Abnormalities in MUC5AC and MUC5B Protein in Airway Mucus in Asthma

To the Editor:

Gene expression for MUC5AC is increased in airway epithelial cells in asthma, and MUC5B expression is decreased (1, 2), but it is uncertain how this abnormal gene expression for gel-forming mucins contributes to mechanisms of formation of pathologic airway mucus gels in asthma exacerbation and stable asthma. Recent studies in transgenic mice show different functions for MUC5AC and MUC5B in the lung. Whereas MUC5B has physiologic functions in the mucus gel that ensure normal mucus clearance (3), MUC5AC has pathologic roles in mechanisms of allergen-induced airway hyperresponsiveness, mucous metaplasia, and airway mucus plugging (4). Changes in the relative proportions of MUC5AC and MUC5B could also affect eosinophil survival in the airway mucus gel because glycoepitopes in the MUC5B glycan coat interact with Siglec-8 on eosinophils to induce apoptosis (5). Thus, a relative deficiency of MUC5B in airway mucus could create an environment that promotes eosinophil survival.

It is unknown whether abnormalities in airway gene expression for MUC5AC and MUC5B in asthma are mirrored in the mucin protein composition of the airway mucus gel. We therefore set out to examine the relative proportions of MUC5AC and MUC5B protein in induced sputum collected from healthy controls (n = 16), patients with stable asthma (n = 34), and patients with exacerbations of asthma (n = 13) (Table 1). Healthy and stable subjects with asthma participated in a research protocol involving at least two visits: one for disease characterization (including measurement of lung function and induced sputum collection for analysis of total and differential cell counts) and another for collection of induced sputum for purification of mucins. The patients with asthma exacerbation were recruited from the emergency room at University of California, San Francisco, Medical Center, using methods previously described (6), and their spontaneously expectorated sputum was divided to allow one aliquot to be processed for total and differential cell counts and one to be processed for mucin purification. Methods of characterization, sputum induction, and sputum processing for cell analysis have been described in detail in our prior publications (7, 8). A previous attempt to quantify sputum mucins by direct-binding ELISAs reported technical difficulties related to high levels of nonmucin proteins that interfered with the immobilization of the mucins on the plate (9). Another potential problem with mucin immunoassays is poor antibody recognition of mucin epitopes in protease-rich airway secretions. To overcome these problems, we developed two-step assays for MUC5AC and MUC5B in which the first step involves purification of mucins from nonmucin proteins and the second step involves MUC5AC- and MUC5B-specific ELISAs that used cocktails of antibodies (9 for MUC5AC and 10 for MUC5B) to maximize epitope recognition. To separate mucins from nonmucins in sputum, we processed sputum in 8 M GuHCl and used a cesium chloride density gradient to separate mucins from proteins, glycoproteins, and nucleic acids, as previously described (7). Carbohydrate-rich fractions, as confirmed by periodic acid–Schiff detection, were subjected to gel filtration on a Sepharose CL4B (GE Health Life Sciences, Pittsburgh, PA) column to yield mucins in the Vo fraction. Mucin-rich material was desalted on Sephadex G25 (GE Health Life Sciences) before freeze-drying and resuspension in a standardized concentration (1 mg/ml). Because the mucins were resuspended in a standardized concentration, our approach allows for quantification of the relative proportions of MUC5AC and MUC5B mucins in sputum, but it does not allow determination of the individual concentrations of MUC5AC and MUC5B.

To quantify the relative proportions of MUC5AC and MUC5B in the mucin preparations from healthy and asthmatic sputum, we immobilized mucins on Nunc amino immobilizer plates (Sigma-Aldrich, St. Louis, MO), which use a surface technology that covalently couples an ethylene glycol spacer and stable electrophilic group with nucleophiles such as the thiols that are abundant in mucins. We then sequentially added primary mucin antibodies (MUC5AC clones: MG-31, SPM297, and 2H7 [Abnova, Taoyuan City, Taiwan] and 45M1, 1-13M1, 2-11M1, 2Q445, CLH2, and 2X123 [SCBT, Santa Cruz, CA]; and MUC5B clones: 6F10-EF4, 87376, and 19.4e [Abcam, Cambridge, MA]; 5B19-2E [Invitrogen, Grand Island, NY]; 8C11 [Sigma]; and H-300, S-20, G-16, Y20, and 4H310 [SCBT]), secondary antibodies (biotin–IgG complexes; Biolegend, San Diego, CA), and detection reagents (ExtrAvidin-AP [Sigma] and OptiBlaze ELISA *femto*-AP [G-Biosciences, St. Louis, MO]). We found a concentration-dependent increase in signal intensity for both MUC5AC and MUC5B proteins (Figures 1A and 1B). We also found that MUC5B is the predominant mucin in healthy mucin preparations, whereas MUC5AC predominates in mucin preparations from patients with asthma (including those with asthma exacerbation) (Figures 1A, 1B, and 1C). Furthermore, compared with stable patients with asthma whose mucin samples are comprised predominantly of MUC5AC, we found that patients with asthma with a predominance of MUC5B in their mucin preparations have significantly lower numbers of eosinophils in their sputum (Figure 1D); there was no difference in the number of neutrophils (Figure 1E). Using sputum eosinophils higher than 2% as a measure of airway type 2 inflammation, we find that 13 of the 34 patients with stable asthma (38%) had airway type 2 inflammation; notably, the ratio of MUC5AC to MUC5B is higher in this type 2 subgroup than in those without type 2 inflammation (2.069 ± 0.6552 vs. 2.876 ± 0.8007; $P = 0.03$).

To explore how MUC5AC and MUC5B interact in the airway mucus gel in sputum from patients with stable asthma, we smeared freshly collected sputum from patients with asthma on glass slides to generate smears of sufficient thickness to allow mucin polymer immunolocalization by confocal microscopy (10). We found that MUC5AC and MUC5B proteins exist in distinct spaces within the asthma mucus gel (Figure 1F) and that the usual

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Table 1. Characteristics of Healthy Subjects and Subjects with Stable Asthma and Asthma Exacerbation

Characteristic	Healthy (*n* = 16)		Stable Asthma (*n* = 34)		Asthma Exacerbation (*n* = 13)	
	n	Values	n	Values	n	Values
Age, yr*	16	32.9 ± 13.0	34	39.5 ± 13.7	13	45.2 ± 12.5
Female sex, n (%)	16	7 (44)	34	20 (59)	13	9 (69)
Body mass index, kg/m^2*	15	22.9 ± 2.4	34	29.1 ± 6.6		n/a
Use of inhaled corticosteroids, n (%)	0	0	31	16 (52)	7	5 (71)
Use of oral corticosteroids for asthma in past 2 yr, n (%)	0	0	28	9 (32)	8	7 (88)
Ever smoked, n (%)	16	5 (31)	22	11 (50)	13	8 (62)
Current smoker, n (%)	16	0	22	0	13	1 (8)
Spirometry data*						
FEV_1, % predicted	16	100.8 ± 11.1	34	75.9 ± 17.1		n/a
FVC, % predicted	15	102.7 ± 10.9	34	93.2 ± 12.9		n/a
Sputum cell counts, %†						
Eosinophils (nonsquamous)	16	0.095 (0–0.35)	34	1.0 (0.6–4.2)	8	1.5 (0.8–4.7)
Neutrophils (nonsquamous)	16	26.7 (18.9–35.8)	34	29.6 (23.4–37.4)	8	45.6 (19.5–48.9)
Squamous cells	16	20.6 (16.4–52.3)	34	19 (11.8–32.1)	8	10 (2.8–35.7)

Definition of abbreviation: n/a = not available.
Asthma was diagnosed by either methacholine hyperresponsiveness or bronchodilator responsiveness.
*Age, body mass index, and spirometry data are presented as mean ± SD.
†Sputum cell count data is presented as the median (range).

mucin profile was one in which MUC5AC surrounds or overlays MUC5B polymers (Figure 1G). We found no evidence of MUC5AC/MUC5B heteropolymers in these smears. The information advances our understanding of how the different mucin products of different mucin genes interact in the airway mucus gel.

To our knowledge, our data are the first to successfully use an ELISA to quantify MUC5AC and MUC5B mucins in sputum and relate differences in mucins in asthma to outcomes of type 2 inflammation. We specifically show that MUC5B is the predominant mucin in healthy airway mucus and that asthma is characterized by a decrease in MUC5B relative to MUC5AC. This relative decrease in MUC5B could alter the mucus gel and decrease mucus clearance (3), or it could contribute to mechanisms of airway eosinophilia because deficiency in MUC5B-associated Siglec8 results in decreased eosinophil apoptosis in the airway lumen (5). The mucin glycoprotein changes that we show in stable asthma are also evident during asthma exacerbations. The changes in mucin gene and protein expression in asthma most likely reflect the activity of IL-13 because IL-13 is well known to cause increases in MUC5AC and decreases in MUC5B in airway epithelial cells *in vitro* and in murine models (3, 4). Our findings thus confirm and extend prior *in vitro* and murine model data to show that human asthma is characterized by pathologically relevant changes in the constituents of MUC5AC and MUC5B protein in the airway mucus gel. Some of the results of these studies have been previously reported in the form of an abstract (11). ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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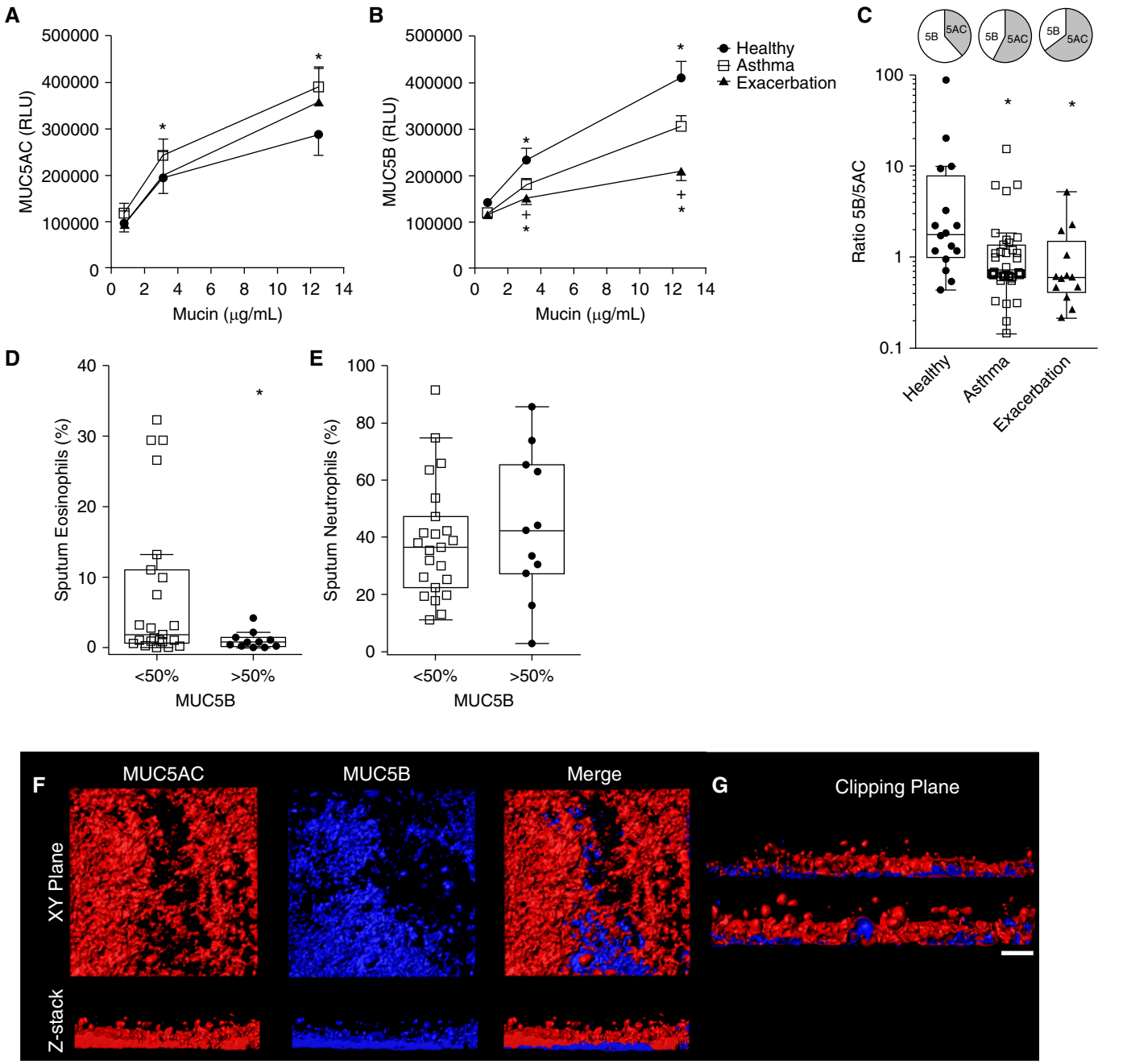

Figure 1. Abnormal mucin glycoprotein expression in asthma. (*A* and *B*) Sputum from patients with asthma (including from those with exacerbation) is characterized by a relative increase in MUC5AC (*A*) and a decrease in MUC5B (*B*). Mucins purified from sputum from healthy subjects and patients with asthma and loaded to Nunc amino immobilizer plates show a concentration-dependent increase in signal intensity for both MUC5AC (*A*) and MUC5B (*B*) protein. These and other optimization experiments (not shown here) indicate that 12.5 μg/ml is the ideal mucin loading concentration for Nunc plates (mucin concentrations >25 μg/ml overload the plate-binding capacity, and concentrations <1 μg/ml are below assay sensitivity threshold). The data are shown as mean ± SEM. *Significantly different from the healthy subgroup, $^{+}$significantly different from the stable asthma subgroup, $P < 0.05$ (unpaired *t* test with Welch's correction). (*C*) The ratio of MUC5B to MUC5AC in sputum from patients with asthma (including patients with asthma in exacerbation) is lower than normal (data generated using the 12.5 μg/ml loading concentration). *Significantly different from the healthy subgroup, $P < 0.05$ (analysis of variance using Kruskal-Wallis test). (*D* and *E*) The sputum eosinophil percentage in stable patients with asthma in whom MUC5B is the minor (<50%) mucin isoform in sputum ("MUC5B-low" subgroup) is higher than the sputum eosinophil percentage in the "MUC5B-high" asthma subgroup (stable patients with asthma in whom MUC5B is the major [>50%] mucin isoform) (sputum eosinophil percentage median value of 1.8 vs. 0.8%). *Significantly different from the <50% MUC5B subgroup, $P < 0.05$ (unpaired *t* test with Welch's correction) (*D*). Sputum neutrophil percentage is not different among these subgroups (*E*). (*F* and *G*) Representative images from immunofluorescence of a sputum smear from a donor with asthma shows that MUC5AC and MUC5B exist in discrete locations in the mucus gel (*F*). This is especially apparent in clipping plane images that reveal three-dimensional structures (*G*). *Scale bar* represents 25 μm. RLU = relative luminescence units.

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The Well-Known Gene *HHIP* and Novel Gene *MECR* Are Implicated in Small Airway Obstruction

To the Editor:

Small airway obstruction is an important phenotype of chronic obstructive pulmonary disease (COPD), which can be induced by a genetic predisposition and/or environmental factors, such as cigarette smoke and occupational exposures (1). Multiple genome-wide association studies have identified genes associated with COPD and predominantly large airway obstruction (FEV_1, FEV_1/FVC). So far, only one genome-wide association study was performed specifically on a spirometric measurement of small airway obstruction. Variants in, among others, *IL6R*, *NID2*, and *SYT10* were associated with forced expiratory flow at 25–75% of forced vital capacity ($FEF_{25–75}$), analyzed as percentage predicted, $FEF_{25–75}$%predicted/FVC, or change over time (2). In addition, we previously showed that occupational exposures affect small airway obstruction, even independent of large airway obstruction (3). Therefore, we hypothesized that specific genes may underlie small airway obstruction, also independent of large airway obstruction or cigarette smoke exposure.

We are the first to investigate the genetic predisposition of small airway obstruction without the potential interference of cigarette smoke exposure by performing a genome-wide association study on $FEF_{25–75}$ in never-smokers. We included 5,070 never-smokers from the LifeLines cohort study (Table 1) and assessed associations between $FEF_{25–75}$ (ml/s) and 227,981 genotyped variants using linear regression models adjusted for sex, age, height, and FVC.

Eight single-nucleotide polymorphisms (SNPs) were associated with $FEF_{25–75}$ in never-smokers with a P value $< 10^{-5}$; the top SNP (rs1512282) being genome-wide significant after correction for multiple testing (Bonferroni-corrected P value $< 2.2 \times 10^{-7}$; Table 2). rs1512282 is located 135 kb from hedgehog-interacting protein (*HHIP*), and the minor allele was associated with a 98 ml/s higher $FEF_{25–75}$ level. This SNP was previously associated with higher FEV_1/FVC levels in never-smokers of the same cohort (4). Of interest, the genetic associations with $FEF_{25–75}$ remained similar after adjustment for asthma and occupational or environmental tobacco smoke exposure, and comparable P values were found for association between $FEF_{25–75}$%predicted and the eight SNPs (data not shown).

To verify our results, we metaanalyzed the effect estimates of the eight SNPs in 1,534 never-smokers of the three cohorts included in the Rotterdam Study (RS; Table 1). The *HHIP* SNP had the same direction of effect in LifeLines and the three RS cohorts, and the association was borderline significant in the metaanalysis (P value = 0.08; Table 2). The other seven SNPs did not have concordant directions of effect in the four cohorts.

To further explore our hypothesis that the identified variants may have an effect on small airway obstruction independent of large airway obstruction, we performed a sensitivity analysis in 4,441 never-smokers of LifeLines with FEV_1/FVC >70% and FEV_1%predicted >80% (i.e., without predominantly large airway obstruction; Table 1). In this analysis, the *HHIP* SNP was no longer genome-wide significant (P value = 2.67×10^{-5}) and had a lower effect estimate (72 ml/s; Table 2). Yet, even though the group size was reduced from n = 5,070 to n = 4,441, rs2452785 in mitochondrial trans-2-enoyl-CoA reductase (*MECR*) was genome-wide significant (P value = 7.61×10^{-8}) and had similar effect estimates (105 and 114 ml/s, respectively). Moreover, the same direction of effect was observed in LifeLines and two of the three RS cohorts.

Interestingly, *cis*-expression quantitative trait loci (eQTL) analysis within a 4 Mb region around the eight SNPs in the lung eQTL database (n = 1,087) showed that the minor allele of rs1512282 was associated with lower mRNA expression levels of *HHIP* (three probesets; P values = 7.89×10^{-10} to 3.38×10^{-5}), which is in line with a previous study showing associations for the same allele with lower *HHIP* expression and lower COPD susceptibility (5). In addition, the minor allele of rs2452785 was associated with lower expression levels of *MECR* (P value = 6.08×10^{-7}) and erythrocyte membrane protein band 4.1 (*EPB41*, P value = 2.44×10^{-5}).

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