

Contribution of Lung Fibroblast Migration in the Fibrotic Process of Airway Remodeling in Asthma

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ABSTRACT

Background: The fibrotic process in airway remodeling of asthma may be characterized by an exaggerated deposition of extracellular matrix (ECM) components such as fibronectin and type I, III and IV collagen. In the present study, we established airway remodeling model mice and examined the mechanism of fibrotic change by measuring chemotactic activity of lung fibroblasts and quantifying collagen content in lung tissues.

Methods: Airway remodeling model mice were made by ovalbumin (OA) sensitization and inhalation. Bronchoalveolar lavage (BAL) and bronchial biopsy were performed. Cell migration was assessed by the Boyden's chamber technique. The collagen content of lung tissue was measured using ELISA.

Results: The chemotactic activity in lung fibroblasts toward the mouse BAL fluid (BALF) was significantly increased in OA-inhaled mice. Total soluble collagen content was significantly increased in OA-inhaled mice. We observed markedly increased collagen deposition around the airway wall in OA-inhaled mice, which was not shown in saline-inhaled mice. Furthermore, fibronectin in the BALF of OA-inhaled mice was significantly higher than that in the control mice.

Conclusions: The total soluble collagen content increased during the fibrotic change of airway remodeling in asthma. Furthermore, migration of fibroblasts may play a key role in this remodeling process, and fibronectin and type I and IV collagen seem to be chemotactic factors for the fibroblasts.

KEY WORDS

airway remodeling, asthma, extracellular matrix, fibroblast, migration

INTRODUCTION

Airway remodeling in asthma is recognized as irreversible structural changes.¹ However, several recent reports have revealed that remodeling might be the process of repair from injury. CysLT₁ receptor blockade has anti-airway remodeling effects including fibrotic change in animal models.²⁻⁴ In addition, Ohta *et al.* demonstrated that the presence of fibrotic change declined and returned to a normal level of airway thickening following treatment with a neutralizing anti-TGF- β antibody.⁵ Another report noted that treatment with anti-platelet-derived growth factor (PDGF) antibody significantly decreased the hydroxyproline content in mice sensitized with silica particles.⁶

The fibrotic process of airway remodeling in asthma may be caused by an exaggerated induction

and deposition of extracellular matrix (ECM) such as fibronectin or type I, III or IV collagen. Part of these structural changes may be induced by migration of fibroblasts activated by fibronectin secreted by bronchial epithelial cells and fibroblasts.^{7,8}

In the present study, we established airway remodeling model mice and evaluated the mechanism of fibrotic change by measuring the chemotactic activity of lung fibroblasts and quantifying the collagen content of lung tissue.

METHODS

ANIMALS

Seven-week-old female BALB/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese As-

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PREPARATION OF AIRWAY REMODELING MODEL

Airway remodeling model mice were made by sensitizing mice with ovalbumin (OA; Sigma, St. Louis, MO, USA) according to the method of Tanaka and coworkers.⁹ Briefly, mice were actively sensitized by intraperitoneal injections of 50 µg OA with 1 mg alum on days 0 and 12. Starting on day 22, they were exposed to OA (1% diluted in sterile physiological saline) for 30 minutes every day for 4 consecutive weeks. Control mice were sensitized and exposed to saline in a similar manner. Bronchoalveolar lavage (BAL) and bronchial biopsy were performed 24 hours after the final inhalation.

SAMPLING OF BAL FLUID

Mice were sacrificed on day 26, 29, 36, 43 or 50 with an intraperitoneal injection of pentobarbital sodium salt (Nacalai Tesque, Inc., Kyoto, Japan). The lungs were washed 4 times with 0.5 ml phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA; Sigma) and 0.05 mM EDTA-2Na (Sigma). This procedure was repeated three times. BAL fluid (BALF) was centrifuged at $1500 \times g$ for 5 minutes. Each supernatant was recovered and stored at -30°C .

PREPARATION OF LUNG FIBROBLASTS

Normal human lung fibroblasts (NHLF) were purchased from Clonetics (Cambrex Bio Science Walkersville, Inc. Walkersville, MD, USA). The cells were cultured on tissue culture dishes (Falcon; Becton-Dickinson Labware, Franklin Lakes, NJ, USA) with FGM-2 media (Clonetics). Cells were incubated at 37°C , 5% CO_2 .

CELL MIGRATION ASSAYS

Cell migration was measured by the Boyden's blind-well chamber technique¹⁰ using 48-well multiwell chambers (Neuroprobe, Inc., Bethesda, MD, USA). An 8-µm pore size membrane (Nuclepore, Pleasanton, CA, USA) was used. The mouse BALF, or an ECM component such as fibronectin or type I, III or IV collagen, was placed into each of the bottom wells. Cells, trypsinized and resuspended at 1×10^6 cells/ml, were placed into each of the top wells. The chamber was then incubated at 37°C , 5% CO_2 for 6 hours. After incubation, cells that had not migrated were scraped off the upper surface of the membrane, and the membrane was air-dried. Cells were stained with Diff-Quick® (International Reagent Corp., Kobe, Japan). Cell migratory activity is shown as the total number of migrated cells counted in ten high-power fields using a light microscope (Olympus, Tokyo, Japan) with $\times 400$ magnification.

FIBRONECTIN ELISA

Fibronectin in BALF was measured using sandwich ELISA.¹¹ Microtest™ 96-well ELISA plates (BD Biosciences, Bedford, MA, USA) were coated with rabbit anti-fibronectin polyclonal antibody (10 µg/ml; Dako, Glostrup, Denmark) in a coating buffer (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2) overnight at 4°C . Each plate was blocked with 2% BSA-PBS for 2 hours at room temperature. Then samples were added to each plate and incubated for 2 hours at room temperature. Polyclonal rabbit anti-fibronectin antibody conjugated with peroxidase (0.22 µg/ml; Dako) was added overnight at 4°C . Chromogen substrate containing 3, 3', 5, 5'-tetramethyl-benzidine (Dako) was added, and optical readings at 492 nm were obtained using an ELISA plate reader (Wako, Osaka, Japan). Mouse fibronectin (Innovative Research, Inc., Southfield, MI, USA) was used to obtain the standard curves for calculation of fibronectin concentration in BALF. The detection limit of this ELISA was 40 ng/ml.

COLLAGEN ASSAYS

Lung collagen content was determined by measuring soluble collagen using the Sircol Collagen Assay kit (Biocolor Ltd., Newtownabbey, Northern Ireland) according to the manufacturer's instructions. Briefly, lungs were extracted with 0.5 M acetic acid containing 1 mg pepsin (Sigma) per 10 mg lung tissues. Each sample was incubated overnight at room temperature with stirring. After centrifugation, 100 µl of each supernatant was mixed for 30 minutes at room temperature with 1 ml of Sircol dye reagents. After centrifugation, each collagen-dye pellet was resuspended in 1 ml of Alkali reagent and optical density was measured at 540 nm using an ELISA plate reader.

HISTOLOGICAL EXAMINATION

Bronchial biopsy was performed 24 hours after the final antigen challenge. The lungs were distended by injection of 10% buffered formalin via the trachea, excised and immersed in the same fixative for 24 hours. Tissues were sliced and embedded in paraffin, and 2–3 µm sections were stained with Elastica van Gieson or Masson-trichrome.

STATISTICAL ANALYSIS

Data were expressed as means $\pm$ SEM. Statistical significance between saline-inhaled and OA-inhaled mice was estimated using the two-tailed Student's *t* test. Probability values of less than 0.05 were considered to indicate significant differences.

RESULTS

MIGRATION OF NHLF TOWARD BALF

To determine whether the migration of lung fibroblasts contributed to the fibrotic process of airway re-

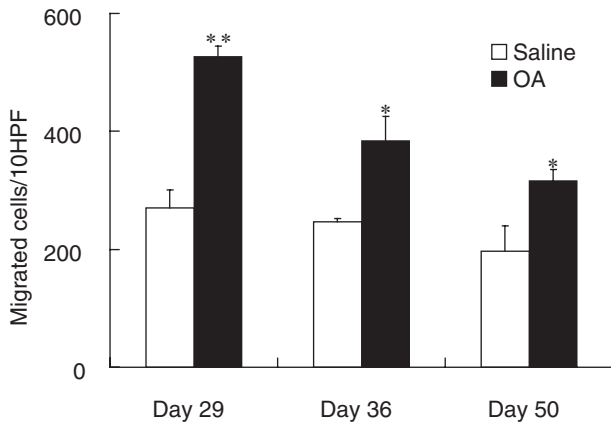


Fig. 1 Migration of NHLF toward BALF. Values represent the means $\pm$ SEM in triplicate. * $p < 0.05$, ** $p < 0.01$ (vs saline-inhaled mice).

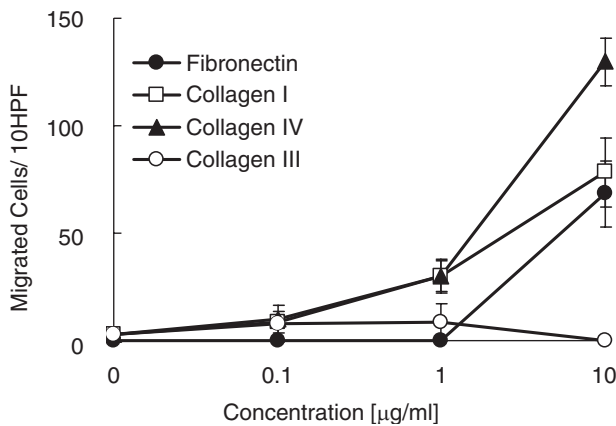


Fig. 2 Migration of NHLF toward ECM components. Fibronectin and type I, III and IV collagen were used as chemoattractants. Values represent the means $\pm$ SEM in triplicate.

modeling, we evaluated the chemotactic activity in the NHLF toward the mouse BALF. The chemotactic activity increased significantly in OA-inhaled mice compared with saline-inhaled mice (Fig. 1). The number of migrated cells declined gradually in both groups.

MIGRATION OF NHLF TOWARD EXTRACELLULAR MATRIX COMPONENTS

As we considered ECM components in the mouse BALF to be chemotactic factors for NHLF, we examined whether there was chemotactic activity in NHLF toward fibronectin or type I, III or IV collagen (Fig. 2). Among the four ECM components, fibronectin and type I and IV collagen showed a dose-dependent increase in chemotactic activity, whereas type III collagen showed little chemotactic activity.

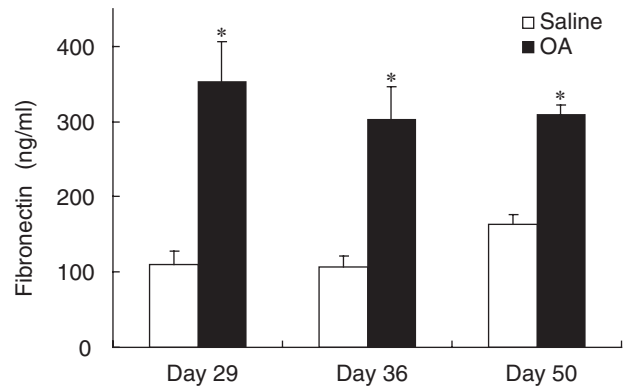


Fig. 3 The concentration of fibronectin in BALF. Values represent the means $\pm$ SEM of 4 mice. * $p < 0.01$ (vs saline-inhaled mice).

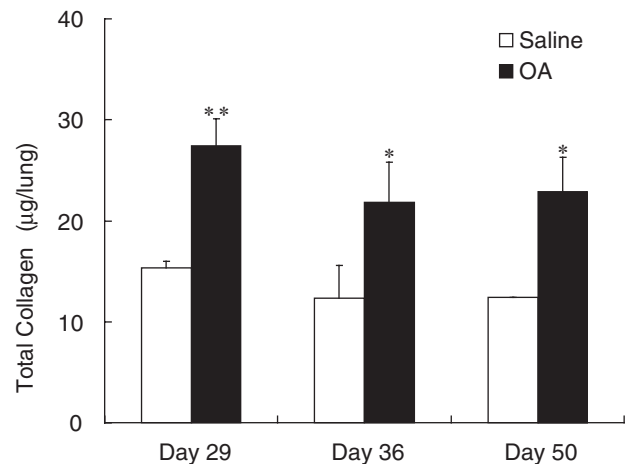


Fig. 4 Total soluble collagen content in lung tissue of mice. Values represent the means $\pm$ SEM of 3–4 mice. * $p < 0.05$, ** $p < 0.01$ (vs saline-inhaled mice).

MEASUREMENT OF MOUSE FIBRONECTIN

We measured fibronectin by sandwich ELISA (Fig. 3). The results demonstrated that BALF of OA-inhaled mice contained a significantly higher concentration of fibronectin than did that of saline-inhaled mice. In addition, fibronectin in the BALF of OA-inhaled mice increased gradually and peaked at day 29, and then declined. In contrast, the fibronectin in the BALF of control mice showed relatively little change throughout the period of OA inhalation.

TOTAL SOLUBLE COLLAGEN CONTENT IN LUNG TISSUE

We measured the total soluble collagen content in lung tissues using the ELISA. Total soluble collagen content increased in OA-inhaled mice, whereas the total soluble collagen content in saline-inhaled mice changed little (Fig. 4).

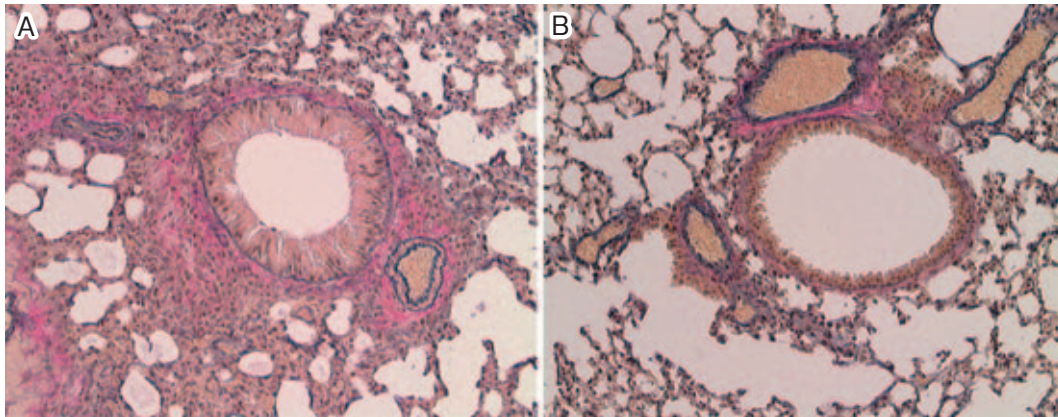


Fig. 5 Elastica van Gieson staining of lung sections from OA-inhaled mice (A) and saline-inhaled mice (B) on day 50. Original magnifications $\times 200$.

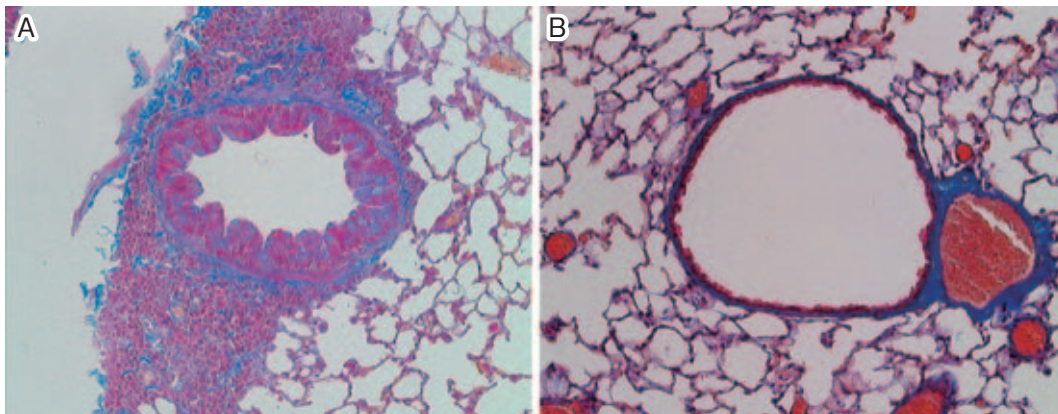


Fig. 6 Masson-trichrome staining of lung sections from OA-inhaled mice (A) and saline-inhaled mice (B) on day 29. Original magnifications $\times 200$.

HISTOLOGICAL ANALYSIS

We evaluated the structural changes in lung tissue. OA-inhaled mice at day 50 (4-week exposure) showed a markedly increased deposition of collagen around the airway wall (Fig. 5A). Masson-trichrome staining of lung tissue at day 29 demonstrated highly increased collagen in OA-inhaled mice (Fig. 6A), which was not observed in the airway wall of saline-inhaled mice (Fig. 6B). Furthermore, in OA-inhaled mice, abundant inflammatory cell infiltration and goblet cell hyperplasia were detected.

DISCUSSION

In the present study, we demonstrated that the chemotactic activity for fibroblasts was significantly increased in the BALF of repeated antigen-inhaled mice. Furthermore, we confirmed that fibronectin and type I and IV collagen had chemotactic activity for fibroblasts. Furthermore, we found that the concentration of fibronectin in the BALF of repeated antigen-inhaled mice increased significantly. It has been reported that fibronectin is included in bron-

chial epithelial cell or fibroblast conditioned medium and chemotactic factors for fibroblasts *in vitro*.^{7,8} Our current study suggests that not only fibronectin but also type I and IV collagen had chemotactic activity for fibroblasts, and they were included in the BALF of repeated antigen-inhaled mice *in vivo*. Moreover, the number of migrated cells over time gradually declined, which suggests that chemotactic factors in the BALF decreased. That is, ECM-involved fibrotic change will occur during the early stage after the injury of epithelial cells and then stimulate the migration of fibroblasts.

Many reports have demonstrated that cell migration occurs beyond the difference between species. Rat lung fibroblasts (RLF) and Swiss mouse 3T3 cells showed chemotactic activity toward human PDGF.¹² Human airway epithelial cells and fibroblasts may release a chemotactic activity for rat basophilic leukemia cells (RBL-2H3) and normal rat peritoneal mast cells (RPMC).^{13,14} Moreover, it has been reported that human lung fibroblasts migrate significantly for bovine bronchial epithelial cell conditioned medium.⁷

Therefore, we conclude that cell migration occurs between different species.

In the airway of asthma patients, the number of myofibroblasts and subepithelial collagen thickness were correlated.^{15,16} Another report demonstrated that myofibroblasts produce ECM more than fibroblasts.¹⁷ Differentiation of myofibroblasts from fibroblasts was induced by TGF- β ,¹⁸ and it was reported that chemotactic activity for fibroblasts was increased by the action of TGF- β .¹⁹ Furthermore, fibronectin increased in the BALF of asthma patients, and the change was positively correlated with the level of TGF- β .²⁰ These findings suggest that not only fibronectin but also TGF- β play an important role in the fibrotic process.

In our experiment, we assayed total soluble collagen content in lung tissues. Although many reports using hydroxyproline content as an indicator of fibrotic change have been published,^{9,11,21} we believe that our current results are in agreement with the results in those reports.

As P. K. Jeffery suggested, the term "fibrosis" was an unfortunate application as a view of airway remodeling in asthma.²² Fibrosis as reticulin indicates that collagen fibers lie relatively deeper in the interstitium of the airway wall. However, collagen fibers increased in the lung tissue in repeated antigen-inhaled mice as shown in our results. Thus, a similar change as fibrosis may occur in airway remodeling processes in asthma.

In previous reports, remodeling has been considered to consist of irreversible structural changes, since dexamethasone treatment had limited effect.^{23,24} However, several studies have demonstrated that CysLT₁ receptor blockade, anti-TGF- β antibody and anti-PDGF antibody have anti-airway remodeling effects including fibrotic change in animal models.²⁻⁶ Moreover, Saetta *et al.* reported that the avoidance of exposure to toluene diisocyanate (TDI) was associated with suppressing the thickness of fibrotic change and the number of fibroblasts in occupational asthma.²⁵ These results suggest that airway remodeling might have repair processes and reversible change.

In summary, we confirmed that total soluble collagen content increased in the fibrotic change in airway remodeling processes in asthma. Moreover, the migration of fibroblasts may play a key role in this process. Thus, understanding the mechanisms of the fibrotic process in airway remodeling is important for asthma therapy.

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