Cigarette Smoke Suppresses Bik To Cause Epithelial Cell Hyperplasia and Mucous Cell Metaplasia

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Rationale: Aberrant regulation of airway epithelial cell numbers in airways leads to increased mucous secretions in chronic lung diseases such as chronic bronchitis. Because the Bcl-2 family of proteins is crucial for airway epithelial homeostasis, identifying the players that reduce cigarette smoke (CS)-induced mucous cell metaplasia can help to develop effective therapies.

Objectives: To identify the Bcl-2 family of proteins that play a role in reducing CS-induced mucous cell metaplasia.

Methods: We screened for dysregulated expression of the Bcl-2 family members.

Measurements and Main Results: We identified Bik to be significantly reduced in bronchial brushings of patients with chronic epithelial cell hyperplasia compared with nondiseased control subjects. Reduced Bik but increased MUC5AC mRNA levels were also detected when normal human airway epithelial cells (HAECs) were exposed to CS or when autopsy tissues from former smokers with and without chronic bronchitis were compared. Similarly, exposure of C57Bl/6 mice to CS resulted in increased numbers of epithelial and mucous cells per millimeter of basal lamina, along with reduced Bik but increased Muc5ac expression, and this change was sustained even when mice were allowed to recover in filtered air for 8 weeks. Restoring Bik expression significantly suppressed CS-induced mucous cell metaplasia in differentiated primary HAEC cultures and in airways of mice *in vivo*. Bik blocked nuclear translocation of phospho-ERK1/2 to induce apoptosis of HAECs. The conserved Leu61 within Bik and ERK1/2 activation were essential to induce cell death in hyperplastic mucous cells.

Conclusions: These studies show that CS suppresses Bik expression to block airway epithelial cell death and thereby increases epithelial cell hyperplasia in chronic bronchitis.

Keywords: Bcl-2 family of proteins; chronic bronchitis; differentiated human airway cultures; former smokers; MUC5AC

Cigarette smoking is the leading cause of disease for 15 to 17 million individuals with chronic obstructive pulmonary disease (COPD) in the United States alone and for more than 200 million people worldwide (1, 2). With 1.25 billion people smoking cigarettes daily worldwide (3), these diseases are expected to reach epidemic proportions in the next decade (1). In humans, epithelial cell hyperplasia and increased mucous cell numbers is a frequent finding in the large and small airways of cigarette smokers (4, 5). Indeed, the amount of intraluminal mucus in the small airways is responsible for airway obstruction and reduced lung function, and excess mucous secretions in the airways are important in the pathogenesis of acute exacerbations of COPD (6, 7). Contact of the airway epithelium with bacteria or viral infectious agents and environmental pollutants

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although it is known that chronic mucous secretion is a hallmark of chronic bronchitis, the mechanisms underlying this condition are unknown.

What This Study Adds to the Field

We show that hyperplastic epithelial cells that secrete mucus are sustained by cigarette smoke suppressing a cell death-inducing protein called Bik. Restoring expression of this protein is a possible therapeutic approach for reducing mucous hypersecretion in chronic bronchitis.

elicits an inflammatory response that recruits polymorphonuclear cells and macrophages to the airways and initiates the proliferation of epithelial cells (8–10). The number of epithelial cells that produce sufficient mucins and protection from further injury is increased by proliferation (11) but when sustained for longer periods can be the basis for airflow obstruction in subjects with asthma and chronic bronchitis (12). Our studies show that after inflammatory responses, up to 30% of hyperplastic cells undergo death to eliminate excess mucous cells and to return to the original cell numbers (13, 14). Disruption of this recovery process may lead to persistent elevation of mucous cell numbers and contribute to chronic epithelial cell hyperplasia and excess mucus hypersecretion and airway obstruction found in chronic lung diseases such as cystic fibrosis (13), asthma (15), and chronic bronchitis (16, 17). In cigarette smokers, hyperplastic airway epithelial cells can also be the source for neoplastic lesions during carcinogenesis (18, 19).

The cell death of airway epithelial cells (AECs) during the resolution of metaplastic mucous cells is regulated by the Bcl-2 family of proteins (20, 21), and involves the intrinsic apoptotic pathway (22). During the resolution of allergen-induced epithelial and mucous cell hyperplasia that is mediated by IFN- γ , we showed that STAT1 and Bik, a proapoptotic Bcl-2 family member, are crucial factors, because unlike wild-type mice, STAT1- and Bik-deficient mice fail to resolve mucous cell metaplasia (MCM). Consistent with these findings, mouse airway epithelial cells (MAECs) from STAT1- and Bik-deficient mice are resistant to IFN γ -induced cell death (23).

Based on these previous findings we wanted to test the hypothesis that a BH3 only-domain protein may be responsible for sustained epithelial cell hyperplasia (ECH) in cigarette smokers. We found that cigarette smoke (CS) suppresses Bik mRNA levels both in differentiated primary normal human airway epithelial cells (HAECs) in culture and in HAECs from patients with chronic bronchitis. Similarly, exposure of mice to CS reduced Bik expression and increased Muc5ac mRNA levels as well as the number of airway epithelial cells. Ectopic expression of Bik using adenoviral vector reversed CS-induced

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MCM by blocking nuclear translocation of activated ERK1/2 to mediate cell death in AECs. We show that in the presence of Bik, phospho-ERK1/2 is retained in the cytosol to facilitate cell death.

METHODS

Lung Autopsy Tissues

Autopsy tissues were obtained from the Lung Tissue Research Consortium, National Heart, Lung, and Blood Institute. The subjects were categorized into four groups based on records obtained by questionnaires administered. For the subgroups classified by chronic bronchitis, the definition of "signs with chronic bronchitis," which is cough and phlegm for 3 consecutive months and for at least 2 years, was used. All who did not answer yes for these questions and answered "do not usually have cough, and do not usually have phlegm" were defined as subjects with no signs of chronic bronchitis. Subjects with less than 15 pack-years of smoking were excluded. The demographic characteristics of the subjects from whom autopsy tissues were used for our studies are shown in Table 1.

Bronchial Brushings

Protocols were approved by the University of New Mexico School of Medicine and the Lovelace Respiratory Research Institutional Review Boards to obtain all bronchial samples. Bronchial brushings 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. Chronic bronchitis was defined as a daily cough with phlegm production for 3 consecutive months, 2 years in a row. Bronchial brushings were obtained from 11 subjects each with chronic bronchitis and 9 control subjects with no evidence of lung disease. Demographics of these subjects are described in Table 2. Bronchial brushings, performed under local anesthesia with 1% lidocaine, contained 0.4 million to 2 million epithelial cells. At least 60,000 cells from each subject were used for quantitative real-time polymerase chain reaction (qRT-PCR) as described previously (15).

Mice and Adenoviral Infection

Male-specific pathogen-free wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in isolated cages under specific pathogen-free conditions. After a 14-day quarantine period, mice were acclimatized for 8 days and entered into the experimental protocol at 8 to 10 weeks of age. *bik*^{+/-} mice on C57BL/6 background were provided by Andreas Strasser (Walter and Eliza Hall Institute, Melbourne, Australia). *bik*^{+/-} with *bik*^{-/-} littermates were bred from the respective heterozygote mice at the Lovelace Respiratory Research Institute under specific pathogen-free conditions and genotyped as described previously (24). All experiments were approved by the Institutional Animal Care and Use Committee and were conducted at Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International. Mice were exposed to 250 mg/m³ CS or filtered air for 6 h/d, 5 d/wk for 3 weeks or were allowed to recover in air for an additional 8 weeks after 3 weeks of CS exposure. Preparation of lung tissues for histopathological examination was performed as described previously (25). After 3 weeks of exposure to CS, mice were anesthetized with isoflurane and intranasally instilled with adenoviral expression vector for hemagglutinin-tagged Bik (HA-

TABLE 2. DEMOGRAPHICS OF BRONCHIAL BRUSHING DONORS

	Control Subjects (n = 9)	Subjects with Chronic Bronchitis (n = 11)
Age, yr, mean ± SD	31.4 ± 12.6	36 ± 9.23
Sex, male/female	4/5	5/6
Smoking status, yes/no	0/9	2/9

Ad-Bik) or adenoviral expression vector for green fluorescent protein (Ad-GFP) as a control in a volume of 50 µl saline on Days 1 and 2 after the last day of exposure. On Day 3, six mice from each group were killed and right lung tissue harvested and immediately examined for expression of HA protein via Western blotting of protein extracted from lung homogenate. Left lungs were inflated and fixed at 25 mm pressure with zinc formalin for preparing tissue sections and evaluating ECH and MCM.

Tissue sections were stained with Alcian blue (AB) and periodic acid Schiff or hematoxylin and eosin as described previously (20). The number of AB-positive cells per millimeter of basal lamina and per total cell count were quantified using a light microscope (BH-2; Olympus, Melville, NY) equipped with the Image analysis system (National Institutes of Health) as described previously (26).

Cells

Mouse airway epithelial cells (MAECs) were harvested and cultured on Transwell membranes (Corning, New York, NY) after seeding with 4 × 10⁴ to 9 × 10⁴ cells as previously described (27). Primary HAECs were purchased from Cambrex Bio Science Walkersville, Inc (Walkersville, MD). The immortalized HAECs, AALEB cells, provided by S. Randell (University of North Carolina Chapel Hill, Chapel Hill, NC), were described previously (28).

Plasmids, Adenoviral Constructs, and Reagents

Adenoviral expression vectors for Bik and Bik^{L61G} were provided by G. Shore (McGill University, Montreal, Quebec, Canada), and cells were infected as described previously (29). The mitogen-activated protein kinase extracellular signal-regulated kinase inhibitor 4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126) was purchased from EMD Chemicals Inc. (Darmstadt, Germany).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

Protein lysates were prepared and analyzed by Western blotting as described previously (20). Cytosolic and nuclear fractions were prepared by lysing cells in NP-40 to obtain the cytosolic fraction and extracting the nuclear proteins with a hypertonic extraction buffer (50 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid, pH 7.8, 50 mM KCl, and 300 mM NaCl) in the presence of protease and phosphatase inhibitors as described previously (22). The following antibodies were used: goat anti-Bik polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-Bik antibody, rabbit anti-phospho-ERK1/2 antibody, and rabbit anti-ERK1/2 antibody (Cell Signaling Technology, Danvers, MA). Equal protein loading was confirmed by subsequent probing with the mouse monoclonal antibody against actin (Santa Cruz Biotechnology, Inc.).

Immunofluorescence

Sections of differentiated primary HAECs and of lung tissues were deparaffinized, rehydrated, and washed. After antigen retrieval, sections were incubated with a 1:1,000 dilution of anti MUC5AC antibody (MAB2011; Chemicon, Billerica, MA) at 4°C overnight followed by 1:500 dilution of secondary donkey anti-mouse-biotin antibody (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hour. Vectastain ABC (Vector Laboratories, Inc., Burlingame, CA) and streptavidin 649 dye (Jackson ImmunoResearch) were used for detection and mounting in 4',6-diamidino-2-phenylindole Fluoromount-G (SouthernBiotech, Birmingham, AL). Immunofluorescence was imaged using Axioplan 2 (Carl Zeiss, Inc., Thornwood, NY) with a Plan-Neofluor 40×/0.75 air objective and a charge-coupled device

TABLE 1. DEMOGRAPHICS OF LUNG TISSUE DONORS

	Never Smokers (n = 5)	Current Smokers (n = 4)	Former Smokers (n = 6)	Former Smokers (n = 6)
Chronic bronchitis	No	Yes	No	Yes
Age, yr	61.6 ± 10.9	61 ± 4.2	64 ± 10	60 ± 8.9
Sex, male/female	2/3	3/1	4/2	6/0
Smoking, pack-years	0	41.8 ± 27.4	38.8 ± 16.5	49.7 ± 24.5
Stop smoking	N/A	No	11.2 ± 6.8	8.7 ± 8.4

Definition of abbreviation: N/A = not applicable.

Data presented as mean ± SD unless otherwise noted.

camera (Hamamatsu Photonics, Hamamatsu, Japan) with the acquisition software Slidebook 5.0 (Intelligent Imaging Innovation, Denver, CO).

qRT-PCR

RNA isolated using the RNeasy Micro kit (Qiagen, Valencia, CA) eluted from the columns subjected to qRT-PCR on the ABI PRISM 7900HT Real-Time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems, Carlsbad, CA). The primer/probe sets (Applied Biosystems) were distributed into each well in duplicates, and target mRNAs were amplified by PCR in 20- μ l reactions. Preamplification efficiency was assessed by performing amplification of nonamplified cDNA with TaqMan Gene Expression Assays (Applied Biosystems). For all reactions, cycle threshold (CT) values greater than 37 were eliminated for evaluation of preamplification efficiency. Uniform preamplification was demonstrated by a $\Delta\Delta$ CT value of -1.5 to 1.5 when comparing the CT values of each gene amplified from preamplified and nonamplified cDNA as described previously (15). Because all results were derived from the linear amplification curve, the use of the $\Delta\Delta$ CT method ensures that only mRNA amplification within the linear range was compared. Normalizing mRNA levels using 18S rRNA or CDKN1B showed similar results.

Statistical Analysis

Grouped results were expressed as means $\pm$ SEM. Data were analyzed using statistical analysis software (Statistical Analysis Software Institute, Cary, NC). Results grouped by time point and genotype were analyzed using two-way analysis of variance. When significant main effects were detected ($P < 0.05$), Fisher least significant difference test was used to determine the differences between groups. A P value of 0.05 was considered to indicate statistical significance.

RESULTS

Exposure to Cigarette Smoke Reduces Bik Expression

Our previous studies in animal models suggest that the intrinsic apoptotic pathway that is responsible for removing excess numbers of mucous cells may be dysfunctional in individuals with asthma (15) and cystic fibrosis (20, 21). In an effort to determine which of the Bcl-2 family of proteins plays a role in sustaining MCM in chronic bronchitis, we screened for the expression of all Bcl-2 family mRNAs in AECs from patients with chronic bronchitis and control subjects without lung diseases. Bronchial brushings were obtained from 11 subjects

each with chronic bronchitis and 9 control subjects with no evidence of lung disease (Table 2). A standard linear model applied to the qRT-PCR data and F-statistics and P values calculated from the linear fit showed that Bik was significantly reduced in bronchial brushings of subjects with chronic bronchitis compared with nondiseased control subjects ($P < 0.05$) (Figure 1A). Levels of mRNAs encoding for Bnip3L, Bok, Bid (Figure 1A) and others (not shown) were not different among these groups. This finding was confirmed by analyzing lung tissues obtained at autopsy from current and never smokers with and without chronic bronchitis, respectively. Although Bik mRNA levels were reduced, MUC5AC mRNA levels were increased in the lung tissues of current smokers with chronic bronchitis compared with control subjects (Figure 1B). This observation was also confirmed in C57BL/6 mice that were exposed to 250 mg/m³ of mainstream CS or filtered air for 6 h/d, 5 d/wk for 10 weeks (Figure 1C). Bik mRNA levels were reduced by 50% and the Muc5ac levels increased approximately threefold in the lungs of CS-exposed mice compared with control mice.

We recently demonstrated that IFN- γ induces Bik expression in AECs within 24 hours of treatment (23). Therefore, we investigated whether CS also blocks Bik expression in IFN- γ -treated cells. Although IFN- γ induced Bik expression in HAECs, CS treatment suppressed this effect both at the mRNA and protein levels (Figures 1D and 1E).

Collectively, these findings demonstrate that CS is a potent suppressor of Bik expression both in airway epithelia of patients with chronic bronchitis and in mouse lungs, even under conditions when a proapoptotic stimulus like IFN- γ is present. To explore the mechanism of suppression, we first examined whether CS blocked STAT1 activation because several of our previous studies had shown that IFN- γ induced Bik expression in a STAT1-dependent manner (22, 23). However, treatment of HAECs with CS did not reduce STAT1 activation (data not shown), suggesting that CS-induced reduction of Bik expression occurs independently of the STAT1 pathway.

Reduction of Bik by CS Causes Irreversible ECH and MCM

Patients with chronic bronchitis have increased numbers of mucous cells compared with control subjects, and this increase

Figure 1. Exposure to cigarette smoke (CS) decreases Bik expression. (A) Bronchial brushings were obtained from 11 patients with chronic bronchitis (CB) and 9 subjects with no lung diseases as control (CTR) and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) for mRNAs of Bcl-2 family members. Box plots represent the median value and 25th and 75th quartile. Whiskers are the 5th and 95th percentile. Open circles are outlier values ($> 1.5 \times$ interquartile range). Results for Bnip32, Bok, and Bid showed no significant differences among groups. (B) Bik

and MUC5AC mRNAs quantified by qRT-PCR in lung autopsy tissues from healthy never-smokers or current smokers with chronic bronchitis. (C) Bik and MUC5AC mRNAs levels in the lungs of C57BL/6 mice exposed to 250 mg/m³ CS or filtered air for 6 h/d, 5 d/wk for 10 weeks. (D) Bik mRNA and (E) protein levels in human airway epithelial cells (HAECs) treated with nothing as control or 1,000 ng/ml cigarette smoke extract (CSE) for 24 hours and with 50 ng/ml IFN- γ for the following 24 hours. Bar = means $\pm$ SEM ($n = 4$ mice/group or $n = 3$ different treatments/group). *Denotes statistically significant difference ($P < 0.05$). CurS-CB = current smokers with CB; NS-NCB = never smokers without CB.

is due to an increased number of hyperplastic epithelial cells (12, 30). Because CS suppressed Bik expression, and our previous studies have shown that Bik plays a major role in the resolution of ECH and MCM during prolonged exposure of mice to allergen (23), we investigated whether CS induces ECH in mice regardless of the presence or absence of Bik. The number of AECs per millimeter of basal lamina was significantly higher in the lung tissues of both CS-exposed *bik*^{+/+} and *bik*^{-/-} mice compared with the filtered air-exposed control groups (Figure 2A). To investigate whether Bik remains suppressed even after cessation of CS, we examined whether the resolution of CS-induced ECH is affected when *bik*^{+/+} and *bik*^{-/-} mice are exposed to CS or filtered air for 3 weeks and allowed to recover in filtered air for 60 days. In these mice, the neutrophilic inflammatory response was resolved and was not different from air-exposed control mice (data not shown). However, the increase in epithelial cell number was sustained both in *bik*^{+/+} and *bik*^{-/-} mice exposed to CS after 60 days in filtered air (Figure 2B). Furthermore, the reduction in Bik mRNA and increase in Muc5ac levels were sustained in *bik*^{+/+} mice that were exposed to CS for 3 weeks and were allowed to recover in filtered air for 60 days (Figure 2C).

These studies suggested that Bik remains suppressed in former cigarette smokers who have persistent chronic bronchitis. Therefore, we compared lung tissues from former smokers with and without chronic bronchitis (Table 1). Bik mRNA levels were reduced, whereas MUC5AC mRNA levels were increased significantly in the lung tissues of former smokers with chronic bronchitis compared with those without (Figure 2D). To further investigate whether Bik is suppressed by CS, primary HAECs from five individuals differentiated in an air-liquid interface culture were treated with a CS extract that contained 0 or 1,000 ng/ml total particulate matter for 24 hours and harvested 5 days later. Similar to what was observed in patients with chronic bronchitis, Bik mRNA levels were reduced threefold in CS-treated cultures compared with nontreated controls. In these CS-treated HAECs, MUC5AC mRNA levels were

significantly increased (Figure 2E), and the percentage of mucous cell numbers per total cell number were increased compared with nontreated controls (Figure 2F). These findings suggest that in mice, CS exposure suppresses Bik expression in a permanent manner. A similar mechanism may cause increased mucous hypersecretion in former smokers with chronic bronchitis as the resolution of CS-induced ECH and MCM was disrupted.

Restoring Bik Expression Reduces CS-Induced ECH and MCM

Bik triggers apoptosis in several tumor cell lines, such as those from breast, lung, prostate, and colon carcinomas, as well as from glioma (31–35). Because Bik levels were reduced in patients with chronic bronchitis and in mice exposed to CS, we explored whether ectopic expression of Bik would reduce CS-induced increases in ECH and MCM. First, we treated primary HAECs that were differentiated in an air-liquid interface culture with 1,000 ng/ml CS extract for 24 hours and infected them with nothing, Ad-Bik, or Ad-Bik^{L61G} as control. Bik^{L61G} is a mutant Bik with the conserved Leu residues in the BH3 domain substituted by Gly. Immunostaining with MUC5AC antibodies showed that Ad-Bik significantly reduced the number of mucous cells in the differentiated HAEC cultures compared with noninfected or Bik^{L61G}-infected cultures (Figure 3A). Similar results were obtained for primary HAECs by AB and periodic acid Schiff staining (data not shown). To investigate whether the expression of Bik would reduce CS-induced ECH *in vivo*, we exposed mice to CS for 3 weeks and intranasally instilled them with 10⁹ pfu HA-tagged Ad-Bik or Ad-GFP or phosphate-buffered saline (PBS) as control on two consecutive days after exposures. Mice were sacrificed 1 day later and lungs from HA-Ad-Bik- and Ad-GFP-instilled mice were analyzed by Western blotting. The HA-tagged Bik was detected in mice instilled with Ad-Bik but not in those instilled with Ad-GFP (Figure 3B). In addition, CS-induced ECH was significantly reduced in the airways of mice instilled

Figure 2. Exposure to cigarette smoke (CS) leads to increased epithelial cell hyperplasia (ECH). Airway epithelial cell numbers in *bik*^{+/+} and *bik*^{-/-} mice exposed to 250 mg/m³ CS or filtered air (FA) for 6 h/d, 5 d/wk for (A) 10 weeks or (B) 3 weeks followed by 8 weeks of recovery in filtered air. The left lungs were fixed under constant pressure perfusion, cut into 4-mm slices from distal to caudal, and slices embedded in paraffin. Tissue sections (5 μ m) were stained with hematoxylin and eosin and the total epithelial cell number in the airways quantified. (C) C57BL/6 mice were exposed to 250 mg/m³ CS or filtered air for 6 h/d, 5 d/wk for 3 weeks followed by 8 weeks of recovery in air and Bik and Muc5ac mRNAs quantified by quantitative real-time polymerase chain reaction (qRT-PCR). (D) Bik and MUC5AC mRNAs in lung tissues from former smokers with and without chronic bronchitis (CB) quantified by qRT-PCR. (E) Primary human airway epithelial cells (HAECs) placed in culture in an air-liquid interface (ALI) culture were left untreated or treated with 1,000 ng/ml CSE for 24 hours and harvested 3 days later. Bik and MUC5AC mRNA levels were quantified from CS-treated and nontreated control subjects by qRT-PCR. (F) Primary HAECs placed in culture in an ALI culture were left untreated or treated with 1,000 ng/ml CS for 24 hours and 3 days later membranes were embedded in paraffin and stained with Alcian blue and periodic acid Schiff (AB/PAS) followed by hematoxylin and eosin staining. The number of mucous cells was significantly increased in CS-treated culture compared with untreated controls. Representative photomicrographs of AB/PAS-stained culture showing increased number of mucous cells in primary differentiated HAECs treated with 1,000 ng/ml CSE for 24 hours and harvested 3 days later, compared with untreated groups. Bars = group means $\pm$ SEM (n = 4 mice/group or n = 3 different treatments/group). *Denotes significant differences among groups (P < 0.05). Ctr = control; FS-CB = former smokers with CB; FS-NCB = former smokers without CB.

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*Denotes significant differences among groups (P < 0.05). Ctr = control; FS-CB = former smokers with CB; FS-NCB = former smokers without CB.

Figure 3. Restoration of Bik expression reduces cigarette smoke (CS)-induced mucous cell metaplasia (MCM) and epithelial cell hyperplasia (ECH). (A) Differentiated human airway epithelial cells (HAECs) were treated with 1,000 ng/ml CSE for 24 hours and harvested 3 days later. Cultures were infected with nothing, Ad-Bik^{L61G}, or Ad-Bik for 24 hours before harvest. Western blot showing expression of Bik in Ad-Bik^{L61G}- or Ad-Bik-infected HAECs. Bars = group means $\pm$ SEM ($n = 3$ /group). (B, C) C57BL/6 mice were exposed to 250 mg/m³ CS or filtered air for 6 h/d, 5 d/wk for 3 weeks and instilled with phosphate-buffered saline (PBS), Ad-GFP, or Ad-Bik in 50 μ L PBS on 2 consecutive days and lungs harvested the following day. (B) Western blot analysis showing expression of HA-tagged Bik in three representative lungs of Ad-Bik- (lanes 1, 2, and 3) but not in Ad-GFP-instilled (lanes 4, 5, and 6) mice. (C) ECH was significantly reduced in the airways of mice instilled with Ad-Bik compared with those instilled with Ad-GFP or PBS. (D) The percentage of Muc5ac-positive cells was significantly reduced in the airways of mice instilled with Ad-Bik compared with those instilled with Ad-GFP or PBS. Bars = group means $\pm$ SEM ($n = 6$ mice/group). *Denotes significantly different among groups ($P < 0.05$).

with Ad-Bik compared with those instilled with Ad-GFP or PBS (Figure 3C). Furthermore, immunofluorescence staining showed that Muc5ac positivity was reduced in CS-exposed mice instilled with Ad-Bik compared with those instilled with Ad-GFP or PBS (Figure 3D). These findings signify that targeted restoration of Bik expression is useful to control CS-induced ECH and MCM.

CS-induced ERK1/2 Activation Enhances Bik-induced Cell Death

Previous studies have shown that CS causes ERK1/2 activation (36, 37), and we recently found that Bik mediates IFN- γ -induced cell death in AECs by blocking nuclear translocation of IFN- γ -activated ERK1/2 (23). In view of the fact that expression of Bik reduced CS-induced MCM in HAECs and ECH in mouse airways, we tested whether ERK1/2 activation enhances Bik-induced removal of CS-exposed AECs.

In HAECs, ERK1/2 was activated within 5 minutes of CS treatment, and this activation was sustained for 15 minutes (Figure 4A). To investigate whether expression of Bik blocks nuclear translocation of CS-activated ERK1/2, HAECs were infected with Ad-Bik or Ad-Bik^{L61G} and 24 hours later treated with CS extract for 15 minutes. Analysis of cytosolic and nuclear fractions showed that activated ERK1/2 was reduced in the nuclear fraction of Ad-Bik- compared with Ad-Bik^{L61G}-expressing cells (Figure 4B). Interestingly, CS enhanced Ad-Bik-induced cell death, whereas Ad-Bik^{L61G} had little effect on CS-treated HAECs (Figure 4C), suggesting that cell death is enhanced when activated ERK1/2 is retained in the cytosol by Ad-Bik and that the conserved Leu residue within the BH3 domain of Bik is necessary for the inhibition of translocation.

To further validate whether retention of activated ERK1/2 in the cytosol enhances Ad-Bik-induced cell death, we treated HAECs with another stimulus that is known to activate ERK1/2. ERK1/2 was activated earlier and with increasing intensity when cells were treated with increasing insulin-like growth factor 1 (IGF-1) concentrations (Figure 4D). ERK1/2 activated using IGF-1 to examine whether ERK1/2 activated by agents other than CS would have a similar effect on Bik-induced cell death. As expected, IGF-1 enhanced Ad-Bik-induced cell death in a dose-dependent manner (Figure 4E). To further confirm the role of CS-activated ERK1/2 in the Ad-Bik-induced cell death, we treated HAECs with 1 μ M ERK1/2 inhibitor, U0126, which effectively reduced the levels of activated ERK1/2 as detected by Western blotting (Figure 4F). Inhibition of ERK1/2 activation using U0126 suppressed Ad-Bik-induced cell death, whereas U0126 alone had no effect on growth of HAEC cells (Figure 4G). Together, these findings show that Bik retains activated ERK1/2 in the cytosol to cause cell death.

DISCUSSION

The present studies show that CS inhibits expression of Bik and thereby increases airway epithelial cell hyperplasia in patients with chronic bronchitis. Restoring Bik expression using adenoviral expression systems together with CS-activated ERK1/2 caused death of epithelial cells to reduce ECH.

Bik remained suppressed in the lungs of mice even 60 days after recovery, suggesting that the miRNA or the RNase that is activated by CS remains intact once induced even after cessation of CS exposure. However, Bik expression was suppressed not only in the biopsy and autopsy lung tissues of cigarette smokers compared with nonsmokers but also in lung tissues of former smokers with chronic bronchitis compared with former smokers without. These findings suggest that not only are Bik levels reduced by cigarette smoking, but the level of reduction is driven by factors that may be directly associated with susceptibility to developing chronic bronchitis. It appears that Bik is suppressed by CS more drastically in former smokers with chronic bronchitis, and this reduction allows increased number of mucous cells to be sustained compared with former smokers without chronic bronchitis. It is possible that airway cells in people who are genetically predisposed to chronic bronchitis either have reduced baseline Bik levels or, once exposed to CS, permanently and more efficiently degrade Bik mRNA compared with smokers without chronic bronchitis. Therefore, Bik may be a useful biomarker that predicts susceptibility to chronic bronchitis, and identification of the factors that differentially regulate Bik expression in subjects with chronic bronchitis may help elucidate the susceptibility factors that cause permanent changes in former smokers with chronic bronchitis.

Interestingly, suppression of Bik expression was associated with significant increases in MUC5AC mRNA levels both in

Figure 4. Bik expression reduces nuclear localization of cigarette smoke (CS)-activated ERK1/2 and phospho-ERK1/2 enhances Bik-induced cell death. (A) Human airway epithelial cells (HAECs) were treated with 1,000 ng/ml CSE, harvested over a period of 5 to 45 minutes and protein lysates analyzed for activated ERK1/2 by Western blotting. ERK1/2 was activated within 5 minutes of treatment, and this activation was sustained for 15 minutes of CSE treatment. (B) Nuclear and cytosolic fractions from HAECs infected with 100 MOI Ad-Bik or Ad-Bik^{L61G} 15 minutes after CSE treatment analyzed by Western blotting for phospho-ERK1/2, total ERK1/2, Bik, lamin, and actin. The figure is representative of three independent experiments. (C) CS enhances Ad-Bik-induced cell death. HAECs infected with Ad-Bik or Ad-Bik^{L61G} at 100 MOI were either treated with CSE or left untreated and counted 24 hours later. (D) HAECs were treated with different concentrations of insulin-like growth factor 1 (IGF-1) for 0 to 30 minutes and protein lysates analyzed for p-ERK1/2, ERK1/2, and actin by Western blotting. (E) HAECs were infected with 100 MOI Ad-Bik or Ad-Bik^{L61G} and treated with different concentrations of IGF-1, and cell viability was quantified. (F) Inhibition of ERK1/2 activation by U0126 reduced in CS-exposed cells. Western blot analysis of extracts from HAECs treated with U0126 and/or CS to reduce CS-induced ERK1/2 activation. (G) HAECs were treated with 1 μ M U0126 and infected with 100 MOI Ad-Bik followed by 1,000 ng/ml CSE treatment 24 hours later. Cell death induced by Ad-Bik in the presence of CS was diminished significantly when ERK1/2 activation was inhibited by 1 μ M U0126. Bars = group means $\pm$ SEM (n = 3 different treatments/group). *Denotes statistically significant difference ($P < 0.05$).

human primary HAECs and in the lungs of mice exposed to CS. Although mucin gene expression and MCM can also occur without epithelial cell proliferation, as shown in cells overexpressing the SAM pointed domain-containing ETS transcription factor (SPDEF) (38, 39), the present studies demonstrate that in mice, MCM is also associated with an increased number of epithelial cells per millimeter of basal lamina compared with those exposed to filtered air as control. These findings suggest that the reduction of this proapoptotic protein allows sustained presence of hyperplastic epithelial cells that synthesize and secrete mucus. The fact that Bik knockout mice show normal epithelial cell numbers when not exposed to CS suggests that the regulation of epithelial cell numbers in normal development is regulated by other mechanisms. However, our findings suggest that once injury occurs, Bik is crucial for the resolution of hyperplastic epithelial cells. Furthermore, these findings are consistent with previous reports that smokers with chronic bronchitis and chronic airflow obstruction have increased levels of MUC5AC mRNA compared with nonsmoking control subjects (40).

The fact that CS-induced MCM and ECH in differentiated primary HAEC cultures and *in vivo* mice were reduced when Bik expression was restored suggests that Bik must be inducing death of mucin-producing cells. Our previous studies showed susceptibility of hyperplastic rather than resting nonproliferating cells to Bik-induced cell death (23). Previous studies showed that proliferating basal cells give rise to a subpopulation of mucous cells that retain the ability to divide, whereas others lose this proliferative phenotype and become fully differentiated (41). When proliferating cells were arrested in mitosis with

colchicines after mechanical injury of the airway epithelium in hamsters, a larger proportion of secretory cells compared with basal cells were found to be in metaphase (42). Therefore, susceptibility of only proliferating AECs to death signals may ensure that only hyperplastic AECs are removed during the resolution process without damaging the barrier function of the airway epithelium. Our previous studies also suggest that hyperplastic cells in airways appear to be primarily mucus-producing cells (8). Hence, restoring Bik expression may reduce hyperplastic epithelial cells, thereby reducing MCM. This selective targeting of hyperplastic cells restores the normal proportions of cell types in airways without destroying the integral barrier function and innate protective mechanism of the airway epithelium. Extensive examination of lung tissues failed to identify epithelial cells with classic apoptotic morphology. Current studies are developing new biomarkers that may be useful to identify the cells undergoing cell death and to characterize the nature and morphology of cell death occurring *in vivo*.

The observation that Bik-induced cell death was enhanced by CS- or IGF-1-induced ERK1/2 activation and that blocking ERK1/2 activation using U0126 alleviated Bik-induced cell death suggests that ERK1/2 activation may be an integral functional part of Bik-induced cell death. The fact that the BH3 domain of Bik was required to inhibit nuclear localization of phospho-ERK1/2 in airway epithelial cells demonstrates that Bik is essential for cytosolic retention of phospho-ERK1/2. Collectively, these findings show that sustained activation of ERK1/2 in the cytosol enhances cell death. ERK activation has generally been associated with cell survival and proliferation

(43); however, a number of studies show that depending on the stimuli and cell types involved, activation of ERK can mediate cell death (reviewed by Mebratu and Tesfaigzi [44]).

In conclusion, our results provide a new paradigm for epithelial remodeling that should be useful for developing a rational basis for therapies aimed at reducing hyperplastic AECs that are involved in mucous hypersecretion in chronic diseases. A strategy that uses an irreversible inhibitor of epidermal growth factor receptor tyrosine kinase activity that showed efficacy in preventing epithelial hyperplasia in a model of intestinal neoplasia (45) is being developed. In that setting, the pharmacologic strategy was aimed at inhibiting epithelial proliferation, but our approach to restore the proapoptotic effect of Bik may prove to be more effective. These studies lay the foundation to investigate peptides derived from Bik that can be used to reduce the number of mucous cells, mucous hypersecretion, and phlegm production and ameliorate the symptoms of chronic bronchitis.

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