

Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation

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Background: Bronchial asthma is characterized by airway structural changes, including mucosal inflammation and subepithelial collagen deposition. An imbalance between the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) is thought to play a critical role in the synthesis or degradation of the extracellular matrix of the airway architecture. However, the relationship between subepithelial basement membrane thickness and these enzymes in asthma has not been determined.

Objective: We compared the thickness of collagen and tenascin deposition and the expression of MMP-9 and TIMP-1 in bronchial biopsy specimens from subjects with asthma and control subjects.

Methods: Bronchial biopsy specimens were obtained from 25 subjects with asthma and 10 healthy control subjects to estimate the extent of collagen and tenascin deposition in subepithelial reticular basement membrane by immunohistochemical staining. Using a computer-assisted image analysis system, we quantitated expression of both epithelial and submucosal MMP-9 and TIMP-1. The numbers of inflammatory cells were also determined.

Results: Subjects with asthma exhibited greater thickness of collagen III ($P < .01$), collagen V ($P < .01$), and tenascin ($P < .01$) deposition in reticular basement membrane than did control subjects. The proportions of epithelium and submucosa immunoreactive to MMP-9 and TIMP-1 were significantly higher in the subjects with asthma than in the control subjects (each $P < .001$). Submucosal expression of MMP-9 was significantly higher than that of TIMP-1 in subjects with asthma ($P < .01$). Significant correlations were found between the number of myofibroblasts and thicknesses of collagen III ($r_s = 0.70$, $P < .001$), collagen V ($r_s = 0.67$, $P < .001$), and tenascin ($r_s = 0.58$, $P < .01$) in subjects with asthma. On the other hand, the number of eosinophils was correlated with degree of mucosal expression of MMP-9 ($r_s = 0.43$, $P < .05$) and TIMP-1 ($r_s = 0.69$, $P < .001$). In subjects with asthma, a significant inverse correlation was found between subepithelial fibrosis and FEV₁ (type III collagen, $r_s = -0.89$, $P < .001$; type V collagen, $r_s = -0.90$, $P < .001$; tenascin, $r_s = -0.88$, $P < .001$), and airway responsiveness

(type III collagen, $r_s = -0.59$, $P < .01$; type V collagen, $r_s = -0.47$, $P < .05$; tenascin, $r_s = -0.48$, $P < .05$).

Conclusion: These findings suggest that collagen III, collagen V, and tenascin deposition in basement membrane in subjects with bronchial asthma are associated with increased expression of MMP-9, which may be produced by eosinophils, and that airway remodeling in subjects with asthma may be related to air-flow obstruction and airway hyperresponsiveness. (*J Allergy Clin Immunol* 1998;102:783-8.)

Key words: Matrix metalloproteinase, tissue inhibitors of metalloproteinase, collagen, tenascin, remodeling, inflammation

Bronchial asthma is a chronic airway inflammatory disease¹ featuring infiltration of airways by eosinophils, mast cells, and T lymphocytes and tissue remodeling that includes subepithelial basement membrane thickening and hypertrophy/hyperplasia of airway smooth muscle.² These findings are also observed in mild asthma, even in patients with a relatively short clinical history.³ The major components of the basement membrane are collagen type IV, laminin, nidogen, and some proteoglycans.⁴ A characteristic feature of airway remodeling in asthma is extensive deposition of interstitial proteins, including collagen III and V and fibronectin, in the subepithelial layer.⁵ Tenascins are a family of large oligomeric extracellular matrix (ECM) glycoproteins with restricted expression in adult human tissues.⁶ Tenascins are abundantly expressed during embryogenesis, and reexpression occurs during wound healing and in tumor stroma.⁷ A recent study showed that tenascin immunoreactivity in airways is increased in patients with asthma.⁸ Airway inflammation in asthma may be associated with a simultaneous wound-repair process in the bronchi. Wound healing is characterized by the appearance of many ECM components in a spatial and sequential pattern.⁹ These repair and developmental processes share common features, suggesting that mechanisms functioning during embryonic development may be recapitulated during inflammation and wound healing.¹⁰

Development, cell migration, infiltration, tissue remodeling, and wound healing are physiologic processes in which the matrix metalloproteinases (MMPs) play crucial roles.¹¹⁻¹³ MMPs are a family of zinc- and calcium-dependent endopeptidases that degrade the various components of connective tissue matrix. In particular, the major *in vivo* role of the gelatinases has been thought to be the digestion of gelatins and type IV collagen, the

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TABLE I. Characteristics of subjects

	Bronchial asthma (n = 25)	Control (n = 10)
Sex ratio (M:F)	14:11	7:3
Age (y)	31 (16-52)	36.3 (26-44)
Duration of asthma (y)	6.6 (1-34)	
FEV ₁ (% pred)	72.4 (54.2-85.6)	86.5 (80.5-91.5)
AHR* (unit)	1.523 (0.184-4.324)	>50

unit, Equal to 1 minute of 1.0-mg/mL aerosol inhalation of methacholine.

Values are mean (range).

*Airway hyperresponsiveness was measured as the minimum cumulative.

Abbreviations used

ECM:	Extracellular matrix
MMP:	Matrix metalloproteinase
RBM:	Reticular basement membrane
TIMP:	Tissue inhibitors of metalloproteinase

major constituent of cellular basement membrane.¹⁴ The coordinated production of MMPs and their tissue inhibitors of metalloproteinases (TIMPs) in a range of development and remodeling situations suggest that TIMPs play a significant role in the regulation of MMP activation in vivo.¹⁵ Four TIMPs have been reported, and TIMP-1 and TIMP-2 have been characterized. Both TIMP-1 and TIMP-2 inhibit the active forms of all MMPs. Interestingly, these inhibitors can also bind to the latent forms of both gelatinases: TIMP-1 binds to the gelatinase B proenzyme, and TIMP-2, to the latent gelatinase A.¹² In this manner, TIMPs may exert additional control of the effects of MMP by inhibiting autoactivation of the gelatinases.

However, little is known about how these enzymes control tissue remodeling. To examine this mechanism, we ascertained whether the degree of subepithelial thickening in patients with asthma differed significantly from that in healthy subjects and examined the expression of MMP-9 and TIMP-1 immunohistochemically in bronchial mucosa. We also examined the relationships between the degree of subepithelial thickness and indices of pulmonary function and airway responsiveness.

METHODS

Subjects

Twenty-five nonsmoking subjects with asthma were recruited from our hospital. Asthma was diagnosed according to the criteria of the American Thoracic Society.¹⁶ The duration of asthma ranged from 1 to 34 years. Of these 25 patients, 6 were nonatopic as determined by clinical history and a skin prick test performed with common aeroallergens generally used on the skin. No patients had received inhaled or oral corticosteroids or any other anti-inflammatory drugs such as sodium cromoglycate or nedocromil sodium in the previous 3 months. Patients used short-acting β_2 agonists only when necessary. Ten healthy subjects were used as a control group. They were nonallergic and nonsmokers; none had any serious lung disease (Table I). No subjects had had any upper or lower respiratory infection for at least 2 weeks before entering the study. The Ethics Committee of Toho University School of Medicine approved

TABLE II. Thickness of subtypes of collagen and tenascin deposition in the subepithelial basement membrane

	Bronchial asthma	Control
Collagen III (μ m)	10.3* (5.3-15.2)	4.5 (3.7-5.7)
Collagen IV (μ m)	5.3 (4.4-6.0)	5.0 (4.4-5.7)
Collagen V (μ m)	9.2* (5.0-12.6)	4.5 (3.7-5.3)
Tenascin (μ m)	10.7* (4.6-16.8)	3.3 (2.3-3.9)

Values are mean (range).

* $P < .01$ vs control.

this study, and written informed consent was obtained from each participant. Airway responsiveness was measured as the minimal cumulative dose of methacholine at which respiratory resistance began to increase during continuous inhalation of methacholine in stepwise incremental concentrations.¹⁷

Bronchoscopy and bronchial biopsy

Premedication with a 0.5-mg intramuscular injection of atropine sulphate and 15 mg of pentazocine was administered to subjects. After the throat had been anaesthetized with 4% lidocaine spray, all patients inhaled 200 μ g of salbutamol to prevent bronchoconstriction. A bronchoscope (BF 1T-30; Olympus Co, Tokyo, Japan) was inserted through the mouth and the pharynx, and the trachea and bronchi were anaesthetized with 2% lidocaine. Oxygen was administered at 4 L/min via nasal cannulae throughout the procedure. Biopsy forceps (FB-15C; Olympus Co) were used to collect 2 or 3 specimens from the spur of a basal segment bronchus of the right lower lobe and from the subcarina of the right upper lobe. After bronchoscopy, inhalation of 200 μ g of salbutamol was administered. All subjects tolerated the procedure well, and there were no instances of bronchoconstriction.

Sample processing and immunohistochemistry

Biopsy specimens were mounted in an OCT compound (Miles, Scientific, Naperville, Ill), frozen rapidly in dry ice-acetone, and stored at -70°C until used. Four-micrometer cryostat serial sections were cut, fixed in acetone, and stained with hematoxylin and eosin for conventional histologic examination. The sections were also immunostained with the alkaline phosphatase anti-alkaline phosphatase system (Dako, Glostrup, Denmark)¹⁸ according to the manufactures instructions and developed with fast red. Control slides were prepared by substitution of PBS and incubation with an irrelevant mouse mAb of the same isotype and concentration.

Detection of subtypes of collagen and tenascin. Sub-basement membrane was stained with several mAbs to determine the thickness of deposition of type III collagen (dilution 1:50; GmbH & Co, Berlin, Germany), type IV collagen (dilution 1:10; Monosan, Uden, The Netherlands), type V collagen (dilution 1:10; Monosan), and tenascin (dilution 1:10; Monosan).¹⁹

Detection of MMP-9 and TIMP-1. Immunolocalization of MMP-9 and TIMP-1 in bronchial tissue was examined with anti-MMP-9 mAb (dilution 1:10; Oncogen Science Inc, Cambridge, Mass)²⁰ and anti-TIMP-1 mAb (dilution 1:10; Fuji Pharmaceutical Co, Toyama, Japan).²¹

Detection of immunoreactive cells. Eosinophils were stained with the mAb EG2 (dilution 1:30; Kabi Pharmacia, Uppsala, Sweden), which recognizes the cleaved form of eosinophil cationic protein. The mAb AA1 (dilution 1:100; Dako), developed against human mast cell tryptase, was applied for detection of mast cells. T

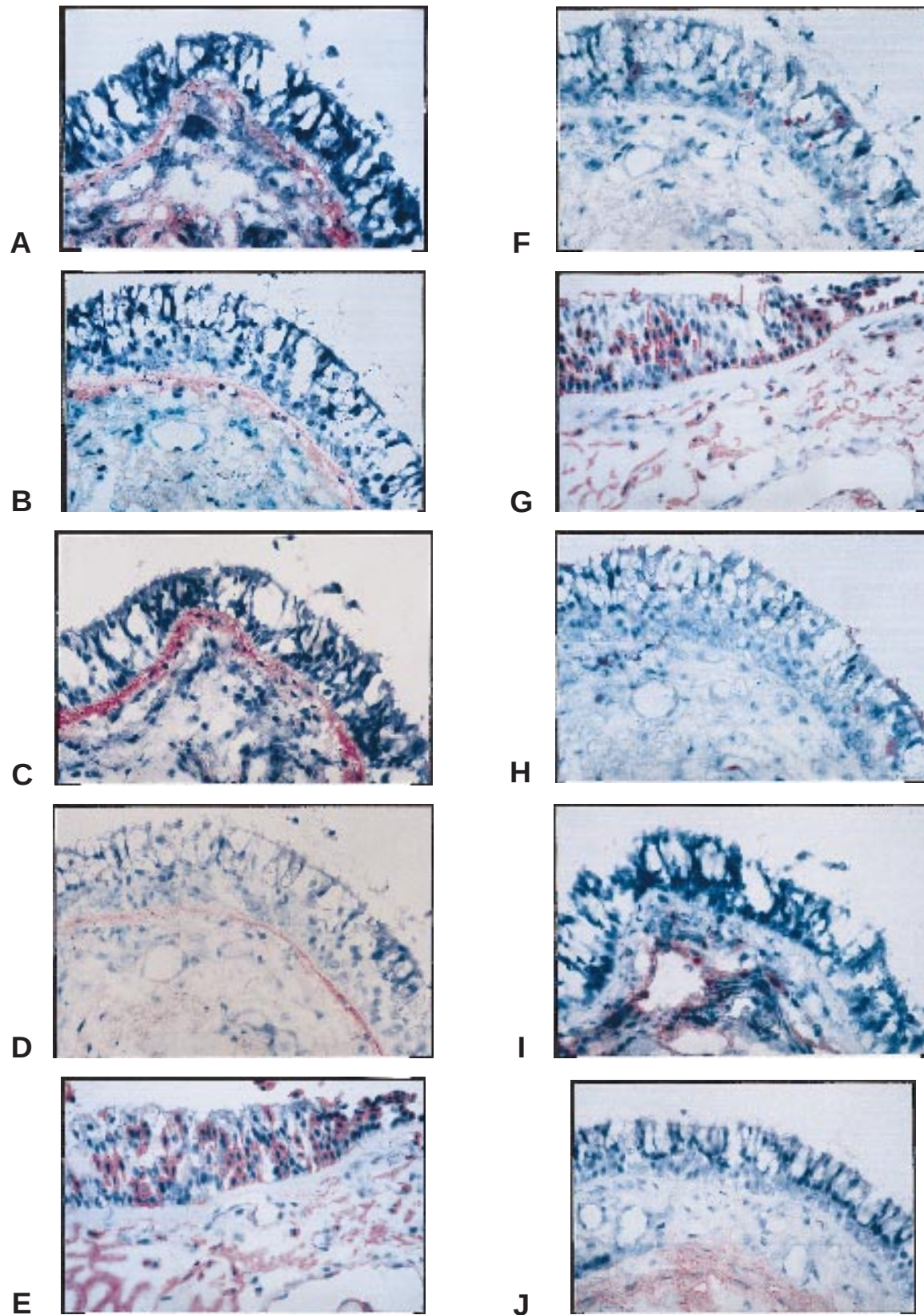


FIG 1. Photomicrographs show staining bronchial mucosal biopsy specimens with each antibody: collagen type III (A, asthma; B, control), tenascin (C, asthma; D, control), MMP-9 (E, asthma; F, control), TIMP-1 (G, asthma; H, control), and myofibroblasts (I, asthma; J, control). (Each original magnification, $\times 400$.)

TABLE III. Percentage of bronchial epithelium and submucosal area stained for MMP-9 or TIMP-1

	Bronchial asthma	Control
MMP-9		
Epithelium (%)	20.0* (6.0-61.0)	6.5 (2.0-12.0)
Submucosa (%)	37.5*† (23.7-70.3)	10.6 (3.1-14.2)
TIMP-1		
Epithelium (%)	16.6* (6.6-50.0)	6.1 (4.0-11.5)
Submucosa (%)	26.9* (7.8-58.1)	6.2 (2.0-14.3)

Values are median (range).

* $P < .001$, compared with control.

† $P < .01$, compared with submucosal expression of TIMP-1.

lymphocytes were stained with anti-CD3 mAb (dilution 1:30; Becton Dickinson, Abingdon, UK), macrophages with anti-CD68 mAb (dilution 1:500; Dako), and myofibroblasts with anti- α smooth muscle actin mAb (dilution 1:5; Immunon, Pittsburgh, Pa).

Quantitation

To avoid observer bias, all microscopic slides were coded before analysis by 1 observer and read blindly. Sections in which the basement membrane zone was not cut crosswise were excluded. Reticular basement membrane (RBM) thickness was quantitated by measurement of the total length of RBM present in the biopsy specimens that was overlaid by the basal epithelial cell layer at least. The length of collagen and tenascin staining was quantitated with a computerized image analysis system (Win ROOF; Mitani Corp, Fukui, Japan) with a resolution of 0.1 μ m. Minimal distances were calculated between each point on the superficial limit and the closest point on the deeper border of the specific immunoreactivity in the basement membrane. The repeatability of results obtained with this technique was assessed at regular intervals of 200 μ m along the length of each section. Mean values were considered the thicknesses of collagen and tenascin staining. Computerized colorimetric analysis (Win ROOF) was used to quantify MMP-9 and TIMP-1 expression in tissue. The total epithelial area was measured after manual delineation. The areas of tissue regions (epithelium: asthma, 0.22 ± 0.30 mm²; control, 0.18 ± 0.05 mm²; submucosa: asthma, 2.45 ± 0.46 mm²; control, 2.16 ± 0.80 mm²) were computed with the image analyzer; there was no significant difference between the 2 groups. Measurements of MMP-9 and TIMP-1 immunoreactivity were then made by expressing the area immunostained as a percentage of total epithelial area. To avoid any influence on epithelial cell counting of differences in the height of the epithelium, hyperplasia, or metaplasia, we used the percentage of positively stained cells. Measurements of the total submucosal area of the field were first recorded. The program allowed for correction of uneven illumination of the field of view and for operator-controlled editing of the image. The threshold for positive staining was determined, and the area of immunostaining was measured. Percentage expression in the submucosa was then calculated. The number of immunoreactive cells was counted for the entire submucosal area beneath the epithelial basement membrane, excluding mucosal glands and vessels, at a magnification of $\times 400$. The total area examined was calculated by delineating the area of the section on a video micrometer system (VM-50; Olympus Co), with software for measurements (Analytical Measuring System; Olympus Co). Results are expressed as the number of positive cells per square millimeter of submucosa.

Statistical analysis

Clinical data are expressed as means (range). Values of clinical parameters were normally distributed and were analyzed using the

2-sample Student *t* test. Percentage MMP-9 and TIMP-1 expression and the number of positive cells exhibited positively skewed distributions. Nonparametric data are expressed as median values (range). Comparisons between groups were made by the Mann-Whitney U test. Correlation coefficients were obtained by Spearman's rank correlation test. *P* values less than .05 were considered significant. Intraobserver reproducibility was assessed with the coefficient of variation, and the same observer performed at least 3 replicate measurements of morphometric parameters. The coefficient of repeatability, as described by Bland and Altman,²² was used to compare measurements. The mean coefficient of variation for repeated measurements ranged from 7% to 19% for the thickness of the RBM, 6% to 13% for percentage expression, and 5% to 17% for the number of cells.

RESULTS

Thickness of the RBM

The thickness of the RBM measured with immunohistochemical staining for collagen type III, type V, and tenascin was significantly larger in patients with asthma than in control subjects, but the thickness of the RBM measured with staining for collagen type IV was not (Table II). Typical examples of bronchial mucosal biopsy specimens from a patient with asthma and a control subject identifying collagen type III and tenascin are shown in Fig 1 (A through D).

Immunoreactivity for MMP-9 and TIMP-1

Immunohistochemical staining revealed MMP-9 and TIMP-1 protein in bronchial biopsy tissue samples from both patients with asthma and control subjects. MMP-9 was localized to epithelial cells, submucosal inflammatory cells, and ECM. TIMP-1 immunoreactivity was localized in the same cells. An example of positive staining for MMP-9 and TIMP-1 is shown in biopsy specimens from a patient with asthma and a control subject in Fig 1 (E through H). The proportions of epithelial cells exhibiting immunostaining for MMP-9 ($P < .001$) and submucosal expression of MMP-9 ($P < .001$) were significantly higher in patients with asthma than in control subjects. In addition, the proportions of epithelial cells expressing TIMP-1 ($P < .001$) and submucosal expression of TIMP-1 ($P < .001$) were significantly higher in patients with asthma than in control subjects. Submucosal MMP-9 expression was significantly higher than TIMP-1 expression in patients with asthma (Table III).

Immunoreactive cells

The numbers of immunoreactive cells in bronchial biopsy specimens are presented in Table IV. In patients with asthma, the numbers of activated eosinophils ($P < .001$), mast cells ($P < .001$), T lymphocytes ($P < .05$), macrophages ($P < .05$), and myofibroblasts ($P < .01$; Fig 1, I and J) in bronchial mucosa were significantly higher than in controls. A significant correlation was found between the number of myofibroblasts and thicknesses of collagen type III ($r_s = 0.70$, $P < .001$), type V ($r_s = 0.67$, $P < .001$), and tenascin ($r_s = 0.58$, $P < .01$) in patients with asthma. There was a positive correlation between submucosal expression of MMP-9 ($r_s = 0.43$, P

TABLE IV. Number of immunoreactive cells in bronchial mucosa

	Bronchial asthma	Control
Cell types		
EG2 (+) eosinophils	22* (7-105)	0 (0-19)
AA1 (+) mast cells	20* (8-46)	7.5 (0-22)
T lymphocytes	160† (70-342)	100.5 (70-245)
Macrophages	135† (48-315)	97.5 (39-200)
Myofibroblasts	23‡ (6-46)	11 (5-16)

Values are the median (range) and represent the number of cells per square millimeter of submucosa. Comparisons between groups were performed with the Mann-Whitney U test.

* $P < .001$.

† $P < .05$.

‡ $P < .01$.

$< .05$) and of TIMP-1 ($r_s = 0.69$, $P < .01$) and the numbers of submucosal eosinophils.

Clinical data

The duration of clinical disease was not related to the thickness of the RBM, but there was a significant inverse correlation between FEV_1 and subepithelial thickness (collagen III, V, and tenascin) in patients with asthma. Moreover, airway responsiveness was inversely correlated to a significant extent with subepithelial thickness (Table V). There was no correlation between any clinical parameter and the expression of MMP-9 or TIMP-1.

DISCUSSION

The present study showed that, by comparison with control subjects: (1) the thicknesses of collagen type III, V, and tenascin deposition were significantly increased in the bronchial RBM in patients with asthma; (2) MMP-9 and TIMP-1 immunoreactivities were significantly increased in both the epithelium and submucosa of patients with asthma; and (3) submucosal expression of MMP-9 was stronger than that of TIMP-1 in patients with asthma. Moreover, in the bronchial biopsy specimens obtained from patients with asthma, the degree of thickening of the RBM was closely correlated with both baseline FEV_1 and airway responsiveness.

A detailed immunohistochemical study showed that collagen III, V, and, to a lesser extent, collagen I contribute to the thickening of subepithelial layer components but that collagen IV and laminin are absent in patients with asthma.⁵ Tenascin immunoreactivity was observed as a narrow variable band under the bronchial epithelium in control subjects.²³ In the present study, subepithelial collagen and tenascin thicknesses were related to the proliferation of myofibroblasts, a finding in agreement with a previous study.²⁴

In this study, we used computerized color image analysis, which permits more objective quantitative assessment of the expression of MMP-9 and TIMP-1 in the epithelium and submucosa. MMP-9 is synthesized and secreted by connective tissue, including fibroblasts, endothelial cells, and inflammatory cells¹²; TIMP-1 is

TABLE V. Correlation between thickness of subepithelial layer and pulmonary function

	Collagen III		Collagen V		Tenascin	
	r_s	P	r_s	P	r_s	P
FEV_1 (% pred)	-0.89	<.001	-0.90	<.001	-0.88	<.001
AHR* (unit)	-0.59	<.01	-0.47	<.05	-0.48	<.05

r_s , Spearman's rank correlation test; unit, equal to 1 minute of 1.0-mg/mL aerosol inhalation of methacholine.

*Airway hyperresponsiveness was measured as the minimum cumulative.

produced by the same cells. The proportions of both epithelial and submucosal areas immunoreactive for MMP-9 and TIMP-1 were significantly increased in patients with asthma. Furthermore, the submucosal expression of MMP-9 was significantly higher than that of TIMP-1 in these patients. A weak but significant correlation was found in patients with asthma only between the thickness of collagen III and submucosal expression of MMP-9 ($r_s = 0.42$, $P < .01$). A dynamic equilibrium between the MMPs and their inhibitors, the TIMPs, maintains tissue integrity. In certain pathologic situations, increase in TIMP concentration may not compensate for a much larger increase in MMP-related degradation, as demonstrated for osteoarthritis²⁵ and cystic fibrosis.²⁶ Even a slight temporal or spatial imbalance between MMPs and TIMPs can lead to tissue remodeling and represents 1 mechanism by which allergic inflammation can be propagated and exacerbated. In the absence of double-staining procedures, we were unable to determine the cellular origins of MMP-9 and TIMP-1 in this study. Ohno et al²⁷ reported that eosinophils are triggered to synthesize MMP-9, because transcription of the MMP-9 gene was up-regulated in the eosinophils of asthmatic airways. On the other hand, Mautino et al²⁸ observed that MMP-9 was significantly increased in supernatants from alveolar macrophages recovered by bronchoalveolar lavage from patients with asthma, compared with healthy subjects. In the present study, positive correlations were found between the submucosal expression of MMP-9 and TIMP-1 and the numbers of eosinophils. Our findings suggest that sources of these enzymes might include inflammatory cells such as eosinophils.

For patients with asthma, no significant correlation was found between the thickening of RBM and duration of disease. This finding suggests that subepithelial collagen deposition is a very early change in asthma. Furthermore, airway remodeling may be caused by acute and repeated exposure to risk factors that may precede the clinical manifestations of asthma.²⁹ Other important findings of our study are the close correlation between the thickness of RBM (collagen III, V, and tenascin) and baseline FEV_1 and the threshold of airway responsiveness. These results agree with previous findings,^{30,31} confirming that airway remodeling caused by increased subepithelial layer thickness is a specific characteristic of asthma. This is consistent with the hypothesis that in

asthma the remodeling of nonmuscular wall components, especially basement membrane,³² in combination with the loss of elastic recoil, is responsible at least in part for bronchial hyperresponsiveness.³³

In conclusion, we have demonstrated increased RBM deposition of collagen type III, collagen type V, and tenascin in patients with asthma and increased expression of both epithelial and submucosal MMP-9 and TIMP-1. Submucosal expression of MMP-9 was significantly stronger than that of TIMP-1 in patients with asthma. Submucosal expression of MMP-9 and TIMP-1 was correlated with numbers of eosinophils. Further studies are needed to determine how the complex equilibrium between MMP-9 and TIMP-1 maintains the integrity of the ECM. Understanding the roles that these 2 groups of enzymes play in chronic inflammatory conditions will undoubtedly lead to new therapies directed at the long-term changes that characterize the asthmatic airway.

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