly to inhaled or oral anti-inflammatory and bronchodilator treatment during exacerbations, and their condition remains clinically stable during maintenance therapy. Subjects with severe asthma, on the other hand, appear to have different airway behavior, as evidenced by persistently low expiratory flow rates and failure to respond adequately to treatment in terms of pulmonary function.³ In addition, they often remain clinically symptomatic, despite maximal medical therapy. The factors that are responsible for the phenotypic difference of subjects with severe asthma are poorly understood and remain a matter of much debate.⁴⁻⁶ Chronic uncontrolled airways inflammation and airway remodeling remain the suggested mechanisms by which severe persistent asthma develops.⁷⁻⁹ Remodeling comprises a number of structural changes, including epithelial detachment, subepithelial fibrosis, and smooth muscle hyperplasia and hypertrophy.¹⁰ These factors lead to increased bronchial wall thickness, which in turn might affect the severity of physiologic and clinical parameters in asthmatic subjects.¹¹ Increase in airway smooth muscle (ASM) mass is potentially of substantial importance for asthma pathophysiology because it could account for airway hyperresponsiveness.¹² It is not entirely known how various stimuli or mediators of the inflammatory response are linked to the processes of airway remodeling. It is also not clear how remodeling contributes to the decrease in lung function as asthma severity progresses. Two recent articles demonstrate the usefulness of endobronchial biopsy specimens from asthmatic subjects in the evaluation of airway remodeling.^{13,14} Evidence for hyperplastic

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Abbreviation used

ASM: Airway smooth muscle

ASM growth was reported for subjects with mild-to-moderate asthma,¹⁴ whereas increases in type III collagen deposition, fibroblast numbers, and ASM cell size (hypertrophy) were reportedly features that distinguished severe asthma from mild asthma and chronic obstructive pulmonary disease.¹³

To better define the structural changes that occur in the airways of subjects with severe asthma, we compared the findings from endobronchial biopsy specimens of subjects with severe asthma with those of subjects with well-controlled moderate asthma. The outcomes of interest included alterations in epithelial integrity and in epithelial and smooth muscle areas with increasing disease severity. We were also interested in the degree of subepithelial fibrosis, as well as changes in the distance between the epithelium and smooth muscle with disease progression. To evaluate ASM for proinflammatory phenotypic change, we examined the expression of 3 chemokines of relevance for asthma, namely IL-8, eotaxin, and RANTES.¹⁵⁻¹⁷

METHODS

Subjects

The subjects for this study were recruited at the Montreal Chest Institute and other hospitals of the McGill University Health Centre and at the Hôpital du Sacre-Coeur of the Université de Montréal, Montreal, Canada. The protocols were reviewed and approved by an institutional review board, and informed consent was obtained. All subjects were nonsmokers or ex-smokers of at least 2 years with a smoking history of less than 15 pack-years.

The inclusion criteria for subjects with severe asthma were based on the American Thoracic Society Workshop for Refractory Asthma.¹⁸ It was necessary for at least one of the 2 major criteria to be present: (1) treatment with daily oral steroids for more than 50% of the previous 12 months or (2) treatment with high-dose inhaled steroid (≥ 1000 μg of fluticasone or equivalent per day) and at least one other add-on therapy (long-acting β -agonist, leukotriene receptor antagonist, or theophylline) continuously over the previous 12 months. In addition, at least 2 or more minor criteria had to be present: (1) daily use of short-acting β -agonist; (2) persistent airflow obstruction, as documented with a prebronchodilator FEV₁ of less than 70% and an FEV₁/forced vital capacity ratio of less than 80% of predicted value; (3) 1 or more urgent care visits in the previous 12 months; (4) 3 or more steroid bursts in the previous 12 months; (5) prompt deterioration with less than 25% dose reduction of steroids; or (6) a near-fatal asthma event in the last 3 years.

Subjects with moderate asthma had to fulfill the following criteria: (1) well-controlled asthma on at least 200 $\mu\text{g}/\text{d}$ of fluticasone or equivalent but not exceeding 1000 $\mu\text{g}/\text{d}$, with or without the use of a long-acting β -agonist; (2) 2 or less steroid bursts in the previous 12 months and none in the past 3 months (the total number of days taking oral steroids could not exceed 30 days in the previous 12 months); (3) FEV₁ of greater than 70% of predicted value and greater than 90% of personal best from the previous

2 years; and (4) no more than 1 nonscheduled urgent care or office visit in the previous 12 months.

It was necessary for each subject to demonstrate an improvement in FEV₁ of 12% or greater, with an absolute increase of at least 200 mL from baseline after administration of a β -adrenergic agonist, or an improvement of FEV₁ of 20% or greater after an oral steroid trial. Attached to each inhaler was an electronic device, the MDIlog (Medtrac technologies, Lakewood, Colo), which permitted us to accurately monitor use of the inhaler.¹⁹ We included only subjects who took at least the prescribed 4 inhalations of fluticasone and salmeterol (Advair; 250/50 μg) per day on at least 70% of days in a 1-month run-in period and were thus considered to be sufficiently compliant to be considered to have refractory asthma in the face of adequate treatment.

Subjects were excluded if they had any other known pulmonary disease, including chronic obstructive pulmonary disease, or any major comorbid disease that might affect asthma disease activity, such as HIV, metastatic cancer, and congestive heart failure.

Bronchoscopy and tissue processing

Bronchoscopy was performed during light sedation. Six endobronchial biopsy specimens were obtained from the right lung at various segmental and subsegmental carinae. Tissues were fixed in formaldehyde and embedded in paraffin before cutting, and staining was performed by using a peroxidase-based method, as previously described.²⁰ Briefly, sections of 5 μm (micrometers) in thickness were deparaffinated in xylene, dehydrated in ethanol, and washed in PBS. Tissues were then permeabilized with Triton (0.2% in PBS) and immersed in a solution of hydrogen peroxide in PBS (5% in PBS). The sections were then washed 3 times for 5 minutes in PBS before incubating with Universal Blocking Solution (DakoCytomation Inc, Mississauga, Ontario, Canada) for 20 to 30 minutes. Collagen deposition was measured by using Van Gieson stain, and ASM was identified by using human muscle-specific α -actin antibody (Novo Castra Laboratories Ltd, Newcastle Upon Tyne, England, United Kingdom).

In addition, we examined the tissues for possible differences in terms of smooth muscle chemokine expression between the 2 groups of asthmatic patients. Tissue sections were stained with mAbs that recognized human eotaxin (R&D Systems Inc, Minneapolis, Minn) and IL-8 (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and a polyclonal antibody directed against human RANTES (R&D Systems Inc). These 3 cytokines have previously been shown to be expressed by ASM.¹⁵⁻¹⁷ Slides were then developed with StreptABComplex/horseradish peroxidase and diaminobenzidine substrate, according to the manufacturer's instructions (DakoCytomation Inc). After immunostaining, the extent of ASM immunoreactivity for eotaxin, RANTES, and IL-8 was assessed by using the following scoring system (which refers to the percentage of the tissue specimen that stained positively): 0, no staining; 1, 0% to 12.5%; 2, 12.5% to 25%; 3, 25% to 37.5%; 4, 37.5% to 50%; 5, 50% to 62.5%; 6, 62.5% to 75%; 7, 75% to 87.5%; and 8, 87.5% to 100%.

Tissue morphometry

Image analysis was performed with the Image Pro-Plus 4.0 system (Media Cybernetics, Silver Spring, Md). Images were recorded on a CCD color video camera (Sony, Montvale, NJ) mounted on a conventional light microscope (Olympus Optical Co., Tokyo, Japan) with a 4 $\times$ objective. The program was used to analyze the percentage of intact epithelium, areas of epithelium and smooth muscle mass, subepithelial fibrosis, and distance between the epithelial layer and smooth muscle. Two sections from 2 of the 6 biopsy specimens taken for each subject were analyzed.

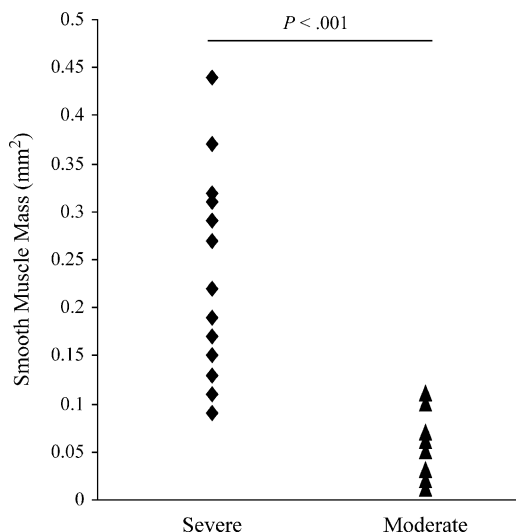


FIG 1. ASM mass in subjects with severe (diamonds, $n = 15$) and moderate (triangles, $n = 13$) asthma. Each point represents the mean area of ASM for a single subject.

Statistical analysis

The values shown are means $\pm$ SEM and ranges unless otherwise stated. Data were analyzed by using unpaired Student t tests, and a P value of less than .05 was considered to be statistically significant.

RESULTS

Patient characteristics

Biopsy specimens were taken from 15 subjects with severe and 13 subjects with moderate asthma. The median age of the severe group was 47 years (range, 25-61 years), with a male/female ratio of 7:8. The moderate group had a median age of 49 years (range, 42-61 years) and a male/female ratio of 7:6. The mean $\pm$ SEM FEV₁ percent predicted after albuterol was $72.7\% \pm 6.5\%$ in the severe group, reflecting persistent airflow limitation despite maximal medical therapy, and $88.6\% \pm 4.8\%$ in the moderate group, reflecting well-preserved pulmonary function.

Assessment of airway remodeling and smooth muscle-specific α -actin immunoreactivity

Airway remodeling was assessed by determining the area and integrity of the epithelium, the smooth muscle mass, the degree of subepithelial fibrosis, and the distance between the epithelium and ASM on bronchial biopsy tissues. The mean total tissue area of the biopsy specimens obtained in the severe group ($0.86 \pm 0.1 \text{ mm}^2$) did not differ significantly from that of the moderate group ($0.79 \pm 0.1 \text{ mm}^2$). The mean smooth muscle area (Fig 1) was $0.24 \pm 0.03 \text{ mm}^2$ in subjects with severe asthma, which was significantly greater ($P < .001$) than that seen in the subjects with moderate asthma ($0.05 \pm 0.01 \text{ mm}^2$). Smooth muscle was present in all sections that were analyzed. The mean epithelial area measured 0.08 ± 0.02

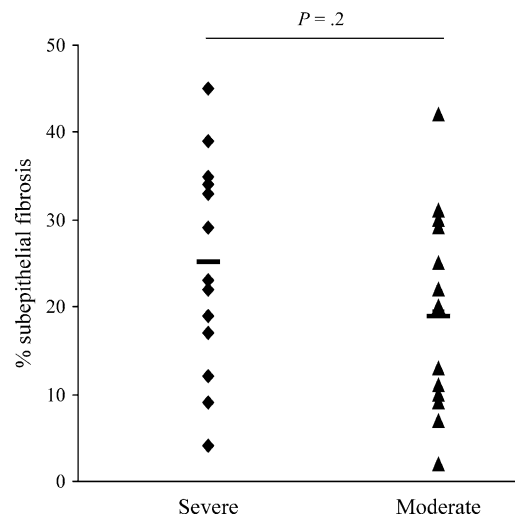


FIG 2. Percentage of subepithelial fibrosis in the airways of subjects with severe (diamonds, $n = 15$) and moderate (triangles, $n = 13$) asthma. Each point represents the percentage for a single subject. Mean values are indicated by horizontal bars.

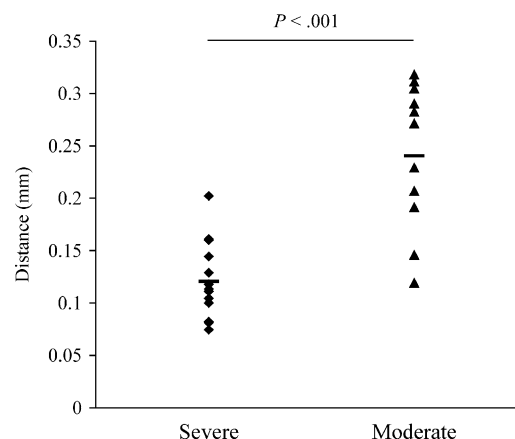


FIG 3. Distance from ASM to epithelium in subjects with severe (diamonds, $n = 15$) and moderate (triangles, $n = 13$) asthma. Each point represents the distance for a single subject. Mean values are indicated by horizontal bars.

mm^2 in the severe group, a value that was not significantly different from that of the moderate group ($0.1 \pm 0.02 \text{ mm}^2$). Subepithelial fibrosis was considered to be the area of tissue beneath the epithelium that contained fibroblasts and collagen. The area was circumscribed and measured with an image analysis program (see the Methods section) and reported as a percentage of the total area of the tissue biopsy. The percentage of subepithelial fibrosis (Fig 2) was also not significantly different ($P = .20$) between the 2 groups: subjects with severe asthma had a mean of $25.0\% \pm 3.0\%$ and subjects with moderate asthma had a mean of $19.3\% \pm 3.3\%$ subepithelial fibrosis. The distance between the luminal aspect of the smooth muscle layer and the epithelium (Fig 3) was significantly different between the 2 groups ($P < .001$); the mean distance measured $0.12 \pm 0.01 \text{ mm}$ in the severe group

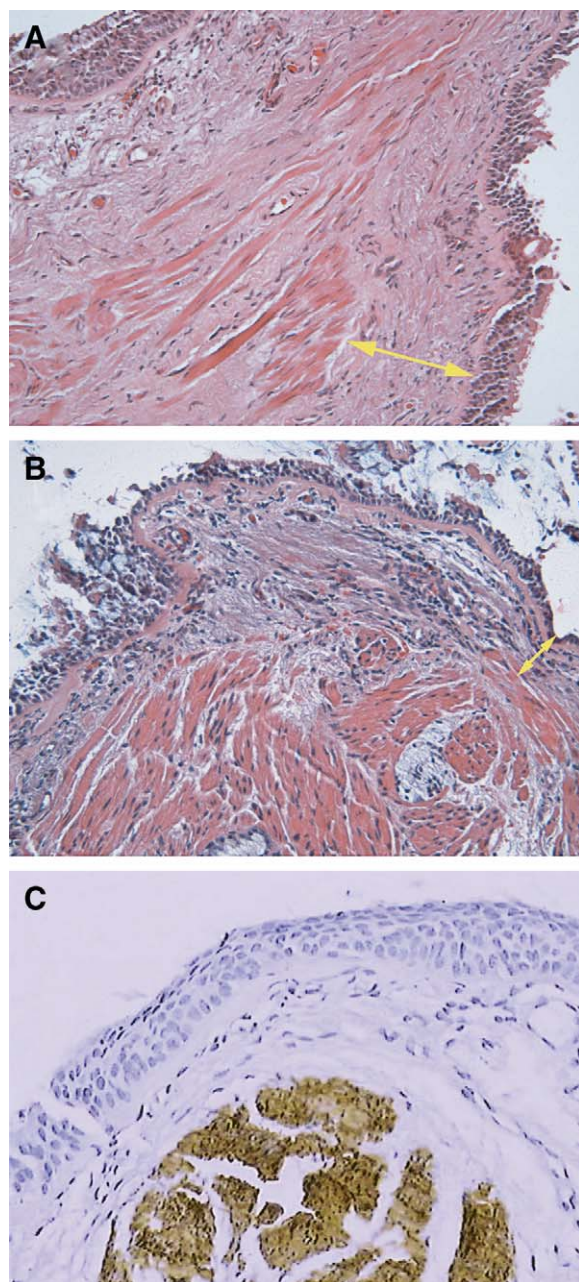


FIG 4. Representative examples of hematoxylin and eosin staining of bronchial biopsy specimens from subjects with moderate (A) and severe (B) asthma showing a greater increase in ASM mass in the subject with severe asthma compared with that in the subject with moderate asthma. Note also the decreased distance between the smooth muscle and epithelial layers in the subject with severe asthma (Fig 4, A). C shows a representative example of immunostaining for eotaxin in the ASM of a subject with severe asthma.

and 0.24 ± 0.02 mm in the moderate group (Fig 4, A and B). We found a significant negative correlation (Fig 5) between FEV₁ percent predicted and smooth muscle area in the severe group ($r = -0.58$, $P < .05$) compared with the moderate group, in which the correlation was not significant ($r = 0.19$, $P > .05$).

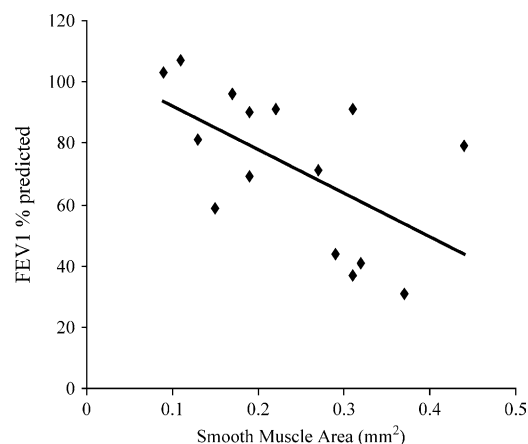


FIG 5. Correlation between FEV₁ percent predicted and smooth muscle area in subjects with severe asthma. Increasing smooth muscle area correlates with a decrease in the FEV₁ percent predicted ($r = -0.58$, $P < .05$).

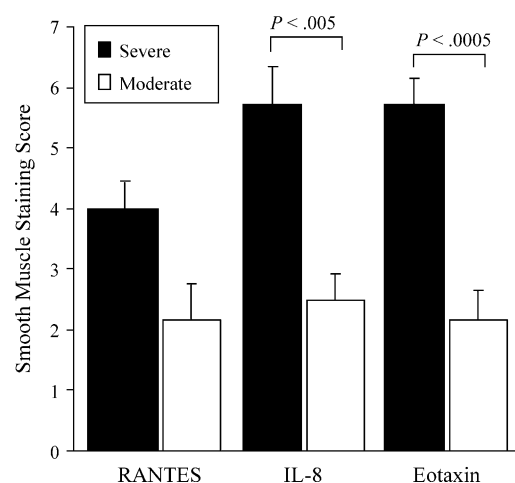


FIG 6. Expression of RANTES, IL-8, and eotaxin by ASM in subjects with severe and moderate asthma. SEM values are indicated by error bars.

There was no significant difference in terms of RANTES expression between the 2 groups. The mean score was 3.3 ± 0.5 in the severe group and 2.2 ± 0.6 in the moderate group (Fig 6). However, the expression of IL-8 and eotaxin in the ASM was significantly increased in the severe group. The mean score for IL-8 expression (Fig 6) in the severe group was 5.7 ± 0.7 , which was significantly higher ($P < .005$) than that in the moderate group (2.5 ± 0.4). The severe group also had a mean score of 5.7 ± 0.5 for eotaxin staining (Fig 4, C, and Fig 6), which was again significantly higher ($P < .0005$) than that of the moderate group (2.2 ± 0.5).

DISCUSSION

To date, few studies have been published on the pathologic changes seen in the airways of subjects with

well-defined severe asthma. Our study groups were characterized from the standpoint of disease severity, and for the severe group, poor compliance with medications was excluded as a cause of lack of disease control. The subjects with moderate asthma were receiving substantial doses of inhaled corticosteroids, as well as other adjunctive treatments, despite receiving lower overall doses of medications than the severe group. Therefore they differed from the severe group predominantly in relation to the control of their asthma. The severe group was comprised of patients who were not optimally controlled, despite documentation of compliance with maximal medical therapy. The severe group was therefore refractory, at least in part, to the usual medications for asthma. In consequence, we were able to address the potential contribution of airway remodeling to severity of disease in these subjects without confounders such as noncompliance.

The nature of airway remodeling in severe asthma has not been adequately documented, and analysis of the changes in the airways has the potential to shed light on the pathophysiology of poor asthma control.²¹ Persistent inflammation in bronchial tissues, along with the ensuing activation of epithelial, subepithelial, and smooth muscle cells, presumably lead to structural changes known as airway remodeling. Since all our subjects were receiving corticosteroid therapy, we chose to focus on the structural abnormalities that might distinguish them from subjects with moderate asthma. A variety of changes have been reported in endobronchial biopsy specimens obtained from a range of asthmatic subjects.^{13,14} Epithelial desquamation has been reported as an injury suffered by the airway lining as a result of allergen exposure. Denudation of the airway epithelium was reported as a significant finding in some early autopsy studies of asthmatic subjects. Indeed, there were early reports of epithelial loss seen on endobronchial biopsy specimens from asthmatic patients that was thought to be a factor in the pathophysiology of asthma.²² However, these findings have been recently challenged. The detachment of epithelial cells from the basement membrane might be an artefactual finding related to the biopsy procedure itself or to the tissue processing.²³ In this study we did not find a difference in total epithelial area between subjects with severe and moderate asthma. Furthermore, the disruption to the integrity of the epithelium was not any greater in the severe group compared with that seen in the moderate group.

Subepithelial fibrosis is a feature of airway remodeling that has received considerable attention. The significance of subepithelial fibrosis for airway function in asthma is uncertain, although it has been argued that it might serve to impede bronchoconstriction.²⁴ Whether the stimulation for production and deposition of extracellular matrix proteins from myofibroblasts comes from signals from the epithelium or from inflammatory cell mediators is not entirely clear.^{25,26} Previous studies have shown that several profibrotic mediators are involved in the formation of subepithelial fibrosis in asthma.²⁷ It has also been demonstrated that there are increased amounts of fibronectin,

tenascin, and collagen I, III, and V deposited beneath the basement membrane, which leads to its thickening and potentially aggravates clinical symptoms by affecting small-airway caliber. However, there was no statistically significant difference between the subjects with moderate and severe asthma in terms of the degree of subepithelial fibrosis present. Benayoun et al²⁸ reported an increase in collagen III expression in their sample of subjects with severe asthma but used a scoring technique to evaluate fibrosis. Interestingly, their patients also demonstrated more severe and possibly irreversible airflow obstruction (FEV₁ approximately 50%). It is conceivable that permanent airflow limitation might result from excessive collagen deposition in the subepithelial space.

There is a lot of interest surrounding the changes in ASM during remodeling. Several publications have shown that ASM cells either undergo hyperplasia²⁹ or become hypertrophied,^{30,31} thereby occupying greater space within the airway wall and thus contributing to mechanical narrowing of the lumen, especially during episodes of bronchoconstriction.³² An increase in force generation by the contracting muscle might also overcome endogenous impedances to airway narrowing, such as lung parenchymal elasticity, to cause excessive airway narrowing. Indeed, an uncoupling of the airway from the parenchyma through an alteration in alveolar attachments or through adventitial edema has been suggested as a potential cause of airway hyperresponsiveness in asthma.³³ Unfortunately bronchoscopic biopsy specimens yield no data on the adventitial portion of the airway wall. Our morphometric studies revealed a markedly increased muscle mass in airway tissues obtained from subjects with severe asthma compared with subjects with moderate asthma. In addition, the smooth muscle was located much closer to the epithelium in subjects with severe asthma, perhaps reflecting a phenomenon of migration of smooth muscle cells toward the epithelial surface. It is not yet known whether migration is a feature of existing smooth muscle cells within the airway or whether it occurs through attraction of circulating precursor stem cell populations. Another explanation for the close proximity of smooth muscle to the epithelium in subjects with severe asthma is that growth factors arising from the epithelium lead to growth closer to the epithelium. However, the growth factors responsible for ASM growth in asthma and their sources are as yet unknown.

Finally, the greater smooth muscle mass seen in the airways of subjects with severe asthma also appears to have a more secretory phenotype than in subjects with moderate asthma, as evidenced by the increased expression of IL-8 and eotaxin. These cytokines are likely to be pivotal in the ongoing inflammatory cycle that characterizes the airways of patients with severe refractory asthma. However, the characterization of the inflammatory cells within the smooth muscle layer seems to be incomplete, having been reported only for mast cells that are present in the muscle layer.³⁴

We have shown in the current study that increased ASM mass and shorter smooth muscle to epithelium distances are selectively seen in airway tissue of subjects with severe

asthma. These findings are significant because they demonstrate a remodeling process that is quite substantial in severe asthma. This permanently altered structure might explain why this population of patients is clinically more symptomatic when compared with subjects with better-controlled moderate asthma. Perhaps the presence of fibrosis in the subepithelial compartment, which was not increased in our subjects with severe asthma, is required for more severe permanent airflow limitation. Our findings provide support for the theory that smooth muscle alteration is the key structural change that distinguishes severe from moderate asthma and suggest that the smooth muscle has a more secretory phenotype. Further studies linking specific causative mechanisms to this remodeling phenomenon are required.

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