

Expression of smooth muscle and extracellular matrix proteins in relation to airway function in asthma

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Background: Smooth muscle content is increased within the airway wall in patients with asthma and is likely to play a role in airway hyperresponsiveness. However, smooth muscle cells express several contractile and structural proteins, and each of these proteins may influence airway function distinctly. **Objective:** We examined the expression of contractile and structural proteins of smooth muscle cells, as well as extracellular matrix proteins, in bronchial biopsies of patients with asthma, and related these to lung function, airway hyperresponsiveness, and responses to deep inspiration. **Methods:** Thirteen patients with asthma (mild persistent, atopic, nonsmoking) participated in this cross-sectional study. FEV₁% predicted, PC₂₀ methacholine, and resistance of the respiratory system by the forced oscillation technique during tidal breathing and deep breath were measured. Within 1 week, a bronchoscopy was performed to obtain 6 bronchial biopsies that were immunohistochemically stained for α -SM-actin, desmin, myosin light chain kinase (MLCK), myosin, calponin, vimentin, elastin, type III collagen, and fibronectin. The level of expression was determined by automated densitometry. **Results:** PC₂₀ methacholine was inversely related to the expression of α -smooth muscle actin ($r = -0.62$), desmin ($r = -0.56$), and elastin ($r = -0.78$). In addition, FEV₁% predicted was positively related and deep inspiration-induced bronchodilation inversely related to desmin ($r = -0.60$), MLCK ($r = -0.60$), and calponin ($r = -0.54$) expression.

Conclusion: Airway hyperresponsiveness, FEV₁% predicted, and airway responses to deep inspiration are associated with selective expression of airway smooth muscle proteins and components of the extracellular matrix. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Actin, desmin, elastin, airway smooth muscle, extracellular matrix, lung function, hyperresponsiveness, deep inspiration-induced bronchodilation, bronchial biopsies

Asthma is characterized by chronic airway inflammation, which is presumed to contribute to variable airways obstruction and bronchial hyperresponsiveness.¹ However, recent studies have led to a reappraisal of the role of airway smooth muscle in asthma pathophysiology.² Because smooth muscle contraction leads to airway narrowing, abnormalities in airway smooth muscle size, mass, or function could easily lead to exaggerated airway narrowing. In addition, mast cells within the airway smooth muscle bundles have been associated with airway hyperresponsiveness,³ and more recently, we observed a similar association with impaired deep inspiration-induced bronchodilation in patients with asthma.⁴

Increased smooth muscle mass has been demonstrated in bronchial biopsies⁵⁻⁷ as well as in resected lung tissue^{8,9} from patients with asthma compared with healthy subjects. Mathematical models have shown that increased smooth muscle mass can explain exaggerated airway narrowing to contractile stimuli in patients with asthma,¹⁰ especially at high lung volumes.¹¹ Interestingly, although increased smooth muscle area in bronchial biopsies has been associated with impaired lung function,^{5,7} no relationship was found with airway hyperresponsiveness. Nevertheless, *in vitro* studies have shown that smooth muscle cells obtained from bronchial biopsies of patients with asthma exhibit an increase in isotonic shortening¹² and shortening velocity compared with controls without asthma.¹³

Smooth muscle cells express several contractile and structural proteins.^{14,15} Cultured airway smooth muscle cells with a contractile phenotype are relatively rich in smooth muscle myosin heavy chain (sm-MHC), α -smooth muscle actin (α -SM-actin), calponin, desmin, and myosin light chain kinase (MLCK), whereas when proliferating they express less sm-MHC, calponin, α -SM-actin, and desmin, and significantly more vimentin. Benayoun et al⁵ examined the expression of some of these contractile proteins in bronchial biopsies in relation to asthma severity. MLCK expression correlated inversely with lung function, but this was not the case for the proteins α -SM-actin or myosin. This suggests that the level of expression of these proteins may

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Abbreviations used

α -SM-actin:	α -Smooth muscle actin
MLCK:	Myosin light chain kinase
Rrs:	Resistance of the respiratory system
Rrs _{Exp} :	Resistance of the respiratory system during tidal expirations
Rrs _{Insp} :	Resistance of the respiratory system during tidal inspirations
sm-MHC:	Smooth muscle myosin heavy chain

have different functional consequences. We selected several contractile and structural proteins that may influence airway responsiveness and function, namely α -SM-actin, myosin, desmin, vimentin, calponin, and MLCK. Furthermore, it has been shown that stretch of smooth muscle cells can increase the expression of contractile proteins.^{16,17} Because smooth muscle cells are most likely stretched during deep inspiration, we analyzed the relationship between protein expression and airway responses to deep inspiration.

In addition, the smooth muscle bundles are embedded in and also contain extracellular matrix. The amount and the composition of the matrix may have functional consequences by altering the physical properties of the airway wall^{18–20} and can also influence the proliferation of smooth muscle cells.^{21–23} Therefore, we analyzed the expression of different extracellular matrix proteins (type III collagen, fibronectin, and elastin) within and surrounding the smooth muscle bundles.

We hypothesized that a higher level of expression of the selected contractile and structural proteins of smooth muscle cells, as well as components of the extracellular matrix, in bronchial biopsies are associated with increased airway hyperresponsiveness and impaired deep inspiration-induced bronchodilation in asthma. The aim of this study was to relate FEV₁% predicted, airway hyperresponsiveness, and deep inspiration-induced changes in resistance of the respiratory system as measured by forced oscillation technique to the level of expression of α -SM-actin, myosin, desmin, vimentin, calponin, and MLCK, as well as type III collagen, fibronectin and elastin, in bronchial biopsies of patients with asthma.

METHODS

Subjects

This study was performed in the framework of a previously published project.⁴ Thirteen patients with mild persistent asthma (Global Initiative for Asthma steps 1 and 2²⁴) were recruited for this study. All patients had a history of episodic chest tightness or wheezing. Their baseline FEV₁ was more than 70% of predicted.²⁵ The PC₂₀ methacholine was less than 8 mg/mL.²⁶ All patients were atopic, as determined by a positive skin prick test result (≥ 3 mm wheal) to 1 or more of 10 common aeroallergen extracts (ALK-Abelló, Nieuwegein, The Netherlands). The patients were clinically stable, nonsmokers or exsmokers with less than 2 pack-years, and did not have a recent (≤ 2 weeks) upper respiratory tract infection or other relevant diseases. None of the patients had used inhaled or oral corticosteroids within 3 months before or during the study. The protocol was approved by the institutional review board for human studies, and before entering the study, the patients gave their written informed consent.

Study design

In this cross-sectional study, measurements were performed on 4 separate days within 3 to 4 weeks. On the first visit, medical history was taken, atopy

was determined, and FEV₁% predicted and resistance of the respiratory system (Rrs) were measured before and after 400 μ g salbutamol. On 2 additional visits, the patients returned for a methacholine challenge to determine airway hyperresponsiveness and deep inspiration-induced bronchodilation after a single dose of methacholine that induced a 20% fall in FEV₁.⁴ Within 1 week of the last visit, a bronchoscopy was performed to obtain 6 bronchial biopsies.

Airway hyperresponsiveness

Methacholine bromide in normal saline was used for the bronchial challenges that were performed by standardized methodology.²⁶ At 5-minute intervals, aerosolized serial doubling concentrations of methacholine (0.15–40 μ mol/L) were inhaled by tidal breathing (DeVilbiss, Somerset, Pa) for 2 minutes with the nose clipped. The challenge was stopped when FEV₁ dropped by more than 20% from baseline, and the response was expressed as the provocative concentration causing a 20% fall in FEV₁ PC₂₀.

Airway responses to deep inspiration

Deep inspiration-induced bronchodilation was measured using a single-dose methacholine challenge to induce a fall in FEV₁ of 20% in the absence of deep inspirations before methacholine inhalation. Baseline measurements of FEV₁ and Rrs were followed by a period of 20 minutes without deep inspirations. A single dose of methacholine (approximately the cumulative dose of the PC₂₀ of the previous challenge) was inhaled, and 2 minutes later, Rrs was measured during tidal breathing, a deep inspiration to total lung capacity, a passive expiration, and again tidal breathing. This was directly followed by spirometry to measure the fall in FEV₁. The forced oscillation technique with an applied oscillation frequency of 8 Hz and an amplitude of ± 1 cmH₂O was used to measure Rrs continuously during tidal breathing and a deep inspiration (Woolcock Institute, Sydney, Australia).⁴ Deep inspiration-induced bronchodilation was expressed as the reduction in Rrs during tidal breathing induced by the deep inspiration.

Bronchoscopy, immunohistochemistry, and image analysis

Bronchoscopy was performed by experienced pulmonologists according to a standardized and validated protocol.²⁷ All patients received 400 μ g salbutamol 30 minutes before bronchoscopy. Six biopsies were taken at the (sub)segmental level using disposable forceps (radial edge; Boston Scientific, Boston, Mass).

The biopsies were fixed for 24 hours in 4% neutral buffered formaldehyde, processed, and embedded in paraffin. Sections 4 μ m thick were cut, and hematoxylin-eosin staining was used to evaluate morphologic quality (intact reticular basal membrane and submucosa without crushing artifacts, blood clots, or only epithelial scrapings). Two sections per subject were selected on the quality of the submucosa, and not on the quantity of smooth muscle area. This was done to avoid a selection bias with regard to the main outcome parameter. In addition, the observers were blinded with regard to the subject number and their disease. The latter was chosen to avoid a selection bias with regard to the main outcome parameter. Antigen retrieval was performed on paraffin-embedded sections with citrate (desmin, myosin, and MLCK) or trypsin (type III collagen). α -SM-actin, calponin, vimentin, and fibronectin did not need antigen retrieval. The sections were incubated with mouse mAbs directed against α -SM-actin (1:50,000, clone 1A; Santa Cruz Biotechnology, Santa Cruz, Calif), myosin (1:40, clone 1A4; Sigma-Aldrich, St Louis, Mo), desmin (1:200, clone D33; Dako UK Ltd, Cambridgeshire, United Kingdom [UK]), vimentin (1:1000, clone V9; Dako), calponin (1:10,000, clone hCP; Sigma), MLCK (1:4000, clone k36; Sigma), type III collagen (1:2000, clone III-53; Merck Calbiochem, Darmstadt, Germany), and fibronectin (1:100, clone 568; Novocastra, Newcastle upon Tyne, UK). As a secondary antibody, Envision-HRP (Dako), was used. Positive cells stained red after development with NovaRed (Vector Laboratories, Burlingame, Vt). Sections were counterstained with Mayer hematoxylin. As a negative control, the primary antibody was omitted from this procedure. For elastin expression, we used Weigert staining with Oxone (Klinipath, BV, Duiven, The Netherlands).²⁸

Morphometry was performed by means of digital image analysis.²⁹ The expression of the smooth muscle proteins (α -SM-actin, myosin, desmin, vimentin, MLCK) was determined in the total biopsy area (including the epithelial layer and glands). Type III collagen, fibronectin, and elastin were measured within the smooth muscle bundles and the area surrounding the smooth muscle bundles separately. We used the desmin-stained adjacent biopsy sections to detect manually the positive stained area that appeared in bundles. Protein expression was quantified by fully automated densitometry (KS400; Zeiss, Oberkochen, Germany).^{27,29} This was performed by using a linear combination of red-filtered and blue-filtered grayscale images to derive a grayscale image (range, 0-255) in which the brown-red staining of interest is highlighted above a uniform background (white = gray value 255). This resulted in a narrow and peaked gray value distribution of background pixels with a longer tail on the left, which represented the positive stained pixels. The distribution was normalized toward the background peak, and subsequently inverted to obtain a zero value for the white background peak (white = gray value 0).

Data and statistical analyses

Airway hyperresponsiveness was expressed as PC₂₀. Reversibility was defined as the change in FEV₁% predicted or Rrs by 400 μ g salbutamol. Rrs was calculated from all the data points, within the 95% CI, during 3 tidal inspirations (Rrs_{Insp}) and during 3 tidal expirations (Rrs_{Exp}) before and after deep inspiration. Deep inspiration-induced bronchodilation was expressed as the difference between Rrs after deep inspiration and Rrs before deep inspiration⁴; thus, a negative value indicates bronchodilation. This was performed for Rrs_{Insp} and Rrs_{Exp} separately because the airways may behave differently during inspiration and expiration.³⁰

Positive staining intensity was expressed as mean density (gray value). The outcome parameters were (log)transformed if necessary to obtain a normal distribution. Within-group differences were analyzed by 2-tailed paired *t* tests or Wilcoxon ranks test. Spearman rank correlation coefficient was used to explore associations between the expression of the proteins and the functional parameters. *P* values <.05 were considered statistically significant.

RESULTS

Smooth muscle protein expression

The density of the smooth muscle protein staining was determined in the whole biopsy section. The mean density (gray value) for each marker is given in Table I. All available sections (2 per patient) were used in the analysis. Fig 1 presents examples of the immunohistochemical staining in the same biopsy section of 1 subject. All markers, except for vimentin, strongly stained the airway smooth muscle cells. Vimentin was negative or weakly expressed in the smooth muscle cells. Outside the smooth muscle bundles, there was staining of the contractile markers, mainly around vessels, around submucosal glands (myoepithelial cells), and in scattered mesenchymal cells (fibroblasts and myofibroblasts).

Extracellular matrix expression

The density of the extracellular matrix protein expression of type III collagen, fibronectin, and elastin was determined in the smooth muscle bundles and the area surrounding the smooth muscle bundles separately. The proteins were expressed both in the lamina propria as well as within the airway smooth muscle bundles, with a fibrillar pattern for type III collagen and elastin and more diffuse pattern for fibronectin. Fibronectin and type III collagen stained the subepithelial basal membrane as well. Airway smooth muscle cells stained negatively for these markers. There were significant differences in density of type III collagen

TABLE I. Mean density of the expression the smooth muscle proteins

Smooth muscle protein	Mean density
α -SM-actin	20.8 (11.9-38.9)
Myosin	14.3 (5.9-18.1)
Desmin	14.4 (6.1-26.7)
Vimentin	13.2 (9.7-19.0)
Calponin	20.2 (9.8-40.4)
MLCK	15.9 (6.5-26.0)

The mean density (gray value) of each marker as measured in the bronchial biopsies. The smooth muscle proteins were measured in the total biopsy area. The data are expressed as median (range).

and fibronectin between the smooth muscle bundles and the area surrounding the muscle (Table II).

Airways obstruction

FEV₁% predicted correlated positively with the mean density of calponin ($r = 0.58$), desmin ($r = 0.61$), and MLCK ($r = 0.55$; $P < .05$; Fig 2, A and B). There was a borderline significant correlation between FEV₁% predicted and the expression of α -SM-actin ($r = 0.58$), myosin ($r = 0.53$), and fibronectin (within the smooth muscle bundles; $r = 0.56$; $P = .06$). None of these correlations was seen for FEV₁% predicted after salbutamol. In addition, mean density of both α -SM-actin and calponin correlated inversely with FEV₁ reversibility ($r = -0.54$ and $r = -0.61$, respectively; $P < .05$). Also, the change in Rrs_{Insp} and Rrs_{Exp} by salbutamol correlated positively with mean density of α -SM-actin and calponin ($r > 0.70$; $P < .01$).

Dynamics of airway narrowing

PC₂₀ methacholine was inversely related to the expression of the contractile smooth muscle protein α -SM-actin ($r = -0.62$; $P < .05$; Fig 2, D), the structural smooth muscle protein desmin ($r = -0.56$; $P < .05$; Fig 2, C), and the extracellular matrix protein elastin outside the smooth muscle bundles ($r = -0.78$; $P < .01$). PC₂₀ methacholine was not significantly related to expression of elastin within the smooth muscle bundles.

Airway responses to deep inspiration

The reduction in Rrs_{Insp} by deep inspiration was inversely related to the expression of desmin ($r = -0.60$; Fig 2, E), MLCK ($r = -0.60$; Fig 2, F), and calponin ($r = -0.54$) in the bronchial biopsies.

DISCUSSION

Our results demonstrate an inverse association between PC₂₀ methacholine and the level of expression of α -SM-actin, desmin, and elastin in bronchial biopsies in patients with asthma. Also, we showed that FEV₁% predicted was positively related, and deep inspiration-induced reduction in respiratory resistance inversely related to calponin, desmin, and MLCK expression. Thus, airway hyperresponsiveness, lung function, and airway responses to deep inspiration are associated with the level of expression of some, but not all, of the smooth muscle contractile and structural proteins, as well as the composition of the extracellular matrix within the airway wall. This suggests that the dynamics of airway

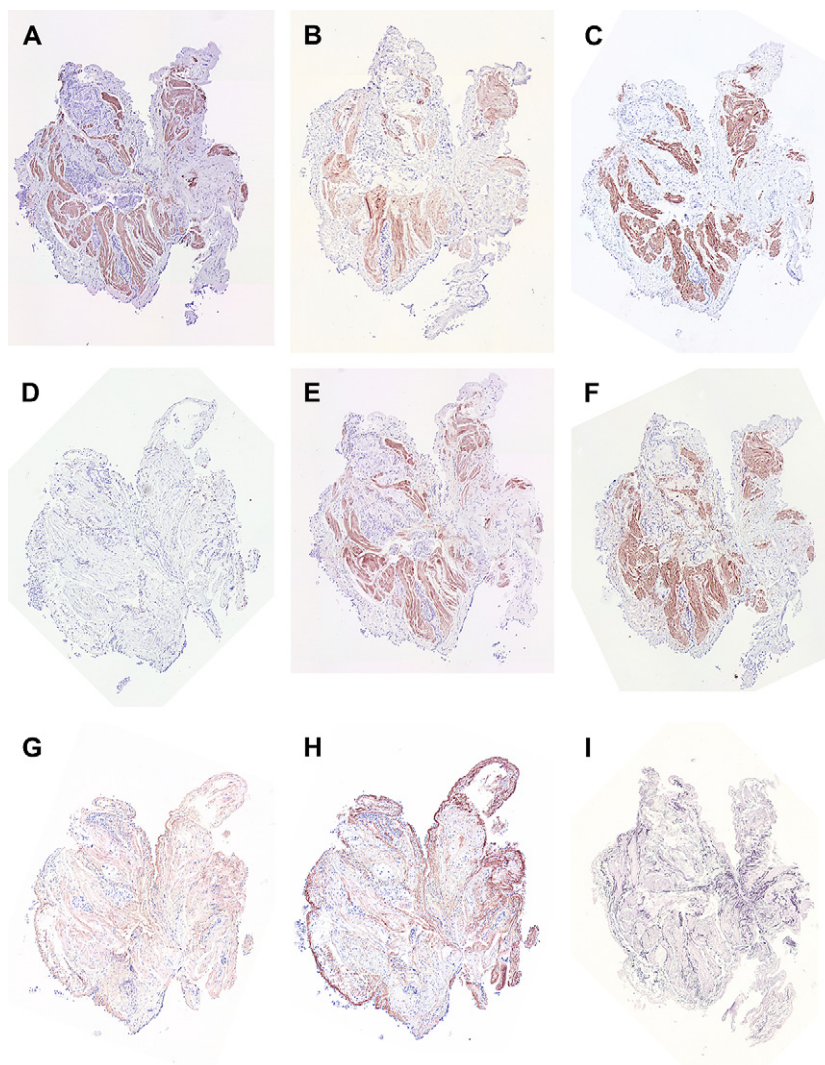


FIG 1. Examples of the immunohistochemical stainings. The same biopsy section is shown for the immunohistochemical staining for α -SM-actin (A), myosin (B), desmin (C), vimentin (D), calponin (E), MLCK (F), type III collagen (G), fibronectin (H), and elastin (I). Original magnification $\times 200$ for all graphs.

TABLE II. Mean density of the expression the extracellular matrix proteins

	Within SM bundles	Outside SM bundles
Type III collagen	20.6 (13.1-35.6)	34.3 (25.9-46.2)*
Fibronectin	42.1 (15.7-64.6)	30.8 (16.5-42.3)†
Elastin	13.1 (9.2-15.2)	10.4 (7.2-16.2)

The mean density (gray value) of each marker as measured in the bronchial biopsies. The data are expressed as median (range). The extracellular matrix proteins were measured in the smooth muscle bundles and the area surrounding the smooth muscle bundles separately. The mean density of type III collagen staining was significantly higher in the area surrounding the smooth muscle bundles compared with within the bundles (* $P = .003$), whereas this was opposite for mean density of fibronectin († $P = .003$). Elastin expression was not significantly different between the compartments.

function are influenced by the expression of several distinct smooth muscle and extracellular matrix proteins.

To our knowledge, this is the first study showing an association between airway hyperresponsiveness and smooth muscle cell

protein expression in patients with asthma. We used markers of different functional components of smooth muscle cells and densitometry to analyze the expression of these proteins in the total biopsy area. An association between airway hyperresponsiveness and smooth muscle area has been shown in bronchial biopsies of both healthy control subjects and patients with asthma. However, no significant association was found in the subanalysis of the patients with asthma only.⁶ We found a relationship between PC₂₀ methacholine and the level of α -SM-actin and desmin expression in bronchial biopsies of patients with asthma. This suggests that airway hyperresponsiveness is associated with the expression of smooth muscle contractile and structural proteins within the airway wall, and not with smooth muscle area *per se*.

In addition, we found a positive relation between FEV₁% predicted and the expression of desmin, myosin, and calponin. Associations between FEV₁% predicted and MLCK expression⁵ or smooth muscle area⁷ have been shown, but with opposite results. This may be due to differences in asthma severity of the

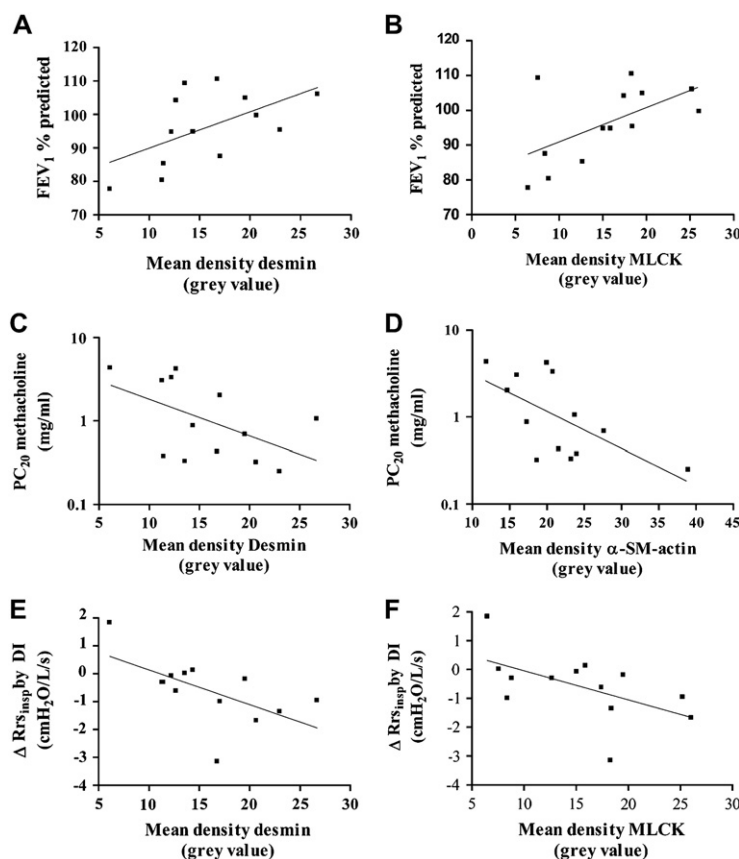


FIG 2. Associations between smooth muscle markers and lung function parameters. The mean density of desmin ($r = 0.61$; $P = .02$) (A) and MLCK ($r = 0.55$; $P = .05$) (B) in relationship with FEV₁% of predicted; desmin ($r = -0.56$; $P = .04$) (C) and actin ($r = -0.62$; $P = .02$) (D) in relationship with PC₂₀ methacholine; and desmin ($r = -0.60$; $P = .03$) (E) and MLCK ($r = -0.60$; $P = .03$) (F) in relationship with the change in Rrs_{exp} by deep breath. DI, Deep inspiration; SM, smooth muscle.

selected patients, because we included only patients with mild disease who were steroid-naïve, whereas patients with more severe disease on steroid treatment were included in the other studies.

In our study, we used densitometry of the immunohistochemically stained sections to quantify the expression of different contractile and extracellular matrix proteins in or surrounding the smooth muscle bundles. Stereological methods have been used to analyze numbers and size of airway smooth muscle cells in bronchial biopsies.⁶ However, densitometry is a reliable and reproducible method,²⁷ and it may also be a valuable tool to examine airway smooth muscle protein expression in bronchial biopsies.

None of the patients included in this study used inhaled or oral steroids within 3 months before or during the study. Therefore, the results were not affected by the effects of glucocorticosteroids. However, as part of the bronchoscopy procedure, all patients received 400 µg salbutamol 30 minutes before the bronchoscopy. We cannot exclude that this may have altered smooth muscle and extracellular matrix protein expression as measured in the biopsy sections. On the other hand, it is feasible to speculate that all patients would be equally affected by the use of salbutamol.^{31,32}

How can we interpret these results? We found an inverse correlation between airway hyperresponsiveness and the level of α-SM-actin, desmin, and elastin expression in asthma, but not

with the other smooth muscle contractile proteins or extracellular matrix components. A higher level of α-SM-actin expression may indicate more actin monomers that can form longer actin filaments by polymerization in the asthmatic inflammatory environment.³³ Smooth muscle cells with longer actin filaments show a more elastic behavior, which can generate force even after being stretched,^{34,35} and may therefore increase airway hyperresponsiveness. Desmin, on the other hand, is an intermediate filament, present in dense bodies, and stabilizes the contractile units and participates in stress transmission between contractile units and anchorage sites linking to the extracellular matrix.³⁶⁻³⁸ An increase in desmin could enhance force transmission between contractile units, which may increase total force generation, and therefore airway narrowing on stimulation. Indeed, in mice lacking desmin, lung stiffness and airway hyperresponsiveness were decreased compared with mice with desmin expression.^{39,40} Increased presence of elastin has been shown in central airways of patients who died of asthma,²⁸ but also a paucity of elastic fibers just underneath the basal membrane was demonstrated in these patients. The mechanical consequences of extracellular matrix, both within and surrounding smooth muscle bundles, is still under debate and most likely depends on the load it provides to airway smooth muscle,⁴¹⁻⁴³ and can either enhance airway narrowing or oppose it. Overall, our data indicate that expression of α-SM-actin and desmin in the airway wall contribute to airway

hyperresponsiveness, further augmented by an increase in elastin expression outside the smooth muscle bundles.

Interestingly, we found a positive relationship between FEV₁% predicted and the positive staining intensity for the smooth muscle proteins calponin, desmin, and MLCK, as well as a negative relationship between these markers and deep breath-induced reduction in respiratory resistance. These proteins are associated with a contractile phenotype of smooth muscle cells.¹⁴ However, a study using ovalbumin-sensitized rats showed a reduction of 50% to 60% in the smooth muscle proteins α -SM-actin, smooth muscle 1 smooth muscle-myosin heavy chain, and smooth muscle-MLCK 24 hours after allergen (ovalbumin) challenge.^{44,45} Expression of smooth muscle-(myosin heavy chain)1, calponin, and sm-MLCK was also reduced after 35 days of allergen challenge.⁴⁴ This suggests that allergen exposure may lead to a change in smooth muscle phenotype from contractile to proliferative, in parallel with impaired lung function. When extrapolating these results, our findings may indicate that the patients, who were all atopic, with lower lung function had more smooth muscle cells of the proliferative phenotype, and thus less expression of contractile proteins. On the other hand, it has been shown that cultured smooth muscle cells with increased tone produce enhanced levels of contractile proteins, such as myosin, MLCK and desmin, when cultured under cyclic stretch conditions.^{16,17,46} The positive correlations between FEV₁% predicted and deep breath-induced bronchodilation could therefore also reflect the effect of stretch on contractile protein production in these patients with asthma, rather than the influence of increased expression of these contractile markers on lung function. However, both suggestions are purely speculative and require further investigation.

What is the clinical implication of our study? Our data show that an increased expression of different components of the contractile unit of the smooth muscle cell, as well as of elastin in the surrounding extracellular matrix, may lead to an increase in the response of the airways to methacholine. Symptoms in mild persistent asthma are most likely the result of airway smooth muscle stimulation by direct or indirect stimuli. Even though it is controversial whether selective destruction of smooth muscle by thermoplasty can improve hyperresponsiveness,^{47,48} it has been shown to reduce symptoms, asthma control, prebronchodilator FEV₁, and exacerbations, and improve quality of life for as long as 12 months.^{48,49} Also, long-acting anticholinergic treatment in a guinea pig model of ongoing asthma prohibited the allergen induced increase in airway smooth muscle cell proliferation and contractility.⁵⁰ In addition, glucocorticosteroids have been shown to influence airway smooth muscle function as well, in particular actin-filament dynamics.^{51,52} Understanding the structure and function of airway smooth muscle cells in asthma could therefore lead to new targeted therapeutic strategies.

We conclude that airway hyperresponsiveness is associated with the level of expression of α -SM-actin, desmin, and elastin within the bronchial wall, but not with myosin, calponin, vimentin, type III collagen, or fibronectin. This suggests that expression of each of the contractile and structural smooth muscle proteins, as well as components of the extracellular matrix, influences dynamic airway function distinctly.

Clinical implications: This study underlines the role of airway smooth muscle in relation to the dynamics of airway function in asthma.

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