NEUTROPHIL ELASTASE INDUCES MUCUS CELL METAPLASIA IN MOUSE LUNG

Judith A. Voynow1*, Bernard M. Fischer1, David E. Malarkey3, Lauranell H. Burch2, Teresa Wong4, Malinda Longphre4, Samuel B. Ho5, W. Michael Foster2

Departments of Pediatrics1 and Medicine2, Duke University Medical Center, Durham, NC 27710

3Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

4Department of Cell and Analytical Biology, Bayer Biotechnology, Berkeley, CA 94710

5Department of Medicine, Veterans Affairs Medical Center, University of Minnesota, Minneapolis, MN 55417

Running Title: NE – induced goblet cell metaplasia

*Corresponding Author:
Judith A. Voynow, M.D.
Division of Pediatric Pulmonary Medicine
Duke University Medical Center
Box 2994
Durham, NC 27710
919.684.6127
919.684.2292 (fax)
voyno001@mc.duke.edu

Copyright © 2004 by the American Physiological Society.
Abstract

Goblet cell hyperplasia in the superficial airway epithelia is a signature pathologic feature of chronic bronchitis and cystic fibrosis. In these chronic inflammatory airway diseases, neutrophil elastase (NE) is found in high concentrations in the epithelial lining fluid. NE has been reported to trigger mucin secretion and increase mucin gene expression *in vitro*. We hypothesized that chronic NE exposure to murine airways *in vivo* would induce goblet cell metaplasia. Human NE (50 µg) or phosphate buffered saline was aspirated intratracheally by male Balb/c (6 wk of age) mice on days 1, 4, and 7. On days 8, 11, and 14, lung tissues for histology, and bronchoalveolar lavage (BAL) samples for cell counts and cytokine levels were obtained. NE induced Muc5ac mRNA and protein expression and goblet cell metaplasia on days 8, 11, and 14. These cellular changes were due to proteolytic activity, since the addition of an elastase inhibitor, methoxysuccinyl Ala-Ala-Pro-Val chloromethylketone (AAPV-CMK), blocked NE-induced Muc5ac expression and goblet cell metaplasia. NE significantly increased keratinocyte-derived chemokine, and IL-5 in bronchoalveolar lavage (BAL), and increased lung tissue inflammation and BAL leukocyte counts. The addition of AAPV-CMK reduced these measures of inflammation to control levels. These experiments suggest that NE proteolytic activity initiates an inflammatory process leading to goblet cell metaplasia.

Keywords: neutrophil elastase, goblet cell metaplasia, Muc5ac
Introduction

Mucus obstruction of the airways causes significant morbidity in cystic fibrosis (CF), chronic bronchitis (CB), and severe, acute exacerbations of asthma. Although these chronic inflammatory airway diseases differ in their etiologies and in the inflammatory mediators present under stable conditions, they share the common pathologic feature of goblet cell hyperplasia (38, 44, 60), a prerequisite for mucus obstruction of airways. Th2 cytokines have been implicated as triggers for physiologic changes (20) and for goblet cell metaplasia in murine models of asthma (12). Th1 cytokines and other non-Th2 classes of inflammatory mediators, present in the airway surface liquid of CF and CB patients, up-regulate mucin expression in human airway cells (51). During acute exacerbations of CF, CB, or asthma due to infections or inhaled pollutants, neutrophils and neutrophil mediators are increased in the airway (37, 39).

*In vivo* studies have also shown that infectious particles, inhaled pollutants, or neutrophil inflammatory mediators may activate goblet cell metaplasia. Endotoxin induces goblet cell metaplasia in mouse airways (65) and in rat nose (47), and potentiates ozone induced goblet cell metaplasia (57) in rat nasal epithelium. Infection with paramyxovirus in mice induces a chronic goblet cell metaplasia (58); this response is partially mitigated by glucocorticoid treatment. Environmental toxins can also stimulate secretory cell remodeling, usually associated with an inflammatory response. Sulfur dioxide induces superficial goblet cell metaplasia in rats (29) and mucous gland hypertrophy in dogs (43). The remodeling in dogs is associated with both acute and chronic inflammation. Cigarette smoke (24, 33) and acrolein (5, 6), increase mucin glycoprotein expression in rat and mouse airways. Ozone induces goblet cell metaplasia in rat nasal airway epithelium (21); this process requires neutrophilic inflammation since neutrophil depletion (8) or topical glucocorticoid therapy (22) inhibits secretory remodeling. A common
theme among these reports is the presence of inflammation following the toxic stimulus, and associated with the development of goblet cell metaplasia.

Although neutrophilic inflammation is a dominant feature of CF and chronic bronchitis, there is limited information on the effect of neutrophil mediators on goblet cell metaplasia. Neutrophil elastase (NE, EC 3.4.21.37), a serine protease, is present in nanomolar to micromolar concentrations in chronic bronchitis (52) and CF (35) airway surface liquid. NE stimulates mucin secretion (15, 26, 32) and increases mucin gene expression (16, 28, 56). In hamsters, NE has been reported to induce goblet cell metaplasia (7) although the mechanism for this cellular transition is not known. We hypothesized that in mice, intratracheal exposure to NE would activate a cascade of events leading to airway goblet cell metaplasia. In the present study, we report that intratracheal aspiration of proteolytically active NE initiates the process of goblet cell metaplasia.
Materials and Methods

Animals. Seventy two, male Balb/c mice (6-8 weeks, 25-30 g) were obtained commercially (Jackson Laboratories, Bar Harbor, ME). The animals were housed in plastic shoebox-type cages suspended over absorbent bedding and were maintained on a 12-h diurnal cycle. Food and water were provided ad libitum. The study protocol conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare Guidelines for the Experimental Use of Animals and was approved by the Duke University Animal Care and Use Committee.

Neutrophil elastase exposures. Animals received NE or phosphate buffered saline (PBS) by oropharyngeal aspiration (18) on days 1, 4, and 7 (Figure 1 – Days 1, 4 and 7). Immediately following inhalational anesthesia with isoflurane (IsoFlo, Abbott Labs, Illinois: Open-Circuit Gas Anesthesia System, Stoelting Corp, Illinois), animals were suspended by their upper incisors on a 60° incline board, and a liquid volume of human NE (50 µg (43.75 U)/40 µl PBS, 42.37 µM; specific activity 875 U/mg protein, Elastin Products, Owensville, MO) or PBS alone was delivered with the animal’s tongue extended to the distal part of the oropharynx. With the tongue extended, the animal was unable to swallow, and the liquid volume was aspirated into the respiratory tract. Mice were euthanized by inhalational exposure to 100% CO₂ gas on 1, 4, and 7 days following the last NE exposure (Figure 1 – Days 8, 11 and 14). Immediately following euthanasia, the chest was opened, the trachea exposed and BAL was performed. The right primary bronchus was tied off and right lung was flash frozen for RNA. The left lung was inflated through the trachea with 10% formalin, fixed in 10% formalin, stored at 4°C for 24 h, and paraffin embedded and sectioned for further study.
To determine whether the effects of NE were due to proteolytic activity, NE was incubated with a specific inhibitor, methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone (AAPV-CMK, Sigma, St. Louis, MO), at a 2-fold higher molar concentration (85 µM), for 1 h, at room temperature. NE proteolytic activity was measured by a spectrophotometric assay using a specific substrate, methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroaniline (Elastin Products, Owensville, MO) according to the manufacturer’s protocol. NE that was preincubated with AAPV-CMK had no detectable \textit{in vitro} proteolytic activity. Four treatment groups of mice (AAPV-CMK alone, PBS alone, NE alone, or NE preincubated with AAPV-CMK) were treated as described above (isoflurane inhalational anesthesia, followed by oro-pharyngeal aspiration) on 3 separate treatment days (Days 1, 4, and 7) with respective delivery of 40 µl liquid volumes of AAPV-CMK (85 µM), PBS, NE (50 µg) or NE (50 µg) +AAPV-CMK (85 µM). Mice were subsequently sacrificed with inhalation exposure to 100% CO₂ gas on 4 and 7 days following the last treatment, and as described for NE-treated mice, underwent BAL and collection of lung tissues for further analyses.

\textbf{Bronchoalveolar lavage (BAL).} Immediately following euthanasia by CO₂ inhalation, the chest was opened, the trachea exposed and intubated with PI-90 tubing (0.86 mm and 1.27 mm, inner and outer diameter, respectively). Three ml of sterile saline were instilled 1 ml at a time into the tracheal catheter at a pressure of 20 cm water and retrieved. Return volume was recorded and was consistently greater than 75% of the instilled volume. Cells were isolated by centrifugation (500 x g, 10 min), and the supernatant was stored at -80°C for assessment of cytokine levels. Cells were resuspended in Hanks’ balanced salt solution (1 ml) and counted via hemocytometer. Cell differential was determined from an aliquot of the cell suspension (100 µl).
by centrifugation onto a slide (Cytospin 3, Shandon, Pittsburgh, PA), and Wright-Giemsa stain (Diff-Quik Stain set, Harleco, Gibbstown, NY). Cell differentials were determined for 200 total cells per slide and expressed as a percentage of total leukocytes. Total and differential cell counts were expressed as mean ± SEM for each group of animals.

**Histology and Immunohistochemistry.** Lungs in paraffin blocks were sectioned in the coronal plane at 5 µm and affixed to slides. After paraffin removal and rehydration, slides were stained with hematoxylin and eosin (H & E) for histology, or alcian blue/ periodic acid Schiff (AB/PAS) for detection of mucin glycoproteins, dehydrated in graded ethanol, and mounted. Overall lung tissue inflammation was semi-quantitatively determined by an examiner (DM) who did not know the treatment conditions for each section, using a 4-tier grading system of inflammation severity. Normal lung was given a Grade 0 (no inflammation) score. Grade 1 (mild) was given to those sections that had mild inflammation as characterized by multifocal perivascular and interstitial infiltrates of granulocytes, and/or lymphocytes and macrophages. Cell infiltrates were scattered and less than 3 cells in depth in inflamed regions. A score of Grade 2 (moderate) was assigned to those sections that had more disseminated inflammatory infiltrates, 3-5 cells in depth, and pigment-laden macrophages were present. A score of Grade 3 (marked) was given to those lung sections that had diffuse perivascular or peribronchial inflammation 5-15 cells in depth and pigment-laden macrophages.

To confirm that AB/PAS positive cells were expressing airway mucins, serial lung sections were immunostained for the murine homologue of MUC5AC, a major respiratory tract mucin glycoprotein. Following paraffin removal and rehydration, an antigen retrieval procedure was performed by incubating slides in a citrate buffer solution, (Target & Retrieval concentrate solution, Dako, Carpinteria, CA), at 85°C, for 20 min. Endogenous peroxidase was quenched
with 3% hydrogen peroxide/methanol for 10 min, and blocked with a universal blocking solution (Background Buster®, Innovex Biosciences, Richmond, CA). Slides were exposed to a chicken polyclonal anti-Muc5ac antibody that detects the mouse Muc5ac tandem repeat polypeptide sequence [H08, 1:4000, (46)], for 90 min at room temperature. Antigen was detected by incubation with secondary antibody (biotinylated rabbit anti-chicken IgY, 1:75 dilution, Zymed, South San Francisco, CA), 20 min, room temperature, followed by incubation with streptavidin-peroxidase conjugate and DAB. As a control, pre-immune chicken antibody was used in place of H08.

**Histological Mucus Index.** A histologic mucus index (62) was performed on AB/PAS-stained sections that included both central and peripheral airways. The slides were examined with a 10x objective and images were captured with a digital camera. A minimum of 4 representative cross- or sagittally-sectioned airways were imaged per animal. Only airways where the complete circumference of the airway could be visualized and included in the image were analyzed. Airways that opened directly into an alveolar space were not included. The extent of PAS positive staining in each airway imaged was semi-quantitatively determined by an examiner (BF) who did not know the treatment conditions for each section, using a 5-tier grading system: Grade (0) – no PAS staining; Grade (1) – 25% or less of the airway epithelium had PAS staining; Grade (2) – 26-50% of the airway epithelium had PAS staining; Grade (3) – 51-75% of the airway had PAS staining; and Grade (4) – greater than 75% of the airway epithelium had PAS staining. This grading system was used to calculate a mucus index score for each group and results were presented as mean ± SEM.

To estimate the diameter of the airways that demonstrated PAS positive staining in response to NE treatment, longitudinally sectioned airways were examined with a 4x objective
and images captured with a digital camera. Nine airways from 7 different animals (two animals had two longitudinal airway sections on each slide) were imaged, which included airways from each time point. Calibrated diameter measurements were done at three different points along each airway using Metamorph Software (Universal Imaging Corp./Molecular Devices, Downington, PA).

**Cytokine analysis.** Concentrations of 16 cytokines in lavage fluid were determined by using a commercial multiplex fluorescent bead-based immunoassay (sensitivity: 0.3-10.5 pg/ml depending on the cytokine; Linco, St. Charles, MO). Cytokines analyzed include murine IL-4, IL-2, IL-1β, IL-5, IL-10, IL-12 (p70), keratinocyte-derived chemokine (KC), monocyte chemoattractant protein – 1, RANTES, macrophage inflammatory protein - 1α, granulocyte monocyte-colony stimulating factor, interferon γ, IL-9, IL-13, IL-6, and tumor necrosis factor α. Briefly, BAL samples (50 µl) and standard concentrations of the respective cytokines (50 µl) were placed in duplicate into wells in a 96-well microtiter plate. Samples were incubated with 25 µl of anti-mouse multicytokine beads, specific for mouse cytokines and mixed on a plate shaker for 18 h, 4°C. Biotin anti-mouse multicytokine reporter was added to each well as a secondary/detection antibody and incubated at 37°C, 2 h while shaking. Streptavidin-phycoerythryin (25 µl) was added to wells and incubated shaking for 2 h, at 37°C, in the dark. Finally the addition of stop solution (25 µl) terminated the reactions. Samples and standards were read using a Luminex instrument (Bio-Plex Workstation, Bio-Rad, Hercules, CA), in which a minimum of 50 beads per cytokine for each sample were analyzed. Blank values were subtracted from all readings. Mouse keratinocyte-derived chemokine (KC) levels for the studies
using AAPV-CMK were measured by a sandwich ELISA kit (R&D Systems, Minneapolis, MN) as per manufacturer’s instructions.

**Quantitative RT-PCR of Muc5ac and actin.** Total RNA was isolated from frozen mouse lung tissues (50 to 100 mg) with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Gene specific primer sets were designed for mouse Muc5ac and β-actin. Sequences and amplified PCR product sizes were as follows: Muc5ac, 5’-AGAGGAGGTTTGATCTGTT-3’ and 5’-CTCTCCGCTCCTCTCAATGTTGCT-3’, 430 bp (46); β-actin, 5’-GACCCAGATCATGTTTGAGACTTCAACAC-3’ and 5’-TAGAGGTCTTTAAGATGATGACGTCAACAC-3’, 524 bp [GenBank accession number, AK078935].

RT-PCR amplification was done by using the Qiagen OneStep RT-PCR reaction mix with the addition of SYBR Green I (Molecular Probes, Eugene, OR) as the fluorescence reporter for quantification. Real-time PCR was performed with an iCycler iQ Detection System (Bio-Rad, Hercules, CA). The reaction was started with incubation at 50°C, 30 minutes for reverse transcription. This was followed by 95°C, 15 minutes to inactivate the reverse-transcriptase and simultaneously activate the HotStarTaq DNA Polymerase. Forty cycles of denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute and extension at 72°C for 2 minutes were then completed. Melting curve analysis of the PCR products was employed to determine the specificity of PCR product from each individual primer set. Both Muc5ac and β-actin specific primer sets were shown to amplify only one gene specific product. The relative expression level of Muc5ac, was normalized with β-actin, and calculated by the formula of $2^{(\Delta\Delta CT)}$, where $\Delta CT$ is the difference in threshold cycle ($C_T$) values between Muc5ac and β-actin as calculated by
(Muc5ac $C_T$)-(β-actin $C_T$). $\Delta C_T - \Delta C_T$ is the difference between the Sample $\Delta C_T$ and the Control $\Delta C_T$. This quantification approach was based on the technical support information provided in the iCycler iQ Detection system manual from Bio-Rad (Hercules, CA).

**Statistics.** The Kruskal-Wallis one-way, non-parametric analysis of variance (ANOVA), and post-hoc comparisons by the Wilcoxon rank sum tests (48) were used to compare cytokine protein, Muc5ac expression levels, BAL cell counts and differentials, tissue inflammation indices, and histologic mucus indices. Significant differences between groups were defined as $p<0.05$ (two tailed analysis).
Results

*Neutrophil elastase induces goblet cell metaplasia in mouse airways.* Following three aspirations of NE (Figure 2), goblet cell metaplasia was observed on days 8, 11, and 14, defined by robust AB/PAS staining (Figure 2A), increased Muc5ac mRNA expression (Table 1), and immunohistochemistry positive for Muc5ac (Figure 2A). In the absence of primary antibody or when pre-immune chicken antiserum was used, no Muc5ac epitope was detected. Quantification of AB/PAS staining (Figure 2B) confirmed that changes in goblet cell number increased significantly on days 8, 11 and 14. Muc5ac mRNA and protein expression qualitatively corresponded to AB/PAS histology.

Evaluation of AB/PAS positive airways also revealed that goblet cells were restricted to larger, proximal airways with diameters greater than 125 µm (Figure 3). This observation was consistent among 9 airways from 7 separate NE-treated animals (two animals had two longitudinal airway sections on each slide). The restriction of goblet cell metaplasia to larger proximal airways may be due to technical artifact of limited distribution of NE by oropharyngeal aspiration. However, the alveolar hemorrhage in response to NE, as evidenced by the hemosiderosis and erythophagia, suggests that NE reached the terminal bronchiole/alveolar compartment.

*NE generates an inflammatory response in vivo.* Starting on day 8 and continuing to day 14, NE provoked increased peribronchial, perivascular and interstitial inflammation in the tissue (Figure 4, Table 2). Inflammation was marked initially by neutrophilic and eosinophilic peribronchial and perivascular inflammation and alveolar hemorrhage. Over time, the intensity of inflammation increased with greater depth of cellular infiltrates. Alveolar hemorrhage resolved and pigment-laden macrophages appeared (Figure 4).
The course of tissue inflammation was mirrored in the bronchoalveolar lavage cell counts and differentials. Following NE treatment, total BAL leukocyte counts were greater than control animal leukocyte counts at all time points (Figure 5A), but decreased over time. BAL cell differential counts revealed no significant difference in the percentage of macrophages between treatment groups (data not shown). In contrast, NE–treated animals had an initial increase in neutrophils (PMN) on day 8, that decreased over time (Figure 5B), and a persistent increase in lymphocytes (Figure 5C) and eosinophils (Figure 5D) at all time points.

Quantification of 16 cytokines in BAL by enzyme immunoassay revealed that NE treatment significantly increased only two cytokines, KC, a neutrophil chemoattractant, and IL-5, an eosinophil chemoattractant (Figure 6). There were no significant changes in other Th2 cytokines, IL-4, IL-9 or IL-13, and no significant changes in other cytokines such as tumor necrosis factor α or IL-1β.

**AAPV-CMK blocks goblet cell metaplasia and inflammation.** To determine whether the effect of NE on airway epithelium was due to proteolytic activity, NE activity was inhibited by a specific pharmacologic inhibitor, methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone (AAPV-CMK). AAPV-CMK alone instilled by intratracheal aspiration, had no effect on airway epithelial cell differentiation, and did not cause inflammation (data not shown). After a one hour incubation with a 2-fold molar excess of AAPV-CMK, NE protease activity was completely inhibited, significantly reducing goblet cell metaplasia (Figure 7) and the inflammatory response (Figure 8). AAPV-CMK blocked the induction of goblet cell metaplasia (Figure 7A), significantly reduced Muc5ac gene expression to control levels (Figure 7B), and reduced Muc5ac protein expression (Figure 7C). In addition, BAL total leukocyte, lymphocyte, and eosinophil counts were significantly reduced (Figure 8), lung inflammation was significantly
reduced to control levels (Grade of inflammation severity: Day 11: NE, 2.25 ± 0.25; NE + AAPV-CMK, 0±0, P < 0.05; Day 14: NE, 2 ± 0; NE + AAPV-CMK, 0.25±0.25, P < 0.05; n=4 animals per treatment). In addition, KC levels were significantly reduced by 60% at day 11 and 68% at day 14 (Day 11: NE, 6.25 ± .75; NE + AAPV-CMK, 2.5 ± 0.6 pg/ml, P < 0.05; Day 14: NE, 15 ± 2.7; NE + AAPV-CMK, 4.7 ± 1.7 pg/ml, P < 0.05; n = 4 animals per treatment). These results suggest that the effect of NE on goblet cell metaplasia *in vivo* is due to proteolytic activity, and not due to an allergic reaction to NE, or contamination of the NE preparation.
Discussion

In this report, we describe an \textit{in vivo} mouse model of goblet cell metaplasia induced by intratracheal aspiration of NE. To our knowledge, this is the first murine model of goblet cell metaplasia induced by NE. This murine model is relevant to the process of goblet cell hyperplasia in chronic inflammatory airway diseases as the concentration of NE aspirated is similar to concentrations found in the BAL of CF and CB patients (35, 52). Our results suggest that NE proteolytic activity is required for this effect since inhibition by AAPV-CMK abrogates goblet cell metaplasia. Some inflammatory proteases, including NE (15, 26), Pseudomonas proteinases (27), cathepsin G (50), and chymase (49), have been reported to stimulate mucin or submucosal gland cell secretion. Also proteases, including NE (16, 28, 56) and metalloproteases (30, 45), have been reported to increase mucin gene expression \textit{in vitro}. However, surprisingly few reports have been published on the effect of proteases on goblet cell metaplasia \textit{in vivo}. Serine proteases, specifically, neutrophil elastase and pancreatic trypsin, induce goblet cell metaplasia in hamsters (7). Tryptase also contributes to goblet cell metaplasia in a mouse model of asthma (36) as ovalbumin-sensitized and challenged mice treated intranasally with a specific tryptase inhibitor have fewer goblet cells in the airways. The mechanism by which proteases induce goblet cell metaplasia is not known. However, our data suggests that inflammation provoked by elastase is temporally related to the development of goblet cell metaplasia.

Goblet cell metaplasia in murine airways is induced by several airway stimuli including ovalbumin challenge, Th2 cytokines, and lipopolysaccharide (LPS). Protocols for ovalbumin sensitization and challenge or direct airway instillation of recombinant murine IL-13 (2, 20) include three challenges prior to detection of murine goblet cell metaplasia. It is not clear from these reports whether goblet cell metaplasia is detectable after only one ovalbumin challenge or
IL-13 instillation. Following ovalbumin challenge or IL-13 exposure, goblet cell metaplasia is observed within the first 24 hours (2, 20), an earlier time point than observed following LPS exposure. Ovalbumin challenge and IL-13 instillation are also associated with increased BAL neutrophils and eosinophils concomitant with goblet cell metaplasia (20). In contrast, only one LPS intratracheal instillation induces BAL inflammation within 2 days, but goblet cell metaplasia is observed after 4 days (65). LPS induces BAL neutrophils, macrophages and lymphocytes, but no eosinophils. In all of these models, the inflammatory responses are observed within 2 days following stimuli, however, the goblet cell metaplasia is observed at different times following stimuli. In the NE – aspiration murine model, goblet cell metaplasia began on day 1 and peaked on day 4 following NE exposure, with the most robust inflammatory response occurring on day 1 following NE aspiration. Although the histologic descriptions suggest that the types of inflammatory cells differ depending on the stimulus, we suggest that differences in the inflammatory responses may also be due in part to genetic variability. Different mouse strains have varying inflammatory responses and airway hyperresponsiveness to allergic stimuli (61). The effect of the murine genetic background on goblet cell metaplasia has not yet been systematically investigated.

We observed that NE induced goblet cell metaplasia was restricted to larger, more proximal airways. The diffuse alveolar hemorrhage in response to NE, suggests that NE was delivered to all airway surfaces, and that the regional distribution of the PAS positive cells is likely related to the different cellular microenvironments of the respiratory tree. In mouse airways, there are different subsets of Clara cell secretory protein (CCSP)-expressing bronchiolar epithelial cells whose function is dependent on the microenvironment. Importantly, a limited population of these cells will serve as progenitor cell populations important for epithelial
regeneration post injury (19, 40). In the proximal airways of mice, cells expressing CCSP have been reported to produce mucus (alcian blue positive) in response to antigen challenge (14). In human airways, there is a subset of the Clara cell population that exhibit both PAS positive staining, characteristic of a goblet cell, and Clara cell 10 kD protein immunoreactivity characteristic of Clara cells (4). Thus, in our model, it is not known whether the goblet cells emanate from Clara cells that undergo transdifferentiation (34), and/or from a transient amplifying cell population that undergoes proliferation (13).

Following NE aspiration, a robust inflammatory response ensued characterized by increased neutrophils, lymphocytes, and eosinophils, and increased concentrations of KC and IL-5 in the BAL. IL-8, a neutrophil chemoattractant similar to KC, has been reported to upregulate MUC5AC in human airway cells in vitro (41), but it is not known whether IL-8 directly induces goblet cell metaplasia in vivo. IL-5 expression has been reported to increase goblet cell metaplasia in vivo via CD4+ T cell activation (25). Importantly, we did not observe increased expression of other Th2 cytokines following NE exposure. Th2 cytokines have previously been reported to induce goblet cell metaplasia in murine models in vivo (53, 54, 63).

NE increases neutrophil chemotaxis via increased expression of IL-8 in human airway epithelial cells (35). A similar mechanism of neutrophil chemotaxis via KC may be occurring in our model. Neutrophilic inflammation is a common inflammatory response associated with the development of goblet cell metaplasia following LPS exposure in mouse lung (65), endotoxin exposure to rat nasal epithelium (47), ozone-exposed rat nasal epithelium (8), combined endotoxin and ozone exposure to rat nasal epithelium (57), or cigarette smoke exposure to mouse lung (33). Importantly, inhibition of neutrophilic inflammation by glucocorticoid treatment (22, 58), or anti-neutrophil antiserum (8, 47, 57), reduces goblet cell metaplasia. Similar to these
other models, we suggest that NE-induced inflammation plays an important role in the development of goblet cell metaplasia.

In our model, we observed an eosinophilic response in addition to increased BAL neutrophilia. The mice responded to NE aspirations with increased levels of IL-5, an eosinophil chemoattractant. Although there are no reports that NE directly regulates IL-5, NE has been reported to degranulate eosinophils (31), which may release eosinophilic mediators that further increase IL-5 release from lymphocytes. Eosinophils infiltrate airways in response to allergic sensitization (2), but importantly, also as part of a general inflammatory response to epithelial injury including ozone-induced tracheobronchial injury (23), and viral infections (10, 55). We do not yet know whether the eosinophils in the lung tissue are contributing to the development of goblet cell metaplasia or are coincidently present as a response to NE exposure.

NE has many direct effects on airway epithelium. NE regulates expression of several airway epithelial genes, including $MUC5AC$ (28, 56), and $MUC4$ (16) respiratory mucin genes, IL-8 (35), ICAM-1 (64), and serine leukoprotease inhibitor (1, 42). NE appears to act at the epithelial cell surface (9) to activate signaling via multiple pathways, including generation of reactive oxygen species (3, 17), activation of Toll-like receptor 4 (11), and activation of the epidermal growth factor receptor (28). Interestingly, NE activates at least two signaling pathways that are required for $MUC5AC$ mRNA regulation: reactive oxygen species (16), and the epidermal growth factor receptor (26). This example supports the concept that NE may activate different signaling pathways with redundant or singular consequences for epithelial functions.

Although mucin gene expression is required to define the goblet cell phenotype (2), there are likely to be unique mechanisms specific to mucin gene regulation that are distinct from those
required for goblet cell metaplasia. There remain major gaps in our understanding of the molecular mechanisms leading to goblet cell metaplasia. The changes in transcriptional programs and intracellular signals are not yet defined. At least two potential mechanisms for NE-induced goblet cell metaplasia include activation of the epidermal growth factor receptor (34), or down-regulation of FOXA2, an embryonic transcription factor responsible for terminal epithelial differentiation in the lung (59). The model of NE-induced goblet cell metaplasia presented here will be useful to investigate these fundamental processes.
Acknowledgements

This work was presented in part at the 100th Annual American Thoracic Society Meeting, Orlando, FL. The authors would like to thank Angela Byrd, Jacob Cuellar, Katherine Berman and Erin Tekippe for technical assistance.

This work was supported by NIH grants HL 65611 (JAV) and NIH HL 62641 (WMF), the March of Dimes Foundation (JAV), and GlaxoSmithKline (WMF).
References


Figure Legends

Figure 1. Treatment protocol for NE aspiration. BALB/c mice receive 3 doses of exogenous NE (50 µg) via intratracheal aspiration (days 1, 4, and 7). BAL and lungs are harvested over 3 time points (days 8, 11, and 14). BAL is performed with 3 aliquots of 1 cc saline.

Figure 2.

A. Histology and Immunohistochemistry of airways following NE or control vehicle treatment. BALB/c mice treated with NE or control vehicle were euthanized on day 11 and the left lung was formalin-fixed and sectioned for histology/immunohistochemistry. AB/PAS staining revealed increased goblet cell metaplasia following NE treatment. Immunohistochemistry for Muc5ac mucin was performed using HO8, an anti-Muc5ac peptide chicken polyclonal antibody, and detected with a peroxidase-conjugated goat anti-chicken antibody and DAB. Histologic sections are characteristic of each treatment condition (n=6 control, and 10 NE-treated animals at each time point, summarizing 2 separate experiments). Bar, 50 µm.

B. Histologic Mucus Index for NE- and control-treated mice. BALB/c mice instilled with NE (black bars) or control vehicle (gray bars) were euthanized on days 8, 11, or 14, and the left lung was formalin-fixed and sectioned for AB/PAS staining. The extent of PAS staining of the epithelium in each airway was graded as described in the Materials and Methods section. A minimum of 4 representative cross- or sagittally-sectioned airways were graded per animal. The results are shown graphically and are expressed as mean ± SEM, n=6 control, and 10 NE-treated animals at each time point, summarizing 2 separate experiments. *, p<0.05, NE-treatment is significantly different from corresponding control-treatment. †, p<0.05, NE-treatment for day 11
animals is significantly different from day 8 animals. Note: All airways evaluated from control animals at day 8 had no evidence of PAS staining yielding a grade of zero.

Figure 3. Histology and PAS staining and their relationship to airway diameter in mouse airways following NE treatment. BALB/c mice treated with NE were euthanized on days 8, 11 and 14 and the left lung was formalin-fixed and sectioned for histology/immunohistochemistry. To estimate the diameter of the airways that demonstrated PAS positive staining in response to NE treatment, longitudinally sectioned airways were examined as described in Materials and Methods section. Airways measuring 125 µm or less did not exhibit PAS staining of the airway epithelium (arrow with A) while larger airways did have PAS staining indicative of goblet cell metaplasia (arrow with B). Representative histologic section shown is from an animal euthanized on day 11. Bar, 50 µm.

Figure 4. Histology of lung inflammation following NE aspiration. BALB/c mice instilled with NE were euthanized on day 11 and the left lung was formalin-fixed, sectioned and stained with H & E. There were moderate (grade 2) inflammatory infiltrates with granulocytes both peribronchial and perivascular (arrowheads). Pigment-laden macrophages were present in the alveolar space (arrow). A – control-treated animal; B – NE-treated animal. Histologic sections were characteristic of each treatment condition (n= 6 control, and 10 NE-treated animals, 2 separate experiments). Bar, 50 µm.
Figure 5. Bronchoalveolar lavage cell count and differential for NE and control-treated mice. Bronchoalveolar lavage fluid (BAL) was obtained from NE-treated (black bars) or control-treated (gray bars) mice using 3 ml of saline. Total leukocyte counts were obtained by hemocytometer (A). BAL cells were cytocentrifuged onto slides, fixed, and stained for differential cell count percentages for neutrophils (PMN, B), lymphocytes (Lymph, C), and for eosinophils (Eos, D). At least 200 cells were counted. Data is expressed as mean ± SEM; n= 6 control, and 10 NE-treated animals, 2 separate experiments. *, p<0.05, NE treatment is significantly different from control-treatment. Note: There were no lymphocytes or eosinophils identified in the BAL from Day 8 control animals.

Figure 6. BAL Cytokine levels in NE and control treated mice. BALB/c mice treated with NE (black bars) or control vehicle (gray bars) were lavaged with 3 ml saline at 8, 11, or 14 days. Cytokine analysis was determined by a multiplex bead-based immunoassay (Linco) for 16 cytokines. Keratinocyte-derived chemokine (KC) and IL-5 were significantly different between treatment groups. Data is expressed as mean ± SEM; n= 6 control, and 10 NE-treated animals, 2 separate experiments. *, p<0.05, NE-treatment is significantly different from control-treatment.

Figure 7. Histologic Mucus Index and Muc5ac expression for mice treated with NE or NE + AAPV-CMK. Mice received intratracheal aspirations of NE or NE + AAPV-CMK. Prior to administration, NE + AAPV-CMK, was found to have no protease activity by spectrophotometric assay with an NE-specific substrate.

(A) AB/PAS staining was performed on lungs harvested on Days 11 and 14. The extent of PAS staining of the epithelium in each airway was graded as described in the Materials and
Methods section. A minimum of 4 representative cross- or sagittally-sectioned airways were graded per animal. The results are shown graphically and are expressed as mean ± SEM (n= 4 animals per treatment condition). All airways evaluated from control and AAPV-CMK-treated animals at both days 11 and 14 had no evidence of PAS staining yielding a grade of zero. *, p<0.05, NE + AAPV-CMK is significantly different from NE alone.

(B) Real-time quantitative PCR was performed using primers specific for mouse Muc5ac and β-actin and mRNA levels are expressed as relative amplification levels corrected for β-actin and normalized to values for one control mouse at each time point, 2^(-ΔΔCT). Values are expressed as mean ± SEM (n= 4 animals per treatment condition; See methods for details). *, p<0.05, NE + AAPV-CMK is significantly different from NE alone.

(C) Immunohistochemistry for Muc5ac mucin was performed using HO8, an anti-Muc5ac peptide chicken polyclonal antibody, and detected with a peroxidase-conjugated goat anti-chicken antibody and DAB. Histologic sections are characteristic of each treatment condition (n= 4 animals per treatment condition). Bar, 50 µm.

Figure 8. Bronchoalveolar lavage cell counts and differentials for NE and NE + AAPV-CMK-treated mice. Bronchoalveolar lavage fluid (BAL) was obtained from NE-treated (black bars) or NE + AAPV-CMK-treated (gray bars) mice using 3 ml of saline. Total leukocyte counts were obtained by hemocytometer (A). BAL cells were cytocentrifuged onto slides, fixed, and stained for differential cell count percentages for neutrophils (PMN, B), lymphocytes (Lymph, C), and for eosinophils (Eos, D). At least 200 cells were counted. Data is expressed as mean ± SEM; n = 4 animals per treatment condition. *, p<0.05, NE + AAPV-CMK treatment is significantly
different from NE alone treatment. *Note:* There were no eosinophils identified in the BAL from animals treated with NE + AAPV-CMK on Days 11 and 14.
Table 1. *Muc5ac/β-actin* mRNA expression in mouse lungs

<table>
<thead>
<tr>
<th>Lung harvest time point</th>
<th>Control</th>
<th>NE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8</td>
<td>0.70±0.16</td>
<td>1.64±0.49</td>
<td>0.07</td>
</tr>
<tr>
<td>Day 11</td>
<td>1.65±0.34</td>
<td>13.53±3.28</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.71±0.17</td>
<td>8.62±1.48</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Real-time quantitative PCR was performed using primers specific for mouse *Muc5ac* and *β-actin* and mRNA levels are expressed as relative amplification levels corrected for *β-actin* and normalized to values for one control mouse at each time point, $2^{-\Delta\Delta CT}$. Values are expressed as mean ± SEM (n= 3 control, and 5 NE-treated animals; See methods for details).
Table 2. Lung tissue inflammation grading

<table>
<thead>
<tr>
<th>Lung Harvest Time Point</th>
<th>Control</th>
<th>NE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8</td>
<td>0.33 ± 0.21</td>
<td>2.3 ± 0.21</td>
<td>0.0014</td>
</tr>
<tr>
<td>Day 11</td>
<td>0 ± 0</td>
<td>2.2 ± 0.13</td>
<td>0.0004</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.33 ± 0.21</td>
<td>1.7 ± 0.15</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

Lung sections were fixed, paraffin-embedded, sectioned and stained with H & E. An observer blinded for treatment conditions graded the intensity of inflammation on a 4-tier scale: 0, no inflammation, 1, minimal to mild, multifocal perivascular and interstitial infiltrates of granulocytes, and/or lymphocytes and macrophages. Cell infiltrates were scattered and less than 3 cells in depth in inflamed regions. Grade 2 (moderate) had more disseminated inflammatory infiltrates, 3-5 cells in depth, and pigment-laden macrophages were present. Grade 3 (marked) had diffuse perivascular or peribronchial inflammation 5-15 cells in depth and pigment-laden macrophages. Values are expressed as mean ± SEM (n=6 control, and 10 NE-treated animals at each time point, summarizing 2 separate experiments).
Figure 1

Harvest BAL/ Lungs

Days
Figure 2

A

CTRL

AB-PAS

Muc5ac

NE

B

PAS GRADE

0.0

0.5

1.0

1.5

2.0

2.5

8

11

14

DAYS

* control

NE

* *
Figure 3
Figure 5

A

TOTAL LEUKOCYTE COUNT (x10^3 cells)

B

PMN (% of total cell count)

C

LYMPH (% of total cell count)

D

EOS (% of total cell count)

* indicates a significant difference between control and experimental groups.

DAYS: 8, 11, 14
Figure 6

**KC**

- Control (gray)
- NE (black)

**IL-5**

- Control (gray)
- NE (black)

*Significant difference.*
Figure 7

(A) PAS GRADE

(B) Muc5ac RT-PCR mRNA Expression (expressed as a % of corresponding NE)
Figure 8

A

B

C

D

Figure 8: Graphs showing the change in Total Leukocyte Count, PMN (Neutrophils), LYM (Lymphocytes), and EOS (Eosinophils) over days 11 and 14 for two conditions: NE and NE + AAPV-CIHK.

- **A**: Total Leukocyte Count with bars indicating the mean and error bars showing standard deviation. Stars (*) indicate statistical significance.
- **B**: PMN (Neutrophils) count with similar representation of mean and error bars.
- **C**: LYM (Lymphocytes) count with similar representation of mean and error bars.
- **D**: EOS (Eosinophils) count with similar representation of mean and error bars.