Modulation of MUC7 Mucin Expression by Exogenous Factors in Airway Cells *In Vitro* and *In Vivo*

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The human MUC7 gene encodes a low-molecular-mass mucin that participates in the maintenance of healthy epithelium in the oral cavity, and possibly in respiratory tracts, by promoting the clearance of various bacteria. We examined whether MUC7 gene is expressed in primary normal human tracheobronchial epithelial cells and whether the expression is modulated by exogenous factors. By assessing MUC7 transcripts, we found that the MUC7 gene was induced by culturing the normal human tracheobronchial epithelial cells at the air–liquid interface, in which the cells were well differentiated. When the cells were treated with a panel of cytokines (IL-1β, IL-4, IL-13, and TNF-α), epidermal growth factor, or a bacterial product (*Pseudomonas aeruginosa* lipopolysaccharide [LPS]), MUC7 transcripts and glycoprotein products were increased 1.7- to 3.2-fold. The effect of LPS on MUC7 gene expression was also studied in the airway tissues of MUC7 gene transgenic mice. In the *in vitro* cultured trachea and lung explants, the LPS-treated tissues showed over 2-fold increased levels of MUC7 mRNA compared with the untreated specimens. These results were confirmed by *in vivo* studies using the lungs and tracheas harvested from the transgenic mice irritated by LPS through the tracheal instillation. By immunohistochemistry, MUC7 glycoprotein was localized in tracheal submucosa within the serous cells. Upon LPS stimulation, the overexpressed MUC7 remains confined to the serous glands. In the lungs, MUC7 seems to be expressed within the respiratory epithelium at the level of the bronchioles. Upon stimulation with LPS, it seems to be overexpressed within the same cells and within the stromal tissue.

Keywords: intratracheal instillation; immunohistochemistry; MUC7 gene expression and regulation; MUC7 gene transgenic mice; real-time PCR; tracheobronchial epithelial cells

The human oral cavity and respiratory, gastrointestinal, and reproductive tracts are coated by a thin film of viscoelastic liquid (mucus) that provides lubrication and protection to the epithelial surfaces. Normal mucus secretion is important in maintaining epithelial integrity and nonimmune mucosal defense (1–3). Excessive mucus is likely associated with inflammatory response and epithelial diseases. Chronic respiratory diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis (CF) are characterized by airway inflammation and mucus hypersecretion. The mucus hypersecretion by itself could result in impaired mucociliary movement and compromised protective functions of mucosa (4). The major components of mucus are mucin glycoproteins, which are also the determinants of the viscoelasticity of mucus secretions. Mucus hypersecretion is the culmination of several complex processes, including mucin overexpression.

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At least 17 human mucin genes have been identified and designated MUC1–4, MUC5AC, MUC5B, MUC6–9, MUC11–13, MUC15–17, and MUC19. These genes are expressed in a wide range of tissues. The expression levels and patterns of mucins are subjected to variation during an inflammatory process, tumor progression, and carcinogenesis. For example, MUC1 overexpression was observed in most adenocarcinomas of the breast, lung, stomach, pancreas, prostate, and ovary (5, 6). MUC2 overexpression was thought to be a common characteristic of colon and pancreatic carcinomas (7). MUC3 was also upregulated in colon cancers (5). Aberrant expression of MUC5B was found in gastric cancer (8). Differential expression of MUC7 was linked to invasive bladder carcinoma (9, 10).

Airway mucins often show an enhanced expression in response to inflammatory conditions. The expression of gel-forming mucins (MUC2, MUC5AC, and MUC5B) was increased during bacterial invasion (11). A similar observation was extended to CF airways (4, 12). The major infectious pathogen in airways is *Pseudomonas aeruginosa*, which produces a virulence factor known as lipopolysaccharide (LPS) that has the ability to initiate a host defense response (13, 14). Airway mucins have also shown to be regulated by cytokines and growth factors (15, 16). These observations have been reported mainly for MUC2, MUC5AC, and MUC5B, and there is no information available about MUC7 regulation in response to exogenous modulations.

The human MUC7 gene encodes a relatively small mucin glycoprotein (MG2; $\sim$ 125 kD) that is secreted mainly by human sublingual and submandibular glands. The functions of MUC7 mucin in the oral cavity have been extensively studied. As a salivary glycoprotein, it is involved in mastication, speech, swallowing, and lubrication of the oral cavity. It also functions as an antimicrobial agent involved in promoting the clearance of various bacteria. It can inhibit bacterial colonization by masking their surface adhesins (3). This mucin has been shown to interact *in vitro* with many oral and respiratory microorganisms, including *Candida albicans*, *P. aeruginosa*, *Staphylococcus aureus*, *Actinomyces viscosus*, *Porphyromonas gingivalis*, *Streptococcus gordonii*, *S. mutans*, *Eikenella corrodens*, *Herpes simplex* virus, and human immunodeficiency virus (3, 17). Recent studies in our laboratory have shown that the N-terminal region of MUC7 mucin exhibits potent fungicidal and bactericidal activities (18–20).

Although MUC7 gene expression was originally detected in salivary glands (21), this gene regulation has not been studied *in vivo* or *in vitro* because there is no appropriate salivary gland cell model available for this purpose. More recently, MUC7 gene expression was found in respiratory tracts (22–24) and likely contributes to the protection of airway epithelium. There is a great upsurge in studies of airway mucin expression with primary cultures of normal human tracheobronchial epithelial (NHTBE) cells. The so-called "air–liquid interface" (ALI) culture was found to be effective in promoting NHTBE cell differentiation and mucin gene expression. NHTBE cells grown in this configuration retain many morphologic and functional characteristics of *in vivo* airway epithelium (25).

In the present study, we examined whether MUC7 is expressed in primary NHTBE cells and whether the same agents

that modulate the expression of MUC2, MUC5AC, and MUC5B (cytokines, growth factors, and *P. aeruginosa* LPS) also modulate MUC7 gene expression. Second, the modulation of the MUC7 gene expression by *P. aeruginosa* LPS was studied in MUC7 transgenic mice airway and salivary gland tissues *in vitro* (cultured tissue explants) and *in vivo*. The modulation of the mouse endogenous mucin gene, muc10 (26), was also examined in cultured tissue explants.

MATERIALS AND METHODS

Human Airway Cells

NHTBE cells were obtained from Clonetics Corp. (San Diego, CA). They were cultured in bronchial epithelial growth medium (BEGM) supplemented with bovine pituitary extract (50 μg/ml), hydrocortisone (0.5 μg/ml), human epidermal growth factor (0.5 ng/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), *all-trans* retinoic acid (0.1 ng/ml), tri-iodothyronine (6.5 ng/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and BSA (1.5 μg/ml; Sigma, Saint Louis, MO). All reagents were from Clonetics Corp. unless otherwise indicated.

Cell Culture and Treatment

Cryopreserved passage-1 stock of NHTBE cells was cultured for expansion following the manufacturer's instructions. The passage-2 cells were seeded onto a Transwell-COL culture insert coated with rat tail collagen type I gel (Costar; Corning Inc., Big Flats, NY) at 1×10^5 cells/insert. The insert was put in a 6-well plate, and the cells were grown in 2 ml of BEGM-DMEM medium (1:1 mixture) in a 37°C, 5% CO_2 incubator. They were submerged in the medium for the first 7 d, during which time the culture medium was changed every other day. The ALI was created on Day 8 by removing the apical medium and feeding the cells from the basal compartment only. The culture medium was changed daily after creation of an ALI. In control cultures, the cells were kept in an immersed condition (squamous culture) (25). The cultures were examined for cell morphology, MUC7 transcription, and MUC7 mucin production every day from Day 1 to Day 10 after ALI creation.

To stimulate cells, at Day 7, the cultures were individually treated with IL-1β, IL-4, IL-13, TNF-α, epidermal growth factor (EGF) (all at 20 ng/ml), and *P. aeruginosa* LPS (10 μg/ml) for 12 h. These single doses are optimal doses, based on the dose–response curves performed for the individual agents (data not shown). All cytokines, EGF, and LPS (serotype 10) were purchased from Sigma and dissolved in PBS (pH 7.4) containing 0.1% BSA.

Animals

Human MUC7 gene transgenic mice were generated on the BCF2 strain by our laboratory in 1998 (27) and were maintained by heterozygous breeding yearly. The progenies were screened for the presence of the human MUC7 gene by PCR. The mice were maintained under environmentally controlled conditions. All procedures of using animals were approved by the Institutional Animal Care and Use Committee at the State University of New York at Buffalo.

LPS Stimulation of Mouse Tissues

Three male transgenic mice (8 wk old) were killed, and their tracheas, lungs, and salivary glands were collected for culturing. The overlying muscles and blood vessels were removed from the tissues under a microscope. The pure tissues were cut into ~ 0.5-mm rings (trachea) or ~ 2-mm^3 pieces. The tissues were cultured in 2 ml of BEGM in a 37°C, 5% CO_2 incubator. For stimulation, *P. aeruginosa* LPS was added into the cultures at 10 μg/ml for 2 h. After 2 h of incubation, the tissues were rinsed with PBS twice and immersed in RNAlater (Ambion Inc., Austin, TX) and stored at −20°C until they were subjected to RNA isolation and immunohistochemistry (*see below*).

Mouse Intratracheal Instillation

Six male MUC7 gene transgenic mice were divided into experimental and control groups. Three male nontransgenic mice were used as MUC7 gene negative controls. All mice were 8 wk old and had similar body weights (~ 25 g). The mice were anesthetized by injection of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5 mg/kg) to circumvent the cough reflex during instillation. The upper portion of the trachea was surgically exposed by making an incision in the skin and separating the thyroid gland, overlying muscles, and connecting tissues. A microsyringe carrying a 25-gauge soft needle filled with 25 μl (2.5 μg) of *P. aeruginosa* LPS solution (dissolved in PBS at 100 μg/ml) was inserted into the exposed trachea, and the solution was injected into the lumen of the mouse trachea. The control mice received 25μl of PBS solution only. The three nontransgenic mice were also given the same amount of the LPS-PBS solution. After injection, the skin was sutured, and the animals resumed their daily activity. The animals were observed for postinjection respiratory changes. They were killed 48 h after surgery, and their tracheas, lungs, and salivary glands were collected for RNA analysis.

RNA Preparation and RT-PCR

Total RNA was isolated from cultured NHTBE cells, and animal airway tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. The quality and quantity of RNA were determined spectrophotometrically.

Reverse transcriptions were performed using M-MLV reverse transcriptase (Promega, Madison, WI) following the manufacturer's instructions. Briefly, first-strand cDNA was synthesized in 25 μl final volume, containing total RNA (2 μg), random hexamer primers (1 μg), dNTP (0.5 mM), rRNasin (25 U), M-MLV (200 U), and 5× first-strand buffer (5 μl). The reactions were incubated at 37°C for 60 min. Template contamination was monitored by including negative control reactions that did not contain reverse transcriptase or total RNA.

PCR was performed on 2 μl of cDNA using specific pairs of primers. The primers for human MUC7 gene are 5′-CTGGACTGCTAGCT CACCAGAAGCCG-3′ and 5′-GGGTGGGGCAGCTGTGGTGTC TTG-3′, with an expected PCR product size of 361 bp (cDNA nucleotide position: 244–605) (21). The primers for mouse muc10 gene are 5′-GGTTTCATTCCAAGCTCTCC-3′ and 5′-TTAGGAGAACGG CGACTGAT-3′, with an expected PCR product size of 161 bp (cDNA nucleotide position: 212–372) (28). The primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are 5′-ACCACAGTC CATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, with an expected PCR product size of 152 bp. PCR reactions were performed using the Advantage 2 PCR Kit (BD Biosciences, Palo Alto, CA) as described in the instructions. Thirty cycles of amplification were performed, and the products were analyzed on 1.2% ethidium bromide–stained agarose gels run in 1× Tris/acetate/EDTA buffer.

Quantitative real-time PCR was performed using Applied Biosystems 7500 (Foster City, CA) following the TaqMan Gene Expression Assay protocol. TaqMan primers and probes for human MUC7 and GAPDH (as an internal control) genes were pre-developed by Applied Biosystems. The primers were designed to span intron/exon boundaries to verify that PCR products were amplified from cDNA. Reactions were run in the presence of a FAM dye-labeled TaqMan probe. To optimize the reaction, serially diluted cDNA templates were tested, and the target gene products were plotted against threshold cycle. The plots of mRNA levels showed an increase in the fluorescent signal as a function of cycle number. PCR amplification was run under the control of SDS software (Sequence Detection Systems; Applied Biosystems). The data were analyzed with Relative Quantification Study Document. The relative quantity of MUC7 mRNA was normalized to GAPDH expression.

Northern Analysis

Northern analysis was performed according to the standard procedures. Briefly, RNA samples (15 μg) were resolved on 2% agarose-formaldehyde gel and transblotted onto Nytran membrane (Schleicher and Schuell, Keene, NH) by capillary blotting. After UV cross-linking (Stratalinker; Stratagene, La Jolla, CA), the membrane was prehybridized in 15 ml of the rapid hybridization buffer (Amersham Biosciences, Piscataway, NJ) at 65°C for at least 15 min. A ^{32}P-labeled cDNA probe was added to the hybridization, and the blot was hybridized for 2–24 h. The cDNA probe was prepared using Rediprime II random primer labeling kit (Amersham Biosciences). The human MUC7 probe was a 403-bp cDNA fragment (nt: 13–415) (21). The GAPDH gene was used as an internal control. The blot was washed twice with 250 ml of 2× saline sodium

citrate/0.1% SDS at room temperature for 30 min and washed twice with 250 ml of 0.1× saline sodium citrate/0.1% SDS at 65°C for 30 min. The hybridization results were visualized by autoradiography. Quantitative analysis was performed using an Image-Store 7500 Scanner and Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). The relative abundance of MUC7 message was normalized with the GAPDH mRNA bands.

Cell Lysate Preparation and ELISA Assays

NHTBE cell lysates were prepared by dissolving the cells in radioimmunoprecipitation assay lysis buffer (1× PBS, 1%Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and complete mini-protease inhibitor cocktail) at 50 μl/well. Protein concentrations of the cell lysates were measured using Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). The cell lysate contains secreted and cellular MUC7 mucin because mucin secretion occurs on the apical side of the biphasic culture condition (29). The lysates were plated in four replicates on 96-well microtiter plates (1 μg protein/well). ELISA was performed with a Protein Detector ELISA Kit (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) following the manufacturer's instructions. The primary antibody (rabbit antisera) was made by Bio-Synthesis (Lewisville, TX); it is specific to a 15-mer peptide derived from MUC7 mucin glycoprotein (aa 39–53). The specificity of the primary antibody was examined by using the cell lysates from squamous cultures that do not express MUC7. The nonspecific interaction was detected in insignificant amounts (~ 5%) by comparing it with that in ALI cultures. The secondary antibody was goat anti-rabbit IgG–alkaline phosphatase conjugate. The purified human MUC7 mucin glycoprotein was used for generating the standard curve.

Immunolocalization of MUC7

Before immunohistochemistry, each histologic sample was incubated in 0.5 M sucrose in PBS (12 h at 4°C) and cryosectioned (7 μm). Immediately after cryosectioning, tissue sections were washed three times in PBS (10 min at room temperature [RT]) and incubated with a blocking agent for 2 h at RT (1% BSA [Sigma], 10% goat serum [Sigma], and 0.1% Triton X-100 in PBS). Tissue sections were then incubated for 1 h at RT with monoclonal mouse anti-MUC7 antibody (diluted 1:1,000 in the blocking agent) (30). After washing three times with PBS (10 min at RT), tissue sections were incubated for 2.5 h at RT with a fluorescent secondary goat anti-mouse IgG (Alexa Fluor 488; Molecular Probes, Eugene, OR) diluted in 1% BSA and 10% goat serum in PBS (1:150). The sections were washed three times in PBS (10 min at RT), air dried for 10 min at RT, and covered with a cover glass using an aqueous mounting medium (Immu-Mount; Thermo-Shandon, Pittsburgh, PA). Fluorescent pictures (original magnification: ×200) were acquired by a digital camera (70-ms exposure time) (SPOT RT-KE; Diagnostic Instruments Inc., Sterling Heights, MI) attached to a Nikon Eclipse TE2000-U inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY). For each sample, some tissue sections immediately adjacent to those used for immunohistochemistry were processed for hematoxylin-EOSIN staining and used for morphologic analysis (original magnification: ×200 and ×400).

Statistical Analysis

Statistical analyses were performed upon comparisons made between the treated group and the control group using the Student's t test. A P value of < 0.05 was considered statistically significant. Data are expressed as mean ± SE.

RESULTS

Differentiation-Dependent Expression of the MUC7 Gene

To establish culture conditions optimal for the MUC7 gene expression in NHTBE cells, we have grown the cells in two ways: ALI culture and squamous culture. In the ALI culture, cells were fed from the basal sides with the apical sides facing air. In the squamous culture, the cells were submerged in medium. Cell morphology was examined microscopically, and MUC7 transcription was detected by RT-PCR each day from Day 1 to Day 10. A MUC7 transcript was detectable only when the cells were grown in ALI, increased in a time-dependent manner, and reached the highest level around Day 7 (Figures 1A and 1B). In contrast, when the cells were immersed in culture medium and maintained in the squamous condition, the MUC7 transcript was not detectable (Figure 1A, lane S, a representative of squamous cultures from Day 1 to Day 10, in which the cells had been grown for 7 d). MUC7 gene transcription was induced by the ALI culture condition. We consider this induction in ALI to be differentiation dependent because the NHTBE cells grown in ALI had a well differentiated appearance (Figure 1C). Microscopy

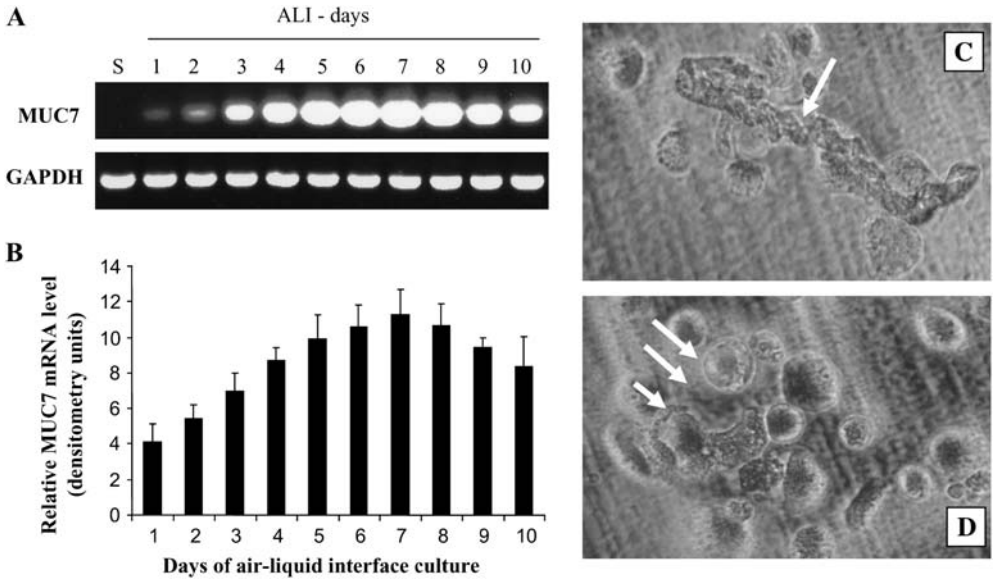

Figure 1. Analysis of NHTBE cells and MUC7 expression in NHTBE cells cultured under different conditions. (*A*) A semiquantitative RT-PCR analysis of the MUC7 gene transcription. NHTBE cells were cultured in ALI or squamous conditions, respectively. Total RNA was isolated from those cultures every day from Day 1 to Day 10. The levels of MUC7 mRNA were assessed by semiquantitative RT-PCR, and analyzed by agarose gel electrophoresis. *Lane S* is a representative of the squamous culture (the cells were submerged in medium for 7 d). *Lanes 1* through *10* represent ALI cultures from Day 1 to Day 10. Human GAPDH gene was used as an internal control. (*B*) Relative MUC7 mRNA levels expressed in densitometry units. The figure shown is a representative of three separate experiments. *Error bars* indicate SDs of the triplicate determinations. (*C* and *D*) Cell morphology of the cultures at Day 7 viewed by light microscopy (original magnification: ×20). (*C*) The cells grown in ALI conditions revealed a basal layer overlaid by columnar cells with goblet morphology. (*D*) The cells grown under squamous conditions revealed a stratified (*three arrows*), nondifferentiated pattern.

examination revealed a basal layer overlaid by columnar cells with goblet morphology. In contrast, the cells grown under squamous conditions showed poor differentiation with a stratified appearance (Figure 1D). Thus, for subsequent experiments, we chose to grow the NHTBE cells in ALI for 7 d.

Cytokines, EGF, and *P. aeruginosa* LPS Modulate MUC7 Expression in NHTBE Cells

To examine the effects of exogenous factors on MUC7 gene expression, the levels of MUC7 mRNA and the mucin glycoprotein in the control and stimulated NHTBE cells were assessed using quantitative real-time PCR and ELISA, respectively. When the cells that had been grown in ALI for 7 d were treated individually with an optimal dose of IL-1β, IL-4, IL-13, TNF-α, EGF, or LPS, the MUC7 transcript increased by 1.7- to 2.8-fold (Figure 2A), and the MUC7 glycoprotein increased by 2.1- to 3.4-fold (Figure 2B). Thus, all of these exogenous factors showed stimulatory effects on MUC7 gene expression, suggesting that MUC7 upregulation may relate to airway inflammation because these factors are relevant to the host inflammatory response (31).

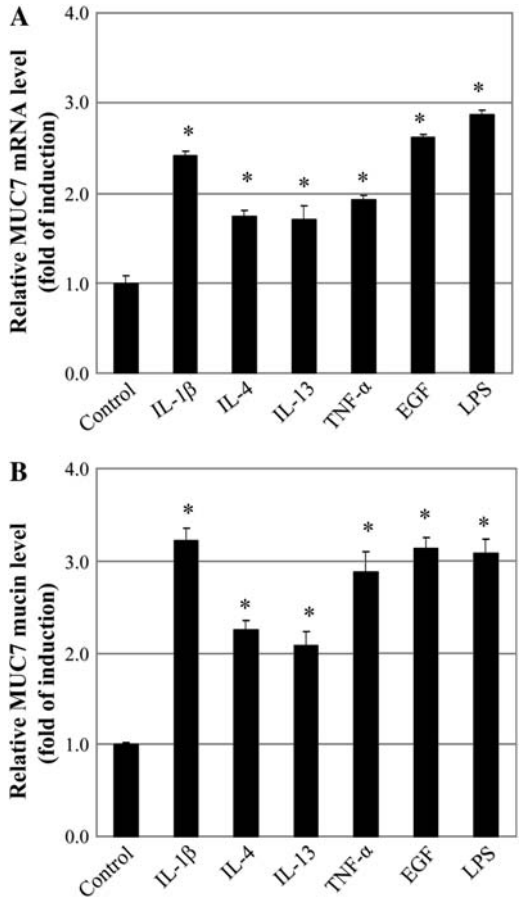

Figure 2. Effect of exogenous factors on MUC7 mRNA and glycoprotein production. NHTBE cells were grown in ALI for 7 d and treated with IL-1β, IL-4, IL-13, TNF-α, EGF, and *P. aeruginosa* LPS for 12 h. MUC7 expression in these cultures was examined at the transcriptional level and protein level. (*A*) Quantitative analyses of MUC7 transcription. Total RNA was isolated from the control cells and treated cells and reverse transcribed. The levels of MUC7 mRNA were assessed by real-time PCR and normalized to those of the GAPDH gene. MUC7 mRNA levels are expressed as fold of induction by comparing with untreated control cultures. Each bar represents the mean ± SE from three independent experiments analyzed in four replicates. $*P < 0.05$. (*B*) Quantitative analyses of MUC7 mucin production. The relative stimulatory effects on MUC7 mucin production by the exogenous factors were determined by ELISA as described in MATERIALS AND METHODS. Cell lysates were made from the control cells and the exogenous factor–treated cells. The MUC7 mucin levels were normalized to standard curve made with purified MUC7 mucin and expressed as percentage of the control. Each bar represents the mean ± SE from three independent experiments analyzed in four replicates. $*P < 0.05$.

Effect of LPS on MUC7 Gene Transcription in Mouse Airways

First, we examined the effect of *P. aeruginosa* LPS on the MUC7 using MUC7 transgenic mice tissue explants that were cultured *in vitro* and irritated with LPS. Up to 2.3-fold enhancement of the MUC7 mRNA level was found in the LPS-treated trachea and lung explants, compared with the control tissues (Figure 3), whereas no increase was observed in the salivary gland tissue (data not shown). For comparison, we determined the transcript levels of endogenous mouse mucin, muc10. Similarly to MUC7, the muc10 transcripts were increased upon the LPS stimulation in the airway tissues; up to 2.7-fold increase was found in the trachea (Figure 3), and no increase was detected in the salivary gland tissue (data not shown).

Next, we examined the LPS effect *in vivo* after administration of LPS to animal airways by intratracheal instillation. MUC7-transgenic mice and nontransgenic mice were used in this study. The mice in the control group received saline solution only. Two days later, breathing difficulties were observed in the mice that

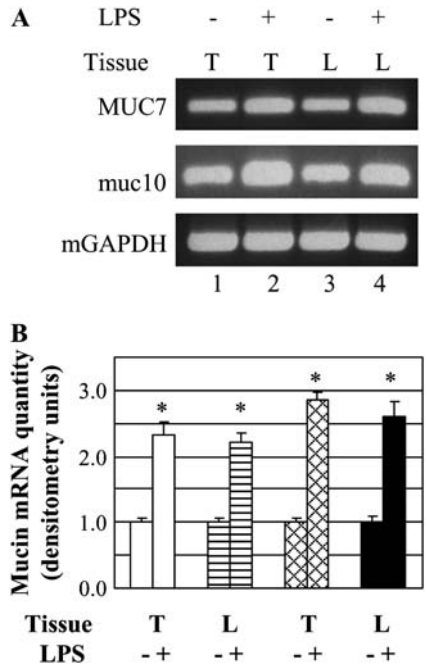

Figure 3. Effect of *P. aeruginosa* LPS on MUC7 transcription in mouse tracheal and lung explants. (*A*) Three human MUC7 gene transgenic mice were killed, and their tracheas and lungs were removed for *in vitro* culture. The tissues were cultured in bronchial epithelial growth medium for 2 h with or without LPS stimulation (10 μg/ml). RNA samples were prepared and the MUC7 (human) and muc10 (mouse) mRNA quantity determined by semiquantitative RT-PCR. Mouse GAPDH gene served as an internal control for RNA integrity and the loading amount. (*B*) Relative levels of MUC7 mRNA and muc10 mRNA expressed in densitometry units. Results shown are representative of triplicate experiments, and the values represent the mean ± SE. $*P < 0.05$. *Open bars* and *bars with horizontal lines*, MUC7; *cross-hatched bars* and *solid bars*, muc10.

received the LPS (transgenic and nontransgenic). Because the single dose of LPS irritation of the mice was sufficient to elicit breathing difficulty, under the suggestions of veterinarians, no further LPS dose was administered. Animals were killed, and tissues were harvested. MUC7 mRNA levels were determined by Northern blot analysis. The MUC7 mRNA levels were increased $\sim$ 2-fold in the tissues of MUC7 transgenic mice that received LPS compared with control animals that received vehicle only (Figure 4). The MUC7 mRNA was not detectable at all in nontransgenic mice, even in those that received LPS, due to the absence of the MUC7 gene (Figure 4). These findings indicate that animal airways are sensitive to the bacterial product LPS and that MUC7 expression can be modulated by LPS in transgenic mice. The upregulation of the MUC7 gene by *P. aeruginosa* LPS may relate to airway inflammation as a consequence of bacterial infection. Consistent with the salivary gland explant experiment, the MUC7 expression was also not altered in the salivary glands of the animals irritated with the LPS by intratracheal instillation (data not shown). The fact that salivary glands are not sensitive to LPS suggests that these glands do not have LPS receptors.

Immunolocalization of MUC7 and Effect of LPS on MUC7 Glycoprotein Production in Transgenic Mice Airways

The immonohistochemistry shows the MUC7 glycoprotein in lung and trachea tissues of MUC7 transgenic mice (Figures 5I–5L).

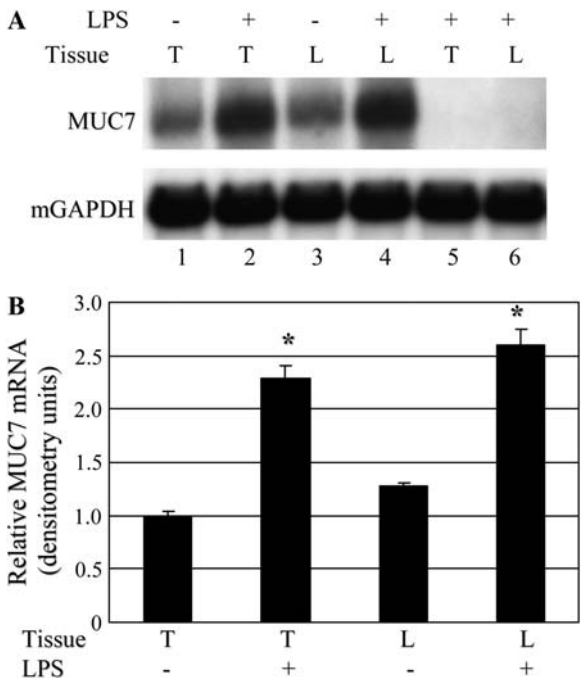

Figure 4. Effect of *P. aeruginosa* LPS on MUC7 transcription in mouse airways. (*A*) MUC7 mRNA was examined by Northern blot analyses. Six human MUC7 gene transgenic mice were divided into two groups (control and *P. aeruginosa* LPS–treated, as described in MATERIALS AND METHODS). Three nontransgenic mice were also treated with LPS. Tissues were collected from the animals 2 d later. The RNA samples were prepared from the tissues, and MUC7 mRNA levels were assessed. Mouse GAPDH gene served as an internal control. The RNA samples in *lanes 1* to *4* were from transgenic mice; the RNA samples in *lanes 5* and *6* were from nontransgenic mice. T, trachea; L lung. (*B*) Relative levels of MUC7 mRNA determined by densitometry. MUC7 mRNA was normalized to GAPDH mRNA bands and expressed as fold of untreated control samples. Results are representative of three independent experiments (mean $\pm$ SE). $^*P < 0.05$.

No MUC7 is detectable in BCF2 nontransgenic mice (Figures 5A–5D). Upon incubation of the transgenic lung and trachea tissues with LPS, an overexpression of MUC7 can be observed (Figures 5M–5P). No MUC7 is detectable upon incubation of nontransgenic tissues with LPS (Figures 5E–5H).

In transgenic trachea, MUC7 glycoprotein is expressed in the submucosa, within the serous glands (Figures 5I and 5J). Also, the overexpression of MUC7 glycoprotein upon LPS stimulation remains confined at the level of the serous glands (Figures 5M, 5N, 5Q, and 5R). In unstimulated and stimulated tissues, our immunolocalization analysis does not show expression of MUC7 within the respiratory epithelium containing goblet cells.

In transgenic lung, MUC7 glycoprotein seems to be expressed within the respiratory epithelium at the level of the bronchioles (Figures 5K and 5L). No MUC7 was detected in the alveolar epithelial cells. Upon stimulation with LPS, MUC7 glycoprotein seems to be overexpressed within the same cells of the respiratory epithelium and within the stromal tissue (Figures 5O, 5P, 5S, and 5T).

DISCUSSION

We examined the expression and regulation of the human MUC7 gene in normal human primary airway cells and in MUC7 transgenic mice. MUC7 gene expression was detectable only in ALI-cultured cells and was modulated by cytokines (IL-1β, IL-4, IL-13, and TNF-α), EGF, and bacterial LPS. Therefore, the MUC7 gene expression and regulation show similar patterns to other airway mucin genes, including MUC2 and MUC5AC. As previously discussed in the review article on control of mucin transcription by diverse injury-induced signaling pathways (32), it maybe speculated that each type of agent stimulates MUC7 transcription through distinct yet intersecting signaling pathways.

Although MUC7 mucin is mainly secreted into human saliva, it is also a part of the respiratory mucin pool (22, 23). It has been proposed that growing NHTBE cells at the ALI provides an appropriate model system for studying airway mucin gene expression because the mucin genes showed a pattern of differentiation-dependent expression (33). In this work, we observed well differentiated morphology of NHTBE cells cultured in ALI and found that MUC7 transcription was also induced under these culture conditions (Figure 1). Thus, like other airway mucins (MUC3, MUC4, MUC5AC, MUC5B, and MUC6) (34), MUC7 expression is differentiation dependent. The expression of mucin genes upon epithelial cell differentiation represents a regulatory mechanism under normal physiologic conditions.

The stimulatory effect of *P. aeruginosa* LPS on MUC7 expression (transcription and glycoprotein production) was also observed in animal airways. The stimulatory effect of LPS was further extended to the endogenous mouse muc10 transcription. Bacterial LPS has a potential to elicit an inflammatory response in epithelial cells. Indeed, bacterial infection of pulmonary airways is often accompanied by mucus overproduction, a phenomenon that could lead to airflow obstruction. Excess mucus in airways can result from mucous cell metaplasia and enhanced production of mucins. *P. aeruginosa* is a primary causative pathogen in respiratory infections of hospitalized, immunosuppressed patients with CF (13). It has been reported that mouse airway mucus hypersecretion was induced by the onset and persistence of *P. aeruginosa* infection (35–37). Several gel-forming mucins (MUC5B, MUC5AC, and MUC2) have shown aberrant expression during bacterial infection and contributed to mucus hypersecretion (13, 38). Bacterial LPS is able to induce a variety of primary inflammatory mediators, such as TNF-α, which can provoke production of secondary mediators (cytokines, reactive oxygen, and nitrogen) by epithelial cells. These secondary

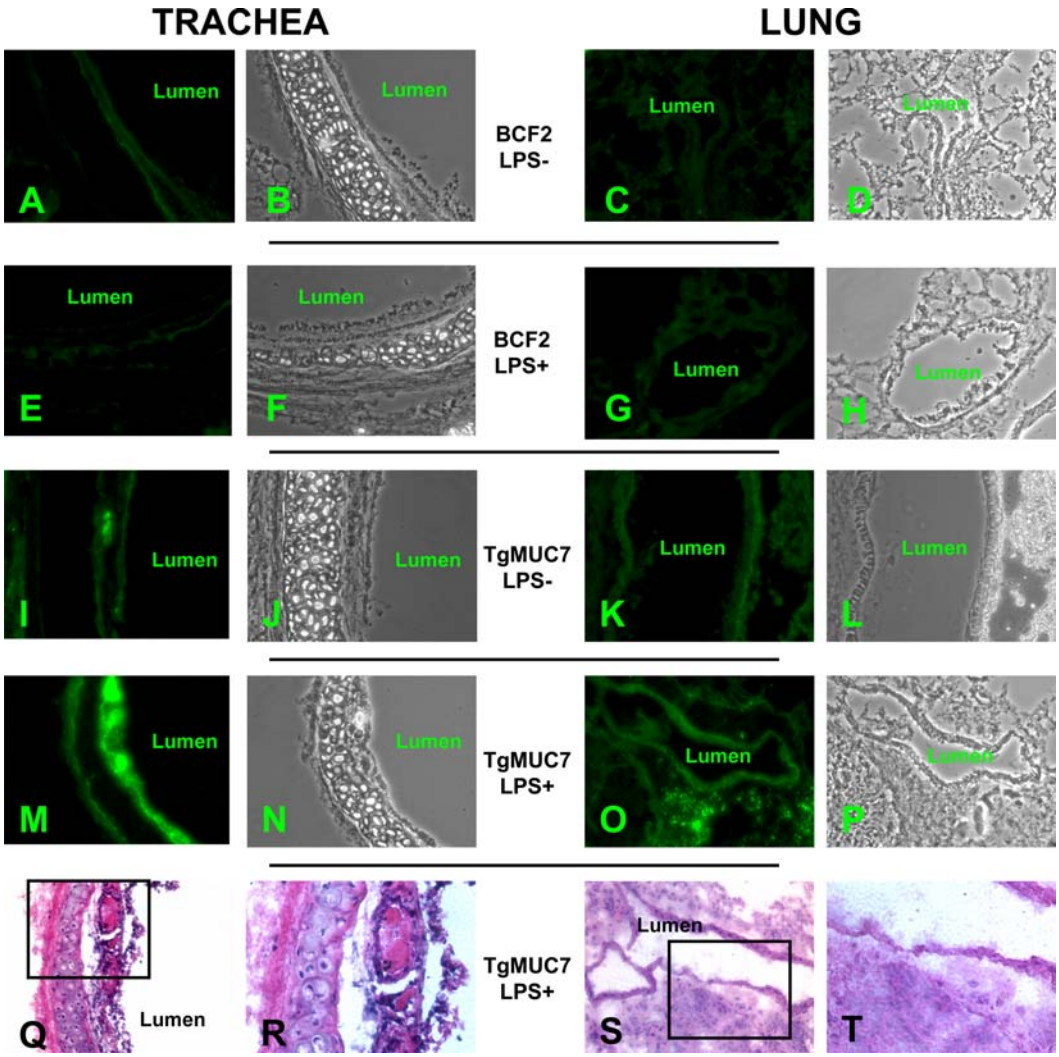

Figure 5. Immunolocalization of MUC7 protein expression in mouse tracheal and lung explants. Fluorescent and phase contrast pictures are shown for each sample. (*A–D*) Trachea and lung of nontransgenic BCF2 mouse. (*E–H*) Trachea and lung of nontransgenic BCF2 mouse incubated with LPS. (*I–L*) Trachea and lung of transgenic TgMUC7 mouse. (*M–P*) Trachea and lung of transgenic TgMUC7 mouse incubated with LPS. (*Q–R*) Hematoxylin-eosin staining of trachea of transgenic TgMUC7 mouse incubated with LPS. (*S–T*) Hematoxylin-eosin staining of lung of transgenic TgMUC7 mouse incubated with LPS. Original magnification in *panels A–P*, *Q*, and *S*: ×200. Original magnification in *panels R* and *T*: ×400.

mediators can further elicit pathophysiologic alterations, such as hypersecretion of mucus and increased inflammation (31). Therefore, they are potent actors in the pathogenesis of respiratory diseases.

Although the adherence of organisms to respiratory mucins maybe important in the pathogenicity of some chronic colonization states or diseases, it also may be a mechanism by which the normal host can remove bacteria from the respiratory tract and can protect the underlying cell surfaces from microbial attachment. For example, rat tracheobronchial mucin has been shown to bind to *P. aeruginosa* and effectively inhibit its ability to adhere to injured tracheal cells in *in vitro* adhesion models (39). Other *in vivo* studies have confirmed that mucus and purified mucin binding to the bacterium inhibited its adherence to injured host tissues (40).

In this study, we show for the first time that MUC7 expression in the primary airway cells and in the MUC7 transgenic mice airways can be upregulated by pathogenic agents. However, we cannot be certain whether the increased production of MUC7 has a protective or harmful (pathogenic) role. MUC7 mucin is a small and non–gel-forming mucin. Its main function is thought to be clearance of various bacteria and thus inhibition of bacterial colonization. It has been shown to interact *in vitro* with many oral and respiratory micro-organisms, including *P. aeruginosa* (3, 17). Thus, we can speculate that the overproduction of MUC7 mucin may reduce the viscosity of the mucus and facilitate mucociliary clearance, thereby having a protective role in the lungs.

Our immunohistochemical study determined which types of secretory cells in trachea and lungs of MUC7 transgenic mice produce MUC7 glycoprotein and respond to the LPS by overproduction of MUC7. We found that in transgenic mouse trachea, MUC7 glycoprotein is expressed in the serous cells of submucosal glands. This finding is consistent with a previous study investigating human mucin gene expression by *in situ* hybridization (23). In that study, MUC7 transcripts were localized to the serous cells of the submucosal glands of normal human trachea, and no hybridization was detected in mucous glands or in the surface epithelium. Our results are consistent also with the study by Sharma and colleagues (24), in which the expression of MUC7 and MUC5B was investigated in human bronchial airways by *in situ* hybridization and immunocytochemistry. By *in situ* hybridization, MUC7 was highly expressed in a subpopulation of serous tubules within submucosal glands and no MUC7 expression was found in the surface epithelium. Their immunocytochemical analysis showed the MUC7 expression also within a subpopulation of serous tubules of submucosal glands. In the present study, we found that the overexpression of MUC7 glycoprotein upon LPS stimulation remains confined at the level of the serous cells of submucosal glands. Our study further found that in transgenic mouse lung, MUC7 glycoprotein seems to be expressed

within the respiratory epithelium at the level of the bronchioles, and no MUC7 was detected in the alveolar epithelial cells. These results are different from the previous *in situ* hybridization study (23), where MUC7 transcripts were not found in bronchioles or in alveolar epithelial cells. We speculate that this difference may be explained by the fact that our study used MUC7 transgenic mouse lung tissue, whereas the hybridization study used human lung tissue. Moreover, the present study showed that upon stimulation with LPS, in addition to the respiratory epithelium at the level of the bronchioles, MUC7 glycoprotein seems to be overexpressed within the stromal tissue. Our findings may give some insights into the MUC7 overexpression by the inflammatory actors in human airways.

Our future studies will focus on examining the effects of other factors (IL-1β, IL-4, IL-13, TNF-α, and EGF) on MUC7 gene expression in MUC7 transgenic mice and on delineating the cellular signaling pathways.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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