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Identification of Circulating Fibrocytes as Precursors of Bronchial Myofibroblasts in Asthma¹

Matthias Schmidt,* Guo Sun,* Martin A. Stacey,* Luca Mori,* and Sabrina Mattoli^{2*†}

The mechanisms contributing to airway wall remodeling in asthma are under investigation to identify appropriate therapeutic targets. Bronchial myofibroblasts would represent an important target because they play a crucial role in the genesis of subepithelial fibrosis, a characteristic feature of the remodeling process, but their origin is poorly understood. We hypothesized that they originate from fibrocytes, circulating cells with the unique characteristic of expressing the hemopoietic stem cell Ag CD34 and collagen I. In this study we show that allergen exposure induces the accumulation of fibrocyte-like cells in the bronchial mucosa of patients with allergic asthma. These cells are CD34-positive; express collagen I and α -smooth muscle actin, a marker of myofibroblasts; and localize to areas of collagen deposition below the epithelium. By tracking labeled circulating fibrocytes in a mouse model of allergic asthma, we provide evidence that fibrocytes are indeed recruited into the bronchial tissue following allergen exposure and differentiate into myofibroblasts. We also show that human circulating fibrocytes acquire the myofibroblast phenotype under in vitro stimulation with fibrogenic cytokines that are produced in exaggerated quantities in asthmatic airways. These results indicate that circulating fibrocytes may function as myofibroblast precursors and may contribute to the genesis of subepithelial fibrosis in asthma. *The Journal of Immunology*, 2003, 170: 380–389.

Asthma is characterized by chronic airway inflammation and remodeling of the normal bronchial architecture in response to the inflammatory tissue injury (1–5). The remodeling process has become an important target for therapeutic intervention (3, 5) because it may contribute to the chronic sequelae of asthma (6, 7), leading to permanently impaired pulmonary function and work disability (8).

Subepithelial fibrosis is a distinctive aspect of the remodeling response (3, 5). The thickened lamina reticularis contains an increased amount of collagens I, III, and V; fibronectin; and tenascin that is not present in other airway inflammatory diseases (9–11). These proteins are thought to be produced by myofibroblasts (12–14). It is therefore crucial to elucidate the mechanisms involved in the accumulation of these cells in the airways of asthmatic individuals.

Although it has been proposed that myofibroblasts originate from airway resident cells, particularly tissue fibroblasts or myocytes (15–20), there may be a circulating precursor still unidentified. Previous studies (21, 22) have described a population of circulating cells, termed fibrocytes, that express fibroblast products as well as the hemopoietic stem cell Ag CD34, enter sites of tissue injury, and localize to areas of extracellular matrix deposition. In culture they acquire structural and functional properties (21–23) similar to those described for myofibroblasts (18, 19, 24). We hypothesized that fibrocytes represent the circulating precursors of bronchial myofibroblasts and are involved in the genesis of subepithelial fibrosis in asthma.

This study was designed to provide an answer to the following questions. 1) Is it possible to detect fibrocyte-like cells in the bron-

chial mucosa of asthmatic patients? 2) If so, does the number of fibrocyte-like cells increase during an asthmatic reaction? 3) Do fibrocyte-like cells express any marker of differentiation into myofibroblasts in vivo? 4) Do fibrocyte migrate from the peripheral blood to the bronchial tissue during an asthmatic reaction and differentiate into collagen-producing myofibroblasts once they have migrated at the tissue site? 5) Can circulating fibrocyte differentiate into myofibroblasts when exposed to stimuli that are known to induce myofibroblast accumulation in the airways of asthmatic patients? Issues 1–3 were addressed by examining bronchial mucosal biopsy specimens from patients with allergic asthma, with or without an acute exacerbation of the disease induced by inhalation of the allergen to which they were sensitized. The issue of fibrocyte migration and differentiation was addressed by tracking labeled circulating fibrocytes in a mouse model of allergic asthma and examining their phenotype. The last issue was addressed by investigating the phenotypic changes induced in cultured human circulating fibrocytes upon exposure to fibrogenic cytokines that are released in exaggerated quantities in the airways of asthmatic patients and that are known to contribute to myofibroblast accumulation in asthma.

Materials and Methods

Bronchial biopsy specimens from patients with allergic asthma

To investigate whether fibrocyte accumulation occurs in allergic asthma, we used a model from a previous study (25) that mimics the inflammatory and functional changes of an exacerbation due to natural allergen exposure in allergic asthmatics, the late asthmatic response induced by allergen inhalation in the laboratory (26, 27). The study protocol and the patient characteristics have been reported in detail previously (25). Briefly, allergic asthmatics were randomly allocated into three groups of eight patients each. They received a control inhalation test with the allergen diluent alone, followed by an inhalation test with the allergen to which they were sensitized 4 wk later. In the three groups of patients we performed fiberoptic bronchoscopy at 2, 4, or 24 h, respectively, after the control and allergen inhalation to obtain samples of bronchial mucosa by endobronchial biopsies. The study protocol was approved by the institutional review board, and patients gave written informed consent. Since we still had frozen tissue specimens available for the detection of fibrocyte-like cells in these groups of well-characterized asthmatic patients, we did not consider

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it ethical to perform an allergen inhalation test and invasive procedures in other allergic asthmatics.

Detection of fibrocytes in the bronchial mucosa of patients with allergic asthma

One of the unique characteristics of circulating fibrocytes is to express both CD34 and collagen I (21, 22). Therefore, these cells can be identified in tissue samples by double immunohistochemistry and in situ hybridization technique using a specific Ab against CD34 and a probe for procollagen I mRNA. For this analysis we prepared serial cryosections (5 μm) of each frozen bronchial tissue available from our previous study (25). Double immunohistochemistry and in situ hybridization were performed sequentially on the same sections. Tissue sections were first incubated with a mouse anti-human CD34 mAb (Accurate Chemical and Scientific, Westbury, NY). Binding sites were revealed using an anti-mouse secondary Ab and the alkaline phosphatase/anti-alkaline phosphatase technique with a chromogenic substrate that stains positive cells red (all from Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. In situ hybridization was performed on the same sections using a synthetic oligodeoxynucleotide probe for the human $\alpha 1(\text{I})$ procollagen (5'-CTG TACGCAGGTGATGGTGGG-3') complementary to the initial 22 nt of exon 2 of human $\alpha 1(\text{I})$ procollagen mRNA (28). This antisense probe and the sense probe were 3' end-labeled with digoxigenin-11-deoxyuridine triphosphate (Roche, Mannheim, Germany) using TdT (Roche). Sections were permeabilized with Triton X-100 and proteinase K solution (1 $\mu\text{g}/\text{ml}$) in 0.1 M TBS containing 50 mM EDTA for 20 min at 37°C. Sections were subsequently incubated with 0.1 M triethanolamine and 0.5% acetic anhydride for 20 min at 37°C to prevent nonspecific binding of the labeled probe. Hybridization was performed by incubating sections with 50 μl of the hybridization mixture containing 300 $\text{pg}/\mu\text{l}$ of the labeled probe at room temperature overnight in a moist chamber as previously described (28). Following hybridization, sections were washed twice with $2\times$ SSC for 60 min each time at room temperature, with $0.1\times$ SSC for 30 min at 50°C, and twice with $0.1\times$ SSC for 60 min each time at room temperature. Sections were then washed in TBS and covered with blocking solution (TBS containing 0.1% Triton X-100 and 1% normal sheep serum; all from Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. At completion of this procedure, sections were incubated sequentially with a mouse anti-digoxin mAb (1/10,000; Sigma-Aldrich), a biotinylated anti-mouse F(ab')₂ (1/200; DAKO, Glostrup, Denmark), and avidin conjugated with alkaline phosphatase (1/100; DAKO). Following each of the first two steps, sections were washed twice with TBS for 20 min each time at room temperature. The probe-mRNA hybrids were visualized by incubation with a solution containing 0.4 mg/ml nitro blue tetrazolium, 0.19 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt, 100 mM TBS (pH 9.5), 50 mM MgSO₄, and 1 mM levamisole (Roche and Sigma-Aldrich). In this way cells expressing the procollagen I mRNA were stained dark blue. Omission or substitution of the primary mAb with an irrelevant, isotype-matched mAb was used as a negative control for immunohistochemistry. To evaluate probe specificity and to validate the in situ hybridization procedure, the following control experiments were conducted: 1) evaluation of the RNA binding specificity: sections were pretreated with 100 $\mu\text{g}/\text{ml}$ RNase A (Roche) in Tris-HCl (pH 7.5) for 1 h at room temperature before incubation with the labeled probe; 2) assessment of specific vs nonspecific binding: sections were incubated with the labeled probe in the presence of a 100-fold excess concentration of unlabeled probe; and 3) evaluation of the sequence specificity: sections were incubated with the labeled sense probe.

The expression of α -smooth muscle actin (α -SMA)³ is a marker of differentiation into myofibroblasts (17, 18, 20), and fibrocytes are the only cell population known to express both CD34 and α -SMA (21–23, 29). To detect fibrocytes that had undergone local differentiation into myofibroblast-like cells, adjacent sections were therefore double labeled with the anti-CD34 mAb indicated above and with an anti α -SMA mAb (DAKO). Binding of the anti-CD34 mAb was revealed as reported above, while binding of the anti- α -SMA mAb was visualized by an enzyme-linked indirect immunoperoxidase method (25, 30, 31) using a chromogenic substrate that labels positive cells blue (Fast Blue, Vector Laboratories). Omission or substitution of each primary mAb with an irrelevant, isotype-matched mAb from the same species was used as a negative control.

To localize fibrocytes in relation to areas of collagen deposition, adjacent tissue sections were stained with a mouse anti-human collagen I mAb (Chemicon, Temecula, CA). Binding sites were revealed by the enzyme-

linked indirect immunoperoxidase method using 3,3'-diaminobenzidine as chromogenic substrate (25, 30, 31). Omission or substitution of the primary mAb with an irrelevant, isotype-matched mouse mAb was used as a negative control.

Animal model of allergic asthma

Some studies (reviewed in Ref. 32) have demonstrated that long term exposure of BALB/c mice to low mass concentrations of allergens elicits specific and chronic inflammatory changes in the airways and remodeling of the bronchial wall similar to those observed in chronic asthma. We reasoned that once mice have developed these chronic lesions, it is possible to investigate the alterations occurring during an acute inflammatory reaction induced by any subsequent allergen exposure. This situation would closely mimic the acute exacerbation of the disease that can be induced in patients with chronic asthma by inhalation of the allergen to which they are sensitized. Therefore, we developed a mouse model of recurrent exposure to low allergen concentrations over the long term in an attempt to reproduce the conditions under which our asthmatic patients were tested.

BALB/c mice were systemically immunized by two i.p. injections of 50 μg of OVA (Sigma-Aldrich) conjugated to aluminum potassium sulfate in 0.9% sterile saline at an interval of 14 days. Sensitized mice were challenged with an aerosolized solution of 2.5% OVA in PBS in a whole body inhalation chamber (aerosol concentration, 10–15 mg/m^3 ; median particle diameter, 3.6 μm) for 20 min three times a week at an interval of 24 h over a period of 8 wk. Control mice were exposed to the OVA vehicle alone (PBS). Response was assessed every week for the first 2 wk and every 2 wk thereafter 24 h after the last OVA or PBS exposure. Animals were sacrificed, and lungs were removed for tissue examination. All experiments were performed in compliance with institutional guidelines that conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the institutional review board.

Examination of mouse airway wall sections

The trachea and left lung lobe were snap-frozen in melting isopentane and stored at -25°C until preparation of cryostat sections. Congo Red stain was used for specific detection of eosinophils (33). To evaluate fibronectin and collagen I deposition, airway wall sections were probed with a rabbit anti-mouse fibronectin IgG fraction or with a rabbit anti-mouse collagen I IgG fraction (both from Accurate Chemical and Scientific). By using the enzyme-linked indirect immunoperoxidase method and 3,3'-diaminobenzidine as chromogenic substrate (Vector Laboratories) (25, 30, 31), Ab-binding sites were stained brown. Omission or substitution of each primary Ab with normal rabbit serum was used as a negative control. Deposition of fibronectin and collagen I below the basement membrane (BM) of the epithelium was examined by light microscopy and was quantified using an image analysis system (Leica Microsystems, Wetzlar, Germany). The intensity of illumination in the image of a stained section was digitized into pixels. Each pixel was divided into the color components: hue, saturation, and intensity. The threshold for each color component for the brown stain of fibronectin and collagen I was defined and kept constant in the analysis. In different fields along the BM, the image analysis system determined the area covered by the brown stain and calculated its value. The area was expressed as square micrometers per length of BM in micrometers. For each group of animals tested, fibronectin and collagen I deposition was measured in three sections of the left main bronchus, and results were averaged.

To detect the presence of fibrocyte-like cells, airway wall sections were processed to reveal cells coexpressing CD34 and procollagen I or CD34 and α -SMA. After sequential immersion in 0.1% glycine/PBS and in 15% FBS/PBS (Vector Laboratories), sections were incubated overnight at 4°C with an anti-CD34 mAb (BD Pharmingen, San Diego, CA), washed repeatedly, and then incubated for 1 h at room temperature with a cyanine Cy2-conjugated secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) according to the manufacturer's instructions. The second single staining for procollagen I was conducted using an anti-procollagen I polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or an α -SMA mAb (Sigma-Aldrich) and a Rhodamine Red-X-conjugated secondary Ab (Jackson ImmunoResearch Laboratories) following the procedure indicated above. The nuclei were stained with 4',6-diamidino-2-phenylindole (Vector Laboratories) according to the manufacturer's instructions. Control staining was performed omitting each primary Ab and using any possible combinations of each primary Ab and both secondary Abs. Mounted sections were analyzed by confocal laser scanning microscopy (MRC-1000; Bio-Rad, Hercules, CA). The fluorescent images were processed and overlaid using Photoshop 5.0 software (Adobe Systems, Mountain View, CA).

³ Abbreviations used in this paper: α -SMA, α -smooth muscle actin; BM, basement membrane; BrdU, bromodeoxyuridine; ET, endothelin; SFM, serum-free medium.

Isolation of circulating fibrocytes

Fibrocytes were isolated from mouse and human PBMC by immunomagnetic depletion of contaminating cells following a published method (23). Cell purity was verified by flow cytometry using both PE-conjugated anti-CD34 (BD PharMingen) and FITC-conjugated anti-collagen I (Chemicon) Abs as described previously (23). The results of preliminary experiments had indicated that these human and mouse CD34- and collagen I-positive cells were also CD11b⁺/CD13⁺/CD45RO⁺/HLA-DR⁺/CD14⁻/CD25⁻ (Fig. 1), as previously reported for human and mouse circulating fibrocytes (21).

We were able to isolate $\sim 0.9\text{--}4.8 \times 10^4$ and $1.2\text{--}5 \times 10^4$ pure fibrocytes/ml of mouse and human blood, respectively.

Tracking and phenotypic evaluation of labeled circulating fibrocytes in mice

The dye PKH-26 has been used repeatedly as a viable red fluorescent membrane stain to track the migration and proliferation of hemopoietic stem cells and mature leukocytes *in vivo* (34–37) and to study the *in vivo* chemotactic response of circulating fibrocytes to chemokines (29). The dye shows little or no toxicity, does not seem to interfere with the phenotype and metabolic activity of labeled cells, and can be used to track cell migration for weeks to months (34–37). Since cellular fluorescence halves following each cell division, the reduction in fluorescence can be readily quantified by flow cytometry to assess cell proliferation (34, 35, 37). We therefore used this membrane-inserting fluorescent dye to track circulating fibrocytes in our mouse model of allergic asthma.

Mouse fibrocytes purified from the peripheral blood were stained with PKH-26 (Sigma-Aldrich) according to the manufacturer's recommendation. Labeling efficiency and viability of cells were checked by flow cytometry and by the trypan blue exclusion method, respectively, and both were >80%. Labeled fibrocytes were injected into the tail vein of BALB/c mice (5×10^5 cells in 100 μ l of PBS) that had been repeatedly exposed to OVA or PBS for 4 wk, immediately before the subsequent OVA or PBS aerosolization at 6 wk.

In some groups of mice we evaluated the phenotype of injected labeled fibrocytes at 2, 4, 12, or 24 h after OVA or PBS exposure. Peripheral blood was obtained by retro-orbital bleed before the animals were sacrificed, and PKH-26-positive cells were sorted by flow cytometry for further processing as reported below.

In other groups of mice we evaluated the recruitment of labeled fibrocytes to the airway wall and examined the phenotype of these cells at the

tissue site. At 24 h after OVA or PBS exposure animals were sacrificed, and the lungs were removed for tissue examination. The trachea and the left main bronchus were dissected and cut into pieces under a surgical dissecting microscope. Some airway tissue samples were snap-frozen in melting isopentane and stored at -25°C until preparation of cryostat sections. Other tissue samples were chopped into small fragments and subjected to proteolytic digestion by incubation in serum-enriched medium containing 2 mg/ml collagenase and 20 μ g/ml DNase I (Sigma-Aldrich) for 1 h at 37°C . The recruitment of labeled fibrocytes to the tissue sites was evaluated by microscopic analysis of frozen tissue sections and flow cytometry of the single-cell suspensions obtained after proteolytic digestion of airway tissue. Enumeration of fluorescent fibrocytes by flow cytometry was performed using Coulter beads (Hialeah, FL) for calibration (37).

The phenotype of labeled fibrocytes in the peripheral blood and the single-cell suspension obtained after proteolytic digestion of airway tissue was examined by analysis of the expression of CD34, collagen I, and α -SMA. After sorting PKH-26-labeled fibrocytes using a FACS (Coulter) with an argon laser tuned at 488, cells were washed twice in PBS containing 3% BSA and 0.1% sodium azide (FACS buffer) and incubated with FITC-conjugated anti-CD34 Ab (BD Biosciences, San Jose, CA), FITC-conjugated anti-collagen I Ab (Chemicon), or FITC-conjugated anti- α -SMA Ab (Sigma-Aldrich) for 10 min in the dark. Cells were washed twice in FACS buffer and analyzed immediately in a FACSCalibur flow cytometer (BD Biosciences) for the expression of CD34, collagen I, and α -SMA vs PKH-26 fluorescence intensity. Cells incubated with irrelevant isotype-matched Abs and unstained cells were used as controls. About 5000 cells were analyzed per condition.

Proliferation and differentiation of human fibrocytes

Human fibrocytes were seeded into the wells of 24-well plates (Corning Costar, Cambridge, MA; 5×10^4 cells/ml), and incubated in DMEM (Life Technologies, Paisley, U.K.) containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) for 12 h at 37°C . The medium was then replaced by Ultraculture serum-free medium (SFM; BioWhittaker, Wokingham, U.K.), and cells were reincubated for the indicated periods of time in the absence or the presence of either endothelin-1 (ET-1; Peninsula Laboratories, San Carlos, CA) or TGF- β 1 (PeproTech, London, U.K.).

Cell proliferation was assessed using the incorporation of bromodeoxyuridine (BrdU; Roche) into DNA as a growth index. BrdU (100 μ M) was added to the cultures for the last 2 h of each incubation period. Immunofluorescent labeling of the resuspended cells was performed with a mouse anti-BrdU FITC-conjugated mAb (Chemicon), and the proportion of labeled fibrocytes was estimated by flow cytometry.

To evaluate the expression of α -SMA as a marker of differentiation into myofibroblasts (17, 18, 20), cells cultured for the indicated periods of time in the absence or the presence of either ET-1 or TGF- β 1 were resuspended and incubated with an FITC-conjugated Ab against α -SMA (Sigma-Aldrich). The proportion of labeled fibrocytes was estimated by flow cytometry using Coulter beads for calibration (37). We also examined the changes in the expression of CD34 in relation to the expression of collagen I and α -SMA. Cultured fibrocytes were double labeled with the PE-conjugated anti-CD34 and the FITC-conjugated anti-collagen I or with the PE-conjugated anti-CD34 and the FITC-conjugated anti- α -SMA and analyzed in a FACSCalibur flow cytometer. Cells labeled with irrelevant PE- or FITC-conjugated isotype-matched Abs and unlabeled cells were analyzed as controls.

The amount of fibronectin released to the culture medium was measured at the indicated time points by ELISA as previously described (38). Analysis of collagen III release was performed by a sandwich ELISA. Nunc-immuno ELISA plates (Nunc, Roskilde, Denmark) were coated with a mouse anti-human collagen III capture mAb (Accurate Chemical and Scientific; 10 μ g/ml) in a coating buffer (0.6 M NaCl, 0.08 M NaOH, and 0.26 M H_3BO_4 , pH 9.6) for 16 h at 4°C . The unbound mAb was washed away, and the plates were incubated with PBS containing 2% BSA for 2 h at room temperature. The plates were then washed with PBS containing 0.05% Tween 20. Samples of the culture medium were added, and the plates were incubated again for 2 h at room temperature. After washing, a biotinylated goat anti-human collagen III-purified IgG (Accurate Chemical and Scientific) was added, and the plates were reincubated for 2 h at room temperature. Immunodetection was obtained using a streptavidin-HRP system with *O*-phenylenediamine as the chromogen substrate, according to the manufacturer's instruction (Vector Laboratories). Optical readings were made with an ELISA plate reader (38). Human collagen III protein (Accurate Chemical and Scientific) was used to generate the standard curves for calculation of the collagen III concentration in each sample of culture medium. The detection limit of this assay was consistently 2 ng/ml.

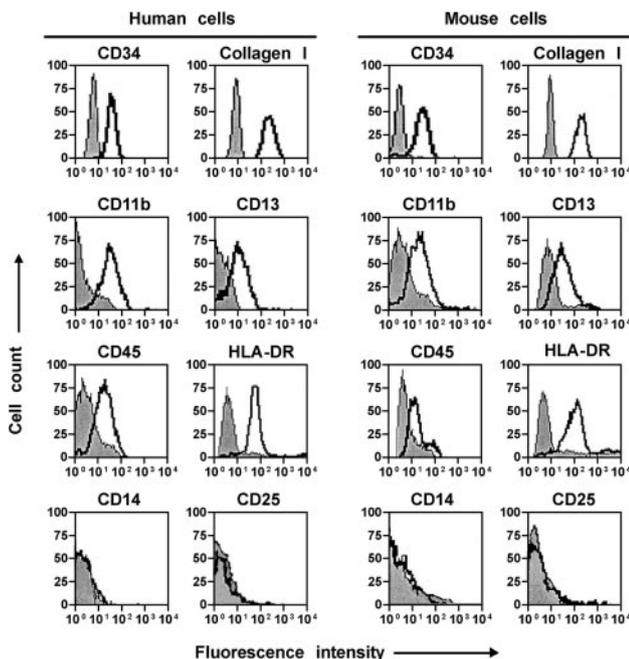


FIGURE 1. Cytofluorometric analysis of the phenotype of human and mouse fibrocytes freshly isolated from the peripheral blood. Each histogram shows staining with the FITC- or PE-conjugated mAb specific for the indicated marker (solid line) and the background nonspecific staining with the irrelevant isotype-matched control (shaded area). For each marker, representative data from 1 of 5–10 preliminary experiments are reported.

For morphological evaluation, fibrocytes were seeded onto polycarbonate filters placed into the wells of 24-well plates (39) and exposed to either ET-1 or TGF- β 1 in Ultraculture SFM as indicated above. At the indicated time points, filters with the cell monolayers were removed and processed for electron microscopic analysis according to our published method (39).

Statistical analysis

Data were compared with the Kruskal-Wallis test and the Mann-Whitney *U* test as appropriate. A value of $p < 0.05$ was accepted as statistically significant. Results are reported as the mean \pm SE.

Results

Detection of fibrocyte-like cells in the bronchial mucosa of asthmatic patients

In the bronchial mucosa of asthmatic patients there were cells expressing both CD34 Ag and procollagen I mRNA, and the number of these cells markedly increased between 4 and 24 h after allergen

exposure (Fig. 2, A–D). The majority of double-labeled cells were detected below the epithelium (Fig. 2A) and appeared to be located near the area of collagen I deposition under the BM (Fig. 2, A and E). At 24 h after allergen exposure, double-labeled cells represented $15.6 \pm 5.4\%$ of all CD34-positive cells, excluding vessels, and $42.6 \pm 9.6\%$ of all cells expressing procollagen I mRNA.

In the bronchial mucosa of asthmatic patients there were also a few cells expressing both CD34 and α -SMA (Fig. 2G). Their number increased dramatically at 24 h after allergen exposure (Fig. 2, F and G), when these cells represented $39.8 \pm 8.1\%$ of all cells expressing α -SMA, excluding vessels and smooth muscle cells. CD34⁺/ α -SMA⁺ cells also expressed procollagen I mRNA, as assessed by comparison with adjacent tissue sections double labeled with the anti-CD34 mAb and procollagen I mRNA probe. At 24 h postallergen exposure these cells represented $46.9 \pm 7.8\%$ of those coexpressing CD34 and procollagen I mRNA.

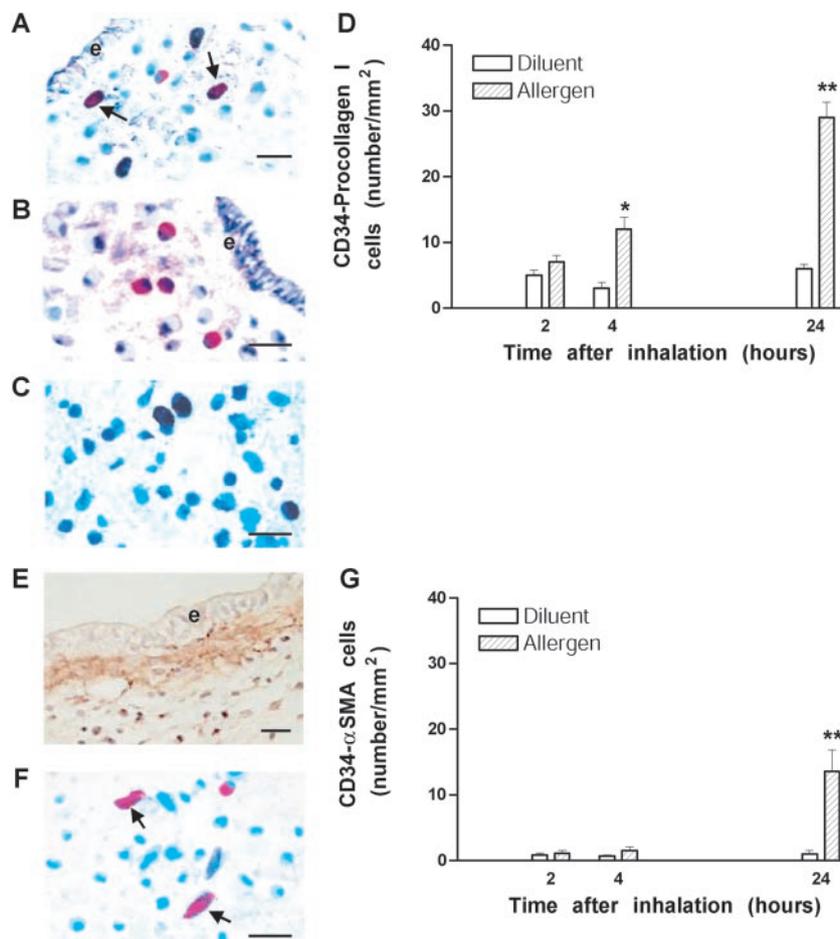


FIGURE 2. Detection of fibrocyte-like cells in the bronchial mucosa of patients with allergic asthma at the indicated time points following the inhalation of the clinically relevant allergen or the allergen diluent alone, and localization of these cells to areas of collagen I deposition. A–C, Adjacent cryostat sections of a bronchial biopsy sample obtained in a representative patient at 24 h following allergen inhalation and labeled with an anti-CD34 mAb (red staining) and with the antisense probe for human procollagen I mRNA (dark blue staining; A), with the anti-CD34 Ab and the sense probe for human procollagen I mRNA (B), or with an irrelevant mAb and the antisense probe for human procollagen I mRNA (C). Arrows in A point to cells double-stained with the anti-CD34 mAb and the antisense probe for procollagen I mRNA (pink-blue staining). D, Counts of cells double-stained with the anti-CD34 mAb and the antisense probe for procollagen I mRNA. Data are expressed as the mean number of cells per square millimeter of bronchial mucosa \pm SE. *, $p < 0.05$; **, $p < 0.025$ (compared with the tissue sections from patients exposed to the allergen diluent alone); $n = 8$ patients/group). E, Cryostat section of a bronchial biopsy sample obtained in a representative patient and 24 h following allergen inhalation and labeled with an anti-collagen I mAb (brown staining). F, Cryostat section of a bronchial biopsy sample obtained in a representative patient at 24 h following allergen inhalation and labeled with an anti-CD34 mAb (red staining) and an anti- α -SMA mAb (blue staining). Arrows point to cells double-stained with the anti-CD34 and anti- α -SMA Abs (red-blue staining). G, Counts of cells double-labeled with the anti-CD34 and anti- α -SMA mAbs. Data are expressed as the mean number of cells per square millimeters of bronchial mucosa \pm SE. **, $p < 0.025$ compared with the tissue sections from patients exposed to the allergen diluent alone ($n = 8$ patients/group). e, airway epithelium. Scale bar = 50 μ m.

Detection of fibrocyte-like cells in a mouse model of allergic asthma

We systemically sensitized BALB/c mice to OVA and exposed them to low concentrations of aerosolized Ag for 20 min three times a week, 24 h apart, over a period of 8 wk. Systemic sensitization effectively induced high initial titers of serum anti-OVA IgE (mean \pm SE, 1120 \pm 430 ng/ml), which increased >4-fold following OVA aerosolization. Repeated OVA exposures elicited inflammatory and structural abnormalities of the airways comparable to those of human asthma (5), including recruitment of eosinophils into the lamina propria (Fig. 3, A–C) and thickening of the subepithelial zone with deposition of fibronectin (Fig. 3, D–F) and collagen I (Fig. 3, G–I). Eosinophil infiltration within and below the airway epithelium was evident after 2 wk of OVA exposure and became widespread thereafter (Fig. 3C), while significant fibronectin and collagen deposition appeared between 4 and 6 wk (Fig. 3, F and D). Similar changes were not present in the airway wall of control mice repeatedly exposed to the OVA vehicle alone (PBS; Fig. 3, C, F, and D).

Repeated Ag exposures were also associated with the accumulation in the airway wall of cells expressing both CD34 and procollagen I (Fig. 4, A–C) and both CD34 and α -SMA (Fig. 4, D and E). Similar changes were not present in the airway wall of mice exposed to PBS (Fig. 4, C and E). At 6 wk of repeated exposure to OVA, many CD34⁺/procollagen I⁺ cells could be localized below the epithelium (Fig. 4A) in the area of collagen I deposition (Fig. 3G) and represented 32.3 \pm 11.9% of all cells expressing

procollagen I. At this time point, 36.2 \pm 9.2% of the CD34⁺/procollagen I⁺ cells also expressed α -SMA, as assessed by comparison with adjacent tissue sections double-labeled with the anti-CD34 mAb and α -SMA mAb. At 8 wk of repeated exposure to OVA, the proportion of CD34⁺/procollagen I⁺ cells that also expressed α -SMA became 44.9 \pm 10.4%. At this time point, the CD34⁺/ α -SMA⁺ cells represented 31.4 \pm 6.5% of all cells expressing α -SMA, excluding vessels and smooth muscle cells.

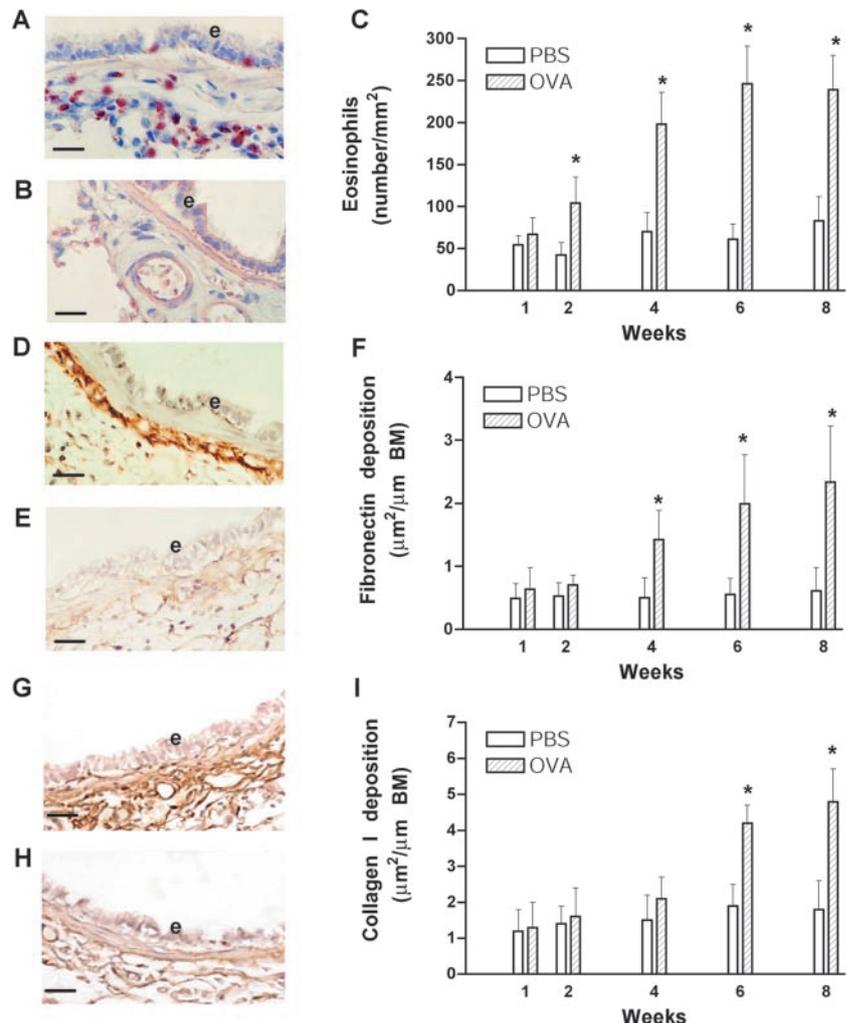
Tracking and phenotypic evaluation of labeled circulating fibrocytes in mice

We isolated circulating fibrocytes from the mouse peripheral blood as a >95% pure population of cells coexpressing collagen I and CD34 (Fig. 5A). Only a minority of these cells expressed α -SMA (Fig. 5B).

Next, we stained the isolated mouse fibrocytes with the fluorescent red dye PKH-26, injected labeled cells into the tail vein of BALB/c mice chronically exposed to OVA or PBS for 4 wk immediately before the next OVA or PBS aerosolization at 6 wk, and tracked fluorescent fibrocytes in the peripheral blood and in the airway wall.

Two hours after OVA (Fig. 5, C–E) and PBS (Fig. 5, F–H) exposure, the phenotype of PKH-26-labeled cells in the peripheral blood was similar to that of freshly isolated and unlabeled cells (Fig. 5, A and B). More than 95% of labeled cells expressed CD34 and collagen I, and only a few cells expressed α -SMA, without

FIGURE 3. Inflammatory and structural abnormalities in the airway wall of mice repeatedly exposed to aerosolized OVA or to aerosolized OVA vehicle alone (PBS). Different groups of five or six mice exposed to OVA or PBS were tested at each of the indicated time-points. *A*, Accumulation of eosinophils (red cells) in the lamina propria and within the airway epithelium in a mouse exposed to OVA for 2 wk. *B*, Absence of eosinophil infiltration in the lamina propria and epithelium of a control mouse exposed to PBS for a similar period of time. *C*, Counts of eosinophils. Data are expressed as the mean number of cells per square millimeter of airway tissue \pm SE. *, $p < 0.05$ compared with the airway tissue from control mice exposed to PBS. *D*, Thickening of the subepithelial zone with deposition of fibronectin (brown staining) in the airway wall of a mouse exposed to OVA for 6 wk. *E*, Absence of a similar thickening of the subepithelial zone and fibronectin deposition in the airway wall of a mouse exposed to OVA for only 2 wk. *F*, Quantification of fibronectin deposition. Data are expressed as the mean area of fibronectin staining (square micrometers) per length of epithelial BM (micrometers) \pm SE. *, $p < 0.05$ compared with the airway tissue from control mice exposed to PBS. *G*, Deposition of collagen I (brown staining) in the airway wall of a mouse exposed to OVA for 6 wk. *H*, Absence of a similar collagen I deposition in the airway wall of a mouse exposed to OVA for 4 wk. *F*, Quantification of collagen I deposition. Data are expressed as the mean area of collagen I staining (square micrometers) per length of epithelial BM (micrometers) \pm SE. *, $p < 0.05$ compared with the airway tissue from control mice exposed to PBS. *e*, airway epithelium. Scale bar = 50 μ m.



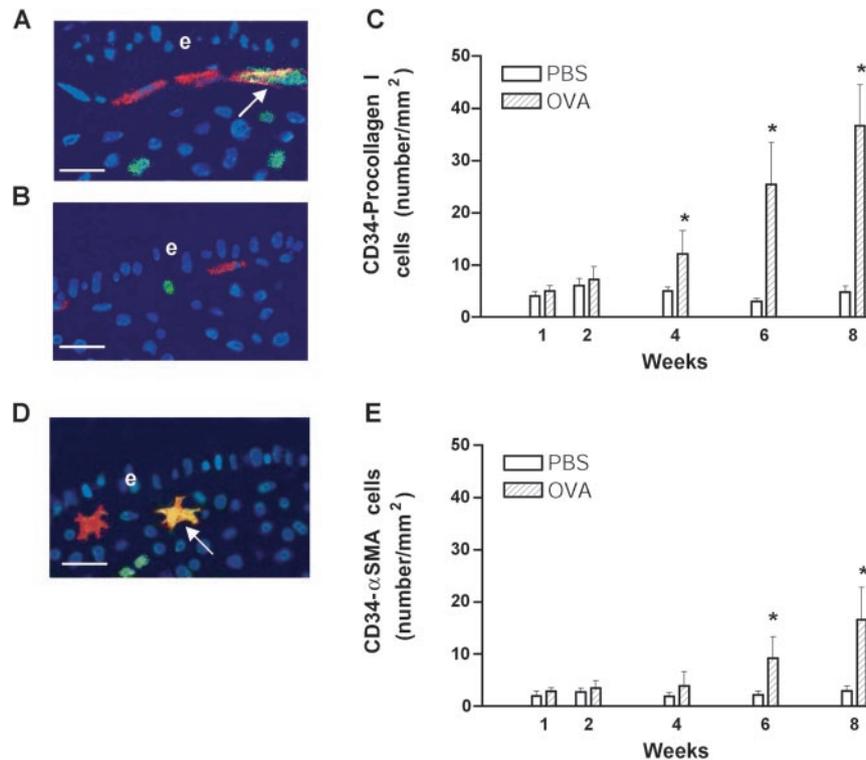


FIGURE 4. Detection of fibrocyte-like cells in the airway wall of mice repeatedly exposed to aerosolized OVA or to aerosolized OVA vehicle alone (PBS). Different groups of five or seven mice exposed to OVA or PBS were tested at each of the indicated time-points. *A* and *B*, Immunofluorescent staining with Abs against CD34 (green) and mouse procollagen I (red) of airway wall sections from representative mice repeatedly exposed to OVA (*A*) or PBS (*B*) for 6 wk. Cell nuclei were stained blue using 4',6-diamidino-2-phenylindole. The arrow in *A* points to a cell double-stained with the anti-CD34 and anti-procollagen I (yellow). *e*, airway epithelium. Scale bar = 50 μ m. *C*, Count of cells double-stained with the anti-CD34 and anti-procollagen I. Data are expressed as the mean number of cells per square millimeter of airway tissue \pm SE. *, $p < 0.05$ compared with the airway wall from control mice exposed to PBS. *D*, Immunofluorescent staining with Abs against CD34 (green) and α -SMA (red) of the airway wall section from a representative mouse repeatedly exposed to OVA for 6 wk. Cell nuclei were stained blue using 4',6-diamidino-2-phenylindole. The arrow in *D* points to a cell double-stained with the anti-CD34 and anti- α -SMA (yellow). *e*, airway epithelium. Scale bar = 50 μ m. *E*, Count of cells double-stained with the anti-CD34 and anti- α -SMA. Data are expressed as the mean number of cells per square millimeter of airway tissue \pm SE. *, $p < 0.05$ compared with the airway wall from control mice exposed to PBS.

differences between mice exposed to OVA or PBS. At any subsequent time point after OVA and PBS exposure (4, 12, and 24 h), the yield of PKH-26-positive cells from peripheral blood was too low to perform any analysis, indicating that most of the labeled fibrocytes had disappeared from the circulation.

By microscopic analysis of airway tissue sections at 24 h following OVA or PBS aerosolization, we found numerous fluorescent cells in the airway wall of animals that had been exposed to the allergen (Fig. 6, *A* and *B*), while there were only a few fluorescent cells in the airway wall of mice exposed to PBS. In OVA-challenged mice most labeled fibrocytes appeared to be located in the subepithelial area (Fig. 6, *A* and *B*) in the zone where we had detected collagen I deposition in another group of animals subjected to chronic OVA exposure for a similar period of time (Fig. 3, *G* and *I*).

To quantify fibrocyte migration to the lung, we prepared single-cell suspensions from airway tissue fragments and enumerated labeled fibrocytes by flow cytometry. This procedure confirmed the results of the microscopic analysis and showed a highly significant increase in the number of labeled fibrocytes in the airway tissue of mice exposed to OVA compared with mice exposed to PBS alone (Fig. 6C). Analysis of the phenotype of PKH-26-labeled cells showed a decrease in the expression of CD34 and an increase in the expression of collagen I and α -SMA in the cells recovered from the airway tissue of mice exposed to OVA (Fig. 6, *D*–*F*) compared with the cells recovered from the peripheral blood 2 h

after OVA exposure (Fig. 5, *C*–*E*). The intensity of the PKH-26 fluorescence was slightly reduced, suggesting that a minority of the cells had divided. The phenotype of the cells recovered for the airway tissue of mice exposed to PBS (Fig. 6, *G*–*I*) was similar to that of labeled cells recovered from the peripheral blood 2 h after PBS exposure (Fig. 5, *F*–*H*), and the intensity of the PKH-26 fluorescence was virtually unchanged.

Proliferation and differentiation of human fibrocytes

We examined whether circulating fibrocytes can proliferate and differentiate into myofibroblast-like cells under stimulation with the fibrogenic cytokines ET-1 and TGF- β 1. Both these cytokines are released in excess in the airways of asthmatic patients (30, 31, 40–42), particularly during natural or induced acute exacerbations of the disease (40, 41). They are important promoters of myofibroblast differentiation in vitro (15, 19, 20, 43, 44) and are considered to play a role in the remodeling response to tissue injury by inflammation in the lung (5, 45).

We cultured human circulating fibrocytes (>96% pure population of cells coexpressing collagen I and CD34) (21–23) in SFM with or without the addition of ET-1 or TGF- β 1, and evaluated at different time points cell proliferation, the production of fibronectin and collagen III, and the expression of α -SMA, a characteristic feature of myofibroblasts (17, 18, 20). Less than 15% of cultured fibrocytes were BrdU-positive cells between 4 and 6 days of incubation in SFM alone

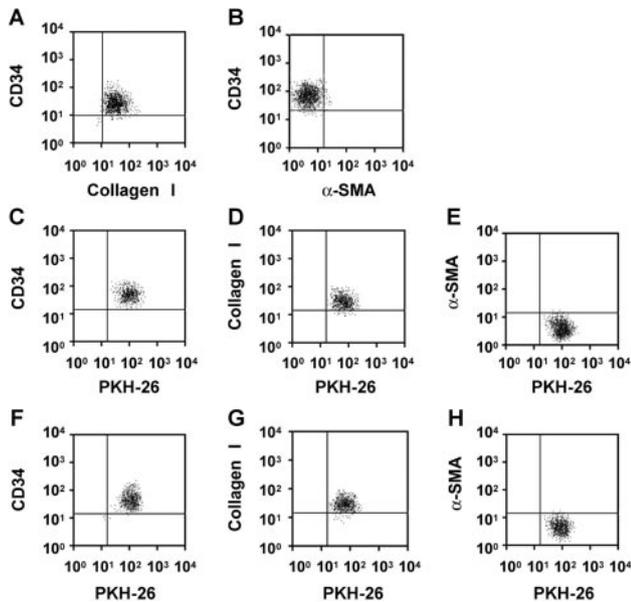


FIGURE 5. Flow cytometric analysis of the expression of CD34, collagen I, and α -SMA in mouse fibrocytes freshly isolated from the peripheral blood (A and B) and in PKH-26-labeled fibrocytes recovered from the peripheral blood of mice exposed to OVA (C–E) or PBS (F–H) at 2 h postexposure ($n = 4$ or 6 mice/group). The horizontal and vertical lines mark fluorescence intensity greater than the background that was observed with irrelevant PE- or FITC-conjugated isotype-matched controls for the anti-CD34 (A, B, C, and F), anti-collagen I (A, D, and G) and anti α -SMA (B, E, and H) Abs or with cells not labeled with PKH-26 (C–H).

(Fig. 7, A and B). ET-1 and TGF- β 1 increased the fibrocyte proliferation rate in a dose- and time-dependent fashion (Fig. 7, A and B). Unstimulated fibrocytes constitutively released detectable amounts of immunoreactive fibronectin (Fig. 7, C and D) and collagen III (Fig. 7, E and F) to the culture medium. TGF- β 1 accelerated and enhanced the release of these proteins in a dose- and time-dependent manner (Fig. 7, C–F). Cells cultured in SFM alone constitutively expressed α -SMA (Fig. 8A), while freshly isolated, uncultured fibrocytes did not (Fig. 8B). The addition of ET-1 and TGF- β 1 to the culture medium resulted in a dose- and time-dependent increase in the proportion of cells expressing this protein (Fig. 8, A and B). After the first 2 days of culture in presence of ET-1 or TGF- β 1, fibrocytes appeared as spindle-shaped cells resembling tissue fibroblasts (17, 18) (Fig. 8C). Between 4 and 6 days of exposure to these cytokines, stimulated fibrocytes showed the presence of cytoplasmic bundles of microfilament with dense bodies running parallel to the cell long axis (Fig. 8D), a finding that confirmed the development of an actin cytoskeleton consistent with the contractile phenotype of myofibroblasts (17, 18). Analysis of the phenotype of uncultured and cultured fibrocytes by flow cytometry revealed that an increased expression of α -SMA in culture was associated with a parallel reduction in the expression of CD34 (Fig. 8, E–H). This phenomenon was already evident in cells cultured for 6 days in SFM alone (Fig. 8F) compared with uncultured cells (Fig. 8E), and was more marked in cells incubated in presence of ET-1 (Fig. 8G) and TGF- β 1 (Fig. 8H) for the same period of time.

Discussion

The coexpression of the hemopoietic stem cell Ag CD34 and collagen I has been detected only in circulating fibrocytes to date (21–23, 29), and the ability of expressing α -SMA while retaining CD34 expression seems to be a unique property of fibrocytes that have entered a phase of differentiation into myofibroblast-like cells (29). We detected cells expressing both the CD34 Ag and procol-

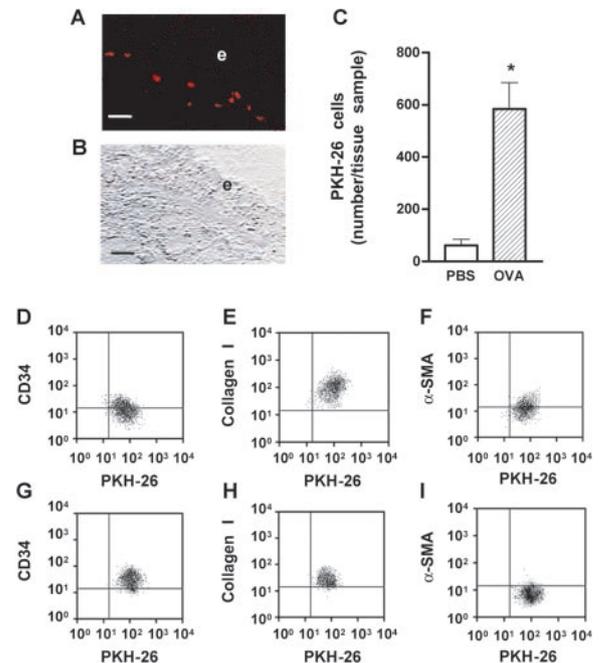


FIGURE 6. Tracking and phenotypic analysis of PKH-26-labeled fibrocytes in the airways of mice at 24 h following the last challenge with OVA or PBS at 6 wk of repeated exposures. The samples of airway tissue and peripheral blood were obtained from different groups of six mice exposed to OVA or PBS and otherwise tested in the same way. A, Detection of PKH-26 labeled cells in the airway wall section (darkfield) of a representative mouse exposed to OVA. B, Brightfield of the same section. *e*, airway epithelium. Scale bar = 50 μ m. C, Quantitative analysis of the number of PKH-26-labeled cells sorted from the single-cell suspensions of digested bronchial tissue fragments from mice exposed to OVA or PBS. Data are expressed as the mean number of cells \pm SE per tissue sample (300 μ g). *, $p < 0.05$ compared with the tissue from mice exposed to PBS. D–I, Flow cytometric analysis of the expression of CD34, collagen I, and α -SMA in PKH-26-labeled cells sorted from the single cell suspensions of digested airway tissue fragments from mice exposed to OVA (D–F) or PBS (G–I). The horizontal and vertical lines mark fluorescence intensity greater than the background that was observed with irrelevant FITC-conjugated isotype-matched controls for the anti-CD34 (D and G), anti-collagen I (E and H), and anti α -SMA (F and I) Abs or with cells not labeled with PKH-26 (D–I).

lagen I mRNA in the bronchial mucosa of patients with chronic allergic asthma. Their number markedly increased during an acute exacerbation of the disease induced by allergen exposure in laboratory, particularly 24 h postallergen inhalation. At this time point many of the cells expressing both CD34 and procollagen I mRNA also were α -SMA-positive cells. They localized to areas of collagen I deposition below the BM, in a zone where myofibroblasts accumulate in chronic asthma (12, 13) and where myofibroblast-like cells have been previously observed to appear in asthmatic patients 24 h following allergen inhalation (16). The characteristics of these cells and their location in the bronchial mucosa strongly suggested that they were fibrocyte-like cells that had migrated from the peripheral blood and were differentiating into collagen-producing myofibroblasts at the tissue site.

To explore this possibility we used an animal model of allergic asthma. Most of the asthmatic patients we tested were sensitized to perennial allergens (i.e., house dust or cat allergens) (25) and chronically exposed to low concentrations of them. When they inhaled the allergen to which they were sensitized in laboratory, they had an acute exacerbation of the disease with an acute exacerbation of a chronic inflammatory reaction (25). To mimic this

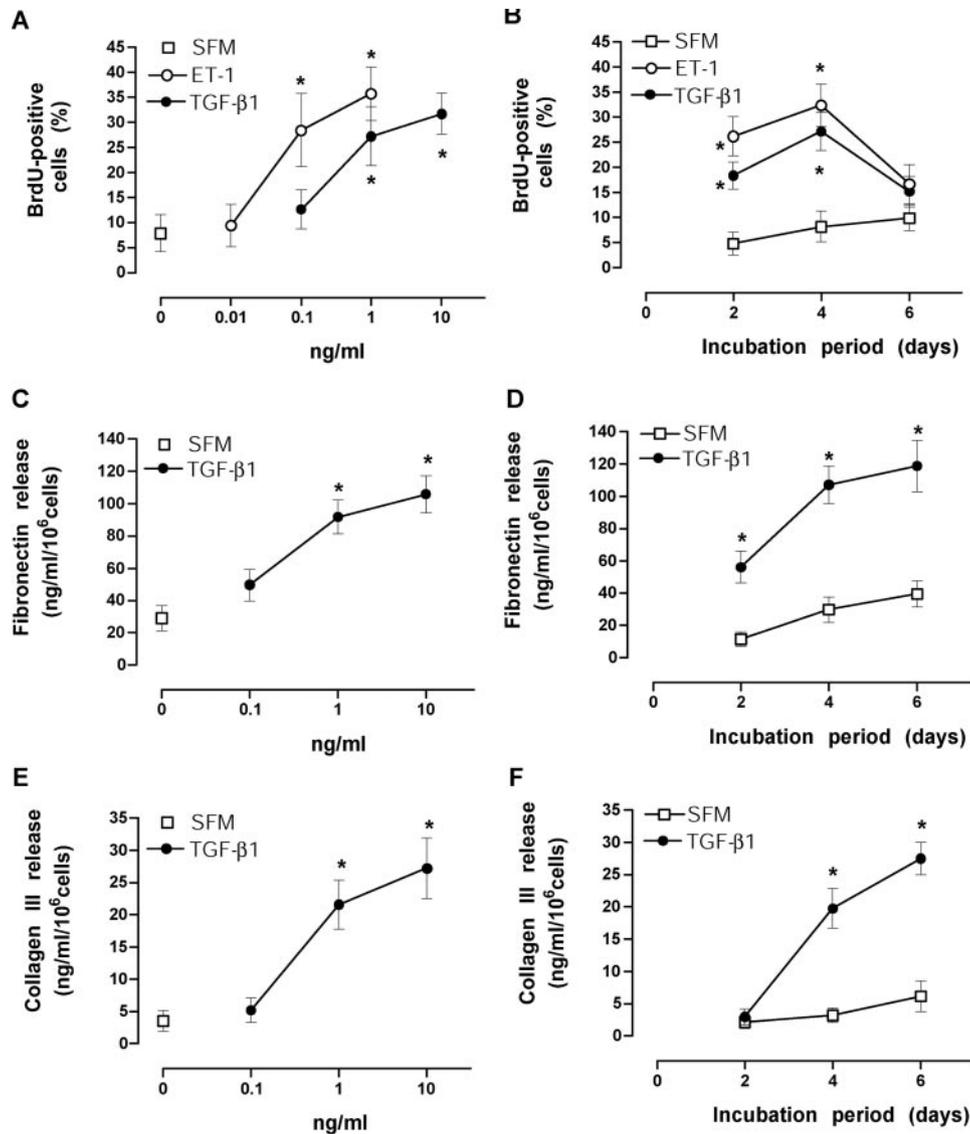


FIGURE 7. Cell proliferation (*A* and *B*) and release of immunoreactive fibronectin (*C* and *D*) and collagen III (*E* and *F*) in cultures of human fibrocytes incubated in SFM with or without the addition of ET-1 or TGF- β 1. *A*, *C*, and *E*, Dose-dependent effect of ET-1 and/or TGF- β 1 in cell cultures incubated for 4 days. *, $p < 0.05$ compared with the effect of SFM alone ($n = 5$ or 6 triplicate experiments/group). *B*, *D*, and *F*, Time-dependent effect of 0.1 ng/ml ET-1 and/or 1 ng/ml TGF- β 1. *, $p < 0.05$ compared with the effect of SFM alone ($n = 5$ or 6 triplicate experiments/group). Fibrocyte proliferation was assessed by the incorporation of BrdU into DNA, and data are expressed as the mean percentage of BrdU-immunoreactive cells \pm SE (*A* and *B*). To avoid the confounding effect of TGF- β 1 on fibrocyte proliferation, data for the release of fibronectin and collagen III were normalized to the cell number and are expressed as the mean nanograms of immunoreactive fibronectin (*C* and *D*) or collagen III (*E* and *F*) released per milliliter of culture medium per 10^6 cells \pm SE.

condition in mice, which are not spontaneously asthmatic, we sensitized BALB/c mice to OVA and repeatedly exposed them to this Ag over the long term. In this way mice developed inflammatory and structural changes of the airways similar to those we observed in asthmatic patients, particularly recruitment of eosinophils into the lamina propria, collagen deposition below the airway epithelium, and the accumulation of CD34-positive cells that coexpressed procollagen I. The accumulation of these fibrocyte-like cells was evident after 4 wk of repeated exposures and was markedly increased thereafter. After 6 and 8 wk of repeated exposures to OVA, a substantial proportion of the same cells also expressed α -SMA, suggesting that many fibrocyte-like cells were differentiating into myofibroblasts at the tissue site. To investigate whether these cells really represented circulating fibrocytes that had migrated from the peripheral blood into the airway tissue, we repeatedly exposed other two groups of sensitized mice to either OVA or

PBS for 4 wk and tracked PKH-26-labeled fibrocytes in the peripheral blood and in the airway wall after the next exposure to OVA or PBS at 6 wk. At 24 h postexposure, OVA-challenged mice showed higher numbers of PKH-26-labeled fibrocytes in their airways than control mice exposed to PBS alone. The phenotype of the PKH-26-positive cells present in the airways of mice exposed to PBS was similar to that of labeled cells recovered from the peripheral blood, suggesting that they were probably PKH-26-positive cells that had lodged in the lung capillary bed after the i.v. injection. On the contrary, the PKH-26-positive cells that accumulated in the airways of mice exposed to OVA had a phenotype different from that of labeled cells recovered from the peripheral blood in that they showed increased expression of collagen I and α -SMA and down-regulation of expression of the surface Ag CD34. Their red fluorescence intensity had only slightly decreased, suggesting that only a minority of the labeled cells had

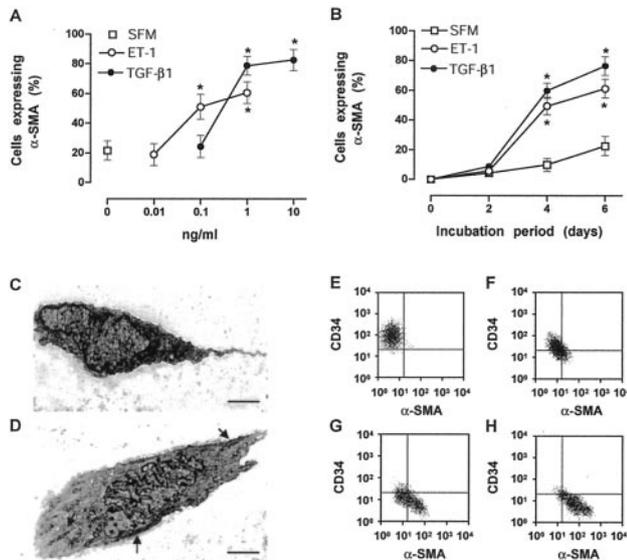


FIGURE 8. In vitro expression of α -SMA in human fibrocytes cultured in SFM with or without the addition of ET-1 or TGF- β 1. **A**, Dose-dependent effect of ET-1 and TGF- β 1 in cell cultures incubated for 6 days. *, $p < 0.05$ compared with the effect of SFM alone ($n = 5$ triplicate experiments/group). **B**, Time-dependent effect of 0.1 ng/ml ET-1 and 1 ng/ml TGF- β 1. *, $p < 0.05$ compared with the effect of SFM alone ($n = 5$ triplicate experiments/group). Data are expressed as the mean percentage of cells expressing α -SMA immunoreactivity \pm SE. **C** and **D**, Representative microphotographs showing the ultrastructural characteristics of fibrocytes cultured in presence of 1 ng/ml TGF- β 1 for 2 (**C**) and 6 (**D**) days. Arrows in **D** point to cytoplasmic bundles of microfilaments with dense bodies that run parallel to the cell long axis. Scale bars = 2 μ m. **E-H**, Flow cytometric analysis of the expression of CD34 and α -SMA in freshly isolated human fibrocytes (**E**) and in human fibrocytes cultured for 6 days in SFM alone (**F**) or in SFM supplemented with 0.1 ng/ml ET-1 (**G**) or 1 ng/ml TGF- β 1 (**H**). The horizontal and vertical lines mark fluorescence intensity greater than the background that was observed with irrelevant PE- and FITC-conjugated isotype-matched controls for the anti-CD34 and anti- α -SMA Abs.

proliferated in the lung. In addition, most of these labeled cells could be localized below the airway epithelium and along the BM by microscopic analysis of airway sections (Fig. 6, **A** and **B**). Taken together these results indicated that labeled fibrocytes were recruited from the circulation into the airways as a result of OVA exposure and had entered into a phase of local differentiation into myofibroblast-like cells. They also suggested that a similar recruitment of circulating fibrocytes may occur in the bronchial mucosa of patients with chronic allergic asthma after any subsequent exposure to the relevant allergen.

However, our findings did not imply that recruited fibrocytes can undergo differentiation into myofibroblasts once they have migrated at the tissue site or that they can actively produce collagen in a way similar to the fibrocyte-like cells we identified in the bronchial mucosa of our asthmatic patients. In another set of experiments we therefore evaluated whether human circulating fibrocytes can differentiate into myofibroblast-like cells under the influence of two fibrogenic cytokines, ET-1 and TGF- β 1, that are known to function as important myofibroblast promoters (15, 19, 20, 43, 44) and are released in exaggerated quantities in the airways of asthmatic patients (30, 31, 40–42). We found that fibrocytes cultured for 6 days in SFM alone were able to grow and constitutively produce fibronectin and collagen III with a pattern similar to mature lung fibroblasts (44, 46). Upon stimulation with either ET-1 or TGF- β 1, their proliferation rate increased, and they showed multiple markers of differentiation into myofibroblasts

(15, 19, 20, 43, 44). In particular, they developed bundles of actin microfilaments indicative of a contractile phenotype and produced increased amounts of fibronectin and collagen III.

The differentiation of human circulating fibrocytes into myofibroblasts in culture was associated with a down-regulation of the expression of the surface Ag CD34. Such a phenomenon was not unexpected because it is well known that the degree of CD34 expression correlates directly with the stem cell activity of most hemopoietic stem cells and that this Ag is down-modulated during the cell commitment along a differentiating lineage (reviewed in Ref. 47). Nonetheless, it was interesting to note that the same phenomenon could be observed with PKH-26-labeled cells recovered 24 h after OVA exposure in the airways of mice chronically exposed to this allergen over the previous 4 wk. This observation, in fact, supported the hypothesis that those cells had entered the differentiation process under the influence of local factors released during the acute inflammatory reaction elicited by the last OVA exposure in the context of a chronic inflammatory status due to repeated allergen challenges. A similar down-regulation of CD34 expression on fibrocytes that have migrated from the peripheral blood to the bronchial mucosa may also explain why the mean number of CD34-positive, fibrocyte-like cells we detected in the bronchial biopsy sections of patients with chronic allergic asthma at 24 h after allergen exposure represented <50% of all cells expressing procollagen I mRNA or α -SMA (excluding vessels and smooth muscle).

In summary, the results of our experiments provide the following new information. It is possible to detect fibrocyte-like cells in the bronchial mucosa of patients with allergic asthma, and the number of fibrocyte-like cells increases during an asthmatic reaction induced by allergen exposure. These fibrocyte-like cells express collagen I mRNA and a marker of differentiation into myofibroblasts in vivo. They may derive from circulating fibrocytes that start their differentiation into collagen-producing myofibroblasts once they have migrated at the tissue site, as demonstrated in the murine model of allergic asthma. Two of the fibrogenic cytokines that are thought to promote myofibroblast accumulation in asthma, ET-1 and TGF- β 1, induce the proliferation of circulating fibrocytes in vitro and promote their differentiation into mature myofibroblasts that produce increasing amounts of fibronectin and collagen III. Taken together these findings strongly suggest that circulating fibrocytes may function as precursors of bronchial myofibroblasts and may contribute to the genesis of subepithelial fibrosis in asthma.

However, it should be taken into account that our experiments do not provide any direct evidence that the fibrocyte-like cells we identified in the bronchial mucosa of asthmatic patients were fibrocytes recruited from the circulation. Therefore, the possibility exists that they originated from an unidentified progenitor that resides in the airways.

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