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Lower Airways Remodeling in Nonasthmatic Subjects with Allergic Rhinitis

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SUMMARY: We analyzed by immunohistochemistry the distribution of types I, II, III, IV, V, and VII collagens, laminin, and fibronectin in the bronchial biopsy specimens of nonasthmatic subjects with seasonal allergic rhinitis ($n = 8$) and compared these results with those found in mild stable allergic asthmatics ($n = 6$) and normal controls ($n = 5$). The content of type I and III collagens was increased in rhinitic subjects compared with controls. These collagens were focally deposited in the reticular basement membrane area. Three subjects with allergic rhinitis had no fibronectin deposition in their basement membrane, as in controls, whereas the other five had a focal fibronectin deposition. In asthmatic patients, type I and III collagens and fibronectin were more abundant and more uniformly distributed underneath the basement membrane than they were in rhinitic subjects. Expression of type II, IV, V, and VII collagens and laminin were similar in the three groups. Electron microscopic and immunohistochemical analyses of bronchial mucosa showed a network of myofibroblasts beneath the epithelium in rhinitis as in asthma subjects. These data show that the irregularly distributed subepithelial fibrosis observed in subjects with allergic rhinitis results from the deposition of type I and III collagens and fibronectin, probably produced by bronchial myofibroblasts. These results suggest the presence of an active structural remodeling in the lower airways of allergic rhinitic subjects that is similar in nature to that seen in asthma, although less marked. (*Lab Invest* 1996; 75:735-744).

Allergic rhinitis is a common inflammatory disease of the nasal mucosa (Naclerio, 1991). Compared with nonatopic individuals, subjects with allergic rhinitis generally show an increased airway responsiveness and may have an increased risk of developing symptomatic asthma (Eggleston, 1988; Settiple et al, 1994; Townley et al, 1975). In a previous study, we reported that allergic subjects with seasonal pollen-induced rhinitis had airway alterations on bronchial biopsy samples somewhat similar to those observed in asthmatic subjects. These changes consisted of cellular infiltration, mucosal edema, increased epithelial desquamation, and focal basement membrane thickening corresponding to an irregularly distributed subepithelial fibrosis (Boulet et al, 1995). These features persisted out of the pollen season.

Ultrastructural and immunohistologic studies of bronchial biopsy specimens obtained from subjects with asthma show that the apparent basement membrane thickening, defined as subepithelial fibrosis, is caused by the deposition of fibrillar collagen, which

leads to an increase in the depth and density of the lamina reticularis (Roche et al, 1989). In normal subjects, the bronchial basement membrane is composed of laminin and type IV collagen, to which epithelial cells are attached. The subepithelial fibrosis seen in asthmatic subjects is mainly caused by the abnormal deposition of type I and III collagens and fibronectin (Roche, 1991). Brewster et al (1990) showed that this collagen layer contained myofibroblasts and suggested that these cells were probably responsible for the subepithelial fibrosis observed in asthma. A good correlation was found between the degree of fibrosis and the number of myofibroblasts.

The pathophysiologic significance of this subepithelial collagen deposition is unknown, but it may represent a long-term and progressive remodeling process secondary to the ongoing airway inflammation. The irregularly deposited collagen observed under the basement membrane of subjects with allergic rhinitis may represent an early stage of this phenomenon and possibly involves constituents somewhat different from those observed in asthma. This study was, therefore, undertaken to evaluate the nature of the subepithelial fibrosis observed in subjects with allergic rhinitis as compared with those found in asthmatic subjects and normal controls.

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Results

Subjects' Characteristics

Rhinitic subjects were aged 20 to 36 years (mean, 27.5 ± 1.9 years) and had normal airway responsiveness to methacholine with a geometric mean PC_{20} of 25.9 ± 1.1 (range, 11 to 128) (Table 1). One subject had a PC_{20} of 11 mg/ml^{-1} , but he had no history of asthma. All had a history of recurrent rhinoconjunctivitis during the pollen season and had positive allergic skin tests to tree, grass, and/or ragweed pollens. Six subjects were also sensitized to house dust. The group of mild allergic asthmatic subjects were aged 20 to 25 years (mean, 21.5 ± 0.8 years). They had normal expiratory flows (mean percentage predicted, 96.3 ± 3.1) and a geometric mean PC_{20} of $4.0 \pm 2.2 \text{ mg/ml}$ (range, 0.56 to 6.3). Normal controls were aged 21 to 27 years with a geometric mean PC_{20} of 99.4 mg/ml .

Collagen Distribution in the Subepithelial Area of Asthmatic, Rhinitic, and Normal Subjects

Bronchial biopsy specimens of asthmatic subjects showed a strong collagen type I staining in the subepithelial area, in which a dense band of collagen was uniformly deposited (Fig. 1A). Collagen type I was also observed in the interstitium, but the staining was less intense. In rhinitic subjects, the distribution of type I collagen was focal under the basement membrane (Fig. 1B). In normal controls, there was no deposition of this collagen in the subepithelial area (Fig. 1C). Basement membrane of asthmatic subjects presented an uniform accumulation of type III collagen (Fig. 2A), whereas in rhinitic subjects, type III collagen was focally observed under the basement membrane (Fig. 2B). In normal bronchial mucosa, there was no deposition of type III collagen (Fig. 2C).

The maximal thickness of the focal collagen deposition observed in rhinitic subjects' bronchial basement membranes was similar to the collagen deposition thickness of those of asthmatic subjects, with respective means of $16.9 \pm 3.6 \mu\text{m}$ and $13.5 \pm 4.5 \mu\text{m}$ for type I collagen and $18.4 \pm 4.4 \mu\text{m}$ and $12.8 \pm 4.6 \mu\text{m}$ for type III collagen. On the other hand, the

minimal thickness of collagen deposition in rhinitic subjects was comparable with normal controls' values, with respective means of $5.4 \pm 1.4 \mu\text{m}$ and $7.8 \pm 1.9 \mu\text{m}$ for type I collagen and $6.5 \pm 1.4 \mu\text{m}$ and $8.4 \pm 3.4 \mu\text{m}$ for type III collagen (Fig. 3). Types II, IV, V, and VII collagens and laminin were present in the same proportion in the three groups (data not shown).

Fibronectin Distribution under the Basement Membrane

Fibronectin was regularly deposited underneath the basement membrane of asthmatic patients (Fig. 4A), and its mean thickness was $13.8 \pm 4.5 \mu\text{m}$. In rhinitic subjects, the distribution of fibronectin under the basement membrane showed two profiles, one similar to normal controls ($n = 3$) with no fibronectin deposition in their basement membrane (Fig. 4B) and one ($n = 5$) with a focal deposition of fibronectin (Fig. 4D) whose maximal thickness ($11.1 \pm 9.2 \mu\text{m}$) was similar to values of asthmatic subjects and minimal thickness ($6.7 \pm 1.0 \mu\text{m}$) close to normal controls' values (Fig. 3). There was no accumulation of fibronectin in the basement membranes of normal subjects (Fig. 4C).

No correlation was found between collagen or fibronectin deposit thickness in the basement membrane and PC_{20} or baseline FEV_1 in the three groups studied.

Myofibroblast Analysis

Electron microscopic analysis of asthmatic and rhinitic biopsy specimens showed elongated myofibroblasts containing microfilament bundles with abundant polyribosomes. These myofibroblasts were found at the deep margin of the collagen layer in the basement membrane in both asthmatic and rhinitic subjects (Figs. 5A and 6). In these subjects, immunoperoxidase staining of sections with PR_2D_3 showed positive cells beneath the bronchial epithelium under the collagenous band (Fig. 5B), which suggests an association between myofibroblasts and collagen deposition. These cells were positive for α smooth muscle actin, and some were also positive for desmin and vimentin.

Table 1. Subjects' Characteristics

	Sex (F/M)	Age (years)	FEV_1 (% pred)	FVC (% pred)	PC_{20} (mg/ml)
Allergic rhinitis	3/7	27.5 ± 1.9^a	111.0 ± 4.6^a	107.9 ± 4.3^a	25.9 ± 1.1^b
Allergic asthma	4/2	21.5 ± 0.8	96.3 ± 3.1	89.2 ± 2.5	4.0 ± 2.2
Normal controls	2/3	24.0 ± 1.1	96.0 ± 5.9	98.2 ± 5.1	99.4 ± 1.2

^aExpressed as mean $\pm$ SEM.

^b PC_{20} expressed as geometric mean $\pm$ SEM.

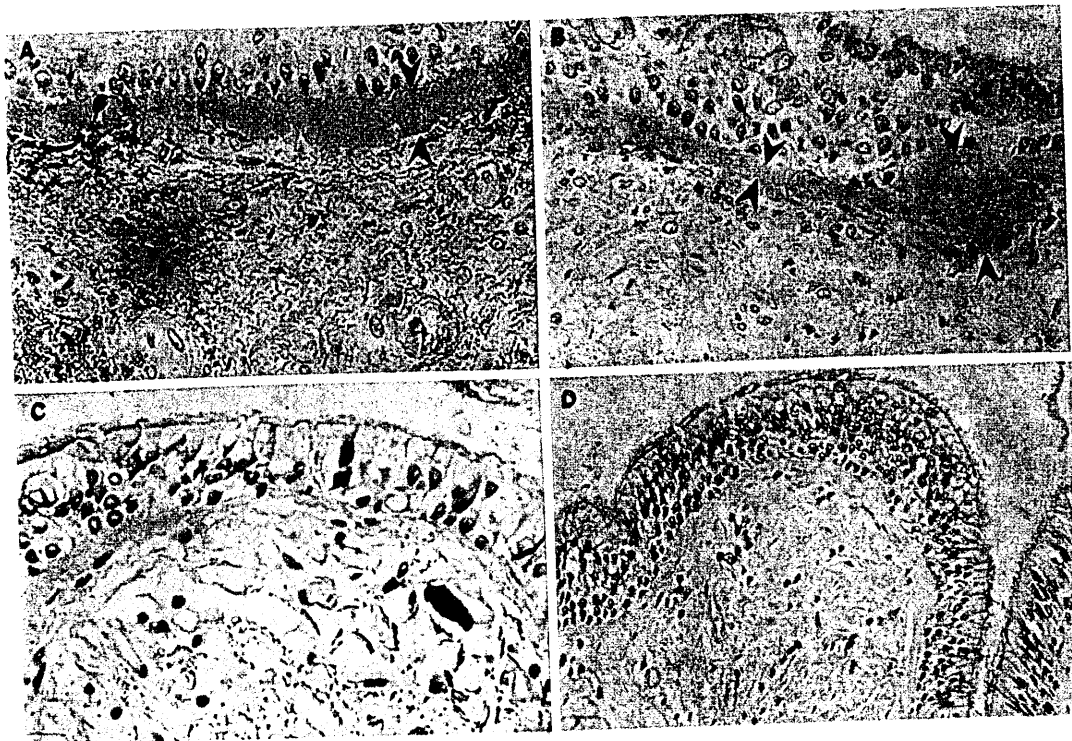


Figure 1.

Type I collagen detection in bronchial mucosa of asthmatic, rhinitic, and normal subjects. Immunoreactivity appears in red. Bronchial biopsies of asthmatic subjects (A) show a strong and uniform type I collagen staining in the basement membrane area (arrows). The staining is present but less intense in the interstitium. In rhinitic subjects (B), the immunoreactivity for type I collagen is focally distributed in the subepithelium (arrows show the thinnest and thickest zones measured on this specimen). Normal controls show no accumulation of collagen type I in the basement membrane (C) ($\times 400$). A control section processed without primary antibody shows no background staining (D) ($\times 200$).

Discussion

The main purpose of this study was to demonstrate that nonasthmatic allergic rhinitic subjects showed structural airway changes and to compare the extent of this phenomenon in allergic asthmatic and normal nonatopic subjects. We found that the content of type I and III collagens was increased and focally distributed in rhinitic subjects compared with controls. Most rhinitic subjects (five of eight) presented an increased focal fibronectin deposition. In the asthmatic subjects, types I and III collagens and fibronectin were abundantly and uniformly distributed underneath the basement membrane. These data show that the focally distributed subepithelial fibrosis observed in rhinitic subjects is similar in nature to the one observed in asthmatic subjects. The structural changes observed in rhinitic bronchial mucosa can be considered intermediate between what is found in normal and asthmatic airways. They may represent the first stage of airway repair and remodeling processes promoted by the underlying airway inflammation, which may contribute to the development of changes in airway function over time.

In a previous study, we showed that, compared with other times, allergic rhinitic subjects had increased airway responsiveness to methacholine during the

pollen season but that the total inflammatory cell counts on bronchial biopsy samples and the airway wall thickness were unchanged (Boulet et al, 1995). In this study, we chose to evaluate the rhinitic group out of pollen season to ensure that all groups were in a stable phase. We believe, however, that subepithelial fibrosis is a long and progressive process that could increase over many years, with minimal fluctuation in and out pollen season within a year.

Types IV and VII collagens and laminin constitute the basic framework of the normal basement membrane. Laitinen et al (1993) reported that asthmatic patients showed a significantly thicker area of collagen VII immunoreactivity in their basement membranes. We found no modification of this component in mild asthmatic subjects, but this observation does not preclude the possibility that this collagen type varies with the severity or duration of asthma.

Interstitial collagens are deposited by activated fibroblasts, namely myofibroblasts. These cells are located in the lamina reticularis of the basement membrane. The number of myofibroblasts correlates with the depth of the basement membrane collagen layer, which suggests that they are involved in the airway repair mechanisms and are probably responsible for the observed subepithelial collagen deposition in

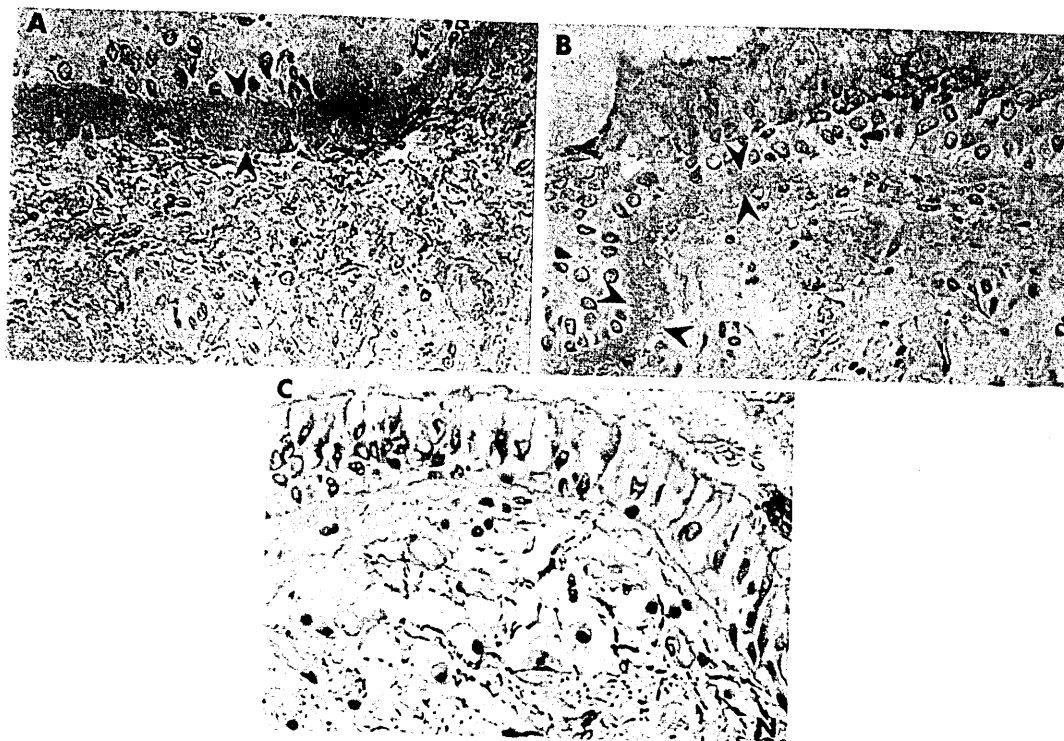


Figure 2.

Type III collagen detection in bronchial mucosa of asthmatic (A), rhinitic (B), and normal (C) subjects. Arrows indicate the deposition of type III collagen in the subepithelial areas of asthmatic and rhinitic subjects. In rhinitic subjects, the immunoreactivity for type III collagen is focally distributed in the basement membrane, and the overall thickness of the collagen band is intermediate between those observed in normal and asthmatic subjects. ($\times 400$).

asthma (Brewster et al, 1990). The increased airway collagen content may, therefore, be the result of the chronic allergic inflammatory process, although its role as a potential contributor to changes in airway function or as a protective mechanism against airway narrowing is uncertain. Microenvironment-derived stimuli, such as cytokines and growth factors released by inflammatory cells, may be responsible for the differentiation and the proliferation of myofibroblasts. Another possibility is that airway changes in collagen and the extracellular matrix contribute to the inflammatory process (Gauldie et al, 1992).

Immunoperoxidase staining of myofibroblasts by PR_2D_3 showed that, in rhinitic subjects, myofibroblasts were localized in the subepithelial area in which the collagen was deposited. Interestingly, these myofibroblasts were positive for desmin, an intermediate filament expressed by smooth muscle cells. This is consistent with the observations of Skalli et al (1989), who reported that myofibroblasts from diverse pathologies were heterogeneous in their content of this intermediate filament protein. They distinguished two groups of lesions: normal healing granulation containing only vimentin-positive cells and hypertrophic scars and fibromatoses in which vimentin- and desmin-positive cells were seen. They suggested that myofibroblasts of pathologic conditions, characterized by a

tissue chronic retraction, express immunochemical features indicative of smooth muscle differentiation.

The accumulation of fibronectin in the basement membrane of asthmatic subjects suggests that this protein may play an important role in the airway repair process. Fibronectin has been reported to mediate fibroblast recruitment (Postlethwaite et al, 1981) and direct cellular attachment (Fukuda et al, 1984), leading to wound healing. It possesses multiple domains recognized by integrins, including the alternately spliced connecting segment domain, which interacts with $\alpha_4\beta_1$ integrin. Neeley et al (1994) demonstrated that $\alpha_4\beta_1$ integrin selectively mediates adhesion of eosinophils to fibronectin and augments stimulated eosinophil degranulation. The relationship between the deposition of fibronectin in the asthmatic basement membrane and eosinophils remains to be determined. In rhinitic subjects, fibronectin content identified two subpopulations, one with no fibronectin accumulation in the basement membrane and the second with a focal deposition of fibronectin. The healing process may be more active in the second group, and the first group possibly represents an early stage of repair with a collagen deposition but no fibronectin.

The focal deposition of types I and III collagens in rhinitic subjects may contribute to the development of

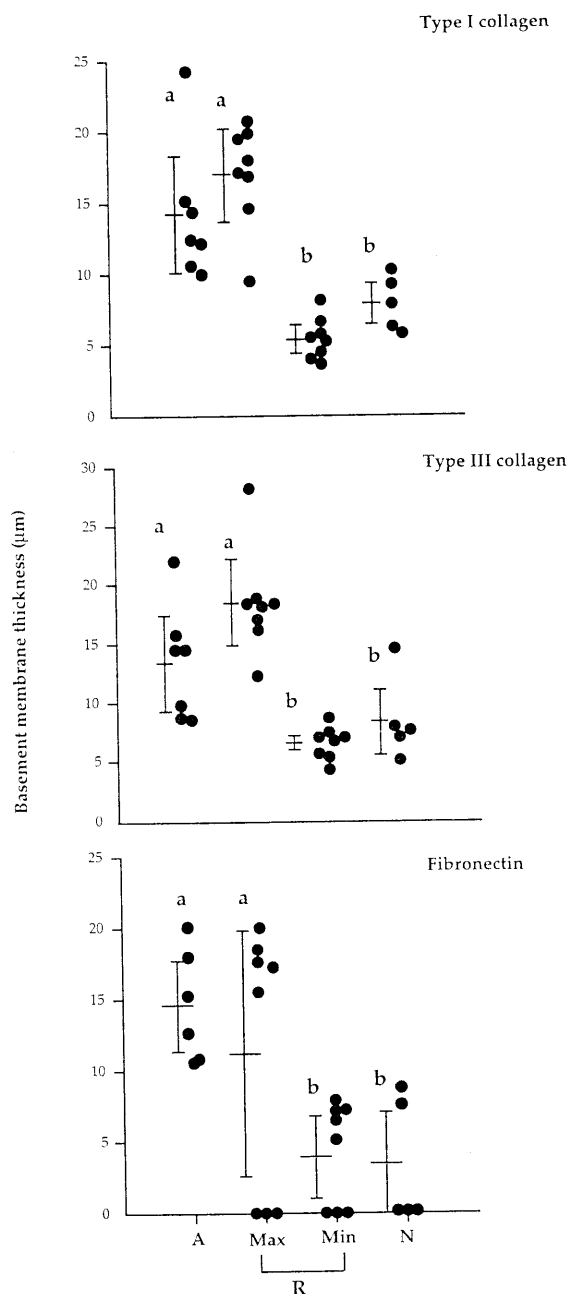


Figure 3.

Thicknesses of types I and III collagens and fibronectin in the bronchial basement membrane area. A, asthma; R, rhinitis; Max, thickest collagen or fibronectin area; Min, thinner collagen or fibronectin area; N, normal. Differences among groups reported using letters, $p < 0.0001$ (ANOVA).

airway hyperresponsiveness that appears in some rhinitic subjects. In a global comparison analysis of subjects with either allergic or occupational asthma, chronic cough, allergic rhinitis, and normal controls, we reported that the degree of subepithelial collagen deposition correlated with the degree of airway responsiveness to methacholine (Boulet et al, 1993). Airway fibrosis may be a determinant or a marker of a process that leads to the development of hyperresponsiveness (Bousquet et al, 1992).

A mild reduction in the apparent basement membrane thickness has been described after withdrawal from sensitizing exposure in asthmatic subjects (Trigg et al, 1994; Jeffery et al, 1992). Corticosteroids are, however, generally considered ineffective in reducing this subepithelial fibrosis although they may reduce inflammation and airway responsiveness (Djukanovic et al, 1991; Juniper et al, 1990). Lundgren (1977) demonstrated that asthmatic subjects who used inhaled corticosteroids for many years had no significant inflammation but still had hyperresponsiveness and subepithelial fibrosis. This suggests that bronchial fibrosis, once established, cannot be significantly reversed. We do not, however, know whether airway wall remodeling of nonasthmatic rhinitic subjects could be reversible or stopped with corticosteroid treatment or adequate allergen avoidance.

Further studies are necessary to evaluate the clinical and histopathologic evolution of nonasthmatic rhinitic subjects to study the relationships between the appearance of airway hyperresponsiveness or symptomatic asthma and the development of bronchial subepithelial fibrosis.

In conclusion, we showed that the irregularly distributed subepithelial fibrosis observed in subjects with allergic rhinitis results from the deposition of types I and III collagens and fibronectin, probably after inflammation-induced bronchial myofibroblasts activation. This suggests the presence of an active structural remodeling in the lower airways of nonasthmatic allergic rhinitic subjects, which may possibly contribute to the development of asthma over time.

Methods

Subjects

Eight subjects (three women, five men) with recurrent seasonal allergic rhinitis and no past or present history of asthma volunteered for this study. They had not used nasal topic corticosteroids within the last 3 months. Bronchial biopsy samples of subjects with rhinitis were compared with those of a group of six subjects with mild seasonal allergic asthma (four women, two men) and a control group of five normal subjects (two women, three men). The diagnosis of asthma was based on the American Thoracic Society (1987) criteria. Asthma was controlled by an inhaled β_2 agonist on demand. None had used inhaled corticosteroids or an other anti-inflammatory medication in the past. The normal subjects had no history of asthma or atopy, negative skin prick tests, and normal pulmonary function tests. All subjects were nonsmokers, and none had a respiratory condition other than

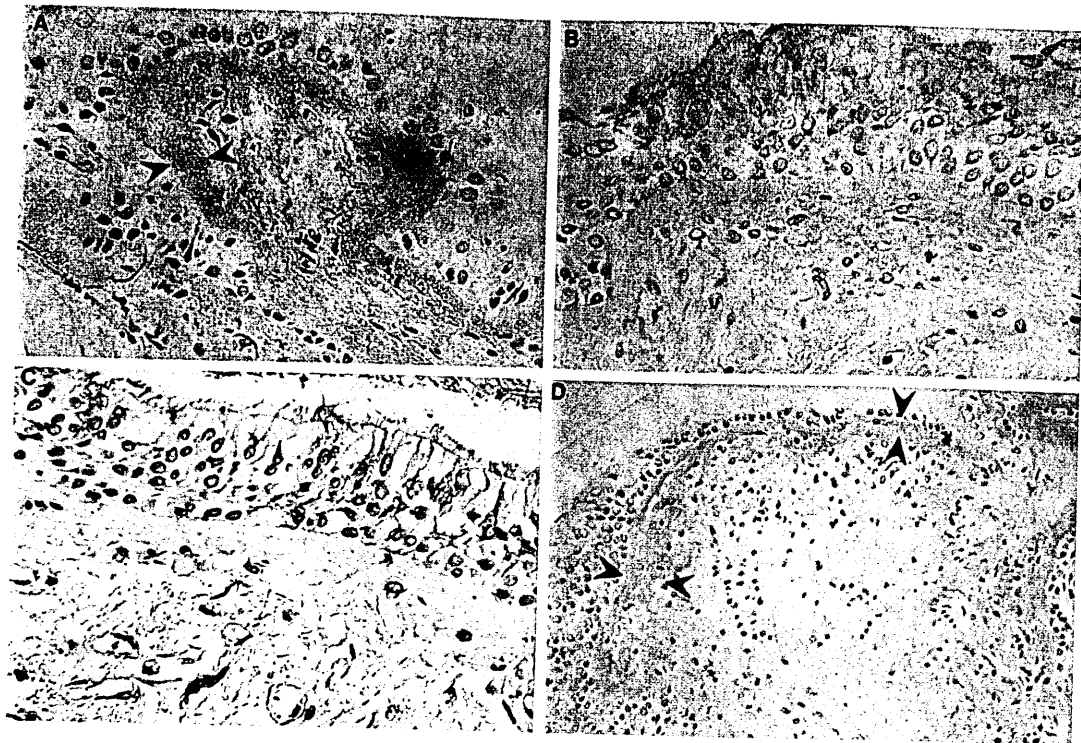


Figure 4.

Fibronectin detection in bronchial tissues of asthmatic (A), rhinitic (B and D), and normal subjects (C). Arrows indicate the distribution of fibronectin underneath the basement membrane of asthmatic subjects. The rhinitic group shows two populations: one (B) without fibronectin accumulation in the basement membrane ($\times 200$), and another (D) with focal deposition of fibronectin (arrows). No fibronectin deposition was detected in the basement membrane of normal subjects (C) ($\times 400$).

asthma or rhinitis. The study was approved by our local ethics committee, and all subjects provided written informed consent.

Baseline Evaluation

All subjects had spirometry and airway responsiveness to inhaled methacholine assessment according to standardized procedures out of the pollen season (Juniper et al, 1991). Briefly, during methacholine challenges, aerosols were generated with a Wright nebulizer (Roxon, Montreal, Quebec, Canada) and inhaled at tidal breathing for periods of 2 minutes (output, $0.13 \text{ ml/minutes}^{-1}$). PC_{20} was determined by interpolation of the last two points of the dose-response curve. Skin prick tests were performed with a battery of common inhaled allergens. Atopy was considered to be present if there was at least one positive response ($\geq 3 \text{ mm}$ wheal at 10 minutes) to the tested allergens.

Bronchoscopy and Bronchial Biopsy Specimens

No subjects had any evidence of respiratory infection for at least 4 weeks before the endoscopies. For rhinitic subjects, biopsies were performed out of the pollen season. Before the bronchoscopy, asthmatic subjects received a $200\text{-}\mu\text{g}$ dose of inhaled salbutamol via a metered-dose inhaler. For all subjects, the

procedure was performed under oxygen at $5 \text{ ml/minutes}^{-1}$ by nasal catheter. After local anesthesia of the throat, larynx, and bronchi with 2% and 4% xylocaine, the flexible bronchoscope (Olympus OES 10 fiberscope, Olympus Optical Company, Ltd, Tokyo, Japan) was introduced into the bronchial tree, and 8 to 10 tissue samples were taken from the carinae of lobar and segmental bronchi on both sides using conventional forceps. Vital signs, electrocardiograph output, and oximetry were recorded throughout the procedure.

Immunohistochemistry

Tissue Processing. Each biopsy sample was immediately placed in acetone containing the protease inhibitors phenylmethylsulfonyl fluoride (2 mM) and iodoacetamide (2 mM) from Sigma Chemical Company (St. Louis, Missouri) and cooled rapidly to -20°C . They were then immersed in acetone at room temperature for 15 minutes and transferred to methylbenzol for another 15 minutes. The biopsy samples were then immersed in glycolmethacrylate (GMA) monomer (Polyscience, Warrington, Pennsylvania) at 4°C for 7 hours. During this time, the GMA solution was changed three times. Finally, the tissue was embedded in GMA resin prepared by mixing GMA monomer and *N, N*-dimethylalanine in PEG 400 and benzoyl peroxide and polymerized overnight at 4°C (Britten et

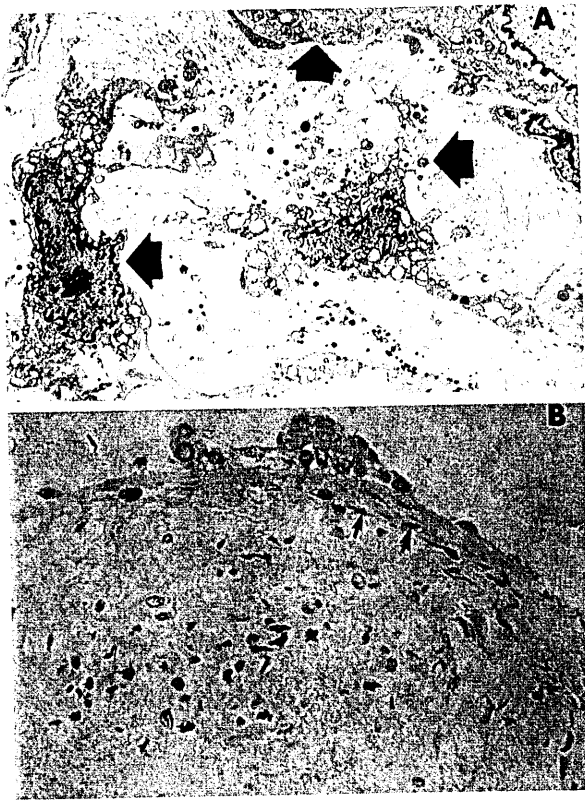


Figure 5.

Electron micrograph of a bronchial biopsy specimen from a rhinitic subject (A) showing elongated myofibroblasts with contractile filaments (arrows) ($\times 2700$). B, Immunoperoxidase staining with PR₂D₃ of bronchial myofibroblasts on bronchial biopsy specimens of a rhinitic subject. PR₂D₃-positive cells are found beneath the bronchial epithelium under the band of collagen ($\times 200$).

al, 1993). Samples embedded in GMA were stored at -20°C in an airtight container. Sections ($2\ \mu\text{m}$) were cut on an ultramicrotome (Leica Reichert Ultracut S, Wein, Austria) and floated on water containing 0.02% ammonia until they were fixed on poly-L-lysine-coated slides.

Immunostaining. The following antibodies (dilutions), from Biodesign (Kennebunk, Maine), were used: rabbit anti-human types I, II, III, IV, and V collagens and mouse anti-human collagen VII (1:50). Mouse anti-human fibronectin (1:400), monoclonal anti- α smooth muscle actin (1:400), monoclonal anti-desmin, a marker of smooth muscle cells (1:400) from Sigma Chemical Company, and PR₂D₃, a monoclonal antibody reported to label a 140-kd membrane component of myofibroblasts and smooth muscle cells (kindly provided by Imperial Cancer Research Technology, London, United Kingdom). Cross-reactions were removed by immune absorption of these antibodies on the other types of collagens, and specificity was tested by immunohistochemistry on sections of human skin.

The first bound antibody was detected using the Vectastain Elite ABC kit from Vector Laboratories (Burlingame, California). Briefly, sample peroxidase

activity background was blocked by incubation with 0.3% H₂O₂, 0.1% sodium azide in Tris buffer solution for 30 minutes and normal serum for 30 minutes. The samples were then incubated overnight with primary antibody (4°C), 1 hour with a biotinylated secondary antibody (23°C), and 30 minutes with the avidin-biotin complex (23°C). The slides were washed three times with Tris buffer solution between steps. Bound antibodies were detected with amino-ethylcarbazole substrate until staining was visible on the microscope. Reaction was stopped by washing in water, and Mayer's hematoxylin was used for counterstaining. Slides were mounted with Crystalmount (Biomed, Foster City, California). Irrelevant isotypic antibodies served as negative controls.

Quantification of Immunohistochemical Stainings

The collagen content of bronchial mucosa was determined for the three groups of subjects by immunoperoxidase staining of serial sections. Each type of collagen was identified using specific antibodies. All slides were assigned a number, and measurements were made independently by two observers. A blinded observer measured the thickness at regular intervals (fixed distance). The thickness of types I and III collagens and of fibronectin in the basement membrane area was measured by calibrated image analysis (Mocha image analysis software; Jandel Scientific, San Raphael, California). Eleven measurements were performed on an unfolded area. The minimal and maximal thickness values from each patient's biopsy specimens were then analyzed and compared with the values obtained from normal and asthmatic subjects.

Electron Microscopy

Samples for electron microscopy analysis were fixed by immersion in Karnovsky's fluid, washed in cacodylate buffer, osmicated, dehydrated in alcohol, and embedded in epoxy resin. Half of the samples were treated en bloc with uranyl acetate. All sections were stained with lead citrate and analyzed using a Jeol 100 CX (Tokyo, Japan) electron microscope. Electron microscopic studies evaluated cellular alterations of epithelial and connective tissue cells and the presence of myofibroblasts. In this study, we did not, however, count these myofibroblasts or correlate their number with the collagen thickness.

Statistical Analysis

Results are presented as the means $\pm$ SEM. PC₂₀ geometric mean values were calculated for each



Figure 6.

Electron micrograph of a bronchial biopsy specimen from a rhinitic subject (A) showing myofibroblasts (MF) along subepithelial collagen (arrow). E, epithelial cells. ($\times 5,000$). B shows high magnification of electron micrograph of myofibroblast cytoplasm with filaments (arrow) ($\times 25,000$).

group. If PC_{20} was $>128 \text{ mg/ml}^{-1}$, it was considered to be 128 mg/ml^{-1} . A one-way ANOVA was applied to the comparison of basement membrane thickness in

our three groups of subjects, and the maximal and minimal thicknesses were also evaluated in the rhinitic groups, as it varied markedly from one site to the

other. Values were transformed by their rank, as normality and homogeneity of variance assumptions were not encountered. Multiple comparisons were preformed using Scheffé's test. Differences between groups were reported using letters. All tests were performed at the 0.05 level of significance.

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