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Number, activation, and differentiation of circulating fibrocytes correlate with asthma severity

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Abstract

Background—A biomarker that predicts poor asthma control would be clinically useful. Fibrocytes are bone marrow–derived circulating progenitor cells that have been implicated in tissue fibrosis and T_H2 responses in asthmatic patients.

Objective—We sought to test the hypothesis that the concentration and activation state of peripheral blood fibrocytes correlates with asthma severity.

Methods—By using fluorescence-activated cell sorting analysis, fibrocytes ($CD45^+$ and collagen 1 $[Col1]^+$) were enumerated and characterized in the buffy coats of fresh peripheral blood samples from 15 control subjects and 40 asthmatic patients.

Results—Concentrations of peripheral blood total ($CD45^+Col1^+$), activated (the TGF-β transducing protein phosphorylated SMAD2/3 $[p\text{-}SMAD2/3]^+$ or phosphorylated AKT $[p\text{-}AKT]^+$), and differentiated (α-smooth muscle actin $[\alpha\text{-}SMA]^+$) fibrocytes were increased in asthmatic patients compared with control subjects. The increase in total and $CD45^+Col1^+CXCR4^+$ fibrocytes was primarily seen in patients with severe asthma (Global Initiative for Asthma steps 4–5) as opposed to those with milder asthma (Global Initiative for Asthma steps 1–3). In addition, numbers of circulating $\alpha\text{-}SMA^+$ and $\alpha\text{-}SMA^+CXCR4^+$ fibrocytes were increased in asthmatic patients experiencing an asthma exacerbation in the preceding 12 months. A significant correlation ($P < .05$) was observed between $CD45^+Col1^+CXCR4^+$ fibrocytes and the activation phenotypes $CD45^+Col1^+p\text{-}SMAD2/3^+$ and $CD45^+Col1^+p\text{-}AKT^+$.

Conclusion—There was correlation between circulating fibrocyte subsets and asthma severity, and there was an increased number of activated/differentiated fibrocytes in circulating blood of asthmatic patients experiencing an exacerbation in the preceding 12 months.

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Keywords

Asthma; asthma severity; fibrocytes; peripheral blood; biomarker

An asthma-related fatality occurs approximately every 2 hours in the United States alone, and approximately 20 million Americans have asthma.[1,2] Phenotypes of severe asthma, which include recurrent exacerbations, refractoriness to treatment, or the presence of fixed or progressive airflow obstruction, are responsible for most asthma-related deaths.[3,4] The ability to identify asthmatic patients with poor control by using a biomarker would be clinically useful.

Fixed airflow obstruction is thought to be an important component of airway remodeling caused by increased collagen deposition and fibrosis in the subepithelium and is evidenced by a greater degree of bronchial fibrosis in patients with severe asthma.[5] A substantial body of evidence exists that airway myofibroblasts develop from cellular precursors located within the lung. However, there is mounting evidence for bone marrow–derived circulating cells with both hematopoietic and mesenchymal characteristics (ie, fibrocytes) in airway remodeling in asthmatic patients. Fibrocytes are present in the circulation in both healthy subjects and patients with disease.[6–8] In animal models they localize to injured skin and change to a phenotype resembling myofibroblasts with loss of CD34 and CD45 and acquisition of α-smooth muscle actin (α-SMA) expression.[7,8] Moreover, several studies have recently reported an increase in peripheral blood fibrocyte numbers in asthmatic patients.[9–11] Saunders et al[9] reported higher numbers of $CD34^+$ collagen 1 $(Col1)^+$ fibrocytes and Wang et al[10] observed a higher number of $CD45^+Col1^+CD34^+$ fibrocytes in patients with asthma compared with control subjects. Wang et al reported correlation between circulating fibrocyte numbers and the rate of decrease in FEV_1 over a 5-year period in patients with fixed airflow obstruction. Bellini et al[11] reported an increase in the percentage of $CD34^+Col1^+$ leukocytes among total leukocytes in the buffy coat fraction of peripheral blood samples from asthmatic patients compared with those of control subjects. Taken together, these recent studies raise the possibility that fibrocytes, fibrocyte subsets, or both in the circulation might be useful biomarkers for persistent asthma phenotypes. We sought to test the hypothesis that the concentration and activation state of peripheral blood fibrocytes correlate with asthma severity. We have developed techniques for enumeration and characterization of fibrocytes in fresh blood samples without intercurrent cell culture, thus avoiding problems of selection by means of adherence and phenotypic changes that occur in culture. Moreover, analysis of cells by using flow cytometry with advanced multicolor flow cytometers allows additional staining to assess for activation and differentiation phenotypes of fibrocytes.

METHODS

The University of Virginia Institutional Review Board for Health Sciences Research approved these experiments (Health Sciences Research no. 15119). Asthmatic patients were recruited from the adult and pediatric Pulmonary Clinics at the University of Virginia and fulfilled the inclusion criteria of (1) a clinical diagnosis of asthma; (2) either a significant

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postbronchodilator increase in forced vital capacity (FVC) or FEV_1 based on American Thoracic Society (ATS) criteria[12] or a significant methacholine provocation challenge with a PC_{20} value of less than 8 mg/mL; and (3) willingness to sign the institutional review board–approved informed consent form. Subjects were excluded if they were actively smoking or had a prior smoking history of more than 10 pack years. Involvement in the study consisted of a single venipuncture to obtain a 10-mL heparinized blood sample that was refrigerated until processing. In the 6 weeks before enrollment, none of the patients were judged to be experiencing an asthma exacerbation based on published criteria.[13]

Asthma severity and exacerbations

Asthma severity was graded based on the intensity of treatment required to control symptoms by using treatment steps 1 through 5 of the Global Initiative for Asthma (GINA).[14,15] Fixed airflow obstruction was identified in patients with a postbronchodilator FEV_1/FVC ratio of less than the predicted value in view of its correlation in asthmatic patients with decreasing lung function[16] and radiographic evidence of remodeling, including increased bronchial wall thickness.[17] Patients were identified as having a severe asthma exacerbation in the 12 months preceding the study based on previously described ATS criteria[13]: (1) increased use of rescue bronchodilator for 48 hours or more; (2) symptoms that required use of systemic corticosteroids for 3 or more days; or (3) symptoms that required either hospitalization or an emergency department visit that resulted in administration of systemic corticosteroids. Additional information on skin test status, comorbid conditions, and absence of effect of age, comorbid conditions, or asthma medications on circulating fibrocyte numbers can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Criteria for identification of fibrocytes

With time in culture, circulating fibrocytes downregulate the progenitor marker CD34 and upregulate entothelin-1 or TGF-β induction of maturation with expression of α-SMA.[18,19] In tissue there is an inverse correlation between CD34 expression and collagen production.[20] Therefore, as we have done in the past,[21] we chose to evaluate fibrocytes in the peripheral circulation based on CD45 and Col1 positivity for identification of total, differentiated, and activated fibrocyte phenotypes.

Fibrocyte analysis

Peripheral blood fibrocytes were characterized by means of fluorescence-activated cell sorting (FACS) analysis, as we have previously reported.[22] Heparinized venous blood samples were processed after overnight refrigeration. The white blood cell–rich buffy coat was harvested after simple centrifugation of the chilled peripheral blood sample at 1200*g* for 10 minutes for rapid isolation of the sample's leukocyte fraction. All antibodies and isotype control antibodies were purchased from BD Biosciences (San Jose, Calif), except anti-CCR2 peridinin-chlorophyll-protein complex (PerCP), anti–α-SMA phycoerythrin (PE; R&D Systems, Minneapolis, Minn), anti-Col1 (Rockland, Gilbertsville, Pa), the TGF-β transducing protein anti–phosphorylated Smad2/3 (p-Smad2/3; Santa Cruz Biotechnology, Santa Cruz, Calif), and anti–phosphorylated AKT (p-AKT) allophycocyanin (APC; Cell Signaling Technology, Danvers, Mass). All the antibodies were purchased conjugated,

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except anti-Col1 and anti–p-Smad2/3. Anti-Col1 and isotype control were conjugated to fluorescein isothiocyanate, and anti–p-Smad2/3 and the isotype control were conjugated to APC by using DyLight Conjugation Kits (Thermo Fisher Scientific, Waltham, Mass). Quantitative FACS analysis was then performed for fibrocytes (defined as $CD45^+Col1^+$ or $CD45^+Col1^+CD34^+$), α-SMA–differentiated fibrocytes ($CD45^+Col1^+$ α-SMA^+), TGF-β–activated fibrocytes ($CD45^+Col1^+$p-$Smad2/3^+$), and p-AKT–activated fibrocytes ($CD45^+Col1^+$p-AKT^+) and fibrocytes expressing chemokines receptors (CXCR4, CCR2, and CCR7). Contaminating red blood cells were then removed, and the cells were washed and brought up to a concentration of 1×10^7/mL in PBS containing 0.1% FBS. The leukocytes were stained for combinations of surface markers by using anti-CD45 AmCyan, anti-CD34 PerCP, anti-CXCR4 APC, anti-CCR2 PerCP, anti-CCR7 PE-Cy7, and the isotype control. Next, the cells were washed and permeabilized with Cytofix/Cytoperm (BD Biosciences) before intracellular staining of anti-ColI fluorescein isothiocyanate, anti–α-SMA PE, anti–p-Smad2/3 APC, or anti–p-AKT APC. Samples were washed, fixed, and read on a FACSCanto II flow cytometer with BD Diva software (BD Biosciences). Flow cytometric gating for quantitation of total fibrocytes and fibrocyte subsets is outlined in Fig E1 in this article's online repository at www.jacionline.org. The inter-assay coefficients of variability for $CD45^+Col1^+$, differentiated $CD45^+Col1^+$ α-SMA^+, and activated $CD45^+Col1^+Smad2/3^+$ fibrocytes were 1.4%, 1.7%, and 2.7%, respectively. The intra-assay coefficients of variability for these fibrocyte subsets were 3.25%, 6.0%, and 3.23%, respectively.

Statistical methods

Data were analyzed with SAS software (SAS Institute, Cary, NC). As we have done before,[23] multiple comparisons of groups were performed by using the false discovery rate procedure.[24] The Mantel-Haenszel χ^2 procedure in SAS software was used to analyze differences between $GINA^{low}$ and $GINA^{high}$ asthmatic patient groups. Correlation of differentiated fibrocytes with activated fibrocytes was evaluated by using the SAS regression procedure.[25] Statistical significance was identified as a P value of less than .05.

RESULTS

The demographics of the control and asthmatic subjects are tabulated in Table E1 in this article's Online Repository at www.jacionline.org. The pool of 15 healthy control subjects ranged in age from 25 to 77 years and consisted of 7 male and 8 female subjects. Study subjects with asthma ranged in age from 7 to 86 years and consisted of 16 male and 24 female subjects. The prebronchodilator FEV_1 percent predicted was significantly decreased based on ATS criteria[12] in 29 of the 40 asthmatic patients (see Table E1). The severity of airflow obstruction varied from mild to moderate in 18 and severe in 11 subjects with a prebronchodilator FEV_1 of less than 50% of predicted values. FEV_1 percent predicted was normal in 11 asthmatic patients. There was evidence of fixed airflow obstruction in 22 of the 40 subjects evidenced by a persistently decreased FEV_1/FVC ratio after bronchodilator. Total serum IgE data were increased in 12 of the 30 subjects with available data (see Table E1).

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Asthmatic patients were grouped by severity of treatment identified by GINA score: 15 patients were in the $GINA^{low}$ group (GINA step 1, 2, or 3), and 25 patients were in the $GINA^{high}$ group (GINA step 4 or 5). There was a significant decrease in prebronchodilator FVC and FEV_1 percent predicted and postbronchodilator FEV_1 percent predicted in the $GINA^{high}$ group compared with the $GINA^{low}$ group (Table I). There was also a significantly lower prebronchodilator and postbronchodilator FEV_1/FVC percentage in the $GINA^{high}$ group compared with the $GINA^{low}$ group. In addition, the percentage increase in FEV_1 after bronchodilator was significantly greater in the $GINA^{high}$ group compared with the $GINA^{low}$ group. Body mass index was significantly increased in the $GINA^{high}$ group compared with the $GINA^{low}$ group, but serum IgE levels, peripheral blood eosinophil counts, and exhaled nitric oxide levels were comparable between the 2 asthmatic groups.

There was no significant difference in the numbers of asthmatic patients with positive skin test results to inhaled allergens in the $GINA^{high}$ versus $GINA^{low}$ groups. There were differences in the number of exacerbations of asthma in the preceding 12 months between the $GINA^{high}$ and $GINA^{low}$ groups (Table I). The daily inhaled corticosteroid (ICS) dose and presence of leukotriene receptor antagonists were comparable between the 2 groups, but significantly more patients in the $GINA^{high}$ group were taking oral corticosteroids (OCSs; Table I). Only 1 asthmatic patient was receiving anti-IgE therapy at the time of the study.

Increased circulating total and differentiated and activated fibrocyte numbers in asthmatic patients

There were low numbers of fibrocytes ($CD45^+Col1^+$) in the peripheral blood of healthy control subjects, but the numbers of total circulating $CD45^+Col1^+$ fibrocytes were significantly increased ($P < .05$) in asthmatic patients (Fig 1). Although the number of $CD45^+Col1^+CD34^+$ fibrocytes was also increased in asthmatic patients compared with that seen in control subjects ($P < .05$), $CD45^+Col1^+CD34^+$ fibrocytes represented less than one third of the numbers of $CD45^+Col1^+$ fibrocytes circulating in the peripheral blood of asthmatic patients (Fig 1). There was an 8-fold significant increase in the number of differentiated $CD45^+Col1^+$ α-SMA^+ fibrocyte phenotypes in the asthmatic patients compared with the control subjects. There were also significantly increased numbers of activation fibrocyte phenotypes $CD45^+Col1^+$p-$Smad2/3^+$ and $CD45^+Col1^+$p-AKT^+ in asthmatic patients compared with control subjects.

Total numbers of $CD45^+Col1^+$ fibrocytes expressing CXCR4, CCR2, or CCR7, irrespective of other chemokine receptor expression, were all significantly increased ($P < .05$) in asthmatic patients compared with those in control subjects (Fig 2), but the predominant chemokine receptor expressed on total circulating fibrocytes in asthmatic patients of the 3 chemokine receptors was CXCR4. The predominant expression of CXCR4 in $CD45^+Col1^+$ fibrocytes was not observed in the $CD45^+Col1^+$ α-SMA^+ cells, with fewer $CD45^+Col1^+$ α-SMA^+ fibrocytes expressing CXCR4 (Fig 2).

Circulating fibrocytes correlate with asthma severity

Total circulating $CD45^+Col1^+$ fibrocyte numbers in asthmatic patients were significantly increased in the $GINA^{high}$ group compared with those in both control subjects and the

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GINA^{low} group (Fig 3, A). Similarly, numbers of CD45^{+}Col1^{+} fibrocytes expressing the chemokine receptor CXCR4 were also significantly increased in the GINA^{high} group compared with those in both control subjects and the GINA^{low} group (Fig 3, B).

Differentiated circulating fibrocytes are associated with asthma exacerbations

Asthmatic patients were grouped into those with or without an asthma exacerbation in the preceding 12 months to determine whether circulating fibrocyte subsets were associated with asthma exacerbations. Numbers of circulating differentiated CD45^{+}Col1^{+} α-SMA^{+} fibrocytes were increased in asthmatic patients experiencing an exacerbation in the preceding 12 months compared with those in control subjects and asthmatic patients without an exacerbation (Fig 4, A). Similarly, numbers of CD45^{+}Col1^{+} α-SMA^{+}CXCR4^{+} fibrocytes in peripheral blood were significantly increased in the asthmatic patients with exacerbations compared with those in both asthmatic patients without exacerbations and control subjects (Fig 4, B).

Differentiated fibrocyte phenotypes associated with activated fibrocyte phenotypes

Because TGF-β can induce α-SMA expression through the SMAD2/3 signaling pathway, cells were stained for p-Smad2/3 in fibrocytes to examine this activation pathway.[26] Because CD45^{+}Col1^{+}CXCR4^{+} fibrocytes were associated with asthma severity based on GINA scores (Fig 3), we examined the correlation between activated fibrocytes and this fibrocyte subset. As depicted in Fig 5, A, regression analysis revealed correlations between CD45^{+}Col1^{+}CXCR4^{+} and CD45^{+}Col1^{+}p-SMAD2/3^{+} in the asthmatic patients (P <.0001, R^2 = .65). We also examined activation of the signaling protein AKT using FACS staining for p-AKT because both platelet-derived growth factor[27] and the chemokine CXCL12 can induce AKTactivation, which can occur as a CXCR4-dependent mechanism.[28] Significant correlation was observed in the asthmatic patients between the numbers of CD45^{+}Col1^{+}CXCR4^{+} fibrocytes and p-AKT^{+} fibrocytes (P < .0001, R^2 = 0.56; Fig 5, B). Correlation was also observed between total numbers of p-Smad2/3^{+} versus p-AKT^{+} fibrocytes (P < .0001, R^2 = 0.62; Fig 5, C), which is consistent with the hypothesis that activation of the Smad and AKT pathways occurs in the same cells.

DISCUSSION

The purpose of this study was to evaluate whether total numbers of activated/differentiated fibrocytes in peripheral blood samples of asthmatic patients were increased in those with severe asthma. Asthmatic patients were found to have higher numbers of circulating CD45^{+}Col1^{+} fibrocytes compared with control subjects and an increase in both p-Smad2/3 or p-AKT activation and α-SMA^{+} differentiation phenotype fibrocytes. Numbers of both total and CXCR4^{+} circulating fibrocytes were significantly increased in patients in the GINA^{high} group compared with those in patients in the GINA^{low} group and control subjects. In addition, numbers of α-SMA^{+} and α-SMA^{+}CXCR4^{+} fibrocytes were significantly increased in asthmatic patients experiencing an exacerbation in the preceding 12 months compared with those in asthmatic patients without an exacerbation and control subjects. Significant correlation was observed between the numbers of CD45^{+}Col1^{+}CXCR4^{+} fibrocytes and fibrocyte expression of the activation proteins p-Smad2/3 and p-AKT.

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These studies expand the existing evidence of circulating fibrocytes in asthmatic patients. Saunders et al[9] reported a significant increase in the number of cultured $CD34^+Col1^+$ fibrocytes in peripheral blood of patients with refractory asthma (1.4×10^4 cells/mL of blood) compared with healthy subjects (0.4×10^4 cells/mL of blood). Wang et al[10] described an increase in $CD34^+CD45^+Col1^+$ cell numbers to $6.4 \pm 2.1 \times 10^4$ cells/mL of blood in patients with chronic obstructive asthma compared with $0.8 \pm 0.2 \times 10^4$ cells/mL of blood in patients with nonobstructive asthma. Significant correlation was found in the patients with chronic obstructive asthma between the number of nonadherent fibrocytes from cultured PBMCs and the decrease in FEV_1 over 5 years.

This study also identifies a correlation of circulating fibrocyte numbers and asthma severity, with the highest numbers of circulating fibrocytes in patients with more severe asthma defined by GINA treatment steps 4 and 5. The present study is the first to identify the increase in activation (p-Smad2/3$^+$ or p-AKT$^+$) and differentiation (α-SMA$^+$) fibrocyte phenotypes in the peripheral blood of asthmatic patients.

There are inherent problems in the original approach used in these previous studies, which assessed fibrocytes in asthmatic patients in that fibrocytes were cultured from PBMCs and selected on the basis of adherence[9,11] or absence of adherence in culture.[10] Although Bellini et al[11] assessed the percentage of $CD34^+Col1^+$ fibrocytes of the total leukocyte fraction in pelleted buffy coat fractions, their evaluation of the responses to cytokine stimulation was performed in fibrocytes selected for adherence at 48 hours of culture and then grown in culture for an additional 72 hours. It is possible that the results of these previous studies underestimated the true number of fibrocyte subsets in peripheral blood or evaluated fibrocytes which phenotypically changed in culture. The yield of $CD34^+CD45^+Col1^+$ cells in patients with chronic obstructive asthma by Wang et al[10] of $6.4 \pm 2.1 \times 10^4$ cells/mL is approximately 1 log lower than the numbers of $4.3 \pm 1.9 \times 10^5$ $CD45^+Col1^+CD34^+$ cells we obtained in the present study using FACS analysis of freshly harvested buffy coats from peripheral blood samples of asthmatic patients in the present study. As shown in Fig 1, the requisite of CD34 positivity in the previous studies underestimates the total number of fibrocytes in the peripheral blood of asthmatic patients by 3-fold. The underestimation of fibrocytes by using CD34 as a requisite marker might be due to diminished expression of CD34 in culture because Phillips et al[19] observed a progressive loss of CD34 with time in culture.

Previous studies have identified increased fibrocyte subsets in the bronchial wall with allergen challenge.[18,29] Schmidt et al[18] found increased accumulation of $CD34^+$ procollagen 1–positive cells in the airways of patients with allergic asthma in endobronchial biopsy specimens at 2, 4, and 24 hours after allergen challenge that was not observed in vehicle control experiments. Fibrocytes localizing to the subepithelial layer near the basement membrane after allergen challenge were in proximity to areas of collagen deposition. At 24 hours after allergen challenge, $CD34^+$ procollagen 1–positive cells represented 15.6% $\pm$ 5.4% of all $CD34^+$ cells. Importantly, additional staining revealed that numbers of $CD34^+$ α-SMA^+ cells in the bronchial mucosa increased after allergen challenge and were increased over those in control subjects treated with vehicle in the absence of allergen.

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We observed correlation between $CXCR4^+$ fibrocytes and expression of the activation signaling proteins p-Smad2/3 and p-AKT. TGF-β induces fibrocyte differentiation with expression of α-SMA. Because TGF-β signals through Smad2/3 and plasma TGF-β levels are increased in asthmatic patients,[10] it is possible that TGF-β contributed to activation and differentiation of these fibrocytes in the circulation of asthmatic patients. It is interesting that p-AKT expression was also associated with α-SMA expression. p-AKT expression can occur through CXCR4 stimulation by CXCL12.[28] It is also possible that p-AKT activation can represent epidermal growth factor receptor (EGFR) transactivation through CXCR4 by the agonist CXCL12 because this has been reported in cancer cells.[27,30] In cultured fibrocytes from peripheral blood samples of asthmatic patients with chronic obstructive asthma, Wang et al[31] observed increased EGFR expression, and EGFR inhibition in these cells attenuated proliferation, including α-SMA expression. p-AKT activation might also have been mediated through platelet-derived growth factor, which has been reported to mediate fibrocyte migration to airway smooth muscle cells in an *in vitro* chemotaxis model.[9] The tightest correlation was observed between fibrocyte expression of p-Smad2/3 and p-AKT, and it is possible that these pathways were activated by a common agonist.

In regard to the question of an effect of corticosteroids on circulating fibrocyte numbers, we found no effect of either ICSs or OCSs on circulating fibrocyte total numbers or numbers of differentiated or activated fibrocyte subsets. An absence of effect might be related to recent *in vitro* observations by Lo et al,[32] who reported the absence of dexamethasone-induced apoptosis on cultured fibrocytes from peripheral blood of patients with severe asthma, which contrasted with the steroid-induced apoptotic effect on fibrocytes cultured from patients with nonsevere asthma and control subjects.

The greater percentage increase in postbronchodilator FEV_1 in $GINA^{high}$ patients could at first glance seem paradoxical because this group had increased numbers of total, differentiated, and activated fibrocytes. However, previous investigators have observed correlation with lower FEV_1/FVC ratios and an accelerated decrease in lung function in asthmatic patients with increased bronchodilator-induced FEV_1 reversibility.[16,33]

The observations in this study identify an increase in fibrocyte subsets in the peripheral blood of asthmatic patients that correlated both with severity of asthma and asthma exacerbations, as well as with an increase in both activation (ie, p-Smad2/3^+ and p-AKT^+) and differentiation (ie, α-SMA^+) fibrocyte subsets. Although asthma has often been viewed as a disease localized to the airways, we speculate that severe asthma is a disease associated with a systemic inflammatory process and that this process can be detected by means of analysis of fibrocyte phenotypes from peripheral blood samples. It is unknown whether these activated and differentiated fibrocytes present in the peripheral blood of asthmatic patients traffic to the airways and contribute to remodeling. In fact, a recent preclinical study in a murine model of bleomycin-induced lung injury suggests that Foxd1 progenitor-derived pericytes and resident fibroblasts in the lung expand into an increased population of lung fibroblasts.[34] Therefore it is possible that increased activation/differentiation fibrocyte phenotypes in peripheral blood of asthmatic patients represent this intrapulmonary event and a systemic inflammatory response rather than trafficking to the airways. Trafficking from the peripheral circulation to the lung aside, circulating fibrocytes might contribute to

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inflammatory events in asthmatic patients through a T_H2 proinflammatory action. Such a role is suggested by Isgro et al,[35] who recently observed that coculturing house dust mite (HDM)–pulsed peripheral blood fibrocytes with HDM-pulsed memory CD4 cells from HDM-sensitized asthmatic patients resulted in enhanced release of the T_H2 cytokines IL-4 and Il-5. The possibility of cross-activation between CD4 cells and fibrocytes was suggested by enhanced formation of α-SMA in the fibrocytes cocultured with HDM-pulsed CD4 cells.

Clearly the relationship of circulating fibrocyte subsets to development of fixed airflow obstruction and decreasing lung function would be enhanced by endobronchial biopsies to quantitate activated and differentiated fibrocytes in the bronchial wall and with serial measurements of lung function over time. This study was cross-sectional, and these measurements will be the focus of future longitudinal studies.

In conclusion, by using FACS analysis on routine clinical samples and without the use of cell selection or culture, this is the first study to identify an increased population of circulating fibrocyte activation/differentiation subsets in the peripheral blood of asthmatic patients. Using CD45 and Col1 as markers rather than CD34 results in identification of a larger pool of fibrocytes than has been previously reported.[9,10] An increase in numbers of $\alpha\text{-SMA}^+\text{CXCR4}^+$ differentiated fibrocytes in patients with asthma exacerbations in the preceding year supports the potential use of fibrocyte subsets as biomarkers for severe asthma. If asthmatic patients at risk for either exacerbations or progression to severe disease were identified, the clinician could be alerted to the necessity of more aggressive therapy earlier in the disease process. In addition, such observations would be a strong impetus to evaluate new and innovative therapeutic approaches to inhibit fibrocyte trafficking and cell-signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	Allophycocyanin
ATS	American Thoracic Society
Col1	Collagen 1

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EGFR	Epithelial growth factor receptor
FACS	Fluorescence-activated cell sorting
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
HDM	House dust mite
ICS	Inhaled corticosteroid
OCS	Oral corticosteroid
p-AKT	Phosphorylated AKT
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
p-SMAD2/3	Phosphorylated SMAD2/3
α-SMA	α-Smooth muscle actin

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Clinical implications

Circulating fibrocyte subsets might represent a novel biomarker for identification of patients at risk for asthma exacerbations, progression to severe asthma, or both.

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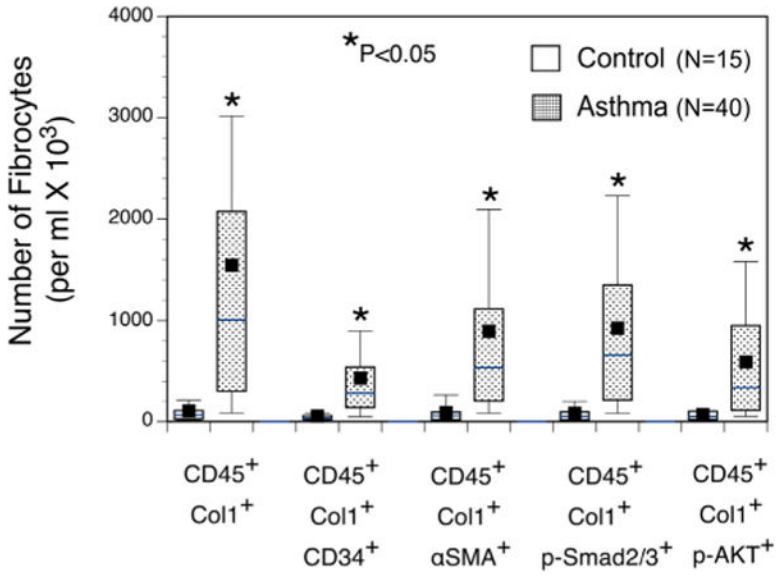

FIG 1.

Increased numbers of total, differentiated, and activated fibrocytes are found in peripheral blood of asthmatic patients. Numbers of $CD45^+Col1^+$, $CD45^+Col1^+CD34^+$, $CD45^+Col1^+$ α-SMA^+, $CD45^+Col1^+$ $Smad2/3^+$, $CD45^+Col1^+$p-$Smad2/3^+$ and $CD45^+Col1^+$p-AKT^+ fibrocytes were increased in asthmatic patients compared with those in control subjects. The *solid box* represents the mean, and the *horizontal line* represents the median for each group. *Significant difference between asthmatic patients and respective control groups, $P < .05$.

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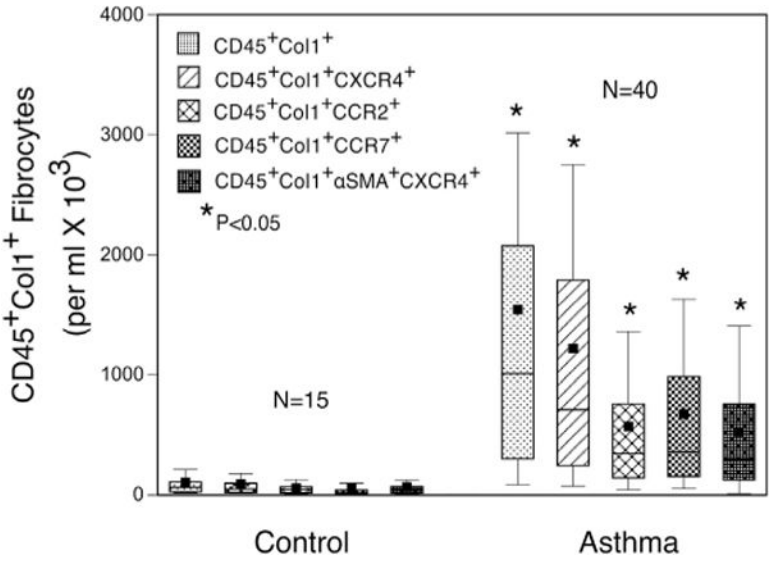

FIG 2.

The predominant chemokine receptor on circulating fibrocytes is CXCR4. The predominant chemokine receptor on peripheral blood $CD45^+Col1^+$ fibrocytes was CXCR4. Numbers of $CD45^+Col1^+CXCR4^+$, $CD45^+Col1^+CCR2^+$, and $CD45^+Col1^+CCR7^+$ fibrocytes were all significantly increased in asthmatic patients to levels greater than in control subjects. There was also expression of CXCR4 on α-SMA^+ fibrocytes. *Significant difference between asthmatic patients and respective control groups, $P < .05$.

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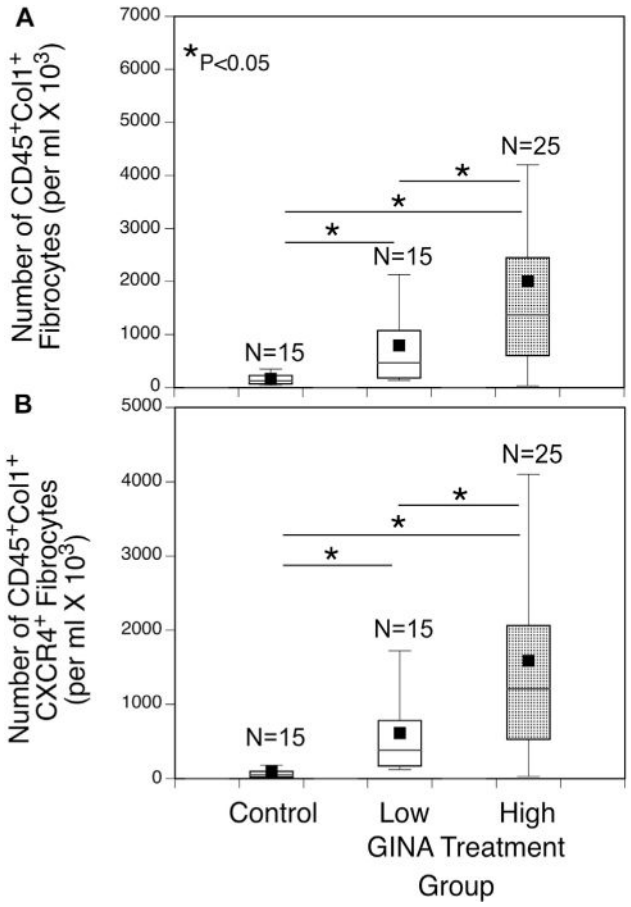

FIG 3.

The concentration of circulating fibrocytes is associated with the increase in asthma severity. Numbers of circulating $CD45^{+}Col1^{+}$ (**A**) and $CD45^{+}Col1^{+}CXCR4^{+}$ (**B**) fibrocytes in the $GINA^{high}$ group (GINA step 4 or 5) were significantly increased compared with those in both control subjects and the $GINA^{low}$ group (GINA steps 1–3). *Significant difference between groups, $P < .05$.

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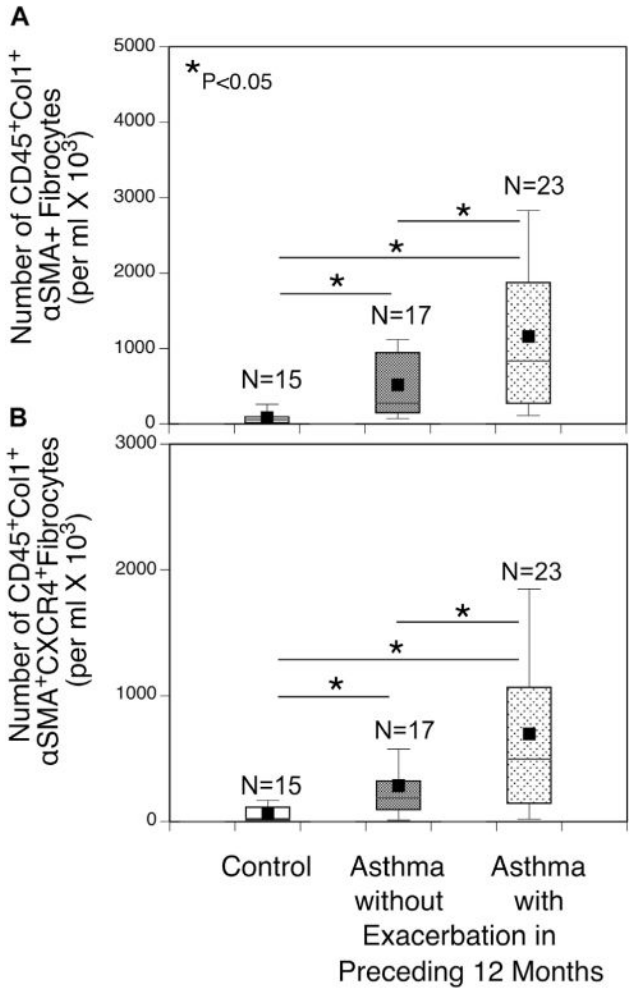

FIG 4.

Increase in differentiated fibrocyte subsets in asthmatic patients with recent exacerbations. Asthmatic patients were divided into those having at least 1 exacerbation in the preceding 12 months and those not having an exacerbation. Numbers of circulating $CD45^+Col1^+$ α-SMA^+ (**A**) and $CD45^+Col1^+$ α-SMA^+CXCR4^+ (**B**) fibrocytes were significantly increased in asthmatic patients with exacerbations to levels greater than those in asthmatic patients without an exacerbation and control subjects. *Significant difference between groups, $P < .05$.

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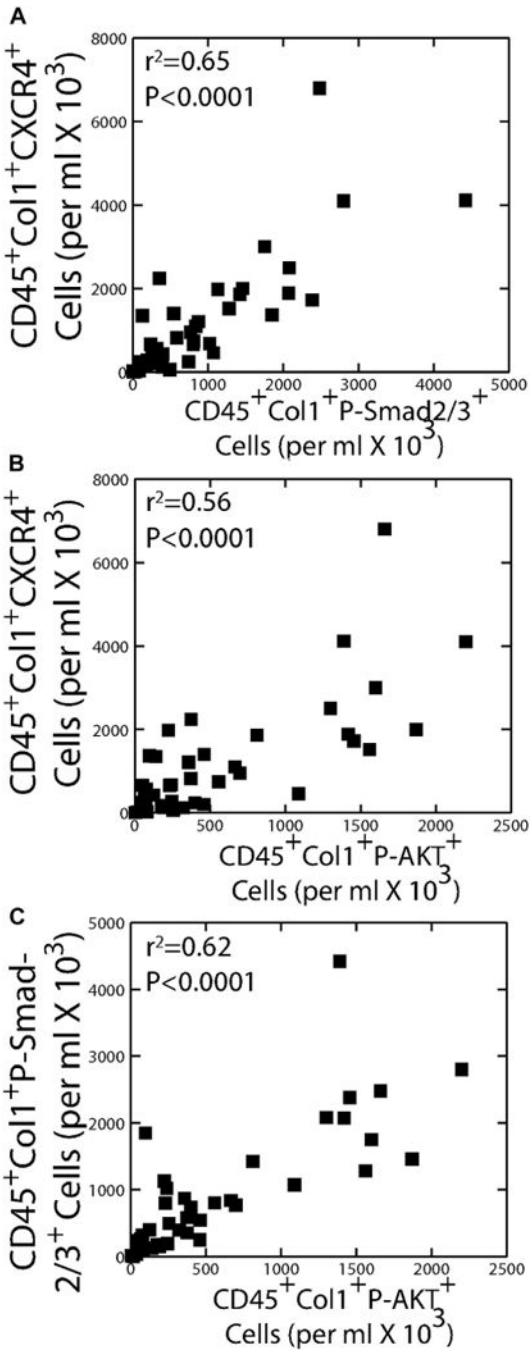

FIG 5.

Correlation between $CD45^+Col1^+CXCR4^+$ fibrocytes and p-Smad2/3$^+$– or p-AKT$^+$–activated fibrocytes in asthmatic patients (n = 40). **A** and **B**, Significant correlation was observed between $CD45^+Col1^+CXCR4^+$ and both $CD45^+Col1^+$p-Smad2/3$^+$ (Fig 5, A) and $CD45^+Col1^+$p-AKT$^+$ (Fig 5, B) fibrocytes. **C**, Correlation was also found between numbers of p-Smad2/3$^+$ $CD45^+Col1^+$ fibrocytes and p-AKT$^+$ $CD45^+Col1^+$ fibrocytes.

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TABLE I

Characteristics of patients in the GINA^{low} and GINA^{high} groups

	Control subjects (n = 15)	GINA^{low} group (GINA steps 1–3 [n = 15])	GINA^{high} group (GINA steps 4 and 5 [n = 25])	*P* value
Age (y)	25–77	7–86	9–79	
Sex (M/F)	7/8	5/10	11/14	
Prebronchodilator				
FVC (L)	4.2 ± 0.03	3.0 ± 0.3 *	2.6 ± 0.2 *	
FVC (% predicted)	100.5 ± 2.9	92.5 ± 4.8	69.5 ± 3.4 *†	
FEV_1 (L)	3.3 ± 0.3	2.1 ± 0.2 *	1.6 ± 0.1 *	
FEV_1 (% predicted)	100.9 ± 3.0	81.2 ± 5.0 *	53.3 ± 3.3 *†	
FEV_1/FVC ratio (%)	79.5 ± 1.5	69.6 ± 2.2 *	52.9 ± 4.4 *†	
Postbronchodilator				
FEV_1 (L)	3.4 ± 0.3	2.4 ± 0.2 *	1.9 ± 0.1 *	
FEV_1 (% predicted)	97.0 ± 7.2	91.9 ± 4.8	66.6 ± 4.0 *†	
FEV_1/FVC ratio (%)	82 ± 1	74 ± 3 *	64 ± 2 *†	
Increase in FEV_1 after bronchodilator (%)	2.5 ± 0.7	15.0 ± 2.3 *	27.3 ± 4.2 *†	
Fixed airflow obstruction (yes/no)	NA	4/11	18/7	<.01
Serum IgE (IU/mL)	56 ± 18	122 ± 59	331 ± 109	
Blood eosinophils (cells/mm^3)	0.14 ± 0.02	0.46 ± 0.08 * (n = 13)	0.66 ± 0.11 * (n = 23)	
FENO (ppb)	NA	18 ± 3 (n = 10)	35 ± 8 (n = 16)	NS
Positive skin test results to inhaled allergens (yes/no)	NA	10/2	17/7	NS
BMI (kg/m^2)	25 ± 1	25 ± 1	31 ± 2 *†	
Asthma exacerbations in prior 12 mo (yes/no)	NA	5/15	18/25	<.05
ICS (μg per 24 h)	NA	426 ± 127	1069 ± 144	NS
OCS (no. of patients)	NA	0/15	10/25	<.01
LTRA (yes/no)	NA	9/6	19/6	NS

BMI, Body mass index; *F*, female; *LTRA*, leukotriene receptor antagonist; *M*, male; *NA*, not applicable; *NS*, not significant.

*Significant difference from control group.

†Significant difference between the GINA^{high} and GINA^{low} groups. Statistical significance in the right column represents Mantel-Haenszel analysis of the GINA^{high} and GINA^{low} groups.