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Fibrocyte localization to the airway smooth muscle is a feature of asthma

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Abstract

Background—Airway smooth muscle (ASM) hyperplasia is a hallmark of asthma that is associated with disease severity and persistent airflow obstruction.

Objectives—We sought to investigate whether fibrocytes, a population of peripheral blood mesenchymal progenitors, are recruited to the ASM compartment in asthma.

Methods—We assessed the number of fibrocytes in bronchial biopsy specimens and peripheral blood from subjects with mild-to-severe refractory asthma versus healthy control subjects. *In vitro* we investigated potential mechanisms controlling fibrocyte migration toward the ASM bundle.

Results—Fifty-one subjects with asthma and 33 control subjects were studied. In bronchial biopsy specimens, the number of fibrocytes was increased in the lamina propria of subjects with severe refractory asthma (median [interquartile range] number, 1.9/mm² [1.7/mm²]) versus healthy control subjects (median [interquartile range] number, 0/mm² [0.3/mm²], $P < .0001$) and in the ASM bundle of subjects with asthma of all severities (subjects with severe asthma, median [interquartile range] number, 3.8/mm² [9.4/mm²]; subjects with mild-to-moderate asthma, median [interquartile range] number, 1.1/mm² [2.4/mm²]; healthy control subjects, (median [interquartile range] number, 0/mm² [0/mm²]); $P = .0004$). In the peripheral blood the fibrocyte number was also increased in subjects with severe refractory asthma (median [interquartile range] number, $1.4 \times 10^4/\text{mL}$ [$2.6 \times 10^4/\text{mL}$]) versus healthy control subjects (median [interquartile range] number, $0.4 \times 10^4/\text{mL}$ [$1.0 \times 10^4/\text{mL}$], $P = .002$). We identified that *in vitro* ASM promotes fibrocyte chemotaxis and chemokinesis (distance of migration after 4.5 hours, 31 μm [2.9 μm] vs 17 μm [2.4 μm], $P = .0001$), which was in part mediated by platelet-derived growth factor (mean inhibition by neutralizing antibody, 16% [95% CI, 2% to 32%], $P = .03$) but not by activation of chemokine receptors.

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Conclusion—This study provides the first evidence that fibrocytes are present in the ASM compartment in asthma and that ASM can augment fibrocyte migration. The importance of fibrocytes in the development of ASM hyperplasia and airway dysfunction in asthma remains to be determined.

Keywords

Asthma; airway smooth muscle; remodeling; mast cells

Asthma is a major cause of morbidity and mortality worldwide, and its prevalence continues to increase.¹ Severe refractory asthma accounts for about 10% of asthma but is particularly important because it leads to debilitating chronic symptoms despite optimal standard asthma treatment and contributes to more than half of the health care costs attributed to asthma.^{2,3} There is increasing recognition that asthma is a heterogeneous condition consisting of several domains, including clinical expression of the disease, disordered airway physiology, and chronic inflammation.² One important feature of severe disease is the development of persistent airflow obstruction, which is refractory to current therapy and contributes significantly to the morbidity of the disease.

This persistent airflow obstruction is considered to be a consequence of airway inflammation together with airway remodeling.² The airway inflammation in asthma is typically eosinophilic, with increased expression of T_H2 cytokines⁴ and mast cell localization within the airway smooth muscle (ASM) bundle.⁵ Airway remodeling encompasses several structural changes in the airway wall, including reticular lamina and basement membrane thickening, an increased number of subepithelial myofibroblasts, and increased ASM mass. This latter feature is due to a combination of ASM hyperplasia⁶ and hypertrophy, which increases with disease severity.⁷ Increased ASM mass is well recognized within both large and small airway walls and is evident in fatal and nonfatal cases of asthma.⁸ It is the most important abnormality responsible for the increased airflow resistance observed in response to bronchoconstricting stimuli⁹ and is a major determinant in the development of persistent airflow obstruction in chronic disease.⁷

The cause of ASM hyperplasia in asthma is unknown, but it is often attributed to increased proliferation. Indeed, ASM proliferation is increased in *ex vivo* asthmatic ASM,^{10,11} but several reports have been unable to demonstrate increased ASM proliferation *in vivo*.^{6,7,12} An alternative explanation is that ASM progenitors either located within the airway wall or derived from peripheral blood mesenchymal cell progenitors (fibrocytes)¹³ migrate to the ASM bundle and differentiate into ASM. In support of this view, myofibroblasts expressing fibrocyte markers have been identified in bronchial tissue after allergen challenge,¹⁴ tissue fibrocyte number has been shown to correlate with subbasement membrane thickening in mild asthma,¹⁵ and a higher percentage of circulating fibrocytes is found in asthmatic subjects with chronic airflow obstruction.¹⁶

We hypothesized that in subjects with asthma the number of fibrocytes is increased in the ASM bundle and mediated by ASM-derived chemotactic factors. We enumerated the fibrocyte number in bronchial biopsy specimens and blood from subjects with mild-to-

severe refractory asthma and healthy control subjects and *in vitro* investigated the mechanisms driving fibrocyte migration.

METHODS

Subjects

Subjects with asthma (n = 51) and healthy control subjects (n = 33) were recruited from respiratory clinics and hospital staff and by means of local advertising. Asthma was defined by using the current Global Initiative for Asthma (GINA) guidelines, and severity was classified based on the GINA treatment steps¹ and the American Thoracic Society criteria for refractory asthma.³ Healthy subjects had no history of respiratory disease and normal lung function. All subjects were nonsmokers with a past smoking history of less than 10 pack-years and were free from exacerbations for at least 6 weeks. The Leicestershire ethics committee approved the study. All patients provided written informed consent.

Clinical characterization

Subjects underwent allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat, and grass pollen; spirometry before and after bronchodilator (400 µg of inhaled salbutamol); exhaled nitric oxide measurement (measured at 50 mL/s; NIOX; Aerocrine, Stockholm, Sweden); and methacholine airway responsiveness with the tidal breathing method (0.03-16 mg/mL) followed by sputum induction on recovery with incremental concentrations of nebulized hypertonic saline (3%, 4%, and 5% each) for 5 minutes.⁵

Subjects either donated a peripheral venous blood sample for fibrocyte isolation or underwent bronchoscopy conducted according to the British Thoracic Society guidelines.¹⁷ Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae and were fixed in acetone and embedded in glycomethacrylate, as described previously.¹⁸

Immunohistochemistry to identify fibrocytes

Sequential 2-µm sections were cut and stained with mAbs against CD34 (R&D Systems, Oxfordshire, United Kingdom), collagen I (Chemicon, Temecula, Calif), α-smooth muscle actin (α-SMA) and tryptase for mast cells or appropriate isotype controls (Dako, Cambridgeshire, United Kingdom). The number of positive nucleated cells was enumerated per square millimeter of lamina propria or ASM bundle by a blinded observer. A minimum area of 0.1 mm² was considered assessable, as described previously.⁵ Fibrocytes were identified as collagen I-positive CD34⁺ cells¹⁴ determined by using the technique of colocalization, as described previously.¹⁹

Cell isolation and culture

Pure ASM bundles were isolated from bronchial biopsy specimens (n = 8 subjects, 4 with asthma) and large airway tissue from subjects undergoing surgical intervention (n = 11). Primary ASM was cultured and characterized as described previously.¹⁹

LAD2 mast cells were a gift from Dr D. Metcalfe (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md) and were cultured as described previously.²⁰

Fibrocytes were isolated from peripheral blood and cultured in fibronectin-coated (40 µg/mL) T25 tissue-culture flasks, as previously described.^{21,22} After 48 hours, nonadherent cells were removed. After 7 to 14 days, adherent cells were washed with HBSS and harvested with Accutase (eBioscience, Wembley, United Kingdom). Cell counts and viability of the initial isolated PBMC fraction and the final adherent cells were determined by using trypan blue. Viability was consistently greater than 95%. In parallel, PBMC preparations were seeded onto fibronectin-coated 8-well chamber slides (2×10^5 cells/well) and cultured as above. Fibrocyte purity and characterization were assessed by means of flow cytometry and immunofluorescence.

Flow cytometry

To assess surface and intracellular expression of CD34, collagen I, and α -SMA, cells were fixed and permeabilized in 4% paraformaldehyde and 0.1% saponin (Sigma, St Louis, Mo), as previously described,²³ and labeled with CD34-RPE mAb (Caltag Laboratories, Burlingame, Calif) and collagen I mAb or α -SMA mAb (Sigma) indirectly labeled with fluorescein isothiocyanate (FITC; Dako). In samples with sufficient cells, receptor expression was assessed by using the mAbs CD13-RPE (Caltag), HLA-DR-RPE (Caltag), CXCR4-RPE (R&D Systems), and CCR7 (gift from Millennium, Cambridge, Mass) indirectly labeled with FITC. All experiments included isotype controls, and analysis was performed with 1- or 2-color flow cytometry, as appropriate, on a FACScan (BD, Oxford, United Kingdom).

Immunofluorescence

The cells in the chamber slides were labeled with the mAbs CD34-RPE, collagen I, α -SMA, CCR3, CCR7, CXCR3, and CXCR4 (R&D Systems) or appropriate isotype controls indirectly labeled with FITC or RPE as required. Nuclei were stained with 4', 6'-diamidino-2-phenylindole dihydrochloride (0.5 µg/mL; Sigma).

Fibrocyte migration

We used a validated chemotaxis assay²⁴ modified to study fibrocyte migration. ASM cells (2.5×10^5 /well) were seeded onto fibronectin-coated (1 µg/ml) 8-rectangular-well plates and allowed to adhere overnight before removal by scraping of half the ASM below a predrawn line on the well underside. Fibrocytes ($4-8 \times 10^4$ /well) were then added for 72 hours before experimentation. Where appropriate, recombinant chemotaxins (CCL11, CCL19, CXCL10, CXCL12 [100 ng/blot] and platelet-derived growth factor [PDGF; 10 ng/blot; R&D Systems) or FBS control media were impregnated onto blotting paper (25 mm $\times$ 6 mm, Sigma), placed along the bottom edge of the multicell layer, and secured in place with silicon grease. Where required, the following blocking or neutralizing antibodies were used: CCR3 (Millennium), CCR7, CXCR3, CXCR4, TGF- β , and PDGF or isotype control (R&D Systems). Further experiments were performed with ASM cocultured with sensitized and nonsensitized mast cells (overnight with human myeloma IgE, 2.5 µg/mL; Calbiochem-

Novabiochem, Nottingham, United Kingdom) at a ratio of 1:4 mast cells/ASM cells. After the addition of fibrocytes, as described above, mast cells were either left unstimulated or activated with goat polyclonal anti-human IgE (1:1000 dilution, Sigma) for 1 hour at 37°C. Photographs of the fibrocytes were taken along a predrawn line 5 mm below the edge of the multicell layer or equivalent position in control wells at 1.5-hour intervals over a 4.5-hour time course. The migration of individual fibrocytes was tracked manually, and the distance and direction of migration in relation to the multicell layer was obtained by a blinded observer.

ASM mRNA expression of PDGF isoforms

mRNA expression by ASM of PDGF isoforms and their cognate receptors was examined by using the Human Genome U133A probe array (GeneChip; Affymetrix, Santa Clara, Calif), as described previously.²⁵

Statistical analysis

Statistical analysis was performed with Prism version 4 software (Graph-Pad Software, Inc, La Jolla, Calif). Parametric data were expressed as means (SEMs), data that had a log-normal distribution were log-transformed and described as geometric means (95% CIs), and nonparametric data were described as medians (ranges). One-way ANOVA and the Kruskal-Wallis test were used for comparisons across groups, with *post hoc* Tukey and Dunn comparisons between groups for parametric and nonparametric data, respectively. χ^2 Tests were used to compare categorical data. Pearson and Spearman rank correlation coefficients were used to assess correlations.

RESULTS

The clinical characteristics of the subjects who underwent bronchoscopy or donated blood for fibrocyte isolation are as shown in Tables I and II, respectively. Subjects with refractory asthma had evidence of airflow limitation and ongoing eosinophilic inflammation, despite receiving more inhaled and oral corticosteroid therapy.

Fibrocyte numbers are increased in the ASM bundle and lamina propria in asthmatic subjects

Representative photomicrographs of CD34⁺ collagen I-positive α -SMA⁺ fibrocytes in the ASM bundle and lamina propria are as shown in Fig 1, A. The ASM bundle area was insufficient to assess fibrocyte numbers in 1 of 13 healthy control subjects and 1 of 12 subjects with severe refractory asthma. The median number (interquartile range) of fibrocytes per square millimeter in the lamina propria was increased in those with refractory disease (1.9 [1.7]) versus that seen in subjects with mild-to-moderate asthma (0 [0.6]) and healthy control subjects (0 [0.3]; $P < .0001$; Fig 1, B). There was a strong correlation between fibrocyte numbers in the lamina propria and asthma severity defined by GINA treatment step ($r = 0.68$, $P = .0003$). The percentage ASM area was increased in those with refractory (19.5% [3.6%]) and mild-to-moderate asthma (23.3% [1.9%]) versus healthy control subjects (10.5% [1.4%]; $P = .0016$, ANOVA). The number of fibrocytes per square millimeter was markedly increased in the ASM bundle in subjects with severe refractory

asthma (3.8 [9.4]) and mild-to-moderate asthma (1.1 [2.4]) versus that seen in healthy control subjects (0 [0], $P = .0004$; Fig 1, C). The number of fibrocytes and mast cells within the ASM bundle was correlated for the whole group ($r = 0.58$, $P = .0002$) and in asthmatic subjects alone ($r = 0.44$, $P = .035$). However, the fibrocyte number in tissue did not correlate with lung function, sputum eosinophil count, or ASM mass.

Peripheral blood fibrocyte characterization

In culture the adherent PBMCs became elongated and spindle shaped, as shown in Fig 2, A. The purity of cultured fibrocyte populations was confirmed by coexpression of CD34 and collagen I. The majority of cells in culture after 7 to 14 days expressed both markers assessed by means of immunofluorescence ($95\% \pm 1\%$; Fig 2, A) and flow cytometry ($86\% \pm 3\%$; Fig 2, B) and also 2 other fibrocyte markers, CD13 ($98\% \pm 0.3\%$) and HLA-DR ($90\% \pm 6\%$; Fig 2, B). Using flow cytometry, we identified that the cells also expressed α -SMA ($66\% \pm 6\%$) and the chemokine receptors CCR7 ($57\% \pm 11\%$) and CXCR4 ($89\% \pm 5\%$; Fig 2, A). This was confirmed by means of immunofluorescence (Fig 2, A).

Peripheral blood fibrocytes are increased in asthmatic subjects

The median (interquartile range) proportion of PBMCs in culture that were identified as fibrocytes was increased in subjects with severe refractory asthma versus healthy control subjects ($P = .01$, Fig 3). There was no significant difference in the number of fibrocytes in subjects with mild-to-moderate asthma versus healthy control subjects or subjects with mild-to-moderate versus refractory asthma. This finding was corroborated by a similar pattern being observed for the number of fibrocytes $\times 10^4/\text{mL}$ peripheral blood, with an increase in those with refractory asthma ($1.4 \times 10^4/\text{mL}$ [$2.6 \times 10^4/\text{mL}$]) versus subjects with mild-to-moderate asthma ($1.0 \times 10^4/\text{mL}$ [$3.1 \times 10^4/\text{mL}$]) and healthy control subjects ($0.4 \times 10^4/\text{mL}$ [$1.0 \times 10^4/\text{mL}$], $P = .005$). There was no correlation between the number or proportion of peripheral blood fibrocytes and the subjects' clinical or physiologic characteristics.

Fibrocyte trafficking to ASM

Example migration paths of fibrocytes from their origin to their position after 4.5 hours are as shown in Fig 4, A. The net distance of fibrocyte migration was increased in the presence of ASM (mean difference, $14 \mu\text{m}$ [95% CI, 8-21 μm]; $P = .0001$; Fig 4, B), and the proportion of fibrocytes that did not migrate was markedly decreased in the presence (21% [3%]) versus the absence (42% [5%]) of ASM ($P < .0001$). The fibrocyte migration toward the ASM cells was increased compared with that seen in those that moved away (mean difference, 7% [95% CI, 1% to 13%]; $P = .02$; $n = 17$; Fig 4, B). The distance and direction of fibrocyte migration was not different between asthmatic and nonasthmatic fibrocytes or ASM donor (mean difference of the distance of fibrocyte migration toward ASM from asthmatic versus nonasthmatic donors, $1.4 \mu\text{m}$ [95% CI, -13 to 16 μm]; $P = .8$).

The recombinant chemokines CCL11, CCL19, CXCL10, and CXCL12 in addition to ASM did not affect the distance or direction of fibrocyte migration (data not shown). PDGF increased the distance of fibrocyte migration in the presence of ASM (mean difference, $5 \mu\text{m}$ [95% CI, 1-9 μm]; $P = .016$; Fig 4, C) and the direction toward versus away from the ASM cells (mean difference, 15% [95% CI, 3% to 26%]; $P = .018$; $n = 10$; Fig 4, C). ASM-

mediated fibrocyte migration was not affected by blockade of the chemokine receptors CCR3, CCR7, CXCR3, and CXCR4 or neutralization of TGF- β ($n = 3$ -5; $<10\%$ inhibition for all conditions; $P > .05$). PDGF inhibition ($n = 5$) attenuated the distance of ASM-mediated fibrocyte migration compared with the isotype control (mean distance of migration, $18 \mu\text{m}$ [95% CI, 16 - $21 \mu\text{m}$] vs $22 \mu\text{m}$ [95% CI, 19 - $25 \mu\text{m}$], respectively; $P = .047$; mean inhibition, 16% [95% CI, 2% to 32%]; $P = .03$; Fig 4, D). Mast cells alone either unstimulated or IgE/anti-IgE stimulated and/or in coculture with ASM did not augment the distance or direction of fibrocyte migration ($n = 5$). All conditions that included ASM showed increased fibrocyte migration ($P < .05$; Fig 4, E).

The mRNA expression compared with β -actin for the PDGF isoforms present in ASM was as follows: A ($0.4\% \pm 0.1\%$), C ($20\% \pm 4\%$), and D ($11\% \pm 5\%$) and receptors A ($75\% \pm 14\%$) and B ($15\% \pm 5\%$). There was no difference between ASM from asthmatic subjects ($n = 3$) and nonasthmatic subjects ($n = 3$).

DISCUSSION

We show, for the first time, an increase in the number of fibrocytes in the ASM bundle in subjects with asthma of all severities and in the lamina propria in subjects with severe refractory asthma. Our observation of an increased number of fibrocytes in the peripheral blood of subjects with severe asthma corroborates a recent report that the percentage of circulating fibrocytes is increased in asthmatic subjects with chronic airflow obstruction.¹⁶ We identified that *in vitro* ASM promotes fibrocyte migration, which is in part mediated by PDGF. This is the first study to provide evidence that fibrocytes are present in the ASM compartment in asthmatic subjects and that ASM can augment fibrocyte migration. However, the fibrocyte numbers in peripheral blood and bronchial biopsy specimens were not correlated with lung function or ASM mass. Therefore the importance of fibrocytes in the development of ASM hyperplasia and airway dysfunction in asthma remains to be determined.

To date, ASM hyperplasia in asthma has been largely attributed to increased ASM proliferation. Although this view finds support from primary culture of ASM from asthmatic subjects^{10,11} there is currently no evidence in bronchial biopsy specimens of increased ASM proliferation.^{6,7,12} Possible explanations for this lack of evidence are that our current tools are too blunt to identify small changes in ASM proliferation or that the proliferation only occurs in response to specific stimuli, perhaps at exacerbations, limiting our opportunities to observe it. We propose an alternative explanation that ASM hyperplasia might, in part, be a consequence of the recruitment of progenitors to the ASM bundle. Our findings support this view because the number of fibrocytes was increased in the ASM bundle in asthmatic subjects compared with numbers seen in healthy control subjects, with this increase most marked in those with severe refractory disease. In addition, we found that the number of fibrocytes in the lamina propria was increased in subjects with severe refractory asthma and was strongly correlated with disease severity. This extends the earlier observations that in subjects with mild disease the number of subepithelial fibrocytes increased after allergen challenge¹⁴ and that tissue fibrocyte number correlated with basement membrane thickness.¹⁵ Our assertion that fibrocyte trafficking might be important in subjects with

asthma is further supported by our finding that there is an increased number of fibrocytes in peripheral blood in subjects with severe refractory disease and a recent study that shows a higher percentage of circulating fibrocytes in asthmatic subjects with chronic airflow obstruction.¹⁶ This suggests that in addition to recruitment to the airway, there is likely to be an increase in the bone marrow production of peripheral blood fibrocytes, as observed for other progenitor cells.²⁶

The view that ASM hyperplasia might be a consequence of fibrocytes trafficking to the airway is consistent with mechanisms proposed in skin wound healing^{13,27} and is analogous to the current concepts of cardiac myocyte progenitors contributing to cardiac repair after myocardial infarction.²⁸ In addition to their role in tissue repair, fibrocytes have been implicated in lung fibrosis²¹ and other fibrotic diseases.²⁹ In mouse models of lung fibrosis, fibrocytes are the major source of matrix protein deposition,³⁰ and blockade of fibrocyte recruitment inhibits the development of lung fibrosis.²¹ Fibrocytes in tissue differentiate into myofibrocytes and express α -SMA mediated largely by TGF- β .¹⁶ In subjects with asthma, they therefore have the potential to contribute to the pool of subepithelial myofibroblasts and have the capacity to migrate to different airway compartments. Whether fibrocytes in the ASM bundle remain as immature progenitors or mature fully into ASM has yet to be determined.

The relationship between airway structure and function is contentious, leading to considerable debate over the clinical importance of airway remodeling.³¹ Indeed, we have recently reported that airway remodeling, including ASM mass, was not associated with airway hyperresponsiveness.³² However, airway wall thickening, assessed by means of computed tomographic imaging, is strongly and consistently correlated with airflow obstruction in asthma and chronic obstructive pulmonary disease.^{33,34} Importantly, mathematic models suggest that thickening of the airway wall is fundamental in the development of disordered airway physiology.³⁵ Features of airway remodeling in asthma, notably increased ASM mass,⁷ increased vascularity,³⁶ thickening of the reticular lamina, and basement membrane and fibroblast numbers in the lamina propria,⁷ have all been shown to correlate with airflow limitation. In most studies these features are studied in isolation, limiting our ability to determine the relative contribution of each component of remodeling with lung function. This shortcoming has perhaps been best overcome by Benayoun et al,⁷ who carefully evaluated several features of airway remodeling and found that subepithelial fibroblast number and increased ASM mass were the major determinants of persistent airflow obstruction. Critically, this earlier report did not distinguish between resident mesenchymal cells and recruited fibrocytes. Hence it is possible that both features associated with persistent airflow obstruction were a direct consequence of fibrocyte recruitment to the airway.

Fibrocyte migration to the ASM bundle is likely to be under the control of important ASM-derived chemotaxins. Previous reports have demonstrated that fibrocytes express the chemokine receptors CCR3, CCR7, and CXCR4, although CCR3 was not functional.^{13,21} We have confirmed and extended this observation to include CXCR3. The CXCR4–CXCL12 axis has been implicated in the recruitment of peripheral blood progenitors to the lung.²¹ The CXCR4 ligand CXCL12 is preferentially expressed by the bronchial

epithelium,¹⁹ and this might provide a mechanism for the enhanced subepithelial and luminal myofibroblasts³⁷ observed in asthma. Here we found that ASM markedly upregulated fibrocyte migration. We have previously proposed that activation of CCR7 by ASM or mast cell–derived CCL19 is important for recruitment of myofibroblasts and ASM toward the ASM bundle. Indeed, in subjects with severe asthma, CCL19 expression by the ASM bundle was increased.²⁴ However, we found that ASM-mediated fibrocyte migration was not augmented by additional recombinant chemokines or mast cells, nor was it attenuated by blocking CCR3, CCR7, CXCR3, and CXCR4. Therefore although the CCR7–CCL19 axis might be important for migration of differentiated cells of the mesenchymal lineage toward the ASM bundle, it is not the predominant pathway involved in recruitment of progenitors. In contrast to the lack of effect by chemokines, we found that ASM-derived PDGF played a role in ASM-mediated fibrocyte migration. PDGF is a powerful stimulator of mesenchymal cell chemotaxis.³⁸ Importantly, fibroblasts derived from subjects with severe asthma have increased expression of PDGF receptor β .³⁹ Although about one fifth of the fibrocyte migration toward ASM was attributable to PDGF, the majority of the migration was PDGF independent. The additional mechanisms involved in fibrocyte trafficking need to be elucidated.

One major criticism of our study was our inability to demonstrate a relationship between fibrocyte infiltration, ASM mass, and measures of lung function. This raises the question of whether the identification of the fibrocytes in the ASM bundle is of real functional importance in asthma or simply an epiphenomenon. We chose a cross-sectional study design, which limits the potential of observing important associations between structural changes and function. Therefore future studies will need to examine the relationship between fibrocyte recruitment and longitudinal outcomes, such as lung function decrease. Similarly, although we found striking differences in fibrocyte numbers between asthma and health, our study design does not enable us to determine whether the differences in severe asthma reflect severity of disease or treatment. Hence later studies will need to study the effect of therapy within individuals. Despite these shortcomings, we are confident that our findings are robust because the fibrocyte cell counts in tissue and peripheral blood were enumerated by observers blinded to the clinical characteristics and the findings were consistent in blood and tissue compartments.

In summary, we show that there is an increase in the number of fibrocytes in the ASM bundle in subjects with asthma of all severities and in the peripheral blood and lamina propria of subjects with severe refractory asthma. We have identified that *in vitro* ASM promotes fibrocyte migration, which is in part mediated through release of PDGF. This is the first study to provide evidence that fibrocytes are present in the ASM compartment in subjects with asthma and that ASM can augment fibrocyte migration. Further studies are required to determine the importance of the recruitment of ASM progenitors to the ASM compartment in the development of the ASM hyperplasia that characterizes asthma.

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Abbreviations used

ASM	Airway smooth muscle
FITC	Fluorescein isothiocyanate
GINA	Global Initiative for Asthma
PDGF	Platelet-derived growth factor
RPE	R-phycoerythrin
α-SMA	α-Smooth muscle actin

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Key messages

- ASM hyperplasia is a hallmark of asthma.
- Fibrocytes are present in the ASM compartment in asthma.
- ASM promotes fibrocyte migration.

FIG 1.

A, Representative photomicrographs of bronchial biopsy specimens showing negative isotype control (i, magnification $\times 200$), fibrocytes (highlighted by *arrows*) from 2 asthmatic subjects in the lamina propria (ii-iii) and ASM bundle (iv-vi) stained for CD34 (ii and v), collagen I (iii and vi), and α -SMA (iv; magnification $\times 400$). **B and C**, Enumeration of fibrocytes in the lamina propria (Fig 1, B) and ASM bundle (Fig 1, C) in bronchial biopsy specimens from asthmatic subjects versus healthy control subjects. *Horizontal bars* indicate

medians (interquartile ranges). $P < .05$, Kruskal-Wallis test. P values for the Dunn post test are shown in the figure.

FIG 2.

A, Representative photomicrographs of cultured fibrocyte morphology (magnification $\times 200$) and collagen I, CD34, α -SMA, CCR3, CCR7, CXCR3, and CXCR4 expression. Insets in the collagen I and α -SMA panels indicate rabbit and mouse isotype controls, respectively (magnification $\times 200$). **B**, Flow cytometric analysis showing example dot plots of CD34⁺ collagen I-positive fibrocytes with corresponding isotype controls and example histograms for CD13⁺, HLA-DR⁺, α -SMA⁺, CCR7⁺, and CXCR4⁺ fibrocytes (black lines) with matched isotype controls (gray lines).

FIG 3.

The proportion of PBMCs identified as fibrocytes in culture is increased in refractory asthma compared with that seen in healthy control subjects. Dot plots of the number of peripheral blood fibrocytes in subjects with or without asthma are shown. Horizontal bars indicate medians (interquartile ranges). $P < .05$, Kruskal-Wallis test. P values for the Dunn post test are given in the figure.

FIG 4.

A, Representative line graphs showing migration paths of fibrocytes. The *arrow* represents migration toward ASM $\pm$ PDGF. **B**, fibrocyte migration alone and in the presence of ASM cells, with the addition of PDGF (**C**) or PDGF neutralizing antibody (**D**). **E**, Distance of fibrocyte migration above spontaneous movement in the presence of mast cells $\pm$ ASM. Data are presented as means $\pm$ SEMs, 1-way ANOVA was used across groups, and *post hoc* Tukey comparisons were used between groups.

TABLE I

Clinical characteristics of subjects who underwent bronchoscopy

	Control subjects (n = 13)	Subjects with mild-to-moderate asthma (n = 12; GINA 1, n = 6; GINA 2-4, n = 6)	Subjects with refractory asthma (n = 12)
Age (y)	38 (18)	33 (24)	49 (6)
Sex M/F	4/9	6/6	5/7
Atopy (%)	46	75	67
Disease duration (y)	NA	14.5 (11.0)	20.2 (15.9)
Smoking (pack-years)	0(0)	4.0 (5.9)	3.4 (1.8)
Inhaled BDP (µg/24 h)	NA	520 (658)	1633 (458)
Oral prednisolone (mg/24 h); n	NA	0; 0/12	5.5 (6.6); 7/12
Long-acting bronchodilator (%)	NA	25	100
FEV ₁ (L)	3.44 (1.14)	2.87 (1.11) *	2.50 (0.78) *
FEV ₁ % predicted	101.9 (9.5)	89.6 (19.9) *	81.4 (6.3) *
FEV ₁ /FVC ratio	86.9 (11.9)	79.9 (11.4)	72.9 (12.0) *
Bronchodilator response (%)	0.6 (2.1)	8.5 (15.9) *	4.6 (14.0) *
PC ₂₀ FEV ₁ methacholine (mg/mL) [†]	>16	0.36 (0.03-2.34) *	0.40 (0.06-2.5) *
FENO (50 mL/s), ppb [‡]	15 (9-28)	20 (12-25)	29 (16-53)
Sputum eosinophils (%) [‡]	0.25 (0.5)	0.25 (4.1)	5.9 (39) *
Sputum neutrophils (%) [‡]	61.3 (63.6)	52.4 (27.5)	50.1 (43.1)
Mast cells/mm ² ASM [‡]	2.7 (5.1)	9.8 (17.8)	11.4 (14.2)

Data are expressed as means (SDs) except for:

[†] geometric mean (95% CI)[‡] median (interquartile range).

Beclomethasone dipropionate equivalents (beclomethasone dipropionate/24 hours): fluticasone, 2:1; budesonide, 1.25:1; mometasone, 1.25:1.

M, Male; F, female; NA, not applicable; BDP, beclomethasone dipropionate; FVC, forced vital capacity; FENO, fraction of exhaled nitric oxide.

* ANOVA or Kruskal-Wallis tests, as appropriate, with *post hoc* tests for multiple comparisons.

TABLE II

Clinical characteristics of fibrocyte donors

	Control subjects (n = 21)	Subjects with mild-to-moderate asthma (n = 12; GINA 1, n = 7; GINA 2-4, n = 5)	Subjects with refractory asthma (n = 17)
Age (y)	34 (9)	35 (15)	48 (17)
Sex (M/F)	9/12	6/6	7/10
Atopy (%)	43	67	71
Disease duration (y)	NA	22 (13)	27 (16)
Smoking (pack-years)	0.5 (2)	1.7 (4)	0.5 (2)
Inhaled BDP (µg/24 h)	NA	317 (439)	1941 (734)
Oral prednisolone (mg/24 h); n	NA	0; 0/12	4.6 (5); 9/17
Long-acting bronchodilator (%)	NA	33	100
FEV ₁ (L)	3.74 (0.64)	3.32 (0.99)	1.89 (0.56)*
FEV ₁ % predicted	95.9 (9.9)	94.9 (17.9)	67.4 (22.1)*
FEV ₁ /FVC ratio	81.9 (3.89)	78.5 (5.1)	71 (15.2)*
Bronchodilator response (%)	1.3 (3.1)	3.3 (3.2)	10.5 (14.9)*
PC ₂₀ FEV ₁ methacholine (mg/mL) [†]	>16	2.1 (0.68-6.28)*	1.1 (0.41-2.8)*
FENO (50 mL/s), ppb [‡]	11.1 (8.6-14.2)	24.7 (16.1-37.9)*	32.5 (22.2-47.5)*
Sputum eosinophils (%) [‡]	0 (0.65)	1.5 (0.93)*	2.11 (8.3)*
Sputum neutrophils (%) [‡]	47.6 (22.9)	56.9 (29.6)	72.5 (15.5)*

Data are expressed as means (SDs) except for:

[†] geometric mean (95% CI)[‡] median (interquartile range).

Beclomethasone dipropionate equivalents (beclomethasone dipropionate/24 hours): fluticasone, 2:1; budesonide, 1.25:1; mometasone 1.25:1.

M, Male; F, female; NA, not applicable; BDP, beclomethasone dipropionate; FVC, forced vital capacity; FENO, fraction of exhaled nitric oxide.

* ANOVA or Kruskal-Wallis, as appropriate, with *post hoc* tests for multiple comparisons.