Morphologic and Functional Properties of Bronchial Cells Isolated from Normal and Asthmatic Subjects

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Recent advances in biomedical sciences have led to the development of various methods for the evaluation of the physiopathology of respiratory diseases. This study reports morphologic and functional features of cells isolated by a new method from bronchial biopsies of normal and asthmatic subjects. Both epithelial and fibroblastic cells were isolated from the same biopsies using collagenase. The cells were cultured for several passages and stored frozen. Two selective culture media were used in order to obtain pure epithelial and fibroblastic cell populations. Immunofluorescence analysis of intermediate filaments, keratins, and vimentin confirmed the type of the isolated cells. The proportions of α-actin-expressing cells varied among the fibroblastic cell populations isolated from normal and asthmatic subjects. Interestingly, the population containing high numbers of α-actin-expressing cells and presenting the fastest collagen contraction kinetic was isolated from bronchial biopsies of an asthmatic subject. Moreover, the fibroblastic cells that showed the best contractile properties 24 h after their seeding in floating collagen gels were isolated from bronchial biopsies of asthmatic patients having PC20 values below 1 mg/ml. On the basis of these data, we propose a new approach to isolate, culture and characterize human bronchial cells in vitro.


Asthma is a bronchial disorder characterized by a mucosal inflammatory process and structural changes which are believed to induce airway hyperresponsiveness (1-3). In an analysis of bronchial biopsies from subjects with asthma or other airway diseases, we showed as others, that an apparent basement membrane thickening was observed in asthmatics and that it correlated with the magnitude of airway hyperresponsiveness (4). Ultrastructural and immunologic studies showed that this thickening of the basement membrane was due to the deposition of fibrillar collagen, leading to an increase in the depth and density of the lamina reticularis and should be referred to as subepithelial fibrosis (1, 2, 5, 6).

In normal subjects, bronchial basement membrane is mainly composed of laminin and type IV collagen, to which epithelial cells are attached. The asthmatic subepithelial fibrosis is due to the abnormal deposition of type III collagen and fibronectin (6). Brewster and colleagues showed that this collagen layer contained myofibroblasts and proposed that these cells were responsible for the characteristic subepithelial fibrosis seen in asthma (7). Their origin is not yet known but these cells may play a crucial role in the healing of damaged bronchial tissues, as it is postulated in the skin (8, 9). There are several reasons to believe that fibroblasts play an important role in the airway structural changes in asthma. First, they may secrete large quantities of collagen, as it is found in asthmatic airways. Secondly, cytokines released by inflammatory and/or epithelial cells are known to modulate fibroblast functions such as proliferation and collagen synthesis (10). It has been suggested that fibroblasts are also capable of producing cytokines and going through phenotypic changes that may perpetuate the bronchial inflammatory and fibrotic processes (11, 12). Nevertheless, the cellular and molecular mechanisms involved in the subepithelial airway fibrosis are still poorly understood.

Fibroblasts isolated from various tissues share a very interesting functional property: they can contract a collagen gel (13, 14). The modulation of this fibroblast function by various exogenous factors added alone or in combination to the culture media can be analyzed and compared upon cul-
In the present study, we report a new method to isolate and culture human epithelial and fibroblastic cells obtained from normal and asthmatic bronchial biopsies. These cell cultures can be useful to better characterize the functional state of these cells in asthma, in order to understand the cellular events involved in the remodeling of bronchial wall in this disease.

Materials and Methods

**Subjects: Selection and Evaluation**

Nonsmoking asthmatic and normal subjects ages 20 to 45 yr, evaluated at the Laval Hospital asthma clinic, were enrolled in the study.

**Asthmatic subjects.** All had a diagnosis of asthma according to the American Thoracic Society criteria (19). All were atopic with at least one positive response to common allergens on allergy skin prick tests. Their asthma required only an inhaled β-2 agonist agent on demand. None of the subjects reported a respiratory infection nor an increase in days with respiratory symptoms reported a respiratory infection nor an increase in days with respiratory symptoms.

**Normal subjects.** They all had a provocative concentration of methacholine inducing a 20% fall in FEV₁ (PC₂₀) over 16 mg/ml. The study was approved by our local Ethics Committee and subjects had given informed written consent.

**Spirometry and response to inhaled methacholine** were measured according to standardized procedures using aerosols generated with a Wright's nebulizer at tidal breathing for periods of 2 min (output = 0.13 ml/min) (20). The PC₂₀ was determined. Skin prick tests were performed with a battery of common airborne allergens. Atopy was considered to be present if there was one or more positive response (> 3 mm wheal) to the inhalant allergens, with a positive reaction to histamine phosphate but not the diluent.

**Bronchoscopy and Bronchial Biopsies**

For asthmatic subjects we obtained the bronchial biopsies by bronchoscopy. Before the bronchoscopy, a 200-μg dose of salbutamol was given using a metered-dose inhaler. All subjects received oxygen at 5 L/min by nasal catheter during bronchoscopy. After local anaesthesia of the throat, larynx, and bronchi with 2% and 4% xylocaine, the flexible bronchoscope (Olympus OES 10 fiberscope; Olympus, Markham, Ontario, Canada) was introduced into the bronchial tree and 10 bronchial biopsies were taken from the carinae of the right upper and middle lobes and the segmental bronchi of the upper and lower lobes on both sides using conventional forceps (21). Vital signs, electrocardiograph, and oximetry were recorded throughout the procedure.

**Bronchial Biopsy Processing**

The biopsies were kept at 4°C until use. The processing was performed within 2 to 3 h after the bronchoscopy. Collagen being the major constituent of bronchi matrix, collagenase was chosen to digest the collagen matrix of the biopsies. The human bronchial biopsies were digested in 0.1% (0.2 U/ml) collagenase H (Boehringer Mannheim, Montréal, Québec, Canada) prepared in Dulbecco-Vogt modification of Eagle's medium (DME) containing 10 mM CaCl₂; without any supplement. Tissues were digested overnight at 4°C. A collagenase digestion performed overnight at 37°C would reduce cell yield and viability, compared with the digestion carried out at 4°C under the same conditions. Shorter digestion periods did not lead to 100% cell isolation (at 4°C and 37°C), as monitored by histologic observations of the undigested tissues under microscope. For normal subjects we obtained bronchial biopsies using conventional bronchoscopy forceps from surgical specimens of subjects without pulmonary or systemic disease and undergoing a lobectomy for a benign noninflammatory lung lesion.

**Homogenates were centrifuged for 10 min at 300 g and the cell pellets were resuspended in DME supplemented with 10% fetal calf serum (FCS).** All the cells were plated in 35-mm petri dishes (Falcon; Becton Dickinson, Oxnard, CA). Half of the cells were cultured in epithelial cell culture medium and the other half was plated in fibroblastic cell culture medium.

**Bronchial Cell Culture**

Bronchial epithelial cells were cultured according to the method for human keratinocytes (22) originally described by Green and coworkers (23). The medium consists of a combination of DME with Ham's F12 in a 3:1 proportion (Gibco BRL; Life Technologies™, Grand Island, NY), supplemented with 10 ng/ml human epidermal growth factor (EGF; Chiron Corp., Emeryville, CA), 24.3 μg/ml adenosine, 5 μg/ml crystallized bovine insulin, 5 μg/ml human transferrin, 2 × 10⁻⁸ M 3,3',5'-triiodo-L-thyronin (Sigma Chemical Co., St. Louis, MO), 0.4 μg/ml hydrocortisone (Calbiochem, La Jolla, CA), 10⁻⁶ M cholera toxin (Schwarz/Mann, Cleveland, OH), 10% FCS (Gibco BRL; Life Technologies Inc., Grand Island, NY), 100 IU/ml penicillin G and 25 μg/ml gentamicin (Sigma). The culture medium was changed three times a week. After 8 to 12 days in culture, bronchial epithelial cells had reached 85% confluency and were ready to be stored and subcultured. Human bronchial fibroblastic cells were isolated using the method described above. The cells were cultured in DME supplemented with 10% FCS (Gibco), 100 IU/ml penicillin G, and 25 μg/ml gentamicin (Sigma). Culture medium was changed three times a week. Cultures of epithelial and fibroblastic cells were both kept in an 8% CO₂ atmosphere at 37°C.

**Human Dermal Fibroblast Isolation and Culture**

Human dermal fibroblasts were isolated from skin biopsies removed during reductive breast surgery of healthy subjects. The skin specimens were processed according to a method previously published (22, 24). These cells were cultured and subcultured like bronchial fibroblastic cells, as described earlier. Each collagen gel contraction experiment was done with cells cultured for five passages.

**Indirect Immunofluorescence**

Confluent cell layers, grown on glass slides, were fixed for 10 min in methanol at −20°C, washed with phosphate-buffered saline (PBS) and overlaid with specific antibody diluted with PBS containing 1% of bovine serum albumin (BSA) (B.D.H. Chemicals, Toronto, Ontario, Canada) for 45 min at room temperature. The cells were then rinsed 3 times with PBS and overlaid with the corresponding fluorescein-conjugated antibody for 30 min. They were rinsed and...
mounted. In negative controls, the primary antibody was omitted. The slides were analyzed under a Nikon Optiphot fluorescence microscope and photographed using Kodak Tmax 400 ASA film. The expression of α-actin was also assessed on all fibroblastic cell populations isolated from normal and asthmatic bronchial biopsies. Human dermal fibroblasts isolated from normal skin were tested for comparative purposes. Positively labeled cells of each population (minimum of 500 cells) were counted directly under the microscope.

Mouse monoclonal anti-vimentin antibody (dilution 1/50) was kindly provided by Dr. Normand Marceau (Hôpital-Dieu de Québec Research Centre, Québec City, Québec, Canada) (22, 25). Mouse monoclonal anti-epithelial basic keratin antibody (AE3) (dilution 1/50) was purchased from ICN (Costa Mesa, CA). Mouse monoclonal antibody directed specifically against the c-terminal portion of α-actin was bought from Sigma. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG-IgM (dilution 1/100) was purchased from Cedarlane Labs (Hornby, Ontario, Canada).

**Comparative Study of the Contractile Properties of Fibroblasts Isolated from Normal and Asthmatic Bronchial Biopsies**

The cell-populated collagen gels were prepared according to the method of Bell and coinvestigators (16). A stock solution of bovine type I collagen (Sigma, type IID) was prepared by dissolving 4.22 mg/ml of the powder overnight at 4°C in sterile 0.017 M acetic acid. A solution of 5.6 ml of DME 2.7 × containing 200 IU/ml penicillin G and 50 μg/ml of gentamicin, pH 8.0, was mixed with a second solution containing 3.7 ml of FCS, 9.5 ml of the stock collagen solution, 0.2 ml of NaOH 0.7N, and 1 ml of a fibroblast suspension (2.5 × 10⁶ cells/ml). This mixture was quickly distributed in 60-mm diameter bacteriologic petri dishes (5 ml/dish). The gels were covered with 2 ml of DME supplemented with 10% FCS, 100 IU/ml penicillin G, and 25 μg/ml gentamicin after collagen polymerization. The final concentration of bovine type I collagen in gels was always 2 mg/ml. Experiments were done 3–4 times in sets of 5 gels per fibroblastic cell line tested.

**Measure of the Gel Surface**

The diameter of each collagen gel was directly measured daily over a period of 9 days, as previously reported (26). The total surface area of each gel was determined using the following formula: \[ \frac{\pi D^2}{4} \] where \( \pi \) is a constant (3.1416) and \( D \) corresponds to the gel diameter. The statistical comparison of the data was performed according to an analysis of variance (ANOVA) profile. Correlation between the values of patients’ \( PC_{20} \) and the contractile properties of their bronchial fibroblastic cells was analyzed using the Pearson’s test.

**Results**

\( PC_{20} \) was < 16 mg/ml in all asthmatic subjects, including two patients in whom \( PC_{20} \) values were below 1 mg/ml (BAF.3003-1: 0.59 and BAF.0610: 0.38). All had positive allergic prick skin test. Collagenase digestion of human bronchial biopsies led to the isolation of cell populations from several volunteers (Table 1). Human epithelial and fibroblastic primary cultures established from bronchial biopsies of asthmatic subjects are shown in Figures 1A and B, respectively. The conditions defined for bronchial tissue enzymatic digestion allowed us to observe in two samples the numerous cilia associated with most of the epithelial bronchial cells, under phase contrast microscopy. Cell populations were stored by cryopreservation after one or two passages and retained their proliferative potential when thawed and put back into culture.

Bronchial epithelial cells (Figure 1A) were cultured under conditions used for human keratinocytes, allowing the maintenance of their proliferative and morphologic properties for at least four passages in culture. Fibroblastic cells isolated from normal and asthmatic bronchial tissues adopted an elongated morphology (Figure 1B) that was maintained for more than 5–9 passages in culture. Interestingly, both epithelial and fibroblastic bronchial cells, isolated from collagenase digestion of human biopsies, showed characteristic cell type features, suggesting that the present method repeatedly led to the isolation of homogeneous cell populations.

Immunofluorescence analyses were done on all populations of epithelial and fibroblastic bronchial cells, at several consecutive passages in culture, using both anti-keratins (AE3) and anti-vimentin antibodies. The epithelial cell populations were all positively labeled with the AE3 antibody (Figure 2A), whereas no keratin labeling was ever detected on fibroblastic cells (Figure 2D). All the fibroblastic cells were positively stained with the anti-vimentin antibody (Figure 2E), in contrast with the negative background observed.

### Table 1

**Asthmatic volunteers’ characteristics and isolated cell types from bronchial biopsies**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>FEV₁ (% pred)</th>
<th>( PC_{20} ) (mg/ml)</th>
<th>Epithelial: 100% Keratins+</th>
<th>Fibroblastic Cells: 100% Vimentin+</th>
<th>α-actin+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>23</td>
<td>83</td>
<td>0.38</td>
<td>None isolated</td>
<td>BAF.0610</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>25</td>
<td>95</td>
<td>2.33</td>
<td>BAF.0710</td>
<td>BAF.0710</td>
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<tr>
<td>F</td>
<td>20</td>
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<td>12.78</td>
<td>BAF.2601</td>
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<tr>
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</tr>
<tr>
<td>M</td>
<td>20</td>
<td>98</td>
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<td>BAF.3003-2</td>
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</tr>
<tr>
<td>M</td>
<td>45</td>
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<td>0.59</td>
<td>BAF.3003-1</td>
<td>BAF.3003-1</td>
<td>16.2</td>
</tr>
</tbody>
</table>
with the epithelial cell labeling (Figure 2B). (Only a few epithelial cells [less than 5%] were weakly positively labeled; data not shown.) Note that immunofluorescence results obtained with all normal and asthmatic cell lines were similar, except for the anti-α-actin labeling that varied among the different fibroblastic cell populations, as shown in Table 1. The percentages of α-actin-expressing cells were assessed in all fibroblastic cell populations isolated from normal and asthmatic tissues. Not more than 5% of the fibroblastic cells isolated from normal bronchial biopsies expressed α-actin. However, the highest percentages of α-actin-positive cells (16.2 and 21.6%) were obtained in two of the populations (BAF.3003-1 and BAF.2601) established from asthmatic bronchial biopsies (Table 1 and Figure 2F).

**Contraction of Collagen Gels by Human Bronchial and Dermal Fibroblastic Cells**

Comparative studies between fibroblasts isolated from normal and asthmatic bronchial biopsies and normal skin were carried out to see if there was any difference between the respective ability of these cells to contract collagen fibrils in floating gels. The kinetics of collagen gel contraction varied...
from one cell population to another among the fibroblastic cells isolated from asthmatic bronchial biopsies (Figure 3A). For instance, some fibroblastic cell populations such as BAF0710 and BAF3003-2 contracted the gels very slowly compared with BAF.2601, BAF.1901, and BAF.3003-1. Nevertheless, all cells originating from the different subject biopsies could reduce the surface of collagen gels to less than 30% of their initial size within 8 days, independently of their relative content in α-actin-labeled cells (varying from 0 to 21.6%, Table 1). Interestingly, one of the cell population that did not express α-actin, BAF0610, stopped contracting the collagen gels 2 days after seeding (Figure 3A). However, the gels seeded with BAF0610 and BAF.3003-1, containing a high percentage (at least 16%) of α-actin-expressing cells contracted the gels more rapidly than all the other cell populations within 24 h. Moreover, both of these fibroblastic cell populations were isolated from bronchial biopsies of asthmatic patients having PC_{20} values below 1 mg/ml.

Kinetics were not really different between human dermal and bronchial fibroblastic cells isolated from healthy subject biopsies (data not shown). However, the means of the surfaces of the gels seeded with asthmatic cells were significantly different than the means obtained from gels populated with normal bronchial cells (Figure 3B), during the two first days of the experiments. Moreover, during the two first days following cell seeding in the gels, a clear correlation was found between the low values of patients' PC_{20} (Figure 3B; insert) and the high contractile properties of their bronchial fibroblastic cells. Photographs of each group of cell-populated collagen gels were taken daily for data records. Figure 4A shows the macroscopic aspect of the gels of all groups, contracted after 1 day in culture. These pictures help at visualizing the final collagen gel surface contracted by the cells, confirming the results expressed in Figure 3.

Histologic Analyses of the Contracted Collagen Gels

Histologic analyses were done on all collagen gels contracted by the various fibroblastic cell populations in vitro. As shown in Figure 4B, most fibroblastic cells within the floating gels were oriented perpendicular to tissue length. No difference was observed between the histologic organization of the collagen gels seeded with fibroblastic cells isolated from dermis and normal or asthmatic bronchial biopsies.

Discussion

Fibroblastic cell lines were previously obtained from patients with clinically active interstitial lung disease (27). Bronchial and lung biopsies were used as explants to isolate epithelial and fibroblastic cells in vitro from hamsters and humans (28, 29). However, with these techniques, cells are selected on the basis of their ability to migrate and there is a possibility of getting clones that are not necessarily representative of the total tissue cell population. In this study, we describe the conditions for enzymatic digestion of bronchial biopsies and cell culture to selectively isolate bronchial epithelial and fibroblastic cells in vitro. These cells were characterized in order to pursue comparative studies between normal and asthmatic human bronchial cells. The data reported here are very promising because they indicate that stable cell populations can be obtained and used as material for studies of the fundamental mechanisms involved in asthma using normal cells as control. The protocol could also be adapted for the isolation of human bronchial cells from biopsies of subjects suffering from other lung diseases where the airway inflammation and remodeling are involved. These data suggest that bronchial biopsy sampling is sufficient to allow isolation of human bronchial cells in vitro. Electron microscopy
centrations in culture can greatly influence the kinetics of contraction of collagen gels (14, 26). These parameters were constant in all the experiments performed and reported here. Dermal and several other types of fibroblasts share common properties: they secrete collagen, reorganize and contract the extracellular matrix components (13, 16, 17, 24, 30, 31). Our data indicated that human bronchial fibroelastic cells can also exert extracellular matrix remodeling by their capacity to reorganize and orient collagen fibers within floating collagen gels. Indeed, Gabbiani and coworkers (9) have shown the existence of myofibroblasts during cutaneous wound healing. These cells generally express higher levels of α-actin and show greater capacity to contract collagen fibrils than fibroblasts (9, 26). Our data indicated that the percentage of α-actin-expressing cells could vary in the various normal (2−5%) and asthmatic (0−21.6%) bronchial cell lines isolated in our laboratory. The population containing high numbers of α-actin-expressing cells and presenting the fastest collagen contraction kinetics was isolated from bronchial biopsies of an asthmatic subject (BAF.3003-1). We also observed that the only cell line that did not express α-actin (BAF.0610) did not succeed at contracting the collagen gels as much as all the other cell lines. However, these cells showed higher contractile properties within the first two days after seeding than the other cell lines, suggesting that high α-actin expression may not be necessary for the cells to have high collagen contractile properties. These observations are limited to a too little number of cell lines to draw any general relation between α-actin expression, collagen contraction kinetics, and the type of tissue of cell origin (normal or asthmatic). Such relations might arise in the future, following cell isolation from bronchial biopsies of several normal and asthmatic subjects. Nevertheless, our data strongly suggest that some functional properties of bronchial cells are modulated with the severity of asthma (PC_{20}). For instance, two cell lines, BAF.3003-1 and BAF.0610 (16% and 0% of α-actin-expressing cells, respectively), were isolated from bronchial biopsies of asthmatic patients having PC_{20} values below 1 mg/ml. Both cell populations contracted collagen gels more rapidly, within 24 h after seeding, than all the other cell lines. These data show a correlation between the low values of asthmatic patients' PC_{20} and the high contractile properties of their bronchial fibroelastic cells during the two first days after cell seeding. Such correlation will be assessed on higher numbers of fibroelastic cell lines that will be isolated from wider groups of subjects in the future.

Our results are promising for the characterization and comparative functional analyses of normal and asthmatic cells in culture. The methodology described here allowed us to obtain precious material to pursue several studies, notably to analyze the epithelial−mesenchyme interactions using normal and asthmatic cells in vitro.

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