

Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: Association with asthma severity, neutrophilic inflammation, and wound repair

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Background: Asthma likely involves an active injury and repair process, including components such as neutrophils and matrix metalloproteinase 9 (MMP-9). Although MMP-9 is increased in lavage fluid and sputum in patients with asthma, controversy exists as to the role of tissue MMP-9.

Objective: The purpose of this study was to determine whether increases in submucosal cellular MMP-9, matrix MMP-9 (subepithelial basement membrane [SBM]), or both would be associated with severe asthma, neutrophilic inflammation, and wound repair.

Methods: Immunohistochemical staining and analyses of MMP-9, inflammatory cells, transforming growth factor β , and collagen I were performed in endobronchial biopsy specimens, bronchoalveolar lavage fluid, or both from 38 patients with severe asthma and compared with results in 10 patients with mild asthma, 8 patients with moderate asthma, and 10 healthy control subjects.

Results: A significantly greater proportion of patients with severe asthma demonstrated MMP-9 staining of the SBM than control subjects ($P = .02$). Bronchoalveolar lavage MMP-9 levels were also increased in patients with severe asthma ($P = .0004$). The numbers of submucosal neutrophils and macrophages, but not eosinophils, were significantly higher in asthmatic individuals with MMP-9 staining of the SBM ($P = .004$ and $P = .01$, respectively). However, the presence of SBM MMP-9 was associated with a high correlation between lavage and tissue eosinophils ($r = 0.58$, $P = .009$). Although the SBM thickness did not differ between groups, higher numbers of transforming growth factor β -positive cells were seen in subjects with SBM MMP-9 staining. Pulmonary function was significantly lower in those asthmatic subjects with SBM staining.

Conclusions: These results suggest that localized tissue MMP-9 might play an important role in wound repair and cell trafficking. (*J Allergy Clin Immunol* 2003;111:1345-52.)

Key words: Asthma, pathology, matrix metalloproteinase, neutrophils

Abbreviations used

BAL: Bronchoalveolar lavage
ECM: Extracellular matrix
MMP-9: Matrix metalloproteinase 9
SBM: Subepithelial basement membrane
TGF- β : Transforming growth factor β
TIMP: Tissue inhibitor of matrix metalloproteinase

In the past 10 to 15 years, inflammation in asthma has been portrayed as a predominantly T_H2 -mediated process. However, more recently, it has been appreciated that T_H2 inflammation alone might not explain the pathologic changes associated with asthma. Asthma also involves a chronic injury and repair response, with classic wound-repair factors, such as neutrophils, growth factors, and metalloproteinases, participating in the disease.

Metalloproteinases (MMPs) encompass a family of over 20 different proteases with various abilities to cleave components of extracellular matrix (ECM), enhance cell trafficking, and activate proteins.¹ The ability of different MMPs to degrade intact and denatured collagen, elastin, and other ECM components has suggested that these enzymes play a prominent role in wound repair.² More recently, MMPs have been suggested to enhance the migration (trafficking) of inflammatory cells through tissue to the site of injury through ECM degradation.³ MMP-9, perhaps the best studied of the MMPs in asthma, is a gelatinase the substrates of which include collagen IV, elastin, fibronectin, and plasminogen.⁴ Although it has been quite extensively evaluated, the role of MMP-9 in asthma is not clear. Studies of bronchial lavage and sputum samples have described increased levels of the latent but not the active form of MMP-9 in acute and chronic asthma.^{5,6} In contrast, asthmatic patients have also been reported to have a lower ratio of MMP-9 compared with its inhibitor, tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), than in healthy control subjects.⁷ Finally, Dahlen et al⁸ reported that there were no differences in submucosal or epithelial MMP-9 immunoreactivity in patients with asthma of varying severity and healthy control subjects. Its specific role in asthmatic wound repair and cell trafficking has not been addressed in these studies.

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Inflammation, repair, and remodeling in asthma might target specific compartments of the airway while leaving other areas less affected. In asthma the abnormally thickened band of ECM just below the true basement membrane (the subepithelial basement membrane [SBM]) is likely such a compartment. We hypothesized that the observed specific immunoreactivity of MMP-9 in that localized region might be more relevant to clinical and physiologic outcomes than more generalized activity. Therefore MMP-9 immunostaining was evaluated in the SBM from 38 patients with severe, steroid-dependent asthma and compared with staining results in patients with mild and moderate asthma and healthy control subjects. The presence or absence of SBM staining for MMP-9 was then compared with the pattern and location of inflammatory cells, elements of tissue repair-remodeling, and lung function. The results suggest that the presence and location of MMP-9 in asthmatic airways might influence injury and repair processes and associated cell migration. These effects might then contribute to worsening of lung function and disease.

METHODS

Subjects

Asthmatic subjects were divided into severity categories by using previously defined criteria.⁹ Briefly, patients with mild asthma all had a prebronchodilator FEV₁ of greater than 80% of predicted value and were treated with as-needed β -agonists alone. Patients with moderate asthma had an FEV₁ of less than 80% of predicted value, were taking low-to-moderate doses of inhaled corticosteroids plus as-needed β -agonists, and did not require oral steroids in the last year. Patients with severe asthma were all referred to the National Jewish Medical and Research Center for evaluation of severe, difficult-to-treat asthma. Despite treatment with oral steroids for greater than 50% of the previous year and high-dose inhaled steroids with at least one other long-term control medication, they continued to have daily symptoms and used urgent health care frequently. Healthy control subjects had normal pulmonary function and no history of any respiratory illness. The study was approved by the National Jewish Institutional Review Board, and all subjects provided informed consent.

Procedures

Bronchoscopy was performed as previously reported.⁹ Endobronchial biopsy specimens were taken from the subcarinae of the third- to fourth-generation bronchi of the left or right lower lobes. All tissue was fixed in acetone for embedding in glycol methacrylate resin. Bronchoalveolar lavage (BAL) was performed in subsegments of the lingula or right middle lobe by using four 60-mL aliquots of warm sterile saline. BAL fluid was pooled and centrifuged at 600g for 10 minutes to separate fluid from cells. One-milliliter aliquots were frozen at -70°C for later analysis of MMP-9.

Spirometry was performed to American Thoracic Society standards. The FEV₁ and forced vital capacity values were obtained before bronchodilator use.

Immunohistochemistry

Serial 2- μm sections from glycol methacrylate resin-embedded tissue were stained with primary antibodies against MMP-9 (R&D), eosinophil major basic protein (BMK-13, Accurate Chemical and Scientific Co), neutrophil elastase, monocyte-macrophage CD68

(both from Dako), collagen I (Biodesign International), and pan-transforming growth factor β (pan-TGF- β ; all subtypes, R&D). Positive and negative isotypic controls were always stained as well. After blocking and incubation with the primary antibody, the sections were then incubated with biotinylated horse anti-mouse or goat anti-rabbit IgG antibody. Avidin-peroxidase complex (Vector Labs) is added, and then aminoethylcarbazole in 0.03% H₂O₂ is applied as a substrate. Slides were counterstained with Mayer hematoxylin and read by a single reader (Dr Balzar) without knowledge of group.¹⁰ The number of positive cells was standardized to square millimeters of tissue area measured by using a National Institutes of Health image analysis program. The thickness of the SBM was measured on tissue stained with collagen type I antibody, as previously described.¹¹ Staining of the SBM for MMP-9 was defined as positive or negative.

MMP-9 levels in BAL fluid

MMP-9 levels in unconcentrated BAL fluid were measured by means of enzyme immunoassay with paired antibodies. The MMP-9 assay used reagents from R&D Systems. The plates were coated with a mouse anti-MMP9 (MAB936) and used the detection antibody from the Quantikine kit (DMP900). This assay detects total MMP-9 (both pro and active forms). Although zymography is not reported as part of this study, less than 10% of the samples had any evidence for the 88-kd (active) form of MMP-9.

Lavage cell counts

Total BAL cells per milliliter were obtained, as previously described.¹¹ Differentials were performed on cytopsin preparations stained with commercial Wright-Giemsa stain (Fisher Scientific Products). The numbers of eosinophils and neutrophils per milliliter of BAL fluid were calculated from the total number of cells per milliliter of BAL fluid according to the percentage of those cells obtained from the differentials.

Statistics

Because the majority of continuous variables were highly skewed to the right, these data were log-transformed. Data sets that contained zero values (cell counts and BAL MMP-9), were modified as follows:

$$y = \ln(x + 1),$$

where x is defined as the original data point and y is defined as the modified data and then log-transformed. SBM staining was categorized as present or absent and analyzed by means of χ^2 analysis. Comparisons of the 4 subject groups were by means of ANOVA, whereas comparisons of the 2 groups (positive or negative MMP-9) were by means of the t test. When multiple groups were compared and found to be significantly different, intergroup comparisons were made by using Tukey-Kramer testing. Data are presented as means $\pm$ SEM after log-transformed data are returned to their original form. Retransforming the data to the original scale produces SEMs that are not equal, with the negative SEM being smaller than the positive SEM. P values of less than .05 were considered significant. Correlation analysis was done by using the Pearson correlation coefficient of the log-transformed data.

RESULTS

Subjects

Thirty-eight patients with severe, 8 patients with moderate, and 10 patients with mild asthma and 10 healthy control subjects were evaluated with endobronchial biopsy and BAL. The baseline characteristics of these 66 subjects are given in Table I.

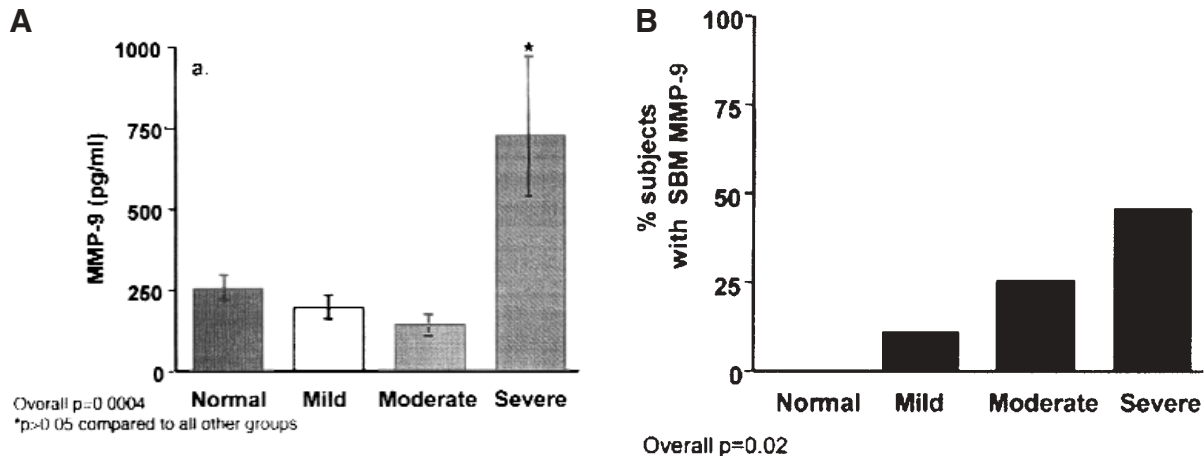


FIG 1. A, BAL fluid levels of MMP-9 in healthy control subjects and patients with mild, moderate, and severe asthma. Patients with severe asthma had significantly higher levels of MMP-9 in BAL fluid than any control group (overall $P = .0004$ and $P < .05$ for comparison with each control group). Values are means $\pm$ SEM reconverted from log-transformed data to original scale. **B**, Percentage of subjects with positive immunoreactivity for MMP-9 in the SBM from healthy control subjects and patients with mild, moderate, and severe asthma. Forty-five percent of patients with severe asthma demonstrated staining of the SBM. One third of these demonstrated more prominent MMP-9 staining than seen in any control group.

TABLE I. Subject characteristics

	Subject characteristics			Steroid dose*	
	Sex (M/F)	Age (y)*	FEV ₁ (%)*	Inhaled (μ g)	Oral (mg)
Normal	5/5	37.3 $\pm$ 7	102 $\pm$ 11	0	0
Mild	4/6	36.5 $\pm$ 9.2	89 $\pm$ 9	0	0
Moderate	3/5	38.5 $\pm$ 13.1	65 $\pm$ 13	660 $\pm$ 110	0
Severe	15/23	34.9 $\pm$ 12.5	50 $\pm$ 18	1620 $\pm$ 220	27 $\pm$ 7

*Mean $\pm$ SD.

Location of lung MMP-9 immunoreactivity in relation to disease severity

BAL fluid MMP-9 levels were increased in subjects with severe asthma and were significantly higher than in any other group (overall $P = .0004$; Fig 1, A). MMP-9 immunoreactivity associated with the SBM was only seen in subjects with asthma, particularly in patients with severe asthma, among whom 45% of the subjects demonstrated positive staining ($P = .02$; Fig 1, B). Although not specifically quantified, patients with severe asthma also had the greatest intensity of staining (Fig 2). There was no statistically significant difference in MMP-9⁺ cells in the submucosa among the 4 groups (overall $P = .79$, data not shown).

Potential determinants of SBM MMP-9 immunoreactivity

Data on potential determinants of SBM MMP-9 immunoreactivity are shown in Fig 3. For these analyses, normal and asthmatic subjects were divided into those with and those without SBM MMP-9 immunoreactivity. Because SBM MMP-9 immunoreactivity was only seen in asthmatic subjects and because inclusions of normal data might make differences between groups inappropri-

ately large, only asthmatic subjects were analyzed in this manner. There were a total of 20 subjects with positive staining out of a total of 56 subjects.

The presence of MMP-9 SBM immunoreactivity in asthmatic patients was associated with increased numbers of MMP-9⁺ cells in the submucosa ($P = .03$; Fig 3, A). In contrast, BAL levels of MMP-9 were not significantly different, suggesting that BAL MMP-9 contributes only marginally to the SBM MMP-9 ($P = .11$; Fig 3, B).

Greater numbers of neutrophils ($P = .004$; Fig 3, C) and macrophages ($P = .01$; Fig 3, D) were seen in the submucosa of asthmatic subjects with SBM immunoreactivity for MMP-9. In contrast, MMP-9 immunoreactivity was not associated with tissue eosinophil numbers ($P = .69$).

There was no difference in corticosteroid use in patients with severe asthma with or without MMP-9 SBM immunoreactivity (MMP-9⁻ = 12 mg/d, upper and lower SEM = 8-19 mg/d; MMP-9⁺ = 16 mg/d, upper and lower SEM = 11-24 mg/d; $P = .71$).

SBM MMP-9 in relation to markers of wound repair-remodeling

Despite the presence of MMP-9 immunoreactivity in the SBM, there was no difference in the thickness of this

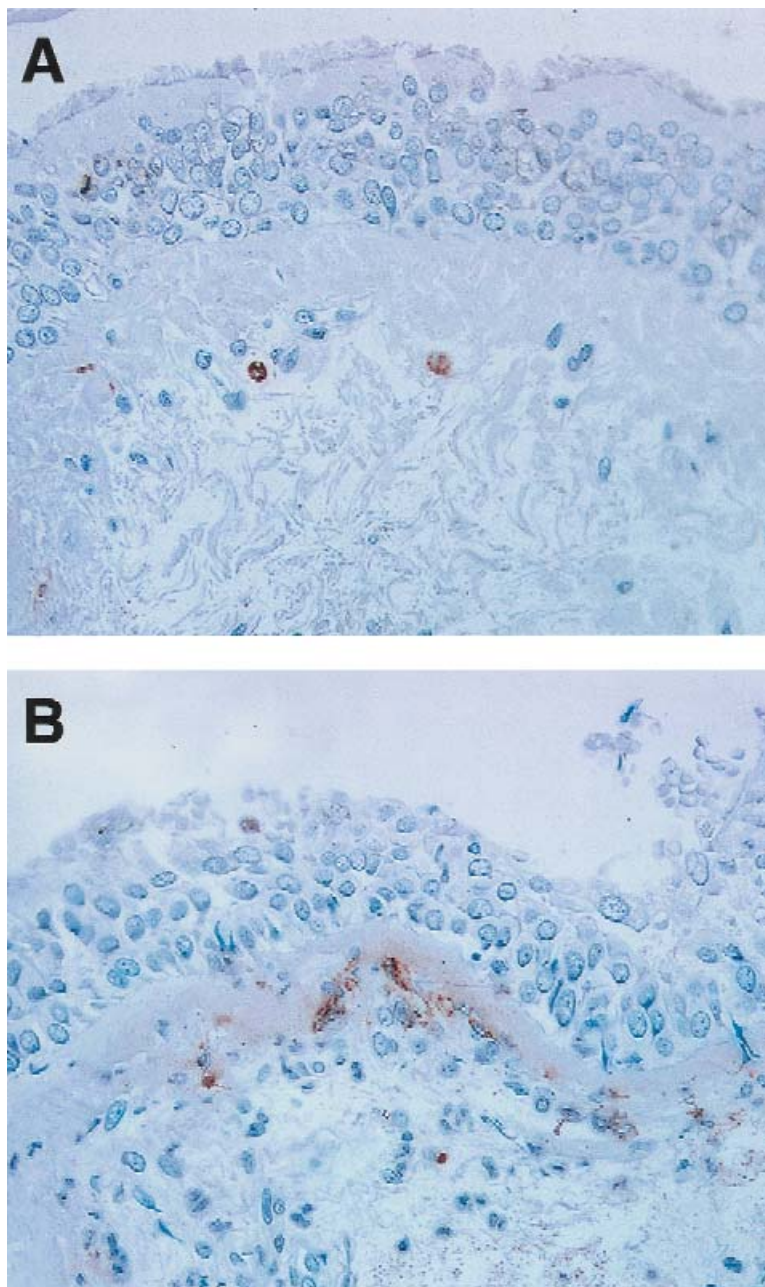


FIG 2. Representative photomicrographs of SBM MMP-9 immunoreactivity from a healthy control subject (A) and a patient with severe asthma (B). Note the intense staining in the tissue from the patient with severe asthma. This was characteristic of nearly 50% of the tissues from patients with severe asthma evaluated and not seen in tissues from patients with milder asthma.

structure between asthmatic subjects with MMP-9 staining and those without ($P = .73$). In contrast, asthmatic subjects with MMP-9 immunoreactivity had higher numbers of TGF- β^+ cells in the submucosa ($P = .01$, Fig 4).

SBM MMP-9 in relation to cell trafficking between tissue and BAL fluid

On the basis of reports that MMPs modify cell trafficking from the submucosa to airway lumen, the influ-

ence of SBM MMP-9 on correlations between the numbers of submucosal cells and luminal (lavage) eosinophils and neutrophils was evaluated. In all asthmatic patients, BMK $^+$ cells per square millimeter of tissue correlated significantly with eosinophils per milliliter of lavage fluid, with an r value of 0.43 ($P = .002$). In the absence of MMP-9 SBM immunoreactivity, the relationship was only marginally significant ($r = 0.31$, $P = .07$; Fig 5, A). However, in the presence of SBM MMP-9 immunoreac-

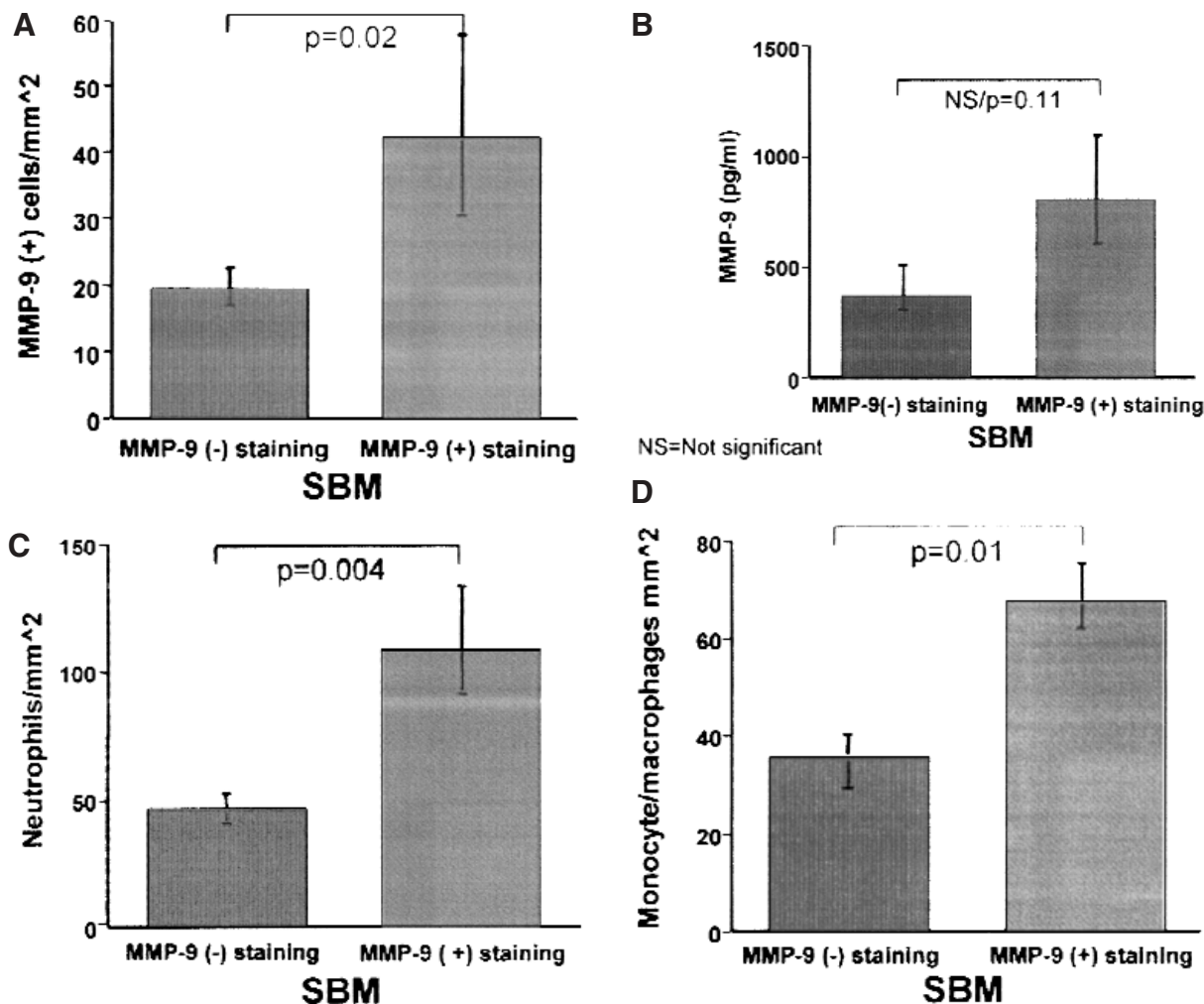


FIG 3. A, Asthmatic subjects with SBM staining had significantly higher numbers of MMP-9⁺ submucosal cells ($P = .02$). B, MMP-9 protein levels in BAL fluid were not different between asthmatic patients with and without SBM staining ($P = .11$). Asthmatic patients with MMP-9 SBM staining had significantly higher numbers of submucosal neutrophils ($P = .004$; C) and monocytes-macrophages than those without staining ($P = .01$; D). Values are means $\pm$ SEM reconverted from log-transformed data to original scale.

tivity, the correlation increased to an r value of 0.58 ($P = .009$; Fig 5, B). These relationships were also significant if eosinophil numbers in tissue were compared with eosinophil percentages in BAL fluid. If the analysis was limited to patients with severe asthma, the observations persisted and became more pronounced, with the correlation for patients with severe asthma (positive SBM staining) increasing to an r value of 0.68 ($P = .004$), whereas that for those without SBM MMP-9 decreased to an r value of 0.29 ($P = .20$). In contrast to eosinophils, the correlation of tissue neutrophils with lavage neutrophils was never significant, regardless of the presence or absence of MMP-9 immunoreactivity ($r < .002$ for all, $P > .89$).

SBM MMP-9 immunoreactivity in relation to FEV₁

As predicted by the association of SBM staining with severity, the asthmatic subjects with MMP-9 immunoreactivity in the SBM had significantly lower lung function,

as manifested by lower prebronchodilator FEV₁ and forced vital capacity values, when compared with those of the group without MMP-9 staining ($P = .01$ and $P = .03$, respectively; Fig 6). When only the 38 patients with severe asthma were evaluated, the difference in FEV₁ still tended toward significance ($P = .1$). BAL MMP-9 levels also negatively correlated with FEV₁ percent predicted ($r = -0.45$, $P = .002$), whereas MMP-9⁺ submucosal cells did not correlate with lung function ($r = 0.07$, $P = .91$).

DISCUSSION

This study suggests that both the location and the amount of MMP-9 immunoreactivity in the lung might be important in modifying wound-repair processes and associated clinical and physiologic outcomes in asthma. In patients with asthma, this MMP-9-mediated process appears to be associated with a specific type of tissue inflammation, with increased numbers of tissue neu-

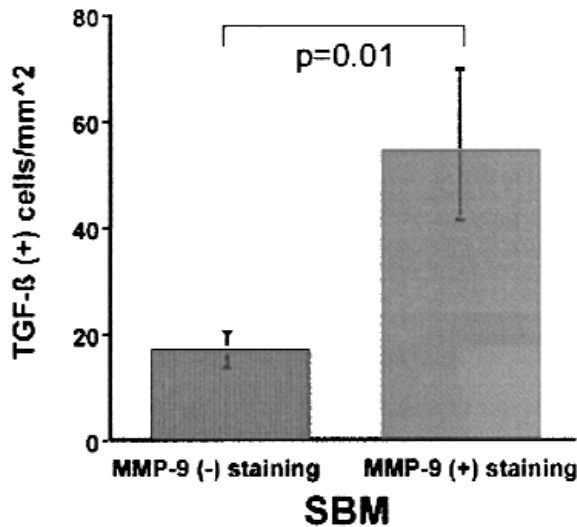


FIG 4. Asthmatic patients with positive SBM staining for MMP-9 had higher numbers of TGF- β ⁺ cells in the submucosa ($P = .01$). Values are means $\pm$ SEM reconverted from log-transformed data to original scale.

trophils and macrophages, increased TGF- β ⁺ cells, and worsened lung function. These associations were present when evaluating asthmatic patients and to a great degree when evaluating patients with severe asthma alone (most data not shown). Therefore this study confirms previous reports of MMP-9 upregulation in asthma but offers new insights into its role in this disease.

The involvement of MMP-9 in any inflammatory-repair process is complex. In addition to transcriptional and secretory regulation, MMP-9 requires further activation and can be inhibited through the presence of TIMPs.¹² Although in situ zymography has been suggested as a way to estimate the tissue activation state of proteases, this method is not applicable to evaluation of small or fragile endobronchial biopsy specimens.¹³ Therefore it is difficult to determine with certainty the activation state of the MMP-9 in the positively stained SBM and whether it is influencing the quality or quantity of the ECM making up that membrane. Alternatively, it is possible the positive staining might be merely a marker of MMP-9 presence.

However, if the MMP-9 is active, it could participate in asthma pathogenesis through involvement in wound injury and repair or through direct or indirect effects on cellular trafficking in response to the injury, altering the migration of inflammatory cells through the thickened SBM. The current data support both of these possibilities and suggest that the 2 processes are closely intertwined.

The interface between the epithelium and the submucosal space occurs in the region of the basement membrane and SBM. Recent articles have suggested that interactions between the epithelium and resident cells in the submucosa (the epithelial-mesenchymal trophic unit) might control much of the tissue injury and repair occurring in asthma.¹⁴ The localized increase in MMP-9 immunoreactivity in asthma occurred at the level of the

SBM at the border between the epithelium and the submucosa. The submucosal tissue compartment, just below the SBM, might be the active front of this wound-repair process. The association of increased submucosal neutrophils, macrophages, and cellular MMP-9 with SBM immunoreactivity suggests that the increased SBM MMP-9 is likely to be arising from these cells. As in other wound-repair settings, neutrophils might be the initial migrating cell.² Neutrophils, known to express both MMP-9 and TGF- β , are capable of both breaking down damaged tissue (MMP-9) and helping to repair it (TGF- β).¹⁵ This dynamic process of breakdown and repair could occur without affecting the overall thickness of the SBM. The more dynamic the process, the more severe the disease, as reflected in the degree of neutrophil-macrophage tissue influx, as well as in FEV₁ and severity categorization. In contrast to submucosal MMP-9, MMP-9 levels in BAL fluid (likely arising from epithelial and other lumenal cells) did not appear to influence SBM MMP-9. Yet similar to SBM MMP-9, BAL fluid MMP-9 correlated negatively with FEV₁ ($r = -0.45$), suggesting that each MMP-9 compartment differentially influences airway obstruction. Finally, in contrast to an earlier report, the use of inhaled (or oral) corticosteroids (especially in severe asthma) appears to have no effect on the process.¹⁶

In addition to repair of the injury, MMP-9 in the SBM might increase the ability of inflammatory cells to migrate from the submucosa through the SBM and true basement membrane into the lumen.¹⁷ MMP control of cellular migration in asthma has received considerable attention in murine systems.¹⁸⁻²⁰ Nonspecifically inhibiting MMPs through their natural inhibitors (TIMPs) decreased inflammatory cell numbers after challenge with ovalbumin in tissue and lavage fluid. Similarly, MMP-9 knockout mice had less inflammation in both the tissue and the lumen and subsequently had an improvement in hyperresponsiveness after ovalbumin challenge.¹⁹ In contrast, mice deficient in MMP-2 had more eosinophils present in airway tissue than in lavage fluid after allergen challenge.²⁰ The authors suggested that the absence of MMP-2 decreased the ability of cells to move through the ECM.

How these studies relate to human asthma is not clear. All of these studies used a relatively simple (by human disease standards) acute allergen (ovalbumin) challenge system. Eosinophil migration varied depending on the system. None of these studies showed effects on neutrophils because no increases in tissue or lavage neutrophils were seen in these models (as they are in chronic human asthma).²¹ Furthermore, there were no chronic remodeling changes present (eg, the thickened SBM).

It is certainly possible that the thickened SBM seen in human asthma could function as a barrier that limits trafficking of cells into the lumen. The increased concentration of MMP-9 in this membrane could be an active effort to promote the movement of inflammatory cells into the lumen. Although in this and other studies the numbers of eosinophils in airway tissue generally corre-

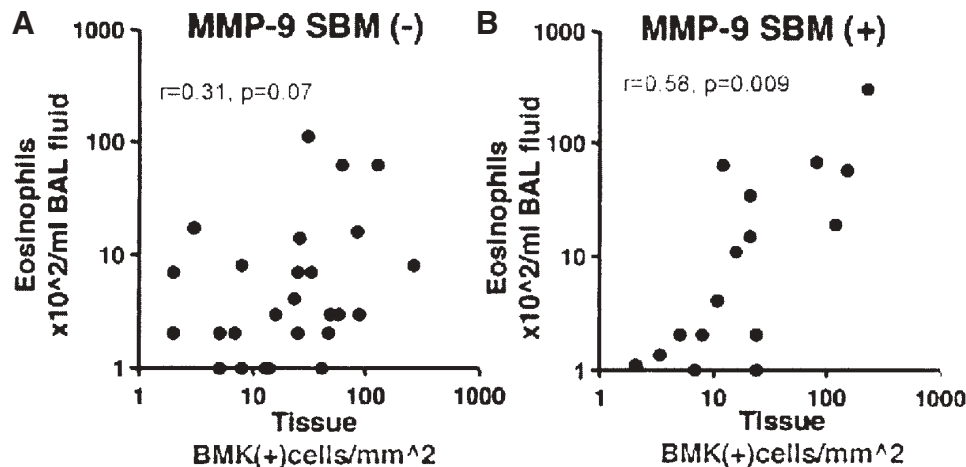


FIG 5. A, Eosinophils in tissue (BMK⁺ antibody staining of eosinophil granule major basic protein) correlate marginally with eosinophils in BAL fluid ($r = 0.31$, $P = .07$) in asthmatic patients without SBM MMP-9 immunoreactivity. **B**, Eosinophils in tissue (BMK⁺ cells) are significantly correlated with eosinophils in BAL fluid in asthmatic patients in the presence of MMP-9 immunoreactivity ($r = 0.58$, $P = .009$).

late with numbers in lavage fluid, the overall correlations are generally weak.^{22,23} Interestingly, in this study the size of the luminal to tissue eosinophil correlations improved when MMP-9 immunoreactivity was present in the SBM. These high correlations of tissue to lavage eosinophils suggest that the presence of SBM MMP-9 might promote the movement of eosinophils into the lumen, possibly contributing to worsened lung function. The MMP-9 in the SBM allows the eosinophils to more easily migrate along a chemotactic gradient through the thickened SBM and epithelium and into the airway lumen. In contrast, the role of the neutrophils, macrophages, or both could be to initially respond to the injury, clear the way for eosinophils to move into the lumen, and remain behind in the tissue to repair the damage through release of both MMP-9 and TGF- β , hence the lack of relationship between BAL and tissue neutrophils. Although this is plausible on the basis of the data, a dynamic model following the migration of eosinophils and neutrophils would be required to confirm this observation.

In summary, it is likely that SBM MMP-9 immunoreactivity derived from a robust macrophage-neutrophil response to injury might be part of an attempt to specifically or nonspecifically heal a T_H2-related injury. Additionally, an alteration in the quality of the ECM in the SBM might improve the egression of eosinophils out of the airway tissue into the lumen. Further studies both in vitro and in vivo are required to better understand the relationship between trafficking of cells to T_H2-type injuries and effective wound repair.

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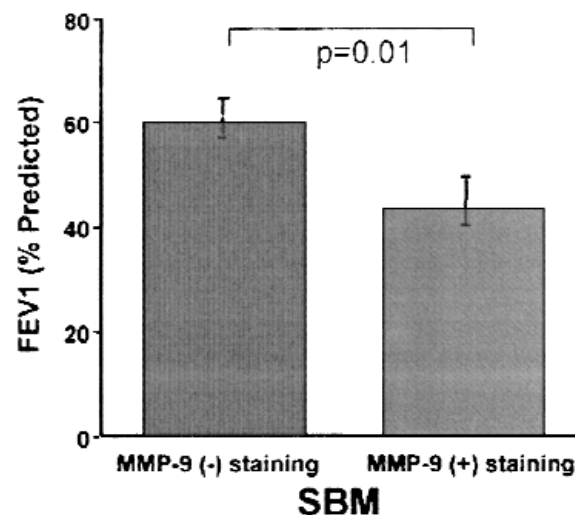


FIG 6. Asthmatic patients with positive SBM staining for MMP-9 had lower lung function, as measured by means of prebronchodilator FEV₁ values, than subjects without staining ($P = .01$).

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