

# Release of Membrane-Associated Mucins from Ocular Surface Epithelia

Timothy D. Blalock, Sandra J. Spurr-Michaud, Ann S. Tisdale, and Ilene K. Gipson

**PURPOSE.** Three membrane-associated mucins (MAMs)—MUC1, MUC4, and MUC16—are expressed at the ocular surface epithelium. Soluble forms of MAMs are detected in human tears, but the mechanisms of their release from the apical cells are unknown. The purpose of this study was to identify physiologic agents that induce ocular surface MAM release.

**METHODS.** An immortalized human corneal-limbal epithelial cell line (HCLE) expressing the same MAMs as native tissue was used. An antibody specific to the MUC16 cytoplasmic tail was developed to confirm that only the extracellular domain is released into the tear fluid or culture media. Effects of agents that have been shown to be present in tears or are implicated in the release or shedding of MAMs in other epithelia (neutrophil elastase, tumor necrosis factor [TNF], TNF- $\alpha$ -converting enzyme, and matrix metalloproteinase-7 and -9) were assessed on HCLE cells. HCLE cell surface proteins were biotinylated to measure the efficiency of induced MAM release and surface restoration. Effects of induced release on surface barrier function were measured by rose bengal dye penetrance.

**RESULTS.** MUC16 in tears and in HCLE-conditioned medium lacked the cytoplasmic tail. TNF induced the release of MUC1, MUC4, and MUC16 from the HCLE surface. Matrix metalloproteinase-7 and neutrophil elastase induced the release of MUC16 but not of MUC1 or MUC4. Neutrophil elastase removed 68% of MUC16, 78% of which was restored to the HCLE cell surface 24 hours after release. Neutrophil elastase-treated HCLE cells showed significantly reduced rose bengal dye exclusion.

**CONCLUSIONS.** Results suggest that the extracellular domains of MUC1, MUC4, and MUC16 can be released from the ocular surface by agents in tears. Neutrophil elastase and TNF, present in higher amounts in the tears of patients with dry eye, may cause MAM release, allowing rose bengal staining. (*Invest Ophthalmol Vis Sci.* 2008;49:1864–1871) DOI:10.1167/iov.07-1081

Mucins are present on the apical surfaces of all wet-surfaced epithelia in either secreted or membrane-associated forms.<sup>1</sup> They are a class of high-molecular weight glycoproteins that contain tandem repeats of amino acids rich in serine and threonine, which serve as sites for O-glycosylation. Secreted mucins have no transmembrane-spanning domains and are produced by goblet cells and specialized epithelial glands.<sup>2</sup> Membrane-associated mucins (MAMs) have a single transmembrane domain, a short cytoplasmic tail, and a large, heavily glycosylated extracellular domain and are found in the glyco-

calyx of apical membranes of wet-surfaced epithelia.<sup>1</sup> They may extend as much as 500 nm from the apical epithelial surface.<sup>3,4</sup>

To date, 10 MAMs have been identified (MUCs 1, 3A, 3B, 4, 12, 13, 15, 16, 17, and 20).<sup>1,5</sup> Most of the wet-surfaced epithelia express several MAMs, but each may have different functions because of differences in cytoplasmic tail sequence, intracellular signaling capability, or presence of binding domains. For example, MUC1, MUC4, and MUC16 are expressed at the ocular surface.<sup>6–9</sup> All three of these MAMs are hypothesized to protect, hydrate, and lubricate the ocular surface through their heavily glycosylated extracellular domains; however, each of the ocular surface MAMs may have additional and unique functions. For example, the MUC1 cytoplasmic tail is capable of signaling and interacting with intracellular molecules such as  $\beta$ -catenin.<sup>10</sup> MUC4 signals through its epidermal growth factor-like domains in its extracellular domain,<sup>11</sup> and the cytoplasmic tail of MUC16 associates with the actin cytoskeleton and has potential sites of phosphorylation.<sup>12</sup>

Soluble forms of MUC1, MUC4, and MUC16 (previously known as CA125 antigen) are constitutively released from the apical surfaces of epithelial cells into luminal fluids *in vivo*, but little is known about the mechanisms of shedding.<sup>13,14</sup> At least three possible mechanisms for release are possible. First, constitutive steady release (shedding) may be brought about by an endogenous protease present normally in either the cell membrane or the extracellular fluids.<sup>1</sup> Second, splice variants of the mucins lacking the transmembrane and cytoplasmic domain may be released from the apical surfaces.<sup>15</sup> Although splice variants have been reported for MUC1 and MUC4, such variants have not been reported for MUC16. Third, proteases or other inflammatory agents in body fluids as a result of disease may induce aberrant “release” of MAMs from the surfaces of affected epithelia. It is the latter aberrant release of MAMs that is the subject of research reported herein.

Soluble forms of MUC1, MUC4, and MUC16 have been detected in samples of normal human tear fluid, indicating their shedding from the ocular surface epithelium.<sup>16</sup> The mechanism and site of proteolytic cleavage in the extracellular domain of MUC1 has been studied in uterine epithelium *in vitro*, where its constitutive shedding is induced by agents such as tumor necrosis factor- $\alpha$  (TNF), TNF- $\alpha$ -converting enzyme (TACE), phorbol-12-myristate-13-acetate (PMA), and membrane-type matrix metalloproteinase 1 (MT1-MMP).<sup>17,18</sup> Agents that have been suggested to induce aberrant MAM release include neutrophil elastase<sup>19</sup> and *N*-acetylcysteine.<sup>20</sup> Release of the extracellular domains of MAMs appears to be independent of the intracellular cleavage and reassociation that occurs in MAMs after protein synthesis in the endoplasmic reticulum during processing and assembly of the full-size protein.<sup>21,22</sup> There is a lack of information on the specific mechanisms of constitutive extracellular domain shedding, or induced release of MAMs, at the ocular surface.

Modification of MAM structure and function is hypothesized to be a contributing factor in dry eye.<sup>2</sup> *In vitro* alteration of MUC16 glycosylation<sup>23</sup> or expression knockdown<sup>12</sup> results in a loss of protection to corneal epithelial cells from rose bengal penetrance, suggesting that, in dry eye, rose bengal staining could be a result of either loss of expression or altered glyco-

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sylation of MUC16. MUC16 localization is altered on the conjunctival epithelium of patients with non-Sjögren dry eye syndrome, as shown by binding of the H185 antibody that recognizes a carbohydrate epitope on MUC16.<sup>8,24</sup> It is unknown whether the alteration in H185 binding is caused by the decreased expression of MUC16, the altered glycosylation of the mucin, or the increased rate of release of its extracellular domain (which carries the H185 epitope) into the tear film. Because MAMs are proposed to function in lubrication, hydration, and protection of the ocular surface, understanding the mechanism of their ectodomain release may help to better clarify the etiology of dry eye syndrome.

The purpose of this study was to determine physiologically relevant agents that induce the release of MAMs on the ocular surface and to determine the possible effects of induced release. As a model for the ocular surface, we used an immortalized human corneal-limbal epithelial cell line (HCLE) optimized to express high levels of the ocular surface MAMs MUC1, MUC4, and MUC16.<sup>25</sup> We identified agents, present in the tear film of patients with dry or inflamed eyes, that induced the release of the extracellular domains of MUC1, MUC4, and MUC16 from HCLE cells, suggesting areas of further study in the pathogenesis and treatment of dry eye.

## METHODS

### Production and Characterization of MUC16 Cytoplasmic Tail Antibody

A peptide corresponding to the entire cytoplasmic tail sequence of MUC16<sup>13</sup> was synthesized by solid-phase methods, as previously described,<sup>12</sup> in the Peptide Synthesis Core at Massachusetts General Hospital. Rabbits were given initial intraperitoneal injection of 500  $\mu$ g of the synthetic peptide to the MUC16 cytoplasmic tail conjugated to keyhole limpet hemocyanin (Rockland Immunochemicals, Gilbertsville, PA) in complete Freund adjuvant.<sup>26</sup> Rabbits were further immunized by weekly intraperitoneal booster injections of 250  $\mu$ g purified MUC16 cytoplasmic tail peptide in incomplete Freund adjuvant. The antibody was purified by affinity chromatography using 10 mg MUC16 cytoplasmic tail peptide coupled to agarose beads. The specificity of the antibody was tested by immunoblot of OVCAR-3 cell lysate, the ovarian carcinoma cell line from which MUC16 was cloned and that expresses high levels of MUC16. Specificity of the antibody was confirmed by loss of binding in immunoblots after preadsorption of the antibody with excess MUC16 cytoplasmic tail peptide. The antibody is designated MUC16CT.

### Tear Collection

This study was conducted in compliance with good clinical practice, institutional review board regulations, informed-consent regulations, and the tenets of the Declaration of Helsinki. Tear washes were collected from normal subjects as previously described.<sup>27</sup> Sixty microliters of sterile saline was instilled onto the unanesthetized ocular surface, and subjects were asked to move their eyes, without blinking, to mix the tear fluid content. Washes were then collected from the inferior fornix of each eye by micropipette. Cellular debris was removed by centrifugation at 14,000 rpm for 30 minutes at 4°C. Collected tear washes were stored at -80°C until used in immunoblot assays. Tears from four subjects, two women and two men, were collected for the individual assays to determine whether the MUC16 in tears contained the cytoplasmic tail. To verify findings from individuals, tear washes from 21 subjects were pooled; both sexes were represented in the pooled sample.

### Cell Culture

Immortalized HCLE cells were maintained at 37°C at 5% CO<sub>2</sub> and grown, as previously reported, to optimize mucin expression.<sup>25</sup> Briefly, HCLE cultures were grown in keratinocyte serum-free medium

(K-SFM; Invitrogen, Carlsbad, CA) to confluence. After reaching confluence, cells were switched to Dulbecco modified Eagle medium (DMEM)/Ham F-12 (Cellgro, Herndon, VA) supplemented with 10% calf serum and 10 ng/mL epidermal growth factor for 7 days, which promotes stratification, differentiation, and mucin gene expression.<sup>25</sup>

### Assays for Release of Mucins in HCLE Cells

Stratified HCLE cells were cultured as described. Culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS) followed by incubation at 37°C with serum-free DMEM/Ham F-12 containing agents to induce mucin release. Incubation times and concentrations of test agents were based on previously published reports of conditions effective in inducing MAM release. HCLE cells were incubated with DMEM/Ham F-12 containing 5  $\mu$ g/mL neutrophil elastase (Biomol, Plymouth Meeting, PA) for 30 minutes (reconstituted in 50 mM sodium acetate and 150 mM NaCl and diluted in DMEM/Ham F-12),<sup>19</sup> 10% *N*-acetylcysteine for 10 minutes,<sup>20</sup> MMP-7 (340 nM)<sup>28</sup> or MMP-9 (2  $\mu$ g/mL) for 6 hours,<sup>29</sup> PMA (1  $\mu$ m)<sup>18</sup> and TACE (0.1  $\mu$ g/mL) for 1 hour,<sup>18</sup> or TNF (10 ng/mL) for 24 hours.<sup>17</sup> After incubation, culture medium was removed and concentrated in filter cartridges (Nanosep 100 K; Pall Life Sciences; East Hills, NY) to remove proteins of smaller molecular weight.

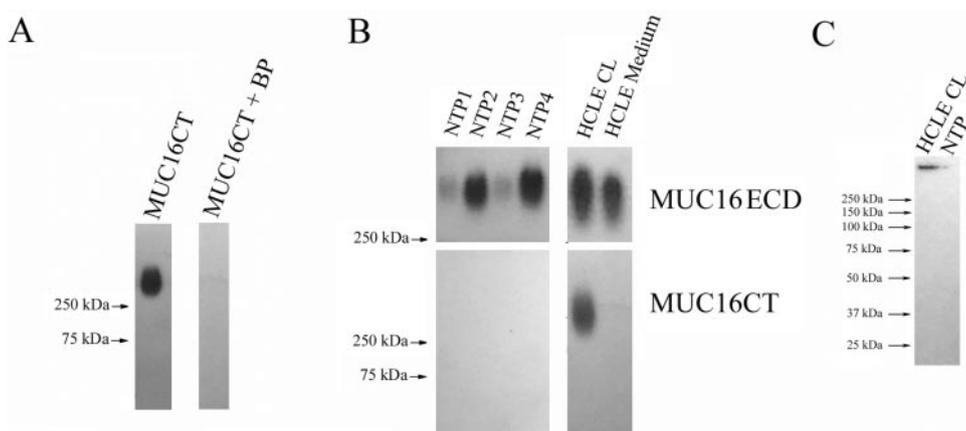
Protein was extracted from HCLE cells with 2% sodium dodecyl sulfate (SDS) plus protease inhibitor cocktail (Pierce, Rockford, IL), denatured in Laemmli sample buffer, and separated on 1% agarose gels or 12% SDS-PAGE gels, followed by transfer to nitrocellulose with vacuum blotting (25  $\mu$ g/lane) or electroblotting of SDS-PAGE gels.<sup>30</sup> Immunoblots were performed as previously described<sup>16</sup> using antibodies specific to the tandem repeats of MUC1 (214D4; Upstate, Lake Placid, NY), MUC4 (8G7; a gift of S. Batra<sup>31</sup>), MUC16 (OC125; Dako, Carpinteria, CA), and the MUC16 CT. Quantification of protein expression was determined by densitometry of immunoblots, as previously described.<sup>16</sup> Results were quantified by densitometry of immunoblots by comparing the total amount of mucin in concentrated culture media samples to 25  $\mu$ g cell lysate after the removal of media.

### Biotinylation of Surface Proteins

Stratified HCLE cultures were treated with neutrophil elastase for 30 minutes. The treated cells were then processed for cell surface biotinylation by incubation with 250  $\mu$ g/mL sulfo-NHS-SS-biotin (Pinpoint-Cell Surface Protein Isolation Kit; Pierce) according to the manufacturer's recommendations. Immobilized gel (NeutrAvidin; Pierce) was used to bind biotinylated proteins on the cell surface from cell lysates. Biotinylated proteins were eluted from the gel with SDS-PAGE sample buffer containing 50 mM dithiothreitol. Protein extract from cultures in which the labeling reagent was omitted was used as control for nonspecific binding to the avidin gel. The amount of MUC16 remaining on the cell surface after protease treatment was expressed as the percentage of the total of biotinylated surface and released MUC16. Statistical analyses were performed with the Fisher protected least-significant difference (PLSD) test or the Mann-Whitney *U* test (StatView 5.0 for Macintosh; SAS Institute, Inc., Cary, NC). *P* < 0.05 was considered significant.

### Real-Time Polymerase Chain Reaction

Total RNA was extracted from HCLE cell cultures using reagent (Trizol; Invitrogen) according to the manufacturer's instructions. cDNA was generated from 1  $\mu$ g DNase-treated RNA, as described previously.<sup>25</sup> Real-time quantitative PCR for MUC1, MUC4, and MUC16 was performed as previously described using a sequence detection system (ABI Prism 7900HT; Applied Biosystems, Foster City, CA) using gene expression chemistry (TaqMan; Invitrogen)<sup>30</sup> with calculations based on  $\Delta$ Ct.<sup>32</sup> Primers and probes were purchased from Applied Biosystems. The amount of cDNA added to each reaction was standardized by normalizing the amount of target gene in each sample to endogenous control (GAPDH). Relative levels of mucin mRNA were then calculated by the  $\Delta\Delta$ Ct method using the nontreated control as the calibrator.



**FIGURE 1.** MUC16 in human tear fluid and HCLE cells. (A) To confirm the specificity of MUC16CT, immunoblots were performed on 25  $\mu$ g HCLE cell lysate with or without the presence of blocking peptide (1 mg/mL MUC16CT antibody diluted from original stock solution 1:2500 in PBS plus 5% reagent [Blotto; BP]). (B) Immunoblots for the MUC16 extracellular domain (MUC16ECD) using the OC125 antibody and the MUC16 cytoplasmic tail (MUC16CT) were performed on normal human tear fluid samples from four subjects (NTP1–4, two men, two women; although there was variation in the amounts of MUC16 in tears, the differences did not correlate to sex), medium from stratified HCLE cultures

(HCLE medium), and 25  $\mu$ g HCLE cell lysate (HCLE CL). (C) To confirm the lack of MUC16CT in tears, excess tear protein (100  $\mu$ g/well) from pooled normal tears (NTP) was separated on a 12% SDS-PAGE gel, transferred to nitrocellulose, and probed with the MUC16CT antibody to confirm the absence of low-molecular weight fragments containing the MUC16 cytoplasmic tail in tear film. Immunoblots using MUC16CT revealed that it binds only to the HCLE cell lysate—not to culture media or tear samples (Fig. 1B)—indicating that the soluble form of MUC16 in HCLE culture medium and in tear samples is the extracellular domain of MUC16 and does not contain the cytoplasmic tail. The absence of lower molecular weight species containing the MUC16 cytoplasmic tail in normal tear film was confirmed in immunoblots using excess tear proteins (100  $\mu$ g) that had been separated by SDS-PAGE (Fig. 1C).

Statistical comparisons of the real-time PCR results were performed using the Fisher PLSD test.  $P < 0.01$  was considered significant.

### Dye-Penetrance Assay

HCLE cells were cultured to stratification and differentiation in 24-well culture plates, as described. Cells were treated for 30 minutes at 37°C with neutrophil elastase (5  $\mu$ g/mL) or vehicle control using nontreated cells as negative controls. Culture medium was aspirated, and cells were washed three times with phosphate-buffered saline (PBS), followed by a 5-minute incubation of 0.1% rose bengal dye in calcium and magnesium-free PBS. The dye solution was removed, and cultures were photographed as previously described.<sup>25</sup> The area of islands of stratified cells that excluded rose bengal was quantified in culture images using ImageJ analysis software (National Institutes of Health, Bethesda, MD).<sup>53</sup>  $P < 0.01$  was considered significant (Fisher PLSD;  $n = 5$ ).

### Hyperosmolarity Assay

Stratified HCLE cells were cultured as described in 24-well culture plates. After stratification, cells were incubated in hyperosmolar serum-free DMEM/Ham F-12 (310 mOsm) for 24 hours, as described previously.<sup>34</sup> DMEM/Ham F-12 containing increasing concentrations of sodium chloride (0–90 mM) was used to adjust osmolarity of the culture medium. The amount of released mucin in culture medium and the remaining cellular mucin was quantified from immunoblots, as described. Cells incubated with neutrophil elastase for 30 minutes were used as a positive-release control.  $P < 0.01$  was considered significant (Fisher PLSD;  $n = 3$ ).

## RESULTS

### Membrane-Associated Mucins in the Tear Film and HCLE Culture Media Lack the Cytoplasmic Tail

Soluble forms of the ocular surface MAMs (MUC1, MUC4, and MUC16) have been detected in samples of normal human tear fluid.<sup>16</sup> The shedding of the extracellular domains of MUC1 and MUC4 has been well documented in cultured epithelial cells.<sup>14,17,18</sup> The extracellular domains of MUC1 and MUC4 do not contain the cytoplasmic tail because it remains associated with the cell membrane.<sup>14,17</sup> However, it is unknown whether the released form of MUC16 is comprised of the extracellular domain alone or if it also contains the cytoplasmic tail. To determine the characteristics of the released form of MUC16

from the ocular surface epithelium, immunoblots were performed on tear proteins using an antibody to the extracellular domain (OC125) and an antibody to the cytoplasmic tail of MUC16 (MUC16CT) developed for this study. Specificity of the MUC16CT antibody was demonstrated by preadsorption with the cytoplasmic tail peptide. Immunoblots of HCLE cell lysate using the MUC16CT antibody with and without preincubation with excess cytoplasmic tail peptide (blocking peptide) confirmed the specificity of the antibody (Fig. 1A). In addition to assay of tears with the OC125 and MUC16CT antibodies, HCLE cells were used to assess MUC16 release because they expressed high levels of MUC16.<sup>25</sup> HCLE cells constitutively release soluble extracellular MUC16 into the culture medium, similar to the release of soluble MUC16 into tear fluid from the native ocular surface (Figs. 1B, 1C).

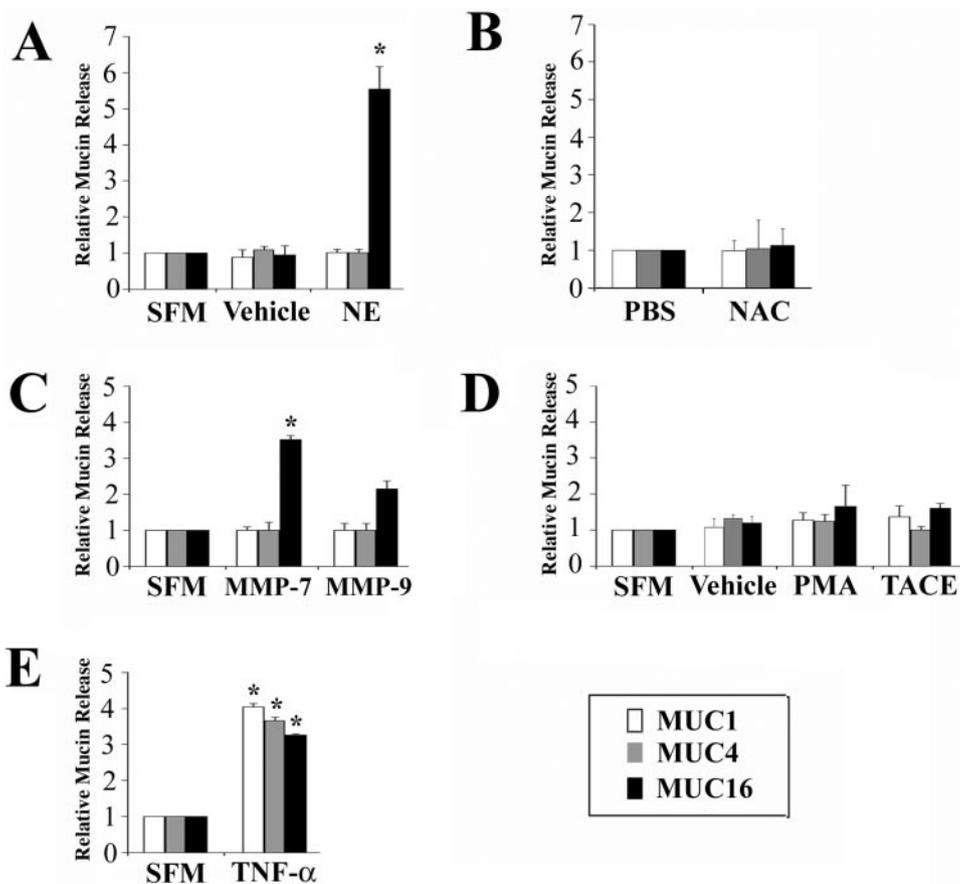
### Agents That Induce Release of MAMs in HCLE Cells

The release of MUC1 on epithelial cells can be induced by a variety of agents, including TNF, TACE, and MT1-MMP.<sup>17,18,35</sup> Little is known about the induction of MUC4 and MUC16 release, and no data are available on the induction of MAM release at the ocular surface epithelia. Thus, potential agents that induce the release of MAMs in other epithelial culture systems were tested in HCLE cells.

Neutrophil elastase is a protease found in azurophilic granules in the cytoplasm of neutrophils that induces the release of the extracellular domain of MUC1 on human and hamster tracheal epithelial cells.<sup>19</sup> The effect of neutrophil elastase on ocular surface epithelial mucins has not been reported. Thirty-minute incubation with neutrophil elastase (5  $\mu$ g/mL) induced a 5.5-fold increase in the release of MUC16 from HCLE cells compared with controls. (Fig. 2A;  $n = 3$ ;  $P < 0.01$ ). No significant effect on MUC1 or MUC4 release was observed. The release of MUC16 was confirmed by immunofluorescence microscopy of nonpermeabilized HCLE cells treated with neutrophil elastase using the OC125 antibody (data not shown), which recognizes epitopes in the tandem repeat region of the extracellular domain.<sup>36</sup>

NAC washings of the ocular surface in normal human subjects have been reported to release soluble MUC1 and MUC4.<sup>20</sup> MAMs are also detected in PBS washes of the human ocular surface, suggesting that there is constitutive shedding of MAMs.<sup>16</sup> In this study, no significant changes in the level of

**FIGURE 2.** Effect of potential shed-dase on membrane-associated mucin release from HCLE cells. Stratified HCLE cell cultures were treated with agents to induce the release of membrane-associated mucins into culture media. Results were quantified by comparing the total amount of mucin in concentrated culture media samples to that in 25  $\mu\text{g}$  cell lysate by densitometry of immunoblots. (A) Effect of 30-minute exposure to 5  $\mu\text{g}/\text{mL}$  neutrophil elastase in media compared with negative control (serum-free DMEM/Ham F-12) and neutrophil elastase vehicle control (50 mM sodium acetate, 150 mM NaCl; pH 5.5). (B) Effect of 10-minute culture with 10% *N*-acetylcysteine (NAC) compared with PBS control. (C) Effect of exposure to MMP-7 (340 nM/mL) or MMP-9 (2  $\mu\text{g}/\text{mL}$ ) for 6 hours compared with negative control (serum-free DMEM/Ham F-12). (D) Effect of exposure to PMA (1  $\mu\text{m}$ ) and TACE (0.1  $\mu\text{g}/\text{mL}$ ) for 1 hour compared with negative control (serum-free DMEM/Ham F-12 and PMA vehicle (0.01% ethanol). (E) Effect of 24-hour culture with TNF (10 ng/mL) compared with negative control (serum-free medium).  $n = 3$ . \* $P < 0.01$  was considered significant.



MAM release were observed when HCLE cells were exposed to 10% NAC (Fig. 2B;  $n = 3$ ).

Matrix metalloproteinases (MMPs) are a class of proteases that cleave extracellular matrix proteins and other bioactive molecules.<sup>37</sup> Several, such as MMP-7 and MMP-9, are expressed by the corneal epithelium.<sup>29,38</sup> In addition, MMP-9 activity is significantly elevated in the tear fluid of patients with dry eye syndrome.<sup>39</sup> The role of MMPs in the release of MAMs has not been well established. MMP-7 may influence the release of MUC1 because they colocalize and are secreted together in lymph node metastases,<sup>40</sup> but definitive data are not available. MMP-7 induced 3.4-fold higher levels of MUC16 release from the surfaces of HCLE cells after 6 hours of treatment, with no significant effect on MUC1 or MUC4 (Fig. 2C;  $n = 3$ ;  $P < 0.01$ ). In addition, 6 hours of MMP-9 treatment induced some MUC16 release in HCLE cells, though this effect was not statistically significant. (Fig. 2C;  $n = 3$ ).

TACE induces MUC1 shedding in human uterine epithelial cells.<sup>18</sup> Additional studies have reported that TACE induces MUC1 shedding in uterine epithelial cells through MT1-MMP, which is expressed in the corneal stroma and basal epithelial cells.<sup>17,41</sup> However, neither TACE (0.1  $\mu\text{g}/\text{mL}$ ) nor PMA (1  $\mu\text{m}$ ),<sup>17</sup> which induces TACE-mediated MUC1 shedding, had a significant effect on MAM release in HCLE cells after treatment for 1 hour (Fig. 2D;  $n = 3$ ).

TNF stimulates MUC1 shedding from the uterine epithelial surface, and its action is blocked by synthetic MMP inhibitors and tissue inhibitors of MMPs.<sup>17</sup> TNF is expressed by the corneal epithelium, and increased levels are found in the tear fluid of mice in an experimental dry eye model.<sup>42</sup> Treatment of HCLE cells with 10 ng/mL TNF for 24 hours significantly increased the release of MUC1, MUC4, and MUC16 from the cell surface (Fig. 2E;  $n = 3$ ;  $P < 0.01$ ). Significant levels of release by TNF at 1 and 4 hours were not

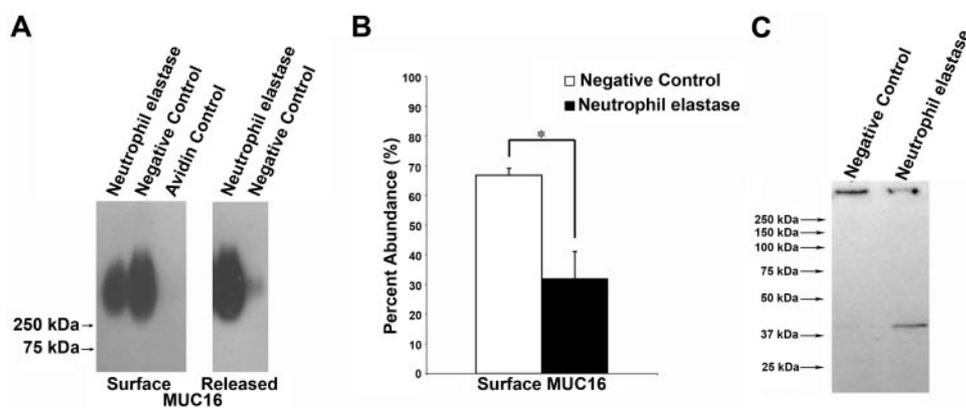
observed (data not shown). The release of MUC1 and MUC16 from the cell surface was confirmed by immunofluorescence microscopy of nonpermeabilized HCLE cells treated with TNF. Cell surface MUC1 and MUC16 localization was reduced on HCLE cells treated with TNF compared with nontreated cells (data not shown). These results correlate with the immunoblot data in Figure 2E.

#### Efficiency of MUC16 Release by Neutrophil Elastase in HCLE Cells

To determine the efficiency of MUC16 extracellular domain release by neutrophil elastase in HCLE cells, the surface proteins were biotinylated after treatment (Fig. 3). Densitometry of immunoblots revealed that 30 minutes of neutrophil elastase treatment of HCLE cells induced the release of 68% of the MUC16 into the culture medium from the cell surface (Fig. 3B;  $n = 4$ ). Nontreated cells constitutively released approximately 33% of cell surface-associated MUC16 within the same treatment time. On treatment of biotinylated HCLE cells with neutrophil elastase, a band at approximately 40 kDa is present in MUC16CT immunoblots (Fig. 3C). The presence of this low-molecular weight form of MUC16 in the membrane indicates that, after 30 minutes of neutrophil elastase treatment, the remaining portion of MUC16 containing a fragment of the extracellular domain, the transmembrane domain, and the cytoplasmic tail are retained at the membrane.

#### Restoration of Surface MUC16

Few data are available on the turnover of MAMs at the ocular surface or the time required for replenishment of mucins after the induced release of the extracellular domain. To assess the restoration of MUC16 on the HCLE cell surface after release,



**FIGURE 3.** Amount of membrane-associated MUC16 remaining on HCLE cells after neutrophil elastase treatment. Stratified HCLE cell cultures were treated with neutrophil elastase to induce MUC16 release. After release, surface proteins were biotinylated and isolated. The amount of MUC16 in the medium was compared with the remaining biotinylated cell surface MUC16 after release and was quantified by densitometry of immunoblots. (A) Representative MUC16 immunoblot of biotinylated HCLE surface and released protein after neutrophil elastase treatment compared with the nontreated negative control. The avidin control confirms that only biotinylated proteins were recovered from the gel. (B) The amount of membrane-associated MUC16 on HCLE cells after neutrophil elastase treatment is significantly reduced to 32% of total mucin compared with untreated negative control ( $n = 4$ ;  $P < 0.05$ ). (C) MUC16CT immunoblot of biotinylated HCLE surface protein after neutrophil elastase treatment compared with the nontreated negative control, demonstrating the appearance of a band at approximately 40 kDa. Samples were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose, and an immunoblot was performed using the MUC16CT antibody. The 40-kDa protein at the cell surface indicates that the cytoplasmic tail, the transmembrane domain, and the remnant extracellular domain are retained at the cell surface after treatment with neutrophil elastase for 30 minutes.

cells were treated with neutrophil elastase followed by biotinylation of surface proteins. Densitometry of MUC16 immunoblots revealed that 24 hours after neutrophil elastase-induced release, MUC16 levels returned to 78% of those present on nontreated cells (Fig. 4A;  $n = 3$ ). MUC16 mRNA levels were not significantly altered in HCLE cells 1, 3, 6, and 24 hours after neutrophil elastase treatment compared with nontreated cells, suggesting that the MUC16 that is replenished on the cell surface in this time period is from intracellular stores and occurs without the induction of increased expression (Fig. 4B;  $n = 3$ ).

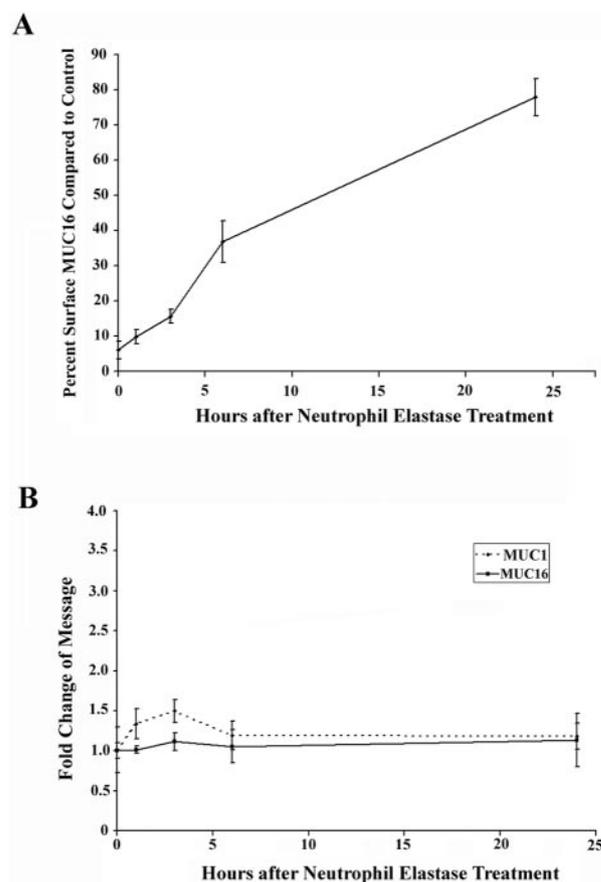
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### Effect of Hyperosmolarity on Membrane-Associated Mucin Release

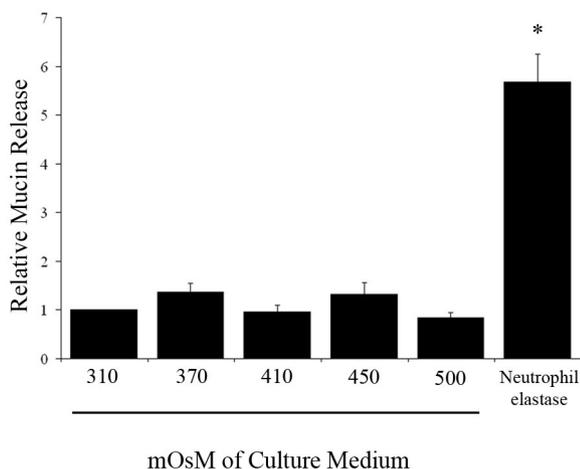
Hyperosmolarity of tear fluid in patients with dry eye syndrome is significantly elevated compared with that in normal patients.<sup>43</sup> Elevated osmolarity induces inflammation at the ocular surface and increases the expression and activity of MMPs in human corneal epithelial cells in vitro.<sup>34</sup> To examine the effect of hyperosmolarity on MAM release at the ocular surface, osmolarity was increased from 310 to 500 mOsm in the culture medium for 24 hours in stratified HCLE cell cultures. Densitometry of immunoblots showed no significant induction of MUC16 release in HCLE cells grown in hyperosmolar conditions compared with the positive control of a 30-minute neutrophil elastase treatment (Fig. 5;  $n = 3$ ). In addition, the release of MUC1 and MUC4 were not altered in stratified HCLE cells grown in hyperosmolar conditions (data not shown).

### Effect of MUC16 Release on Dye Penetrance

Rose bengal, an anionic dye used to assess damage to the ocular surface epithelium,<sup>44</sup> was previously shown to be excluded by differentiated islands of stratified HCLE cells that express MUC16.<sup>12,23</sup> Furthermore, rose bengal penetrance is significantly increased in HCLE cells with altered MUC16 extracellular domain glycosylation or when MUC16 is knocked down by RNAi methods.<sup>12,23</sup> To determine whether the induction of MUC16 release results in changes in the uptake of rose bengal, stratified HCLE cells were treated with rose bengal after induced MUC16 release by neutrophil elastase and were compared with vehicle-treated and control cells. Islands of stratified HCLE cells that excluded the rose bengal dye were observed in nontreated and vehicle-treated cells (Fig. 6A, 6B). However, significantly fewer islands excluding the dye were seen in



**FIGURE 4.** Restoration of surface MUC16 on HCLE cells after release occurs from cytoplasmic stores. MUC16 ( $78\% \pm 5\%$ ) is restored on the surfaces of HCLE cells by 24 hours after treatment, without concurrent increases in message. (A) The percentage of MUC16 present on the surface of stratified HCLE cells was measured by densitometry of immunoblots of biotinylated surface proteins over time (1, 3, 6, and 24 hours) after neutrophil elastase-induced release and was expressed relative to nontreated control ( $n = 3$ ). (B) MUC1 and MUC16 message were measured in similarly treated cultures by real-time PCR to nontreated cells ( $n = 3$ ). No significant difference in expression level of either MUC1 or MUC16 mRNA was found up to 24 hours after treatment with neutrophil elastase.



**FIGURE 5.** Effect of hyperosmolar culture medium on MUC16 release in HCLE cells. No change was seen in the amount of released MUC16 in response to hyperosmolarity. Stratified HCLE cells were cultured for 24 hours in media of increasing osmolarity. Cells incubated with neutrophil elastase for 30 minutes were used as a positive control. Results were quantified by comparing the total amount of mucin in concentrated culture media samples to 25  $\mu$ g cell lysate by densitometry of immunoblots. Each treatment was normalized to serum-free medium control of normal osmolarity (310 mOsm). For comparison, the release induced by neutrophil elastase is shown.  $n = 3$ . \* $P < 0.01$  was considered significant.

stratified HCLE cells in which MUC16 release was induced by neutrophil elastase (Fig. 6C). Areas of islands of dye exclusion were quantified (Fig. 6D), showing that cells treated with neutrophil elastase had 66% less island area per field, whereas there was no significant effect in vehicle-treated cells ( $n = 5$ ;  $P < 0.01$ ). These data indicate that when MUC16 release is induced, the dye penetration barrier on HCLE cells is reduced.

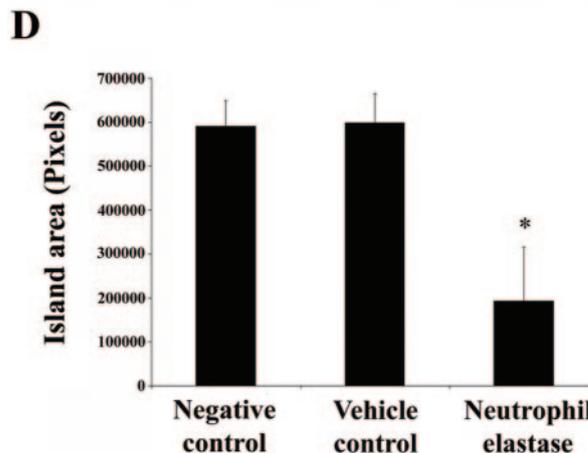
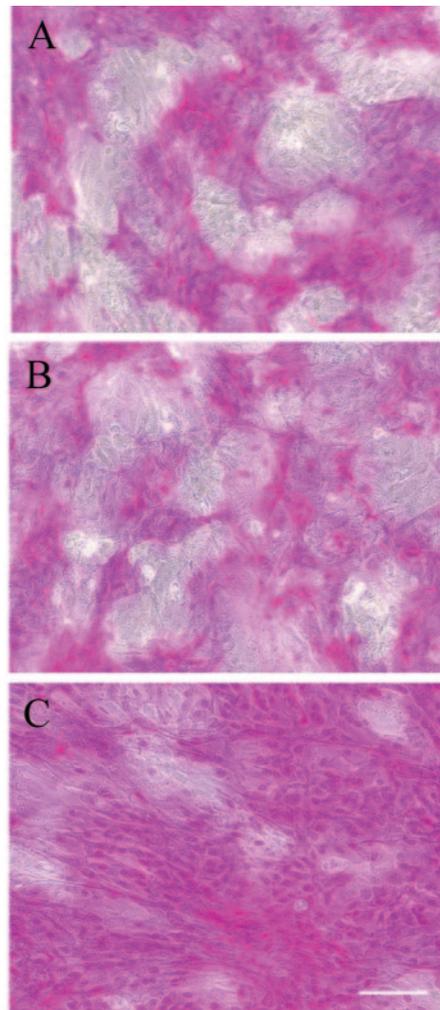
## DISCUSSION

In this report, we have demonstrated that several physiologically relevant agents—neutrophil elastase, TNF, and MMP-7—induce the release of the extracellular domains of MAMs on HCLE cells, a model for the native corneal epithelium. We have demonstrated that, after the induction of MUC16 extracellular domain release in HCLE cells by one of these agents, neutrophil elastase, MUC16 is restored to the cell surface from cytoplasmic stores within 24 hours without increased gene expression. We have also presented data showing that the induction of MUC16 release by neutrophil elastase results in a loss of barrier protection from rose bengal penetration.

As noted, when HCLE cells were treated with neutrophil elastase, release of the MUC16 extracellular domain was observed, but MUC1 and MUC4 were not affected. During inflammation or infection, numerous neutrophils are present in the tear film.<sup>45</sup> Degranulation of neutrophils could cause local release of neutrophil elastase onto cells and into the tear film, directly affecting the release of MUC16 on the ocular surface. In this study, biotinylation of HCLE surface proteins after neutrophil elastase treatment showed that the majority of surface MUC16 was released but returned to the surface after 24 hours. This effect was seen in spite of the fact that the mucin message did not increase in the 24 hours after the induction of release, suggesting that MUC16 returns to the surface through intracellular stores during this time. Similarly, TNF, an inflammatory cytokine elevated in tears of patients with dry eye,<sup>46</sup> induced the release of MUC1, MUC4, and MUC16. However, release was only observed after a 24-hour exposure, suggesting the

activation of other pathways downstream of TNF binding to its receptors.

The question arises as to whether the levels of neutrophil elastase, TNF, and MMP-7 used in the assays reported here are



**FIGURE 6.** Rose bengal penetration of HCLE cells treated with neutrophil elastase. Bright-field micrographs of stratified (A) nontreated, (B) vehicle-treated, and (C) neutrophil elastase-treated HCLE cells show changes in the binding pattern of the anionic vital dye rose bengal. Cells were photographed using phase-contrast microscopy. Scale bar, 50  $\mu$ m. (D) Quantification of decrease in the amount of surface area that excludes rose bengal. Areas of islands of dye exclusion were quantified using image analysis software.  $n = 5$ . \* $P < 0.01$ .

comparable with those found in tear fluid. With regard to neutrophil elastase, given that neutrophils act locally and release their enzymes locally, it is difficult to define physically relevant concentrations. The concentration of neutrophil elastase in the sera of normal subjects ranges from 1.3 to 9.35  $\mu\text{g/mL}$ ,<sup>47</sup> and these levels increase with diseases such as pneumonia and bronchitis.<sup>48,49</sup> Although neutrophil elastase is present in closed-eye tears,<sup>50</sup> to our knowledge the amounts of the enzyme per unit of tear volume are unavailable. In our experiments, neutrophil elastase was used at 5  $\mu\text{g/mL}$  media, which is comparable to serum levels, particularly in lung diseases.

We used 1 ng/mL TNF levels based on previous studies that used 1, 10, 25, and 100 ng TNF to study MUC1 shedding in uterine epithelial cells.<sup>17</sup> Serum levels of TNF in normal subjects is  $13.78 \pm 7.52$  pg/mL, with a doubling in disease.<sup>51</sup> Reports of tear levels of TNF vary widely, ranging from less than 0.5 to 286 pg/mL in normal subjects, with increases in dry eye and allergic eye disease.<sup>46,52</sup> In any event, the amount of TNF used in tissue culture experiments appears to be 100- to 1000-fold higher than in body fluids and should be taken into account when considering the data. Levels of MMP-7 in tear film have, to our knowledge, not been reported.

The effect of induced MAM release in epithelial cells appears to be tissue specific. For example, neutrophil elastase induced the release of the extracellular domain of MUC16 in HCLE cells but not of MUC1. These results contrast with previous reports of the induced release and degradation of Muc1 in hamster airway epithelia by neutrophil elastase.<sup>19</sup> Variations in response to induced release may be explained by differential glycosylation in different epithelia. For example, the glycosylation states of MUC1 vary between epithelial and tumor cell lines.<sup>53</sup> Differences in the degree and nature of glycosylation of the MAM extracellular domains could result in altered accessibility of sheddases to the protease cleavage sites.

We investigated the possibility that the hyperosmolarity observed in tear fluid of patients with dry eye<sup>43</sup> contributes to MAM release at the ocular surface. Increased osmolarity in culture medium of corneal epithelial cells increases MMP expression through specific signaling pathways known to be induced under cellular stress.<sup>34</sup> We report here that the hyperosmolarity of the culture medium alone did not induce the release of MAMs in HCLE cells to a greater extent than constitutive levels of MAM release, even after 24 hours of exposure, suggesting that mucin release on the ocular surface is caused by specific agents of release, not by pathways activated on ionic changes in tear film. However, if the hyperosmolarity of the tear film induces inflammation *in vivo*, this may lead to the expression of agents that induce MAM release.

MAMs are components of the ocular surface glycocalyx and have been hypothesized to participate in providing a barrier to the epithelial surface. Given that the extracellular domain of MUC16 was specifically cleaved from the cell surface by neutrophil elastase, we chose to examine the result of this effect on the epithelial barrier using rose bengal, a dye that is believed to penetrate regions of the ocular surface epithelium in which protection has been compromised.<sup>44</sup> Previously, the production of glycosylated MUC16 by stratified HCLE cells was hypothesized to provide protection to the epithelial surface from rose bengal penetration.<sup>23</sup> The fact that release of the extracellular domain of MUC16 from HCLE cells reduces rose bengal protection further supports the hypothesis that MUC16 is a component of the protective barrier to the epithelial surface and that enhanced release may be detrimental to the ocular surface. This correlates with previously reported data that altered MUC16 glycosylation and reduced MUC16 expression result in increased rose bengal penetration.<sup>12,23</sup>

Some limitations exist that may affect the interpretation of the results in this study. The agents that induced specific MAM

release in HCLE cells may induce the release of other membrane-bound proteins on the apical surface, though the sizes of these molecules are likely to be much smaller and not to play as important a role in creating a barrier to the cell surface. In addition, sheddases of MAMs in HCLE cells do not completely remove the extracellular domains of every MUC16 molecule, as shown here by biotinylation of surface proteins after neutrophil elastase treatment. Another concern is that although HCLE cells express the same MAMs found in native ocular surface epithelia (MUC1, MUC4, and MUC16), expression is not uniform throughout the culture. Islands of protection from rose bengal penetration expressing MUC16, rather than complete surface exclusion as seen in intact native ocular surface epithelium, are observed.<sup>23</sup> Although there are limitations to the culture model, there is sufficient similarity to native epithelium to test our hypothesis on the alteration of barrier function by MAM release.

This article presents the first direct evidence that physiologic agents in tear film involved in inflammation induce MAM release at the ocular surface epithelia. We also report neutrophil elastase as the first specific inducer of MUC16 release on the corneal epithelial surface. These data suggest that dry eye symptoms may result from the release of MAMs through an increase in the presence or activity of sheddase. To further understand the function of the MAMs on the ocular surface, the effects of release on cytoplasmic tail phosphorylation and intracellular signaling must be studied. Based on these results, agents that block the action of MAM release may be a possible therapeutic treatment for dry eye.

## References

- Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer*. 2004;4:45-60.
- Gipson IK. Distribution of mucins at the ocular surface. *Exp Eye Res*. 2004;78:379-388.
- Hilkens J, Ligtenberg MJL, Vos HL, Litvinov SV. Cell membrane-associated mucins and their adhesion-modulating property. *Trends Biochem Sci*. 1992;17:359-363.
- Bramwell ME, Wiseman G, Shotton DM. Electron-microscopic studies of the CA antigen, epitectin. *J Cell Sci*. 1986;86:249-261.
- Higuchi T, Orita T, Katsuya K, et al. MUC20 suppresses the hepatocyte growth factor-induced Grb2-Ras pathway by binding to a multifunctional docking site of met. *Mol Cell Biol*. 2004;24:7456-7468.
- Inatomi T, Spurr-Michaud S, Tisdale AS, Gipson IK. Human corneal and conjunctival epithelia express MUC1 mucin. *Invest Ophthalmol Vis Sci*. 1995;36:1818-1827.
- Inatomi T, Spurr-Michaud S, Tisdale AS, Zhan Q, Feldman ST, Gipson IK. Expression of secretory mucin genes by human conjunctival epithelia. *Invest Ophthalmol Vis Sci*. 1996;37:1684-1692.
- Argueso P, Spurr-Michaud S, Russo CL, Tisdale A, Gipson IK. MUC16 mucin is expressed by the human ocular surface epithelia and carries the H185 carbohydrate epitope. *Invest Ophthalmol Vis Sci*. 2003;44:2487-2495.
- Pflugfelder SC, Liu Z, Monroy D, et al. Detection of sialomucin complex (MUC4) in human ocular surface epithelium and tear fluid. *Invest Ophthalmol Vis Sci*. 2000;41:1316-1326.
- Yamamoto M, Bharti A, Li Y, Kufe D. Interaction of the DF3/MUC1 breast carcinoma-associated antigen and beta-catenin in cell adhesion. *J Biol Chem*. 1997;272:12492-12494.
- Singh AP, Moniaux N, Chauhan SC, Meza JL, Batra SK. Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. *Cancer Res*. 2004;64:622-630.
- Blalock T, Spurr-Michaud S, Tisdale A, et al. Functions of MUC16 in corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2007;48:4509-4518.
- Yin BW, Lloyd KO. Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. *J Biol Chem*. 2001;276:27371-27375.

14. Komatsu M, Arango ME, Carraway KL. Synthesis and secretion of Muc4/sialomucin complex: implication of intracellular proteolysis. *Biochem J.* 2002;368:41–48.
15. Hattrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol.* 2008;70:7.1–7.27.
16. Spurr-Michaud S, Argueso P, Gipson IK. Assay of mucins in human tear fluid. *Exp Eye Res.* 2007;84:939–950.
17. Thathiah A, Brayman M, Dharmaraj N, Julian JJ, Lagow EL, Carson DD. Tumor necrosis factor alpha stimulates MUC1 synthesis and ectodomain release in a human uterine epithelial cell line. *Endocrinology.* 2004;145:4192–4203.
18. Thathiah A, Blobel CP, Carson DD. Tumor necrosis factor-alpha converting enzyme/ADAM 17 mediates MUC1 shedding. *J Biol Chem.* 2003;278:3386–3394.
19. Kim KC, Wasano K, Niles RM, Schuster JE, Stone PJ, Brody JS. Human neutrophil elastase releases cell surface mucins from primary cultures of hamster tracheal epithelial cells. *Proc Natl Acad Sci U S A.* 1987;84:9304–9308.
20. Berry M, Brayshaw D, McMaster T. Dynamic molecular resolution imaging of precocular fluid impressions. *Br J Ophthalmol.* 2004;88:1460–1466.
21. Soto P, Zhang J, Carraway KL. Enzymatic cleavage as a processing step in the maturation of Muc4/sialomucin complex. *J Cell Biochem.* 2006;97:1267–1274.
22. Levitin F, Stern O, Weiss M, et al. The MUC1 SEA module is a self-cleaving domain. *J Biol Chem.* 2005;280:33374–33386.
23. Argueso P, Tisdale A, Spurr-Michaud S, Sumiyoshi M, Gipson IK. Mucin characteristics of human corneal-limbal epithelial cells that exclude the rose bengal anionic dye. *Invest Ophthalmol Vis Sci.* 2006;47:113–119.
24. Danjo Y, Watanabe H, Tisdale AS, et al. Alteration of mucin in human conjunctival epithelia in dry eye. *Invest Ophthalmol Vis Sci.* 1998;39:2602–2609.
25. Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci.* 2003;44:2496–2506.
26. Gipson IK, Moccia R, Spurr-Michaud S, et al. The amount of MUC5B mucin in cervical mucus peaks at midcycle. *J Clin Endocrinol Metabol.* 2001;86:594–600.
27. Argueso P, Baram M, Spurr-Michaud S, Keutmann HT, Dana MR, Gipson IK. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjögren's syndrome. *Invest Ophthalmol Vis Sci.* 2002;43:1004–1011.
28. Lin HC, Chang JH, Jain S, et al. Matrilysin cleavage of corneal collagen type XVIII NC1 domain and generation of a 28-kDa fragment. *Invest Ophthalmol Vis Sci.* 2001;42:2517–2524.
29. Sobrin L, Liu Z, Monroy DC, et al. Regulation of MMP-9 activity in human tear fluid and corneal epithelial culture supernatant. *Invest Ophthalmol Vis Sci.* 2000;41:1703–1709.
30. Hori Y, Spurr-Michaud S, Russo C, Argueso P, Gipson IK. Differential regulation of membrane-associated mucins in the human ocular surface epithelium. *Invest Ophthalmol Vis Sci.* 2004;45:114–122.
31. Moniaux N, Varshney GC, Chauhan SC, et al. Generation and characterization of anti-MUC4 monoclonal antibodies reactive with normal and cancer cells in humans. *J Histochem Cytochem.* 2004;52:253–261.
32. Fink L, Seeger W, Ermert L, et al. Real-time quantitative RT-PCR after laser-assisted cell picking. *Nature Med.* 1998;4:1329–1333.
33. Abramoff M, Magelhaes P, Ram S. Image processing with ImageJ. *Biophoton Intl.* 2004;11:36–42.
34. Li DQ, Chen Z, Song XJ, Luo L, Pflugfelder SC. Stimulation of matrix metalloproteinases by hyperosmolarity via a JNK pathway in human corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2004;45:4302–4311.
35. Thathiah A, Carson DD. MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17. *Biochem J.* 2004;382:363–373.
36. O'Brien TJ, Beard JB, Underwood LJ, Dennis RA, Santin AD, York L. The CA 125 gene: an extracellular superstructure dominated by repeat sequences. *Tumour Biol.* 2001;22:348–366.
37. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol.* 2004;16:558–564.
38. Lu PC, Ye H, Maeda M, Azar DT. Immunolocalization and gene expression of matrilysin during corneal wound healing. *Invest Ophthalmol Vis Sci.* 1999;40:20–27.
39. Solomon A, Dursun D, Liu Z, Xie Y, Macri A, Pflugfelder SC. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. *Invest Ophthalmol Vis Sci.* 2001;42:2283–2292.
40. Ajisaka H, Fushida S, Yonemura Y, Miwa K. Expression of insulin-like growth factor-2, c-MET, matrix metalloproteinase-7 and MUC-1 in primary lesions and lymph node metastatic lesions of gastric cancer. *Hepatogastroenterology.* 2001;48:1788–1792.
41. Ye HQ, Maeda M, Yu FS, Azar DT. Differential expression of MT1-MMP (MMP-14) and collagenase III (MMP-13) genes in normal and wounded rat corneas. *Invest Ophthalmol Vis Sci.* 2000;41:2894–2899.
42. Song XJ, Li DQ, Farley W, et al. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci.* 2003;44:4223–4229.
43. Gilbard JP, Rossi SR. Changes in tear ion concentrations in dry-eye disorders. *Adv Exp Med Biol.* 1994;350:529–533.
44. Tseng SC, Zhang SH. Interaction between rose bengal and different protein components. *Cornea.* 1995;14:427–435.
45. Asano-Kato N, Toda I, Fukumoto T, Asai H, Tsubota K. Detection of neutrophils in late-onset interface inflammation associated with flap injury after laser in situ keratomileusis. *Cornea.* 2004;23:306–310.
46. Yoon KC, Jeong IY, Park YG, Yang SY. Interleukin-6 and tumor necrosis factor-alpha levels in tears of patients with dry eye syndrome. *Cornea.* 2007;26:431–437.
47. Johnson RM. Low levels of serum elastase are not associated with mutations in ELA-2 elastase encoding gene in chronic neutropenia. *Blood.* 2003;101:2898–2899.
48. Matsuse H, Yanagihara K, Mukae H, Tanaka K, Nakazato M, Kohno S. Association of plasma neutrophil elastase levels with other inflammatory mediators and clinical features in adult patients with moderate and severe pneumonia. *Respir Med.* 2007;101:1521–1528.
49. Emboriadou M, Hatzistilianou M, Magnisali C, et al. Human neutrophil elastase in RSV bronchiolitis. *Ann Clin Lab Sci.* 2007;37:79–84.
50. Sakata M, Sack RA, Sathe S, Holden B, Beaton AR. Polymorphonuclear leukocyte cells and elastase in tears. *Curr Eye Res.* 1997;16:810–819.
51. Bonotis K, Krikki E, Holeva V, Aggouridaki C, Costa V, Baloyannis S. Systemic immune aberrations in Alzheimer's disease patients. *J Neuroimmunol.* 2008;193:183–187.
52. Leonardi A, Curnow SJ, Zhan H, Calder VL. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy.* 2006;36:777–784.
53. Poland PA, Kinlough CL, Rokaw MD, Magarian-Blander J, Finn OJ, Hughey RP. Differential glycosylation of MUC1 in tumors and transfected epithelial and lymphoblastoid cell lines. *Glycoconjugate J.* 1997;14:89–96.