Morphological and histochemical characterization of the secretory epithelium in the canine lacrimal gland

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ABSTRACT

In the present study, the expression of secretory components and vesicular transport proteins in the canine lacrimal gland was examined and morphometric analysis was performed. The secretory epithelium consists of two types of secretory cells with different morphological features. The secretory cells constituting acinar units (type A cells) exhibited higher levels of glycoconjugates, including β-GlcNAc, than the other cell type constituting tubular units (type T cells). Immunoblot analysis revealed that antimicrobial proteins, such as lysozyme, lactoferrin and lactoperoxidase, Rab proteins (Rab3d, Rab27a and Rab27b) and soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins (VAMP2, VAMP4, VAMP8, syntaxin-1, syntaxin-4 and syntaxin-6), were expressed at various levels. We immunohistochemically demonstrated that the expression patterns of lysozyme, lactoferrin, Rab27a, Rab27b, VAMP4, VAMP8 and syntaxin-6 differed depending on the secretory cell type. Additionally, in type T cells, VAMP4 was confined to a subpopulation of secretory granules, while VAMP8 was detected in almost all of them. The present study displayed the morphological and histochemical characteristics of the secretory epithelium in the canine lacrimal gland. These findings will help elucidate the species-specific properties of this gland.

Key words: dog; lacrimal gland; glycoconjugate; Rab protein; SNARE protein; electron microscopy.

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Introduction

In humans, tear is composed of a complex mixture of various components, including numerous proteins, such as lysozyme, lactoferrin, secretory IgA, lipocalin and growth factors, and plays an essential role in maintaining the integrity of the ocular surface.[1-4] The secretory cells of the lacrimal gland serve as a main source of tear proteins and fluid onto the ocular surface. These secretory proteins are secreted by a regulated or constitutive pathway.[2] Rab proteins are important regulators of intracellular vesicle trafficking. More than 60 Rab proteins have been identified in mammalian cells, and they exhibit ubiquitous or tissue-specific expression.[5,6] According to previous studies, Rab3d, Rab27a and Rab27b are expressed in exocrine cells, such as acinar cells of the pancreas,[7-9] parotid gland,[10-12] and lacrimal gland.[13-16] Soluble *N*-ethyl-maleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins mediate intracellular membrane fusion and can be functionally classified as v-SNARE and t-SNARE.[17-19] Vesicle-associated membrane proteins (VAMPs), known as v-SNAREs, are associated with transport vesicles, while syntaxins, known as t-SNAREs, are restrictedly distributed in the target membranes. These proteins have been shown to play an important role in neurotransmitter exocytosis of neuronal cells and to be involved in regulating exocytosis in several exocrine glands, including the lacrimal gland.[20-25] However, most of the findings concerning Rab and SNARE proteins in exocrine glands are based on analyses of human and rodent tissues or cultured cells of exocrine gland origin. There are few reports on the expression of these proteins in the exocrine glands of other mammalian species, except for our recent studies.[26,27] The lacrimal gland has been described as compound tubuloacinar or tubuloaveolar in structure, but the secretory unit distribution differs among mammalian species. The canine lacrimal gland is generally regarded as a seromucous gland.[28-30] However, Kuhnel showed this gland to be purely a mucus gland.[31] Therefore, the structural characteristics of this gland remain controversial due to the inconsistency in these findings. In dogs, the lacrimal gland and third eyelid gland are present as lacrimal apparatuses in the orbit, but the former is the main source gland of tears. Thus, the present study investigated the detailed ultrastructural features and distribution of glycoconjugates in the lacrimal gland of dogs. In addition, we attempted to elucidate the expression of antimicrobial, Rab and SNARE proteins by immunoblotting, immunohistochemistry and/or immunocytochemistry. The results obtained may be important not only for elucidating the morphological and functional characterization of the secretory epithelium in the canine lacrimal gland but also for understanding the differences with other mammalian species.

Materials and Methods

Animals and tissue preparation

Following euthanization under deep anesthesia, four male beagle dogs (aged 12, 14, 15 and 28 months; purchased from Kitayama Labes Co., Ltd., Nagano, Japan) were processed for sample preparation. For light microscopy, the tissue samples of the lacrimal gland located dorsolateral to the eyeball, the so-called main lacrimal gland, were fixed in Bouin's solution for 24 h at room temperature and then carefully dehydrated in a graded series of ethanol. These materials were embedded in paraffin wax and cut at a thickness of 5 μm. For general cytological observation, the tissue pieces were fixed in 2.5% glutaraldehyde (GA) in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. They were postfixed in 2% osmium tetroxide solution for 2 h and embedded in Epon 812 in accordance with routine techniques.[32] From these tissue blocks, ultrathin sections were cut on an ultramicrotome and mounted on copper grids. For cytochemical demonstration, the tissue specimens were fixed in 4% paraformaldehyde and 0.2% GA solution in 0.1 M phosphate buffer (pH 7.4) for 2 hr at 4°C and embedded in LR White resin.[33] Ultrathin sections were cut as described above and placed on nylon or nickel grids. For immunoblotting, the lacrimal and parotid gland tissues were frozen on dry ice and stored at -80°C until use.

General morphology

Deparaffinized sections were stained with hematoxylin-eosin (H-E) and Mallory-Azan for histological purposes. For electron microscopy, copper grid-mounted ultrathin sections were stained with 0.2% oolong tea extract (OTE) (Nisshin EM, Tokyo, Japan), 10% TI Blue (Nisshin EM) and lead citrate. The ultrathin sections prepared were examined under an electron microscope (JEM 1011; JEOL, Ltd., Tokyo, Japan). The size and number of secretory granules were analyzed by ImageJ 1.53 software.

Glycoconjugate histochemistry and cytochemistry

For light microscopy, the Alcian blue pH 2.5-periodic acid Schiff (AB-PAS) method was performed to demonstrate the presence of glycoconjugates. For lectin histochemical analysis, deparaffinized sections were treated with an Avidin/Biotin Blocking Kit (Abcam Plc., Cambridge, UK) and Dako REAL Peroxidase-Blocking solution (Agilent Technologies, Inc., Santa Clara, CA, USA). Then, the samples were incubated with different biotinylated lectins (J-Chemical, Inc., Tokyo, Japan) at concentrations of 10-20 μg/ml in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) overnight, followed by preincubation with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) in PBS. After PBS rinses, the sections were soaked in peroxidase-labeled streptavidin (Nichirei Co., Tokyo, Japan) for 30 min at room temperature and then visualized using the Diaminobenzidine (DAB) Peroxidase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA, USA). In addition, these sections were counterstained with hematoxylin. The lectins used were concanavalin A (Con A), peanut agglutinin (PNA), wheat germ agglutinin (WGA) and *Sambucus sieboldiana* agglutinin (SSA). Their specific sugar residues are listed in Table 1.[34,35] For glycoconjugate cytochemistry, nylon grid-mounted ultrathin sections were subjected to the periodic acid-thiocarbohydrazide-silver proteinate-physical development procedure (PA-TCH-SP-PD) for the demonstration of vic-

Table 1. The lectins used and their carbohydrate binding specificities.

Lectins	Abbreviation	Carbohydrate binding specificity
Concanavalin A	Con A	α-Man, α-Glc
Peanut agglutinin	PNA	Galβ1-3GalNAc
Wheat germ agglutinin	WGA	β-GlcNAc
Sambucus sieboldiana agglutinin	SSA	Siaα2-6Gal/GalNAc

Gal, galactose; GalNAc, N-acetyl-glactosamine; Glc, glucose; GlcNAc, N-acetyl-glucosamine; Man, mannose; Sia, sialic acid.

inal diols of carbohydrates.[36] The ultrathin sections on the nickel grids were pretreated with 1% BSA in PBS and then incubated with different biotinylated lectins (WGA, SSA) at concentrations of 5-10 µg/ml in PBS for 12 h at 4°C. After PBS rinses, the sections were incubated with 15 nm colloidal gold-labeled streptavidin (BBI solutions, Crumlin, UK) at a dilution of 1:20 in PBS and then counterstained with OTE, TI Blue and lead citrate.

For specificity controls of the histochemical and cytochemical lectin procedures, sections were incubated with unconjugated lectins (5-20 µg/ml) instead of biotinylated lectins or with PBS without lectins.

Immunoblotting

Tissue sample preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was performed as described previously.[37] The parotid gland tissue was used as a control. Each protein extract, which was normalized to the total protein content (25 µg), was subjected to SDS-PAGE and transferred to Immobilon-P or Immobilon-PSQ transferred membranes (Merck Millipore, Darmstadt, Germany). The primary antibodies used and their dilutions are listed in Table 2. For the analysis of antimicrobial proteins, primary antibodies against lysozyme, lacto-

ferrin and lactoperoxidase were used. Primary antibodies against Rab3d, Rab27a, Rab27b, VAMP2, VAMP4, VAMP8, syntaxin-1, syntaxin-4 and syntaxin-6 were used to analyze Rab and SNARE proteins. Anti-α-tubulin antibody was used as the loading control. The blocking treatment was performed by incubation with 5% skim milk in Tris-buffered saline (pH 7.4) supplemented with 0.05% Tween 20 for 1 h. The peroxidase-conjugated secondary antibodies (diluted 1:5000) used were as follows: goat anti-rabbit IgG, goat anti-mouse IgG and donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoblot analysis was performed as described previously.[26,37]

Immunohistochemistry and immunocytochemistry

For light microscopy, primary antibodies against lysozyme, lactoferrin, Rab3d, Rab27a, Rab27b, VAMP4, VAMP8, syntaxin-4 and syntaxin-6 were used. Their dilutions are listed in Table 2. Deparaffinized sections were pretreated with microwave irradiation (500 W, 6 min) in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. The sections were immersed in Dako REAL Peroxidase-Blocking solution and blocked with 3% BSA in PBS. After incubation with primary antibodies overnight at 4°C, the sections were rinsed in PBS and then incubated with secondary antibodies using
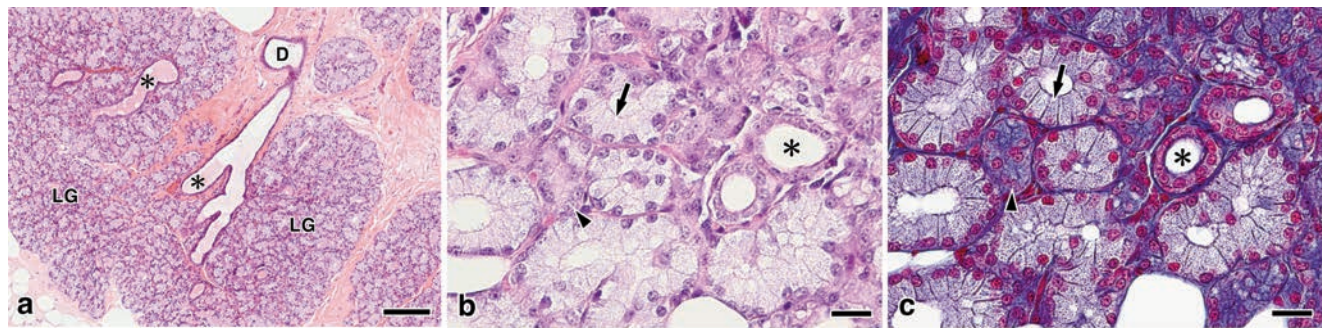

Figure 1. General histology of the canine lacrimal gland. a) The secretory portion forms a number of lobules; H&E; scale bar: 150 µm. b) Higher magnification of the secretory epithelium stained with H&E; scale bar: 20 µm. c) Higher magnification of the secretory epithelium stained with Mallory-Azan; scale bar: 20 µm. Arrows, type T cells; arrowheads, type A cells; asterisks, intralobular ducts; D, interlobular duct.

Table 2. Primary antibodies used for immunoblotting, immunohistochemistry and immunocytochemistry.

Specificity	Host	Clonality	Dilution (usage)	Product* (catalog no.)
Lysozyme	Rabbit	Polyclonal	1:1000 (IB)/1:100 (LM)	Thermo (PA5-16668)
Lactoferrin	Mouse	Monoclonal	1:500 (IB, LM)	Abcam (ab10110)
Lactoperoxidase	Goat	Polyclonal	1:7500 (IB)	Abcam (ab82150)
Rab3d	Rabbit	Polyclonal	1:50 (IB)/1:100 (LM)	Proteintech (12320-1-AP)
Rab27a	Rabbit	Polyclonal	1:200 (IB)/1:100 (LM)	Proteintech (17817-1-AP)
Rab27b	Rabbit	Polyclonal	1:600 (IB)/1:100 (LM)	Proteintech (13412-1-AP)
VAMP2	Rabbit	Polyclonal	1:500 (IB)	Thermo (OSS00035w)
VAMP4	Rabbit	Polyclonal	1:200 (IB)/1:1000 (LM)1:500 (EM)	Abcam (ab80989)
VAMP8	Rabbit	Polyclonal	1:300 (IB)	Aviva (ARP59536-P050)
VAMP8	Rabbit	Polyclonal	1:400 (LM, EM)	Novus (NBP1-20232)
Syntaxin 1	Mouse	Monoclonal	1:300 (IB)	Sigma-Aldrich (S0664)
Syntaxin 4	Rabbit	Polyclonal	1:200 (IB, LM)	Atlas (HPA001330)
Syntaxin 6	Mouse	Monoclonal	1:200 (IB)/1:100 (LM)	Abcam (ab56656)
α-Tubulin	Mouse	Monoclonal	1:3000 (IB)	Sigma-Aldrich (T6199)

IB, immunoblotting; LM, light microscopic immunohistochemistry; EM, electron microscopic immunocytochemistry. *Products from Abcam Plc. (Cambrige, UK); Atlas Antibodies AB (Stockholm, Sweden); Aviva System Biology (San Diego, CA, USA); Novus Biologicals (Littleton, CO, USA); Proteintech Group, Inc. (Rosemont, IL, USA); Sigma-Aldrich, Inc. (St. Louis, MO, USA); Thermo Fisher Scientific (Rockford, IL, USA).

the horseradish peroxidase (HRP)-labeled polymer technique (Envision+ Dual Link System-HRP; Agilent Technologies) for 1 h at room temperature. The immunoreaction was visualized using the DAB Peroxidase Substrate Kit and counterstained with hematoxylin. For electron microscopy, nickel grid-mounted ultrathin sections were incubated with primary antibodies against VAMP4 and VAMP8 overnight at 4°C, followed by pretreatment with 3% BSA in PBS. After PBS rinses, these ultrathin sections were incubated with biotinylated secondary antibody (dilution 1:500, donkey anti-rabbit IgG; Jackson ImmunoResearch Laboratories) and then with 15 nm colloidal gold-labeled streptavidin. Counterstaining was performed by the abovementioned procedure.

For negative controls for immunohistochemical and immunocytochemical procedures, the sections were incubated with a mixture of diluted primary antibodies that were pretreated with the respective absorbing antigens (2-10 µg/ml) or with normal rabbit or mouse IgGs (1 µg/ml; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) instead of the primary antibodies.

Results

General morphological observation and morphometric analysis of secretory granules

In the dog, the secretory portion of the lacrimal gland formed a number of lobules that were connected to the interlobular duct (Figure 1a). The lacrimal gland was tubuloacinar in configuration and found to consist of two types of secretory cells, which showed differential H-E and Mallory-Azan staining (Figure 1 b,c). Therefore, we classified the secretory cells constituting tubular units as type T cells and those constituting acinar units as type A cells. The type A cells were slightly more predominant (approximately 55%) than in the type T cells (approximately 45%). For general cytological observation, both cell types contained a well-developed Golgi apparatus and a large number of secretory granules within their cytoplasm, and the type T cells had a more promi-
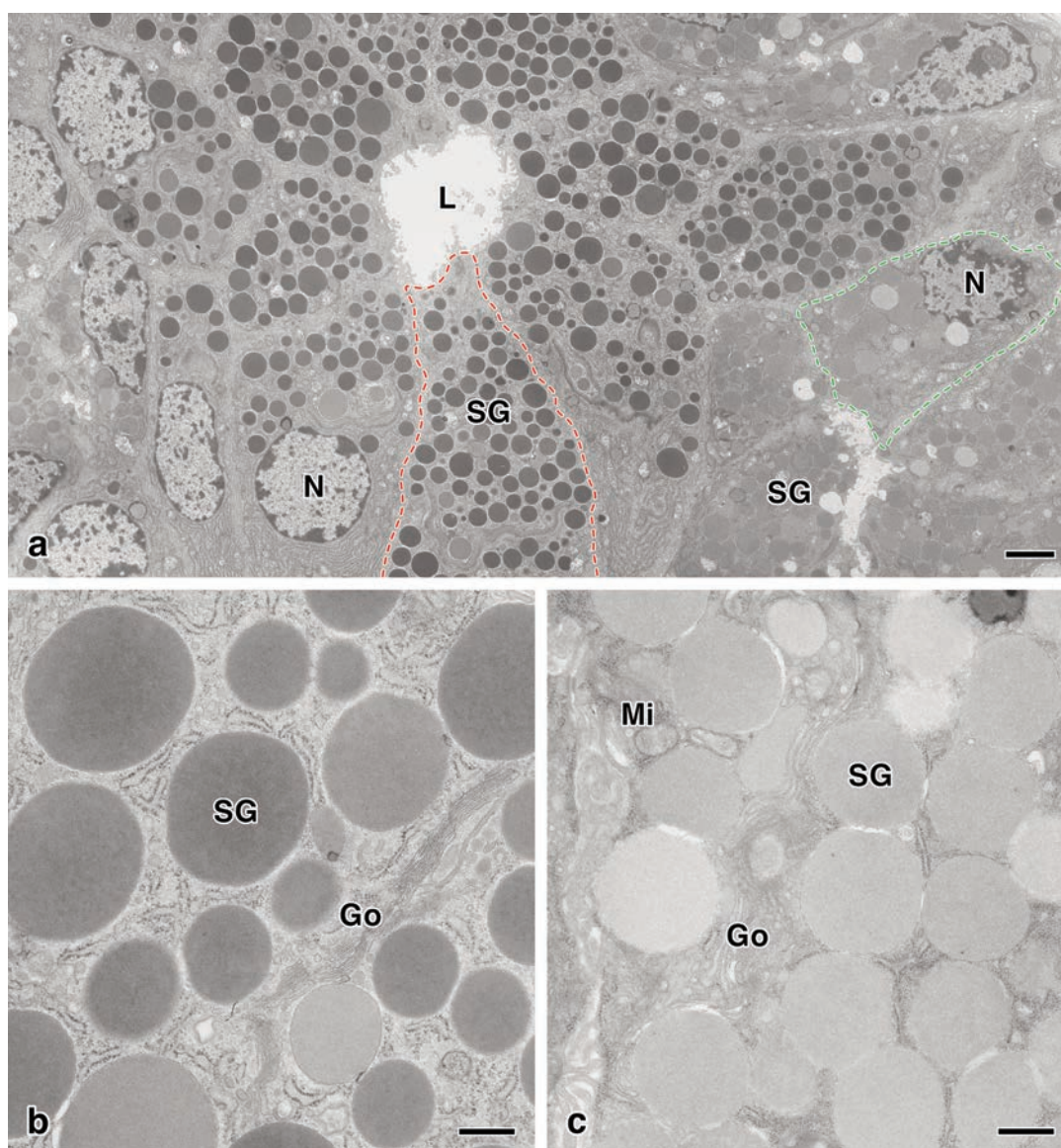

Figure 2. General ultrastructure of the lacrimal gland. a) The secretory portion consists of type T cells (red dashed line) and type A cells (green dashed line); scale bar: 2 µm. b) Higher magnification of type T cells; scale bar: 0.5 µm. c) Higher magnification of type A cells, scale bar: 0.5 µm. Go, Golgi apparatus, L, lumen, Mi, mitochondrion, N, nucleus, SG, secretory granule.

nent rough endoplasmic reticulum than the type A cells (Figure 2 a-c). The secretory granules of the type T cells possessed relatively high electron density (Figure 2 a,b), while those of the type A cells showed moderate electron density (Figure 2 a,c). In addition, intercellular canaliculi were visualized between neighboring type A cells. For morphometric analysis, we examined the size and number of secretory granules in both cell types. The percentage of secretory granules >0.8 μm^2 was higher in type T cells than in type A cells (Figure 3a). The mean secretory granular size in the former cell type was also larger than that in the latter cell type (mean ± SEM, 0.738±0.01 μm^2 in type T cells *vs* 0.585±0.008 μm^2 in type A cells). However, in both cell types, there was a variation in the mean of these sizes in each cell (Figure 3b). The mean granule density of the type A cells was higher than that of the type T cells (0.61±0.03 number/μm^2 in type T cells *vs* 0.82±0.04 number/μm^2 in type A cells).

Glycoconjugate histochemical and cytochemical observation

In the lacrimal glandular epithelium, the AB-PAS procedure gave rise to a weak positive reaction in the type T cells and a moderate or strong positive reaction in the type A cells (Figure 4a). For lectin histochemical methods, both cell types were found to exhibit positive reactions after treatment with Con A, PNA and SSA (Figure 4 b,d). When incubated with WGA, however, a positive reaction was clearly confined to the type A cells (Figure 4c).

In ultrathin sections stained with the PA-TCH-SP-PD procedure, the secretory granules of the type T cells revealed a weak to moderate positive reaction, whereas a distinct positive reaction was observed in those of the type A cells (Figure 5 a,b). Other prominent PA-TSH-SP-PD reactive structures were detected in the secretory cells at the surface coat of the plasma membrane and in cisternae of the Golgi apparatus. After the WGA-gold procedure, reactive particles were scarcely detectable in the type T cells
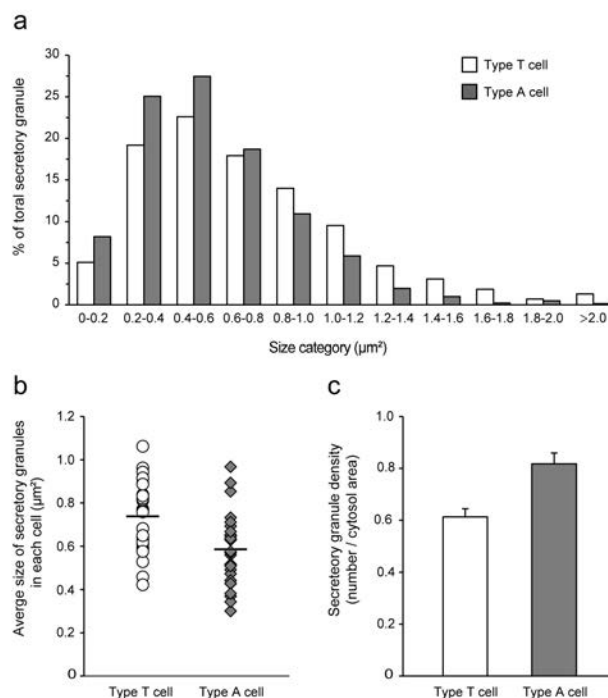

Figure 3. Morphometric analysis of secretory granules in the lacrimal gland. For each cell type, 10 randomly selected cells from three different animals were analyzed. a) Morphometric analysis of size distribution for secretory granules; the total number of secretory granules analyzed was 1606 from type T cells and 1428 from type A cells. b) Average size of secretory granules in each cell. c) Average number of secretory granules per cytosol area (μm^2). The results are provided as the mean ± SEM.
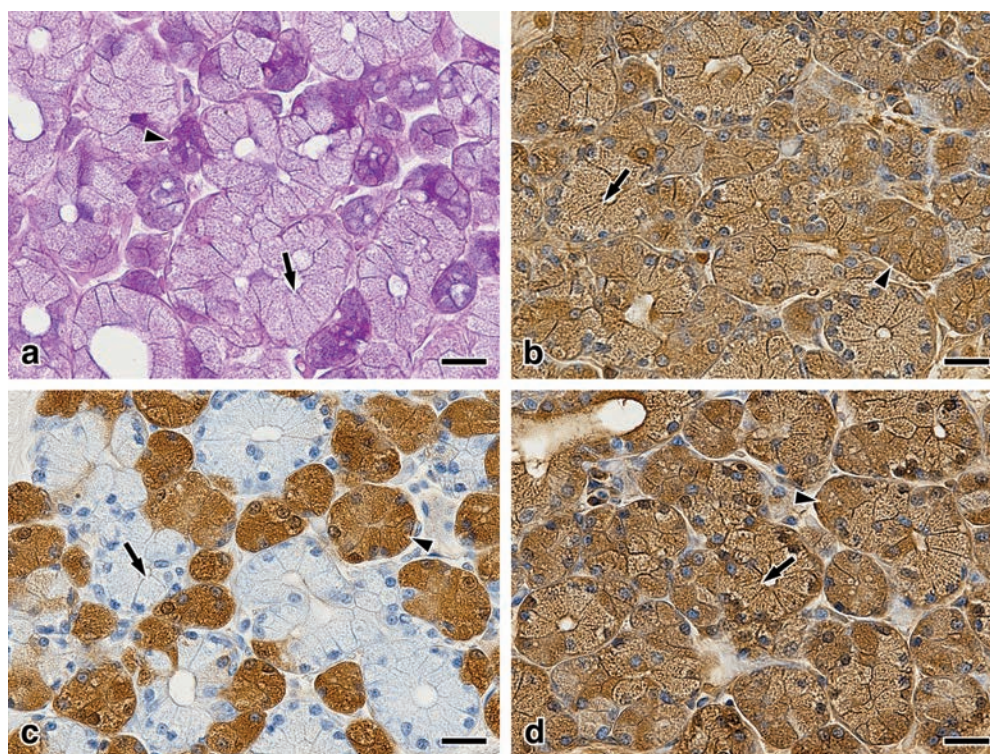

Figure 4. General glycoconjugate and lectin histochemical appearance of the lacrimal gland. a) AB-PAS. b) Con A. c) WGA. d) SSA. Arrows: type T cells, arrowheads: type A cells; scale bars: 20 μm.

(Figure 5c). In contrast, the type A cells showed a distinct positive reaction in the secretory granules, Golgi apparatus and surface coat of the plasma membrane facing the lumen and intercellular canaliculus (Figure 5d). In the SSA-gold technique, both cell types displayed identical staining images in which gold particles were associated with the secretory granules, Golgi apparatus and surface coat of the plasma membrane (Figure 5 e,f).

In all of the control experiments for the lectin histochemical and cytochemical procedures, the positive reactions were greatly diminished or nearly abolished in the glandular structures of the lacrimal gland.
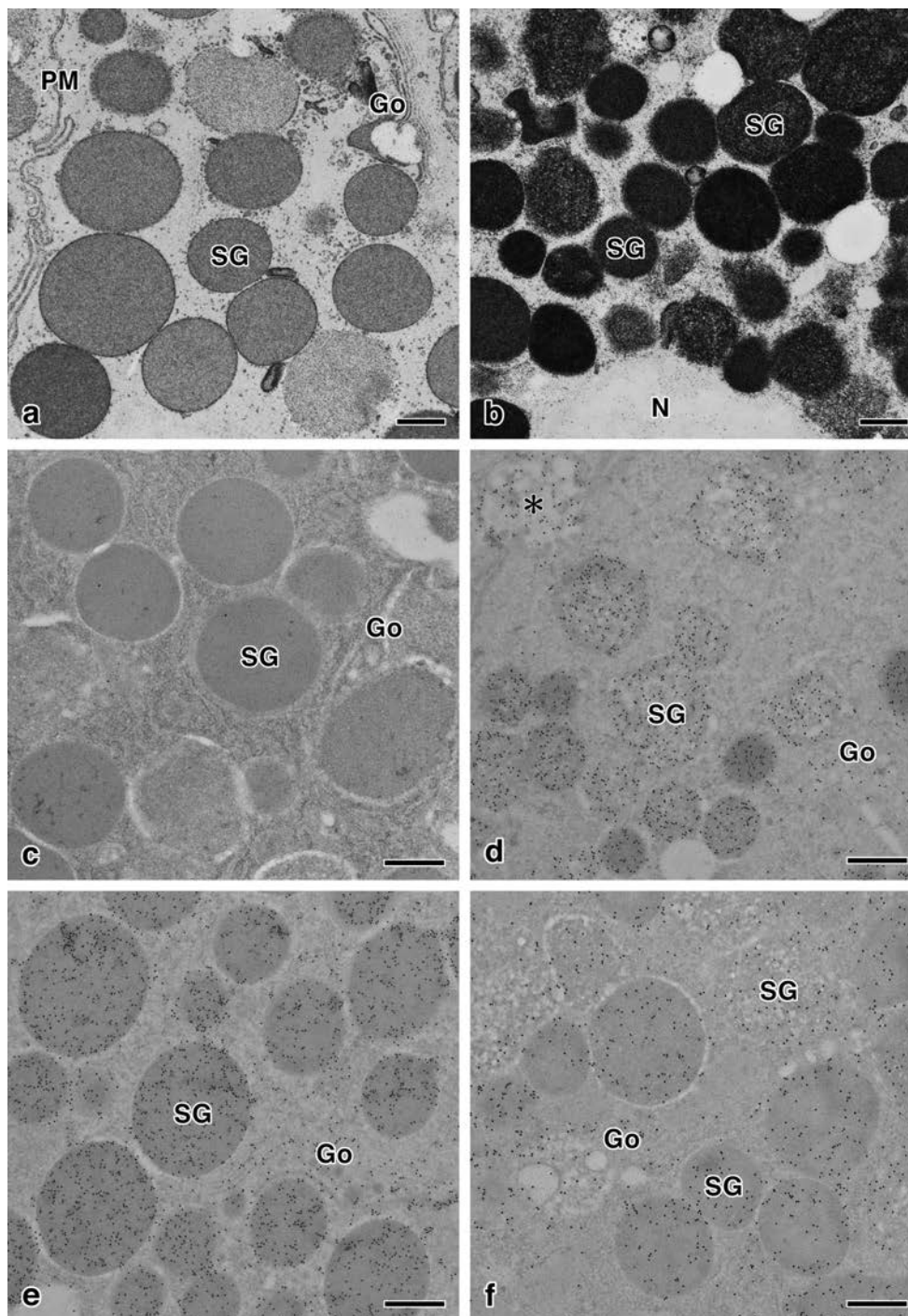

Figure 5. Glycoconjugate cytochemical staining in the lacrimal gland. a) Cytochemical PA-TCH-SP-PD staining of type T cells. b) Cytochemical PA-TCH-SP-PD staining of type A cells. c) WGA-gold staining of type T cells. d) WGA-gold staining of type A cells. e) SSA-gold staining of type T cells. f) SSA-gold staining of type A cells. Go: Golgi apparatus, N: nucleus, PM: plasma membrane, SG: secretory granule, asterisk: intercellular canaliculus; scale bars: 0.5 μm.

Expression of antimicrobial, Rab and SNARE proteins using immunoblotting

The expression of antimicrobial, Rab and SNARE proteins was analyzed by immunoblotting (Figure 6). The expression pattern of antimicrobial proteins in the lacrimal gland was different from that in the parotid gland. In the lacrimal gland, lysozyme and lactoferrin were expressed, while the expression of lactoperoxidase could not be detected. With regard to Rab and SNARE proteins, their expression levels in the lacrimal gland were broadly similar to those in the parotid gland except for Rab3d, VAMP8 and Syntaxin-6. The signals for these proteins were more strongly detected in the lacrimal gland than in the parotid gland, especially for Rab3d and VAMP8.

Immunohistochemical and immunocytochemical observation

For the immunohistochemical analysis of antimicrobial proteins, antibodies to lysozyme produced a weak positive reaction of the type T cells, whereas a relatively strong positive reaction was detected in the type A cells (Figure 7a). In contrast, a positive reaction for lactoferrin was restricted to type T cells (Figure 7b). Regarding the immunohistochemical labeling for Rab and SNARE proteins, in both cell types, the secretory cells showed varying reaction intensities after treatment with antibodies to Rab3d (Figure 7c). Positive reactions for Rab27a and Rab27b were mainly observed in the type T cells (Figure 7d). A clear positive reaction for VAMP4 was also detected in the type T cells (Figure 7e), and the staining images of VAMP8 were similar to those of VAMP4 (Figure 7f). Incubation with antibodies against syntaxin-4 led to a staining pattern similar to that obtained with Rab3d (Figure 7g). Antibodies against syntaxin-6 gave rise to a weak positive reaction in the type T cells (Figure 7h).

With regard to the immunocytochemical approach for VAMP4, a subpopulation of the secretory granules (approximately 23% of total secretory granules) in the type T cells showed a clear positive reaction in which reactive particles were associated with their membranes (Figure 8a). In addition, a few gold particles were occasionally detected in the Golgi apparatus of this cell type. Type A cells were almost all negative for antibodies to VAMP4. The immunocytochemical technique for VAMP8 demonstrated that reactive particles were observed in almost all the secretory granule membranes of the type T cells (Figure 8b). Specific labeling for VAMP8 was also found to be almost absent in type A cells, similar to VAMP4.

In the control experiments for the immunohistochemical and immunocytochemical procedures, the significant immunoreactivity of all the formerly reactive structures was strongly suppressed or abolished.

Discussion

The ocular surface is covered and protected by tear film possessing various functions, such as nutrition for the cornea, antimicrobial activity and lubrication.[38] According to recent studies, the tear film is considered a bilayered structure, which consists of an aqueous mucin layer containing fluid, various proteins and secretory mucin, under a thin lipid layer. The lacrimal gland acts as a major contributor to the aqueous mucin layer.[39-41] In dogs, the lacrimal gland and third eyelid gland are the two intraorbital glands, and it has been reported that approximately 70% of tear production originates from the former gland.[42] The histological structures of the canine lacrimal gland (H-E and Mallory-Azan staining) were similar to those of seromucous glands such as salivary glands. However, the cytological and glycoconjugate histo-

chemical observations of the secretory cells obtained differed from those of the salivary glandular cells.[26,43,44] According to Martin et al., three types of secretory granules may be present in the acinar cells of canine lacrimal and third eyelid glands.[30] The structural features of the granule type in tubular cells and the most common granule type in acinar cells correspond to our results except for the electron density of the latter. Electron-lucent secretory granules in the acinar cells, which are consistent with those shown by Kuhnel,[31] could not be observed in this study. This discrepancy may be influenced by fixation or staining reagents. In addition, clusters of acinar cells containing lipid granules are present in the periphery of the third eyelid gland, but not in the lacrimal gland.[30,45]

The present study showed that both secretory cell types, classified as type T cells and type A cells, had different morphological and morphometrical features of the secretory granules.
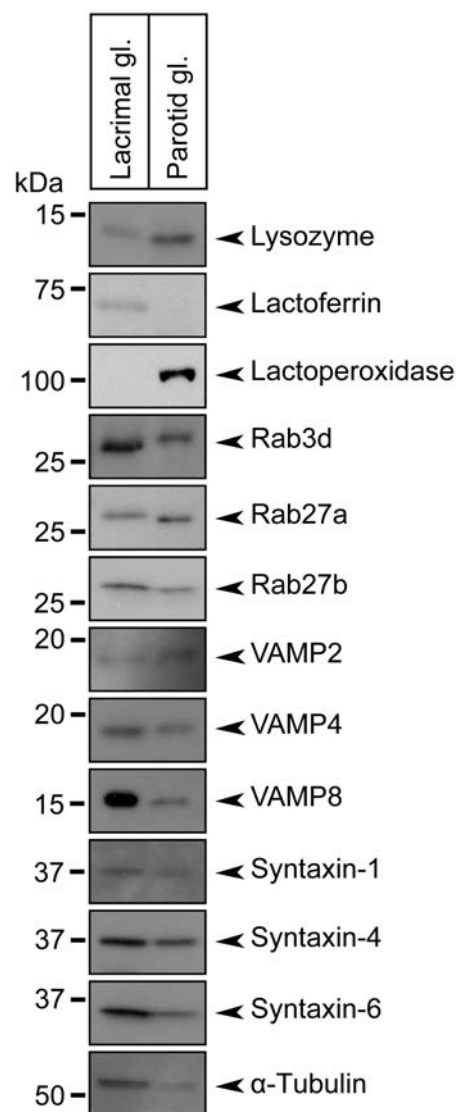

Figure 6. Expression of antimicrobial, Rab and SNARE proteins in the lacrimal gland. Equal amounts of protein extracts (25 μg) from the lacrimal and parotid glands were subjected to SDS-PAGE, followed by immunoblotting. The bottom panel shows the control α-tubulin immunoblots.
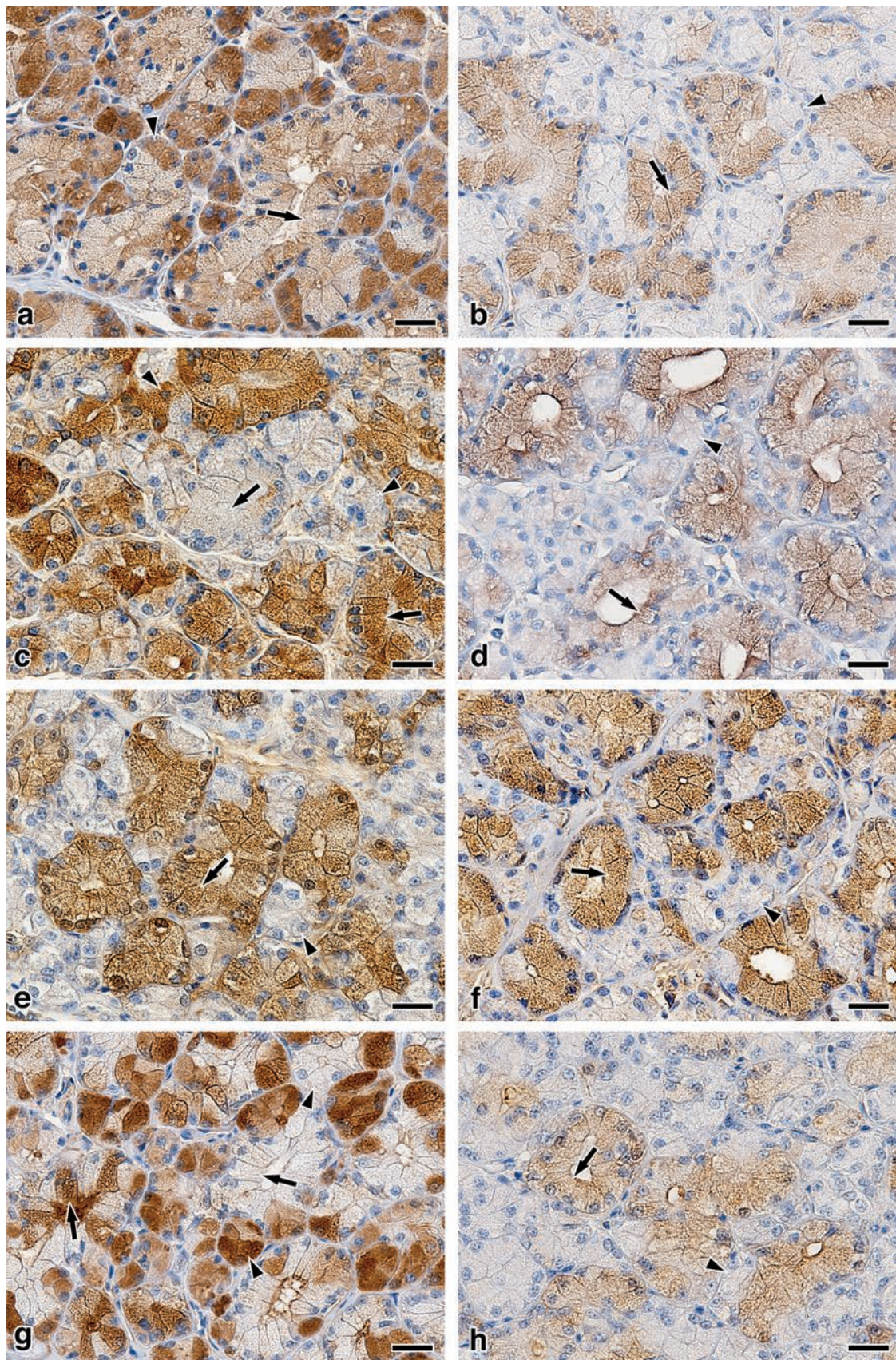
Figure 7. Immunohistochemical staining for the detection of antimicrobial, Rab and SNARE proteins in the lacrimal gland. a) Lysozyme. b) Lactoferrin. c) Rab3d. d) Rab27b. e) VAMP4. f) VAMP8. g) Syntaxin-4. h) Syntaxin-6. Arrows, type T cells; arrowheads, type A cells; scale bars: 20 μm.

Furthermore, the secretory granules of the A cell type had a higher concentration of glycoconjugates than those of the T cell type, as shown by the staining ability of the PA-TCH-SP-PD procedure.[36] Although the presence of glycoconjugates with saccharide residues, such as α-Man, Galβ1-3GalNAc and Siaα2-6Gal/GalNAc, was detected in both cell types by lectin histochemical and cytochemical procedures, the distribution of β-GlcNAc residues was confined to type A cells. Therefore, the glycoconjugate histochemical results obtained suggest that there is a qualitative difference in secretory products between both cell types. The ocular surface is constantly exposed to environmental pathogens, and thus human tear film includes antimicrobial proteins, such as lysozyme, lactoferrin, lipocalin and defensins, which are mainly secreted by the lacrimal gland.[2,38,41,46,47] In this study, lysozyme and lactoferrin were expressed in the canine lacrimal gland, as shown using immunoblotting, whereas immunohistochemical analysis revealed that the localization of these proteins varied depending on the cell type. These findings are consistent with the abovementioned suggestion.

We demonstrated that Rab27a and Rab27b were predominantly expressed in type T cells of the canine lacrimal gland. Previous studies have provided evidence that Rab27a and Rab27b, which are localized on the secretory granule membranes of rat parotid and mouse pancreatic acinar cells, may regulate the secretion of digestive enzymes.[8,9,48,49] Additionally, these isoforms seem to be involved in the release of secretory granules in rabbit primary cultured cells of the lacrimal gland.[15,16] Functionally, Rab3d appears to play an important role during regulated exocytosis in exocrine secretion.[7,11] However, the secretory granular size of parotid and pancreatic acinar cells in Rab3d knockout mice was significantly increased compared with that in wild-type mice, although the change in release kinetics in response to secretagogue stimulation was not detected, suggesting that Rab3d may be important for preventing homotypic fusion of secretory granules rather than for regulating exocytosis.[50] Thus, the role of Rab3d in exocrine glands is not conclusive. In the present study, the immunoreactivity of Rab3d was inconsistent even in the same cell type, in addition to the variance in the mean size of the secretory granules in each cell.

Our results support the view that Rab3d participates in granule formation of exocrine glands.

This study also detected the expression of VAMP4, VAMP8, syntaxin-4 and syntaxin-6 in the canine lacrimal gland using immunoblotting. In addition, immunohistochemical analysis demonstrated that VAMP4, VAMP8 and syntaxin-6 were predominantly expressed in type T cells. The expression of VAMP2 was observed in rabbit primary lacrimal acinar cells.[13] However, in the present study, the expression level of VAMP2 was very weak, notwithstanding that the immunogenic peptides of the anti-VAMP2 antibody used correspond exactly to the canine amino acid sequence. The weak signal for syntaxin-1 in immunoblot analysis is assumed to originate from the nerve fibers distributed in this gland, since this protein is known as an important t-SNARE in the release of a neurotransmitter from the synaptic vesicles of neurons.[51] VAMP4, which colocalizes with syntaxin-6, is localized in the trans Golgi network (TGN) membranes, medial compartment of the Golgi apparatus, clathrin-coated and noncoated vesicles, endosomes and immature secretory granules in PC12 cells. Therefore, the interaction of VAMP4 with syntaxin-6 may be involved in TGN-to-endosome transport.[52] Additionally, in rat parotid acinar cells, it has also been suggested that VAMP4 and syntaxin-6 are transported from immature secretory granules to early endosomes during the process of granule maturation.[53,54] The present study provided the first information, to our knowledge, on the localization of VAMP4 in exocrine gland tissues at the cytological level. The formation of the VAMP4/syntaxin-6 complex may have an important role in the maturation of secretory granules in the canine lacrimal gland based on the expression pattern of these proteins. Although VAMP8 was originally identified as an endosomal SNARE,[55,56] this protein may be implicated in regulated granule secretion of platelets and mast cells.[57,58] Previous studies using knockout mice have suggested that VAMP8 plays an important role in regulated exocytosis of pancreatic acinar cells, salivary glands and lacrimal glands by serving as a v-SNARE that may interact with syntaxin-4 and SANP-23.[21,22] Additionally, there are alterations in the distribution and mRNA level of VAMP8 in the lacrimal gland with impaired secretory function, such as Sjögren's
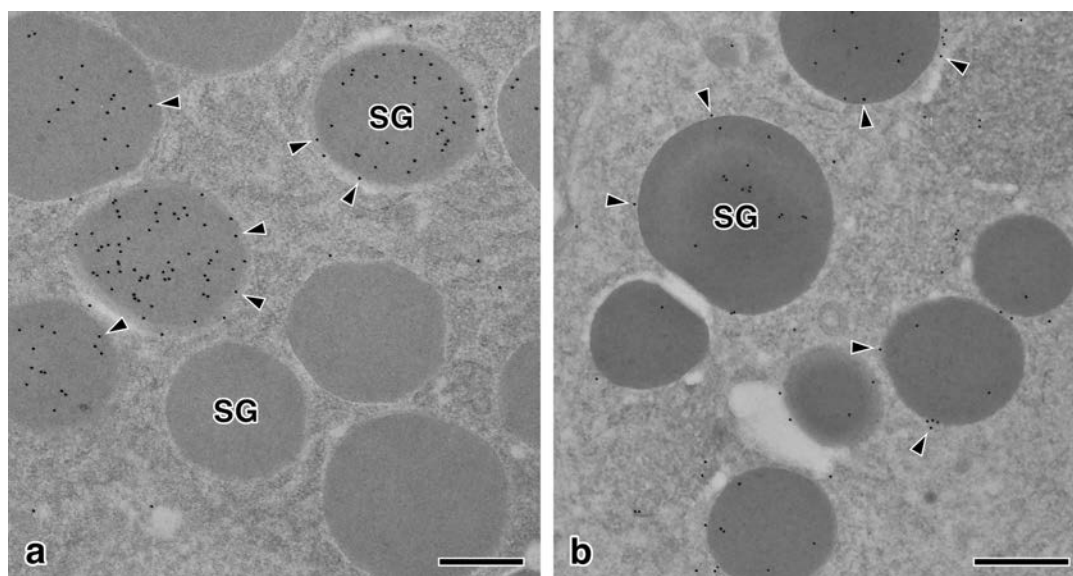

Figure 8. Immunocytochemical staining for the detection of VAMP4 and VAMP8 in the lacrimal gland. a) Immunogold labeling for VAMP4 of type T cells. b) Immunogold labeling for VAMP8 of type T cells. Arrowheads, reactive particles associated with secretory granule membranes; scale bars: 0.5 μm.

syndrome, visual display terminal syndrome and chronic graft-versus-host disease.[59,60] The localization of VAMP8 was observed in the membrane of the secretory granules in the type T cells of the canine lacrimal gland, identical to previous results indicating its presence in the zymogen granule membranes of pancreatic acini.[21] VAMP8 may also be involved in regulated exocytosis in the canine lacrimal gland, even though the expression patterns of VAMP8 and syntaxin-4 in the secretory epithelium were not necessarily consistent.

In conclusion, we demonstrated, for the first time in domesticated animals, that the secretory epithelium of the canine lacrimal gland displayed differences in the expression of secretory components and vesicular transport proteins between secretory cell types in addition to their morphological features. Although some have suggested that the ultrastructural variation of the seromucous and mucous cells in the human labial gland may reflect their different maturation stages,[61] it is unclear whether the morphological and histochemical heterogeneity of the canine lacrimal gland is due to the different lineages or maturation stages of the secretory cells. Our findings indicate that this gland is composed of two types of secretory cells with different functional roles and secretory regulation, unlike humans.[59,60,62] Such aspects may be related to the rate of tear evaporation and blinking, because a higher lipid level of tear film in dogs compared to humans seems to induce the prevention of tear evaporation, resulting in a slower blinking rate.[63] Thus, the present study is indispensable for understanding the species-specific properties of the lacrimal gland and provides valuable information for elucidating the pathological mechanisms of diseases caused by secretory system disorders in dogs, such as keratoconjunctivitis sicca.

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