Evaluation of *MUC5AC* expression and upregulation in airway epithelial cells of horses

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**Objective**—To isolate and culture primary equine airway epithelial cells in vitro and elucidate the major cytokines involved in expression of the gel-forming mucin gene *MUC5AC* in horses.

**Sample Population**—12 tracheas obtained within 5 hours after euthanasia from horses free from respiratory tract disease.

**Procedures**—Tracheal rings were digested overnight in 0.2% protease, and dissociated airway epithelial cells were grown in a serum-free defined medium at an air-liquid interface until confluence was achieved. Differentiated airway epithelial cells were treated with a panel of recombinant equine cytokines followed by quantitative reverse transcriptase PCR assay for mRNA of equine *MUC5AC* and the control gene glyceraldehyde 3-phosphate dehydrogenase. Cultures were incubated in the presence of isohelenin, a nuclear factor κB–DNA-binding inhibitor, to investigate transcriptional regulation of *MUC5AC*.

**Results**—Light and electron microscopy revealed a differentiated epithelium with ciliated cells, nonciliated mucous cells, and basal-like cells. Recombinant equine tumor necrosis factor-α was the major mediator in the cytokine panel that significantly increased *MUC5AC* mRNA by a factor of 5 in a dose- and time-dependent manner. This enhancement was attenuated by isohelenin.

**Conclusions and Clinical Relevance**—Data suggested that a nuclear factor κB-based transcriptional mechanism is involved in induction of *MUC5AC* expression by tumor necrosis factor-α. Understanding the molecular mechanism of cytokine-enhanced *MUC5AC* expression in horses may lead to better treatment options and understanding of the pathogenesis of equine pulmonary diseases. (Am J Vet Res 2010;71:690–696)

In humans, airway remodeling with mucus cell metaplasia—hyperplasia and mucus hypersecretion is a hallmark of many chronic inflammatory pulmonary diseases, including chronic obstructive pulmonary disease, chronic bronchitis, and asthma.1 Horses are also susceptible to a variety of pulmonary diseases, many of which are associated with mucus hypersecretion. Airway inflammation has been correlated with mucous cell metaplasia and an increase in the amount of stored mucins in equine lung biopsy specimens.2 Additionally, clinically important mucus hypersecretion has been associated with airway inflammation.3,4 Similar to humans, the major gel-forming mucins produced in the equine lung are muc5ac and muc5B.5

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**Abbreviations**

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<th>Abbreviation</th>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>MEM</td>
<td>Minimum essential medium</td>
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<td>NF-κB</td>
<td>Nuclear factor κB-light-chain-enhancer of activated B cells</td>
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<td>PAS</td>
<td>Periodic acid—Schiff</td>
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<td>RAO</td>
<td>Recurrent airway obstruction</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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Recurrent airway obstruction in horses is an allergic airway disease characterized by bronchoconstriction, airway wall thickening, and increased mucus secretion caused by exposure to dust or allergens, such as endotoxin or fungi, which are present in an indoor barn area. Goblet cell hyperplasia and metaplasia with mucus hypersecretion are typical features associated with RAO.6,7 Increased amounts of mucin glycoproteins in bronchoalveolar lavage fluid have been identified in horses with this disease, some of which remain increased even in affected horses in clinical remission.8 Gerber et al identified increased concentrations of muc5ac in different airways of RAO-affected horses and concluded that *MUC5AC* upregulation is likely a primary mechanism of mucus hypersecretion. However, the nature of the major cytokines that are involved in this pathogenesis has not been elucidated.
Methods to isolate and culture human airway epithelial cells in vitro are well established and have been instrumental in understanding the molecular mechanisms of mucus secretion.9 In human airway epithelial cultures, maintenance of cells at an air-liquid interface in the presence of retinoic acid is necessary for the development of well-differentiated mucociliary epithelium. Presently, 2 publications of studies11,12 that used different culture conditions have described the isolation and growth of equine airway epithelial cells in culture. However, neither method maintained cells in an air-liquid interface manner and neither study reported development of primary equine tracheal epithelial cells into ciliated or mucus-secreting cells in vitro.

The purpose of the study reported here was to isolate and culture primary equine airway epithelial cells at an air-liquid interface in vitro and elucidate the major cytokines involved in expression of the gel-forming mucin gene MUC5AC in horses.

Materials and Methods

Cell isolation and growth—Equine tracheas (n = 12) were isolated within 5 hours of death from horses that were euthanized for clinical reasons other than respiratory tract disease at the William R. Pritchard Veterinary Medical Teaching Hospital, University of California-Davis. Connective tissue was dissected from the trachea, and the dorsal tracheal ligament was removed under sterile conditions. After extensive cleaning and washing in MEM,4 individual tracheal rings were incubated in 0.2% protease in MEM supplemented with 50 µg of gentamicin/mL, 30 U of penicillin-streptomycin/mL, and 250 ng of amphotericin B/mL at 4°C overnight. The next morning, the epithelial cells were gently dislodged by washing with MEM plus 10% fetal bovine serum. The resulting pellet was washed twice with MEM plus 10% fetal bovine serum and plated on 100-µm2 hydrophilic tissue culture–treated plates6 for adhesion and expansion of epithelial cells. The cells were maintained in a serum-free medium of Dulbecco modified Eagle medium–Ham F12 (1:1) supplemented with insulin (4 µg/mL), transferrin (5 µg/mL), epidermal growth factor (5 ng/mL), dexamethasone (0.1 µM), cholera toxin (20 ng/mL), bovine hypothalamus extract (15 µg/mL), bovine serum albumin (0.5 mg/mL), penicillin-streptomycin (30 U/mL), gentamicin (50 µg/mL), and amphotericin B (250 ng/mL). Clusters of cells that did not attach to the tissue culture plates within 24 hours were aspirated and subjected to a 10-minute incubation with trypsin-EDTA (1 mg/mL) at 37°C to further dissociate the cell clumps. Following inactivation with trypsin inhibitor (2 mg/mL), the dissociated cells were plated on 100-µm2 tissue culture plates8 for further growth and expansion of the epithelial cells in the serum-free defined medium as described. Enhanced cell attachment and growth were seen in these trypsinized clusters.

Once confluent, primary equine tracheal epithelial cells from both plates of primary plating and secondary plating after trypsinization were obtained by use of standard trypsin-EDTA dissociation, and 50,000 cells were plated in each well onto 12-well polycarbonate filters with a pore size of 0.4 µm and maintained in a submerged condition with the same serum-free media. Following confluence on the filters (about 7 to 10 days), cells were subsequently maintained at an air-liquid interface and supplemented with 30 nM retinoic acid for 1 week prior to the start of the experiments.

At confluence, transepithelial resistance was measured by use of a voltmeter in 8 cultures that were not used in subsequent experiments. Briefly, 1 electrode was immersed in the basal side of the culture and the other electrode was immersed in the apical side in PBS solution to assess tight junction formation. The transepithelial resistance was measured 3 times in 8 cultures.

Morphological cell characterization—Cells on filters were fixed with either a zinc-based formalin fixative9 or Karnovsky fixative for light and electron microscopy, respectively. For light microscopy, filters with the attached cells were removed from the inserts and embedded in paraffin. Five-micron sections on glass slides were stained with H&E. Some cultures were treated with 0 or 50 ng of TNF-α/mL for 48 hours followed by staining with PAS–Alcian blue by use of standard techniques. For electron microscopic evaluation, fixed filters were processed as specified in instructions regarding preparation of inserts for electron microscopy1

The membrane was either cut in pieces for cross-sectional orientation or left intact for on-face orientation. For on-face orientation, the filter was sandwiched between 2 squares of polychlorotrifluoroethylene film9 with a drop of fresh resin. The sandwich technique of embedding has been used for embedding of 30- to 100-µm sections of brain.13

Care was taken to label the film on the cultured side of the filter. A weight was added to ensure flat polymerization at 70°C. The film was peeled from the noncultured side of the insert and glued to a prepolymerized flat block. Once the glue hardened, the film was peeled from the cell-side of the filter, trimmed, and thin sectioned on face. Grids9 of cross sections and on-face sections were stained with uranyl acetate and lead citrate prior to viewing on an electron microscope. Images were taken with a digital camera.10 The only digital manipulation was contrast and brightness adjustment before the image was saved.

Treatment of differentiated cells—Triplicate wells of differentiated airway epithelial cells were treated with a panel of equine recombinant cytokines for 48 hours at 50 ng/mL following overnight incubation in basal cell culture media (Dulbecco modified Eagle medium–Ham F12, 1:1) devoid of hormonal and growth factor supplements. The following recombinant equine cytokines were used: TNF-α, IL-2, IL-4, IL-5, IL-6, IL-8, and IL-10.14 After 48 hours' treatment, RNA was isolated4 according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase by use of random primers for 90 minutes at 42°C in 20 µL of reaction mixture. The reaction mixture was further diluted to 80 µL with nuclease-free water for the subsequent procedure. Three microliters of cDNA was analyzed by use of 2X PCR mixture by use of a thermocycler following the manufacturer's protocol. Gene-specific primers were designed (equine MUC5AC forward: 5′-GGCTGTGGTTTGGACACGTG-3′; reverse: 5′-AAGGAGCATGTGGTTTGGT-3′; equine GAPDH
Development of a differentiated equine airway epithelium in vitro—Initially, isolated cells were plated on 100-mm² tissue culture plates to expand cell numbers. Clusters of cells attached to the plate with growth of cells around the periphery (Figure 1). After passage onto filters, equine tracheal epithelial cells were cultured in the presence of retinoic acid at an air-liquid interface to mimic in vivo conditions. Following at least 1 week in these conditions, the cells polarized and differentiated. Polarization was supported by an increase of transepithelial resistance (mean of 8 cultures, 1,410 Ω/cm², range, 1,240 to 1,570 Ω/cm²) suggested the formation of tight junctions in the confluent cultures.

Differentiation was determined via light and electron microscopy. Examination of a cross-section via light microscopy (Figure 2) revealed development of a pseudostratified epithelial cell layer. The pseudostratification resembled that observed in cross section of an airway epithelial layer obtained from a tracheal specimen (Figure 3). Via scanning electron microscopy, cilia bundles on the cell surface of cultured epithelial layer were seen (Figure 4), which was further confirmed via transmission electron microscopy that revealed basal bodies with characteristic cilia morphology (Figure 5). Transmission electron microscopy also revealed mucous-secretory granule-like morphology in some of the cells (Figure 6). Maintenance of the cultures for longer times (up to 1 month) resulted in subjectively increased proportion of mucous cells in the cultures.

Statistical analysis—Data from the panel of equine cytokines were analyzed by use of a Student t test. The dose response and isohelenin data were analyzed via ANOVA followed by a Bonferroni multiple comparison test. Values were considered significant at P < 0.05.
Upregulation of equine MUC5AC—Differentiated airway epithelial cells had the highest induction with equine recombinant TNF-α (Figure 7). After 48 hours of treatment, TNF-α stimulated equine MUC5AC mRNA by a factor > 5.

After these results were obtained, subsequent experiments were focused on equine recombinant TNF-α. A dose response was determined regarding induction of MUC5AC mRNA by TNF-α, with significant upregulation at the 50 ng/mL dose, compared with the control condition (Figure 8). After a 24-hour stimulation,
50 ng of TNF-α/mL induced a 2.3-times induction of equine MUC5AC mRNA. The highest dose of TNF-α did not induce any toxic effects in the cells as assessed via trypan blue and ethidium homodimer staining.

**TNF-α stimulation increases mucous cell phenotype**—To determine morphological changes associated with stimulation by TNF-α, some cultures were treated with 50 ng of TNF-α/mL for 48 hours and stained with PAS–Alcian blue to demonstrate acidic (Alcian blue positive) and neutral (PAS positive) mucins. More PAS- and Alcian blue–positive cells were determined in a culture treated with TNF-α, compared with a nontreated control culture (Figure 9).

**NF-κB dependency**—To investigate the transcriptional regulation of TNF-α-mediated MUC5AC upregulation, cultures were treated with isohelenin, an NF-κB DNA-binding inhibitor, in the presence of TNF-α for 48 hours. Both 5μM (P = 0.003) and 20μM (P = 0.001) isohelenin significantly inhibited TNF-α-mediated MUC5AC induction (data not shown). These doses of isohelenin had no cytotoxic effect on the cells as evidenced via trypan blue and ethidium homodimer staining.

**Discussion**

Development of sophisticated techniques for in vitro culture of human primary airway epithelial cells that simulate in vivo conditions has led to an explosion of research investigating the basic molecular mechanisms of airway cells. In 2 reports,11,12 methods for the isolation and culture of equine airway epithelial cells are described. In those studies, equine airway epithelial cells were maintained in submerged conditions with no evaluation of mucociliary differentiation. Both studies revealed cytokeratin staining of the cultured cells but did not detect further differentiation. In the present study, the first objective was to develop methods to grow a pseudostratified differentiated airway epithelium from equine tracheal epithelia. We adapted the techniques our laboratory uses for isolation and culture of human primary airway epithelial cells with maintenance of the cultures at an air-liquid interface in the presence of retinoic acid to maximize mucociliary differentiation. Light microscopy revealed a pseudostratified epithelium, and increased transepithelial resistance was determined, similar to in vivo conditions. Both scanning electron microscopy and transmission electron microscopy revealed cilia and mucus-secreting granules. Alcian blue and PAS staining revealed stained cells. These results indicated differentiation of equine cultures with the development of ciliated cells, nonciliated mucous cells, and basal-like cells after 1 week. Establishment of these techniques provides a basis for studying the basic biology and molecular regulation of equine airway epithelial cells.

By use of this culture system, differentiated airway epithelial cells were treated with a panel of cytokines and TNF-α was found to be most potent for stimulation of equine MUC5AC mRNA expression and enhancement of PAS–Alcian blue staining. This effect was time and dose dependent and sensitive to isohelenin inhibition, suggesting NF-κB–based transcriptional regulation. Importantly, we intended to detect upregulation of equine MUC5AC by TNF-α at the protein level as well but were unsuccessful in finding a commercially available MUC5AC antibody that cross-reacted with equine protein for use in a western blot or immunohistochemical test.

The cytokines IL-4 and IL-6, which stimulate airway mucin gene expression in mouse and human airway epithelial cells and cell lines, were ineffective in this study. The reasons for this were unclear, but it could be attributable to innate differences in the percentage of mucous cells present in airway epithelium. Results of a previous study15 indicate species differences among humans, monkeys, and mice in the MUC5AC mRNA expression levels associated with use of IL-6. Monkeys and mice have fewer mucous cells in airways, compared with humans, which results in a lower expression level of MUC5AC mRNA, a need for greater cytokine stimulation for MUC5AC expression, or both. We have also observed fewer mucous cells in equine airway epithelium, compared with humans, which could result in similar innate species differences in MUC5AC expression.

Tumor necrosis factor-α is a major pleiotropic proinflammatory cytokine primarily produced by pulmonary macrophages in response to activation of the innate immune system. In humans, increased numbers of TNF-α–positive cells are detected in bronchoalveolar lavage and bronchial mucosal biopsy specimens from asthmatics,15,16 and substantial amounts of TNF-α have been measured in exhaled breath condensates and sputum of patients with chronic obstructive pulmonary disease.17,18 Clinical trials of TNF-α antagonists reveal improved symptoms in some asthma patients who are refractory to corticosteroid treatments.19 Likewise, high

Figure 9—Photomicrographs of cultured equine tracheal epithelial cells. A—Control cells not exposed to TNF-α for 48 hours. Arrow indicates a stained cell. PAS–Alcian blue stain; bar = 200 μm. B—Cells exposed to 50 ng of TNF-α/mL for 48 hours. Notice the increased number of stained cells (arrows), compared with A. PAS–Alcian blue stain; bar = 200 μm.
concentrations of TNF-α transcripts are detected in alveolar macrophages and from bronchoalveolar lavage fluid isolated from RAO-susceptible horses after challenge with allergens. A recent study did not detect any differences in TNF-α, IL-6, or IL-10 by use of quantitative PCR assay or immunohistochemical analysis of bronchial biopsy specimens from RAO-challenged horses, compared with healthy horses. However, that study primarily examined airway epithelial cells present in the biopsied tissue and did not examine alveolar macrophages, which may be a primary source of TNF-α.

Being one of the primary proinflammatory cytokines, TNF-α is likely produced early in other equine pulmonary diseases that are characterized by mucus hypersecretion, such as viral and bacterial bronchitis and pneumonia; however, documentation of this is scant in the literature. One study revealed increased mRNA for TNF-α as well as IL-1β, IFN-γ, and IL-12 p40 in the lungs of foals inoculated with Rhodococcus equi, an important pulmonary pathogen in foals. Interestingly, this same study also revealed increased pulmonary activation of NF-κB, likely mediating the TNF-α upregulation. Another study revealed that the pathogenic equine herpesvirus-1 strain RacL11 increased transcripts of TNF-α in bronchoalveolar lavage fluid on day 3 after inoculation of the virus in mice, suggesting a role for the early induction of proinflammatory cytokines in the pathogenesis of equine herpes virus pneumonia.

Muc5ac is a major gel-forming mucin in the lung produced by goblet cells in the airway epithelium in humans and is also a major mucin in the lung in horses. Tumor necrosis factor-α–induced MUC5AC upregulation has been detected in primary human nasal epithelial cells and NCI-H1292 cells, a pulmonary-derived mucoepidermoid carcinoma cell line. The signal transduction pathway of this induction in human airway epithelial cells is incompletely described, but studies have indicated a role for NF-κB–mediated upregulation, possibly mediated by the MAPK p38 and ERK. In vivo, TNF-α–induced NF-κB activation is localized to the airway epithelium as seen in mice by use of whole body bioluminescence with subsequent morphological evidence of goblet cell metaplasia and mucus production. The study reported here found that TNF-α–mediated MUC5AC mRNA upregulation with evidence of NF-κB involvement also occurs in equine airway epithelial cells. Nuclear factor κB is composed of p65 and p50 homodimers and heterodimers that are sequestered in an inactive form in the cytoplasm by the inhibitor protein IκBα. Upon activation, the IκB kinase proteins (IκKα and IκKβ) phosphorylate IκBα, resulting in translocation of NF-κB to the nucleus, where it acts as a transcription factor regulating the expression of many proinflammatory genes. Isohelenin, used in this study, acts as an NF-κB inhibitor by irreversibly preventing IκBα degradation. Interestingly, increased activity of NF-κB, which is primarily composed of p65 homodimers, has been detected in bronchial epithelial cells and bronchoalveolar lavage fluid from RAO-affected horses and has been correlated with clinical disease.

References

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