

Epithelial Cell Proliferation Contributes to Airway Remodeling in Severe Asthma

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Rationale: Despite long-term therapy with corticosteroids, patients with severe asthma develop irreversible airway obstruction.

Objectives: To evaluate if there are structural and functional differences in the airway epithelium in severe asthma associated with airway remodeling.

Methods: In bronchial biopsies from 21 normal subjects, 11 subjects with chronic bronchitis, 9 subjects with mild asthma, and 31 subjects with severe asthma, we evaluated epithelial cell morphology: epithelial thickness, lamina reticularis (LR) thickness, and epithelial desquamation. Levels of retinoblastoma protein (Rb), Ki67, and Bcl-2 were measured, reflecting cellular proliferation and death. Terminal deoxynucleotidyl-mediated dUTP nick end labeling (TUNEL) was used to study cellular apoptosis.

Measurements and Main Results: Airway epithelial and LR thickness was greater in subjects with severe asthma compared with those with mild asthma, normal subjects, and diseased control subjects ($p = 0.009$ and 0.033 , respectively). There was no significant difference in epithelial desquamation between groups. Active, hypophosphorylated Rb expression was decreased ($p = 0.002$) and Ki67 was increased ($p < 0.01$) in the epithelium of subjects with severe asthma as compared with normal subjects, indicating increased cellular proliferation. Bcl-2 expression was decreased ($p < 0.001$), indicating decreased cell death suppression. There was a greater level of apoptotic activity in the airway biopsy in subjects with severe asthma as compared with the normal subjects using the TUNEL assay ($p = 0.002$), suggesting increased cell death.

Conclusions: In subjects with severe asthma, as compared with subjects with mild asthma, normal subjects, and diseased control subjects, we found novel evidence of increased cellular proliferation in the airway contributing to a thickened epithelium and LR. These changes may contribute to the progressive decline in lung function and airway remodeling in patients with severe asthma.

Keywords: epithelium; desquamation; airflow obstruction

Airway remodeling in asthma refers to structural changes in the airway, including subepithelial fibrosis, smooth muscle hypertrophy, and blood vessel hyperplasia. Ongoing inflammation, airway injury, and healing are part of the remodeling process in asthma and perhaps lead to disordered repair and fibrosis as a consequence. It has been suggested that the subepithelial fibrosis in asthmatic lungs may contribute to thickening of the airway wall,

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Airway remodeling in asthma refers to structural changes in the airway, including subepithelial fibrosis, smooth muscle hypertrophy, and blood vessel hyperplasia. Limited knowledge exists on airway remodeling in severe asthma.

What This Study Adds to the Field

In severe asthma, there is increased cellular proliferation in the airway contributing to a thickened epithelium and lamina reticularis.

which in turn may result in physiologic alterations, such as chronic airflow obstruction and airway hyperresponsiveness (1). However, histopathologic studies (including ours) have shown that subepithelial fibrosis is present even in subjects with newly diagnosed asthma (2–5). The association of airflow obstruction, as measured by FEV₁, with subepithelial fibrosis has been demonstrated in some studies (2, 6) but not others (5, 7, 8). Therefore, there are likely other components of the remodeling process that are contributing to the physiologic consequences of chronic asthma.

Previously, we have shown that airway epithelial cells provide critical signals for epithelial-immune cell interaction resulting in airway inflammation and remodeling (9–12). We hypothesized that cellular proliferation and cell death of airway epithelial cells may explain, at least in part, the structural remodeling changes noted in severe asthma. The proliferative response in the airway epithelium may be triggered by viral infection or injury in the genetically susceptible individual. For epithelial cells to proliferate, they must move from the G1 to S phase of the cell cycle and this step requires inactivation of retinoblastoma (Rb) or Rb-related proteins (5, 13).

The death response in the airway epithelium may be promoted by proapoptotic factors (or viral infection) resulting in epithelial desquamation and damage. Bcl-2 is a family of genes involved in the regulation of cell death (cell death promoters, such as Bax, Bcl-XS, Bak, and Bad) and survival (cell death suppressors, such as Bcl-2, Bcl-XL, and MCL-1) without affecting cell proliferation. It has been shown that selected members of the Bcl-2 family also have cell cycle effects. The ability of Bcl-2 to delay cell cycle progression is most obvious during entry into the S phase from quiescence (14). In transgenic Bcl-2 mouse models, S phase entry after T-cell activation is retarded, whereas Bcl-2^{-/-} T cells exhibit accelerated activation-induced cell cycle entry (15, 16). Therefore, the Bcl-2 family of genes may play a

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role in the balance of proliferative and death response in the airway epithelium of subjects with asthma.

If either the proliferative or death response in the airway epithelium is unchecked in asthma or uncontrolled with agents such as corticosteroids, this may manifest as epithelial hyperplasia, thickening, and desquamation, resulting in airway mucus and plugging. This dysregulated epithelium may contribute to airway remodeling in severe persistent asthma and result in persistent physiologic abnormalities.

METHODS

Subjects

The extended version of the methods is available in the online supplement. Adult subjects with severe and mild persistent asthma, normal subjects, and subjects with chronic bronchitis were recruited for a protocol approved by the Washington University Human Research Protection Office. Subjects with severe asthma met the American Thoracic Society (ATS) workshop criteria (17) for refractory severe asthma. The subjects with mild persistent asthma had symptoms three to six times weekly or four or fewer nocturnal awakenings due to asthma per month and an FEV₁ of 80% or more of predicted, consistent with National Asthma Education and Prevention Program guidelines (18). The diagnosis of asthma was also confirmed by bronchial hyperreactivity to methacholine (19). Criteria for normal subjects were as follows: age, 18 to 60 years; good overall health; and nonallergic. Subjects with chronic bronchitis were 30 to 60 years of age, had a history of "chronic bronchitis" as defined by the ATS (20), and were current or previous smokers with a minimum of 20 pack-years.

Histologic Processing

Twelve to 15 endobronchial biopsies of proximal airway tissue were frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA) or formalin and embedded in paraffin. Fifteen to 20 endobronchial brushings were obtained and placed in RPMI 1640 media. Formalin-fixed endobronchial biopsies were cut into 5- μ m sections and prepared as previously outlined (21). The tissue sections were examined under light microscopy ($\times 200$) for areas of intact epithelium that were cut perpendicular to the epithelium. Measurements of the thickness of both the epithelium and underlying lamina reticularis (LR) were performed using NIH ImageJ software (National Institutes of Health, Bethesda, MD). The two-dimensional thickness was calculated by dividing the area by the length of the corresponding basement membrane (BM) as previously described (22). Three measurements were taken from each evaluated area of epithelium (where the epithelium and submucosa were intact), with a minimum of three different biopsy specimens per subject, and quantified by two independent reviewers. Desquamation was quantified as previously described (23) using NIH ImageJ software into percentages of intact, basal, and denuded epithelium.

Immunohistochemistry and TUNEL Analysis

Paraffin-fixed endobronchial biopsy sections were incubated overnight with anti-Rb protein (Invitrogen, Carlsbad, CA), anti-Bcl-2 (Dako, Glostrup, Denmark), anti-Ki67 (Dako), and anti-Fas (Santa Cruz, Santa Cruz, CA) antibodies (Abs). Primary Ab binding was detected with biotinylated anti-mouse IgG Ab, streptavidin-conjugated alkaline phosphatase or horseradish peroxidase, and red chromogenic substrate or diaminobenzidine (Vector, Burlingame, CA). Isotype control Abs were used as negative controls. Grading was performed as previously described (9). Differences in immunohistochemical staining was quantified (for Rb and Bcl-2) as previously described (24, 25). The ApopTag Plus Fluorescein *In Situ* Detection Kit (Millipore, Billerica, MA) was used to label apoptotic cells. The sections were quantified by two independent reviewers for TUNEL (terminal deoxynucleotidyl-mediated dUTP nick end labeling)-positive cells per millimeter squared using fluorescence microscopy.

Immunoblot

Immunoblotting for Rb and Bcl-2 expression were performed as described previously (21). Briefly, samples from 10 to 16 bronchial brush-

ings were pooled. Twenty micrograms of whole cell lysate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-Rb and anti-Bcl-2 monoclonal Abs (mAbs) conjugated to horseradish peroxidase (PY20-H; BD Biosciences, San Jose, CA). Binding was detected by an enhanced chemiluminescence method (Amersham International, Buckinghamshire, UK). Polyvinylidene difluoride (PVDF) membranes were reprobbed with anti- β -actin mAb (Sigma Chemical, St. Louis, MO).

Statistical Analysis

Clinical data and results were analyzed using SAS statistical software (Version 9; SAS Institute, Cary, NC). Group comparisons were made using an analysis of variance. All pairwise comparisons were performed using statistical contrasts as part of the analysis of variance. The following data were transformed before analysis: logarithmic transformation, FEV₁ PC₂₀ (concentration of methacholine causing a 20% decrease in FEV₁) and serum IgE; square root transformation, Ki67. Correlation analysis was performed using a Pearson test. A *p* value of less than 0.05 was considered to indicate statistical significance. Because subjects with chronic bronchitis were older than the subjects with severe asthma and normal subjects, comparisons between groups were performed using analyses of covariance that adjusted for age. All results are presented as mean \pm standard deviation.

RESULTS

Seventy-two subjects (39 females, 33 males) were enrolled: 31 with severe, persistent asthma; 9 with mild, persistent asthma; 21 normal control subjects; and 11 diseased control subjects with chronic bronchitis (*see* Table 1 for subject characteristics). The subjects with severe asthma were more severely obstructed than the subjects with mild asthma and normal control subjects, and also displayed a more reactive airway (lower PC₂₀) than the normal subjects. The subjects with severe asthma were treated with a higher dose of inhaled corticosteroid than those with mild disease. All subjects with severe asthma and two of the nine subjects with mild persistent asthma were receiving an inhaled corticosteroid. Serum IgE levels were significantly higher in those subjects with severe asthma. The subjects with chronic bronchitis were older and diagnosed at an older age than the subjects with severe asthma, subjects with mild asthma, and normal control subjects. Subsequently, analysis was age adjusted due to this baseline difference.

Epithelial Layer and LR

We observed that the epithelium was thicker in the subjects with severe, persistent asthma (37.3 ± 14.3 mm²/mm BM length [BML]) than in those with mild, persistent asthma (25.9 ± 8.3 mm²/mm BML), normal control subjects (28.0 ± 9.7 mm²/mm BML), and subjects with chronic bronchitis (27.5 ± 10.1 mm²/mm BML) (*p* = 0.019) (Figures 1A and 1B). The epithelial thickness was not significantly different between those with mild persistent asthma, those with chronic bronchitis, and normal individuals. The LR was also significantly thicker in those subjects with severe persistent asthma (14.9 ± 6.0 mm²/mm BML) than in subjects with mild persistent asthma (10.7 ± 1.7 mm²/mm BML), normal subjects (8.2 ± 4.0 mm²/mm BML), and subjects with chronic bronchitis (8.9 ± 1.6 mm²/mm BML) (*p* < 0.0001). The LR was not significantly different between those with chronic bronchitis and normal individuals but there was a trend when comparing those with mild asthma to subjects with severe asthma (*p* = 0.067). There was a significant positive correlation between epithelial and LR thickness (*r* = 0.51, *p* < 0.0001). There was a significant inverse correlation between epithelial and LR thickness and FEV₁% predicted (*r* = -0.36, *p* = 0.01, and *r* = -0.41, *p* = 0.007, respectively). We also confirmed our measurement of LR thickness using Picro-Sirius red, a collagen stain, in a subset that demonstrated a high correlation with the measurements

TABLE 1. BASELINE CHARACTERISTICS OF SUBJECTS

	Normal (n = 21)	Chronic Bronchitis (n = 11)	Mild Asthma (n = 9)	Severe Asthma (n = 31)	p Value*
Age, yr	30 ± 8	53 ± 15	32 ± 7	39 ± 14	< 0.0001
Age at diagnosis, yr	—	39 ± 12	12 ± 12	18 ± 18	0.034
Sex, n (M/F)	12/9	5/6	6/3	17/14	NS
Race, n (white/other [‡])	18/3	8/3	6/3	15/16	0.03
IgE, IU/ml	82 ± 111	50 ± 64	276 ± 313	473 ± 794	0.006 [†]
Inhaled corticosteroid dose, μg [§]	NA	NA	142 ± 307	2416 ± 1184	< 0.0001
FEV ₁ ± SD, L/min	3.52 ± 0.70	1.67 ± 0.68	3.42 ± 0.65	2.34 ± 0.58	< 0.0001
FEV ₁ % predicted ± SD (range)	98 ± 10 (81–123)	58 ± 22 (19–90)	98 ± 13 (80–114)	74 ± 17 (51–116)	
FEV ₁ PC ₂₀ ± SD, mg/ml (range)	—	9.74 ± 10.2 (3.5–25)	2.4 ± 2.8 (0.06–8.0)	2.2 ± 4.1 (0.01–8.0)	0.026

Definition of abbreviations: FEV₁ PC₂₀ = concentration of methacholine causing a 20% decrease in FEV₁; M/F = male/female; NA = not applicable; NS = not significant.

* Comparing groups by analysis of variance or χ^2 .

[†] Kruskal-Wallis test.

[‡] Other = African American, Asian, mixed race.

[§] Inhaled corticosteroid dose is expressed in beclomethasone equivalents.

made on the hematoxylin–eosin slides ($r = 0.91$, $p < 0.0001$) (data not shown).

We then measured whether the differences we had observed in the epithelium were due to differences in desquamation of the epithelium. The percentages of intact, basal only, and denuded epithelium were not significantly different across all groups (mean ± SD): 38.2 ± 21.4, 42.8 ± 26.8, and 19.0 ± 16.0 in subjects with severe asthma; 41 ± 13.9, 33.2 ± 9.9, and 25.8 ± 10.2 in subjects with mild asthma; 28.0 ± 18.1, 46.0 ± 14.7, and 26.0 ± 19.5 in normal subjects; and 36.8 ± 13.0, 33.0 ± 8.5, and 30.2 ± 10.8 in subjects with chronic bronchitis, respectively (all $p > 0.05$) (Figure 1C).

Proliferative Changes in Airway Epithelium

Rb protein was measured using a monoclonal Ab that recognizes the hypophosphorylated form of Rb (localized to an epitope between amino acids 444 and 621 of the Rb gene) with no cross-reaction with other Rb members. Rb expression in the epithelial layer was significantly decreased in the subjects with severe asthma (3.9 ± 3.7), compared with subjects with mild persistent asthma (14.3 ± 1.5), normal control subjects (13.0 ± 4.6), and subjects with chronic bronchitis (22.8 ± 6.5) ($p < 0.0001$) (Figure 2). The expression of Rb was significantly different between subjects with mild persistent asthma and normal control subjects ($p = 0.0007$) but not compared with subjects with chronic bronchitis. The expression of Rb was also significantly different between subjects with chronic bronchitis and normal subjects ($p = 0.009$). This would indicate that, in subjects with severe asthma, active hypophosphorylated Rb expression is diminished, leading to an increase in epithelial cell cycling and proliferation. We then confirmed our findings by performing immunoblots of lysates from epithelial cells obtained from endobronchial brushings. Western blots of Rb protein from subjects with severe asthma were significantly decreased in comparison to normal control subjects (Figure 3).

Furthermore, we confirmed our findings with Rb as a marker of proliferation by using Ki67 immunostaining, a marker of cellular proliferation (26). The number of epithelial cells expressing Ki67 was 386 ± 116 cells/mm² in the subjects with severe asthma, 99 ± 119 cells/mm² in the subjects with mild persistent asthma, 144 ± 94 cells/mm² in subjects with chronic bronchitis, and 61 ± 32 cells/mm² in the normal subjects group ($p = 0.017$) (Figure 2). These results support the findings of Rb immunostain and immunoblots.

We then evaluated a marker of cell death in the epithelium because an increase or decrease in cell death may affect the thickness of the epithelial layer. We used Bcl-2, which is a marker of cell death suppression. The epithelial expression of Bcl-2 was 4.3 ± 3.9 in the subjects with severe asthma, 10.3 ± 3.1 in the subjects with mild persistent asthma, 14.5 ± 3.3 in the normal subjects group, and 12.7 ± 2.8 in the chronic bronchitis group ($p < 0.0001$) (Figure 2). The expression of Bcl-2 was not significantly different between subjects with mild persistent asthma, normal control subjects, and subjects with chronic bronchitis. We then confirmed our findings by performing immunoblots of lysates from epithelial cells obtained from endobronchial brushings. Western blots of Bcl-2 protein from subjects with severe asthma showed significantly decreased expression than those from normal control subjects (Figure 3). This decrease in Bcl-2 expression noted in the subjects with severe asthma would indicate increased ability for cell cycling and therefore cellular proliferation.

Interestingly, we found a significant inverse correlation between Rb expression and epithelial ($r = -0.46$, $p = 0.02$) but not LR thickness. There was a trend to a correlation between Bcl-2 and epithelial thickness ($r = -0.38$, $p = 0.06$) but not with LR thickness. In addition, Rb and Bcl-2 were positively correlated with each other ($r = -0.57$, $p = 0.006$). This suggests that the thickness of the epithelium or LR is associated with increased cellular proliferation (demonstrated by a decrease in Rb immunostaining).

These findings together may also imply a diminished antiapoptotic path and potentially greater apoptosis in the epithelium. TUNEL analysis was used to confirm this hypothesis.

TUNEL Immunofluorescence

The evaluation of apoptosis via DNA fragmentation was performed by TUNEL assay. The DNA strand breaks are detected by enzymatically labeling the free 3'-OH termini with modified nucleotides. These new DNA ends that are generated upon DNA fragmentation are typically localized in morphologically identifiable nuclei and apoptotic bodies. The number of positive TUNEL cells was 18.93 ± 16.4 cells/mm² in the subjects with severe asthma and 1.35 ± 2.92 cells/mm² in the normal control subjects ($p = 0.002$) (Figure 4). TUNEL immunofluorescence was not performed in normal subjects or subjects with chronic bronchitis due to a limited number of samples available. We also examined the expression of Fas protein, a member of the apoptotic pathway, in the airway epithelium. We found no

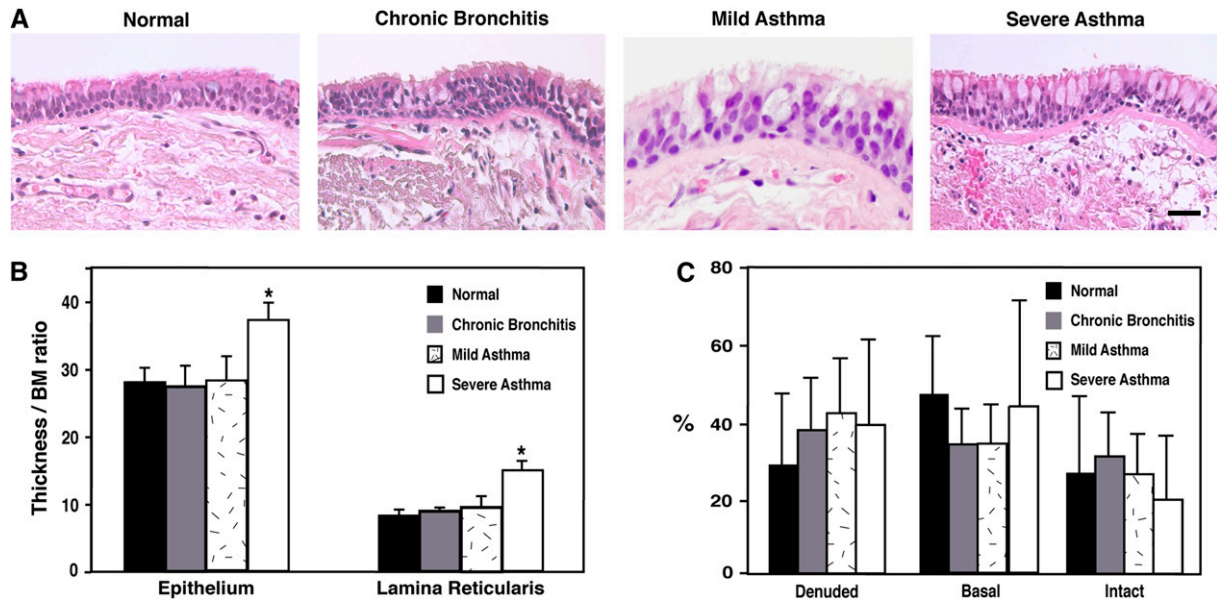


Figure 1. Increased epithelial and lamina reticularis (LR) thickness in bronchial biopsies in subjects with severe asthma. (A) Endobronchial biopsies were obtained from subjects with severe or mild persistent asthma, subjects with chronic bronchitis, and normal subjects, and subjected to hematoxylin–eosin staining. Representative photomicrographs are demonstrated for each condition. Bar = 20 μ m. (B) Corresponding quantitative analysis of results in (A). Values for epithelial and LR thickness represent mean of at least three measured sections from each individual divided by the area by the length of the corresponding basement membrane (BM). (C) Quantitative analysis of epithelial desquamation from subjects with severe asthma, subjects with chronic bronchitis, and normal subjects. Values represent the percentage of BM covered by intact epithelium (Intact), a single layer of basal cells (Basal), or completely denuded (Denuded). Values represent the mean \pm SD of at least three measured sections from each individual. A significant difference ($p < 0.05$) for the severe asthma group compared with the mild persistent asthma, normal, or chronic bronchitis groups is indicated by an *asterisk*.

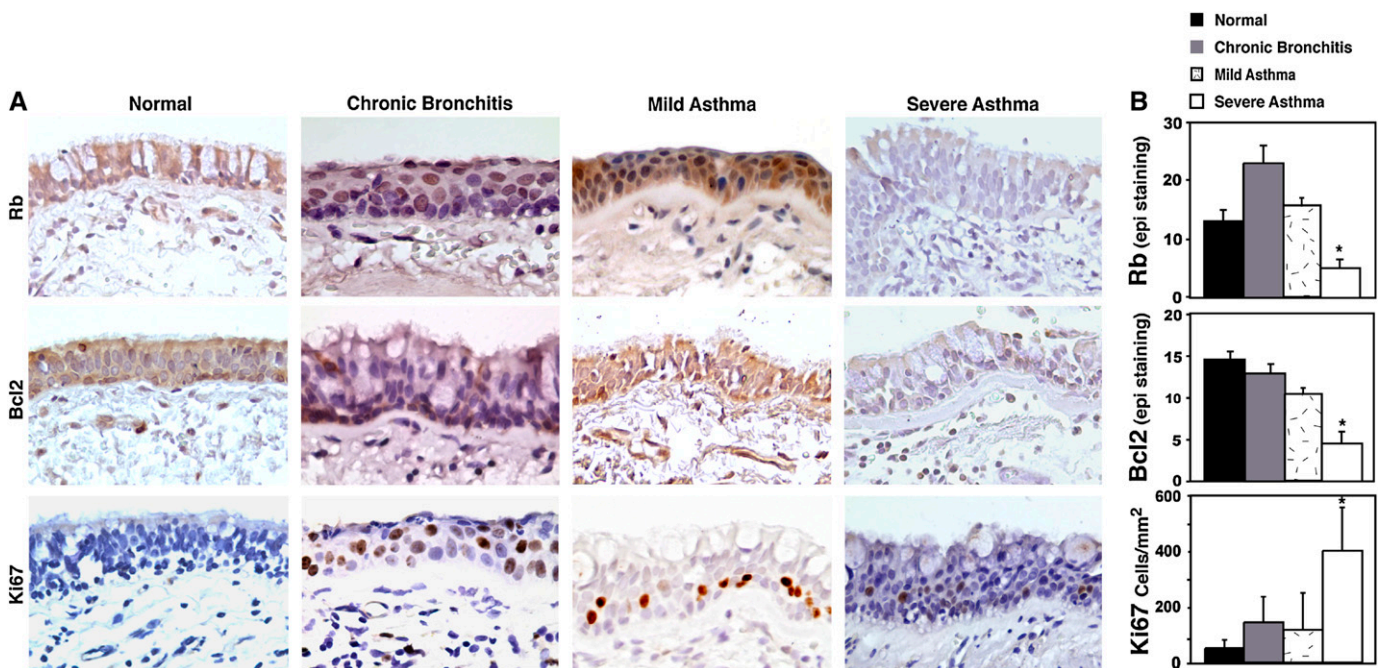


Figure 2. Increased epithelial cell proliferation and decreased cell death suppression in bronchial biopsies in subjects with severe asthma. (A) Endobronchial biopsies were obtained from subjects with severe or mild persistent asthma, subjects with chronic bronchitis, and normal subjects, and were immunostained with anti-retinoblastoma (anti-Rb), anti-Bcl-2, or anti-Ki67 monoclonal antibody. Representative photomicrographs are shown for each condition. Control staining with nonimmune IgG gave no detectable signal above background (data not shown). Bar = 20 μ m. (B) Corresponding quantitative analysis of changes in (A). Values for Rb and Bcl-2 immunostaining were derived by quantitating the brown intensity of epithelial (epi) immunostaining using an image analysis system. Values for Ki67 immunostaining were derived by quantitating the positive cells over the area of the corresponding tissue in mm². Values represent mean \pm SEM. A significant difference ($p < 0.05$) for the severe asthma group compared with the mild persistent asthma, normal, or chronic bronchitis groups is indicated by an *asterisk*.

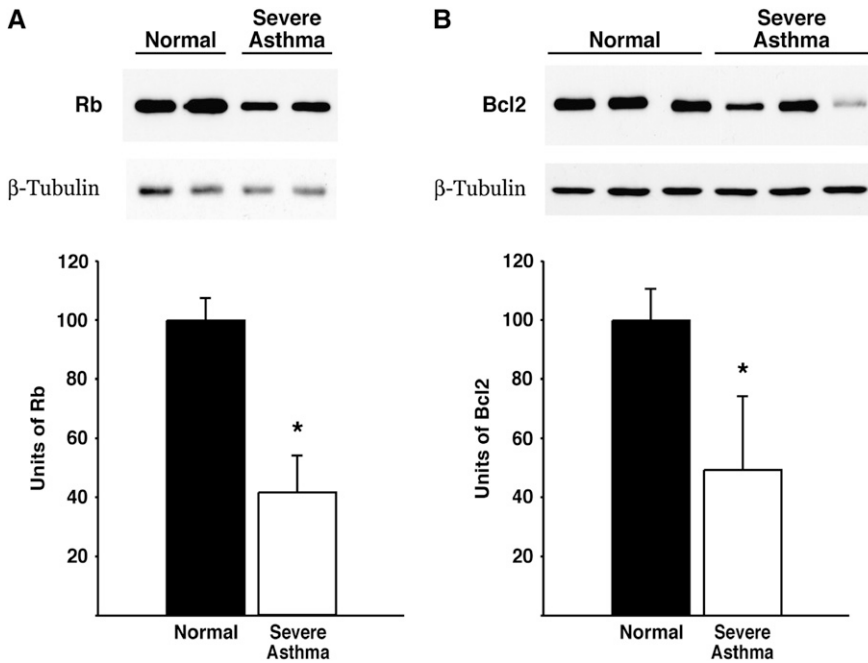


Figure 3. Increased epithelial cell proliferation and decreased cell death suppression in bronchial epithelial brushings in subjects with severe asthma. Representative immunoblot results for Rb and Bcl-2 or control β -tubulin monoclonal antibody from endobronchial brushings obtained from subjects with severe asthma and normal subjects. (B) Corresponding quantitative analysis of changes in (A). Values represent the mean percent \pm SEM of Rb or Bcl-2: β -tubulin ratio in 12 subjects with severe asthma compared with Rb or Bcl-2: β -tubulin ratio in 12 normal subjects. * $p < 0.05$

significant difference in Fas expression between the subjects with severe asthma and normal control subjects (Figure 4). The increase in TUNEL-positive cells in the airway epithelium of subjects with severe asthma indicates a greater rate of epithelial apoptosis.

DISCUSSION

In subjects with chronic asthma, it has been shown that there is a progressive decline in lung function that is greater than in normal subjects (27). A number of studies demonstrated a variety of structural changes in asthma that are likely not reversible, including subepithelial fibrosis, smooth muscle hypertrophy, and goblet cell and blood vessel hyperplasia, which led to the use of the term “remodeling” (2, 28–33). It has been suggested that the remodeling process in asthmatic lungs may contribute to

thickening of the airway wall, which in turn may result in physiologic alterations, such as chronic airflow obstruction and airway hyperresponsiveness (1). Histopathologic studies (including ours) have shown that subepithelial fibrosis is present even in subjects with newly diagnosed asthma (2–5) and children (34, 35); therefore, it appears that this is not responsible for the progressive decline in lung function. The association of airflow obstruction, as measured by FEV₁, with subepithelial fibrosis has been demonstrated in some studies (2, 6) but not others (5, 7, 8). Therefore, we reasoned that there may be other processes leading to airway remodeling in severe asthma.

The current study provides evidence of abnormal epithelial and LR thickening in subjects with severe asthma in comparison to subjects with mild persistent asthma, subjects with chronic bronchitis, and normal individuals. This difference in the epithelium does

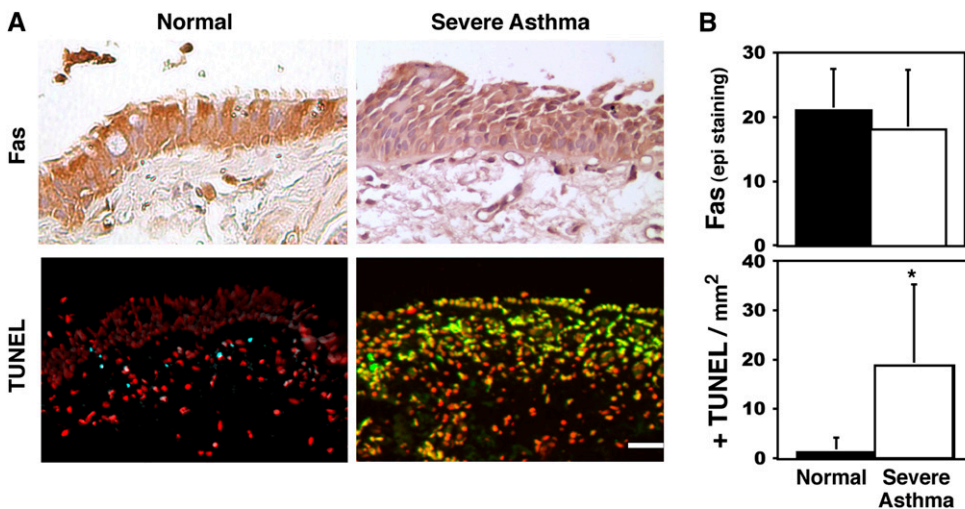


Figure 4. Increased cellular apoptosis in bronchial biopsies in subjects with severe asthma. Endobronchial biopsies were obtained from subjects with severe asthma and normal subjects and were immunostained with anti-Fas monoclonal antibody or TUNEL (terminal deoxynucleotidyl-mediated dUTP nick end labeling) immunofluorescence (reflecting cellular apoptosis by DNA fragmentation). Representative photomicrographs are shown for each condition. Control staining with nonimmune IgG gave no detectable signal above background (data not shown). Bar = 20 μ m. (B) Corresponding quantitative analysis of changes in (A). Values for Fas immunostaining were derived by quantitating the brown intensity of epithelial immunostaining using an image analysis system. Values for TUNEL posi-

tivity were derived by quantitating the positive cells over the area of the corresponding tissue in mm². Values represent mean \pm SEM. A significant difference ($p < 0.05$) for the severe asthma group compared with the normal subjects group is indicated by an asterisk.

not appear to be due to the presence or lack of epithelial desquamation (Figure 1 and Reference 36). Ricciardolo and colleagues demonstrated that allergen challenge appears to increase epithelial proliferation in subjects with mild asthma (37). Few studies have examined differences in airway remodeling in subjects with severe asthma. Benayoun and coworkers previously reported in a small group of subjects with severe asthma ($n = 15$) that there was a difference in subepithelial BM thickness and epithelial integrity in comparison to normal subjects and subjects with mild-moderate asthma (38). Vignola and colleagues demonstrated in oral corticosteroid-dependent subjects with asthma ($n = 19$) that there was increased epithelial thickness in comparison to subjects with untreated asthma, but there was no difference in comparison to normal control subjects (39). Pepe and associates also demonstrated a trend to greater subepithelial fibrosis in subjects with severe asthma ($n = 15$) compared with those with moderate asthma that did not reach statistical significance, but they also found no difference in epithelial area (40). Our results may differ from those of Pepe and colleagues and Vignola and coworkers due to differences in technique (we corrected our epithelial measurements for the length of the BM) and a larger sample size.

We found that, in severe asthma, the airway epithelium appears to be proliferating at a much higher rate as demonstrated by the decreased Rb expression. Furthermore, we found that, in concert with this proliferation, there is increased epithelial apoptosis and decreased cell death suppression, as demonstrated by a decrease in Bcl-2 expression. We believe this abnormal proliferative response in the airway epithelium is unchecked in severe asthma, resulting in epithelial hyperplasia and thickening that appears uncontrolled despite treatment with high doses of corticosteroids. The finding of epithelial hyperplasia and thickening is a manifestation of remodeling in chronic and severe asthma and may lead to a progressive decline in lung function. However, there are likely other processes contributing to this decline in lung function, such as distal airway remodeling and air trapping. For example, Gono and colleagues compared chest computed tomography expiratory and inspiratory (E/I) lung density measurements to assess air trapping as a measure of small airway disease. Subjects with asthma with irreversible airflow obstruction (without emphysema) had significantly higher E/I ratios compared with subjects with asthma whose expiratory flows normalized after bronchodilation and normal control subjects. In addition, wall thickness and air trapping were inversely correlated with post-bronchodilator FEV₁% predicted (41). These findings suggest that airway remodeling in more proximal airways manifested as airway wall thickening reflects similar changes that are occurring in more distal airways, resulting in measurable airflow limitation and air trapping.

Understanding the pathogenesis and mechanisms driving airway remodeling may lead to new approaches to therapy and, specifically, retard the progressive decline in lung function that starts in this disease at an early age (27, 35). Healing of the airways after injury begins in the early stages of inflammation and results in repair. Repair usually involves two distinct processes: replacement of injured tissue (dead/apoptotic cells) by normal cells of the same type, leaving minimal trace of the previous injury, and replacement by connective tissue (42). Both processes that contribute to healing can be followed by a wide variety of consequences, from complete or partial restitution of airway structure and function, to fibrosis. Ongoing inflammation and new injury means that there are processes of injury and healing overlapping each other on an ongoing basis, perhaps increasing the possibility of disordered repair and fibrosis as a consequence. In addition, this abnormal matrix may contribute to integrin-mediated epithelial detachment and apoptosis (43). These pro-

cesses may lead to several of the features of airway remodeling seen in severe asthma.

What is driving this proliferative epithelial remodeling process in severe asthma? This proliferative response may be triggered by persistent airway inflammation, viral infection, or injury in the genetically susceptible individual. Some researchers have demonstrated an increase in epithelial proliferation in conjunction with eosinophilic inflammation after allergen challenge (37). We have previously demonstrated a prominent proliferative response associated with persistent airway hyperreactivity that developed in the setting of paramyxoviral infection (44). This proliferative response may be mediated by an Rb suppressor gene, which encodes a nuclear phosphoprotein (pRB) that regulates cell cycle. pRB is dephosphorylated during mitosis, and the active, hypophosphorylated form (recognized by anti-Rb antibody used in this study) inhibits cell cycle progression during early S and G1 phases (45–47). Hyperphosphorylation leads to progression into the S phase. Therefore, a down-regulation of the active, hypophosphorylated Rb, as seen in the epithelium of our subjects with severe asthma, indicates cell cycling or proliferation. The key factors leading to this heightened epithelial proliferation in severe asthma are unclear at this time.

The current findings demonstrate an abnormal death response in the airway epithelium in severe asthma. The epithelial cell death by apoptosis may represent a host response for controlling infection without a destructive inflammatory response (48) or may represent a homeostatic response to the loss of epithelial cell contact with the BM matrix (43, 49). Apoptosis in response to inappropriate cell and extracellular matrix interactions is called anoikis. Loss of anoikis or anchorage dependency is believed to be a critical step in malignant transformation. In addition, anoikis plays a role in tissue homeostasis as demonstrated at least in one report in the bronchial epithelium (50) and another in the intestine (51). Intestinal epithelial cells die of apoptosis as they reach the luminal surface where they lose cell anchorage and are shed into the intestinal lumen. This process is mediated by alterations in cell-cell anchorage, cell-matrix anchorage, and the expression of pro/antiapoptotic proteins, such as Bcl-2 (49, 52).

Up-regulation of a death factor (e.g., Bcl-2) may cause death, but down-regulation of the same factor may mediate cell survival (53). It has been shown that selected members of the Bcl-2 family also have cell cycle effects (15). Bcl-2 has also been shown to function through the pRB family member p130 in a complex with the transcription factor E2F4 to negatively regulate E2F1, thus inhibiting cell cycle progression (54, 55). Lastly, Bcl-2 proteins, in part, mediate apoptosis as Bcl-2 overexpression prevents anoikis after epithelial cell detachment from matrix (56–58). Therefore, Bcl-2 appears to play a critical role in regulating both cell cycling and survival in the airway epithelium.

The current study suggests that the increase in epithelial cell proliferation noted in severe asthma may lead to a loss of epithelial cell contact with the BM, resulting in anoikis. This would explain the increased apoptosis, potentially in a subpopulation of cells in the airway epithelium, found in airway biopsies from subjects with severe asthma. This homeostatic mechanism of inducing apoptosis maintains tissue homeostasis but may be dysregulated in severe asthma. We propose that the dysregulated epithelium in severe asthma is due to greater epithelial proliferation occurring rather than apoptosis, resulting in a thicker remodeled epithelium.

In the current study, we also found a significant increase in thickness of the LR in severe asthma. The “true” BM, consisting of the lamina rara and lamina densa, in asthma is of normal dimensions (59). This thickening (as previously demonstrated in fatal asthma [60]) is likely due to the deposition of collagen type III and V (5), fibronectin, laminin, tenascin, and elastin.

One study found a significant correlation between the number of epithelial cells expressing transforming growth factor (TGF)- β and the thickness of the BM, suggesting an interaction between epithelial cells, fibroblasts, and collagen deposition (61). Furthermore, it appears that corticosteroids do not significantly decrease the collagen deposition and that this might be mediated by persistent TGF- β effect (62).

The present report demonstrates that part of the remodeling process in severe asthma involves a thickening of the airway epithelium and LR due to an abnormal proliferative process that is not sufficiently counterbalanced by the death pathway. We recognize that there are other components of remodeling that we have not accounted for in the present study which may be equally important to the remodeling process in severe asthma. Smooth muscle hypertrophy and increased airway vascularity appear to contribute to airway remodeling and physiologic changes in severe asthma (38, 40, 63). We (3, 64) and others (23) have also noted significant changes in the goblet cells and mucin products in subjects with asthma and chronic obstructive pulmonary disease. A combination of these remodeling features likely contributes to the chronic airflow obstruction seen in severe asthma.

Morphometric studies in fatal asthma cases have demonstrated marked thickening and folding of the epithelium relative to the relaxed external area (1, 65). Modeling studies by Lambert and colleagues and Wiggs and coworkers demonstrate that this change in airway geometry markedly reduces the degree of narrowing required to cause airway closure (66, 67). Furthermore, in a murine ovalbumin-sensitized model, airway hyperresponsiveness of the inflamed airways was entirely explained by the thickening of the airway mucosa and increased propensity of the airways to close (68). The current study demonstrates that there is an inverse relationship between epithelial and LR thickening and the degree of airflow obstruction at baseline. In combination, these studies suggest that the epithelial and LR remodeling noted may contribute to the airflow obstruction seen in severe asthma.

In conclusion, we have found novel evidence of increased epithelial cell proliferation in the airways of subjects with severe asthma, contributing to a thickened epithelium and LR that is not present in subjects with mild persistent asthma, subjects with chronic bronchitis, or normal individuals. This dysregulated epithelium may contribute to the progressive decline in lung function and airway remodeling seen in patients with severe asthma. Because corticosteroids appear to be ineffective in preventing these changes, cellular proliferation in the airway epithelium may present a new target for disease-modifying therapies.

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