Development of Conjunctival Goblet Cells and Their Neuroreceptor Subtype Expression

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PURPOSE. To investigate expression of muscarinic, cholinergic, and adrenergic receptors on developing conjunctival goblet cells.

METHODS. Eyes were removed from rats 9 to 60 days old, fixed, and used for microscopy. For glycoconjugate expression, sections were stained with Alcian blue/periodic acid-Schiff's reagent (AB/PAS) and with the lectins *Ulex europeus agglutinin* I (UEA-I) and *Helix pomatia agglutinin* (HPA). Goblet cell bodies were identified using anti-cytokeratin 7 (CK7). Nerve fibers were localized using anti-protein gene product 9.5. Location of muscarinic and adrenergic receptors was investigated using anti-muscarinic and β-adrenergic receptors.

RESULTS. At days 9 and 13, single apical cells in conjunctival epithelium stained with AB/PAS, UEA-I, and CK7. At days 17 and 60, increasing numbers of goblet cells were identified by AB/PAS, UEA-I, HPA, and CK7. Nerve fibers were localized around stratified squamous cells and at the epithelial base at days 9 and 13, and around goblet cells and at the epithelial base at days 17 and 60. At days 9 and 13, M_2- and M_3-muscarinic and β_2-adrenergic receptors were found in stratified squamous cells, but M_1-muscarinic and β_1-adrenergic receptors were not detected. At days 17 and 60, M_2- and M_3-muscarinic receptors were found in goblet cells, whereas M_1-muscarinic receptors were in stratified squamous cells. β_1- and β_2-Adrenergic receptors were found on both cell types. β_3-Adrenergic receptors were not detected.

CONCLUSIONS. In conjunctiva, nerves, M_2- and M_3-muscarinic, and β_1- and β_2-adrenergic receptors are present on developing goblet cells and could regulate secretion as eyelids open. (*Invest Ophthalmol Vis Sci.* 2000;41:2127–2137)

The tear film mucus layer consists of high molecular weight glycoconjugates including mucins, which are secreted mainly by conjunctival goblet cells. This layer plays an important role in protecting the ocular surface from exogenous agents (bacterial or chemical) and provides lubrication during all types of eye movements.[1] Goblet cells can release their secretory granules in a reflex response mediated by the activation of either parasympathetic or sympathetic nerves that surround them.[2,3] Previous reports from this laboratory showed the localization of nerve fibers adjacent to goblet cells in rat conjunctiva.[4] Recently we found that the parasympathetic neurotransmitters acetylcholine and vasoactive intestinal peptide (VIP) stimulate glycoconjugate secretion from goblet cells in vitro.[5] Use of immunofluorescence techniques demonstrated that M_2- and M_3-, but not M_1-muscarinic acetylcholine receptors (MAchRs), are present on goblet cells and are located on membranes subjacent to secretory granules. VIP type 2 receptors (VIPR2s) are located in the basolateral

membranes of goblet cells.[3] Although the role of the sympathetic agonists in stimulating goblet cell secretion is unknown, β_1- and β_2-adrenergic receptor (βAR) subtypes appear to be present in goblet cells as well as in stratified squamous cells.[6] This evidence suggests that parasympathetic and perhaps sympathetic nerves regulate goblet cell secretion in response to external stimuli. The neural regulation of goblet cell secretion is essential to provide protection for and maintenance of the ocular surface ensuring clear vision.

Morphologic studies in developing conjunctiva suggest that based on changes in the acidity of glycoproteins in the secretory granules, goblet cells may differentiate from basal epithelial cells in the forniceal zone.[7] The existence of bipotent precursor cells (stem cells) in the forniceal zone has been hypothesized because these stem cells can give rise to both goblet and nongoblet cells.[8–12]

Based on several studies, goblet cell differentiation and mucin secretion appear to be directly related to the eyelid opening. In humans, the eyelids are fused until the 5th–6th month of intrauterine life, and goblet cells appear in the fornix extending toward the palpebral and bulbar regions from the 8th to 9th week of gestational age.[13,14] In chicks, goblet cells are absent at hatching and then appear in the fornix at 2 days after hatching.[15] In rats, the eyelids remain adherent and then begin to separate between the 12th and 15th day after birth.[16] In rat conjuctiva goblet cells form clusters. The density of clusters is highest in the forniceal zone with a gradual decrease toward the bulbar and tarsal zones.[7] Watanabe et al.[16] demonstrated that the expression of glycoconjugates in rat ocular
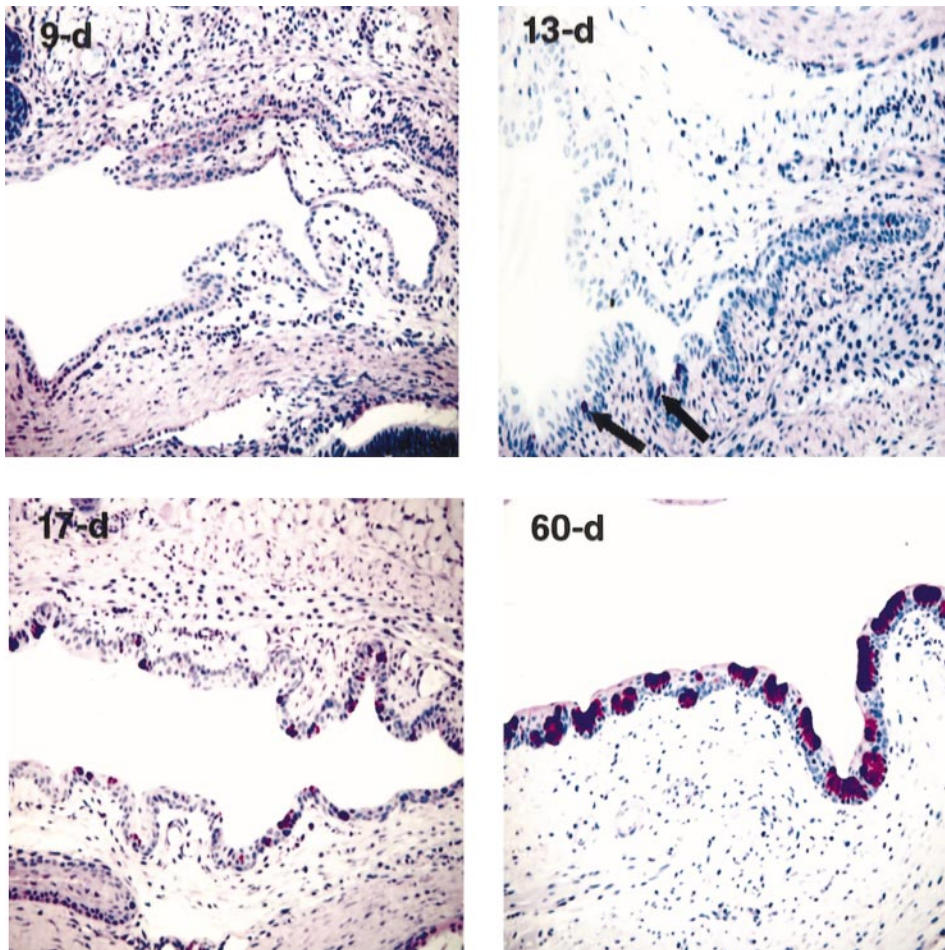
FIGURE 1. Methacrylate sections of 9-, 13-, 17-, and 60-day-old rat conjunctiva were stained with Alcian blue/periodic acid–Schiff's (AB/PAS), producing *magenta* and *dark purple* staining, and counter-stained with hematoxylin to indicate cell nuclei. At 9 days, only a few layers of stratified squamous cells were distributed throughout the entire conjunctiva, with no appreciable positivity to AB/PAS stain. At 13 days, a few round-shaped cells of conjunctival epithelium were AB/PAS–positive (*arrow*) in the forniceal region. At 17 days, single and small clusters of goblet cells were AB/PAS–positive and were distributed throughout the forniceal and palpebral region. At 60 days, conjunctival epithelium contained several layers of stratified squamous cells and goblet cell clusters that are AB/PAS–positive. Similar results were obtained in at least two other experiments. d, days. Magnification, ×100.

surface epithelium is induced by the eyelid opening. Recently, Tei et al.[17] demonstrated that the expression of the stratified squamous cell mucin ASGP (rMuc4) and goblet cell mucin rMuc5AC is correlated with the eyelid opening and goblet cell differentiation. In mice a heavy surface coat of presumably mucins is present after eyelid opening.[18,19]

It is not known when neural regulation of goblet cell secretion becomes functional. Little is known about the expression of MAchRs or βARs in the conjunctival goblet cells during postnatal development. Thus, the objective of this study was to localize nerves and determine the expression of MAchR and βAR subtypes in goblet cells during postnatal development of rat conjunctiva to compare this data with differentiation of goblet cells.

MATERIALS AND METHODS

Materials

Monoclonal antibody against cytokeratin 7 (CK7) was obtained from ICN (San Francisco, CA). *Ulex europeus agglutinin* lectin, (UEA-I) and *Helix pomatia agglutinin* lectin (HPA) directly conjugated with fluorescein isothiocyanate (FITC) or rhodamine, and streptavidin conjugated with rhodamine were obtained from Pierce (Rockford, IL). The polyclonal antibody, protein gene product (PGP) 9.5, was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). Poly-

clonal antibodies against M_1-, M_2-, and M_3AchR subtypes were obtained from Research and Diagnostics Laboratory (Berkeley, CA). Polyclonal antibodies against β_1-, β_2- and β_3AR subtypes were obtained from Santa Cruz (Santa Cruz, CA). The inhibitor sugars galactose and L-fucose and all other chemicals were obtained from Sigma (St. Louis, MO).

Tissue Collection and Preparation

All experiments conformed to the guidelines established by the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

Male Sprague–Dawley rats between 9 to 60 days old were used in this study and obtained from Taconic Laboratory (Germantown, NY). The animals were divided into four groups: 9 days (eyes closed), 13 days (eyes beginning to open), 17 days (eyes fully open), and 60 days (adult). Each group consisted of three animals ($n = 3$). On the appropriate day after birth, animals were anesthetized for 1 minute in carbon dioxide and decapitated, and both eyes were removed and fixed for 4 hours at 4°C in 4% buffered formaldehyde solution or in 10% buffered formalin. The tissue was cryopreserved overnight in 30% sucrose at 4°C in phosphate-buffered saline (PBS) containing 145 mM NaCl, 7.3 mM Na_2HPO_4, and 2.7 mM $NaH_2 PO_4$, pH 7.2 and frozen in OCT or embedded in methacrylate. Tissue used for CK7 was not fixed before freezing. Cryostat sections (6 μm) were placed on gelatin-coated slides and air-dried for 2 hours.
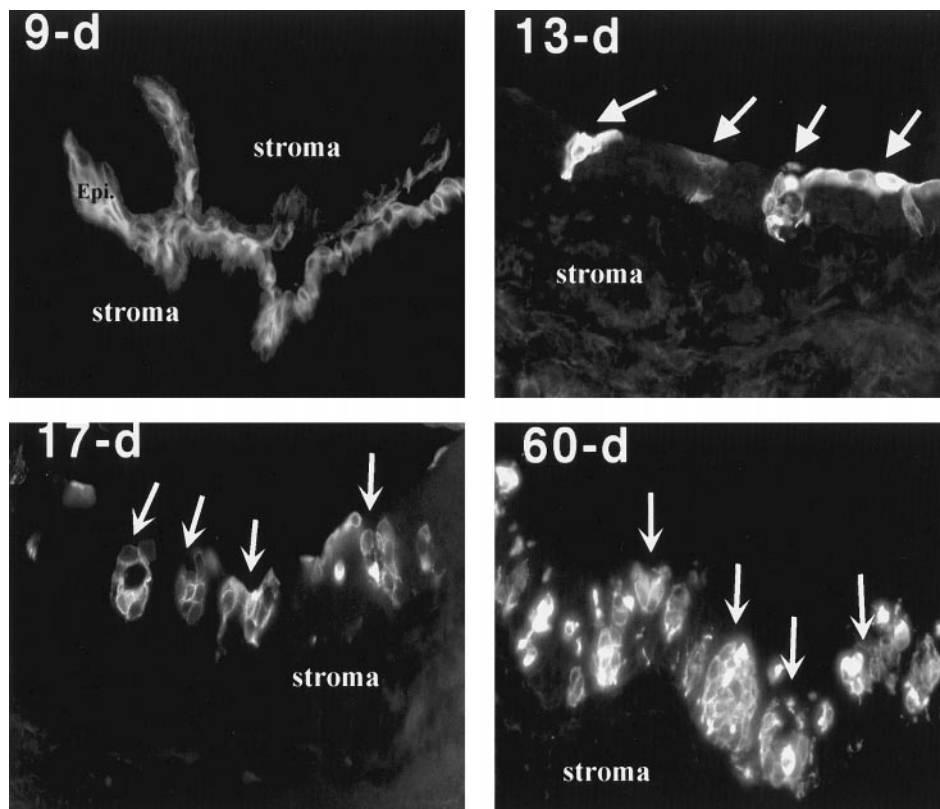
FIGURE 2. Immunolocalization of CK7 in sections from postnatal, developing rat conjunctiva. At 9 days, CK7 was detected in the cytoplasm of squamous epithelial cells in the fornix. At 13 days, CK7 stained cell bodies of developing goblet cells and patches of apical epithelial cells in the fornix (*flat arrows*). At 17 and 60 days, CK7 indicated the cell bodies of goblet cells in clusters (*bowed arrows*). Similar results were obtained in at least two other experiments. d, days. Magnification, ×420.

Sections were processed by conventional techniques for lectin histochemistry or for immunohistochemistry.

Histochemistry

To detect neutral and acidic glycoconjugates in goblet cells as well as for general observation of conjunctival morphology, sections were processed with Alcian blue/periodic acid–Schiff's reagent (AB/PAS) and counterstained with hematoxylin. Consecutive sections were stained with AB/PAS and then with each lectin used in the present study to compare the glycoconjugate-staining pattern with lectin staining. Sections were processed for lectin histochemistry using UEA-I or HPA, each directly conjugated with rhodamine and diluted 1:1000. The sections were blocked in 1% BSA for 1 hour and incubated with lectin for 30 minutes at room temperature. As a negative control, the lectins were preabsorbed overnight with the competing sugar. No positive binding was detected in these controls.

Immunohistochemistry

Air-dried sections were incubated in blocking buffer that contained 4% goat serum and 0.2% Triton X-100 for 1 hour at room temperature. The polyclonal antibody against human PGP 9.5, which indicates nerve fibers, was diluted 1:400 in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) and incubated overnight at 4°C. For MAchR subtypes, sections were incubated in blocking buffer that contained 1.5% normal goat serum and 0.2% Triton X-100 in PBS for 30 minutes at room temperature. Polyclonal antibodies against M_1-, M_2- and M_3AchR subtypes were each diluted 1:2000 in PBS and incubated overnight at 4°C. For βAR subtypes, sections were incubated in blocking buffer that contained 1% BSA and 0.2% Triton X-100 in PBS for 1 hour.

Polyclonal antibodies against β_1-, β_2-, and β_3AR subtypes were each diluted 1:800 in PBS, and incubated overnight at 4°C. The monoclonal antibody against CK7 was diluted 1:50 in PBS and incubated for 1 hour at room temperature. The secondary antibodies, conjugated to FITC or rhodamine, were diluted 1:200 in PBS and incubated for 1 hour at room temperature. Sections were mounted with Vectashield alone or containing DAPI to indicate nuclei. The negative control was the omission of the primary antibody. Sections were viewed using a Nikon UFX II microscope equipped for epi-illumination and differential interference-contrast (DIC) microscopy.

RESULTS

Identification of Developing Goblet Cells by Glycoconjugates in the Secretory Product

To elucidate the morphogenesis of rat conjunctival goblet cells, sections of 9-, 13-, 17-, and 60-day-old rat conjunctivas were stained with AB/PAS, which stains the secretory granules of goblet cells, and counterstained with hematoxylin (Fig. 1). At 9 days old (eyelids closed), few cell layers of squamous cells with picnotic nuclei were observed in all areas of the conjunctiva. At 13 days old (eyelids beginning to open), a few large round shaped cells, presumably immature goblet cells, which were AB/PAS–positive, were found in the forniceal region. At 17 days old (eyelids open), single goblet cells and small clusters of goblet cells were AB/PAS–positive and distributed from the forniceal to palpebral regions. At 60 days old (adult), approximately four cell layers of squamous cells with picnotic nuclei were observed. Goblet cells formed clusters that were AB/PAS–positive and distributed throughout the entire conjuncti-
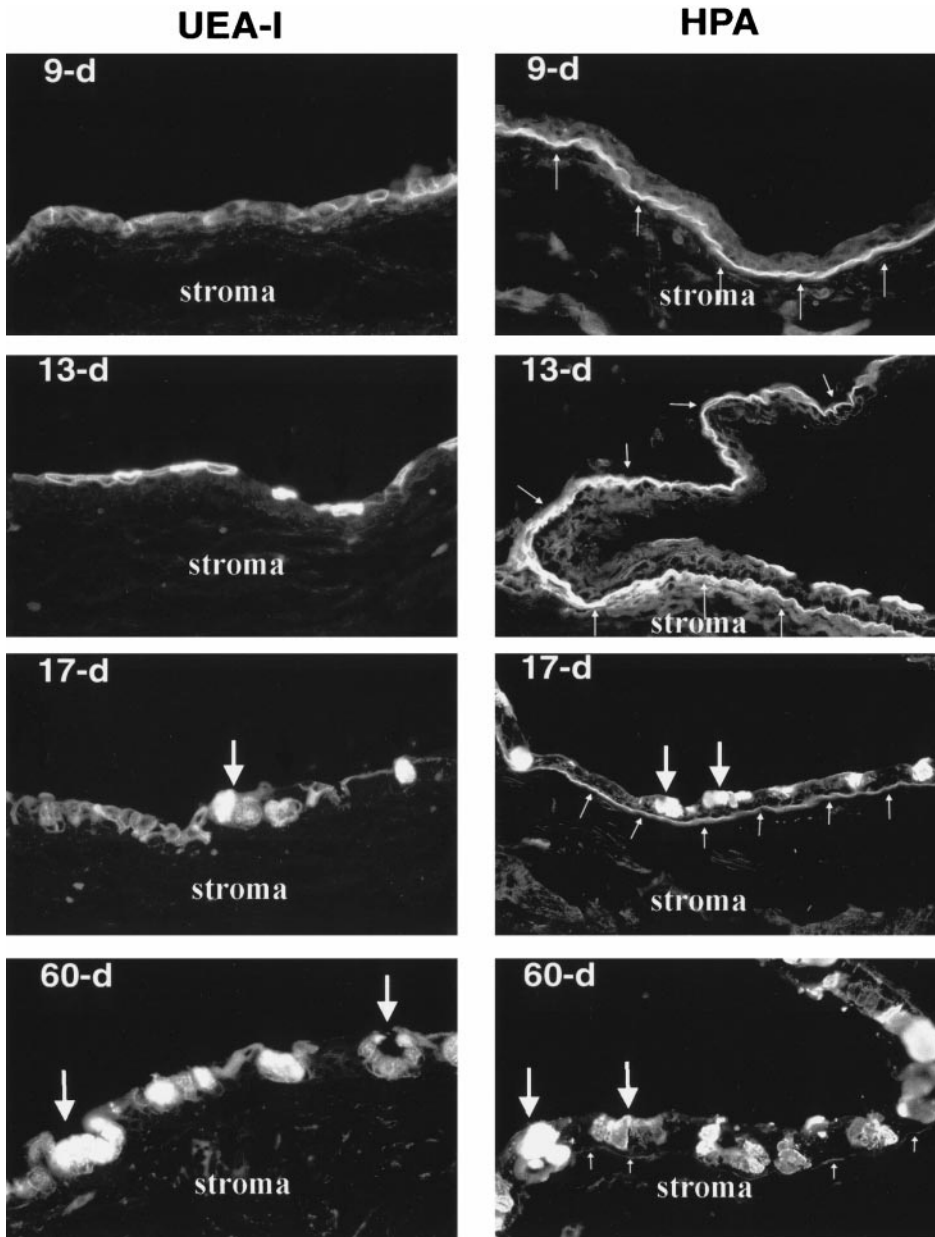
FIGURE 3.   Localization of goblet cell secretory product in postnatal developing rat conjunctiva. Sections of 9-, 13-, 17-, and 60-day-old conjunctiva were stained with biotinylated UEA-I (*left*) or HPA (*right*). At 9 days, UEA-I stained the cytosol and plasma membrane of stratified squamous cells in the fornix, which became patches of apical cells at 13 days. At 17 and 60 days, UEA-I stained only secretory products of goblet cells. At 9 days, HPA stained the basement membrane of stratified squamous cells. At 13 days, HPA stained the basement membrane of stratified squamous cells and the cytosol of some apical squamous cells in the forniceal zone. At 17 days, HPA stained the stromal–epithelial junction and stained secretory product in goblet cell clusters. At 60 days, HPA stained faintly the epithelial–stromal junction and strongly stained the secretory product in goblet cell clusters. *Large arrows,* goblet cell clusters; *small arrows,* basement membrane. Similar results were obtained in at least two other experiments. d, days. Magnification, ×300.

val surface, including the nictitating membrane. The highest number of goblet cell clusters was observed in the fornix.

Identification of Developing Goblet Cells Using Cytokeratins Present in the Cell Bodies

In adult rat conjunctiva cytokeratins 7, 8, 18, and 19 are present only in goblet cells.[20,21] In the present study, we used a monoclonal antibody to CK7, which stains the cell bodies of goblet cells, to determine the presence of goblet cells independent of secretory product before and after the eyelid opening. Our results show that at 9 days old, CK7 stained the cytosol of the apical layer of squamous epithelial cells in the fornix (Fig. 2). However, at 13 days old, when the eyelids begin to open, CK7 stained the cytoplasm of the cell bodies of individual cells, which spanned the entire epithelium and are presumably immature goblet cells. CK7 also stained selected apical cells. At 17 days old, when the eyelids are fully opened, CK7 predom-

inantly stained goblet cell bodies, which were now in clusters although a few apical cells still stained. At 60 days old, CK7 selectively recognized cell bodies of goblet cell clusters. These results show that in rat conjunctiva, single immature goblet cells are present during eyelid opening and then begin to form clusters after eyelids are fully open. Results obtained by identifying goblet cell bodies confirm the findings in Figure 1 using a marker for goblet cell secretory products.

Identification of Developing Goblet Cells by Carbohydrates in the Secretory Product

In previous studies, we showed that in the adult conjunctiva, the lectins UEA-I, and HPA selectively recognize carbohydrates in goblet cell secretory products, especially those in secretory granules.[3,5] In this study, we used these lectins to elucidate changes in carbohydrate composition of secretory product during the morphogenesis of goblet cells. At 9 days old, UEA-I

FIGURE 4. Immunolocalization of PGP 9.5 in postnatal developing rat conjunctiva. Sections were stained with anti-PGP 9.5 (a pan-neuronal marker; green), UEA-I (indicates UEA-I– detectable carbohydrate, red), and DAPI (stains nuclei, blue). Immunofluorescence micrographs were digitized and overlaid. At 9 and 13 days, PGP 9.5-containing nerves were located in the stratified squamous cells in either a basal or apical location as well as the stroma of the fornix. At 9 and 13 days, red indicates UEA-I– detectable carbohydrate in the stratified squamous cells. At 17 and 60 days, intense green staining indicated the distribution of nerve fibers along the epithelial–stromal junction and around the basolateral aspect of goblet cell clusters as well as the conjunctival stroma. At 17 and 60 days, intense red staining by UEA-I indicates the location of the secretory granules in goblet cell clusters. Arrows, nerve fibers. Similar results were obtained in at least two other experiments. Magnification, ×350.
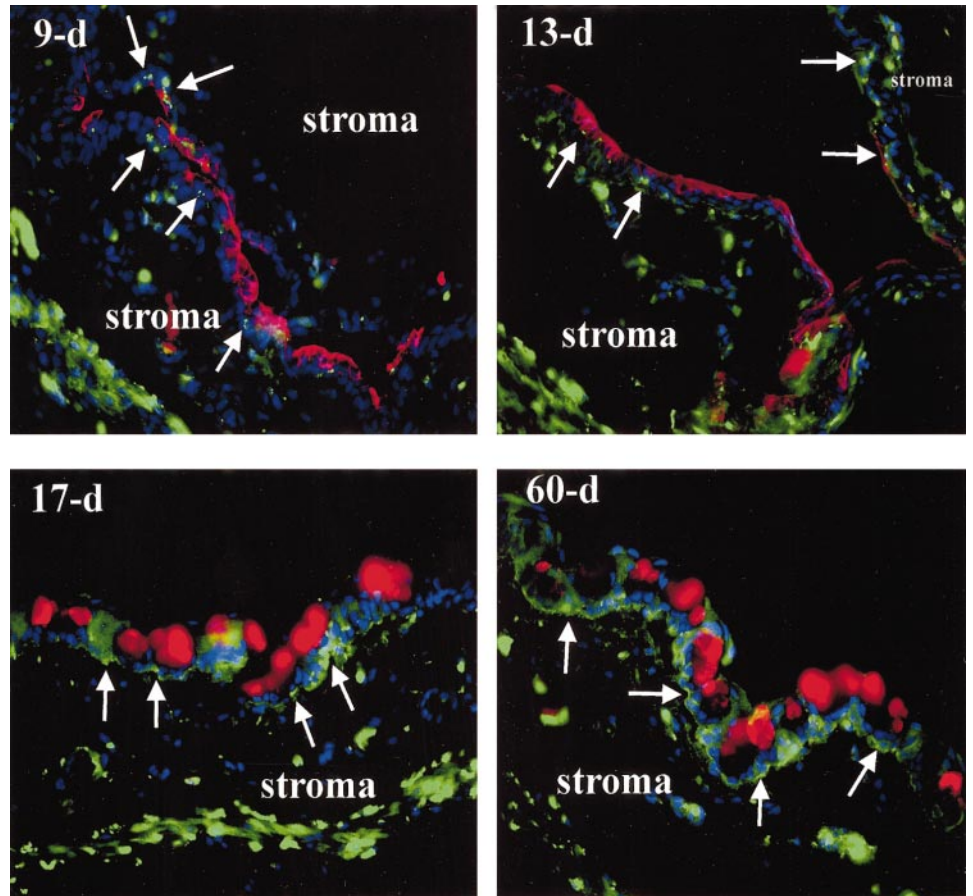

FIGURE 5. Immunolocalization of M_1-muscarinic acetylcholine receptor (AchR) subtype in postnatal developing rat conjunctiva. At 9 days, no M_1AchR were detected in the conjunctival epithelium or stroma. At 13 days, M_1AchRs were found in all cell layers of the conjunctiva. At 17 and 60 days, M_1AchRs were detected in the cytoplasm of stratified squamous cells. No immunoreactivity to M_1AchRs was detected in goblet cells. Similar results were obtained in at least two other experiments. d, days. Magnification, ×400.
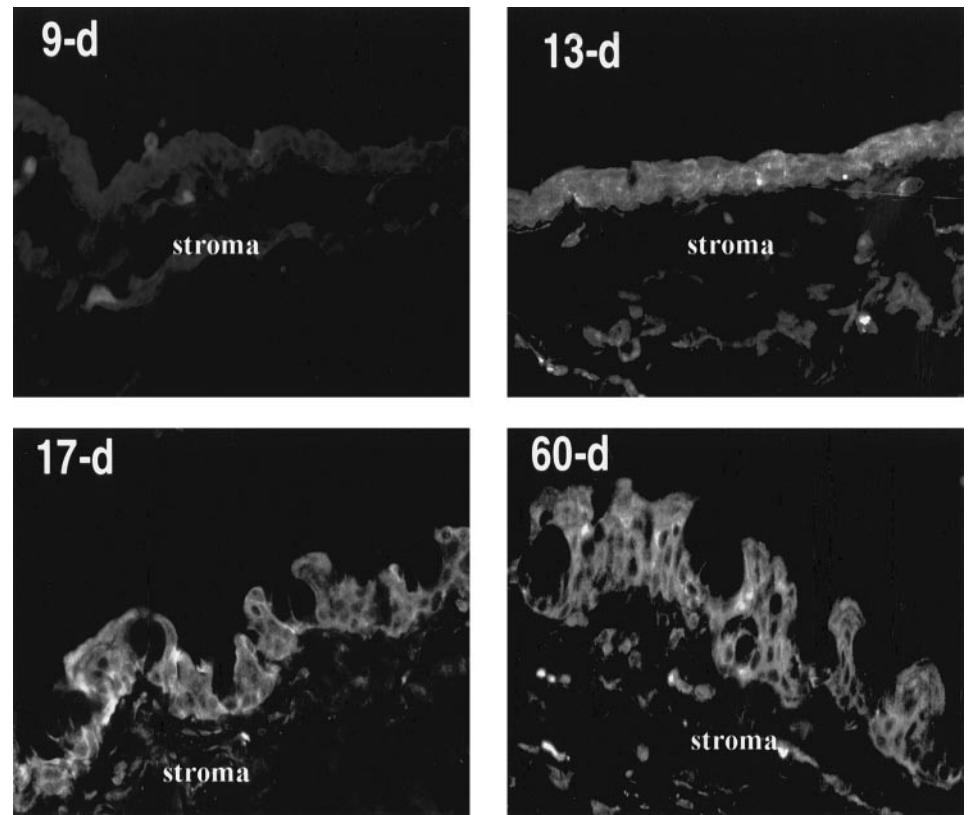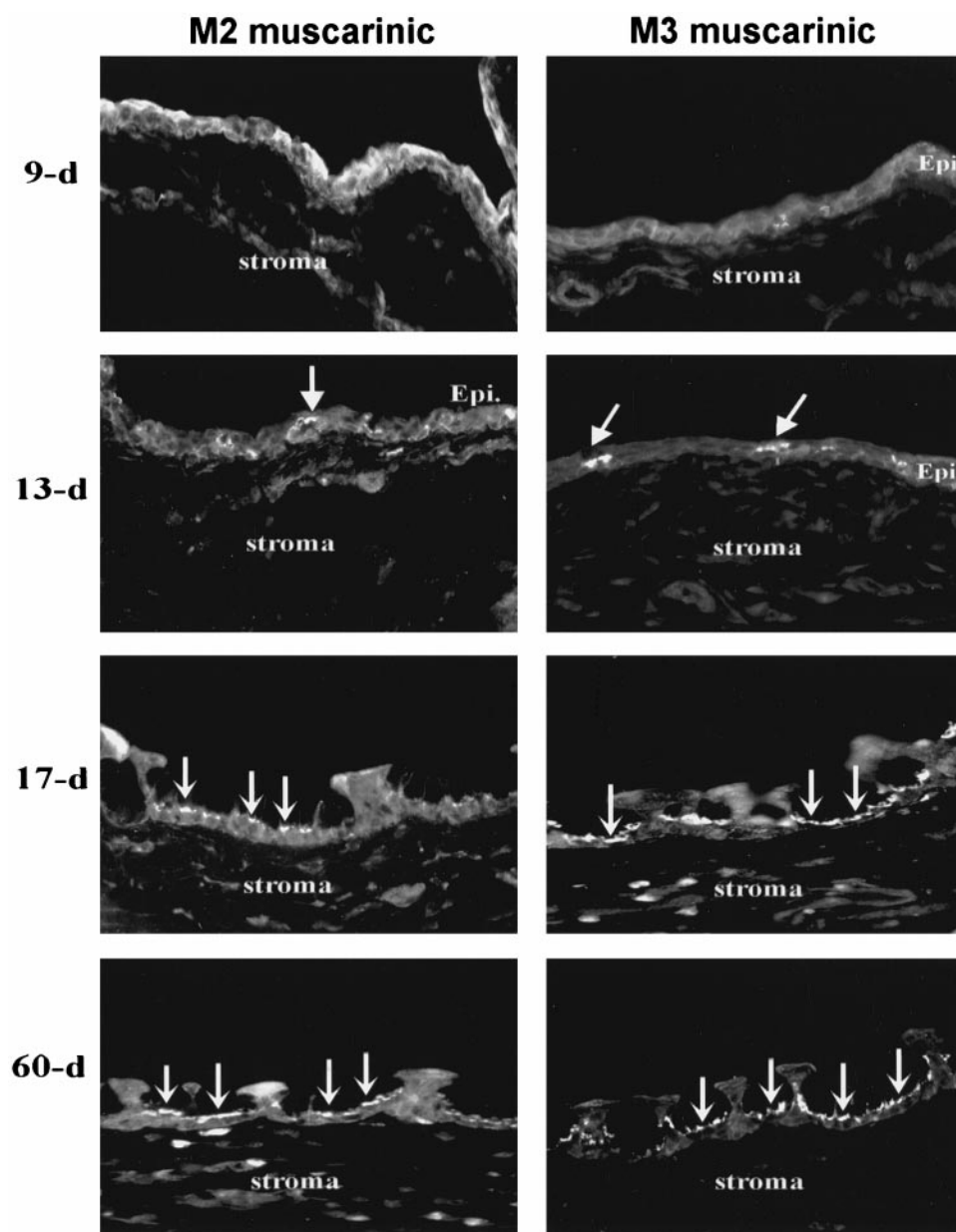
M2 muscarinic   M3 muscarinic

FIGURE 6. Immunolocalization of M_2- and M_3-muscarinic acetylcholine receptor (AchR) subtypes in postnatal developing rat conjunctiva. At 9 and 13 days, immunoreactivity to M_2- and M_3AchRs was detected in the cytoplasm of squamous cells, and at 13 days M_2- and M_3AchR also were found to intensely stain developing goblet cells. At 17 and 60 days, immunoreactivity to M_2- and M_3AchRs was detected in the stratified squamous cells as well as in the goblet cell clusters. At 60 days, M_3AchRs were predominantly in the goblet cells. Similar results were obtained in at least two other experiments. *Flat arrows* indicate M_2- and M_3AChRs on immature goblet cells. *Bowed arrows* indicate M_2- and M_3AChRs on goblet cells in clusters. d, days. Magnification, $\times$ 300.

intensely stained the plasma membranes of cells in a single layer on the epithelial surface (Fig. 3). A similar pattern was observed at 13 days old as the eyes were opening, except that only patches of apical cells were stained. At 17 days old, UEA-I bound strongly to single goblet cells and goblet cell clusters. At 60 days old, all the goblet cell clusters reacted with UEA-I, although the pattern (shape and density) of staining varied between clusters. Minimal binding of UEA-I was observed in the stroma or in the stratified squamous cells at this age. In contrast to UEA-I, at 9 days old, HPA strongly stained the basement membrane and the basal layer of cells with moderate staining of the squamous epithelial cells (Fig. 3). A similar staining pattern of HPA was observed at 13 days old, although occasional patches of apical epithelial cells were also stained. At 17 days old, HPA intensely stained almost all the goblet cell clusters, whereas the basement membrane was faintly stained. At 60 days old, HPA bound intensely to almost all the goblet

cell clusters with minimal binding to the basement membrane, stratified squamous cells, or the stroma. These results suggest that the immature goblet cells differentiating during eyelid opening do not express the same carbohydrates in their secretory products as do mature goblet cells. The carbohydrates recognized by UEA-I and HPA are present in cells other than goblet cells before and during eyelid opening.

Localization of Nerves in the Developing Rat Conjunctiva

In the present study, we used an antibody to the neuron-specific pan-neuronal marker, PGP 9.5 to localize nerve fibers, during postnatal development of the rat conjunctiva. Tissue sections were labeled with both anti-PGP 9.5 and UEA-I lectin (Fig. 4). At 9 days old, nerve fibers immunoreactive to PGP 9.5 were found in the basal and apical cell layers of the conjunctival epithelium and in the conjunctival stroma (Fig. 4). These
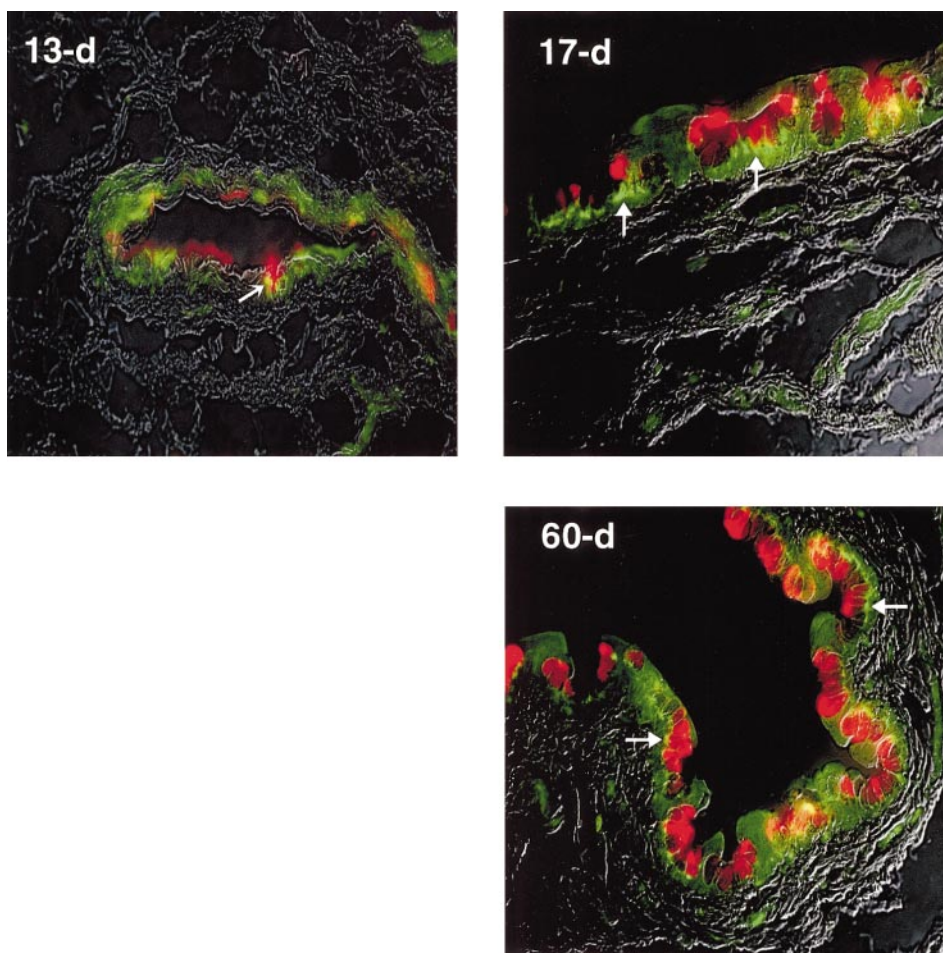
FIGURE 7. Colocalization of M_3-muscarinic acetylcholine receptor (AchR) subtype and UEA-I– detectable carbohydrates in postnatal-developing rat conjunctiva. Sections were double-labeled with anti-M_3AchR and UEA-I directly conjugated to rhodamine and photographed with differential interference-contrast microscopy (DIC). *Green* represents FITC-labeled M_3AchRs. *Red* represents rhodamine-labeled UEA-I, which indicates the location of secretory granules in mature goblet cells. At 13 days, M_3AchRs colocalized with UEA-I in some immature goblet cells in the fornix (*arrow*). At 17 and 60 days, goblet cell clusters of the fornix were visualized with DIC and with UEA-I. Note that M_3AchRs are subjacent to the secretory granules (*arrows*). Similar results were obtained in at least two other experiments. d, days. Magnification, ×400.

fibers appear as punctate staining as the nerves course in and out of the plane of focus because the conjunctiva is elastic and contracts unevenly on removal. At 13 days old, anti-PGP 9.5 labeled nerve fibers in basal and apical epithelial cell layers. At 17 and 60 days old, PGP 9.5-immunoreactive nerve fibers were found in blood vessels of conjunctival stroma, along the epithelial–stromal junction and around the basolateral aspect of goblet cell clusters. These results show that the conjunctival epithelium is innervated before eyelid opening. Nerve fibers located in the basolateral aspect of goblet cell clusters could thus regulate goblet cell secretion as these cells develop during eyelid opening.

Immunolocalization of Cholinergic Muscarinic Acetylcholine and β-Adrenergic Receptor Subtypes in Developing Rat Conjunctiva

Recently we showed that adult rat conjunctival goblet cells expressed the M_2- and M_3- but not M_1,MAchR subtypes.[5] However, little is known about the expression of these receptor subtypes during postnatal development. To address this, we performed immunohistochemical studies using antibodies against M_1-, M_2-, and M_3AchR subtypes. These antibodies were generated against peptide sequences from the nonhomologous regions of the carboxyl terminus of M_1-, M_2-, and M_3AchR subtypes.[22–24] The specificity of these antibodies was determined by enzyme-linked immunosorbent assay and Western

blot analysis, with elimination of the primary antibody and preincubation of the primary antibody with the immunogenic peptide serving as controls.[22]

At 9 days old, immunoreactivity to M_1AchRs was not detected in the conjunctiva, but at 13 days old, immunoreactivity against M_1AchRs was located in the cytosol and plasma membranes of all layers of the stratified squamous cells (Fig. 5). At 17 and 60 days old, immunoreactivity to M_1AchRs was seen predominantly in the stratified squamous cells. At 9 days old, immunoreactivity to M_2- and M_3AchR subtypes were found located in the cytosol and membranes of the stratified squamous cells (Fig. 6). At 13 days strong immunoreactivity to M_2- and M_3AchR subtypes was located in the midportion of presumably immature goblet cells. In 17- and 60-day-old conjunctivas, immunoreactivity to M_2AchRs was located in both stratified squamous cells and goblet cells, whereas M_3AchRs were present predominantly in goblet cells and located immediately subjacent to the secretory granules. We confirmed this localization by the simultaneous incubation of M_3AchR antibody and biotinylated UEA-I lectin, which indicates goblet cell secretory granules (Fig. 7). Especially at 13 days as goblet cells are developing, M_3AchRs were present on several of these goblet cells. At days 17 and 60, M_3AchRs were present subjacent to the secretory granules in almost all goblet cells.

In another set of experiments, we used antibodies against $β_1$-, $β_2$-, and $β_3$AR subtypes to determine their expression and
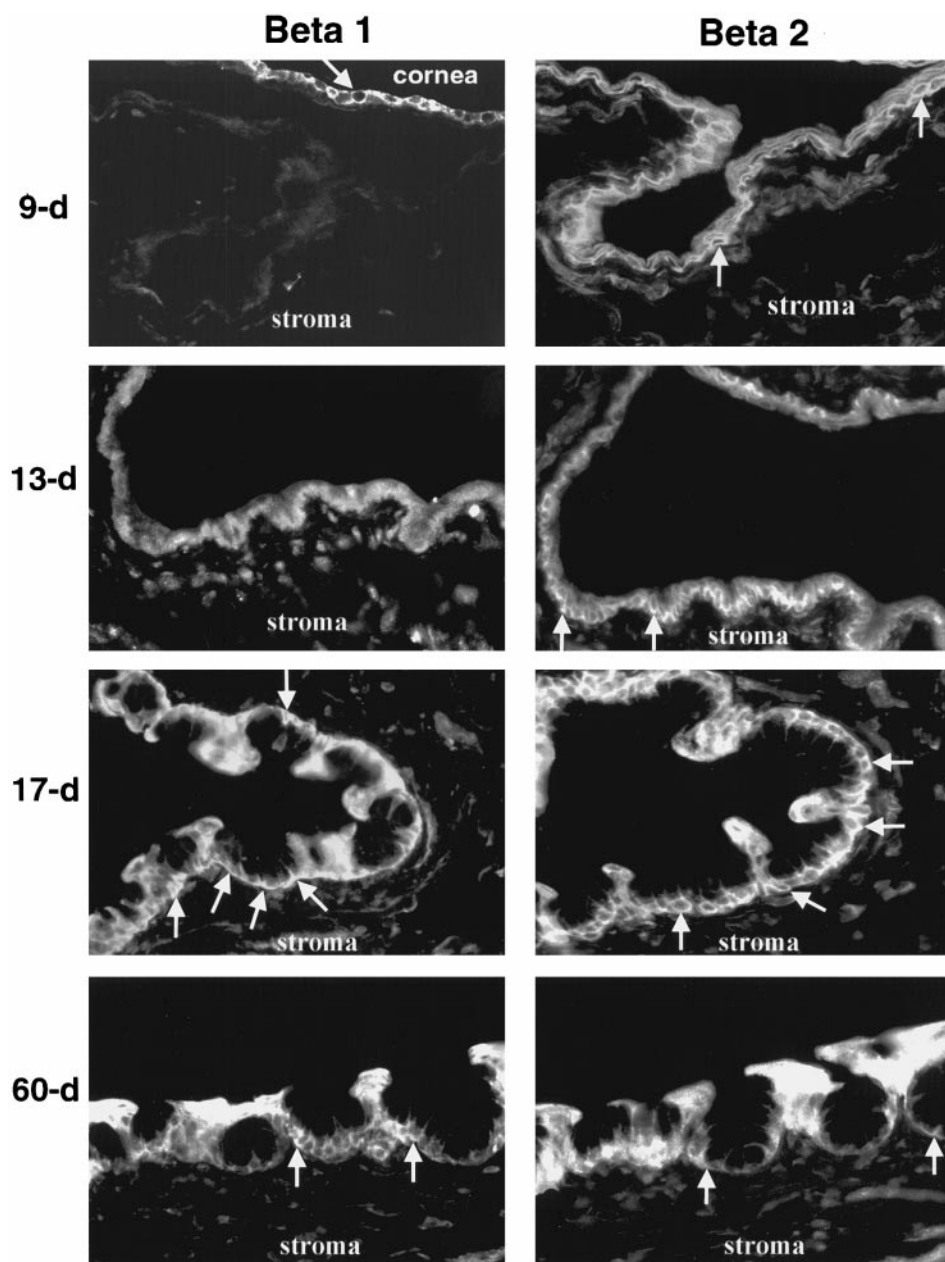
Beta 1   Beta 2

FIGURE 8. Immunolocalization of β_1- and β_2AR subtypes in postnatal developing rat conjunctiva. *Left:* At 9 days, no apparent immunoreactivity to β_1ARs was detected in the conjunctival epithelium or stroma, but was present in the plasma membranes of corneal basal cells. At 13 days, β_1ARs were detected diffusely in the cytoplasm of stratified squamous cells of the fornix. At 17 and 60 days, immunoreactivity to β_1ARs was detected in the cytoplasm and plasma membranes of stratified squamous cells and in the basolateral membranes of goblet cells in clusters. *Right:* At 9 days, immunoreactivity to β_2ARs was detected in the plasma membranes of apical and basal squamous cells. At 13 days, β_2ARs were detected in the plasma membrane of basal squamous cells and diffusely in the cytoplasm of apical squamous cells of the fornix. At 17 and 60 days, immunoreactivity to β_2AR was detected in the cytoplasm and plasma membrane of stratified squamous cells and in the basolateral membrane of goblet cells in clusters. Similar results were obtained in at least two other experiments. d, days. *Arrows* indicate positive immunoreactivity of basolateral and intracellular membranes. Magnification, ×350.

cellular distribution. These antibodies had been generated against peptide sequences from the carboxyl terminus of the β_1-, β_2- and β_3AR.[25] At 9 days old, immunoreactivity to β_2- but not β_1ARs was detected in the plasma membrane of the layers of squamous cells (Fig. 8). As a control, β_1ARs were present in the corneal epithelium. At 13 days old, β_1- and β_2ARs were expressed in almost all the stratified squamous cells, but binding was most intense in the basal cell layers. This labeling could include the immature goblet cells. At 17 and 60 days old, β_1- and β_2ARs were expressed in the plasma membranes of both stratified squamous cells and goblet cells in clusters. No immunoreactivity against β_3ARs was observed at any of the ages studied (data not shown). These results show that M_2- and M_3AchR and β_2AR subtypes are expressed in the conjunctiva before eyelid opening. During and after eyelid opening M_1-, M_2-, M_3AchR as well as β_1- and β_2AR subtypes are present in the conjunctiva. All receptors except M_1AchRs are present in goblet cells.

DISCUSSION

In the present study, we showed that the neural components for the regulation of goblet cell secretion are present before rat eyelids open. Nerve fibers as well as MAchR and βAR subtypes are differentially expressed around or on goblet cells during postnatal development. These findings are summarized in Table 1. PGP 9.5, a marker of nerves, is a developmentally regulated and neuroendocrine cell–specific ubiquitin carboxyl-terminal hydrolase[26,27] that is expressed in the conjunctiva before eyelids open. The expression of M_2- and M_3AchR and

Marker/Cell Type	Postnatal Age			
	9 Days	13 Days	17 Days	60 Days
PGP 9.5				
Epithelial cells	++	++	++	++
Goblet cells	−	+/−	+++	+++
M_1AChR				
Epithelial cells	−	+++	+++	+++
Goblet cells	−	−	−	−
M_2AChR				
Epithelial cells	++	++	++	++
Goblet cells	−	+	+++	+++
M_3AChR				
Epithelial cells	++	++	+/−	−
Goblet cells	−	+	+++	+++
β_1AR				
Epithelial cells	−	++	+++	+++
Goblet cells	−	+	+++	+++
β_2AR				
Epithelial cells	+	++	+++	+++
Goblet cells	−	+	+++	+++
β_3AR				
Epithelial cells	−	−	−	−
Goblet cells	−	−	−	−

−, not detectable; +, detectable.

β_2AR subtypes begin in the stratified squamous cells before eyelid opening and then localize in the plasma membrane of goblet cells as eyelids open and as these cells develop. In contrast, M_1AChRs and β_1ARs are not present before eyelid opening. M_1AChRs are expressed in stratified squamous cells during and after eyelid opening, and β_1AR are expressed in stratified squamous cells and goblet cells after eyelid opening. These data suggest that nerves, M_2- and M_3AChR and β_2AR subtypes are present on goblet cells as they develop and could regulate secretion by these cells as eyelids open. In addition, the presence of nerve fibers close to the cells in the developing conjunctival epithelium before eyelids open and in the basolateral aspect of goblet cells after eyelids open confirms previous reports about the neural regulation of the goblet cell secretion.[2-4]

Differences in subcellular distribution of M_1AChR and β_1- and β_2AR subtypes in rat conjunctiva may indicate that those receptors are derived from cytoplasmic structures or are redistributed between cytoplasmic pools to the cell surface membrane during nerve stimulation. This evidence suggests a process of receptor internalization and recycling in stratified squamous cells and goblet cells of rat conjunctiva. Evidence supporting these processes has been obtained in rat lacrimal gland and for G protein–linked receptors in many tissues.[28-30]

In the adult nervous system, neurotransmitters mediate cellular communication within neuronal circuits. However, in embryonic and postnatal developing tissues, it has been hypothesized that neurotransmitters play an additional role in regulating growth and morphogenesis.[31-33] Experimental studies in nonneural cells such as human epidermal keratinocytes demonstrated that MAchR agonists exert distinct biological effects through MAchRs at different stages of cell differentiation.[22,34,35] Several functional roles of acetylcholine have been

proposed in corneal epithelium, including stimulation of corneal epithelial cell growth, proliferation, and wound healing.[36-38] It has also been hypothesized that the ocular elongation is mediated by direct stimulus from the retinal cells in scleral chondrocytes via the muscarinic system.[39,40] In recent investigations, M_1AChRs have been found primarily in chick scleral chondrocytes and MAchR antagonists have inhibited the proliferation and extracellular matrix production of these cells in culture.[41] These findings raise the possibility that neurotransmitter secretion may contribute to conjunctival goblet cell morphogenesis during postnatal development as MAchRs and βARs are present in immature goblet cells.

The differentiation of conjunctival goblet cells during development is controversial. Our morphologic studies using AB/PAS or CK7 showed that goblet cells appear in the fornix at 13 days of age and form clusters at 17 days. CK7 is a type II intermediate filament protein that is found in most glandular and transitional epithelial cells and is not usually present in stratified squamous epithelial cells.[42,43] However, in our study, we found that CK7 was present in stratified squamous epithelial cells in the fornix before eyelid opening, in single goblet cells during eyelid opening, and then in all goblet cells after eyelid opening. Kasper et al.[20] suggested that rat conjunctival goblet cells develop from ectodermally derived epithelium. Thus, in our results, the pattern and distribution of CK7 in the postnatal developing conjunctiva suggests that goblet cells are more highly differentiated than stratified squamous cells.

Changes in glycosylation pattern have been associated with the state of cell differentiation.[44-46] For the ocular surface, an increase in glycoprotein expression is directly related to eyelid opening and artificial opening of the eyelid induces an increase in the presence of a carbohydrate-rich glycoprotein on the ocular surface.[16] In the present study, the use of UEA-I and HPA lectins revealed differential distribution of glycoconjugates in the conjunctiva, which changed during postnatal development. UEA-I is a lectin that binds strongly to α-L-fucosyl residues, whereas HPA binds strongly to N-acetyl-D-galactosyl residues.[47,48] Before eyelid opening, the two lectins recognized carbohydrate epitopes in different locations of the conjunctiva. However, after eyelid opening, both recognized carbohydrates in the secretory product of goblet cells. These data suggest that terminal glycosylation of glycoproteins in developing conjunctiva is closely associated with the differentiation of goblet cells. The developmental differences in glycoconjugate location may represent changes in activity of particular glycosyltransferase as the conjunctiva and its goblet cells develop. In addition, it is also possible that downregulation of a glycosyltransferase in the apical epithelial cells as well as upregulation of this enzyme in the goblet cells can account for the observed changes in UEA-I labeling pattern.

Based on UEA-I lectin and CK 7 staining, which appears first on apical cells and then extends basally as goblet cells appear, the goblet cell precursor appears to be in the apical surface. This suggests that a signal for differentiation of goblet cells comes from the apical side (tear side) of the conjunctival epithelium rather than from the basal side. One possible source for the signal is a diffusible factor from the tears. A second possible source is stimuli from the environment such as light or O_2 that reaches the ocular surface on eyelid opening.[16]

In summary, we confirmed that the morphogenesis of goblet cells in rat conjunctiva is correlated with eyelid opening. We showed that MAchR and βAR subtypes are expressed

in the conjunctiva as an early event of goblet cell development. Thus, we conclude that nerves, M_2- and M_3AchR, and β_1- and β_2AR subtypes are present on goblet cells as they develop and could regulate their secretion as eyelids open.

Acknowledgments

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