Expression of the proapoptotic protein Bax is reduced in bronchial mucous cells of asthmatic subjects

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1Lovelace Respiratory Research Institute, Albuquerque, New Mexico; 2Applied Biosystems, Foster City, California; 3University of New Mexico School of Medicine, General Clinical Research Center, Departments of Medicine and Biochemistry and Molecular Biology, Albuquerque, New Mexico; 4Department of Cell and Molecular Physiology, The University of North Carolina, Chapel Hill, North Carolina; and 5Respiratory Research Group, University of Calgary, Calgary, Alberta, Canada

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Schwalm K, Stevens JF, Jiang Z, Schuyler MR, Schrader R, Randell SH, Green FH, Tesfaigzi Y. Expression of the proapoptotic protein Bax is reduced in bronchial mucous cells of asthmatic subjects. Am J Physiol Lung Cell Mol Physiol 294: L1102–L1109, 2008. First published April 4, 2008; doi:10.1152/ajplung.00424.2007.—The present studies were designed to determine whether our findings in mice showing that the Bcl-2-associated protein X (Bax), which plays a role in the resolution of allergen-induced mucous cell metaplasia, can be applied to asthma in humans. Immunostaining of autopsy tissues from mild and severe asthmatic subjects showed a significant reduction in the percentage of Bax-positive mucous cells compared with those from nonasthmatic controls. To exclude the possibility that postmortem changes may have affected Bax expression, Bax mRNA levels in airway epithelial cells obtained from nonsmoking asthmatic subjects were compared with those from nonasthmatic controls. Because the number of cells obtained by bronchial brushings is limited, we developed a robust preamplification procedure of cDNA before quantitative real-time PCR to allow detection of 100 gene targets from limited sample size, even when it was prepared from partially degraded RNA. cDNA was prepared by reverse transcription from RNA isolated from bronchial epithelial cells obtained by bronchial brushings from well-characterized subjects without lung disease and from subjects with mild asthma. Quantitative analysis showed that Bax mRNA levels were significantly reduced in samples obtained from asthma patients compared with nonasthma controls. Furthermore, Bax mRNA levels were reduced when primary airway epithelial cells from 10 individuals were treated in culture with the T helper 2 cytokine IL-13. These studies show that Bax expression is reduced in airway epithelial cells of even mild asthmatic subjects and suggest that restoring Bax expression may provide a clinical approach for restoring the normal numbers of epithelial cells and reduced mucous hypersecretion in asthma.

epithelial cell apoptosis; Bcl-2 family of proteins; lung airway epithelia; mucous cell hyperplasia; quantitative real-time polymerase chain reaction

THE BCL-2 FAMILY OF PROTEINS is involved in regulating the resolution of inflammation-induced epithelial cell hyperplasia (26, 33, 35). Bcl-2-associated protein X (Bax)-deficient mice have a delayed resolution of mucous cell hyperplasia during prolonged exposure of mice to allergen (36), and overexpression of Bax using adenoviral vectors causes resolution of allergen-induced mucous cell metaplasia (MCM) in mice (42). Increased numbers of epithelial cells in asthma are a dysfunctional source of innate immune response that can perpetuate increased mucous production or activate the adaptive immune response and mesenchyme (13, 27). The purpose of the present study was to determine whether Bax expression is reduced in airway mucous cells of asthma patients.

Understanding the genes involved in pathological change requires the quantification of altered patterns of gene expression in normal and disease states. Differential expression of these molecules between normal and pathological tissues can provide indications for specific mechanisms of diseases. Protein expression can be analyzed by immunohistochemistry, and mRNA expression can be assessed in airway cells obtained by bronchial brushings. In general, cell samples obtained by bronchial brushings from humans are limited, making reliable analysis of multiple gene expression profiles difficult. The most common method for analyzing multiple mRNAs from a small sample has been the microarray approach (39). Small samples of RNA can be amplified to provide sufficient target for hybridization to cDNA microarrays that display a large number of probes. However, microarray assays have limitations in sample throughput and analytical performance and require stringent protocols for RNA labeling, hybridization, data acquisition, and data normalization for reproducibility of results between laboratories (3).

Real-time RT-PCR, or quantitative RT-PCR (qRT-PCR), is, in general, the method of choice to validate data obtained by microarray hybridization, because of its high accuracy, precision, and dynamic range (15). In many cases, qRT-PCR is efficient for comparing specific mRNA levels in samples that are available in limited quantities. qRT-PCR has become a common technique, no longer limited to specialist core facilities. Despite the advantages of qRT-PCR, it has limitations in the number of targets that can be analyzed in a small sample. High-throughput qRT-PCR strategies use 5- or 20-µl reactions in 96- or 348-well microplates, but are, by and large, still restricted to single-gene targets. We have developed a method that allows reliable analysis of up to 100 genes in each sample simultaneously. This method allows users to increase the cDNAs of interest over 1,000-fold and analyze the quantity of each gene to a reference sample.
In the present study, we found that Bax expression was significantly reduced in mucous cells of mild to severe asthmatic subjects compared with controls when analyzing autopsy samples by immunohistochemistry. To validate this finding, we used the preamplification method to screen for mRNA levels encoding for the major Bcl-2 family proteins in airway epithelial cells of patients with asthma and to compare those levels with those found in subjects with no lung diseases. Because of the limited number of cells obtained by brushings, previous studies have placed the cells in culture before analyzing the properties of the cells (6, 16, 20, 30). However, placing cells in culture changes the state of cell differentiation, which can introduce major changes in gene expression. The present study analyzed expression of the Bcl-2 family of genes in cells obtained from patients to minimize gene changes and to more closely reflect the situation in vivo. Bax mRNA levels were found to be significantly reduced in the cells obtained by bronchial brushings from mild asthmatic subjects compared with controls. Furthermore, Bax mRNA levels were reduced when primary airway epithelial cells from 10 individuals were treated in culture with the T helper 2 (Th2) cytokine IL-13. These studies suggest that findings from the mouse model of asthma may apply to human asthmatic subjects, and restoring Bax expression may be a useful approach for reducing mucous hypersecretion in asthma.

**MATERIALS AND METHODS**

**Autopsy tissues.** Autopsy tissues were obtained through the Prairie Provinces Fatal Asthma Study, which has been described previously (11). The subjects were categorized into three groups based on records obtained by questionnaires administered to the next of kin as follows. Nonasthmatic controls (NAC) had no history of asthma, wheezing, use of asthma medication, or other lung disease. Nonfatal asthma (NFA) subjects died of nonrespiratory causes, but were subsequently found to have a definite history of asthma from relatives or medical examiner’s files. Fatal asthma (FA) subjects were cases in which asthma was determined to be the cause of death, and the events before death were consistent with acute asthma. These classifications were supported by full autopsies and toxicological analysis of postmortem serum. Information on asthma history (age at onset, asthma duration, triggers, and drug use for asthma treatment), smoking, and occupation were obtained from the questionnaire and supplemented with Medical Examiner and recent pharmacy records. Only lifelong nonsmokers were included in the study reported here. With consent of the next of kin, the left lungs of all study subjects obtained at autopsy were fixed with 2.5% gluteraldehyde at 20-cmH2O pressure via the main stem bronchus. The airways of the anterior basal and posterior basal segments of the left lower lobe and apical segment of the upper lobe were systematically sampled, as described previously (11), and 5-μm sections were stained with hematoxylin and eosin for routine examination and Alcian Blue/periodic acid Schiff pH 2.5 (AB/PAS) for quantification of muco-substances. An asthma severity score (absent, mild, moderate, or severe), based on the pathology, was derived for each subject (11).

**Bronchial brushings.** All samples were obtained under the auspices of protocols approved by the University of New Mexico School of Medicine and the Lovelace Respiratory Research Institutional Review Boards. These samples were obtained by bronchoscopy at the University of New Mexico Health Sciences Center. All participants were recruited by advertising in local newspapers and in the University newspaper. Age was limited to 18–45 yr. Asthma was defined according to American Thoracic Society criteria (1).

To be included in the study, the asthmatic subjects had to exhibit a >15% increase in forced inspiratory volume in 1 s (FEV1) in response to 200 μg albuterol, or increased sensitivity to inhaled methacholine. Increasing concentrations (0.156–25 mg/ml) of methacholine were inhaled via a nebulizer through a Rosenthal-French dose-metering device until the FEV1 decreased by at least 20% from baseline values. Increased methacholine reactivity was defined as >20% decrease of FEV1 after inhalation of <25 mg/ml methacholine.

Normal subjects had no history of asthma or upper respiratory tract atopy, negative allergy skin tests, and normal spirometry. None of the asthmatic or control subjects was a smoker. Subjects who were exposed to chronic medications, allergy immunotherapy, or corticosteroid therapy (oral or inhaled) during the past 6 mo, or had experienced symptomatic asthma or upper respiratory tract infection within 4 wk before enrollment, were excluded from the study. All subjects had a chest roentgenogram, electrocardiogram, urinalysis, and complete blood count and were excluded for any clinically significant abnormality. Female subjects had a negative pregnancy test within 1 wk before bronchoscopy.

Bronchial brushings, performed under local anesthesia with 1% lidocaine, contained 0.4–2 million epithelial cells. From each brushing, cytospins were prepared with ~5,000 cells for staining with Wright Giemsa, and at least three cell aliquots with ~60,000 cells each were frozen at ~80°C.

**Quantification of mucous glands and surface MCM.** The area of bronchial wall occupied by mucous glands was quantified on the AB/PAS-stained sections using a modified point-counting technique (22). The point counting was done at a magnification of ×10 or ×20, depending on the size of the airway, and the membrane intersection counts were at ×2.5. The airway epithelial basement membrane length was calculated from the horizontal and vertical grid intersections with the airway basement membrane. Point counts for the mucous glands were converted to areas using the formula, area = Z^2 (number of points), and basement membrane (BM) intersection counts were converted to lengths using the formula, BM length = Z (π/4) (number of intersections). In both cases, Z represented a magnification constant, which was obtained by measuring the distance between two grid points at a specific magnification.

Airway surface MCM was evaluated semiquantitatively using a grading system based on evaluation of all (n = 17) AB/PAS-stained sections using a scoring system (absent = 0, mild = 1, moderate = 2, or severe = 3).

**Quantification of serum IgE.** Postmortem sera were taken after death and stored at ~20°C until assayed. Total IgE was quantified using the Pharmacia CAP System IgE FEIA TEMPO, as previously described (29). The samples were coded, and the analyst was unaware of case details. Reproducibility was determined on duplicate samples submitted to the laboratory in separate batches.

**Cell culture.** Primary (normal) human bronchial epithelial cells (NHBECs) were obtained from 10 different lungs under the protocol and consent form approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. Epithelial cells were removed from the lower trachea and bronchi by protease XIV digestion, and cells were plated in BEGM medium on collagen-coated dishes, as described previously (9). Approximately 5 × 10^4 passage 2 cells were seeded onto 5-mm-diameter type VI collagen (Sigma) coated Millicell CM inserts (0.4 μM pore size, Millipore, Bedford, MA), and, following confluence at day 4–5, were maintained at an air-liquid interface for an additional 14 days. NHBECs from each individual either were left untreated or were treated for 4 days with 10 ng/ml IL-13 from the basal side of the Transwell system before harvesting for RNA isolation.

**Immunohistochemistry.** Bax and Bcl-2 protein detection by immunohistochemistry was performed as previously described (8, 36). The anti-Bax antibody, a gift from Dr. John Reed, The Burnham Institute, La Jolla, CA, and the anti-Bcl-2 antibody (BD-Pharmingen, San Diego, CA) were used at a dilution of 1:1,000. The number of Bax-
and Bcl-2-positive cells as a percentage of all mucous cells was recorded.

**RNA samples and reverse transcription.** Stratagene universal human reference (UHR) total RNA was used for testing the preamplification procedure using various RNA amounts. From bronchial brushings, RNA was isolated from 60,000 cells using RNeasy Micro Kit (Qiagen, Valencia, CA) and eluted with 16 µl of RNase-free water. RNA samples were then reverse transcribed to cDNA using ABI’s High-Capacity cDNA Archive kit (Applied Biosystems; P/N 4322171).

**Degraded RNA samples.** For testing the effect of RNA degradation, Stratagene UHR total RNA was degraded by adding 10 mM NaOH and heating at 37°C for varying lengths of time, ranging from 1 to 5 min. At every minute interval, samples were removed and immediately neutralized with 10 mM Tris, pH 7.0. The RNA integrity number (RIN) was determined by running samples on the Agilent 2100 Bioanalyzer.

**Preamplification.** Approximately 1–250 ng of cDNA were used for 50–µl preamplification reactions. After converting degraded and intact RNA samples to cDNA, 25 ng of cDNA were used for preamplification. Preamplification of the cDNA prepared from Stratagene UHR total RNA was carried out using TaqMan Gene Expression Assays and the 2× TaqMan PreAmp Master Mix in the ABI PRISM 7900HT Real-Time PCR System. The assays that represent primers and probes specific for the 100 genes are listed in Supplemental Table S1. (The online version of this article contains supplemental data.)

**Preamplification of the cDNA prepared from the bronchial brushings was carried out using primers specific for MUC5AC and 15 Bcl-2 family gene members, including Bcl-2, Bax, Bak, Mcl-1, Bcl-xL, Blk, Bid, Bok, Bad, Bnip3L, Hrk, Noxa, Bmf, and Puma. To determine whether cytokines from inflammatory cells are detectable, probes and primers for IL-9 and IL-13 were included. We also amplified several genes that are used for normalizing mRNA levels, including 18S rRNA, cyclophilin, GAPDH, and CDKN1B. The ABI premade primer/probe sets were pooled into a mixture that underwent 10 cycles of 1.5 min at 95°C for 15 s and 60°C for 4 min.

**Real-time PCR.** Before proceeding to single-target sequence detection, the preamplified products were diluted 1:20 to eliminate any downstream assay inhibition, mixed with the TaqMan Universal PCR Master Mix, and distributed into the 96-well plate. The primer/probe sets (Applied Biosystems, Foster City, CA) were distributed into each well in duplicates, and target mRNAs were amplified by real-time PCR in 20-µl reactions. Preamplification efficiency was assessed by performing amplification of nonamplified cDNA with TaqMan Gene Expression Assays. For all reactions, threshold cycle (CT) values >37 were eliminated for evaluation of preamplification efficiency. Uniform preamplification was demonstrated by a ΔΔCT value of 1.5–1.5 when comparing the CT values of each gene amplified from preamplified and nonamplified cDNA. Because all results were derived from the linear amplification curve, the use of ΔΔCT method ensures that only mRNA amplification within the linear range was compared.

**Statistical analysis.** The ΔCT or ΔΔCT values from each qRT-PCR data for each gene from 20 bronchial brushings, consisting of 9 control and 11 asthma samples, were analyzed after normalization using the free R statistical packages version 2.3.0 (http://www.r-project.org/). To reduce the influence of possible outliers in the sample, ΔCT values for each gene were scaled using the formula [sample – median (samples)/MAD (samples)], such that the median equals 0 and the median absolute devation (MAD) equals 1. Welch’s test of equality of means, in which R is formulated as a linear model test, was used to assess differences in qRT-PCR from the samples of asthmatic subjects and controls, and P values of <0.05 were considered statistically significant.

For the data obtained from bronchial brushings and autopsy samples, ANOVA was used to test the effects of theophylline, inhaled steroids, oral steroids, /β/-agonists, and the presence or absence of asthma on the Bax variables after preliminary analysis demonstrated normal distribution of the Bax variables. Using the variable percent Bax, weighted least squares, with weights equal to the number of replicates per subject, was performed.

**RESULTS**

**Subjects.** Autopsy tissues suitable for immunostaining were obtained from five NAC, five NFA, and seven FA subjects. Demographic and clinical information on these subjects are shown in Table 1. Subjects in all groups were life-long non-smokers. The causes of death for the five FA cases were congenital heart disease, asphyxia in an avalanche, morbid obesity, coronary artery disease, and diabetic coma. For the five NAC cases, dissecting aneurysm, subarachnoid hemorrhage, carbon monoxide poisoning, and renal failure were recorded as cause of death. Comorbid conditions were seen in 3 of the 17 cases. Two asthma cases had obesity, and one asthma case had anaphylaxis, as comorbid conditions. The time from death to autopsy was not significantly different between the groups. The serum IgE in the NAC group (10.5 ± 10.6) was significantly less (P < 0.05) than in the NFA (132.6 ± 218.8) and FA (396.0 ± 281.6) groups. All seven FA subjects had reported that both atopic and nonatopic factors triggered their asthma. Only one of the NFA group had reported both atopic and nonatopic factors as triggers for his or her asthma, two had reported purely atopic asthma, and two purely nonatopic asthma. All subjects with asthma and none of the control subjects had been taking regular inhaled /β/-agonists. All seven FA cases had received a course of oral corticosteroids in the year before death. Information on other asthma medications was incomplete.

Bronchial brushings were obtained from 11 subjects each with mild asthma (8 women and 3 men, with an average age of 29 ± 9 yr), and 9 controls with no evidence of lung disease (5 women and 4 men with an average age of 31 ± 12 yr). Demographic and clinical information on these subjects are shown in Table 2.

**Mucous gland size and surface epithelial MCM.** Mucous gland size (Fig. 1A) and surface MCM (Fig. 1B) were both significantly (P < 0.05) increased in the asthma groups (NFA and FA) compared with the NAC group. Furthermore, MCM was significantly (P < 0.05) increased in the FA group compared with the NFA group.

**Bax expression in airway epithelia of asthmatic subjects and controls.** Expression of Bax was analyzed by immunohistochemistry in autopsy tissues from patients with mild to severe
Table 2. Demographic information of subjects from whom bronchial brushings were obtained

<table>
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<tr>
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<th>Control</th>
<th>Asthma</th>
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<tr>
<td>n</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Age, yr</td>
<td>31.4±12.6</td>
<td>29.0±8.9</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>4/5</td>
<td>3/8</td>
</tr>
<tr>
<td>Smoking status (yes/no)</td>
<td>0/0</td>
<td>0/1</td>
</tr>
<tr>
<td>FEV₁, %predicted</td>
<td>96.0±7.7</td>
<td>94.1±11.7</td>
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<tr>
<td>FVC, %predicted</td>
<td>99.7±9.9</td>
<td>105.4±8.7</td>
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<tr>
<td>FEV₁/FVC</td>
<td>81.1±2.4</td>
<td>75.3±5.8</td>
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Values are means ± SD; n, no. of subjects. FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; FEV₁/FVC, ratio of FEV₁ to FVC.

asthma and compared with the NACs (Fig. 1, B and C). The percentage of Bax-positive mucous cells was greater in NACs compared with both groups with asthma (NFA and FA) (Fig. 1A). The differences between groups were significant (ANOVA, P = 0.001), whereby the percentage of Bax-positive cells in the NAC group was higher than that in the asthma groups combined; there was an estimated difference of 36.9 (95% confidence interval, 8.8–65.0). Use of theophylline, inhaled steroids, oral steroids, or (95% confidence interval, 8.8 – 65.0). Use of theophylline, inhaled steroids, oral steroids, or β-agonist grouped as daily (high) and occasional (low) showed no significant effect on percent Bax. No differences were observed in the percentage of Bcl-2-positive mucous cells among these groups (data not shown).

Uniformity of preamplified cDNA. Because expression of genes can occur in autopsy tissues due to postmortem changes before fixation and processing, we wanted to validate these findings in bronchial brushings from living asthma and control subjects. In animal models of inflammation, the Bcl-2 family of proteins is crucial for maintaining the hyperplastic airway epithelial cells (12, 36, 37). Therefore, we wanted to analyze expression of transcripts for Bcl-2 family members in the bronchial brushings, including main mucin mRNA, MUC5AC. For this purpose, we developed a robust method to analyze mRNA levels quantitatively.

Initially, we developed a method to assess uniformity of preamplification, i.e., whether the gene expression profile of the original cDNA sample would be retained following the preamplification procedure. In each sample, target mRNAs were analyzed by running the qRT-PCR in duplicate; therefore, CT values for each target were normalized by subtracting average CT value for the uniformity reference gene from the average CT value for the specific target to obtain ΔCT. In general, CDK1NB was used as a uniformity reference gene because of its consistent gene expression profile, while normalizing to cyclophilin resulted in similar results. The same procedure was used to obtain ΔCT values for the preamplified products and for targets amplified from the cDNA template, because the difference between the two ΔCT values (ΔΔCT = ΔCT preamp − ΔCT cDNA) is expected to be close to zero, if the preamplification yielded uniform detection without modifying the target amplified from the unamplified cDNA. Therefore, ΔΔCT values that were between ±1.5 were considered acceptable, denoting similar amplification efficiency. Interestingly, 90% of TaqMan Gene Expression Assays fulfilled that criterium (Fig. 2). In addition, using cDNA prepared from Stratagen’s UHR total RNA at 1, 25, and 250 ng showed reliable uniformity of amplification for the 100 gene targets that were tested (Fig. 2).

Because acquisition of human samples is often faced with the presence of degraded RNA, we further investigated whether RNA degradation affected uniformity of amplification from preamplified cDNA products compared with nonamplified cDNA. Degradation of Stratagen’s UHR total RNA was caused by incubation in 10 mM NaOH for 1 and 3 min, RNA degradation was assessed after running the sample on Agilent

Fig. 1. Mucous gland size and surface mucous cell metaplasia were significantly increased, but the percentage of Bcl-2-associated protein X (Bax)-positive mucous cells was significantly reduced in fatal asthmatic (FA) and nonfatal asthmatic (NFA) groups compared with nonasthmatic controls (NAC). Tissue sections were stained with Alcian Blue/periodic acid Schiff. Mucous gland size (A) and surface mucous cell metaplasia (B) were scored, and the mean of the scores ± SE are shown. Tissue sections were immunostained for Bax and counterstained with Alcian Blue, and the percentage of Bax-positive mucous cells was quantified. C: the mean percentage within groups is shown by a horizontal line. Quantifications were performed by a person unaware of slide identity. Significant differences among groups are shown (*P < 0.05). Representative photomicrographs are shown of mucous cells that are immunopositive for Bax (arrows) in NAC controls (D) and immunonegative in patients with asthma (E).
The mRNA levels for MUC5AC, Bcl-2, Bak, Mcl-1, Bad, Bid, calculated from the linear fit for each of the target transcripts. Those in the control group. The mRNAs in the asthma group were significantly different from to determine whether expression levels of any Bcl-2 family levels for each of the Bcl-2 family transcripts.

shows the feasibility of preamplifying and testing the mRNA tested were within the acceptable
diseases. Except for the actin mRNA, all other gene targets
brushings from patients with asthma or controls without lung
tested were described above for each of the samples obtained by bronchial
were not detected in any of the bronchial brushings analyzed.

expression profile of the Bcl-2 family of genes. To exclude the possibility that mRNA from contaminating inflammatory cells may affect the results, probes and primers for IL-9 and IL-13 were included in the analyses. IL-9 and IL-13 mRNAs were not detected in any of the bronchial brushings analyzed.

The uniformity of preamplification was assessed by comparing the ΔC_T data from real-time relative quantitation of cDNA with the ΔC_T data from preamplified amplicon as a starting template. The normalization was carried out as described above for each of the samples obtained by bronchial brushings from patients with asthma or controls without lung diseases. Except for the actin mRNA, all other gene targets tested were within the acceptable ±1.5 range (Fig. 4B), which shows the feasibility of preamplifying and testing the mRNA levels for each of the Bcl-2 family transcripts.

Standard linear model was applied to the qRT-PCR data to determine whether expression levels of any Bcl-2 family mRNAs in the asthma group were significantly different from those in the control group. The F statistics and P values were calculated from the linear fit for each of the target transcripts. The mRNA levels for MUC5AC, Bcl-2, Bak, Mcl-1, Bad, Bid, Bnip3L, and Bok were unchanged in the asthma group compared with controls; mRNAs for Blk and Hrk were essentially not detected in all samples. Results for Bcl-xL, Bmf, Bik, and Puma will be reported elsewhere. Box plots of three mRNAs (Bax, Bcl-2, and MUC5AC) are shown as examples representing fold change relative to the control group (Fig. 4B). For Bax mRNA levels, the differences in the median and mean between asthmatic subjects and controls were −0.76 and −1.23, respectively, and the P value from the Welch test was 0.027 (Fig. 4C). IL-13 is a major Th2 cytokine that causes MCM in asthma (34, 40). To further confirm that Bax mRNA levels are reduced in asthma, we treated differentiated NHBEC cultures from 10 individuals with IL-13 and determined the levels of Bax mRNA by qRT-PCR. Bax mRNA levels were significantly reduced in IL-13-treated cultures compared with untreated controls (Fig. 4D).

**DISCUSSION**

Reduced Bax mRNA levels, as detected by quantitative PCR in cells from the bronchial brushings, correlated with the reduced expression of Bax proteins in airway mucous cells in autopsy tissue from asthmatic subjects compared with NACs. Autopsy material is prone to artifacts due to postmortem changes in tissues before and after fixation and processing. Furthermore, we introduced a selection bias by only studying airways with intact bronchial epithelium. Nonetheless, the complimentary information derived from fresh (brushings) and fixed (postmortem) tissues suggests that the findings are real and occur in vivo.

2100 Bioanalyzer, and extent of degradation was expressed as RIN (Fig. 3A). Both the intact RNA (RIN number of 9.0) and the partially and more extensively degraded RNA (RIN numbers of 7.1 and 3.3, respectively) were converted to cDNA, and 25-ng cDNA were subjected to preamplification. Comparison of amplification uniformity to nonamplified cDNA showed that essentially all 100 genes were amplified similarly in both the intact RNA and the degraded RNA (Fig. 3B). These results suggested that this procedure should be useful for reliably analyzing expression of the Bcl-2 family of genes from bronchial brushings that were frozen back as 60,000 cell pellets.

**Characterization of cells obtained by bronchial brushings.**

To determine the percentage of inflammatory cells present in the bronchial brushings, cytospins prepared with ~5,000 cells were stained with Wright Giemsa. White blood cells accounted for 3–6% of all cells in the samples that were obtained from subjects with asthma or from the control group (Fig. 4A). No differences in the percentages of macrophages (2.8–3.4%), lymphocytes (0.8–1.2%), or polymorphonuclear cells (0.1–0.4%) were observed among the groups. The rest of the cells were airway epithelial cells.

**Expression profile of the Bcl-2 family of genes.** To exclude the possibility that mRNA from contaminating inflammatory cells may affect the results, probes and primers for IL-9 and IL-13 were included in the analyses. IL-9 and IL-13 mRNAs were not detected in any of the bronchial brushings analyzed.

The uniformity of preamplification was assessed by comparing the ΔC_T data from real-time relative quantitation of cDNA with the ΔC_T data from preamplified amplicon as a starting template. The normalization was carried out as described above for each of the samples obtained by bronchial brushings from patients with asthma or controls without lung diseases. Except for the actin mRNA, all other gene targets tested were within the acceptable ±1.5 range (Fig. 4B), which shows the feasibility of preamplifying and testing the mRNA levels for each of the Bcl-2 family transcripts.

Standard linear model was applied to the qRT-PCR data to determine whether expression levels of any Bcl-2 family mRNAs in the asthma group were significantly different from those in the control group. The F statistics and P values were calculated from the linear fit for each of the target transcripts. The mRNA levels for MUC5AC, Bcl-2, Bak, Mcl-1, Bad, Bid,
Clinical samples used for RNA analysis are often small and may contain compromised or degraded RNA, due to the disease process or the lag time between sample collection and preservation. Therefore, it is imperative to develop methods that can utilize these valuable samples to study genetic changes associated with pathology.

The present study shows that the preamplification procedure for analyzing multiple transcripts in a limited number of cells obtained by bronchial brushings is highly efficient. Our findings suggest that the best results are obtained when a minimum of 1 ng cDNA is used and C values >37 are excluded from final analyses, because C values >37 represent marginal detection and, therefore, provide inconsistent results. The 18S rRNA or actin TaqMan assays were not useful as reference, as they introduced amplification bias, because of their high expression levels and the lack of a reliable TaqMan assay, respectively. Furthermore, when analyzing a set of genes using this method, it is important to verify the uniformity of PCR results by examining the preamplified and the nonamplified cDNA using the de method.

The optimized cycling parameters with the TaqMan PreAmp Master Mix allowed nearly 100% efficient amplification of target sequences, even when partially degraded RNA is used as starting material. Therefore, this preamplification procedure can be reliably used to amplify transcripts from minute samples or identify low abundant mRNA from limited samples. These studies lay the foundation for successfully analyzing expression of a large number of genes in samples of very limited availability, obtained from either archived slides by laser microdissection, or as described in the present study by bronchial brushings.

Using this method, we found no differences in the MUC5AC mRNA levels between the NACs and the group with asthma. There was no evidence of immunologically mediated comorbid disease (other than asthma) in these populations that would be likely to affect the extent of MCM. However, subjects with asthma had significantly increased serum IgE levels compared with the NAC group, and the FA group had significantly increased serum IgE levels compared with the NFA group. Furthermore, both asthmatic groups had significantly more MCM compared with the NAC group, and the FA group had significantly more MCM than the NFA group. These findings suggest that the FA group was more atopic and had more severe asthma compared with the NFA group. Consistent with our study, two previous studies reported that MUC5AC mRNA levels were not significantly increased in mild-to-moderate asthmatic subjects compared with controls (25, 41), while MUC5AC protein was increased in the subjects with asthma (25). Because the small airways in humans are not accessible by bronchoscopy, all human studies using bronchoscopy procedures to obtain airway epithelia from human subjects are limited to sampling large airways, whereas MCM may be more evident in airways smaller than 2 mm in diameter. It is also possible that Bax is crucial in regulating hyperplastic airway epithelial cells that may be involved in dysfunctional, innate immune responses other than increased mucous production, such as perpetuating adaptive immune responses or producing mediators that affect the underlying mesenchyme (13). It is also possible that epithelial cell hyperplasia may be present and MCM could rapidly develop in these epithelia. A study of smokers with chronic bronchitis and chronic airflow obstruction showed increased levels of MUC5AC mRNA compared with nonsmoking controls (14). The differences in MUC5AC gene expression between subjects with asthma and chronic bronchitis shown by these studies may reflect the influence of cigarette smoke. Current exposure to cigarette smoke may directly induce MUC5AC mRNA expression, as shown in cell culture studies (10). None of the asthmatic subjects in this...
Bax EXPRESSION IN AIRWAYS FROM ASTHMATIC SUBJECTS

study had ever smoked. Therefore, future studies need to compare MUC5AC mRNA levels in subjects with asthma or chronic bronchitis, having varying degree of disease severity and age, and in current and former cigarette smokers.

It could be argued that the presence of 3–6% white blood cells in the bronchial brushing samples was the source of the Bc1-2 family of mRNAs detected. IL-13 mRNA can be detected in lavaged inflammatory cells from asthmatic subjects (5, 7). IL-9 mRNA has been detected in the lavage of asthmatic children (23) and in bronchial biopsy specimens of subjects with bronchial hyperresponsiveness and increased eosinophilic infiltrates (38). However, the lack of detection of IL-9 or IL-13 in the bronchial brushings of the present study supports the hypothesis that inflammatory cells were not the source of the major portion of mRNA. Furthermore, the finding of decreased Bax mRNA levels in the epithelial cells of asthmatic subjects compared with controls was confirmed by immunohistochemical analysis. This finding indicates that results obtained from bronchial brushings by and large reflect the status of the bronchial epithelial cells, which were the predominant cells in the brushings.

The percentage of Bax-positive mucous cells, as detected by immunohistochemistry, was significantly reduced in asthmatic subjects compared with controls. Similarly, Bax mRNA levels were significantly reduced in bronchial brushings from asthmatic subjects compared with controls. Lidocaine is not known to affect Bax expression, and it is highly unlikely that 1% lidocaine applied 1–3 min before bronchoscopy could affect gene expression within the 10 min that were required to remove the cells from the brushes into the culture media. Cells obtained by bronchial brushings represent all types of airway epithelial cells, but only a subset of epithelial cells expresses Bax, as shown by immunohistochemistry. Therefore, it is possible that Bax mRNA levels are diluted by nonexpressing cells, leading to the minimal differences that were observed by qRT-PCR compared with the dramatic differences when Bax expression in mucous cells was quantified by immunohistochemistry. In addition, the findings that Bax mRNA levels were significantly reduced in NHBECs treated with IL-13 further support the finding that Bax expression is reduced in airway epithelia of human asthmatic subjects.

How Bax expression is affected by inflammatory cytokines is largely unknown. Bax expression is increased along with caspases-3, -6, -7, and -9 in the lungs of IL-13 transgenic mice (4). In those studies, expression of Bax was analyzed in the whole lung and was not focused on the epithelia. Furthermore, the levels of IL-13 in these mice may be highly increased compared with what is found during allergic inflammation. Because IL-13 has different effects, depending on its levels of expression, it is conceivable that increased Bax expression in IL-13 transgenic mice does not reflect the situation in allergic asthma. We recently reported that Bax mRNA levels are not affected by treatment with IFN-γ, but rather the translocation of Bax to the endoplasmic reticulum is observed in cells undergoing IFN-γ-induced apoptosis (31). Whether, in addition to IL-13, other cytokines in asthma may reduce Bax expression in airway cells of the bronchial epithelium, as was shown by both qRT-PCR and immunohistochemical staining in the present study, needs further investigation.

The findings in the present study are consistent with our laboratory’s previous findings in mice that Bax is involved in the resolution of MCM during prolonged exposure to allergen (36). In mice, the loss of Bax is associated with lymphoid hyperplasia (17). Reduced expression of Bax has been attributed to many human diseases, primarily tumors. Lower expression of Bax in ovarian (32), cervical (18), and in metastatic breast (19) carcinomas is associated with poor response to chemotherapy and shorter disease-free survival. The mechanisms underlying aberrant expression of Bax include structural changes in the gene and p53-mediated downregulation of Bax expression. A single-nucleotide polymorphism in the Bax gene promoter affects its expression and is associated with a higher stage of disease and failure to achieve complete response to treatment in chronic lymphocytic leukemia (24). Mutations in the second and third exons of Bax gene affect protein expression (21, 28, 43). Whether polymorphisms or mutations in the Bax gene affect Bax expression in asthma needs to be studied in the future.

In summary, we have shown that Bax expression and Bax protein are significantly reduced in airway epithelial cells derived from both stable and symptomatic asthmatic subjects compared with NAC subjects. These findings, taken in conjunction with animal studies, indicate that downregulation of Bax expression is important for sustained epithelial cell hyperplasia and that restoring Bax expression may restore normal homeostasis and proper function of the airway epithelium in subjects with asthma.

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