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

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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. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *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

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Abbreviations used
APC: Allophycocyanin
ATS: American Thoracic Society
Col1: Collagen 1
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

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 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, 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 ($CD45^+Col1^+\alpha\text{-}SMA^+$), 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 $CD45^+Col1^+\alpha\text{-}SMA^+$, 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