Sialomucin complex at the rat ocular surface: a new model for ocular surface protection

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The ocular surface, which is among the most accessible and vulnerable tissues in mammals, is protected by a complex tear film composed of lipid, aqueous and mucin layers. In spite of its importance, the molecular nature of the mucin contribution remains uncertain. Since membrane mucins have been implicated in the protection of other epithelia, we have analysed rat corneal and conjunctival tissues for sialomucin complex (SMC), a membrane mucin found at the apical epithelial cell surfaces in the airway and uterus. Using Northern and Western blot analyses, SMC expression was found in both ocular tissues, being particularly abundant in the cornea. In contrast with the other known membrane mucin, MUC1, SMC was localized more heavily towards the apical surface of the epithelial cells. SMC in

ocular surface epithelia was produced in both soluble and membrane forms, the latter being found predominantly in the most superficial cells and at apical surfaces. The soluble form was found loosely adsorbed to apical cell surfaces, particularly of the cornea, as indicated by a mild rinsing protocol. Finally, the tear fluid contained substantial amounts of SMC. From these results, we propose a new model for tear mucin components in which SMC is expressed at the apical ocular surface in both membrane-bound and adsorbed soluble forms to provide a direct protective barrier. SMC secreted into the tear fluid may also participate in maintaining the stability of the precocular tear film by acting with other secreted mucins to determine the physical properties and protective behaviour of the tear film.

INTRODUCTION

The surface of the eye is overlaid by a tear film which covers and protects the corneal and conjunctival epithelia. The tear film is biochemically complex, being composed of three layers: (1) an outer lipid layer, secreted by meibomian glands; (2) a middle aqueous layer, secreted mainly by lacrimal glands; and (3) an inner mucin-containing gel layer, produced by and associated closely with apical cells of the ocular surface [1]. In rodents the mucin gel layer is believed to comprise a major fraction of the tear film thickness [2,3]. The complex constitution and interactions of the tear film assure optical clarity, lubricate the ocular surface, and protect against pathogenic and noxious agents. In the ocular epithelium, secreted mucins appear to play an important role in the maintenance of the ocular surface and tear film and in protection against external pathogens and foreign debris [4].

Mucins are large glycoconjugates with molecular masses ranging from 3×10^5 to over 4×10^7 kDa [4,5]. The most important characteristic of these molecules is that they contain serine- and threonine-rich domains which can be extensively O-glycosylated [6–8]. Roughly 70–80% of mucin dry weight is carbohydrate. Mucins are subdivided into secretory, soluble and membrane-bound forms. Secretory gel-forming mucins, such as MUC2 and MUC5AC, make up the viscous mucus of the tracheobronchial, gastrointestinal and reproductive tracts, and form large oligomers through linkage of protein monomers via disulphide bonds at their N- and C-termini. Soluble mucins such as MUC7 [9], which is found in the salivary gland, have smaller polypeptide backbones and do not form gels by disulphide cross-linking. Membrane-bound mucins, such as MUC1, have a hydrophobic membrane-spanning domain and also do not form

disulphide-linked oligomers [4]. MUC1 is produced by some cells in a soluble form that lacks the transmembrane and C-terminal domains. Because of its large extended structure and cell surface location, MUC1 has been assigned a number of possible functions. In normal and tumour tissue MUC1 may create a barrier to cell–cell interactions, reduce the adhesion of macromolecules to the cell surface, and provide resistance to natural killer and cytotoxic T-cell killing [10]. Several different mucins have been reported in ocular tissues, and it was once thought that all ocular mucin was produced by conjunctival goblet cells. However, several reports indicate that both corneal and conjunctival epithelia produce mucins [11–14]. These include the secreted mucins MUC5AC, MUC4 and MUC2 [15–17], and the membrane mucin MUC1 [18].

We have described another membrane mucin, called sialomucin complex (SMC), which is composed of two subunits: an O-glycosylated mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and an N-glycosylated integral membrane glycoprotein ASGP-2 [19,20]. The mucin subunit, ASGP-1, is tightly but non-covalently bound to the transmembrane subunit, ASGP-2. SMC was originally isolated and characterized as a heterodimeric glycoprotein complex from the surface of highly metastatic 13762 rat mammary adenocarcinoma ascites cells [19]. Recently, SMC has been shown to be expressed in a number of normal secretory epithelial tissues in the adult rat, including small and large intestine, trachea, uterus and lactating mammary gland [21,22]. SMC is transcribed from a single gene as a 9 kb transcript [23,24] and is translated into a polypeptide precursor that is proteolytically cleaved into the ASGP-1 and ASGP-2 subunits early in its transit to the cell surface [25]. Mature glycosylated ASGP-1 has a molecular mass of > 500 kDa [26] and contains three domains: an N-terminal unique sequence, a large tandem

Abbreviations used: SMC, sialomucin complex; ASGP, ascites sialoglycoprotein; EGF, epidermal growth factor; mAb, monoclonal antibody.

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Figure 1 Domain structure of SMC and locations of epitopes for antibodies used in this study

See Table 1 for further details of antibodies. α -ASGP-2 and α -C-pep indicate anti-ASGP-2 and anti-C-pep respectively. TM, transmembrane; C-term, C-terminal.

repeat region rich in serine and threonine residues similar to that of other mucins, and a C-terminal unique sequence (Figure 1) [24].

SMC is similar to MUC1 in that both are expressed in a number of normal secretory epithelial tissues, including the lactating mammary gland, trachea and uterus [10,21,22]. Both proteins are found as heterodimeric complexes containing a transmembrane subunit [19,27]. In addition, SMC and MUC1 can be found in both membrane and soluble forms; the latter lack the C-terminal and transmembrane domains [10,21]. The soluble form of MUC1 may be generated either by alternative RNA splicing [28] or by proteolytic cleavage [29]. The soluble form of SMC is produced by proteolytic cleavage in the tissues that we have analysed. Both MUC1 and SMC have been shown to disrupt cell-cell and cell-matrix interactions and to provide resistance to cell killing by natural killer cells [30]. However, SMC is uniquely expressed in some tissues where MUC1 is not detected, such as the colon and small intestine [10,21]. In addition, the transmembrane subunit ASGP-2 contains two epidermal growth factor (EGF)-like domains which contain all of the consensus residues found in EGF-like regions of proteins possessing growth factor activity (Figure 1) [23], and has been shown to act as a ligand for the receptor tyrosine kinase ErbB-2 [22,31]. Thus SMC may be bifunctional, serving both as a protective, lubricating mucin and an active growth factor.

To determine whether SMC may be important in ocular surface protection, we have characterized SMC expression in normal rat corneal and conjunctival tissue. SMC is expressed in both corneal and conjunctival epithelia. SMC in conjunctival tissue is larger than in other tissues studied, possibly due to glycosylation differences. By immunofluorescence, SMC is localized to all layers of the corneal and conjunctival epithelia. Significantly, it is not found in conjunctival goblet cells. Moreover, in both corneal and conjunctival epithelia SMC is found as both soluble and membrane forms. Interestingly, the membrane form is predominantly expressed in the most superficial layers of the stratified epithelia, consistent with a role in the direct protection of the apical surface. In contrast, MUC1 appears to be present predominantly in the more basal layers, as also shown previously [18]. From these results we have proposed a new molecular model for the tear film and ocular surface protection.

EXPERIMENTAL

Materials

Polyclonal antisera against whole ASGP-2 and its C-terminal domain [21,25], monoclonal antibodies (mAbs) against ASGP-2

[21] and the AM-3 mAb against conjunctival goblet cell mucin were produced as previously described [21,32]. The CT-1 mAb against the C-terminal region of Muc-1 [33] (where Muc-1 is the designation for non-human homologues of MUC1) was a gift from Dr. Daniel D. Carson (M. D. Anderson Cancer Center, Houston, TX, U.S.A.). The polyclonal HA-1 antibody, which recognizes a peptide in the C-terminal region of ASGP-1, was prepared by Quality Controlled Biochemicals (Hopkinton, MA, U.S.A.) from a rat ASGP-1 sequence (CAGYRPPRPAWT-FGD). See Figure 1 and Table 1 for a summary of antibodies used. The MAT-C1 ascites subline of the 13762 rat mammary adenocarcinoma was maintained by weekly passage as described previously [34].

Preparation of tissue samples

Corneal and conjunctival tissues dissected from female Fischer 344 rats were pulverized with a mortar and pestle in liquid N₂ and stored as a powder at -80°C . For immunoblotting, tissue powders were solubilized in 1% SDS in water, boiled and clarified by centrifugation at 2000 g. Protein concentrations were determined by Lowry assay of the cleared SDS lysates. Cleared cell lysates were diluted in SDS/PAGE sample buffer to a concentration of 1 mg/ml, and 5 μg of total protein was loaded for SDS/PAGE unless otherwise indicated.

Northern blotting

For Northern blots, total RNA was isolated from whole conjunctival or corneal tissue with TRI-REAGENT (Molecular Research Center, Cincinnati, OH, U.S.A.) and 25 μg portions were electrophoresed on 1% formaldehyde/agarose gels. Resolved RNAs were transferred to ZetaProbe positively charged nylon membranes (Bio-Rad, Hercules, CA, U.S.A.) and cross-linked using a Stratalinker (Stratagene, La Jolla, CA, U.S.A.). The membranes were prehybridized for at least 2 h at 42°C in prehybridization solution (50% formamide, $5\times\text{SSC}$, $5\times\text{Denhardt's reagent}$, 0.1% SDS and 0.5 mg/ml salmon sperm DNA). The probe A2G2-#9, which spans the 5' unique region of ASGP-1 cDNA, was random-prime-labelled with [^{32}P]dCTP according to the protocol of a Random Primed Labeling kit (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). The membranes were hybridized overnight at 42°C in prehybridization solution containing 0.1 g/ml dextran sulphate and the labelled probe. Following hybridization, membranes were washed once at room temperature in $2\times\text{SSC}/0.1\%$ SDS for 15 min, twice at 50°C in $2\times\text{SSC}/0.1\%$ SDS for 20 min each, and once at 50°C in

Table 1 Antibodies used in the present study

Name	Type	Epitope sequence or location	Cross-reactivity	Original reference
Anti-ASGP-2	Polyclonal	Total protein	Rat	25
Anti-C-pep	Polyclonal	SMNKFSYPDSEL	Rat	21
HA-1	Polyclonal	AGYRPPRPAWTFGD	Rat/human	Unpublished*
4F12	Monoclonal	First 53 amino acids of ASGP-2	Rat	21
13C4	Monoclonal	N-gly-1/Cys-rich domain of ASGP-2	Rat	21
CT-1	Monoclonal	SSLSYTNPAVAATSANL	Rat/multiple species	33
AM-3	Monoclonal	Goblet cell mucin protein (rabbit ocular mucin)	Rat/human/rabbit/pig	32

* E. A. Rossi, S. A. Price-Schiavi and K. L. Carraway, unpublished work.

0.1 × SSC/0.1 % SDS for 15 min. Signals were detected by exposure to Kodak XAR-5 X-ray film.

Western blotting

For Western blots, SDS/PAGE was performed under reducing conditions using 6 % polyacrylamide gels and the mini-Protein II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes, which were blocked with 5 % (w/v) non-fat dry milk in Tris-buffered saline/0.5 % Tween 20. After a 1 h incubation in primary antibody diluted in 1 % BSA/Tris-buffered saline/0.5 % Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG Fc specific (Pierce, Rockford, IL, U.S.A) diluted 1:20000 in 1 % BSA/Tris-buffered saline/0.5 % Tween 20. Signals were detected with the Renaissance Enhanced Chemiluminescence kit (DuPont NEN, Boston, MA, U.S.A).

Immunoprecipitation

For immunoprecipitation, corneal and conjunctival tissues were solubilized in RIPA buffer (150 mM NaCl, 1 % Nonidet P-40, 0.5 % deoxycholate, 0.1 % SDS, 50 mM Tris/HCl, pH 8.0), homogenized with a probe sonicator, and centrifuged at 2000 g for 10 min to clarify the sample. The supernatant (1 ml) was added to Protein A-agarose with bound antibody [21]. The samples were rotated overnight at 4 °C, washed six times in RIPA buffer for 10 min each, and resuspended and boiled in 50 µl of SDS/PAGE sample buffer. Immunoprecipitated proteins were subjected to SDS/PAGE and anti-ASGP-2 immunoblotting.

Immunofluorescent staining

Eyes, including the eyelids, were dissected from adult Sprague-Dawley rats (Charles River, Wilmington, MA, U.S.A), embedded in OCT (Tissue-Tek, Sakura Fine TEK, Torrance, CA, U.S.A.) and frozen in liquid nitrogen, and 6 µm thick frozen unfixed sections were prepared with a cryostat. The sections were incubated with goat serum in PBS (1:1000) to prevent non-specific staining. Following two 5 min rinses with PBS (pH 7.4), the sections were incubated with primary antibody for 2 h. Sections were washed twice with PBS for 5 min each followed by incubation in FITC-conjugated secondary antibody, goat anti-mouse IgG (1:100) or goat anti-rabbit IgG (1:100) (Caltag Laboratories, San Francisco, CA, U.S.A). Sections were washed with PBS, mounted with slow fade mounting solution (Molecular Probes, Eugene, OR, U.S.A), and photographed under a Zeiss AxioPhot fluorescence microscope.

Analysis of corneal and conjunctival rinses

Corneal and conjunctival tissues were dissected from six rats and rinsed sequentially with 1 × PBS (twice), 2 × PBS (twice), 1 M NaCl in 1 × PBS (twice) and 0.05 % Triton-X 100 in 1 × PBS (twice). Sequential rinses were performed by adding 1 ml of the solution to a 2 ml centrifuge tube containing the dissected tissue, gently agitating for 1 min, and collecting the solution. All rinse samples were concentrated to 0.1 ml by centrifugation in Centricon 50 kDa cut-off filters (Amicon, Beverly, MA, U.S.A). The volumes were brought to 2 ml with 0.05 % Triton-X 100 in 1 × PBS, and the concentration cycle was repeated twice. The remaining rinsed tissue was solubilized in RIPA buffer, and the tissue lysate was cleared by centrifugation at 2000 g for 10 min. All samples were immunoprecipitated with anti-ASGP-2, and the precipitates were subjected to immunoblot analysis with mAb 4F12. Alternatively, an aliquot of concentrated rinse solution was collected and diluted 1:1 (v/v) in SDS sample buffer; all samples were subjected to immunoblot analysis with mAb 4F12 without immunoprecipitation.

Collection of tear samples

Normal rat tears were collected with TRANSORB wicks R-15643 (Filtrona Richmond Inc., Richmond, VA, U.S.A). The wicks were pre-soaked overnight in PBS, placed in a pipette tip and then in a 1.7 ml Eppendorf centrifuge tube, and centrifuged at 1000 g for 1 min. The pre-moistened wicks were placed in contact with the surface of the eyes of anaesthetized rats for 10 s to absorb tear fluid. After absorption of the tear fluid, the wicks were placed into pipette tips and then into 1.7 ml Eppendorf centrifuge tubes and centrifuged at 1000 g for 1 min. Collected tear fluid was diluted 1:1 (v/v) with SDS sample buffer and boiled for 5 min prior to SDS/PAGE.

RESULTS

Expression of SMC in rat corneal and conjunctival tissues

To determine whether the ocular surface epithelia express SMC, total RNA was isolated from whole corneal and conjunctival tissue and subjected to Northern blot analysis with probe A2G2-#9, which spans the 5' unique region of SMC cDNA. Total RNA from mammary epithelial cells, which express SMC message at relatively high levels, was used as a positive control for hybridization. SMC transcript was present in abundance in corneal tissue, but was found at a much lower level in conjunctival tissue,

Figure 2 Detection of SMC transcript in normal rat cornea and conjunctiva

Total RNA was isolated from normal rat cornea and conjunctiva, and 25 μ g portions were subjected to Northern blot analysis with probe A2G2-#9. (A) Detection of SMC transcript in normal rat cornea and conjunctiva; (B) ethidium bromide-stained agarose gel of blot shown in (A) for loading comparison. Normal mammary RNA was used as a positive control.

confirming that SMC is expressed in normal ocular surface tissues (Figure 2).

To analyse SMC protein levels in ocular surface tissues, corneal and conjunctival tissue lysates were subjected to immunoblot analysis with mAb 4F12, which recognizes the transmembrane subunit ASGP-2. A mammary epithelial cell lysate (Figure 3A) was included as a positive control. ASGP-2 (SMC) was stained heavily in corneal samples and more faintly in conjunctival samples, confirming that SMC protein is present in both tissues (Figure 3A). Furthermore, SMC protein and transcript levels appear to correlate directly in these tissues, as both SMC transcript and protein stain strongly in corneal tissue and weakly in conjunctival tissue. Interestingly, the ASGP-2 subunit in normal rat conjunctiva appears to be larger than those in normal rat corneal and mammary epithelial cells. This may be due to a difference in glycosylation that causes conjunctival ASGP-2 to migrate more slowly in the gel than mammary epithelial or corneal ASGP-2.

To confirm that both SMC subunits ASGP-1 and ASGP-2 are present in ocular surface tissue, the corneal and conjunctival cell lysates were subjected to immunoblot analysis with polyclonal antibody HA-1, which recognizes a peptide in the C-terminal region of ASGP-1 (SMC). For this immunoblot, a 13762 ascites tumour cell lysate was included as a positive control. ASGP-1 was stained heavily by the HA-1 antiserum in the ascites cell and corneal cell samples, confirming that both subunits of SMC are present in the normal rat cornea (Figure 3B). ASGP-1 was not detected in normal rat conjunctiva by immunoblotting with the HA-1 antiserum, although it could be detected by immunofluorescence.

Localization of SMC in normal rat cornea and conjunctiva

There appears to be a large difference in the amounts of SMC protein expressed in normal rat corneal and conjunctival tissues. This difference could be due to actual differences in the amount

Figure 3 Detection of SMC (ASGP-1 and ASGP-2) protein in normal rat cornea and conjunctiva

Tissue lysates were prepared from whole, dissected corneal and conjunctival tissues and subjected to SDS/PAGE and immunoblot analysis with (A) anti-ASGP-2 mAb 4F12 or (B) anti-ASGP-1 polyclonal HA-1 antiserum. A normal mammary epithelial cell (MEC) lysate was included as a positive control for (A), and a 13762 ascites cell lysate was included as a positive control for (B).

of SMC protein in the two tissues, or to dilution of SMC signal by the presence of other cell types in the tissue preparations, since the epithelia were not isolated. Cross-sections of rat eyes (lid plus globe) were analysed by immunofluorescent staining to determine the location of SMC and possible reasons for differences in apparent SMC protein levels between corneal and conjunctival tissues. Sections were labelled with antibodies HA-1, CT-1 (which recognizes the C-terminal peptide of Muc-1) and AM-3 (which recognizes a goblet-cell-specific mucin) [32]. In cornea and conjunctiva, SMC was localized in the whole epithelial layer, with stronger staining towards the most superficial cell layers (especially apparent in the cornea) (Figure 4). SMC staining in the corneal and conjunctival epithelia appeared to be of similar intensity, suggesting that these two epithelial layers produce similar amounts of SMC protein. Thus the apparent differences in SMC protein levels between cornea and conjunctiva on immunoblots are probably due to dilution of the signal in the conjunctival samples by other non-SMC-expressing cell types, including the goblet cells.

The pattern of SMC staining in corneal and conjunctival epithelia was different from that of Muc-1, which is also localized throughout the epithelial layer, but whose strongest staining is close to the basement membrane and not the superficial cell layers (Figure 4). Neither SMC nor MUC1 was found in conjunctival goblet cells. When stained with HA-1, the stratified epithelial cells stained strongly and conjunctival goblet cells were

Figure 4 Immunofluorescent staining of SMC in normal rat cornea and conjunctiva

Cross-sections of normal rat eyes were subjected to immunofluorescent staining with anti-ASGP-1 polyclonal antiserum HA-1, anti-Muc-1 mAb CT-1, or mAb AM-3 against goblet-cell-specific mucin, as indicated on the left. The corneal and conjunctival epithelial layers are indicated. The space between the cornea and conjunctiva is a result of the eye dissection. The apical surfaces of the corneal and conjunctival epithelia are indicated by arrows on the right.

Figure 5 Analysis of SMC forms in rat corneal and conjunctival tissues

RIPA-buffer-solubilized corneal and conjunctival tissues were subjected to serial immunoprecipitation (Ip) with polyclonal anti-C-pep and polyclonal anti-ASGP-2 antisera. Tissue lysates were immunoprecipitated with anti-C-pep antisera twice to clear the lysate of the membrane form of SMC, which contains the C-terminal peptide recognized by anti-C-pep. The supernatant was then immunoprecipitated with anti-ASGP-2 antiserum to precipitate any remaining soluble SMC that was not recognized by the anti-C-pep antibodies (because it lacks the C-terminal peptide recognized by anti-C-pep). These immunoprecipitates were analysed by immunoblotting with anti-ASGP-2 mAb 4F12. Abbreviation: NRS, non-immune rabbit serum (negative control).

clearly negative. However, conjunctival goblet cells but not the stratified corneal epithelium stain strongly when stained with AM-3, an antibody that specifically recognizes conjunctival goblet cell mucin [32]. Thus SMC protein is expressed throughout both corneal and conjunctival epithelia, and its distribution is different from those of MUC1 or goblet-cell-secreted mucin.

Figure 6 Localization of the membrane form of SMC in normal rat cornea and conjunctiva

Cross-sections (globe plus lid) of normal rat eyes were subjected to immunofluorescent staining with anti-C-pep polyclonal antiserum. Corneal and conjunctival epithelial layers are indicated on the right. Magnification: top and middle panels, $\times 65$; bottom panel, $\times 260$.

Characterization of SMC forms in rat ocular surface tissues

Corneal and conjunctival tissue lysates were subjected to serial immunoprecipitation analysis with anti-C-pep and anti-ASGP-2 polyclonal antisera to analyse the isoforms of SMC (soluble and membrane-bound) present in ocular surface tissue. Anti-C-pep recognizes the C-terminal peptide of ASGP-2 (SMC), which is not present in the soluble form. Corneal and conjunctival lysates were immunoprecipitated with anti-C-pep antibody to remove membrane-bound SMC. Any unprecipitated membrane SMC was cleared by re-immunoprecipitating the supernatant with anti-C-pep antiserum. Soluble SMC, which is not recognized by anti-C-pep, was immunoprecipitated from the membrane-SMC-cleared supernatant by immunoprecipitation with anti-ASGP-2 polyclonal antiserum. A considerable amount of membrane SMC was precipitated from both corneal and conjunctival tissues (Figure 5). After clearance of membrane SMC from these tissues, a significant amount of soluble SMC was precipitated from both tissues, indicating that corneal and conjunctival epithelia produce both soluble and membrane-bound forms of SMC in similar relative amounts.

Localization of the membrane-bound form of SMC on rat ocular surface tissues

In order to act as a protective barrier for the ocular surface, membrane-bound SMC must be localized at least at apical surfaces of the cornea and conjunctiva. SMC was detected throughout the epithelial layers of both cornea and conjunctiva by HA-1 (Figure 4), which recognizes both the membrane and soluble isoforms. To study the distribution of membrane SMC in

Figure 7 Analysis of SMC in normal rat tears

Normal rat tears were collected by absorption to a TRANSORB wick, diluted 1:1 (v/v) in SDS sample buffer, and subjected to immunoblot analysis with anti-ASGP-2 mAb 13C4. An ascites cell lysate was included as a positive control.

Figure 8 Analysis of corneal and conjunctival rinses

Corneal and conjunctival tissues were rinsed as described in the Experimental section. The rinse samples were analysed by immunoprecipitation followed by immunoblotting with anti-ASGP-2 mAb 4F12.

corneal and conjunctival epithelia, cross-sections of normal rat eyes (globe plus lid) were subjected to immunofluorescent staining with anti-C-pep antiserum. Membrane SMC was stained in all layers of the corneal and conjunctival epithelia, as well as the lid margin mucosal surface, but not the cutaneous surface epithelium (Figure 6). Membrane SMC was found to be expressed on the apical surface of the corneal and conjunctival epithelia and on membrane projections from the apical corneal surface. Thus the membrane form of SMC is localized on the ocular surface, where it may form part of the protective glycocalyx.

Analysis of SMC in normal rat tears

Because SMC can be produced in a soluble form, it may also be present in the tear fluid. To determine if normal rat tears contain SMC, tear fluid was collected from rat eyes by absorption to a

TRANSORB wick and subjected to immunoblot analysis with anti-ASGP-2 mAb 13C4. A 13762 ascites cell lysate was included as a positive control. Both antibodies stained SMC strongly, indicating that both SMC subunits are present in normal rat tears (Figure 7).

Analysis of corneal and conjunctival rinses

When corneal and conjunctival tissues were subjected to a sequential rinsing protocol, substantial amounts of SMC were removed by the mildest rinse, particularly from the cornea (Figure 8). Additional SMC, possibly including some of the membrane form, was removed by a mild Triton X-100 treatment. However, even the Triton treatments failed to remove all of the SMC, particularly in the case of the conjunctiva (Figure 8). These results demonstrate the presence of SMC in an adsorbed form as part of the apical surface glycocalyx, providing a protective layer at the apical surface of the vulnerable corneal epithelium.

DISCUSSION

Although mucins have been recognized as components of the ocular surface for many years [4], information about the mucins of the ocular surface and tear film is still rather limited. Many analyses of mucin expression in tissues are performed with nucleic acid probes, which determine the cellular source of the mucins, but not their ultimate location. Such studies have shown the expression of the *MUC4* and *MUC5AC* genes, encoding secreted mucins, by human conjunctival epithelia [4,17]. *In situ* hybridization showed *MUC4* transcripts in the stratified conjunctival epithelium and *MUC5* transcripts in conjunctival goblet cells. These analyses are consistent with previous observations of conjunctival goblet cells as a primary locus of synthesis of secreted mucins [4]. However, they do not provide information about the roles of these mucins in the tear film or at the ocular surface. Even less is known about membrane mucins at the ocular surface. Previous studies have shown that *MUC1* is produced by both corneal and conjunctival epithelia [18]. By immunohistochemistry this mucin is localized primarily in the basal cell layers of the stratified conjunctival epithelia, although it also appears at the conjunctival apical surfaces and along the apical surfaces of the corneal epithelium.

In the present studies we have concentrated on SMC, which was originally described as a membrane mucin of the 13762 mammary ascites tumour [26], but has more recently been observed in both membrane and soluble forms in numerous simple epithelia [22]. At the ocular surface SMC is expressed by both corneal and conjunctival epithelia, as indicated by Northern and Western blot analyses. By immunofluorescence SMC is observed throughout these two stratified epithelia, although it is more heavily concentrated in the superficial layers. This location contrasts with that of *MUC1*, which appears to be more abundant in the basal layers, as reported previously [18] and shown here in Figure 4. Thus ocular surface epithelia represent the first example of tissues in which *MUC1* and SMC may have distinctly different functions based on their locations. SMC is produced by ocular surface epithelia in both soluble and membrane-bound forms. The membrane form is most abundant in the superficial epithelial layers and is observed by immunofluorescence at apical surfaces, supporting its protective role. Soluble SMC can be removed from the ocular surface by gentle rinsing, as previously reported for the uterus [35] and trachea [36], suggesting that it contributes, along with the membrane form, to the formation of an apical surface glycocalyx as part of the mucin protective layer of these two ocular surface epithelia [22]. SMC is also found as a

component of the tear fluid, and presumably along with other gel-forming and secreted mucins contributes to the physical properties of the fluid.

These results permit us to propose a molecular model for the role of SMC at the ocular surface. In this model, membrane SMC provides a direct barrier as a component of the plasma membrane of the ocular surface epithelial cells and presumably of the glycocalyx associated with the apical surface. In addition, soluble SMC, possibly produced by the same epithelial cells, is adsorbed to the cell surface as a second component of this glycocalyx. Whether other mucin components are part of the glycocalyx remains to be resolved. SMC is also a component of the tear fluid, along with other mucins. The source of this SMC is uncertain. It could be desorbed from the glycocalyx, as part of the turnover of the cell surface protective barrier. Alternatively, it could be produced in the lacrimal glands, along with other components of the tear fluid [1]. Our data indicate that SMC is not produced by conjunctival goblet cells.

A second important role of SMC could be in stabilizing the tear film. The stability of this film is achieved due to its unique composition and structure. The first description of the tri-layered tear film envisioned a sandwich of aqueous fluid between lipid and mucus layers [37]. Surface tension measurements of mucin and lipid mixtures led to the proposal of a multi-layered tear film, including an adsorbed mucin layer, a mucin coacervate, a dilute mucin aqueous layer and a mucin-lipid boundary layer [38]. Experiments using optical methods estimate a 10–30 μm tear film (the depth being species dependent) [39,40]. Moreover, ultrastructural analysis following *in vivo* cryofixation suggests a 2–6 μm mucin layer [3] and that a pure aqueous layer may not be present. Dilly [41] has suggested that membrane-attached cell surface mucin from subsurface vesicles of superficial epithelial cells aid in stabilizing the tear film by binding goblet cell mucin in the overlying aqueous layer. Our model is consistent with these observations and interpretations, and provides a molecular identification of one of the components contributing to this tear film-epithelial layer interface. Interestingly, there is a striking similarity between the appearance by electron microscopy of cell surface mucin on microvilli at the ocular surface [41] and that of SMC on the microvilli of ascites tumour cells [42].

Although little is known about the structure and composition of the precocular tear film, even less is known about the differentiation-dependent biosynthesis, glycosylation, intracellular processing and secretion of mucins by the ocular surface epithelium. Herein, we have shown that SMC is a mucin component of the tear film, and we believe that additional studies of its production and expression should help to delineate further its roles in ocular surface protection and tear film stability.

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