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GP2-expressing cells in the conjunctiva and tear ducts of mice: identification of a novel type of cells in the squamous stratified epithelium

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ABSTRACT

GP2 is a membrane-associated secretory protein originally identified in zymogen granules of pancreatic acinar cells. Recently, this glycoprotein has attracted attention as a marker substance of M cells of Peyer's patches and for its involvement in the selective uptake of pathological bacteria via M cells. When we stained the conjunctiva and tear ducts of mice using a GP2 antibody, all goblet cells in the squamous stratified epithelium of the conjunctiva were intensely immunolabeled, while goblet cells in the intestine and airway were devoid of the immunoreactivity, indicating that the conjunctiva contains a special type of goblet cell. Further immunostaining for GP-2 labeled dispersed cells of peculiar shapes within the stratified squamous epithelium in the lacrimal canaliculi, lacrimal sac, and nasolacrimal duct. The GP2-immunoreactive cells in the tear duct projected arched or branched processes toward the basement membrane. Electron-microscopically, immunogold particles for GP2 outlined the basolateral plasma membrane of both the conjuntival goblet cells and the peculiarly shaped cells in the tear duct. Intracellularly, GP2 products of the goblet cells were localized around secretory granules in the apical cytoplasm and those of the tear duct cells inside the vesicles. The luminal contents close to apical plasma membrane were heavily labeled with immunogold particles, suggesting an exocytosis-based targeting of GP2 to the plasma membrane and its release into the lumen. The possible function of GP2 in tear ducts is discussed in relation to a defense system against invasive microoranisms and antigens.

Tears contain various substances, such as lipids, mucins, ions, enzymes, and immunoglobulins. Mucins originate mostly in conjunctival goblet cells and the lacrimal glands (1, 13, 36), the latter being mucous in nature in some animals. The main and accessory lacrimal glands also secrete antimicrobial substances including lysozyme, lactoferrin, and lipocalin in both basal and reflex tear secretions (for review, 24). Secretory immunoglobulin A, the major antibody present in the tear film and tear fluid, is produced by plasma cells in cooperation with lacrimal glands and conjunctival lymphoid tissues (24). The antimicrobial enzymes, immunoglobulins, and complement factors assure immunologic functions against microorganisms and antigens. It is generally believed that conjunctival goblet cells are not directly involved in the ocular defense system. In the intestine, however, morphological studies for longer than 100 years have reported the existence of intermediate cells between goblet cells and Paneth cells, the latter a predominant cell source of antimicrobial substances in the small intestine (3). Numbers of the intermediate cells increased in the intestine of mice with abnormal Notch signaling (37) or with the infection of parasitic nematodes (15). Furthermore, goblet cells in the small intestine possess an ability to uptake luminal antigens and present them to sub-

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epithelial dendritic cells (25).

GP2, a glycoprotein with a molecular weight of 75~92,000, was originally identified as the major protein in zymogen granules of pancreatic acinar cells (22, 28, 33). GP2 is bound to the limiting membrane of secretory granules, targeted to the apical plasma membrane, and mostly released into pancreatic secretions (5–7, 35). Recently, GP2 appeared as a candidate for a transcytotic receptor during a genome-wide survey of genes specific for M cells of Peyer's patches, which are known to actively ingest macromolecules and microbes residing in the gut lumen (8, 40). GP2 in M cells functions in the detection and transcytosis of pathogenic bacteria and bacteria-derived toxin in the gut lumen (9, 23). Among many marker molecules for M cells, GP2 is recognized as a reliable indicator for mature and functional M cells (17).

The conjunctiva is the first defense line of ocular tissues against microbial and nonmicrobial pathogens. The conjunctiva-associated lymphoid tissue (CALT) including lymphoid follicles is seen in several mammals (18). In the laboratory mouse, the nictitating membrane (plica semilunaris) has been reported to contain organized lymphoid tissue (34). Although the murine CALT was rarely present under normal conditions, the direct administration of *Chlamydia* and nonmicrobial antigens into eyes increased the incidence (34, 39). Tears are consistently secreted to wet the conjunctiva and cornea, and leaked to the nasal cavity via the lacrimal canaliculi, lacrimal sac, and nasolacrimal duct. The lacrimal sac in the mouse develops lymphoid follicles which are classified as part of the tear duct-associated lymphoid tissue (TALT) (27). The lymphoid follicles in mouse TALT are covered by the thinner stratified squamous epithelium which contains dispersed M cell-like cells, as detected by lectin histochemistry and a monoclonal antibody (27). When we tried to find M cell-like cells in the TALT by use of a GP2 antibody, GP2-immunoreactive cells were scarce in the follicle-associated epithelium (FAE) of TALT. Instead, we found a broad, dense distribution of GP2-immunoreactive cells with a unique morphology in the epithelium of the tear duct. Furthermore, goblet cells in the conjunctiva also expressed GP2, unlike other goblet cells in other organs, such as the intestine and airway. Here, we report the distribution, morphology, and ultrastructure of the GP2-expressing cells in the conjunctiva and tear ducts of mice.

MATERIALS AND METHODS

Eight-week-old male mice of the ddY strain were supplied by Japan SLC (Hamamatsu, Japan). For immunohistochemistry at the light and electron microscopic levels, deeply anesthetized mice were perfused via the aorta with a physiological saline, followed with 4% formaldehyde plus 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The heads were removed, immersed in the same fixative for an additional 48 h, and decalcified with 5% EDTA for 3 weeks at 4°C. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

Immunohistochemistry. The decalcified tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections, about 14 μm in thickness, were mounted on poly-L-lysine-coated glass slides and stained by the avidin-biotin complex (ABC) method. After pretreatment with 0.3% Triton X-100-containing phosphate buffered saline (PBS, pH 7.2) for 1 h, sections were immersed in 0.3% H_2O_2/methanol for the inhibition of endogenous peroxidase activities. After preincubation with normal goat serum, the sections were incubated with a rat anti-GP2 monoclonal antibody (D278, 1 μg/mL; MBL, Nagoya, Japan) overnight. The sites of the antigen-antibody reaction were detected by incubation with biotin-conjugated goat anti-rat IgG, followed by incubation with the avidin-peroxidase complex (Nichirei, Tokyo, Japan). The reactions were visualized by incubation in 0.01 M Tris–HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.001% H_2O_2. The specificity of immunoreactions on sections was confirmed according to a conventional procedure, including absorption tests. The immunoreactivity with the GP2 antibody was completely abolished using the primary antibodies preabsorbed with the corresponding antigen.

For single and double immunofluorescence staining, the sections were incubated with the rat anti-GP2 antibody overnight, followed by incubation with Alexa Fluor 488-labeled anti-rat IgG (Invitrogen, Carlsbad, CA) or Cy3-labeled anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). The stained sections were further incubated with either rabbit anti-substance P (1 : 1,000, Y151; Yanaihara Institute, Shizuoka, Japan), rabbit anti-human PGP9.5 antibody (1 : 2,000, RA-95101; Ultraclone, Isle of Wight, UK), goat anti-high-affinity choline trans-

porter-1 (CHT1) antibody (1 μg/mL; Frontier Institute, Sapporo, Japan), rat anti-MHC class II antibody (1 μg/mL; Santa Cruz Biotechnology (Santa Cruz, CA), or hamster CD11c antibody (1 μg/mL; AbD Serotec, Kidlington, UK). The antigen-antibody reaction was visualized by incubation with Cy3-labeled anti-guinea pig or goat IgG (Jackson ImmunoResearch), or Alexa Fluor 488-labeled anti-hamster IgG (Invitrogen). Some of the immunostained sections were counterstained with SyTO 13 (SYTOX, Invitrogen) for observation of the nuclei. The stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

PAS reaction and lectin histochemistry. For the detection of goblet cells, frozen sections from decalcified heads were stained with conventional periodic acid-Schiff (PAS) reaction and histochemistry using *Ulex europaeus* agglutinin-I (UEA-I) for M cells. The latter staining used Cy3-labeled UEA-I lectin (Vector laboratories, Barlingame, CA, USA) diluted at 1 : 1,000. The UEA-I histochemistry is known to label goblet cells in the intestine and nasal cavity as well as M cells in Peyer's patches and nasal lymphoid tissues (9, 40).

Silver-intensified immunogold method for electron microscopy. Frozen sections from the formaldehyde-fixed tissues were mounted on poly-L-lysine-coated glass slides, incubated with the rat anti-GP2 antibody (1 μg/mL) overnight, and subsequently reacted with goat anti-rat IgG covalently linked with 1-nm gold particles (1 : 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmificated, dehydrated, and directly embedded in Epon (Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan). The specificity of the immunoreactions was confirmed by the disappearance of the immunolabeling when the antibody was pre-incubated with the antigen.

In situ hybridization. Two non-overlapping antisense oligonucleotide DNA probes (45 mer in length) were designed for mRNA of mouse glycoprotein 2 (Gp2): 421–465 and 1331–1375 of Gp2 mRNA (accession number: NM_025989). The probes were labeled with ^{33}P-dATP using terminal deoxynucleotidyl transferase (Invitrogen). Fresh frozen sections, 14-μm-thick, were fixed with 4% formaldehyde in 0.1 M phosphate buffer for 15 min and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 10 h with a hybridization buffer containing ^{33}P-labeled oligonucleotide probes (10,000 cpm/mL in concentration). The sections were rinsed twice at 55°C for 40 min in 0.1×SSC (1×SSC: 150 mM sodium chloride, 15 mM sodium citrate) containing 0.1% *N*-lauroylsarcosine sodium, dehydrated through a graded series of ethanol, and air-dried. Sections were dipped in an auroradiographic emulsion (Kodak NTB-2; Carestream Health, Inc., Rochester, NY) at 4°C for 8–10 weeks. The hybridized sections used for autoradiography were counterstained with hematoxylin after development.

The *in situ* hybridization technique using the two non-overlapping antisense probes for the mRNA exhibited identical labeling in all the tissues examined. The specificity of the hybridization was also confirmed by the disappearance of signals upon the addition of an excess of an unlabeled antisense probe.

RESULTS

Distribution of GP2-expressing cells in the conjunctiva and tear ducts

Goblet cells in the conjunctiva were intensely immunolabeled with the GP2 antibody. The GP2-immunoreactive goblet cells often gathered to form gland-like structures within the squamous stratified epithelium (Fig. 1a, b). When the same sections were stained for PAS reaction, all the conjunctival GP2-immunoreactive cells were positive in the reaction, indicating that they are mucus-secreting goblet cells in nature (Fig. 1c, d). Apart from the conjunctiva, numerous solitary cells in the stratified squamous epithelia of the lacrimal canaliculi, lacrimal sac, and nasolacrimal duct were intensely immunoreactive for GP2 (Fig. 2a–d). Unlike the conjunctiva, these cells were negative in PAS reaction (Fig. 2e, f). The PAS-negative GP2-expressing cells in the tear duct were characterized by their peculiar shapes, shown in Fig. 2d, e and Supplementary Figure-1: they extended arborized or arched processes toward the basement membrane. Essentially, all of the cells were attached to the basement membrane; on the other side, they reached the lumen via the apical cytoplasmic process. The luminal side of the apical cytoplasm frequently displayed condensed and dot-like immunoreactivities for GP2 (arrows in Fig. 3c). The specificity of the GP2 immunoreactivities in the conjunctiva and tear duct was confirmed at the mRNA level by an *in situ* hybridization technique

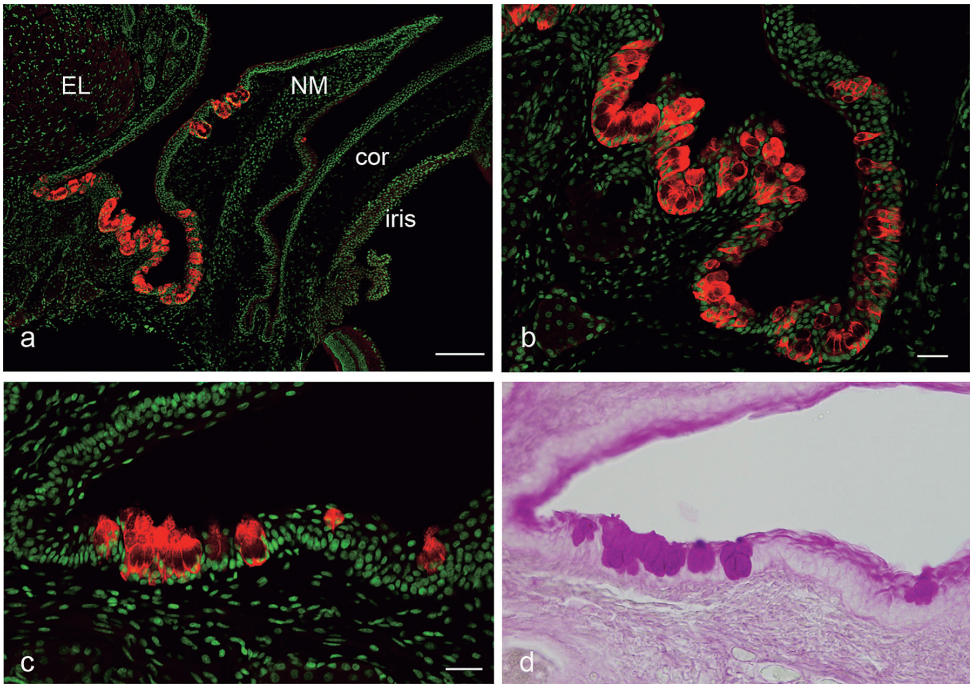

Fig. 1 Immunostaining of GP2 in the conjunctiva of mice. Intensely immunoreactive goblet cells gather or are scattered in the squamous stratified epithelium (a, b). After taking a picture of GP2 immunostaining (c), the same section was stained for PAS reaction (d). Nuclei are stained green with a nucleic acid dye, SYTOX. cor: cornea, EL: eyelid, NM: nictitating membrane *Bar* 50 μm (a), 20 μm (b, c)

(Supplementary Figure-2).

Some M cell-like cells, which were identifiable by UEA-I lectin histochemistry, were present in the FAE (follicle-associated epithelium) of lymphoid follicles in the lacrimal sac, as reported previously (27). However, the UEA-I-labeled cells in the tear duct including the lacrimal sac were almost immunonegative for GP2 (Supplementary Figure-3). Noteworthily, our GP2-immunoreactive cells were less numerous in the FAE than ordinary epithelium covering other regions of the lacrimal sac (Fig. 2a, b). Langerhans cells dispersed in the epidermal squamous stratified epithelium are known to express CD11c and MHC class II molecules; however, no cells were immunoreactive for these markers (data not shown). Thus, the histochemical examination indicates that GP2-expressing cells in the tear duct are a novel type of cells in the squamous stratified epithelium.

Relation with nerve fibers

Immunostaining of PGP9.5, a pan-neuronal marker, in the conjunctiva displayed a dense distribution of intraepithelial nerve fibers. Although some PGP9.5-positive nerve fibers were found around GP2-immunoreactive goblet cells in double staining (Fig. 3a), the intraepithelial nerve fibers were fewer than those in the epidermis of eyelids. A small number of substance P-immunoreactive fibers invaded the epithelium and appeared to contact GP2-expressing goblet cells (arrows in Fig. 3b). PGP9.5-immunoreactive nerves were abundant in the subepithelial connective tissue in the lacrimal canaliculi, lacrimal sac (Fig. 3c), and nasolacrimal duct, but only a few nerve fibers entered the epithelium. Here again, nerve fibers immunoreactive for substance P seemed to have an intimate relation with GP2-expressing cells in the tear duct (Fig. 3d). Immunostaining for CHT1, a marker of cholinergic nerves, failed to detect any topographical relationship to GP2-expressing cells (data not shown). These findings indicate that the GP2-expressing cells have an intimate relation with substance P-containing sensory fibers rather than with the autonomic nervous system.

Ultrastructure of GP2 cells

The silver-intensified immunogold method revealed the subcellular localization of GP2 in the conjunctiva and tear duct. In the conjunctiva, immunogold particles showing the existence of GP2 were localized along the plasma membrane of goblet cells (Fig. 4a). Although most secretory granules were

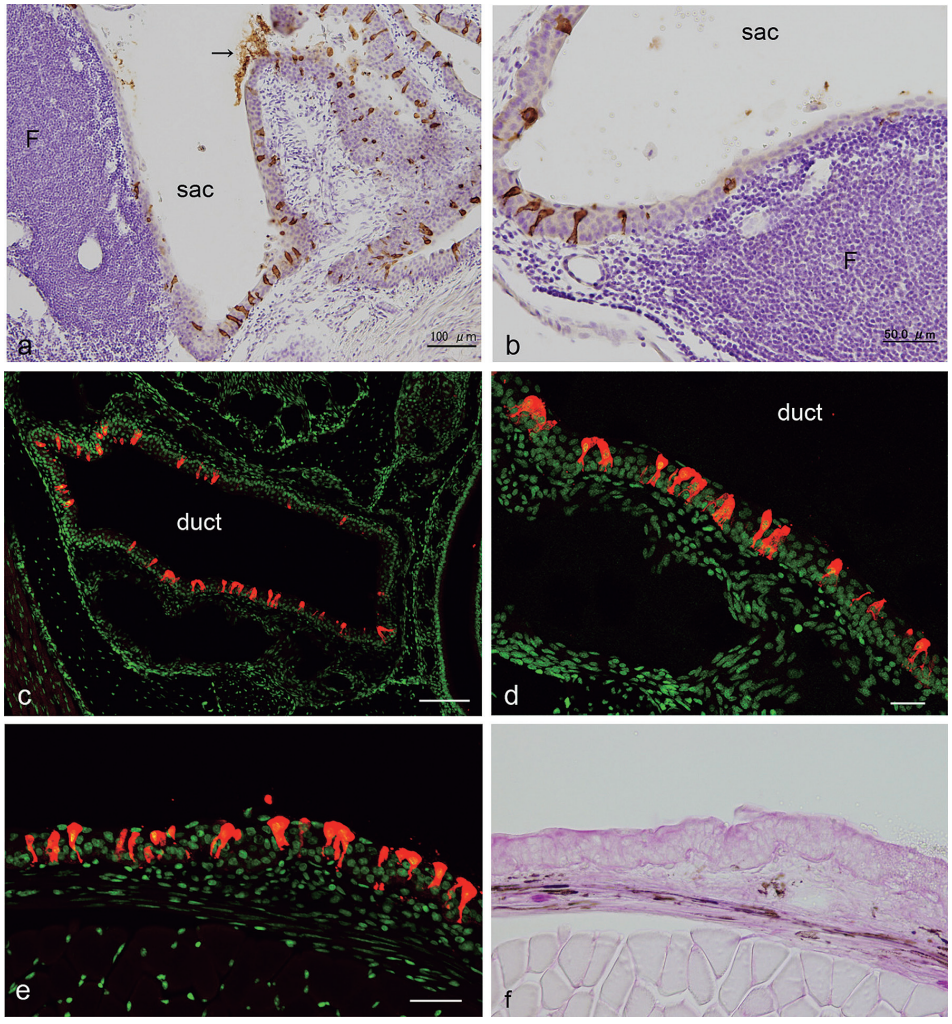

Fig. 2 Immunostaining of GP2 in the lacrimal sac and nasolacrimal duct. GP2-immunoreactive cells in the lacrimal sac are densely distributed in the folded epithelium of the sac but fewer on the follicle-associated epithelium (a, b). An arrow in Fig. 2a indicates an immunoreactivity of the luminal content. Immunofluorescence method shows Cy3-labeled GP2 cells along the nasolacrimal duct (c). In a closer view of Fig. 2c, the peculiar shapes with arborized processes are visible (d). In Figs. 2 e and f, the same section is stained for GP2 and PAS reaction. The GP2-expressing cells are completely negative for PAS reaction. F: lymphoid follicle *Bar* 100 μm (a, c), b (50 μm), 20 μm (d, e)

free from the immunolabeling, gold particles for GP2 condensed around secretory granules only at the apical cytoplasm, where the limiting membrane of granules appeared to be densely labeled (Fig. 4b). Also in the tear duct (lacrimal canaliculi, lacrimal sac, and nasolacrimal duct), the immunogold particles outlined the basolateral plasma membrane of dispersed cells (Fig. 4c), while the apical plasma membrane—equipped with short microvilli—displayed less intense or faint labeling for GP2 (Fig. 5a, b). Frequently, amorphous heavy labeling for GP2 was found in the contents of the lumen (asterisk in Fig. 5b). Another immunoreactivity for GP2 appeared inside the membrane-bound vesicles/vacuoles at the apical cytoplasm (arrows in Fig. 5a, b). Furthermore, the GP2 antibody immunolabeled the Golgi apparatus and insides of endoplasmic reticulum in some cells (Supplementary Figure-4). The immunolabeled basolateral plasma membrane in GP2 cells of the tear duct was fimbriated and sometimes equipped with filopodia (Fig. 5c); this image was able to be captured at a light microscopic level (arrowheads in Fig. 3c).

DISCUSSION

GP2-producing goblet cells

GP2, a GPI (glycosylphosphatidyl inositol)-anchored

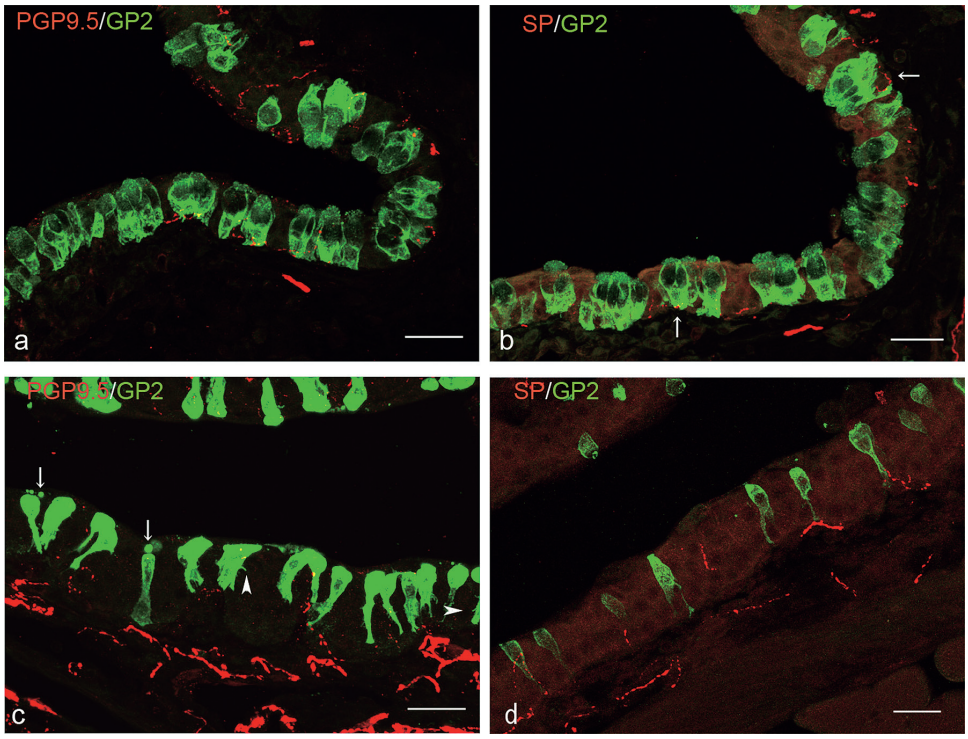

Fig. 3 Relationship of GP2-expressing cells with nerve fibers. In the conjunctiva (a and b), PGP9.5-immunoreactive nerve fibers (red) are distributed in an area containing GP2-positive goblet cells (green); some of them appear to contact GP2 cell bodies (a). A similar intimate relationship is seen between substance P (SP) nerves and GP2-immunoreactive goblet cells (b). Arrows indicate contact by nerves with GP2-immunoreactive goblet cells. In the nasolacrimal duct, PGP9.5-immunoreactive nerves are numerous in the lamina propria but fewer within the epithelium (c). Arrows and arrowheads indicate dotted immunoreactivities on the apical side of cells and filopodia on the lateral side, respectively. SP-immunoreactive fibers show frequent contact with GP2 cells in the nasolacrimal duct (d). *Bar* 20 μm

membrane protein, is targeted to the apical plasma membrane via regulated exocytosis in the pancreatic acinar cells (6, 35). Besides the membrane protein form, GP2 in the pancreas exists as a soluble form and is abundantly released into the pancreatic juice (5, 6, 10, 31). The present study revealed that solitary and grouped goblet cells in the squamous stratified epithelium of the conjunctiva produce and secrete GP2. This function is unique to the conjunctival goblet cells, since goblet cells in the airway and intestine are devoid of any GP2 immunoreactivity (our unpublished data). The intracellular GP2 immunoreactivity was not found in the content of secretory granules but was mainly associated with the limiting membrane of secretory granules present only at the apical cytoplasm. If GP2 produced by goblet cells is released into tears, the subcellular localization may secure the existence of the same releasing mechanism of GP2 in the ocular goblet cells as that in pancreatic acinar cells. This releasing episode shall be further certificated in another type of GP2-producing cell in the tear ducts in the next section.

Secretion from conjunctival goblet cells may be induced by the following three events: *1*) constitutive secretion, *2*) mechanically stimulated secretion, and *3*) neuronal control including reflex. The neuronal regulation is regulated by the autonomic nervous system and/or sensory neuron-associated reflex. Diebold *et al*. (4) described how vasoactive intestinal polypeptide (VIP)-containing parasympathetic nerves as well as sympathetic nerves were present around conjunctival goblet cells in the mouse and human; the direct effect of neural pathways on mucin secretion from conjunctival goblet cells has been obtained during stimulation of parasympathetic nerves (32). Also, capsaicin-sensitive neurons containing substance P caused goblet cell secretion in the rat conjunctiva (20). In the present study, GP2-positive goblet cells co-localized with nerve fibers within the conjunctival epithelium; however, intraepithelial nerves are generally numerous in ocular integuments such as the eyelids. Nerve fibers in the conjunctiva were less numerous around the GP2-secreting goblet cells as shown by PGP9.5 immunohistochemistry,

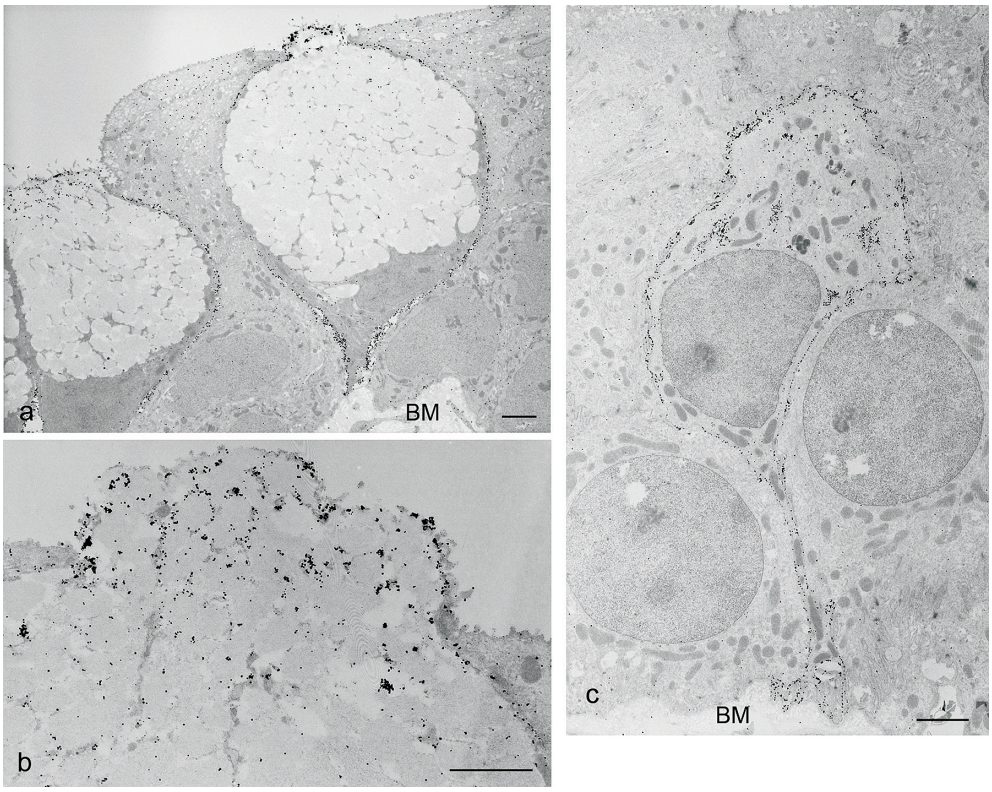

Fig. 4 Electron microscopy of GP2-immunoreactive cells (silver-intensified immunogold method). Gold particles showing the existence of GP2 are localized along the basolateral membrane and gather at the apical pole of conjunctival goblet cells (a). At a higher magnification, the gold particles are condensed around secretory granules in the apical cytoplasm of goblet cells (b). In a GP2-positive cell of the nasolacrimal duct, the gold particles are mainly localized along the basolateral membrane (c). BM: basement membrane *Bar* 2 μm (a, c), 1 μm (b)

and the GP2 goblet cells preferred to make contact with substance P-containing nerves. Thus, it is safe to say that, in the case of mice, the secretion of GP2 from conjunctival goblet cells—and also GP2 cells in the tear duct—may be regulated partially by sensory neurons containing substance P.

GP2-producing cells in the lacrimal canaliculi, lacrimal sac, and nasolacrimal duct

The lacrimal sac develops lymphoid follicles, termed the tear duct-associated lymphoid tissue (TALT). The FAE in the mouse TALT is thinner than the squamous epithelium covering other regions of the lacrimal sac; it contains M cell-like cells but not goblet cells (27). The existence of M cell-like cells in the FAE was detectable with UEA-I lectin histochemistry and an M cell-specific antibody (NKM16-2-4) (27). However, our GP2-producing cells were scarce in the FAE and were not stained with UEA-I, which is a classical M cell marker. In spite of the finding that GP2 is an established novel marker of intestinal M cells (8, 9), the GP2-producing cells in the tear duct were not associated with any lymphoid tissues and distributed broadly and densely in the ordinary epithelium of the lacrimal sac. Therefore, GP2-expressing cells throughout the tear duct including the lacrimal sac may be not equivalent to intestinal M cells, suggesting different functions in the tear duct.

The GP2-immunoreactive cells in the epithelium of the tear duct showed peculiar shapes, characterized by the branched processes toward the basement membrane. The stratified squamous epithelium of the epidermis contains Langerhans cells and melanocytes as non-keratinocytes. The possibility that GP2-producing cells are Langerhans cells or melanocytes is refused by their unique morphology contacting both the basement membrane and lumen. Furthermore, the GP2-positive cells in the tear duct were immunonegative for MHC class II and CD11c, both of which are marker molecules of dendritic cells including Langerhans cells. At present, we consider that the GP2-expressing cells in the tear duct must be a novel type of cell appearing within

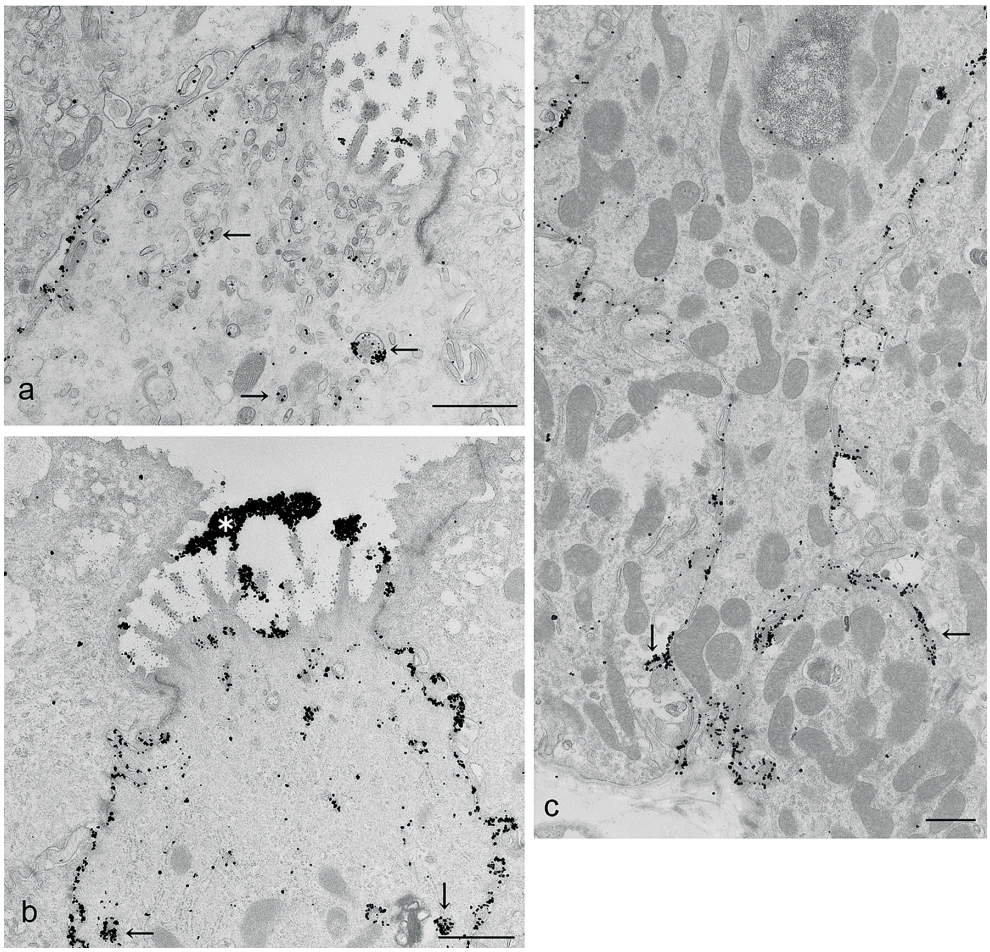

Fig. 5 Electron microscopy of GP2-immunoreactive cells in the nasolacrimal duct. On the apical side of GP2-immunoreactive cells, the gold particles accumulate on small vesicles or vacuoles indicated by arrows but are fewer on the luminal plasma membrane (a, b). A heavy labeling is seen in the contents of the free luminal space (asterisk) as well as lateral cell membrane (b). A basolateral view (c) of a GP2-immunoreactive cell shows many filopodia of various lengths indicated by arrows. *Bar* 1 μm

the stratified squamous epithelium, though it has been described as "a multilayered epithelium" in the rat (30).

Epithelium of the human nasolacrimal ducts is known to have an active secretory function of antimicrobial peptides, such as defensins, secretory phospholipase A_2, lysozyme, and lactoferrin (29). The present study added another peptide to the list. The finding concerning the subcellular localization of GP2 may explain the secretory mechanism of GP2 in the tear duct. It is reasonable to consider that GP2 or its precursor is synthesized in the rough endoplasmic reticulum, structurally modified in the Golgi apparatus, transferred to the apical cell membrane by the use of vesicular and vacuolar cargos, and then spread over the whole plasma membrane, though we cannot deny the direct movement of GP2 to the basolateral membrane mentioned below. The plasma membrane on the luminal side lacked the GP2 immunoreactivity, and instead, a huge aggregation of immunogold particles often was found in the lumen, suggesting that the membrane-bound GP2 is finally released into the lumen. The membrane trafficking of GP2 is identical to that seen in the pancreatic acinar cells (35). Immunohistochemistry for GP2 in the pancreatic exocrine cells has demonstrated that GP2 is present along the whole cell membrane as well as the Golgi apparatus and zymogen granule membrane but also occurs as secretions in the luminal space (7). Gueze *et al.* (7) considered that the broad distribution of GP2 along the entire length of the plasma membrane is explained by two mechanisms: GP2 originates from exocytosis-derived apical GP2 or is added directly to the basolateral

plasma membrane by means of vesicles coming from the Golgi complex. Taken together, these notions suggest that GP2 in the conjunctiva and tear duct may be released into tear secretions by the same mechanism as in pancreatic acinar cells.

Possible functions of GP2

GP2-producing cells are found in several organs other than the pancreas. The epithelium of the paranasal sinuses (16), palatine gland, lingual gland, and duodenal gland (our unpublished data) secrete GP2 into the nasal cavity, oral cavity, and duodenal lumen, respectively. Furthermore, GP2 in the pancreatic juice should supply a great amount of GP2 to the intestinal lumen. Thus, the nasal cavity and alimentary tract have an ability to hold abundant amounts of secreted GP2 in the lumen. The present study was able to add the conjunctiva and tear duct to the panel of tubular organs rich in GP2. In the tear duct, the volume of secreted GP2 is sufficient to irrigate the narrow space of the tear duct up to the nasal cavity. These tubular organs share a special situation that is exposed to the external milieu and is always attacked by pathogenic microorganisms.

Tamm-Horsfall protein (THP), structurally related to GP2 with more than 50% homology in the C-terminal regions, is a GPI-anchored membrane protein produced in the kidney. THP in the urinary tubules is intracellularly present in membrane-bound clear vesicles in the apical cytoplasm (2, 6, 11, 38) and also distributed along the entire plasma membrane of epithelial cells in the thick ascending limb of the loop of Henle (12). Immunohistochemistry at the electron microscopic level has demonstrated morphological signs for the active release of THP into the lumen of urinary tubules (2). In fact, THP is a predominant glycoprotein present in mammalian urine (19, 21). THP has a tendency to self-aggregate and form filament-like polymers (14), a characteristic important for trapping bacteria. THP specifically binds to uropathologic *E. coli* and promotes bacterial clearance; THP inhibited type-1-fimbriated *E. Coli* to colonize on the bladder epithelium but did not affect the bladder colonization of P-fimbriated *E. coli* (26). Based on ultrastructural observations of the pancreas, it is proposed that GP2 forms a fibrillar meshwork with mucoprotein in the lumen of pancreatic acini and ducts to provide a physiological barrier to bacterial invasion (35). Actually, we could find an intimate relationship between secreted GP2 and bacteria in the gut lumen under the light and electron microscopes (our unpublished data). One of the functions of GP2 secreted from the palatine gland, duodenal gland, and pancreas may be to bind and trap bacteria to inhibit their adhesion to the luminal surface of the gut, except M cells. This may hold true for GP2 derived from the conjunctiva and tear duct. However, this idea must be certificated by further studies, including an inoculation of bacteria on the surface of eyes, in the near future.

In conclusion, we applied the immunohistochemistry of GP2 to find unique goblet cell populations in the conjunctiva and a novel type of cells with dendritic shapes—which has been not documented—in the stratified squamous epithelium of the tear duct. They produce a glycoprotein GP2, possibly to contribute to the defense against microorganisms and antigens.

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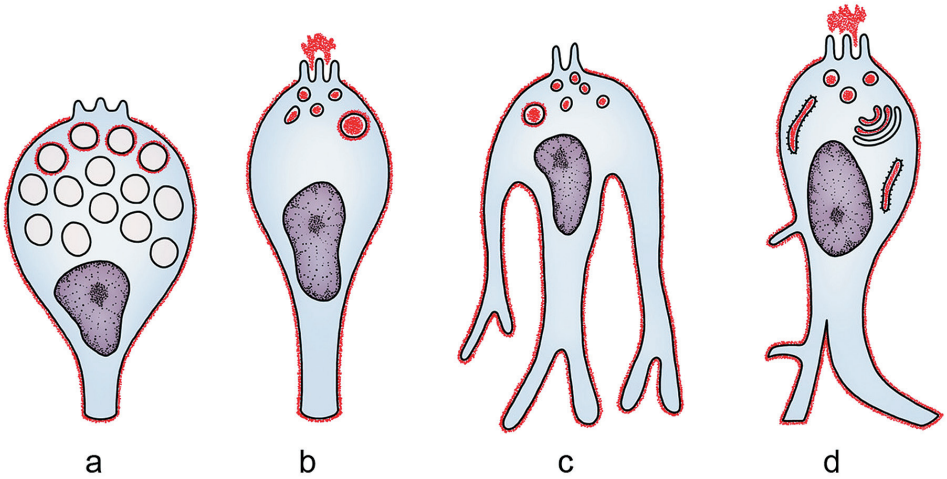
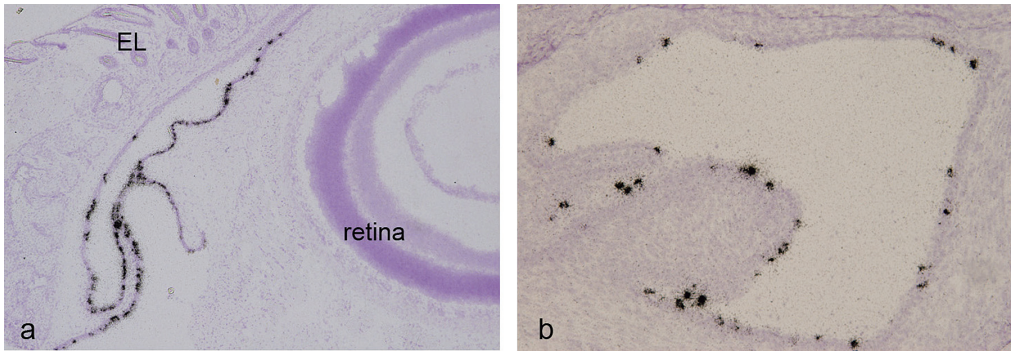
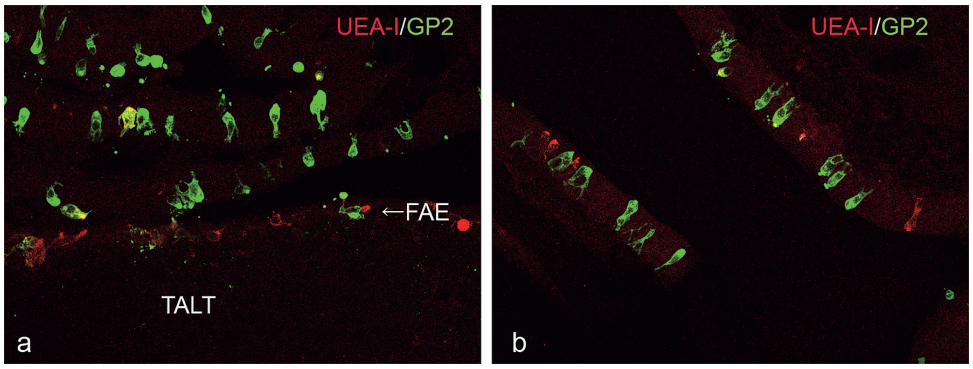


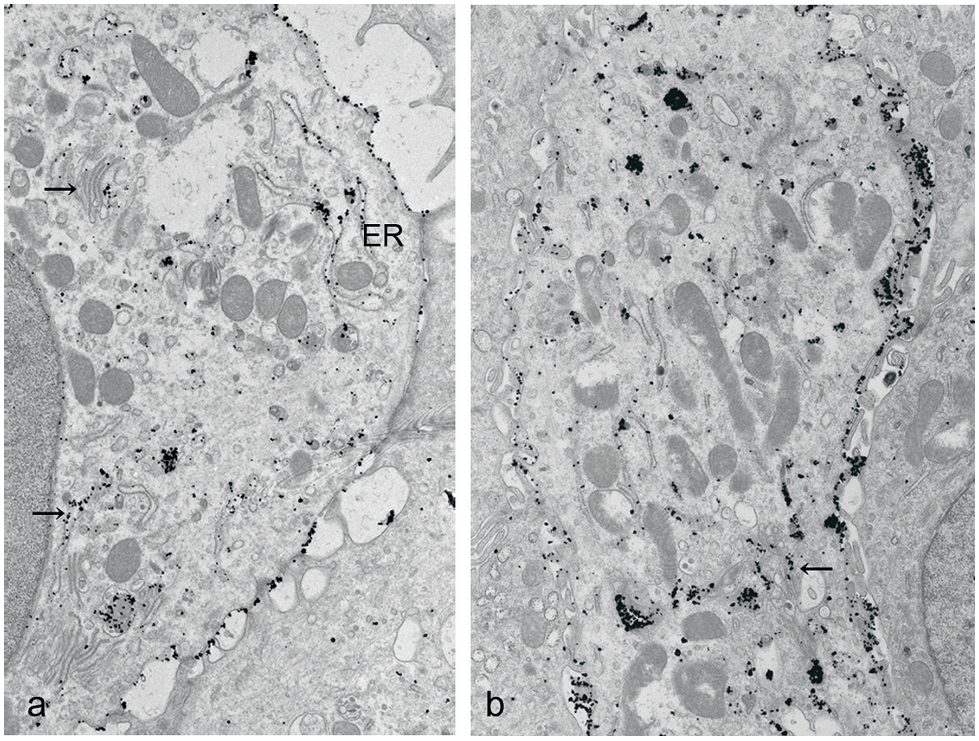
Supplementary Figure 1 A scheme showing the morphology of GP2-expressing goblet cells in the conjunctiva (a) and peculiarly shaped cells in the tear duct (b–d). Localization of GP2 is shown in red.



Supplementary Figure 2 In situ hybridization analysis of GP2 mRNA using a 33P-labeled oligonucleotide DNA probe (45 mer in length). Condensed signals are dispersed along epithelia of the conjunctiva (a) and nasolacrimal duct (b). EL: eyelid



Supplementary Figure 3 Double staining using GP2 antibody and UEA-I, a classical marker of M cells, in the lacrimal sac (a) and nasolacrimal duct (b). UEA-I-labeled cells (red) are seen in the follicle-associated epithelium (FAE) of tear-associated lymphoid tissue (TALT) but are fewer in the ordinary epithelium, where GP2-immunoreactive cells (green) are numerous (a). In the nasolacrimal duct, UEA-I labeling and GP2 immunoreactivity are separately contained in different cells (b).



Supplementary Figure 4 Intracellular localization of GP2 in the lacrimal sac. Immunogold particles are seen inside the endoplasmic reticulum (ER) (a) and are associated with the Golgi apparatus (arrows in a and b).