Human Corneal and Conjunctival Epithelia Produce a Mucin-like Glycoprotein for the Apical Surface

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Purpose. The authors have determined that the corneal and conjunctival epithelia of the rat produce a mucin-like glycoprotein at the apical surface of the epithelium. The purpose of this study was to determine if human ocular surface epithelium produces similar glycoproteins.

Methods. Because our initial attempts at production of monoclonal antibodies yielded blood type A-specific antibodies, corneal epithelia from blood type O donor eyes were used for the production of monoclonal antibodies. Screening of hybridoma products was accomplished by immunofluorescence microscopy of cryostat sections of blood type O donor eyes. The monoclonal antibody produced was used for immunofluorescence microscopy and immunoelectron microscopy to determine tissue and cellular distribution, respectively. Immunoblot analysis of SDS–PAGE-separated proteins from corneal epithelial tissue and from cultured human corneal epithelium was used to determine molecular weight range and epitope binding after periodate oxidation, N-glycanase, and O-glycanase treatment.

Results. A monoclonal antibody, designated H185, that binds to apical cell layers of human corneal, conjunctival, laryngeal, and vaginal epithelium was produced. The antibody binds to apical membranes of apical cells, particularly at the tips of microvilli. In subapical cells, the antibody binds to small cytoplasmic vesicles. Cultured human corneal epithelium produces H185 antigen. By immunoblot analysis, H185 binds a high molecular weight protein, >205 kD, from corneal epithelium and cultured corneal epithelium. The protein band visualized by immunoblot analysis cannot be stained by Coomassie or silver stain on SDS–PAGE, but it does stain with Alcian blue followed by silver stain, indicating that the protein is highly glycosylated. H185 binding to blotted proteins is destroyed by sodium periodate treatment and O-glycanase incubation, suggesting that the epitope of H185 is an O-linked carbohydrate.

Conclusion. Human corneal and conjunctival epithelia produce a molecule, similar in size, cellular localization, and distribution to the mucin-like glycoprotein of rat ocular surface epithelium. These data suggest that the entire ocular surface epithelium produces mucins for the tear film. Invest Ophthalmol Vis Sci. 1995;36:337–344.

The presence of a stable tear film is essential to provide a smooth refractive surface for the corneal epithelium. To be stable, the tear film with its three major components, lipid, aqueous, and mucus, must maintain an intimate association with the apical cells of the ocular surface epithelium. Little is known about how the tear film associates with membranes of the apical cells of the epithelium. This relationship must be a dynamic one because apical cells are shed from the surface. Several reports have shown that the glycocalyx of the apical membrane of the ocular surface is rich in polyanions and in carbohydrate moieties; however, specific information about the molecular nature of the cell–tear film interface is not available. Clarifying the molecule(s) that play a role in the maintenance of a stable tear fluid may lead to a better understanding of ocular surface disease resulting from dry eye syndrome.

Recently, we reported the production of a monoclonal antibody, R339, that binds to apical cell mem-

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branes of the ocular surface epithelium of the rat. Immunelectron microscopic study has shown that the antigen recognized by the antibody is present along the apical–microvillar membrane in the region of the glycocalyx. In subapical cells, the antigen is in cytoplasmic vesicles. The antigen appears to be stored along the membrane of cytoplasmic vesicles and then moves to the apical membrane of the ocular surface cells when cells differentiate to the apical-most position. The antigen is prominent at the tips of microplacae, where it interface with the mucin layer of the tear film. It is hypothesized that the antigen recognized by R339 antibody facilitates tear-film spread over the surface of the epithelium. R339 antigen has been isolated and shown to have mucin-like characteristics (manuscript submitted for publication, 1994). Approximately 60% of the mass of the molecule is carbohydrate. Carbohydrate and amino acid analyses indicate high levels of N-acetyl galactosamine and serine and threonine, respectively. R339 antibody is a useful research tool; however, because it is species specific, its usefulness in the study of the human ocular surface epithelium is limited.

To begin to investigate the specific molecular composition of the apical membrane of the human ocular surface epithelium, we developed a monoclonal antibody against the antigen expressed in the apical membrane of human ocular surface epithelium. In this article, we demonstrate the development of a human-specific antibody, designated H185, that has characteristics similar to those of R339, which binds to apical cells of human ocular surface epithelium. We describe several characteristics of the antigen recognized by this antibody and report that the antigen can be produced by cultured human corneal epithelium as well as by native tissue.

MATERIALS AND METHODS

All investigations conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the tenets of the Declaration of Helsinki.

Monoclonal Antibody Production

Preparation of Immunogen and Immunization. Previous attempts to produce monoclonal antibodies to mucin-like molecules produced by ocular surface epithelium yielded blood type A-specific antibodies. To obtain antibodies that recognize all blood types, corneal epithelial cells were obtained by debridement of epithelium from eye bank eyes of donors with blood group type O. Eyes were obtained less than 24 hours after donor death. The epithelial cells were resuspended in equal amounts of phosphate-buffered saline (PBS) and Ribi adjuvant system (RIBI Immunochem Research, Hamilton, MT). The human corneal epithelial cell suspension was injected intraperitoneally into 6-week-old BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME).

Cell Fusion and Hybridoma Cloning. Cell fusion was performed 4 days after the second boost, as previously described. The supernatant of growing cells was screened on cryostat sections of human corneas of blood group type O by immunofluorescence microscopy to choose hybridomas of interest. Hybridomas showing apical cell binding by immunofluorescence microscopy were cloned by limiting dilution (0.5 cell/well) two consecutive times. The clones were similarly screened, and clone H185.4.6, herein referred to as H185, was chosen for further characterization. H185 was concentrated from tissue culture medium by ammonium sulfate precipitation. The isotype was checked using the Pierce Immunopure Monoclonal Antibody Isotyping Kit I (Pierce, Rockford, IL) and found to be IgG1, with kappa light chain.

Tissue Preparation and Cell Culture

Normal human tissues were supplied by the National Disease Research Interchange (NDRI, Philadelphia, PA). They were obtained less than 24 hours after donor death and were frozen in OCT compound (Miles, Elkhart, IN). Human corneal epithelial cell cultures were derived from limbal epithelium of discarded rim of donor corneas used for penetrating keratoplasty. Cells were cultured as described by Lindberg et al. Primary cultures were propagated on irradiated 3T3 fibroblasts. Cells at passages 3 to 6 were used for H185 characterization.

Immunohistochemistry and Immunelectron Microscopy

Immunofluorescence microscopy was performed as previously described. Briefly, unfixed 6-μm cryostat sections of human cornea and conjunctiva of A, B, and O blood groups were incubated for 1 hour at room temperature with H185. Sections were washed with PBS and incubated for 1 hour at room temperature with fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson Immuno Research, West Grove, PA). Sections were then washed with PBS and coverslipped using a mounting medium containing PBS, glycerol, and paraphenylenediamine. In addition, cryostat sections of human larynx, vagina, skin, esophagus, trachea, buccal mucosa, large intestine, bladder, ileum, liver, and mammary gland were screened by immunofluorescence for H185 binding. To check for cross-reactivity with other species, cryostat sections of corneas of owl monkey, calf, sheep, rat, and rabbit were similarly screened for binding of H185. Cultures of human corneal epithelium were fixed in ice-cold methanol and rinsed with PBS before the usual immunofluorescence protocol to check for
H185 binding. Negative control tissue sections (primary antibody omitted) were run with each experiment. Sections were viewed and photographed on a Zeiss Photomicroscope III (Zeiss, Oberkochen, Germany) equipped for epilumination.

**Immunoelectron Microscopy.** Post-embedding immunoelectron microscopic localization of H185 on fixed (0.2% glutaraldehyde, 4% paraformaldehyde, 0.1 M phosphate buffer) and LR White (Ernest F. Fullam, Latham, NY)-embedded human cornea, conjunctiva, and cultured human corneal epithelium was performed as previously described, except that the secondary antibody used was goat anti-mouse IgG conjugated to 10-nm gold (Ted Pella, Redding, CA). Binding of H185 to human ocular surface epithelium in the LR White-fixed samples was confirmed by immunofluorescence microscopy.

**Electrophoresis and Immunoblotting**

Corneal epithelium debrided from human eye bank eyes and cultured human corneal epithelium were solubilized in 2% sodium dodecyl sulfate (SDS). Protein concentration was determined using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL). The proteins were then diluted with Laemmli sample buffer (containing SDS, β-mercaptoethanol) and run on 6% separating, 4% stacking SDS-polyacrylamide gel electrophoresis (PAGE) using the Laemmli buffer system. After SDS–PAGE, gels were stained with Coomassie blue, silver stain, periodic acid-Schiff, or by a modification of an Alcian blue–silver staining method. For immunoblotting, proteins were electrophoretically transferred to nitrocellulose and probed for reactivity with H185, as previously described.

**Periodate Treatment**

Periodate Oxidation of Immunoblots. To examine if the H185 epitope is carbohydrate, proteins transferred onto nitrocellulose membranes were pretreated with varying concentrations (0, 0.1, 1, 5, 10, and 20 mM) of sodium periodate in 50 mM sodium acetate buffer, pH 4.5, for 1 hour at room temperature in the dark. After rinses in 50 mM sodium acetate buffer, the membranes were incubated in 50 mM sodium borohydride in PBS for 30 minutes at room temperature. After further rinsing in PBS, the membranes were probed with H185 as usual.

**Enzyme Treatment**

N-glycanase Treatment. To determine if the carbohydrate epitope of H185 is O-linked, denatured corneal protein was digested with neuraminidase (Genzyme, Cambridge, MA) followed by O-glycanase (Genzyme, Cambridge, MA) according to manufacturer's protocols. To determine if digestion removed H185 binding, immunoblot analysis was used. Cultured human corneal epithelial cells were solubilized in 15 mM sodium phosphate buffer, pH 7.5, with 0.2 mM phenylmethylsulfonyl fluoride. After centrifugation at 3000 rpm for 30 minutes to spin down any insoluble components, the supernatant was denatured and NP-40 was added to protect the enzyme as above for N-glycanase treatment; 10 mM sodium acetate and 1 U neuraminidase was added, and the sample was incubated for 60 minutes at 37°C. After this, 4 mU of O-glycanase was added and incubated overnight at 37°C. As controls, one sample was treated with neuraminidase alone and a second was treated with heat-inactivated O-glycanase after neuraminidase digestion. The reactions were stopped by the addition of SDS-reducing sample buffer, and samples were run on 6% SDS–PAGE for analysis by immunoblotting with H185.

**RESULTS**

**Immunolocalization**

By immunofluorescence microscopy, we observed that the binding of H185 was to all the flattened cell layers, or squames, along the entire ocular surface epithelium of the cornea (Fig. 1B). In the conjunctiva (Fig. 1C), binding was intense along the apical surface of the epithelium and extended down four cell layers. Binding ended abruptly at the lid margin at the junction with keratinized epidermal epithelium (Fig. 1C, inset). In conjunctiva in a portion of the specimens, the goblet cells also bound the antibody (Fig. 1C). This finding was not consistent between tissue samples. No binding of H185 was observed in basal cells in either the cornea or the
conjunctiva. In comparison with our previous attempts at making human-specific antibodies, which were blood type A specific, the binding of H185 showed the same pattern in human corneal epithelium regardless of blood group (A, B, or O). Binding of H185 was not found in other species tested (owl, monkey, rabbit, rat, cow, sheep) (data not shown). Interestingly, as in rat studies with R339 antibody, H185 binding was observed in human vaginal and laryngeal epithelium. It was present along the apical surface and cell layers below the apical cells, similar to that in ocular surface epithelium (Figs. 1E, 1F). Other epithelia found to be devoid of binding include buccal mucosa, esophagus, ileum, colon, mammary epithelium, and liver. H185 binding was seen in several human glandular tissues, including eccrine sweat glands and submucosal glands of the esophagus and large intestine (data not shown).

By immunoelectron microscopy, binding was on the outer apical membrane adjacent to the tear film (Fig. 2A). Binding was prominent at the tips of the microvilli (Figs. 2A, 2B). In the squames below the apical-most
FIGURE 2. Immunoelectron microscopic localization of H185 in human corneal epithelium in situ (A, B) and in cultured human corneal epithelium (C). In (A), tissue has not been counterstained with lead or uranyl acetate to demonstrate more clearly the gold particles that indicate sites of H185 binding. (B) Binding on counterstained tissue. Note that in subapical cells, binding is primarily associated with cytoplasmic vesicles (small arrows), whereas in apical cells, binding is particularly prevalent along the tips of the microplicae (large arrows). Cultured cells in (C) show intense binding to microplicae and to the periphery of cytoplasmic vesicles. Note also the two adjacent cells (1 and 2) vary in the degree of H185 binding. (Inset) H185 binding material in the lumen of a large vacuole suggests that H185 is secreted from the cell membrane. H185 binding could also be detected in the culture media by immunoblot analysis. Bars = 0.2 μm.
FIGURE 3. Immunoblot analysis of H185 binding to proteins of debrided human corneal epithelium from blood type O (lane 1) and blood type A (lane 2) proteins. (lane 3) Immunoblot of proteins from cultured corneal epithelium. (lane 4) Alcian blue–silver stain of the SDS–PAGE of proteins from cultured corneal epithelium, indicating a highly glycosylated molecule in the same molecular weight range as the immunoreactive band. (arrow) 205-kD molecular weight.

cells, the antigen was present and was associated with small vesicles in the cytoplasm (Fig. 2B).

In cultures of human corneal epithelium derived from limbal tissue, islands of cells develop within cultures. These islands are stratified, and it is the apical cells of the islands that bind H185 (Fig. 1D). Immunoelectron microscopy of H185 on these cultured cells demonstrates binding to cytoplasmic vesicles, as well as to the tips of microvilli on the cell membrane (Fig. 2C). Binding in the cell cultures was more intense with less scatter than in tissue samples (Figs. 2A, 2B). This may be due to the fact that cultured cells are fixed immediately, whereas tissue was obtained up to 24 hours after donor death. Scatter in tissue may be due to postmortem degradation. Cells within the culture show varying degrees of binding of H185 (Fig. 2C) and within large vacuoles within cultures, H185 binding material was present. This observation indicates that H185 is secreted or shed from cell surfaces.

Electrophoretic Mobility and Immunoblots

H185 reacted with a prominent band with a molecular weight greater than 205 kD (Fig. 3). No staining was observed in the area of the immunoreactive band with either Coomassie blue or silver staining of the gel. PAS staining (data not shown), as well as Alcian blue followed by silver staining (Fig. 3, lane 4), stained a band in the region of molecular weight similar to that of the immunoreactive band. These data suggest that the antigen is a highly glycosylated glycoprotein. A similarly reactive band was found in cell-free, spent culture media from corneal epithelial cultures.

Periodate Incubation

To determine whether the epitope recognized by H185 is to the carbohydrate portion of the glycoprotein, we used sodium periodate oxidation on blots of proteins from both native and cultured human corneal epithelium (Fig. 4). There was a significant decrease in the binding of H185 at a concentration of 0.1 mM sodium periodate and a complete loss of binding at concentrations of 1 mM or more of sodium periodate. These data indicate that the epitope recognized by H185 is carbohydrate.

Enzyme Treatments

To determine whether the epitope of the antigen recognized by H185 is an N- or O-linked carbohydrate, we assessed the binding of H185 to blots of proteins solubilized from cultured human corneal epithelium treated with either N- or O-glycanase. N-glycanase treatment had no effect on the binding of H185, indicating that the carbohydrate epitope recognized by H185 is not N-linked to the protein core (Fig. 5A).

In contrast, H185 binding was lost after O-glycanase treatment (Fig. 5B). This result indicates that the carbohydrate epitope recognized by H185 is O-linked to the protein core, indicative of mucins.

DISCUSSION

Data obtained through the use of H185 antibody indicate that, as in the rat,9 the stratified squamous epithelia of the human ocular surface produces mucin-like glycoproteins for the apical surface–tear film interface. The H185 antigen has an identical cellular and tissue distribution to the rat R339 antigen. Both H185 and R339 antibodies bind to the flattened apical squamous cells; they both bind to cytoplasmic vesicles in subapical squames and

FIGURE 4. Periodate oxidation of nitrocellulose-blotted SDS–PAGE-separated proteins from both debrided corneal epithelium (CE) and cultured human corneal epithelium (CHCE). Higher concentrations of sodium periodate completely removed the epitope to which H185 binds. The 205 indicates molecular weight marker (MWM), and mM concentrations indicate the amount of sodium periodate used.
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FIGURE 5. Immunoblot analysis of N-glycanase-treated (A) and O-glycanase-treated (B) proteins from cultured human corneal epithelium. (A) Active N-glycanase treatment (lane A) did not destroy H185 binding more than heat-inactivated enzyme (lane In). Untreated control (lane Un) is an equivalent sample incubated for a comparable length of time without the added enzyme aliquot. (B) Active O-glycanase treatment proceeded by neuraminidase treatment (lane A) destroyed H185 binding, whereas head-inactivated O-glycanase did not (lane N). Neuraminidase alone did not destroy H185 binding (lane Ne). Untreated control (lane Un) is an equivalent sample incubated a comparable length of time without enzyme additions or boiling after enzyme additions. Diminutions in binding in lanes NE and In may reflect degradation due to boiling. MWM also contains untreated control proteins, as seen above the 205-kD protein.

to the apical membranes of the apical cells. Both bind prominently to the tips of the microvilli of the zone of the glycocalyx. H185 and R339 antigen are of similar molecular weight, and both stain with PAS or Alcian blue followed by silver stain. The R339 antigen has been isolated and has been shown to have an approximately 60% carbohydrate:40% protein ratio, placing the molecule in the mucin class of glycoproteins (manuscript submitted for publication, 1994). Although H185 has not been isolated to the purity that allows such assay, the similarity in cellular localization, size, and H185 epitope removal by O-glycanase suggests that H185, like R339, is a mucin.

The past few years has seen significant progress in understanding the molecular nature of mucins. To date, seven different mucins have been cloned. One of these mucins, designated MUC-1, has been reported to be present in the stratified squamous epithelium of the rat vagina.  This so-called "epithelial" mucin is the only mucin cloned that has a membrane-spanning region in its protein backbone.  Mucins 2 to 7 are "secreted" mucins and have only been demonstrated in simple epithelia. MUC-1 may be a candidate mucin for the H185 antigen. The identification of R339 and H185 as one of the cloned mucins, or a unique mucin, awaits molecular cloning.

The data from studies of the rat and now human suggest that, besides goblet cells, the entire ocular surface epithelium, including both conjunctival and corneal epithelia, produce mucins. The observation that the corneal epithelium produces the mucin is, to our knowledge, a new finding. This is proven by the fact that H185 antigen is produced by corneal epithelium in culture, away from a potential conjunctival or goblet cell source. That conjunctival epithelium provides a second source of mucin for the tear film is not a new concept. Greiner et al. proposed—based on PAS or Alcian blue followed by PAS staining of vesicles that appeared to discharge to the apical surface of human conjunctival epithelial cells—that the stratified squamous epithelium provided mucin for the tear film. Dilly and Mackie have also provided histochemical and morphologic evidence that vesicles filled with highly glycosylated material were discharged onto the conjunctival epithelial cells' apical surfaces. Our data corroborate and extend these studies in that we demonstrate that the cornea, as well as the conjunctiva, produce mucin-like glycoproteins. Designation of mucin-like glycoproteins, in this instance, is based on biochemical characteristics.

Two questions arise: What proportion of the mucus layer of the tear film is provided by goblet cells, and what proportion is provided by the stratified epithelium? Answers await probes that discriminate between products of the two cell types. Recent data suggest that goblet cells are innervated and secrete in response to neural stimulation. Perhaps epithelium supplies tear film mucus and goblet cells provide bolus mucus secretion in response to sensory stimulation.

Data from the rat studies using R339 antibody suggest that R339 is a component of the glycocalyx layer. The glycocalyx, so elegantly demonstrated in rapid freeze-prepared electron micrographs by Nichols et al., is the layer directly adjacent to the epithelium. These micrographs demonstrate the glycocalyx as an electron-dense zone, particularly prominent along the tips of the microvilli, which meshes gradually and without abrupt interface with the mucus layer of the tear film in the guinea pig cornea. Both H185 and R339 bind avidly to tips of microvilli in tissues and in cells in culture. Such localization suggests that the function of the mucin-like glycoprotein may be
facilitate tear film spread, providing an interface with the mucus layer. The availability of probes to human ocular surface epithelial mucins will allow study of ocular surface disorders in which tear film spread is disrupted because of various dry eye diseases.

In summary, we have developed a monoclonal antibody-designated H185 that recognizes a highly glycosylated O-linked glycoprotein produced by the stratified epithelium of the cornea and conjunctiva. The antigen in the human appears to be an analog of a recently described mucin-like glycoprotein discovered in rat ocular surface epithelium.

We conclude that the entire ocular surface epithelium produces mucin, and we hypothesize that the epithelial mucins facilitate tear film spread and stabilization.

Key Words
ocular surface, monoclonal antibody, tear film spread, mucus

References


