

Abbreviations used

BAL: Bronchoalveolar lavage
 ELLA: Enzyme-linked lectin assay
 HSPBS: High-salt PBS
 LO: Lipoxigenase
 LXA₄: Lipoxin A₄
 RBM: Reticular basement membrane

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 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 FEV₁ of $\geq 15\%$ 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 $\times 400$ 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 cm²) 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 semiquantitative 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 post-fixed 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 Ultratracut 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 $\mu\text{g}/\text{mL}$) was then incubated for 30 minutes at 37°C. After washes with HSPBS, the substrate (*o*-phenylenediamine dihydrochloride; SIGMAFAST OPD) was added to each well at room temperature, followed by H₂SO₄ (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 LXA₄ levels in apical washes

IL-8 and LXA₄ 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 (Diacclone, Stamford, Conn). Cells were incubated with 250 ng/mL

TABLE I. Patients' characteristics

	Control subjects (n = 5)	Patients with mild asthma (n = 7)	Patients with severe asthma (n = 12)	P value
Age (y)*	48.5 (20-64)	34 (21-63)	54 (37-76)	NS
Sex (% female)	33	14	42	NS
Control of asthma (%)	NA	100	0	.0001
Exacerbations (last year)*	NA	0	5 (3-12)	.0003
Best FEV ₁ (% predicted)*	100 (98-122)	100 (93-117)	89 (45-110)†	.008
Blood eosinophils/mm ³	170 (130-220)	370 (186-430)	344 (100-1800)	NS
Allergy (%)	0	100	58	.001
Sinusitis (%)	0	100	100	.001
GERD (%)	0	0	15	NS
Comorbid condition, ≥1 (%)‡	0	14	50	NS
Long-term OCS (%)	0	0	75	.007

Comparisons were made by using the Kruskal-Wallis test with adequate posttest corrections.

GERD, Gastroesophageal reflux disease; NA, not applicable; NS, not significant; OCS, oral corticosteroid.

*Values are presented as medians (minimums-maximums).

† $P < 0.01$ vs control subjects and patients with mild asthma.

‡Systemic blood hypertension, osteoporosis, sleep apnea syndrome, and obesity.

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 LXA₄ production. LXA₄ levels were measured with a specific ELISA (Interchim, Montlucon, France).

PCR analysis of gene expression

Total RNA was purified from cell lysates (RNeasy; Qiagen, Valencia, Calif), and cDNA was synthesized (Ready-to-Go RT-PCR beads; Amersham, Piscataway, NJ). Semiquantitative analysis of expression of human *MUC5AC*, *MUC5B*, *15LO1*, *15LO2*, and *5LO* was determined by using specific primers (Eurofins MWG Operon, Nantes, France), with β -actin as the internal control.

Statistical analysis

Statistical analysis was performed with the SPSS statistical system (IBM SPSS Statistics, version 15; SPSS, Inc, Chicago, Ill). Nonparametric statistical tests were used when data followed nonnormal distribution. Differences between 2 groups were analyzed by using the Mann-Whitney *U* test, and differences among the 3 groups were analyzed by using the Kruskal-Wallis test, followed by a *post hoc* Bonferroni analysis. The χ^2 test was used to evaluate dichotomous characteristics of patients.

RESULTS

Demographic data

Bronchial biopsy specimens were obtained from 19 subjects (7 with mild and 12 with severe asthma). Demographic characteristics are reported in Table I. Patients with severe asthma had greater airflow impairment (FEV₁ percentage) than those with mild asthma, and their symptoms were uncontrolled (daily symptoms and exacerbation rate) despite treatment. Biopsy specimens were analyzed from 5 control subjects (ie, no asthma, no allergies, nonsmokers, with normal results from chest radiographs and pulmonary function tests who underwent bronchoscopy for various purposes: 3 for foreign body removal, 1 for an unconfirmed hypothesis of hemoptysis, and 1 for a peripheral nodule found in a computed tomographic scan). Control samples had normal macroscopic appearances of the airways. Patients were matched with control subjects for sex and age.

Morphologic characteristics of bronchial biopsy specimens

Samples from patients with severe asthma had significant increases compared with those with mild asthma or control

subjects, respectively, in RBM thickness (8.3 ± 0.317 vs 6.08 ± 0.373 and 4.178 ± 0.694 μ m, $P = .0024$), epithelial thickness (34.4 ± 4.23 vs 8.42 ± 1.817 and 18.75 ± 2.635 μ m, $P = .003$), and percentages of goblet cells ($16.89\% \pm 1.84\%$ vs $7.03\% \pm 1.76\%$ and $5.415 \pm 1.84\%$, $P = .008$; Fig 1).

Semiquantitative analysis of cultured epithelia

The morphology of *ex vivo* reconstituted epithelium was characterized by the integrity of the epithelial barrier, as demonstrated by the absence of leakage in cell cultures; the presence of ciliated cells, which was scored according to the presence of beating cilia; and mucus production, which was evaluated by the density of the plug at the apical surface of the epithelium (Table II). The apparent tight junction formation of epithelia and the presence of ciliated cells were similar among the 3 groups (patients with severe asthma, patients with mild asthma, and control subjects). However, patients with severe asthma had increased production of mucus in the epithelia compared with the other groups ($P = .04$).

Morphology of cultured epithelia

In transmission electron microscopic analysis bronchial epithelia from all groups were well pseudostratified, comprising basal cells and columnar ciliated cells (Fig 2, A and B). Samples from patients with severe asthma had increased numbers of goblet cells, which were rich in mucus granules, and mucus at the apical surface of the epithelium (Fig 2, C). Tight junctions were observed at the apical surface (Fig 2, D). Ciliated cells were also observed with intact cilia ultrastructure. We detected longitudinal sections of cilia with radial spokes (Fig 2, E). Cross-sections of cilia showed the axoneme, which was composed of 9 microtubular doublets, arranged in a ring around 2 central single microtubules (Fig 2, F).

Mucin release and expression

ELLA analysis of mucin secretion (Fig 3, A) showed that patients with severe asthma produced more mucin than patients with mild asthma (2.794 ± 0.512 vs 0.032 ± 0.376 μ g/mL, $P = .018$). However, the severity of asthma did not affect gene expression levels of *MUC5AC* or *MUC5B* (Fig 3, B and C).

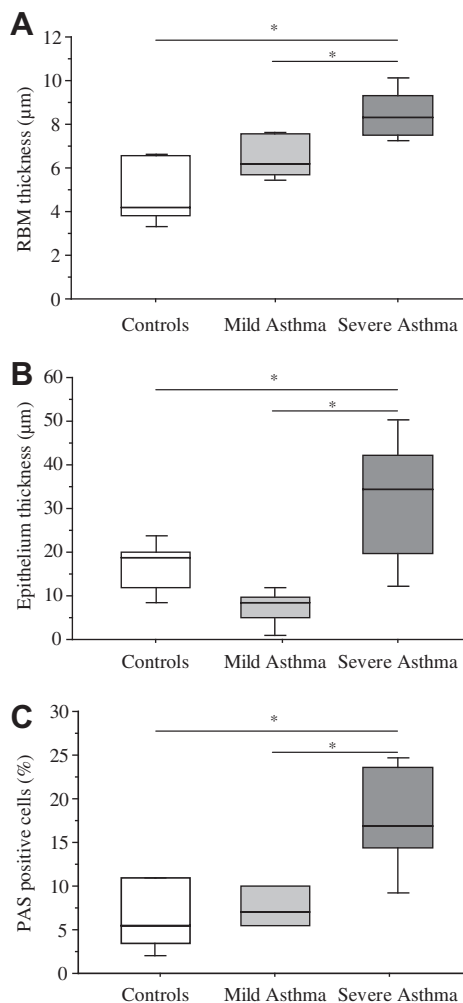


FIG 1. RBM and epithelial thickness and goblet cell numbers were increased in patients with severe asthma. **A** and **B**, RBM (Fig 1, A) and epithelial thickness (Fig 1, B), expressed in micrometers, were measured in endobronchial biopsy specimens from control subjects and patients with mild or severe asthma. **C**, The number of goblet cells, expressed as a percentage of positive cells, was obtained by using periodic acid–Schiff (PAS) staining. Results are expressed as medians and interquartile ranges with 10th and 90th percentiles. * $P < .05$, Kruskal–Wallis followed by *post hoc* test.

Production of IL-8 and lipoxins

IL-8 levels were significantly greater in wash samples from the air-liquid interface of the apical surface of cells from patients with severe asthma (6.713 ± 2.342 ng/mL) than those seen in patients with mild asthma (2.330 ± 0.562 ng/mL) or control subjects (3.823 ± 0.585 ng/mL; Fig 4, A). LXA₄ levels were significantly higher in patients with mild asthma (57 ± 4.06 pg/mL) than in those with severe asthma (36.5 ± 3.4 pg/mL) or control subjects (29.5 ± 5.95 pg/mL; Fig 4, B).

Expression of factors in the lipoxin biosynthetic pathway

We analyzed expression of genes involved in lipoxin biosynthesis, including *15LO1*, *15LO2*, and *5LO* (Fig 5). There were no differences in *15LO1* mRNA levels in epithelial cells from patients with mild or severe asthma (Fig 5, A), but *15LO2* mRNA levels were increased in epithelial cells from patients with severe

TABLE II. Semiquantitative analysis of epithelial cultures

	Control subjects	Patients with mild asthma	Patients with severe asthma	P value
Epithelial cohesion*	3.5 (3-4)	3 (2-4)	4 (2-4)	NS
Ciliated cells*	2 (0-3)	1 (0-3)	2.5 (0-3)	NS
Mucus production*	1.5 (0-2)	1 (0-3)	3 (0-4) [†]	.04

Comparisons were made by using the Kruskal–Wallis test with adequate posttest corrections.

NS, Not significant.

*Values are medians (minimums–maximums).

[†] $P < .01$ vs control subjects and patients with mild asthma.

asthma ($P < .05$; Fig 5, B). Moreover, reconstituted epithelia of patients with mild and severe asthma had similar *5LO* mRNA levels (Fig 5, C).

DISCUSSION

We developed air-liquid interface cultures of bronchial epithelial cells and demonstrated that they maintained characteristics specifically associated with mild and severe asthma. Functional and morphologic analyses of these cultures revealed epithelial differences associated with disease severity. Samples from patients with severe asthma differed in mucus production, cytokine production (sustained IL-8 release), and resolution mechanisms (a defect in LXA₄ production associated with overexpression of *15LO2*).

These findings are consistent with previously reported epithelial features of patients with severe asthma. Our culture system therefore provides an original way to assess *ex vivo* the role of the epithelium in patients with chronic severe airway diseases.

Epithelial changes frequently associated with asthma include cell activation, excessive shedding, and goblet cell hyperplasia, which leads to mucus hypersecretion. We and others have previously associated thickness of the RBM with severe asthma.⁹ Cohen et al¹¹ proposed that epithelial hyperplasia and thickening were manifestations of remodeling in patients with chronic and severe asthma that progressively reduced lung function. Most epithelial changes associated with severe asthma have been described at a steady state, using endobronchial biopsy specimens as a snapshot of the situation. The morphologic changes observed in the bronchial epithelia of patients with severe asthma might be related to long-term treatment with steroids (inhaled high doses and possibly oral), which is a topic of debate.²⁸⁻³⁰

In the present study we demonstrate that abnormalities of the epithelia associated with severe asthma are maintained when the tissue is cultured *in vitro*, forming a fully differentiated airway epithelia. The cells retained these characteristics for at least 6 weeks under the same *ex vivo* culture conditions, indicating that the severe asthma phenotype is an intrinsic characteristic of the bronchial epithelia rather than an effect of corticosteroids. This phenotype is associated with goblet cell hyperplasia, mucin overproduction, and increased *MUC5AC* mRNA levels.

MUC5AC and *MUC5B* encode polymeric mucins that are expressed by airway epithelium and are found in airway secretions.^{31,32} We used ELLA analysis to measure total mucin production and observed increased secretion in patients with severe asthma. We also observed goblet cell hyperplasia, which was observed in the endobronchial biopsy specimens at baseline. *MUC5AC* and *MUC5B* mRNA levels did not increase with asthma severity; Zhao et al²⁴ also reported that there were no

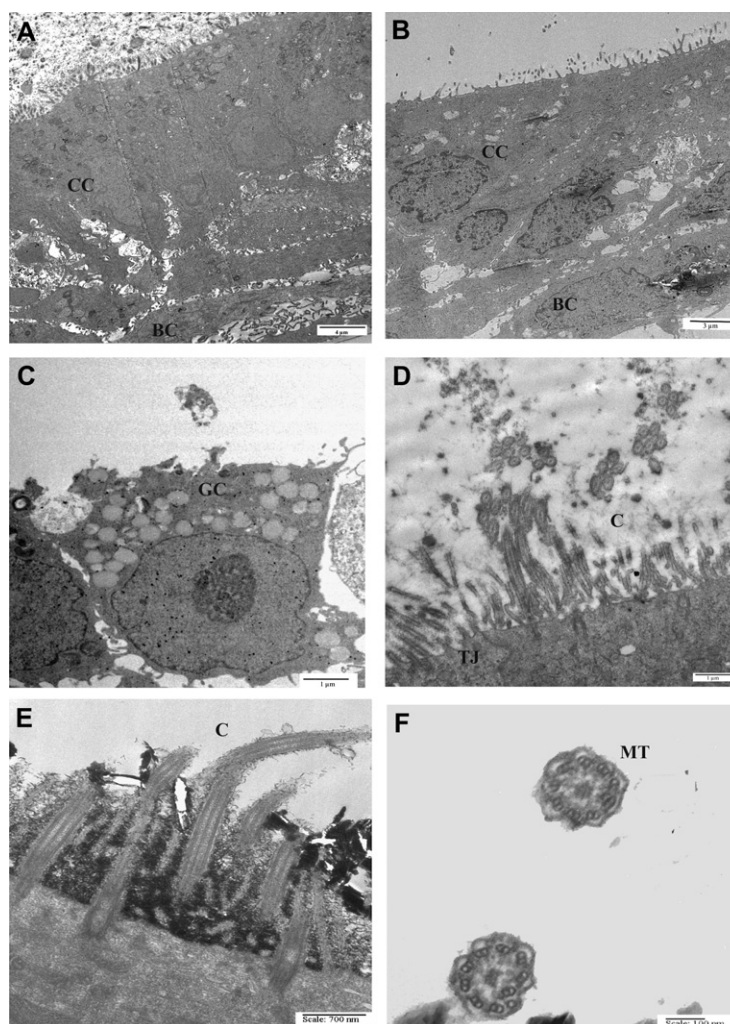


FIG 2. Morphologic analysis of bronchial epithelia in air-liquid interface cultures. **A** and **B**, Representative transmission electron microscopy of fully differentiated epithelium from patients with severe (Fig 2, **A**) and mild (Fig 2, **B**) asthma (original magnification $\times 4000$). **BC**, Basal cell; **CC**, ciliated cell; **GC**, goblet cell. **C**, Goblet cell containing electron-lucent secretory granules (original magnification $\times 12,000$). **D**, Apical surface of the epithelium showing cilia (**C**) and tight junction (**TJ**; original magnification $\times 12,000$). **E** and **F**, Longitudinal sections (Fig 2, **E**) and cross-sections (Fig 2, **F**) of cilia showing microtubular doublets (**MT**; original magnification $\times 50,000$).

difference in *MUC5AC* mRNA levels in patients with more severe asthma. Although differences in *MUC5AC* and *MUC5B* levels have been reported, the size and complexity of these glycoproteins make quantitative analysis difficult.

The persistence of epithelial changes *ex vivo* in a controlled environment supports the role of the bronchial epithelium in the pathophysiology of severe asthma. Although specific genetic factors have not been associated with asthma severity, gene variants could affect immunomodulatory signals that maintain specific epithelial phenotypes and determine the clinical presentation of asthma. Pathways in the innate and acquired immune responses should be compared among patients with different levels of asthma severity.

IL-8 is a chemoattractant for neutrophils and other inflammatory cells, such as granulocytes³³; it was detected in BAL fluid samples from asthmatic patients.¹⁶ Patients with neutrophilic severe asthma have higher IL-8 levels in supernatants of induced sputum compared with those seen in patients with eosinophilic

asthma (control subjects).^{17,18,20} Whereas IL-8 is a proinflammatory cytokine, lipoxins are produced at inflamed tissue sites to downregulate inflammation and to promote its resolution.³⁴ Lipoxins are metabolites produced by LO interactions, resulting from cellular cooperation.^{21,22,35,36} Two 15-LO isoenzymes have been described (15-LO1 and 15-LO2)^{37,38}; human bronchial epithelial cells express high levels of 15-LO, with differential expression of each isoenzyme.³⁹ We previously reported that induced sputum from patients with severe asthma has lower levels of LXA₄ than that of patients with mild asthma. In fact, in samples from patients with severe asthma, there is an inverse correlation between IL-8 and LXA₄ levels^{18,19}; this is in contrast to patients with mild asthma, in whom increased levels of inflammation are counterbalanced by increased production of lipoxins. Severe asthma is characterized by a nonresolved inflammatory process associated with a persistence of inflammatory airway disease. We reproduced these findings in an epithelial air-liquid interface culture model: the increased production of IL-8 and

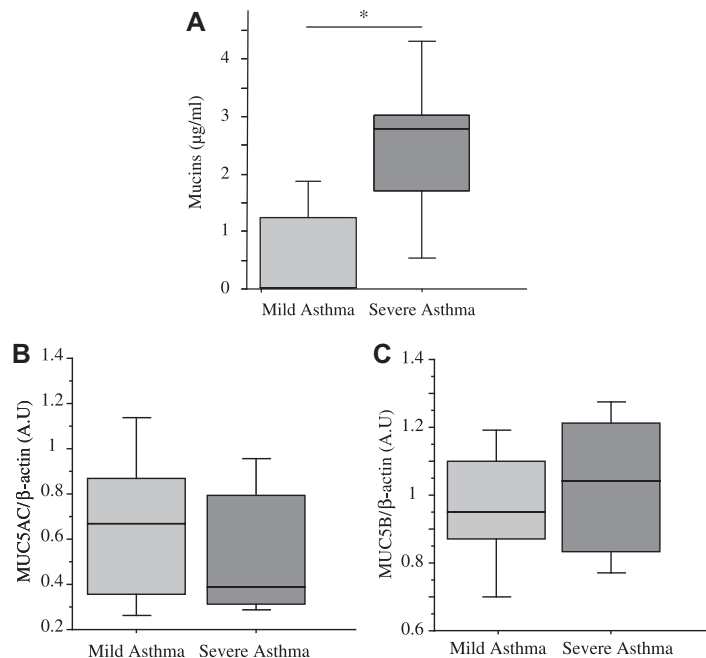


FIG 3. Mucin secretion and gene expression in air-liquid interface cultures of bronchial epithelium from patients with asthma. **A**, Analysis of total mucin secretion by using ELLA. **B** and **C**, Semiquantitative analysis of *MUC5AC* (Fig 3, **B**) and *MUC5B* (Fig 3, **C**) mRNA, with β -actin as a control. Results are expressed as medians and interquartile ranges with 10th and 90th percentiles. * $P < .05$, Mann-Whitney U test.

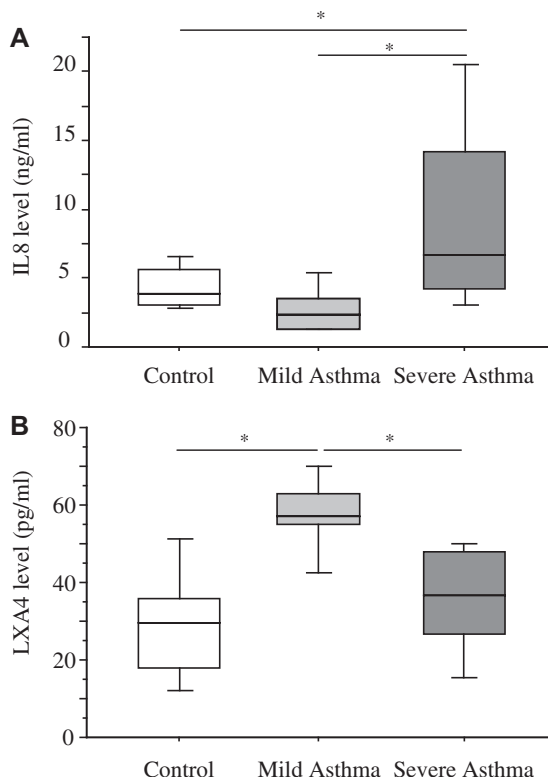


FIG 4. IL-8 and LXA₄ levels in apical wash samples from air-liquid interface cultures. **A**, Quantitative analysis of IL-8 production in epithelia from control subjects, patients with mild asthma, and patients with severe asthma (in nanograms per milliliter). **B**, Quantitative analysis of LXA₄ production in epithelia from control subjects, patients with mild asthma, and patients with severe asthma (in picograms per milliliter). Results are expressed as medians and interquartile ranges with 10th and 90th percentiles. * $P < .05$, Kruskal-Wallis test followed by *post hoc* test.

decreased production of LXA₄ in samples from patients with severe asthma indicated that cultured epithelium can be used to detect inflammation and its resolution.

Furthermore, bronchial epithelial cells in air-liquid interface culture expressed both isoforms of 15-LO. However, only *15LO2* mRNA expression was increased in samples from patients with severe asthma. These results were previously reported, although the effects of altered expression of *15LO2* are debated.⁴⁰ The absence of a difference in *15LO* mRNA levels indicates that the reduced level of LXA₄ in epithelia from patients with severe asthma results from altered LO activity rather than changes in LXA₄ expression.

There are limitations to our study. We did not intend to study *ex vivo* inflammation using the analysis of mediators in sputum supernatants or BAL fluid. We did not focus on eosinophilic inflammation *per se* because we did not observe any differences in levels of eosinophilic inflammation in the blood or endobronchial biopsy samples or in cytokines produced by eosinophils (GM-CSF and macrophage inflammatory protein 1 α , data not shown) in our culture model. Furthermore, some patients included in this study had been previously exposed to high levels of steroids, which might have reduced the eosinophilic phenotype. Finally, we did not assess the kinetics of the restoration of our model because this would not allow sufficient numbers of fully differentiated epithelia to sustain our research hypothesis. Future studies should investigate the dynamics of markers of epithelial tight junction or apoptosis in this culture system.

In summary, reconstituted epithelium derived from endobronchial biopsy specimens has many properties of the *in vivo* airway epithelium, indicating its potential for the study of biological pathways and therapeutic interventions. Our findings could be an important reference tool and starting point for more in-depth investigations of airway epithelium in patients with chronic

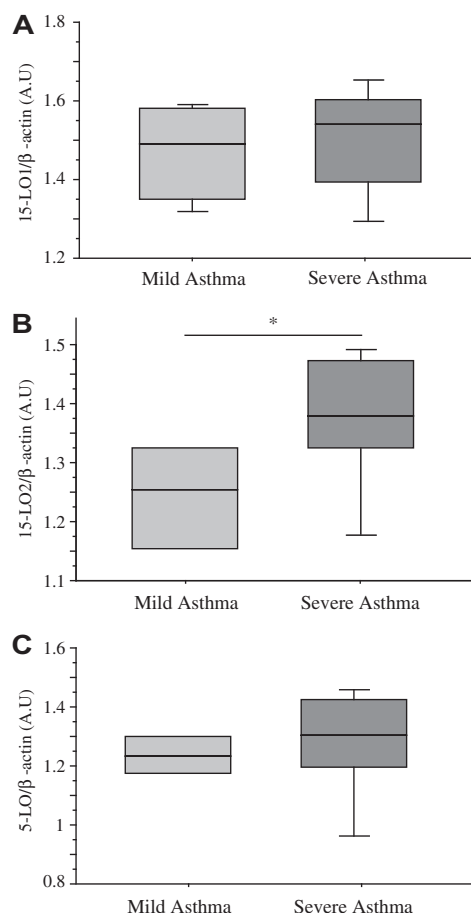


FIG 5. Expression of genes that regulate lipoxin biosynthesis in air-liquid interface epithelial cell cultures. Semiquantitative analysis of *15LO1* (A), *15LO2* (B), and *5LO* (C) mRNA is shown, with β -actin as a control. Results are expressed as medians and interquartile ranges with 10th and 90th percentiles. * $P < .05$, Mann-Whitney U test.

diseases of the bronchi. The development of air-liquid interface epithelial cultures from patients with asthma has provided valuable insight into the processes that occur in the bronchial epithelium during the development of severe disease. The differences observed in well-differentiated epithelial cultures from patients with mild versus severe asthma support the concept that epithelial cells from these groups of patients have inherent differences *in vivo*.

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Clinical implications: The airway epithelium becomes inflamed and undergoes remodeling in patients with severe asthma. Reconstituted bronchial epithelium provides a model for this process that improves our ability to study asthma pathogenesis and test new treatment approaches.

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METHODS

Grading used in Table II

Epithelial cohesion

1. Medium leakage
2. Several holes
3. Few holes
4. No medium leakage and no holes

Ciliated cells

0. No beating
1. Weak cilia beating in periphery
2. Significant cilia beating in periphery
3. General cilia beating

Mucus production

0. No mucus visible

1. Few granules of mucus at the surface
2. Thin layer of mucus
3. Thick layer of mucus
4. Thick layer of mucus with plug

Expression of *MUC5AC*, *MUC5B*, *15LO1*, *15LO2*, and *5LO* mRNA by means of PCR

The sequences for primers were as follows:

MUC5AC—forward 5' ATGAGTGTGGCCGGAGGAA 3', reverse 5' TCGAAGGTCTTGTAAGTGGGAAGCTG 3'; *MUC5B*—forward 5' CACATCC ACCCTTCCAAC 3', reverse 5' GGCTCATTGTCGTCTCTG 3'; *15LO1*—forward 5' CCGACCTCGCTATCAAAGAC 3', reverse 5' GGATGAC-CATGGGCAAGAG 3'; *15LO2*—forward 5' TGCCTCTCGCCATCCA GCT 3', reverse 5' TCATGGAAGGAGAACTCGGCAT 3'; *5LO*—Forward 5' CCCGGGGCATGGAGAGCA 3', reverse 5' GCGGTCGGGCAGCGT 3'.