An *ex vivo* model of severe asthma using reconstituted human bronchial epithelium

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Background: Structural changes to the airways are features of severe asthma. The bronchial epithelium facilitates this remodeling process. Learning about the changes that develop in the airway epithelium could improve our understanding of asthma pathogenesis and lead to new therapeutic approaches.
Objective: We sought to determine the feasibility and relevance of air-liquid interface cultures of bronchial epithelium derived from endobronchial biopsy specimens of patients with different severities of asthma for studying the airway epithelium.
Methods: Human bronchial epithelial cells derived from endobronchial biopsy specimens of patients with mild and severe asthma were maintained in culture for 21 days in an air-liquid interface to reproduce a fully differentiated airway epithelium. Initially, features of remodeling that included epithelial and subepithelial layers, as well as mucus production, were assessed in paraffin-embedded endobronchial biopsy specimens to evaluate morphologic characteristics of asthmatic patients' epithelia. *Ex vivo* differentiated epithelia were then analyzed for morphology and function based on ultrastructural analysis, IL-8 release, lipoxin A_4 generation, mucin production, and lipoxygenase gene expression.
Results: Morphologic and inflammatory imbalances initially observed in endobronchial biopsy specimens obtained from patients with severe or mild asthma persisted in the air-liquid interface reconstituted epithelium throughout the differentiation process to 21 days. Epithelium from patients with severe asthma produced greater levels of mucin, released more IL-8, and produced lower levels of lipoxin A_4 than that from patients with mild asthma. Expression of 15-lipoxygenase 2 was increased in epithelium from patients with severe asthma, whereas expression levels of MUC5AC, MUC5B, 5-lipoxygenase, and 15-lipoxygeanse 1 were similar to those of patients with mild asthma.
Conclusion: *Ex vivo* cultures of fully differentiated bronchial epithelium from endobronchial biopsy specimens maintain inherent phenotypic differences specifically related to the severity of asthma. (J Allergy Clin Immunol 2012;129:1259-66.)

Key words: *Epithelium, inflammation, resolution, asthma severity*

Asthma is caused by inflammation of the airways and has high prevalence worldwide.[1,2] However, the specific mechanisms that mediate its induction and persistence are unclear. Patients with severe asthma have major handicaps in their daily life, and their care accounts for most of the health care costs associated with asthma.[3] Although airway inflammation,[4] structural changes of the bronchi,[5] and impairments in mechanisms that resolve inflammation[6] are important features of severe asthma, little is known about the mechanisms responsible for the severity and persistence of the disease. We developed an *ex vivo* model of asthma using endobronchial biopsy specimens from patients with severe asthma to study epithelial alterations associated with asthma severity.

We focused on the bronchial epithelium in developing a model system because, in addition to its barrier function, the bronchial epithelium regulates inflammatory and immunomodulatory responses of the innate and adaptive immune systems.[7] Activation and damage of the epithelium, differentiation of goblet cells, and increased production of mucus are features of asthma that vary with disease severity.[8] We previously reported that patients with severe asthma had a thickened epithelial layer with cells that express markers of activation and repair.[9,10] These findings were confirmed in a study of endobronchial biopsy specimens by the Severe Asthma Research Program (SARP).[11] Biopsy specimens from patients with severe asthma had thickened airway epithelium and reticular basement membrane (RBM),[9] goblet cell hyperplasia, mucus hypersecretion,[12,13] and increased expression of MUC5AC and MUC5B (the major proteins of airway mucus) compared with tissues from patients with mild asthma.[14,15]

Other changes in patients with asthma, such as the recruitment of inflammatory cells, are thought to be partly mediated by chemokines produced by epithelial cells, such as IL-8. Severe asthma has been associated with persistent activation of inflammation by factors such as IL-8, levels of which are increased in bronchoalveolar lavage (BAL) samples[16] and supernatants of induced sputum from patients.[17,18] This increase in inflammatory activity was associated with reductions in endogenous anti-inflammatory factors; levels of lipoxin A_4 (LXA_4) were reduced in samples from patients with severe asthma compared with levels seen in patients mild asthma.[19,20] Lipoxins are produced in a process that involves lipoxygenase (LO) enzymes: 5-LO is produced by inflammatory cells, and 15-LO is produced by structural cells, such as bronchial epithelial cells.[21-23] Other studies have shown bronchial epithelial cells from patients with severe asthma obtained by brushing have increased expression of 15-LO1, which is associated with overproduction of mucus.[24]

Structural differences in tissues and imbalances between proinflammatory and anti-inflammatory factors in patients with severe asthma have been identified in cross-sectional studies that analyzed BAL fluid, induced sputum, and bronchial biopsy

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Abbreviations used
BAL: Bronchoalveolar lavage
ELLA: Enzyme-linked lectin assay
HSPBS: High-salt PBS
LO: Lipoxygenase
LXA_4: Lipoxin A_4
RBM: Reticular basement membrane

samples. These have also been reported in animal models of airway inflammation and remodeling.

We aimed to confirm these features of severe asthma using *ex vivo* reconstituted epithelia derived from endobronchial biopsy specimens obtained during bronchoscopies of patients with severe asthma. We investigated morphologic and functional properties of the epithelial tissue to assess differences associated with asthma severity. We examined whether these characteristics persisted in air-liquid interface cultures.

METHODS

Additional details are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Patients with mild (n = 7) and severe (n = 12) asthma were recruited at the Arnaud de Villeneuve Hospital, Montpellier, France. Asthma was diagnosed based on the presence of clinical features consistent with asthma and objective measures of variable airflow obstruction investigated 15 minutes before bronchoscopy (improvement in FEV_1 of ≥15% after inhalation of 200 μg of salbutamol). Asthma severity was classified as mild or severe by using the current Global Initiative for Asthma guidelines (www.ginaasthma.org). Patients with severe asthma met the American Thoracic Society criteria for refractory asthma.[10] At the time of collection of endobronchial biopsy specimens, patients were free from respiratory tract infections and asthma exacerbations for at least 6 weeks. All patients were current nonsmokers; those who smoked in the past had a history of less than 5 pack years.

The study protocol was approved by the ethics committee of our institution, and all patients agreed to participate by reading and signing written informed consent forms. All measurements and experiments were performed in a blinded fashion. Assessment of bronchial biopsy specimens and cultured epithelia were performed by 2 independent observers.

Endobronchial biopsy specimens

All subjects underwent flexible bronchoscopy, and 2 biopsy specimens were collected from each patient by using alligator forceps (Olympus, Tokyo, Japan) on a subsegmental bronchus of the left lower lobe, as previously described.[25] Briefly, after subcutaneous atropine (0.25 mg) and midazolam (5 mg) premedication, nasal and buccal cavity local anesthesia was administered (Lidocaine [AstraZeneca, Paris, France] 1% to 5%; maximal quantity, 300 mg), followed by insertion of a fiberoptic bronchoscope (Olympus BF20; Olympus, Tokyo, Japan) into the trachea through the nose.

Measurements of basement membrane and epithelial thickness and mucus production

One biopsy specimen from each patient was fixed in neutral 4% formaldehyde buffer and embedded in paraffin. Four to 6 sections were fixed on a slide and stained with hematoxylin and eosin or Periodic acid–Schiff. RBM and epithelial thicknesses were expressed as the average area/length ratio by using the Wilson method, as previously described,[26] and the number of goblet cells was quantified as a percentage of total epithelial cells. All measurements were generated with a light microscope at ×400 magnification (Olympus TH2) with a CCD camera (Sony DXC950P; Sony, Tokyo, Japan) and image analysis program (ImageJ; National Institutes of Health, Bethesda, Md). The pixel resolution was approximately 0.02 μm.

Human bronchial epithelial cell primary cultures and the air-liquid interface

Primary normal human bronchial epithelial cells were obtained from bronchial biopsy specimens and cultured under air-liquid interface conditions, as described previously.[27] Briefly, bronchial epithelial biopsy tissue was dissociated and suspended in bronchial epithelial growth medium (Lonza, Basel, Switzerland). After seeding in multiwell plates coated with a solution of fibronectin and collagen, cells were expanded in a flask (0.75 cm^2) and then plated (200,000 cells per well) on uncoated nucleopore membranes (24-mm diameter, 0.4-μm pore size, Transwell Clear; Costar, Cambridge, Mass) in a 1:1 mixture of bronchial epithelial growth medium and Dulbecco modified Eagle medium (Invitrogen, Carlsbad, Calif) applied at the basal side only to establish the air-liquid interface. Cells were maintained in culture for 21 days to obtain a differentiated cell population with a mucociliary phenotype.

We performed a semiqualitative analysis of each epithelial sample obtained from the bronchial biopsy specimens using light microscopy, assessing epithelial morphology, the presence of ciliated cells, and mucus production.

Transmission electron microscopy

Inserts with *ex vivo* differentiated epithelium were processed to observe cell morphology by using transmission electron microscopy. Cells were immersed in a solution of 3.5% glutaraldehyde in phosphate buffer (0.1 mol/L, pH 7.4) overnight at 4°C. They were then rinsed in phosphate buffer and postfixed in a 1% osmic solution with 0.8% potassium ferrocyanide for 2 hours in the dark at room temperature. After 2 rinses in a phosphate buffer, cells were dehydrated in a graded series of ethanol solutions (30% to 100%). Cells were embedded in EmBed 812 DER 736. Thin sections (85 nm; Leica-Reichert Ultracut E; Leica, Wetzlar, Germany) were collected at different levels of each block. These sections were counterstained with uranyl acetate and lead citrate and observed with a Hitachi 7100 transmission electron microscope (Hitachi, Tokyo, Japan) in the Centre de Ressources en Imagerie Cellulaire de Montpellier (Montpellier, France).

Measurement of mucin secretion in apical washes

After the reconstituted epithelium was cultured for 21 days, the apical surface was washed once rapidly with warmed PBS (500 μL) to mimic BAL, and the solution was removed immediately. Mucin concentration in the wash samples was assessed by using an enzyme-linked lectin assay (ELLA), which is a test based on mucin's affinity to lectin. Briefly, 96-well plates (Nunc MaxiSorp, Dutscher, France) were coated with *Helix pomatia*–lectin for 1 hour at 37°C and then washed with high-salt PBS (HSPBS; PBS, 0.5 mol/L NaCl, and 0.1% Tween 20). Samples were added for 30 minutes at 37°C before washing the plate with HSPBS. Porcine stomach mucin was used as a standard. *H pomatia*–lectin conjugated to horseradish peroxidase (1 μg/mL) was then incubated for 30 minutes at 37°C. After washes with HSPBS, the substrate (*o*-phenylenediamine dihydrochloride; SIGMA*FAST* OPD) was added to each well at room temperature, followed by H_2SO_4 (0.2 mol/L) to terminate the reaction. The OD was measured at 492 nm. All products used in the ELLA were from Sigma-Aldrich (St Louis, Mo).

Measurement of IL-8 and LXA_4 levels in apical washes

IL-8 and LXA_4 levels were measured in wash samples from the apical surface of air-liquid interface cultures to determine the inflammatory or anti-inflammatory state of the epithelium. IL-8 levels were measured with an ELISA kit (Diaclone, Stamford, Conn). Cells were incubated with 250 ng/mL 5(S),6(R)-dihydroxy-7,9-trans-11,14-cis eicosatetraenoic acid (5(S),6(R)-diHETE (Cayman Chemical, Ann Arbor, Mich) for 15 minutes at 37°C in the basal compartment and then washed to measure LXA_4 production. LXA_4 levels were measured with a specific ELISA (Interchim, Montlucon, France).