Impaired ciliary differentiation of airway epithelial cells from asthmatics with and without exercise-induced bronchoconstriction

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Abstract

Rationale
In the pseudostratified airway epithelium, basal cells act as progenitor cells to repopulate ciliated, goblet and basal cells. In severe asthma, there are increased numbers of basal cells and loss of columnar cells, suggesting a defect in differentiation, which has been linked to inflammation. Close to half of asthmatic patients experience exercise-induced bronchoconstriction (EIB), but little is known of the epithelial phenotype in these patients. We hypothesized that primary human airway epithelial cells (pHAECs) from EIB(+) and EIB(-) asthmatics would display defective differentiation compared to non-asthmatic donors.

Methods
pHAECs were obtained by bronchial brushing of non-asthmatic, EIB(+) and EIB(-) asthmatic donors (n=5 each) and cultured at air-liquid interface (ALI) for 20 days. At days 0, 5, 11, 15 and 20, ALIs were fixed for histology and cell types were analyzed using Alcian blue to distinguish goblet and ciliated cells, and cytokeratin (ck)-5 and p63 to identify basal cells. Release of inflammatory mediators interleukin (IL)-6 and IL-8 were measured by ELISA.

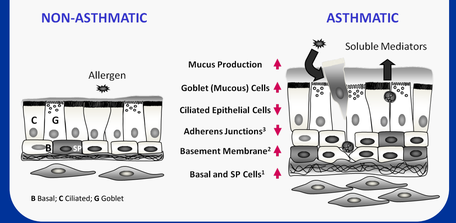
Results
At day 20, ALIs generated from EIB(-) and EIB(+) donors had significantly fewer ciliated cells at the apical surface compared to those from non-asthmatic donors ($p<0.05$). However, only in the EIB(-) ALIs were cilia significantly shorter compared to non-asthmatic cultures ($p<0.05$). We also confirmed that the number of CK-5+ basal cells was significantly greater in ALIs from EIB(-) asthmatic donors compared to non-asthmatic donors ($p<0.05$). Interestingly, the levels of IL-8 significantly decreased during differentiation ($p<0.0001$, two-way ANOVA), and ALIs from asthmatic donors released significantly less IL-8 ($p<0.0001$). IL-6 release did not change over time, but asthmatic cultures released more IL-6 overall ($p<0.01$).

Conclusions
We describe the first evidence of abnormal ciliary differentiation of the epithelium in EIB(+) asthma *in vitro*, and that features of epithelial remodeling are more pronounced in pHAECs from EIB(-) donors.

Introduction

- Asthma is a respiratory syndrome characterized by periods of reversible airflow obstruction, often in response to inhaled stimuli such as allergens or gases.
- In asthma, the epithelium lining the airways displays numerous changes, resulting in an "immature" phenotype.
- Many of the phenotypic differences observed *in vivo* are also evident *in vitro*, when cells are cultured at an air-liquid interface to induce 3-dimensional differentiation.
- Close to half of asthmatic patients experience exercise-induced bronchonstriction (EIB)[4,5].
- EIB(+) patients have more pro-inflammatory mediators and columnar epithelial cells in induced sputum[5] and greater numbers of intraepithelial mast cells[6] suggesting epithelial differences compared to EIB(-) patients.
- Very little is known about the phenotype of EIB(+) vs. EIB(-) asthmatic epithelial cells during mucociliary differentiation.

THE AIRWAY EPITHELIUM IS REMODELLED IN ASTHMA



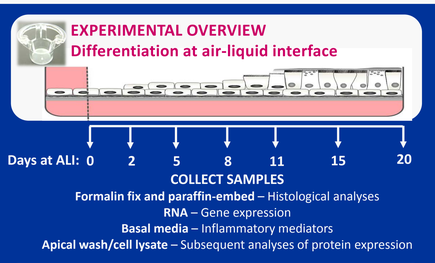
Hypothesis

We hypothesized that the differentiation process is altered in airway epithelial cells from both EIB(+) and EIB(-) asthmatic donors compared to healthy control donors.

Specific Aim

The aim of this study was to phenotype the *in vitro* differentiation process of human airway epithelial cells from non-asthmatic control donors and asthmatic donors with and without EIB.

Materials & Methods



	Asthma			*Statistics*		
	Control (n=5)	EIB(-) (n=5)	EIB(+) (n=5)	Con vs. EIB(-)	Con vs. EIB(+)	EIB(-) vs. EIB(+)
Age(y)						
Mean ± SD	31 ± 15	27 ± 6	30 ± 10	ns	ns	ns
Median (Range)	25 (22-58)	25 (23-38)	24 (21-44)			
Sex (F:M)	4:1	3:2	4:1	ns	ns	ns
Ethnicity						
Caucasian:Asian	4:1	5:0	5:0			
Baseline spirometry						
FEV_1/FVC	0.91 ± 0.05	0.73 ± 0.08	0.75 ± 0.09	p<0.01	p<0.05	ns
Methacholine challenge						
PC_{20} (mg/mL)	> 8	1.08 ± 0.9	0.24 ± 0.3	p<0.0001	p<0.0001	ns
Exercise challenge results						
Max % fall in FEV_1	1.3 ± 2.6	2.4 ± 2.1	25.9 ± 5.2	ns	p<0.001	p<0.001
Area under FEV_1 curve	7.1 ± 72	-26.1 ± 83	573.5 ± 158	ns	p<0.001	p<0.001

Air-Liquid Interface Culture of Primary Human Airway Epithelial Cells
Airway epithelial cells were collected by endobronchial brushing of asthmatic and non-asthmatic patients. All patients were never-smokers and gave informed consent for the study as required by the human protocol accepted by the University of Washington ethics committee. Control patients had no asthma history, baseline $FEV_1 \geq 80\%$ predicted and methacholine $PC_{20} > 8$ mg/mL. Asthmatic patients had a physician diagnosis for ≥1 year and disease of mild to moderate severity. EIB was classified by changes in FEV_1 after exercise challenge; EIB(+) ≥ 15% max fall in FEV_1 after exercise, EIB(-) ≤ 7% max fall in FEV_1 after exercise. Donor demographics for ALI cultures can be found in Table 1. Briefly, 2 x 10^5 cells were seeded in 12-well plates in permeable inserts with 0.4 µm pore size (Corning, New York), submerged in media containing 10 ng/mL EGF and 30 ng/mL retinoic acid[7]. To induce differentiation, apical media was removed, and media in the basal compartment was replaced with media containing 0.5 ng/mL EGF and 30 ng/mL retinoic acid. Basal media was changed every 48 hours, and apical surfaces were washed with PBS to remove mucus build-up twice per week, starting at day 10. Samples were collected at days 0, 2, 5, 8, 11, 15 and 20 for RNA, protein, histology, basal media, and apical wash.

Histology & Image Analysis
Five micron sections were cut from paraffin blocks, deparaffinized and rehydrated. Sections were stained with either Alcian blue to visualize mucins, or for specific proteins by immunohistochemistry. Antigens were retrieved by autoclaving in citrate buffer (Dako Cytomation, Burlington, ON), endogenous peroxidase blocked in 3% H_2O_2, and non-specific binding blocked with appropriate sera. Expression of cytokeratin-5 (Dako Cytomation), p63 (Santa Cruz Biotechnology, Santa Cruz , CA) was visualized using DAB (Dako Cytomation). Nuclei were counterstained with Harris Hematoxylin (Sigma-Aldrich, St Louis, MO). All donors were blinded by random coding to ensure no observer bias was introduced to the image analysis. For each donor, five images were captured by phase-contrast digital microscopy (Spot Imaging Solutions, Sterling Heights, MI) and analyzed by point counting using ImagePro Plus (Media Cybernetics, Rockville MD).

RNA expression
RNA expression was analyzed by RNA sequencing at days 0, 5, 11 and 20. Expression of candidate genes specific for basal, goblet and ciliated cells were analyzed by two-way ANOVA during differentiation with Bonferroni post-test. Expression was normalized as reads per kilobase of transcript per million reads aligned (RPKM) to adjust for sequencing depth and transcript length. Data are shown as mean ±SEM at day 20 only.

Enyzme-Linked Immunosorbent Assay (ELISA)
Inflammatory mediators interleukin-6 (IL-6) and IL-8 were assessed by ELISA (R&D Systems, Minneapolis, MI) of conditioned media from the basal compartment of air-liquid interface cultures at each time point. Samples were analyzed in duplicate and expressed as pg/mL per 24 hours in culture.

Statistics
Kruskal-Wallis with Dunn's post-test was used for comparison of all histological data. Gene expression and ELISA data were assessed by two-way ANOVA with Bonferroni post-test to compare all groups to one another. All statistical analyses were done using Prism Version 5.04 (GraphPad Inc., San Diego, CA), with *P* values less than 0.05 considered statistically significant.

Results

Figure 1. Differentiation of ciliated cells is impaired in airway epithelial cells from asthmatic donors

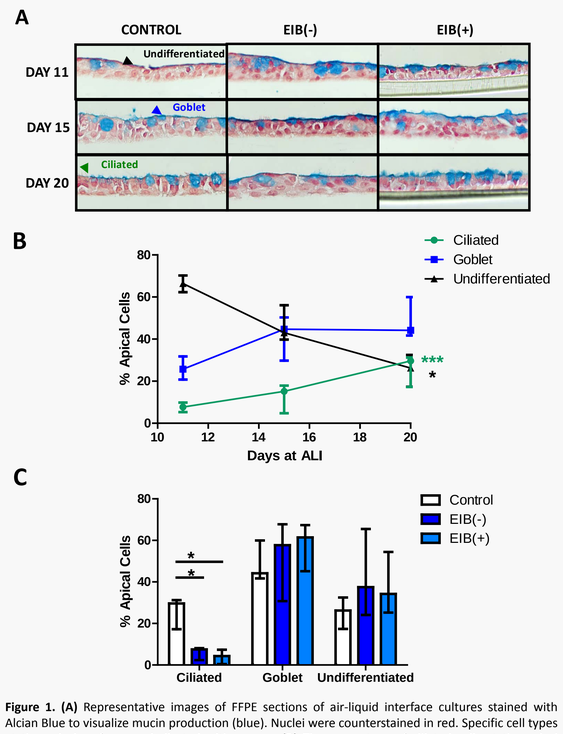


Figure 1. (A) Representative images of FFPE sections of air-liquid interface cultures stained with Alcian Blue to visualize mucin production (blue). Nuclei were counterstained in red. Specific cell types at the apical surface are indicated with arrows. **(B)** The percentage of ciliated cells at the apical surface of control cultures increases over time, while undifferentiated cells significantly decrease. Significance was assessed by two-way ANOVA with Bonferroni post-test (* p<0.05; *** p<0.001 compared to day 11). **(C)** EIB(-) and EIB(+) asthmatic cultures are markedly deficient in ciliated cells at day 20. Significance was assessed with Kruskal-Wallis with Dunn's post-test (*p<0.05). All data are expressed as median with interquartile range.

Figure 2. Cilia are significantly shorter in EIB(-) cultures

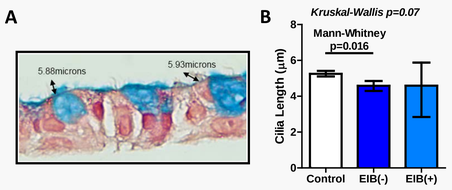


Figure 2. (A) Cilia length was measured in Alcian blue-stained FFPE cultures at day 20. **(B)** Cilia were significantly shorter in EIB(-) compared to control cultures by Mann-Whitney U-test. There were no significant differences between donor groups by Kruskal-Wallis test.

Figure 3. EIB(-) asthmatic air-liquid interface cultures have significantly more basal cells

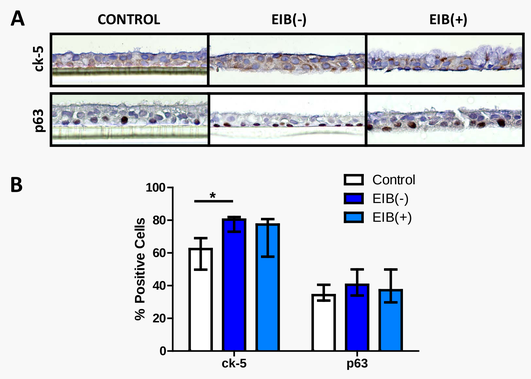


Figure 3. (A) Representative images of FFPE sections of air-liquid interface cultures at day 20 stained for the basal cell markers cytokeratin (ck)-5 and p63 (brown). Nuclei were counterstained with hematoxylin (blue). **(B)** There were significantly more ck-5-expressing basal cells in ALI cultures from EIB(-) asthmatic donors at day 20 by manual point counting. Data are expressed as median with interquartile range. * p<0.05 by Kruskal-Wallis with Dunn's post-test.

Figure 4. Expression of ciliogenesis genes is correlated with histology findings

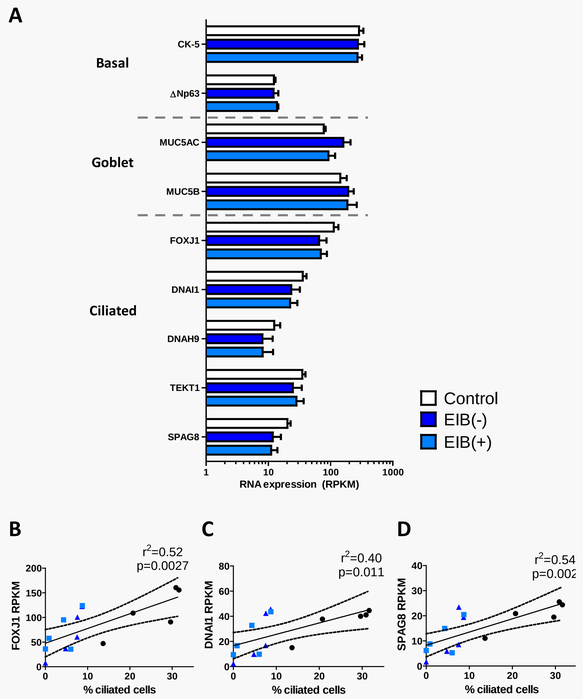


Figure 4. (A) Expression of genes specific to basal cells (CK-5 and ΔNp63), goblet cells (MUC5AC and MUC5B) and ciliated cells (FOXJ1, DNAI1, DNAH9, TEKT1, and SPAG8) were assessed at day 20. MUC5AC was more highly expressed in EIB(-) compared to control cultures but there were no significant differences in basal cell gene expression. There was a trend toward decreased expression of ciliogenesis-related genes in EIB(-) and EIB(+) asthmatic cultures. Data are shown as mean ± SEM of reads per kilobase of transcript per million reads (RPKM). Expression of **(B)** FOXJ1, **(C)** DNAI1 and **(D)** SPAG8 were correlated with percentage of ciliated cells on the apical surface of ALI cultures at day 20 by linear regression across all samples (solid lines). Dashed lines indicate 95% confidence intervals. The correlation measure r^2 and the p-value are indicated for each graph.

Figure 5. Release of inflammatory mediators IL-6 and IL-8 differs between disease states and during differentiation

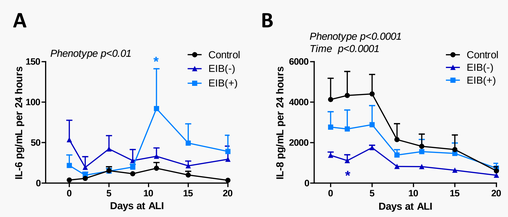


Figure 5. (A) IL-6 and **(B)** IL-8 in media from the basal compartment of air-liquid interface cultures throughout differentiation were measured using ELISA. Statistical significance was assessed using two-way ANOVA and p-values for phenotype and time are shown. *Indicates p<0.05 compared to control cultures by Bonferroni post-test. Data are shown as mean ± SEM of pg/mL per 24 hours.

Summary

- **Ciliary differentiation is impaired in ALI cultures of airway epithelial cells from EIB(-) and EIB(+) asthmatic patients.**
- **EIB(-) cultures have shorter cilia and an expanded ck-5 expressing basal cell population.**
- **Expression of ciliogenesis-related genes FOXJ1, DNAI1, SPAG8 are significantly correlated with the percentage of ciliated cells at the apical surface of ALI cultures.**
- **Asthmatic cultures release less IL-8 but more IL-6 than control cultures.**
- **Future work will aim to elucidate the mechanisms underlying aberrant mucociliary differentiation in asthma through mRNA and miRNA sequencing.**

Acknowledgments

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References

[1]Hackett TL et al. Stem Cells 2008 [2]Barbato et al. AJRCCM 2006 [3]Hackett TL et al. AJRCMB 2011
[4]Crapo et al. AJRCCM 2000 [5]Hallstrand et al. AJRCCM 2005. [6]Hallstrand et al. JACI 2013
[7]Fulcher et al. Methods Mol Med. 2005;107183-206.