Expression of Membrane-Bound Mucins in Human Nasal Mucosa

Different Patterns for MUC4 and MUC16

Hyun-Jae Woo, MD; Chang Hoon Bae, MD; Si-Youn Song, MD; Heung-Man Lee, MD; Yong-Dae Kim, MD, PhD

Objective: To acquire basic information concerning the function of the membrane-bound mucin MUC16 in nasal mucosa compared with the best-characterized membrane-bound mucin, MUC4.

Design: In vitro study using semiquantitative reverse transcription–polymerase chain reaction analysis and immunoassay.

Setting: Yeungnam University College of Medicine.

Subjects: We examined the nasal polyps obtained during endoscopic sinus surgery in 10 patients, the normal ethmoid sinus mucosa obtained from 10 patients, and human nasal polyp epithelial (HNPE) cells.

Main Outcome Measures: Gene expression of MUC4 and MUC16 in nasal polyps and normal nasal mucosa. In addition, we evaluated the effect of 4 physiologically relevant agents, including retinoic acid, interleukin 1β, phorbol 12-myristate 13-acetate (PMA), and dexamethasone, on the expression of MUC4 and MUC16 in HNPE cells at the gene and protein levels.

Results: In nasal polyps, MUC4 was upregulated compared with normal nasal mucosa ($P=.009$), whereas MUC16 expression did not differ between nasal polyps and normal nasal mucosa. Retinoic acid and interleukin 1β increased MUC4 expression at the gene and protein level in HNPE cells, whereas MUC16 expression was not affected. Unlike retinoic acid and interleukin 1β, PMA and dexamethasone increased MUC16 expression, whereas they had no significant effect on MUC4 expression.

Conclusions: Expression of MUC4 and MUC16 are regulated differently in nasal mucosa. Dexamethasone and PMA are potent mediators for the expression of MUC16 in nasal polyps.


MUCINS ARE HIGH-MOLECULAR-WEIGHT GLYCOPROTEINS WITH DIVERSE BIOLOGICAL FUNCTIONS. IN THE RESPIRATORY TRACT, MUC5AC AND MUC5B ARE THE MAIN SECRETED MUCINS THAT CONTRIBUTE TO THE MUCUS GEL; MUC1 AND MUC4 ARE THE PREDOMINANT MEMBRANE-BOUND MUCINS PRESENT ON EPITHELIAL CELL SURFACES. ALTHOUGH THE MEMBRANE-BOUND MUCINS ARE LOCATED PRIMARILY AT THE CELL SURFACE, THEIR RESPECTIVE GENES MAY ENCODE SPlicing VARIANTS FOR SECRETED PROTEINS, AND THEIR HEAVILY Glicosylated EXTRACELLULAR PORTION MAY BE RELEASED INTO THE MUCUS GEL BY PROTEOLYSIS. THE SECRETED MUCINS EXHIBIT A RESTRICTED PATTERN OF EXPRESSION AND CONSTITUTE THE KEY ELEMENTS OF THE MUCUS GEL, WHEREAS THE MEMBRANE-BOUND MUCINS DEMONSTRATE A WIDE EXPRESSION PATTERN AND ARE CONSIDERED TO HAVE DIVERSE ROLES BEYOND BEING CONSTITUENTS OF MUCUS GEL.

The best-characterized membrane-bound mucin is MUC4, which is engaged in interaction with ErbB2, signal transduction, cancer cell proliferation, and cell survival.

The CA125 antigen, recently known as MUC16, has been cloned from an ovarian cancer cell line. As a serum marker, it is used principally to monitor patients with ovarian cancer. Owing to its use as an ovarian cancer marker, much of the work performed to date on CA125 has focused on its potential involvement in the development and progression of cancer. However, CA125 has also been detected in cervical mucus and corneal epithelial cells, suggesting that it may be a component of normal mucosal secretions at some site. In 2007, Davies et al first reported that MUC16 was produced in human tracheal surface epithelium and bronchial epithelial cells. Until now, however, the function of MUC16 in the nasal mucosa has not been studied, to our knowledge.
The expressions of mucin genes in airway epithelial cells are regulated by inflammatory mediators and various factors, such as retinoic acid (RA), interleukin 1β (IL-1β), phorbol 12-myristate 13-acetate (PMA), and dexamethasone. Retinoic acid, a metabolite of vitamin A, has an essential role in the development and maintenance of mucociliary differentiation of the airway epithelium.7 Interleukin 1β is one of the most important multifunctional proinflammatory cytokines and upregulates expression of the major secretory mucins in human airway epithelium.3 By acting as an alternative stimulus to diacylglycerol, PMA can induce protein kinase C (PKC) activation. Hence, it is widely used as a PKC activator. Protein kinase C is involved in a number of exocytotic events in different cell types, including secretion of mucin, insulin, neurotransmitters, and platelet-dense granules.8,9 Dexamethasone has potent anti-inflammatory effects and is known to be the most effective drug for airway mucus hypersecretion.2,10

The purpose of this study was to gather basic information concerning the function of MUC16 by comparing it with MUC4 in the nasal mucosa. We investigated the expression pattern of MUC4 and MUC16 in the normal nasal mucosa and in the nasal polyps. In addition, we evaluated the effect of 4 physiologically relevant agents (RA, IL-1β, PMA, and dexamethasone) on the expression of MUC4 and MUC16 in human nasal polyp epithelial (HNPE) cells.

**METHODS**

**TISSUE COLLECTION AND PREPARATION**

The nasal polyps were obtained during endoscopic sinus surgery in 10 patients with chronic rhinosinusitis (5 men and 5 women; mean age, 35.2 years). Normal ethmoid sinus mucosa was obtained from 10 patients undergoing endoscopic resection for orbital wall fractures (5 men and 5 women; mean age, 38.0 years). The patients and control subjects had no personal or family history of allergy and had negative results on skin-prick tests to 20 common airborne allergens and on multiple simultaneous allergen tests. All subjects discontinued oral and topical corticosteroid and antibiotic therapy for at least 4 weeks before surgery. The study protocol was approved by the institutional review board for human studies at the Yeungnam University College of Medicine.

**CELL CULTURE AND TREATMENT**

For the primary culture of HNPE cells, the nasal polyp tissue was washed with phosphate-buffered saline (PBS) solution and immersed in dispase (Boehringer Mannheim Biochemica, Mannheim, Germany) for 90 minutes. After the tissue was scraped off of the surface of the nasal polyp with a scalpel, it was added to 1% PBS solution and filtered through a mesh. The suspension was centrifuged at 3000 rpm for 5 minutes before the PBS was removed. The cells were seeded in a 24-well plate at 2.5 × 10⁴ cells/well and were then incubated with EpiLife medium (Cascade Biologics, Portland, Oregon) and human keratinocyte growth supplement (Cascade Biosciences, 5 mL/500 mL of medium). When the cultures were confluent, the cells were exposed to the indicated concentrations of all-trans RA, PMA, dexamethasone (Sigma Aldrich Corp, St Louis, Missouri), or IL-1β (R & D Systems, Minneapolis, Minnesota) for different periods.

**REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION ANALYSIS**

The total RNA from tissues and cells was extracted using a commercially available reagent (TRIZol; Gibco BRL, Gaithersburg, Maryland). Fifteen micrograms of the RNA underwent reverse transcription (RT) into complementary DNA (cDNA) at 37°C for 70 minutes in 60 µL of a volume reaction mixture that contained 150 U of reverse transcriptase (Superscript II; Invitrogen Life Science Technologies, Emeryville, California), 10 µL each of 10mM deoxyribonucleotide triphosphate, and 5 µL of 50µM oligo-dT primer (Amersham International plc, Little Chalfont, England). The reactions were stopped by heat inactivation at 85°C for 10 minutes. Two microliters of each cDNA sample from the RT were amplified by means of polymerase chain reaction (PCR) (PTC-200; MJ Research Inc, Watertown, Massachusetts) in a volume of 50 µL containing 0.5 U of Taq DNA polymerase, 2 µL of 50mM magnesium chloride, 1 µL of 10mM deoxyribonucleotide triphosphate, and 1 µL each of the 10µM primers. We performed PCR analysis for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on each individual sample as a positive internal control. Primers specific for MUC4 (sense: TTC TAA GAA CCA CCA GAC TCA GAC G; antisense: GAG ACA CAC CTT GAG AGA ATG AGC) and MUC16 (sense: GCC TCT ACT TTA ACC GTT ACA ATG AA; antisense: GGT ACC CCA TGG CTG TTT TG) were designed to produce 467- and 114-base pair PCR fragments, respectively. The amplification protocol consisted of 23 cycles (for MUC4) or 30 cycles (for MUC16) of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Semi-quantitative analysis of the RT-PCR product was performed on the scanned gel images, and the intensity of the PCR product was measured using commercially available software (Scion Image; Scion Corporation, Frederick, Maryland).

**IMMUNOASSAY FOR INTRACELLULAR PRODUCTION AND EXTRACELLULAR RELEASE OF MUCINS**

Levels of MUC4 and MUC16 proteins were determined using an enzyme-linked immunosorbent assay. Samples of superna-
or cell lysates from HNPE cells were prepared in PBS solution at several dilutions, and each sample was incubated at 40°C in a 96-well plate until dry. Plates were then washed 3 times with PBS solution, blocked with 2% bovine serum albumin for 1 hour at room temperature, washed again 3 times with PBS solution, and incubated with MUC4 or MUC16 antibodies (Santa Cruz Biotechnology, Santa Cruz, California) diluted with PBS solution containing 0.05% polysorbate 20 (Tween 20; Guangzhou Hanglian Chemical Industry Co, Ltd, Guangzhou City, China) for 1 hour. The wells were then washed 3 times with PBS solution and treated with horseradish peroxidase–conjugated antimouse secondary antibody (Santa Cruz Biotechnology). After 4 hours, the plates were washed 3 times with PBS solution. Color was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution and stopped with 1M sulfuric acid. Absorbance was read at 450 nm.

**STATISTICAL ANALYSIS**

Statistical analysis was performed with commercially available software (SPSS, version 10.0; SPSS Inc, Chicago, Illinois). The mean was calculated for each of the obtained quantitative values. Statistical analyses were performed with the Mann-Whitney test (for the tissue studies) or the unpaired t test (for the cell culture studies). For all tests, \( P < .05 \) was considered statistically significant.

**RESULTS**

**EXPRESSION PATTERNS OF MUC4 AND MUC16**

On the RT-PCR study, MUC4 messenger RNA (mRNA) was scantly expressed in the normal nasal mucosa, whereas it was strongly expressed in the nasal polyps. Messenger RNA of MUC16 was strongly expressed in the normal nasal mucosa; however, there was no difference in MUC16 expression between nasal polyps and normal nasal mucosa (Figure 1). All samples were normalized with respect to GAPDH. Semiquantitative analysis of the PCR samples showed a statistically significant difference only in the MUC4:GAPDH mRNA ratios between the nasal polyps and the normal nasal mucosa (\( P = .009 \)), but not in MUC16:GAPDH mRNA ratios.
MODULATION OF MUC4 AND MUC16 EXPRESSION

To determine whether RA, IL-1β, PMA, and dexamethasone may be involved in the modulation of MUC4 and MUC16 expression in nasal polyps, we treated the HNPE cells with each agent for different periods of time or with different concentrations for 24 hours.

As shown in Figure 2 and Figure 3, RA and IL-1β increased the MUC4 mRNA level in a time- and dose-dependent manner, but they did not affect MUC16 mRNA expression. Consistent with the increased gene expression data, the immunoassay also showed a dose-responsive increase of protein level in only MUC4 in both the cell lysate and the supernatant.

Unlike RA or IL-1β, treatment with PMA increased MUC16 mRNA levels in a time- and dose-dependent manner but had no significant effect on MUC4 mRNA expression. Induction of MUC16 by PMA was confirmed at the protein level by immunoassaying cell lysates and supernatants using specific antibodies (Figure 4).

The addition of dexamethasone caused a time- and dose-dependent increase in the MUC16 mRNA and protein levels in the lysate and supernatant; the steady-state MUC4 mRNA level was slightly reduced by treatment with dexamethasone in a dose-dependent manner. We also investigated the involvement of glucocorticoid receptor–dependent signaling on the elevated MUC16 level induced by dexamethasone. Mifepristone (RU-486), which functions as a glucocorticoid receptor antagonist, suppressed the effect of dexamethasone on MUC16 expression at the gene and protein levels (Figure 5).

COMMENT

This study showed that MUC4 and MUC16, large membrane-bound mucins, were expressed differently in nasal mucosa. Compared with normal nasal mucosa, MUC4 was upregulated in nasal polyps, whereas MUC16 expression...
did not differ between nasal polyps and normal controls. Mucosal inflammation is probably the most important factor in the development of nasal polyps; many inflammatory factors, such as cytokines, chemokines, and growth factors, play an important role in the persistence of mucosal inflammation associated with nasal polyps. Therefore, MUC4 upregulation in nasal polyps, like major gel-forming mucins, might result from activation and secretion of these inflammatory factors. Furthermore, because stromal and epithelial cell proliferation is a typical histological characteristic of nasal polyps, and because MUC4 is implicated in intracellular signaling pathways related to cell proliferation, MUC4 might be involved in the proliferative process in nasal polyp formation. However, the lack of difference in MUC16 expression between nasal polyps and normal nasal mucosa in the present study suggests that MUC16 is not related to nasal mucosal inflammatory disease or the proliferative process in nasal polyp formation. Therefore, we suggest the possibility that MUC16 is involved in physiologic mucin production, not inflammation-related hypersecretion.

We found that RA upregulated MUC4 but not MUC16 expression in HNPE cells. When normal human tracheobronchial epithelial cells are cultured in medium deficient in RA, they undergo squamous differentiation. The addition of RA restores mucous differentiation and induces mucin gene expression. A study of cultured human nasal and bronchial epithelial cells demonstrated that RA is necessary for mucociliary differentiation and expression of MUC4, MUC5AC, and MUC5B. The MUC4 findings in the present study are in agreement with the established role of RA in these cells; these findings suggest that MUC16 is not involved in the RA-dependent mucociliary differentiation of human nasal epithelial cells.

Several studies have indicated that IL-1β upregulates the expression of major gel-forming mucin genes in airway epithelial cells. Recently, Bai et al. reported that IL-1β and lipopolysaccharide increased MUC4 expression at the gene

Figure 4. Effect of phorbol 12-myristate 13-acetate (PMA) on the expression of membrane-bound mucins MUC4 and MUC16 in human nasal polyp epithelial cells. A, Time- and dose-dependent expression of MUC4 and MUC16 messenger RNA (mRNA) were analyzed by means of reverse transcription–polymerase chain reaction. B, Dose-dependent production and secretion of MUC4 and MUC16 protein were measured by means of enzyme-linked immunosorbent assay. Representative blots are shown. Bars represent the averages (SDs) of 3 independent experiments. *P<.05 compared with untreated control (C). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; T, treatment with retinoic acid.
and protein levels in HNPE cells. To date, however, it has not been determined whether IL-1β affects MUC16 expression in human respiratory epithelium. Our present study showing constant expression of MUC16 even after IL-1β treatment suggests that MUC16, unlike MUC4, is not involved in IL-1β–induced mucus hypersecretion in HNPE cells.
The role of PMA in the induction of mucin expression has been demonstrated in MUC2, MUC5AC, and MUC5B, the 3 major gel-forming mucins, using the following various human airway epithelial cells: NCI-H292 cells, primary human bronchial cells, HBE1 cells, and A549 cells. In contrast to gel-forming mucins, there is very little information regarding the effect of PMA on membrane-bound mucins. In the present study, PMA induced an increase in MUC16 expression at the gene and protein levels, whereas MUC4 expression was not increased. These findings indicate that PMA, widely used as a PKC activator, is a potent mediator of MUC16 upregulation of major gel-forming mucins has been well demonstrated in airway epithelial cells, little is known regarding its effect on the membrane-bound mucins of the respiratory tract. The regulation of membrane-bound mucin expression by glucocorticoids has been previously characterized in various cell types, but the patterns of regulation have varied. Dexamethasone upregulates MUC1 gene expression in human conjunctival epithelial cells, but it downregulates it in human gastric mucosal cells. Similar to our results, Seo et al reported that dexamethasone has an inhibitory effect on MUC4 expression and a stimulatory effect on MUC1 and MUC16 expression in human corneal epithelial cells. Moreover, our study showed that these stimulatory effects of dexamethasone were suppressed by pretreating HNPE cells with the glucocorticoid receptor antagonist mifepristone. This result suggests that the glucocorticoid receptor could be involved with dexamethasone-induced MUC16 production. Dexamethasone has been shown to regulate gene expression in humans at several different levels through initial interactions with specific receptors. Several mechanisms need to be proposed for the glucocorticoid-mediated upregulation of MUC16 gene expression in human airway epithelial cells.

The tissue-specific patterns of MUC4 and MUC16 expression and variations in response to induced expression may be explained by those structural differences between MUC4 and MUC16 mucin. In addition, it is possible that MUC4 and MUC16 have distinct functions based on their expression sites. The MUC4 mucins are not synthesized by the goblet cells or the mucous cells in submucosal glands but by the ciliated cells; in contrast, MUC16 is located in the cytoplasm of the goblet cells and the submucosal gland cells.

Most studies of respiratory disease to date have focused on the secreted mucins, but it is increasingly apparent that the membrane-bound mucins contribute to airway disease through anchoring obstructive mucus, modulating adhesion, influencing the manner and extent of inflammation, and regulating cellular signaling. In conclusion, the function of membrane-bound mucins in the regulation of the airway epithelium may be quite complex and decidedly requires further study. Our findings could be basic information to determine the exact roles of membrane-bound mucin in respiratory disease.

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Correspondence: Yong-Dae Kim, MD, PhD, Department of Otorhinolaryngology–Head and Neck Surgery, Yeungnam University College of Medicine, 317-1, Dae-myung 5-Dong, Nam-Gu, Daegu 705-717, Korea (ydkim@med.yu.ac.kr).

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