

Original article

Vascular remodeling is a feature of asthma and nonasthmatic eosinophilic bronchitis

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Rationale: Increased vascularity and expression of vascular endothelial growth factor (VEGF) are recognized features of the asthmatic airway. The association of vascular remodeling with airway hyperresponsiveness (AHR) is unclear.

Objective: To assess vascular remodeling and sputum VEGF concentration in subjects with asthma, subjects with nonasthmatic eosinophilic bronchitis (EB), and healthy controls.

Methods: In cohort 1, 19 patients with asthma (Global Initiative for Asthma [GINA] 1-2, $n = 9$; GINA 3-5, $n = 10$), 10 patients with EB, and 11 healthy matched controls were recruited. Expression of the endothelial marker EN4 was assessed in bronchial biopsy samples. Vessels were counted using the validated mean Chalkley count by a blind observer. For cohort 2, a second independent cohort of 31 patients with asthma (GINA 1-2, $n = 11$; GINA 3-5, $n = 20$), 14 patients with EB, and 15 matched controls was recruited. Induced sputum supernatant VEGF was measured by ELISA.

Results: The mean chalkley count was significantly greater in GINA 3-5 asthma (5.2 [0.4]) and EB (4.8 [0.3]) compared with controls (3.5 [0.5]) and demonstrated a significant inverse correlation with the postbronchodilator $FEV_1\%$ predicted in patients with asthma ($R^2 = 0.28$; $P = .02$). Sputum VEGF concentration was also increased in GINA 3-5 asthma (2365 [1361-4110] pg/g) and EB (4699 [2818-7834] pg/g) compared with controls (1094 [676-1774] pg/g) and was inversely related to postbronchodilator $FEV_1\%$ predicted in asthma ($R^2 = 0.2$; $P = .01$).

Conclusion: Vascular remodeling is a feature of asthma, and EB and is inversely associated with the postbronchodilator

FEV_1 in asthma, suggesting that vascular remodeling is associated with airflow obstruction but not AHR.

Clinical implications: Vascular remodeling is dissociated from AHR in asthma and associated with airflow limitation.

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Key words: Asthma, eosinophilic bronchitis, vascular remodeling, airway hyperresponsiveness, VEGF

Asthma is characterized by symptoms, variable airflow obstruction, airway hyperresponsiveness (AHR), and inflammation. In addition, there are a number of changes to the structural components of the airway wall collectively known as *remodeling*.^{1,2} Vascular remodeling and increased expression of associated growth factors such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF) are well-recognized features of asthma.³⁻¹⁰ This vascular remodeling is likely to be functionally important because enhanced vascularity in the inner wall of the medium airways correlates with airflow obstruction in asthma.¹¹ However, the relationship between vascular remodeling and AHR is uncertain.

Nonasthmatic eosinophilic bronchitis (EB) is an airway disease that has emerged from the study of chronic cough and airway inflammation and accounts for approximately 10% of referrals to a specialist cough clinic.¹² EB is characterized by eosinophilic airway inflammation demonstrated by sputum, bronchoalveolar lavage, and bronchial biopsy, without any evidence of variable airflow obstruction or AHR.^{12,13} The EB phenotype remains stable over time with few patients developing asthma; however, some patients develop fixed airflow obstruction, particularly if eosinophilic airway inflammation is severe and not controlled by inhaled corticosteroids.¹⁴ EB is therefore a powerful disease control model to study potential mechanisms of AHR. Importantly, overexpression of IL-13 by mast cells and eosinophils in the bronchial submucosa¹⁵ and peripheral blood¹⁶ and mast cell colocalization to airway smooth muscle¹⁷ are immunopathological features of asthma that are not shared by EB and have therefore been implicated in the pathogenesis of AHR. In addition, Kanazawa et al¹⁸ demonstrated that VEGF was increased in the sputum in asthma but not in EB and correlated with increased airway vascular permeability. VEGF levels

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Abbreviations used

AHR: Airway hyperresponsiveness
 EB: Nonasthmatic eosinophilic bronchitis
 GINA: Global Initiative for Asthma
 MCC: Mean chalkley count
 RBM: Reticular basement membrane
 VEGF: Vascular endothelial growth factor

in sputum and airway vascular permeability showed significant inverse correlations with AHR in asthma, suggesting that vascular remodeling and airway wall edema are important causes of AHR in asthma. However, the selection of patients with chronic disease is critical to the study of airway remodeling,¹⁹ and the duration of disease was unclear in the study by Kanazawa et al.¹⁸ Therefore, whether vascular remodeling is a feature shared by asthma and EB or is indeed a characteristic unique to asthma needs to be clarified.

We sought to assess the relationship between vascular remodeling and induced sputum VEGF with AHR, airflow obstruction by comparison of asthma with EB in patients with chronic disease.

METHODS

Subjects

Subjects were recruited from Glenfield Hospital outpatients, staff and by local advertising. All subjects were nonsmokers with a smoking history of <10 pack years, and had been free of exacerbations and on stable treatment for 8 weeks before entry into the study. EB was defined according to the American College of Chest Physicians criteria.²⁰ Asthma was defined and severity classified using the current Global Initiative for Asthma (GINA) guidelines²¹ based on the GINA treatment steps (GINA 1-2, intermittent to mild persistent asthma; GINA 3-5, moderate to severe persistent asthma) and the American Thoracic Society criteria for refractory asthma.²² Normal subjects had no history of respiratory disease and normal spirometry. The Leicestershire ethics committee approved the study, and all patients gave their written informed consent.

Two independent cohorts were recruited to assess vascular remodeling in bronchial biopsies (cohort 1, 19 subjects with asthma, 10 with EB, and 11 controls) and VEGF in sputum (cohort 2, 31 subjects with asthma, 14 with EB, and 15 controls). Nine of 24 of the subjects with EB, 8 of 50 of the subjects with asthma, and 11 of 26 controls had taken part in previous studies comparing the immunopathology of asthma and EB.^{13,17}

Protocol and clinical characterization

All subjects underwent spirometry; allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat, and grass pollen; measurement of exhaled nitric oxide concentration (measured at 50 mL/s NIOX; Aerocrine, Stockholm, Sweden); a methacholine inhalation test²³; and sputum induction.²⁴ One week later, patients in cohort 1 underwent bronchoscopy conducted according to the British Thoracic Society guidelines.²⁵ Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae and were fixed in acetone and embedded in glycolmethacrylate.²⁶

Cohort 1: Immunohistochemistry and Chalkley counts

Two-micrometer glycomethacrylate sections were cut and stained by using mAbs for EN4 (anti-PECAM-1), major basic protein (Monosan, Uden, The Netherlands), or appropriate isotype control.

Chalkley counts were performed twice by a single observer blind to clinical characteristics.²⁷⁻²⁹ The chalkley count is surrogate for vascular density and area. The chalkley graticule is a circle with 25 randomly placed dots. The chalkley eyepiece graticule was applied to 4 subjectively predetermined, nonoverlapping vascular hotspots (1-2/section) at $\times 200$ magnification (corresponding to an area of 0.196 mm²) and rotated until the maximum number of vessels coincided with dots. All stained separate vessels with a lumen were included in the counts. The mean chalkley count (MCC) was derived. Lamina reticularis and reticular basement membrane (RBM) thickness were measured as previously described.¹⁷

Cohort 2: Sputum VEGF

Induced sputum supernatant VEGF was measured by using a commercial ELISA (R&D Systems, Abingdon, United Kingdom [UK]). Spiking experiments were performed throughout the range of the assay to assess recovery, and VEGF standards were analyzed to assess the effects of dithiothreitol. Dithiothreitol did not affect the VEGF standard curve, and spiking experiments revealed a mean recovery (range) of 88% (84-93; n = 6). The minimum limit of detection was 7.8 pg/mL. The interassay/intra-assay variabilities were 6.6% and 10%, respectively.

Analysis

Statistical analysis was performed by using PRISM Version 4 (GraphPad, San Diego, Calif). Parametric data were expressed as mean (SEM), data that had a log normal distribution were log-transformed and described as geometric mean (95% CI), and nonparametric data were described as median (range). One-way analysis of variance and Student *t* tests were used for across-group and between-group comparisons, respectively. χ^2 Tests were used to compare categorical data. We chose to examine the relationship between disordered airway physiology (postbronchodilator FEV₁ and AHR) and vascular remodeling by univariate analysis Pearson and Spearman rank correlation coefficients as appropriate. Stepwise linear regression was performed to assess which variables best correlated with vascular remodeling in each cohort. Independent variables entered into the regression model were age, daily equivalent dose of inhaled beclomethasone dipropionate, duration of disease, log-transformed percent sputum eosinophil count and exhaled nitric oxide, submucosal eosinophil count (cohort 1), RBM thickness (cohort 1), and FEV₁ % predicted log-transformed methacholine PC₂₀. Regression data are presented as model-adjusted Pearson correlations alongside the standardized correlation coefficient (β) of the modeled independent variable. A *P* value of <.05 was taken as the threshold for statistical significance.

RESULTS

Vascular remodeling is a feature of moderate and severe persistent asthma, refractory asthma, and EB

Clinical characteristics for cohort 1 are shown in Table I. The asthma and EB cohorts were well matched for age, sex, atopy, postbronchodilator spirometric values, disease duration, and the degree of eosinophilic airway inflammation in sputum and within the bronchial submucosa.

TABLE I. Cohort 1: clinical characteristics

	Control (n = 11)	EB (n = 10)	Asthma (n = 19) GINA 1-2, n = 9 GINA 3-5, n = 10 (Refractory asthma, n = 5)	
Age (y)	36 (6.1)	45.8 (4.1)	46.9 (3.9)	NS*
Sex (M:F)	7:4	6:4	6:13	NS†
Atopy (%)	27	50	58	NS†
Disease duration (y)	0	5.5 (1.6)	10.3 (2.7)	NS‡
Inhaled BDP (μg/24 h)	0	0	821 (177)	$P < .0001$ ‡
Oral prednisolone	0	0	4/19	—
Prebronchodilator FEV ₁ (L)	3.53 (0.25)	2.86 (0.23)	2.1 (0.22)	$P = .001$ *
				$P < .05$ §
Postbronchodilator FEV ₁ (L)	3.55 (0.25)	2.89 (0.24)	2.45 (0.20)	$P = .007$ *
				$P < .05$ §
Postbronchodilator FEV ₁ %	100 (4.3)	94 (3.5)	91 (4.0)	NS*
FEV ₁ /forced vital capacity postbronchodilator	81 (2.1)	79.5 (2.5)	76 (3.0)	NS*
PC ₂₀ (mg/mL)	>16	>16	0.86 (0.03-4.6)	$P < .0001$ *‡
ENO (ppb)	7.3 (5.5-9.6)	19.5 (13.6-28.0)	23 (15.7-33.4)	$P < .0001$ *; NS‡
Induced sputum				
Eosinophils (%)	0.45 (0.2-0.9)	7.0 (4.5-10.9)	4.0 (1.5-10.3)	$P < .0001$ *; NS‡
Neutrophils (%)	31 (17.4-55.2)	45.9 (28.5-73.8)	35.7 (22.0-57.5)	NS*‡
Submucosal eosinophils/mm ² ¶	9.9 (5.3-20.5)	30.2 (15.5-71.4)	14.9 (5-25)	NS*‡
RBM thickness (μm)	7.35 (0.42)	12.0 (1.41)	11.20 (0.84)	$P = .006$ *; NS‡

NS, Not significant; BDP, beclomethasone dipropionate.

Data expressed as means (SEMs).

*ANOVA.

† χ^2 Test.

‡Asthma versus EB.

§Asthma versus control.

||Geometric mean (95% CI).

¶Median [interquartile range]. BDP equivalents: fluticasone, 2:1; budesonide, 1.25:1; mometasone, 1.25:1. Intergroup comparisons: *t* test and 1-way ANOVA with Bonferroni correction for multiple comparisons.

Examples of immunohistochemical staining are shown in Fig 1, A to D. The MCC was found to be repeatable with a close correlation between blind observer counts 2 weeks apart (Spearman $r = 0.83$; $P < .0001$).

The MCC was significantly elevated in patients with moderate to severe persistent asthma (GINA 3-5; 5.2 [0.4]; $P = .02$), refractory asthma alone (4.8 [0.3]; $P = .04$), and EB (5.1 [0.20]; $P = .01$) in comparison with the control group (3.5 [0.5]; Fig 2). There was no significant difference between the MCC in refractory asthma compared with patients with GINA 1-2 asthma (4.1 [0.54]; $P = .33$) and GINA 3-5 (nonrefractory) asthma (5.6 [0.70]; $P = .37$).

Increased sputum VEGF is a feature of moderate and severe persistent asthma, refractory asthma, and EB

Clinical characteristics of cohort 2 are shown in Table II. The asthma and EB cohort were well matched for age, sex, atopy, and postbronchodilator lung function. The asthma cohort had a significantly longer disease duration compared with the EB cohort; however, both cohorts had chronic disease. Both groups were matched for the daily dose of inhaled corticosteroid and for the induced sputum eosinophil count.

The median (range) induced sputum VEGF concentration was significantly elevated in subjects with moderate to severe persistent asthma (GINA 3-5; 2.4 [1.4-4.1] ng/mL; $P = .04$), refractory asthma (3.7 [1.8-7.5] ng/mL; $P = .004$) and EB (4.7 [2.8-7.8] ng/mL; $P = .0001$) compared with the healthy controls (1.1 [0.7-1.8] ng/mL; Fig 3).

Vascular remodeling is associated with postbronchodilator lung function and disease duration in asthma

Univariate analysis revealed a significant inverse correlation between the postbronchodilator FEV₁ % predicted in the subjects with asthma and the MCC ($R^2 = 0.28$; $P = .02$; Fig 4, A) and log VEGF sputum concentration ($R^2 = 0.21$; $P = .01$; Fig 4, B). There was no significant relationship between postbronchodilator FEV₁ % predicted in the subjects with EB and MCC ($R^2 = 0.02$; $P = .72$) or sputum VEGF concentration ($R^2 = 0.02$; $P = .64$). We did not find any association between the log-transformed methacholine PC₂₀ and the MCC ($R^2 = 0.04$; $P = .46$) or sputum VEGF concentration ($R^2 = 0.013$; $P = .7$) in subjects with asthma.

Stepwise linear regression demonstrated that postbronchodilator FEV₁ % predicted in combination with the

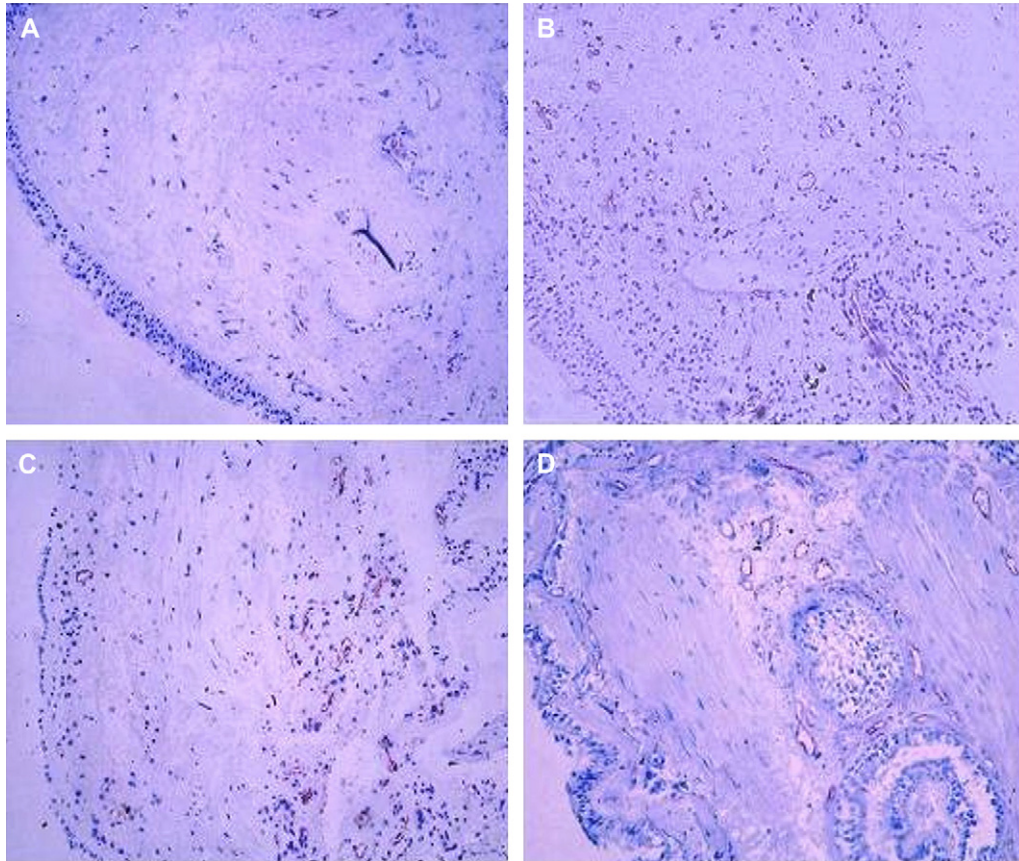


FIG 1. Immunohistochemistry vascular remodeling. Representative photomicrographs ($\times 200$) show bronchial biopsy sections stained with monoclonal antibody against PECAM-1/EN4. **A**, Control subject: relatively few vessels. **B**, Patient with mild intermittent asthma (GINA 1): increased vascularity. **C**, Subject with EB: multiple vessels in the subepithelium. **D**, Subject with refractory asthma: multiple vessels surrounding submucosal glands. RBM thickening is evident in subjects with asthma and EB.

submucosal eosinophil count best modeled the MCC in asthma (model adjusted $R^2 = 0.43$; $\beta = -0.46$, postbronchodilator FEV_1 % predicted, $P < .0001$; $\beta = -0.42$, submucosal eosinophils, $P = .001$). Disease duration was the best predictor of sputum VEGF in asthma (model adjusted $R^2 = 0.24$; $\beta = 0.50$, $P < .0001$).

DISCUSSION

We have shown for the first time that sputum VEGF concentration and vascularity in the bronchial submucosa were increased in EB to a similar intensity observed in moderate to severe persistent and refractory asthma. Our assessment of sputum VEGF and vascular remodeling in bronchial biopsies was made in 2 independent cohorts, and the consistency of our findings in each cohort strengthens our conclusions. The presence of vascular remodeling in EB demonstrates that vascular remodeling and AHR can be dissociated and therefore questions its role in the pathogenesis of AHR in asthma.

Our findings indicate that vascular remodeling is a feature of EB. This is in contrast to the findings of an

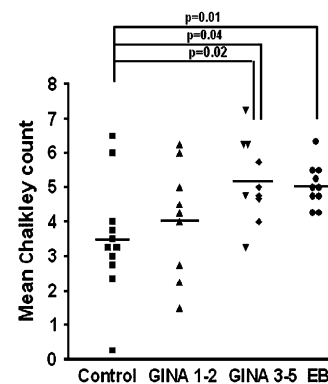


FIG 2. MMCs, cohort 1: control group; GINA 1-2 (intermittent to mild persistent asthma); GINA 3-5 (moderate to severe persistent asthma), including refractory asthma alone and nonrefractory; EB.

earlier study by Kanazawa et al¹⁸ demonstrating that increased sputum VEGF and vascular permeability were not features of EB. One possible explanation for the discrepancy between these studies is disease duration. We carefully selected only patients who had EB for at least

TABLE II. Cohort 2: clinical characteristics

	Control (n = 15)	EB (n = 14)	Asthma (n = 31) GINA 1-2, n = 11 GINA 3-5, n = 20 (Refractory asthma, n = 12)	
Age (y)	36.5 (4.3)	56.6 (3.7)	45.3 (2.7)	$P = .003^*$; NS ‡
Sex (M:F)	7:8	10:4	19:12	NS ‡
Atopy (%)	33	50	66	$P < .0001^\ddagger$; NS ‡
Disease duration (y)	NA	7.8 (1.4)	18.2 (14.6)	$P = .02^\ddagger$
Inhaled BDP (μ g/24 h)	NA	1043 (236)	1342 (224)	NS ‡
Oral prednisolone	NA	0	8/31	—
Intramuscular triamcinolone	NA	0	4/31	—
Prebronchodilator FEV ₁ (L)	3.53 (0.27)	2.82 (0.19)	2.47 (0.17)	$P = .003^*$
Postbronchodilator FEV ₁ (L)	3.52 (0.28)	2.92 (0.19)	2.65 (0.15)	$P < .05^\S$
Postbronchodilator FEV ₁ %	100 (4.3)	94 (3.5)	91 (4.0)	$P = .01^*$
FEV ₁ /forced vital capacity	84.6 (3.1)	78.8 (2.5)	74.5 (1.9)	$P < .05^\S$
postbronchodilator				NS *
PC ₂₀ (mg/mL)	>16	>16	1.06 (0.39-2.93)	$P = .02^*$; NS ‡
Exhaled nitric oxide (ppb)	14.0 (9.9-19.8)	26.1 (15.8-43.1)	30.3 (20.1-45.8)	$P < .0001^*$
Induced sputum				$P < .0001^\ddagger$
Eosinophils (%)	0.53 (0.25-1.2)	3.3 (1.2-9.2)	3.0 (1.4-6.5)	NS *
Neutrophils (%)	43.3 (26-72)	44.8 (21-65)	36.5 (20-65)	NS *

NS, Not significant; NA, not applicable; BDP, beclomethasone dipropionate.

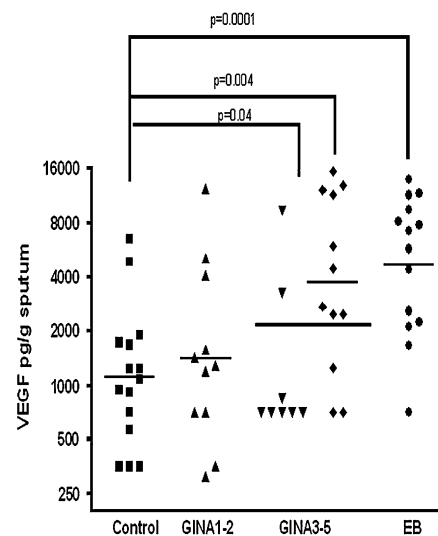
Data expressed as means (SEMs).

*ANOVA.

 $^\ddagger\chi^2$ Test. ‡ Asthma versus EB. § Asthma versus control.

||Geometric mean (95% CI).

1 year, and the median length of disease was more than 5 years in both cohorts. Interestingly, the view that sputum VEGF and disease duration are associated finds support from our finding that using stepwise linear regression disease duration was the strongest predictor of sputum VEGF in asthma. In addition, we have confirmed our findings in 2 independent cohorts of patients by using different methods to assess vascular remodeling. Therefore, we are confident that our findings are robust. The view that AHR and features of remodeling are independent is consistent with several other lines of evidence. We report here and in an earlier study¹⁷ that thickening of the RBM is a feature shared by EB and asthma and was not related to AHR. Indeed, Milanese et al³⁰ have demonstrated a positive relationship between RBM thickening and the dose of methacholine to cause a 20% fall in FEV₁ in asthma, suggesting that remodeling may protect against AHR. Similarly, Ward et al³¹ demonstrated that δV_d the change in anatomical dead space with lung volume, which has been proposed as an index of airway distensibility or stiffness correlates inversely with RBM thickening in asthma. It is important to note that the measurement of RBM thickening alone is not an overall assessment of airway wall remodeling. However, using measurements of airway wall geometry derived from high-resolution computed tomography images, airway wall thickening was inversely related to airway reactivity.³² Thus, taken together, these

**FIG 3.** Induced sputum supernatant VEGF (pg/g), cohort 2. Sputum VEGF concentration in the control group; GINA 1-2 (intermittent to mild persistent asthma); GINA 3-5 (moderate to severe persistent asthma) including refractory asthma alone and nonrefractory; EB.

findings support the view that airway remodeling, instead of contributing to AHR, may indeed protect against AHR.

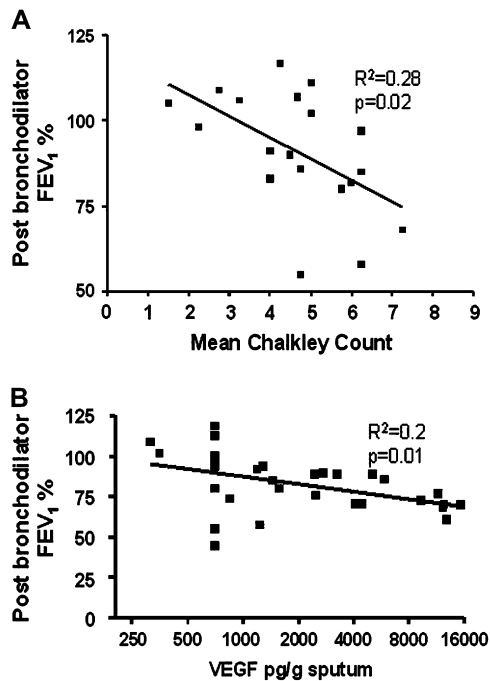


FIG 4. Vascular remodeling and airflow obstruction. In the subjects with asthma, there was an inverse association between the postbronchodilator FEV₁ % predicted and the MCCs in cohort 1 (A) and sputum VEGF concentration in cohort 2 (B).

To date, comparisons of EB and asthma have been very informative about the potential mechanisms of AHR. The cardinal immunopathological feature that distinguishes EB and asthma is the presence of mast cells within the ASM bundle in asthma.¹⁷ Several studies have confirmed that mast cell numbers in the ASM bundle are increased,³³⁻³⁶ and the intimacy of these cell types is illustrated by studies using electron microscopy.³⁷ Importantly, the number of mast cells in the ASM bundle is related to the degree of the AHR.¹⁷ The mast cells are activated in the ASM bundle with increased degranulation,³⁸ and they express the cytokines IL-4 and IL-13.³⁹ There is evidence to suggest that the ASM phenotype in asthma is altered,^{40,41} and it is likely that these intrinsic abnormalities in ASM function together with interactions with mast cells rather than airway remodeling that are the key determinants of AHR.

The lack of association between vascular remodeling and AHR does not exclude the possibility that vascular remodeling is critical in the development of other aspects of disordered airway physiology in asthma. We found that the bronchial submucosal vascularity and sputum VEGF concentration correlated inversely with postbronchodilator lung function in asthma, suggesting that vascular remodeling contributes to persistent airflow obstruction. This is entirely consistent with a previous report demonstrating an association between vascular remodeling and airflow obstruction in asthma.¹¹ We and others have demonstrated that fixed airflow obstruction may develop in EB,^{14,42} particularly in subjects with poorly controlled eosinophilic inflammation. However, we did not observe

an association between submucosal vascularity or sputum VEGF concentration and postbronchodilator lung function in the subjects with EB. Our subjects with EB had normal lung function, and some in cohort 2 were treated with inhaled corticosteroids, leading to a reduction in the intensity of their eosinophilic inflammation. Both of these factors are likely to have reduced the chance of observing a relationship between lung function and vascular remodeling in this group. Therefore, the relative contribution of vascular remodeling to the development of airflow obstruction in some patients with EB requires further study.

The focus of our study was the role of vascular remodeling in the development of the disordered airway physiology in asthma. We have therefore not undertaken an extensive investigation of other features of remodeling such as glandular hyperplasia, matrix deposition, or ASM hyperplasia and hypertrophy. Importantly, others have demonstrated that increased ASM mass was associated with severity of airflow obstruction in asthma,⁴³ and therefore there is a need to extend our observations in later studies to include several features of airway remodeling. One possible weakness of our study was the cross-sectional design. There is a paucity of longitudinal studies investigating the relationship between remodeling and disordered airway physiology. We have attempted to address this shortcoming partly by including subjects with chronic disease, and the inclusion of EB as a disease control group strengthens our assertions that vascular remodeling was not associated with AHR. Intriguingly, the submucosal eosinophil count was inversely related to vascular remodeling in asthma as assessed by the MCC. The cross-sectional design of this study means that we do not have a measure that reflects eosinophilic inflammation over time. A possible explanation for this apparent anomaly is that subjects with increased vascular remodeling are those subjects that had persistent eosinophilic inflammation, which is now controlled by corticosteroid therapy. Future studies need to examine longitudinally the natural history of airway remodeling, its relationship to airway function, airway inflammation, and response to treatment.

In conclusion, we have shown for the first time that vascular remodeling and increased expression of VEGF are features of nonasthmatic eosinophilic bronchitis and moderate to severe persistent and refractory asthma and are dissociated from AHR but are associated with airflow limitation. Our work provides further evidence of the dissociation of airway remodeling and AHR in asthma.

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