Histochemical studies on sialic acids and antimicrobial substances in the modified glands of snout and carpal skin in the pig

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CONTENTS

1 PREFACE

The mammalian skin, which is the largest organ and covers the surface of the body, has an important functional role in the protection of the organism against mechanical injuries, noxious agents and irradiation, the reception of sensory impulses from outside and the secretion of various substances. Furthermore, this organ is provided with appendages, such as hair, hair follicles and skin glands (Bloom and Fawcett, 1962; Calhoun and Stinson, 1981). The skin surface of most animals is a biotope for numerous microorganisms. Thus, an effective defense strategy against microbes and proliferation control of microflora are basic needs of mammalian skin biology.

In humans, eccrine glands are distributed widely throughout the majority of skin regions of the body. On the other hand, the distribution of apocrine glands is confined to a limited number of particular skin regions, for example, the axillar, circumanal and nasoalar regions (Hashimoto et al., 1986). In contrast, apocrine glands in domestic animals are present throughout the common integument, whereas eccrine glands are found only in a few regions of the skin such as the foot pads of dogs and cats, the frog of ungulates (*Cuneus ungulae*), the carpus of pigs and the nasolabial region of ruminants and pigs (Ellis, 1968; Calhoun and Stinson, 1981; Hashimoto et al., 1986). It is widely accepted that the secretory portion of eccrine glands is composed of two distinct cell types, dark

cells and clear cells. The dark cells contain numerous secretory granules and secrete mucous substances with glycoproteins. The clear cells, which are involved in the production of the hypertonic or isotonic precursor fluid, are equipped with large numbers of glycogen particles and many mitochondria (Kurosumi et al., 1984).

The secretory products of the eccrine glands in domestic mammals exhibit high levels of glycoproteins with various saccharide residues, especially in the dark cells. Such glycoconjugates play a major part in different functions, such as protection of epidermal surfaces against bacterial invasion, intraspecies communication, signaling of sexual activity and water retention (Tsukise et al., 1988a, b; Meyer and Bartels, 1989; Meyer and Tsukise, 1989, 1995; Yasui et al., 2004, 2005a, 2010). The porcine snout skin is a highly specialized body region, which has undergone several morphological and functional adaptations, and can be regarded as a delicate sense organ (Halata, 1993; Schwarz and Meyer, 1994). The eccrine glands of this region were found to contain considerable levels of neutral glycoproteins. Their products may be closely related to specific integumental functions (see only Tsukise et al., 1983). On the other hand, several studies have histochemically revealed the localization of glycoconjugates in the carpal glands, which are modified eccrine glands of pigs. Regarding their function, these glands are considered to be odorous glands (Gargiulo et al., 1989; Hraste and Stojković, 1995; Pedini et al., 1999). Nevertheless, in pig, the precise functions of the eccrine glands in snout skin and

carpus are mostly unexplained.

Within various saccharide residues, sialic acids mainly occupy the terminal position of glycoproteins as individual monosaccharides. They constitute components of cell surface glycoproteins and are frequently found in secreted glycoconjugates as well as in oligosaccharides. Their functional properties are manifold and depend on the degree of O-acetylation and on the linkage of sialic acid to the acceptor sugars (Kelm and Schauer, 1997; Schauer, 2004, 2009; Varki and Schauer, 2009). Sialic acids are involved in the regulation of many cellular and molecular interactions, for example, a dual role in masking recognition sites or recognition determinants. Therefore, the great variety of sialic acids may play a particularly important role in the defense against pathogenic agents (Suzuki, 2005; Parillo et al., 2009; Schauer, 2009). Additionally, they participate in the binding and transport of molecules, and contribute to the high viscosity of mucins, owing to their negative charge (Schauer, 2004, 2009; Varki and Schauer, 2009).

In mammals, it is known that antimicrobial substances are widely elaborated by epithelial cells, and are thus contained in various secretions, such as tears, saliva, colostrum and secretions from the genitourinary tract and respiratory epithelium. They serve as a component of non-specific defense against various microorganisms in the different parts of the integument (Meyer et al., 2003; Meyer, 2007; Stoeckelhuber et al., 2004, 2006, 2008; Yasui et al., 2007), including the eccrine glands (Metze et al., 1989; Ali et al.,

2001; Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2010; Park et al., 2011). The antimicrobial substances, such as lysozyme, IgA, lactoferrin and the peptide group of defensins, are generally part of the innate immune system and of great importance for skin integrity owing to their antibacterial activities. Rab proteins have functions in the tethering or docking of vesicles to their target compartment, leading to membrane fusion (Zerial and McBride, 2001). Rab3D, a member of the Rab3 subfamily, is associated with secretory vesicles and implicated in the control of regulated exocytosis in exocrine glands (Millar et al., 2002; Valentijin et al., 2007; Williams et al., 2009). However, in mammalian skin glands, the immunohistochemical expression of this regulatory protein has not been elucidated.

In view of the possible importance of sialic acids, antimicrobial substances and Rab3D for glandular functions, the present author investigated the histochemical and immunohistochemical characteristics of these moieties in the eccrine glands of the snout skin and carpal region in pig using light and electron microscopic methods. The morphochemical data obtained may be indispensable for understanding the functional properties of the porcine eccrine glands.

2 HISTOCHEMICAL LOCALIZATION OF SIALIC ACIDS AND ANTIMICROBIAL SUBSTANCES IN ECCRINE GLANDS OF PORCINE SNOUT SKIN

2. 1. Introduction

In most mammals except for humanoid primates, apocrine glands are extensively distributed throughout the common integument, whereas eccrine glands are confined to the skin of a series of specific body regions, for example, the foot pads of carnivores, the frog of ungulates (*Cuneus ungulae*), the carpus of pig and the nasolabial region of ruminants and pig (Ellis, 1968; Calhoun and Stinson, 1981; Tsukise et al., 1988a, b; Meyer and Bartels, 1989; Meyer and Tsukise, 1989, 1995). These specific regions have been modified by structural and functional adaptations that enable the animal to meet the biological requirements of its environment. The snout skin of the pig, for example, one of such structures that have undergone modifications, can function as a delicate sense organ. The eccrine glands of this region were found to contain considerable amounts of neutral glycoproteins, especially in the dark cells. It is considered that these products are closely related to the specific integumental functions (Tsukise et al., 1983).

It is widely known that sialic acids are mainly located at the terminal position of glycoproteins. They have different functional properties depending on the degree of acetylation and on the linkage of sialic acid to the acceptor sugars. Sialoderivatives seem to

perform a key role as the most versatile function modulators in cell biology and pathology (Schauer, 2004, 2009). The skin is a first barrier against pathogens. In this context, in several mammalian groups, recent studies have revealed that different antimicrobial substances are distributed also in eccrine glands (Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2010). Furthermore, the immune system has developed a special integumental subsystem to bind all of the possible microbial invaders and their products, to present them and to eliminate them rapidly (Schröder, 1999; Bos et al., 2001; Yang et al., 2001).

The present study on the eccrine glands of porcine snout skin investigates the localization of sialic acids and antimicrobial substances using lectin histochemical and immunohistochemical methods. Thus, our findings may be important for understanding the normal functions of snout skin in the pig independent of its abilities as a sensory organ.

2. 2. Materials and Methods

All experiments were performed in accordance with the guidelines for the care and use of laboratory animals at the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University. Five male miniature pigs (potbelly, 1-2 years, 40-50 kg) were deeply anesthetized and then exsanguinated from the common carotid arteries. After bloodletting, the snout skin was

removed surgically (Fig. 1). These skin samples were fixed in Bouin's solution for 24 h at room temperature, then carefully washed and dehydrated through a graded series of ethanol. The materials were embedded in paraffin wax and then sectioned at a thickness of 5 μm. Sections were stained with haematoxylin-eosin (H-E), Mallory–Azan and alcian blue (AB) pH 2.5-periodic acid Schiff (PAS) (Mowry, 1963) methods for general histological purposes, as well as with sialoglycoconjugate histochemical and immunohistochemical procedures.

For the localization of sialic acid residues, after deparaffinization, the histochemical staining method employed was AB pH 2.5 (Pearse, 1968) and periodic acid-phenylhydrazinium chloride-thiocarbohydrazide-silver proteinate-physical development (PA-P-TCH-SP-PD) (Ueda et al., 1995). Selective periodate oxidation (0.4 mM PA in 1 M HCl) of sialic acid residues yields monoaldehyde at C-7 or C-8 because it oxidizes vicinal diols at C-7 and C-8 or at C-8 and C-9. Then, monoaldehyde condenses with phenylhydrazine to give rise to the corresponding phenylhydrazone. This compound can undergo solvolysis to yield TCH-SP-PD-reactive monoaldehydes. On the other hand, the dialdehydes of neutral sugars oxidized by selective periodate oxidation condense with phenylhydrazine to result in TCH-SP-PD-unreactive azido or morpholine. In addition, sialic acids with O-acetyl substitutions at C-8 and/or C-9 are undetected by this procedure. Therefore, chemical modification by saponification (KOH) (0.5% potassium

hydroxide-70% ethanol) (Spicer, 1960) was performed on some sections, prior to PA-P-TCH-SP-PD staining, since KOH treatment is known to remove the acetyl groups on C-4, C-7, C-8 and C-9. Enzyme digestion with sialidase (from *Arthrobacter ureafaciens*, Nacalai Tesque Inc., Kyoto, Japan; 1.0 U/ml in 0.1 M phosphate buffer, pH 5.3, at 37˚C for 18 h) (Spicer et al., 1967) after saponification was conducted before the AB pH 2.5 and PA-P-TCH-SP-PD stainings. These physical development procedures for detection of sialic acids and their moieties are summarized in Table 1.

Additionally, the sections were incubated with biotinylated lectins at concentrations of 10-20 μg/ml in 0.05 M phosphate-buffered saline (PBS) (pH 7.2) for 60 min at room temperature, following treatment with 0.3% hydrogen peroxide to suppress endogenous peroxidase (PO) activity and then preincubation with 1% bovine serum albumin (BSA) (Sigma, MO, USA) in PBS for 30 min at room temperature. The lectins used were wheat germ agglutinin (WGA), *Sambucus sieboldiana* agglutinin (SSA) and *Maackia amurensis* agglutinin (MAM) (Seikagaku Kogyo Co., Tokyo, Japan). Their specificities and inhibitory sugars are listed in Table 2 (for lectin specificities see Danguy, 1995). After rinsing with PBS, the sections were soaked in PO-labeled streptavidin (Nichirei Co., Tokyo, Japan) for 30 min at room temperature. The activity of the PO employed for labeling was evaluated with a solution containing 0.05 M Tris-HCl buffer (pH 7.6),

diaminobenzidine (DAB) and hydrogen peroxide. Lectin controls were prepared by addition of inhibitory sugars at a final concentration of 0.01 M to the respective lectin solutions, by substitution of unconjugated lectins for biotinylated lectin conjugates or by exposure of sections to the PO-DAB system without lectin.

For the immunohistochemical methods, the sections were pretreated with 0.3% hydrogen peroxide for 30 min for the blocking of endogenous PO activities. Then, they were incubated with primary antibodies diluted in 0.01 M PBS (pH 7.3) against lysozyme (polyclonal, from rabbit) (Dako, Glostrup, Denmark), IgA (polyclonal, from rabbit) (Dako), lactoferrin (polyclonal, from rabbit) (MP Biomedicals, Ohio, USA), human β-defensin 2 (polyclonal, from rabbit) (Biolog, Kronshagen, Germany) and Rab3D (polyclonal, from rabbit) (Proteintech, Illinois, USA) for 24 h at 4°C, following preincubation with 1% BSA in PBS for 30 min at room temperature. The primary antibodies were diluted as follows: lysozyme 1:100, IgA 1:100, lactoferrin 1:100, human β-defensin 2 1:400, Rab3D 1:50. For antigen retrieval, some of the sections were incubated with Proteinase K solution (Dako) for 3 min at room temperature before incubation with the primary antibodies to lysozyme, IgA, lactoferrin and β-defensin 2. With regard to anti-Rab3D, the sections were treated with microwave irradiation (500 W, for 6 min) in 0.01 M citrate buffer at pH 6.0. After rinsing with PBS, these sections were incubated with prediluted dextran-polymer-conjugated secondary

antibodies (EnVision+ Dual Link System-HRP, raised goat against mouse and rabbit immunoglobulins, Dako) for 30 min at room temperature. The immunoreaction employed for labeling was revealed using a PO-DAB system, as described above. Controls for immunohistochemical methods were performed by incubation with PBS without primary antibodies or exposure of sections to the PO-DAB system without primary or secondary antibodies.

For general cytology, tissue pieces were fixed in 2.5% glutaraldehyde (GA) solution in 0.1 M phosphate-buffered solution (PB) (pH 7.4) for 2 h at 4°C, post-fixed in 2% osmium tetroxide solution and embedded in Epon 812 (Luft, 1961). From these tissue blocks, ultrathin sections were cut on an ultramicrotome, mounted on copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). For the cytochemical demonstration, the tissue specimens were fixed in a mixture of 4% paraformaldehyde (PFA) and 0.5% GA in 0.1 M PB (pH 7.4) for 2 h at 4°C, and embedded in LR-White resin (Newman et al., 1983). For LR-White-embedded materials, ultrathin sections were cut as stated above and placed on nylon grids. The sections on nylon grids were subjected to the periodic acid-thiocarbohydrazide-silver proteinate-physical development procedure (PA-TCH-SP-PD), which is an efficient and sensitive cytochemical method for the detection of vicinal diols of neutral carbohydrates (Yamada, 1993). These sections were then counterstained with uranyl acetate and lead citrate. For the cytochemical identification of glycogen in the

cytoplasm, enzyme digestion with α-amylase (from *Bacillus subtilis*, Seikagaku Kogyo Co., 1 mg/ml in 0.1 M PBS pH 6.0, at 37°C for 4 h) (Casselman, 1959) was carried out on some sections prior to the PA-TCH-SP-PD procedure.

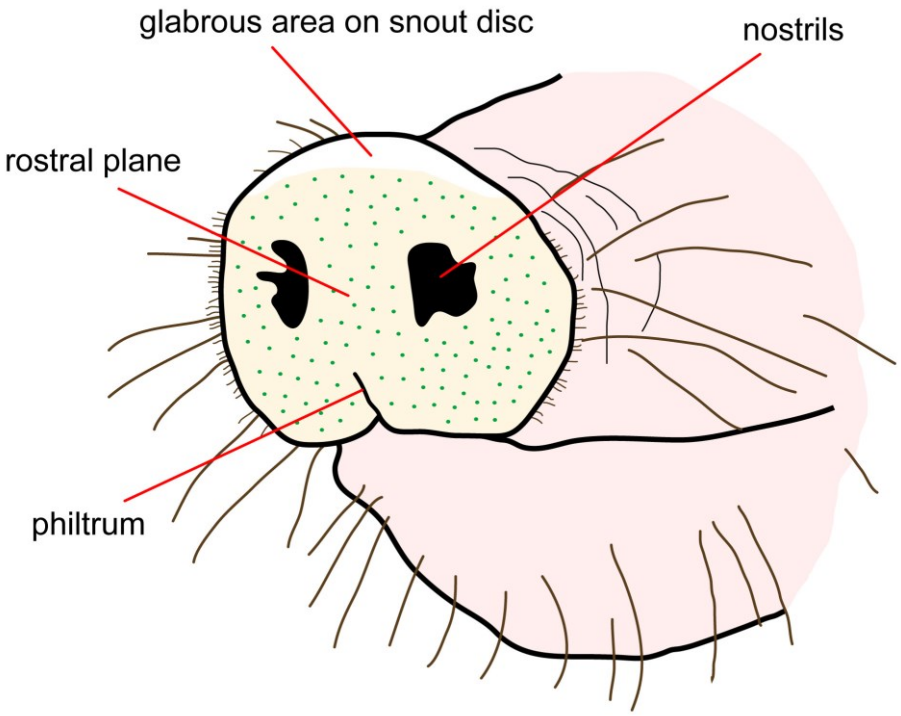

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Fig. 1 Schematic illustrations of porcine snout skin. The eccrine glands of the snout skin are distributed in porcine rostral plate. Their excretory ducts directly open onto skin surface (Green dots).

Table 1. Physical development procedures for detection of sialic acids and their moieties

Staining method	Moiety detected by the reaction
AB pH 2.5	Carboxyl groups and some sulphate esters in glycoconjugates
KOH-Sial-AB pH 2.5	Carboxyl groups and some sulphate esters in glycoconjugates except for sialglycoconjugates
PA-P-TCH-SP-PD	Sialic acids without side-chain substituent and with O-acetyl substitutions at C-7
KOH-PA-P-TCH-SP-PD	Sialic acids without side-chain substituent and with O-acetyl substitutions at C-7, C-8 and/or C-9
KOH-Sial-PA-P-TCH-SP-PD	Not detected

Table 2. The lectins used and their sugar-binding specificities and inhibitory sugars

	Lectins	Sugar-binding specificity	Inhibitory sugar
WGA	Wheat germ agglutinin	Sia, β-D-GlcNAc	β-D-GlcNAc
SSA	*Sambucus sieboldiana* agglutinin	Siaα2-6Gal/GalNAc	α2-6sialyllactose
MAM	*Maackia amurensis* agglutinin	Siaα2-3Galβ1-4GlcNAc	α2-3sialyllactose

2. 3. Results

In pig snout skin, the eccrine glands were visualized in the connective tissue of the hypodermis. Their secretory epithelium was commonly distinguished from dark cells and clear cells (Fig. 2a, b). In the sections stained with AB pH 2.5-PAS, however, different reaction intensities were clearly detected among the dark cells, which showed moderate or strong reactions (Fig. 3a, b). The excretory duct penetrated toward the epidermis and directly opened onto the skin surface.

In the eccrine glandular acini, the AB pH 2.5 procedure gave rise to a weak to moderate reaction of the dark cells and a very weak reaction of the clear cells (Fig. 4a). Enzyme digestion with sialidase following saponification caused a weaker AB pH 2.5 reaction (Fig. 4b). The PA-P-TCH-SP-PD procedure produced a weak to moderate reaction in the dark cells and a very weak reaction in the clear cells (Fig. 4c). Saponification clearly increased the intensity of the PA-P-TCH-SP-PD reaction, in that the reaction intensity of the dark cells was markedly altered (Fig. 4d, e). The PA-P-TCH-SP-PD staining was nearly negative after sialidase digestion following saponification (Fig. 4f). As for lectin histochemical methods, the WGA and SSA techniques resulted in a moderate to strong reaction of the dark cells, and a very weak reaction of the clear cells (Fig. 5a, b). The luminal secretions exhibited moderate reactions after the application with WGA and SSA. When incubated with MAM, a distinct reaction was detected in the luminal surface of some of the

dark cells. Additionally, the cytoplasm of this cell type showed a weak reaction. On the other hand, the other dark cells and clear cells were almost negative (Fig. 5c). In all of the control experiments for the lectin histochemical procedure, positive reactions were greatly diminished or nearly abolished in the eccrine glands (Fig. 5d). The results obtained from the sialoglycoconjugate histochemical procedures are summarized in Table 3.

With regard to the immunohistochemical approach for lysozyme, some of the dark cells were found to exhibit a moderate to strong positive reaction, while the other dark cells and clear cells showed very weak reactions (Fig. 6a). A weak to moderate positive reaction was observed in the luminal secretions with antibodies to lysozyme. Antibodies to IgA produced a very weak to weak positive reactions in some of the dark cells and in the luminal secretions, whereas the secretory epithelial cells except for these dark cells were almost negative (Fig. 6b). In addition, a positive reaction was clearly detectable in the lumina of the blood vessels surrounding the secretory portion of the eccrine glands. Incubation with antibodies to lactoferrin and β-defensin 2 led to the same staining images as those obtained with antibodies to lysozyme in that the reaction intensity of some of the dark cells showed distinct positive reactions (Fig. 6c, d). Antibodies to Rab3D gave rise to a weak to moderate reaction of some of the dark cells, while the clear cells and luminal secretions were seen to react very weakly (Fig. 6e).

In control experiments for the immunohistochemical methods

performed by incubation with PBS without primary antibodies or exposure of sections to the PO-DAB system without primary or secondary antibodies, no glandular structures exhibited any positive reactions (Fig. 6f). These results are summarized in Table 4.

Transmission electron microscopy of the eccrine glands of porcine snout skin confirmed that the secretory portion consisted of dark cells and clear cells with associated with myoepithelial cells. The secretory cells rested on a basement membrane and their nuclei were located in the central or basal region of the cytoplasm. Additionally, different morphological characteristics were detected among the dark cells. Therefore, we expediently classified the respective dark cells as type I dark cells and type II dark cells (Fig. 7a, b). Both cell types of the dark cells contained a well-developed Golgi apparatus and rough-surfaced endoplasmic reticulum within their cytoplasm. Although varying numbers of secretory granules possessing medium electron density were visible in the apical cytoplasm of these dark cells, the secretory granules of the type II dark cells were smaller than those of the type I dark cells (Fig. 7b). On the other hand, the clear cells, the cytoplasm of which exhibited a lower electron density, were equipped with a Golgi apparatus, and contained large amounts of glycogen particles distributed throughout the cytoplasm. The cytoplasmic matrix of these secretory epithelial cells, especially of the clear cells, was studded with many mitochondria of varying morphology.

In the secretory cells stained with the PA-TCH-SP-PD

procedure, the surface coat of the plasma membrane and the basement membrane exhibited positive reactions (Fig. 8a-c). The prominent PA-TCH-SP-PD-reactive structures in the dark cells were the secretory granules and cisternae of the Golgi apparatus. However, in the dark cells, the secretory granules of the type I cells showed a weak to moderate reaction, whereas a distinct positive reaction was observed in those of the type II cells (Fig. 8a, c). The PA-TCH-SP-PD-reactive ultrastructures in the clear cells were glycogen particles and also cisternae of the Golgi apparatus (Fig. 8a, b). Digestion with α-amylase abolished the PA-TCH-SP-PD-reactive glycogen particles, found particularly in the cytoplasm of the clear cells.

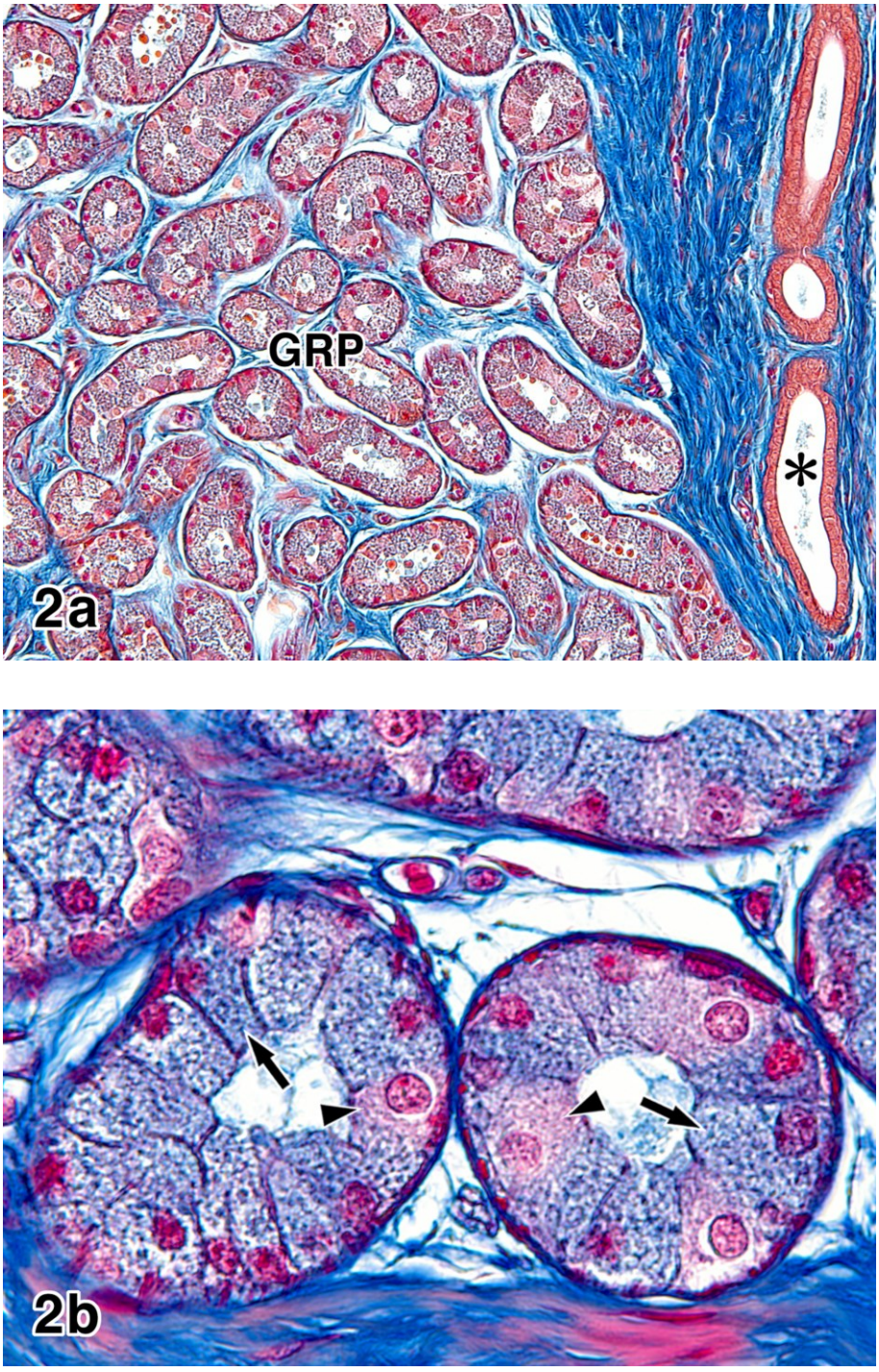


Fig. 2 General structure of the eccrine glands in the porcine snout skin. a) Mallory-Azan, ×160. GRP: gland of rostral plate, asterisk: excretory duct; b) higher magnification of the eccrine glandular acini, Mallory-Azan, ×640, arrows: dark cells, arrowheads: clear cells.

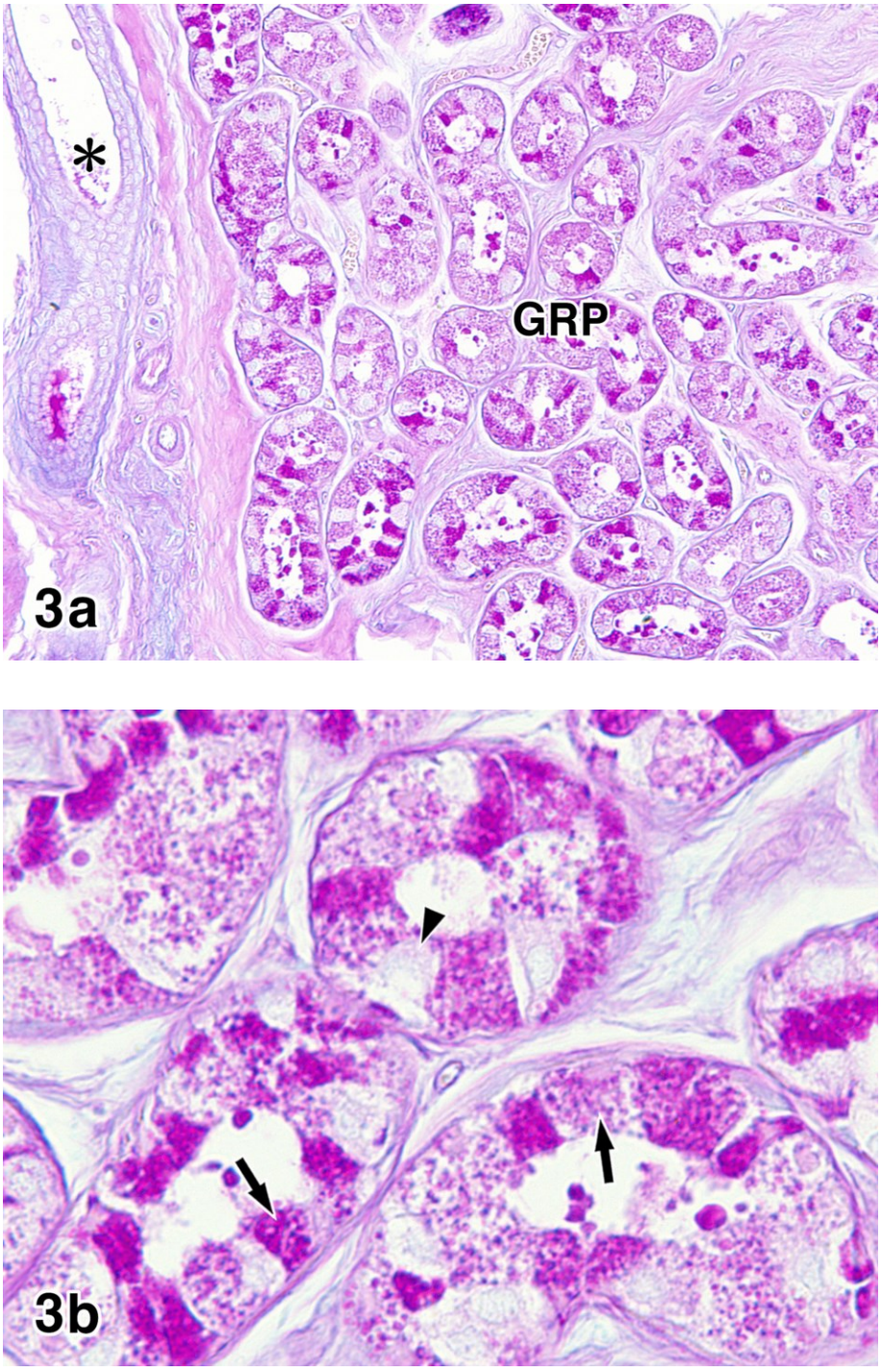


Fig. 3 General carbohydrate histochemical appearance of the eccrine glands in the snout skin. a) AB pH 2.5-PAS, ×160, GRP: gland of rostral plate, asterisk: excretory duct; b) AB pH 2.5-PAS, ×640, arrows: dark cells, arrowhead: clear cell.

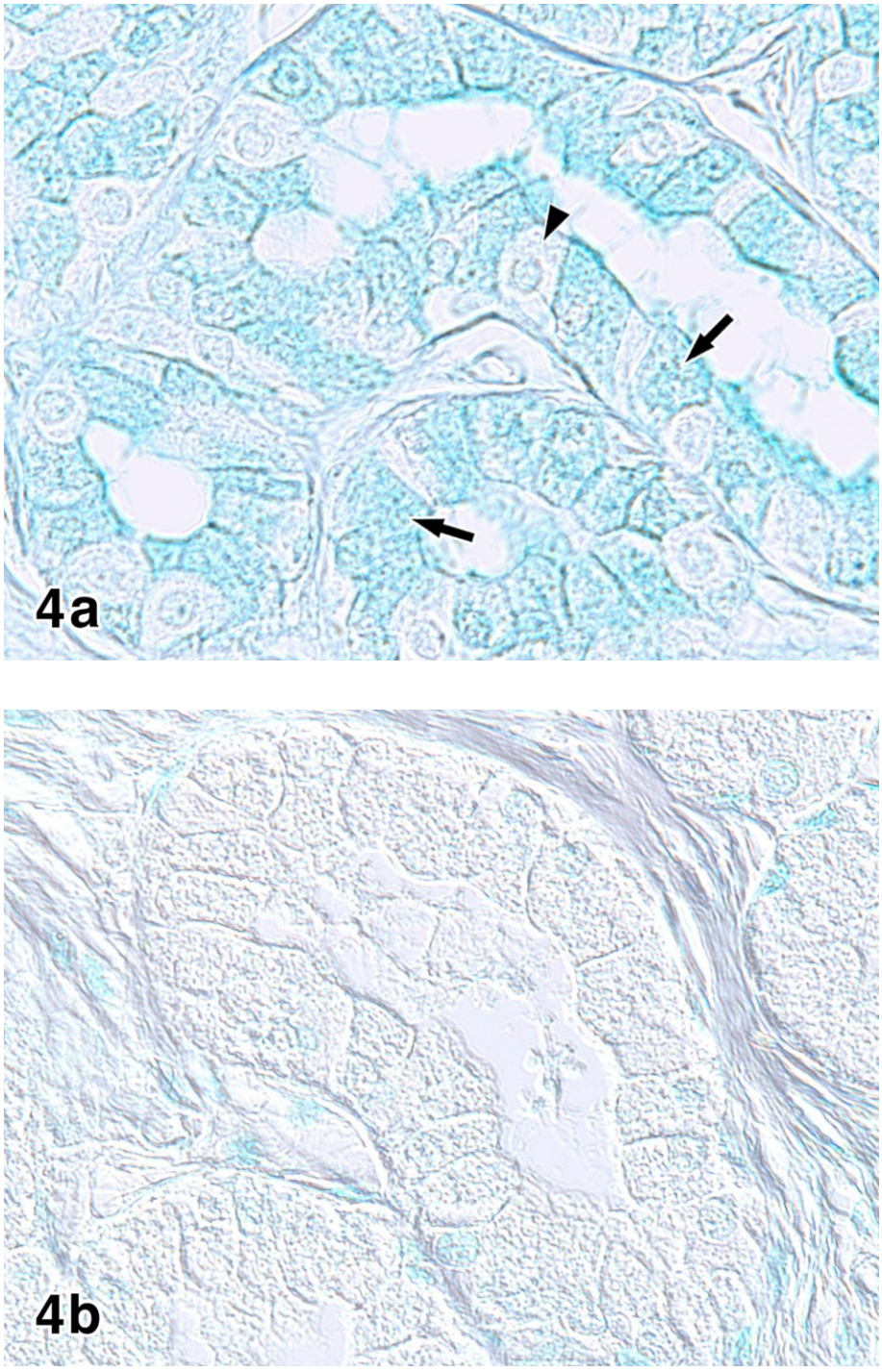


Fig. 4 Histochemical visualization of sialic acids in the eccrine glands of the snout skin. a) AB pH 2.5, ×640, arrows: dark cells, arrowhead: clear cell; b) KOH-Sial-AB pH 2.5, ×640.

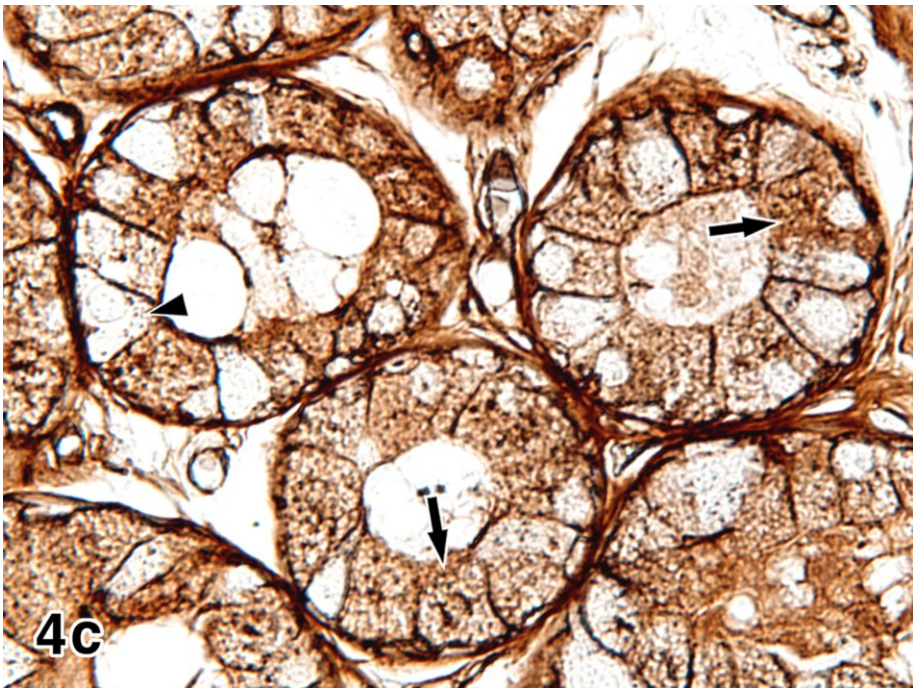
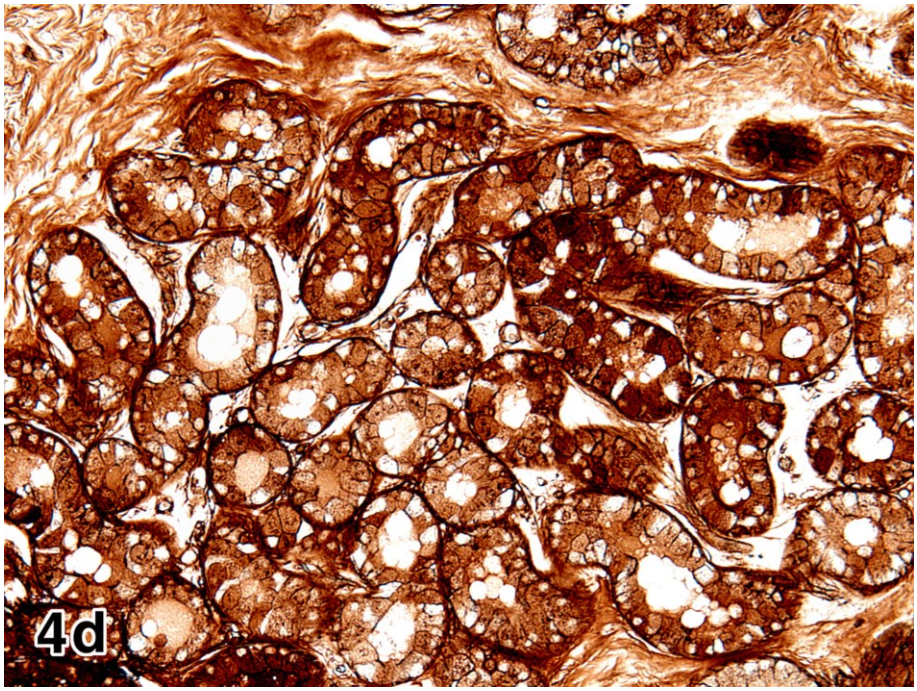


Fig. 4 Histochemical visualization of sialic acids in the eccrine glands of the snout skin. c) PA-P-TCH-SP-PD, ×640, arrows: dark cells, arrowhead: clear cell.



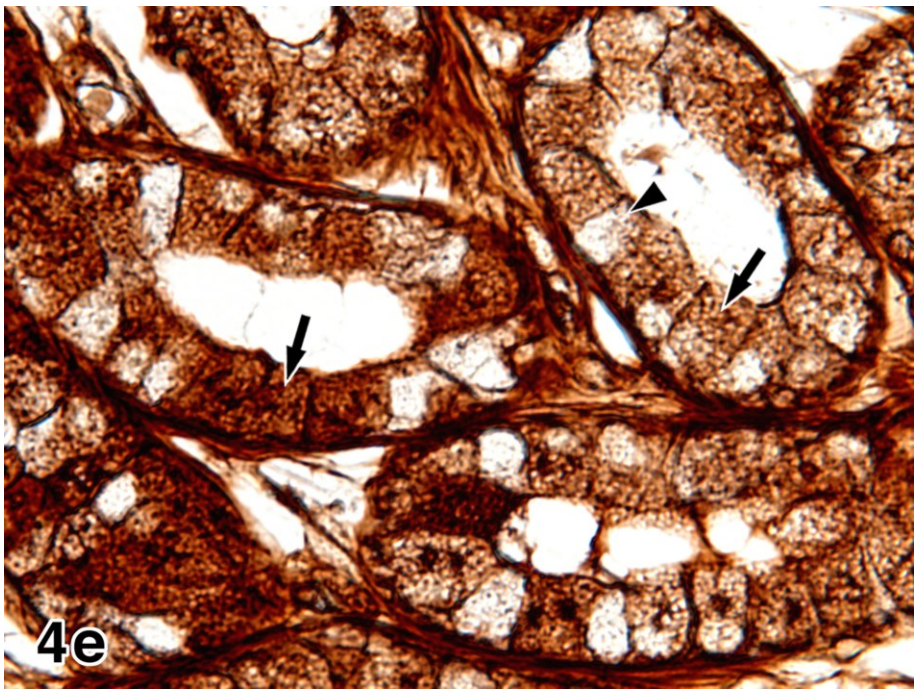


Fig. 4 Histochemical visualization of sialic acids in the eccrine glands of the snout skin. d) KOH-PA-P-TCH-SP-PD, ×160; e) KOH-PA-P-TCH-SP-PD, ×640, arrows: dark cells, arrowhead: clear cell.

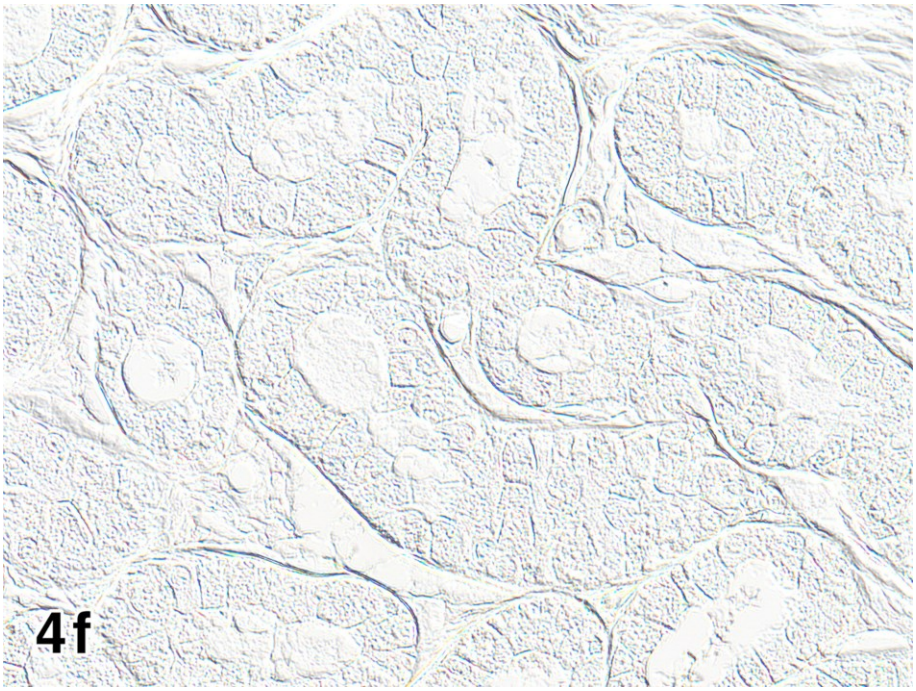


Fig. 4 Histochemical visualization of sialic acids in the eccrine glands of the snout skin. f) KOH-Sial-PA-P-TCH-SP- PD, ×320.

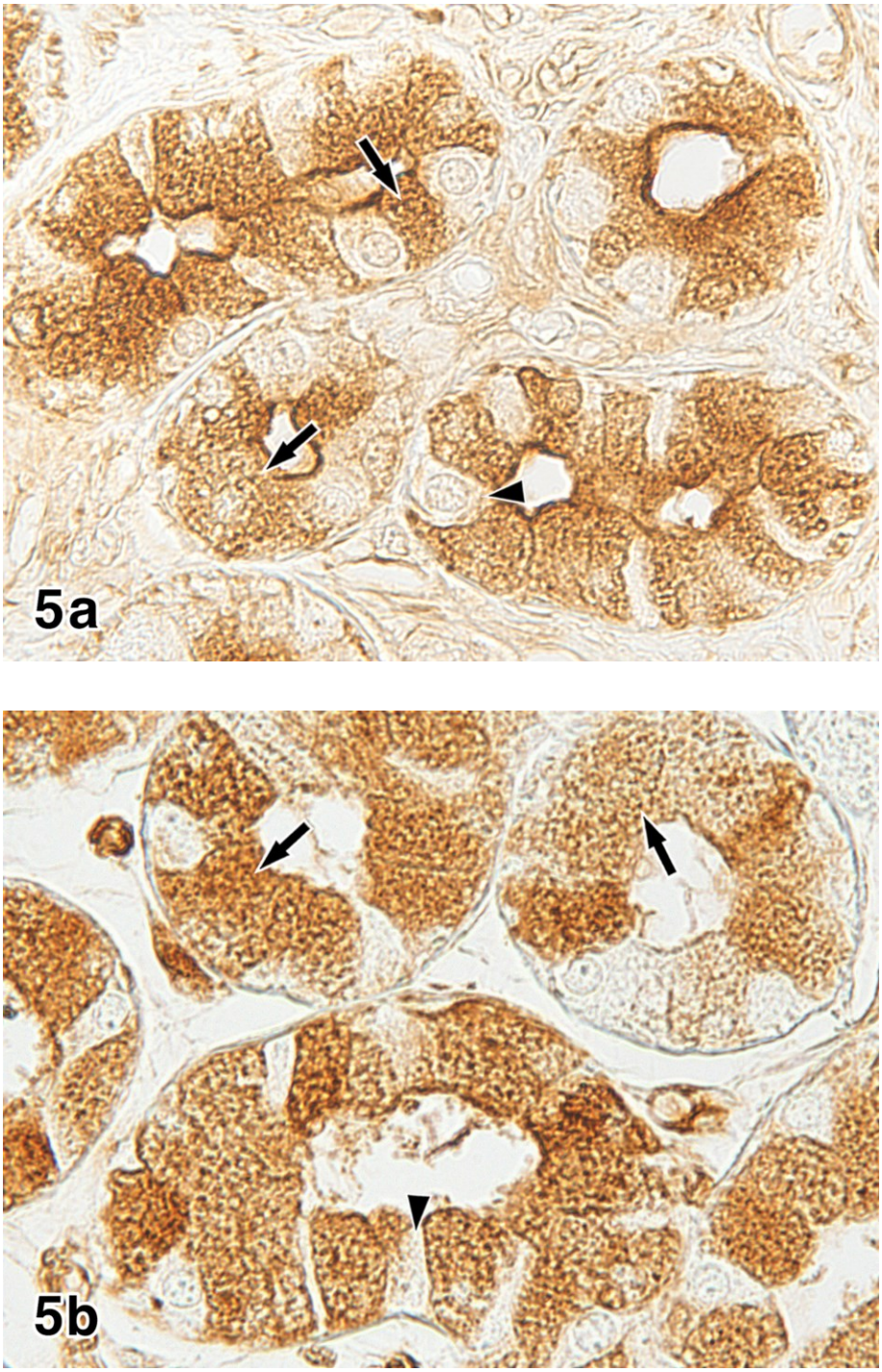


Fig. 5 Lectin histochemical staining in the eccrine glands of the snout skin. a) WGA, ×640, arrows: dark cells, arrowhead: clear cell; b) SSA, ×640, arrows: dark cells, arrowhead: clear cell.

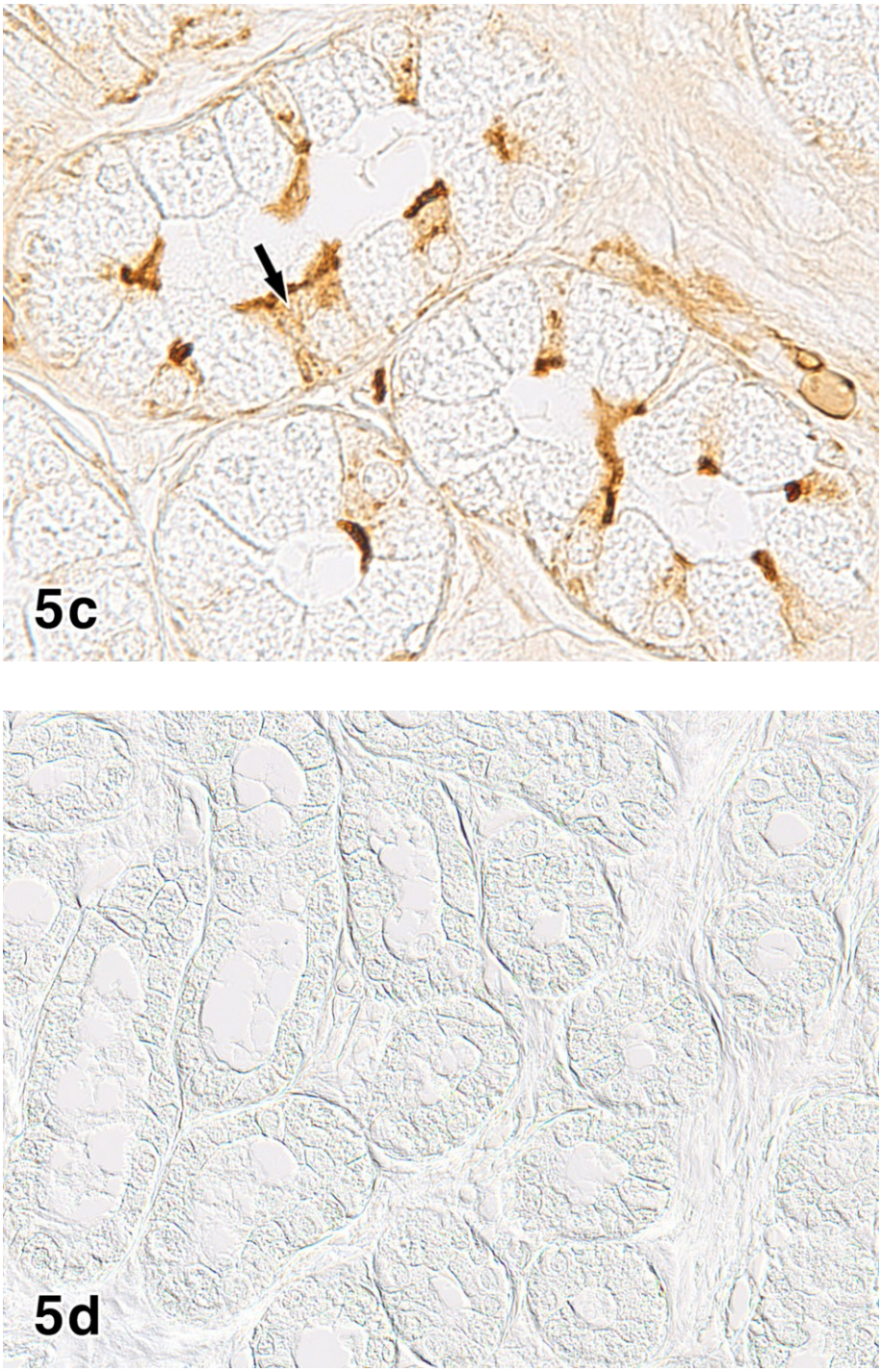


Fig. 5 Lectin histochemical staining in the eccrine glands of the snout skin. c) MAM, ×640, arrow: dark cell; d) control, ×320.

Table 3. Histochemical reactions of sialoglycoconjugates in the eccrine glands of porcine snout skin

Stainings	Secretory cells		Luminal secretions
	Dark cells	Clear cells	
AB pH 2.5	2-3	0-1	2-3
KOH-Sial-AB pH 2.5	0-1	0-1	0-1
PA-P-TCH-SP-PD	2-3	0-1	2-3
KOH-PA-P-TCH-SP-PD	3-4	0-1	3-4
KOH-Sial-PA-P-TCH-SP-PD	0	0	0
WGA	3-4	0-1	3
SSA	3-4	0-1	3
MAM	0-4*	0	1

Reaction intensities: 0: negative, 1: very weak, 2: weak, 3: moderate, 4: strong

* Some dark cells were only stained

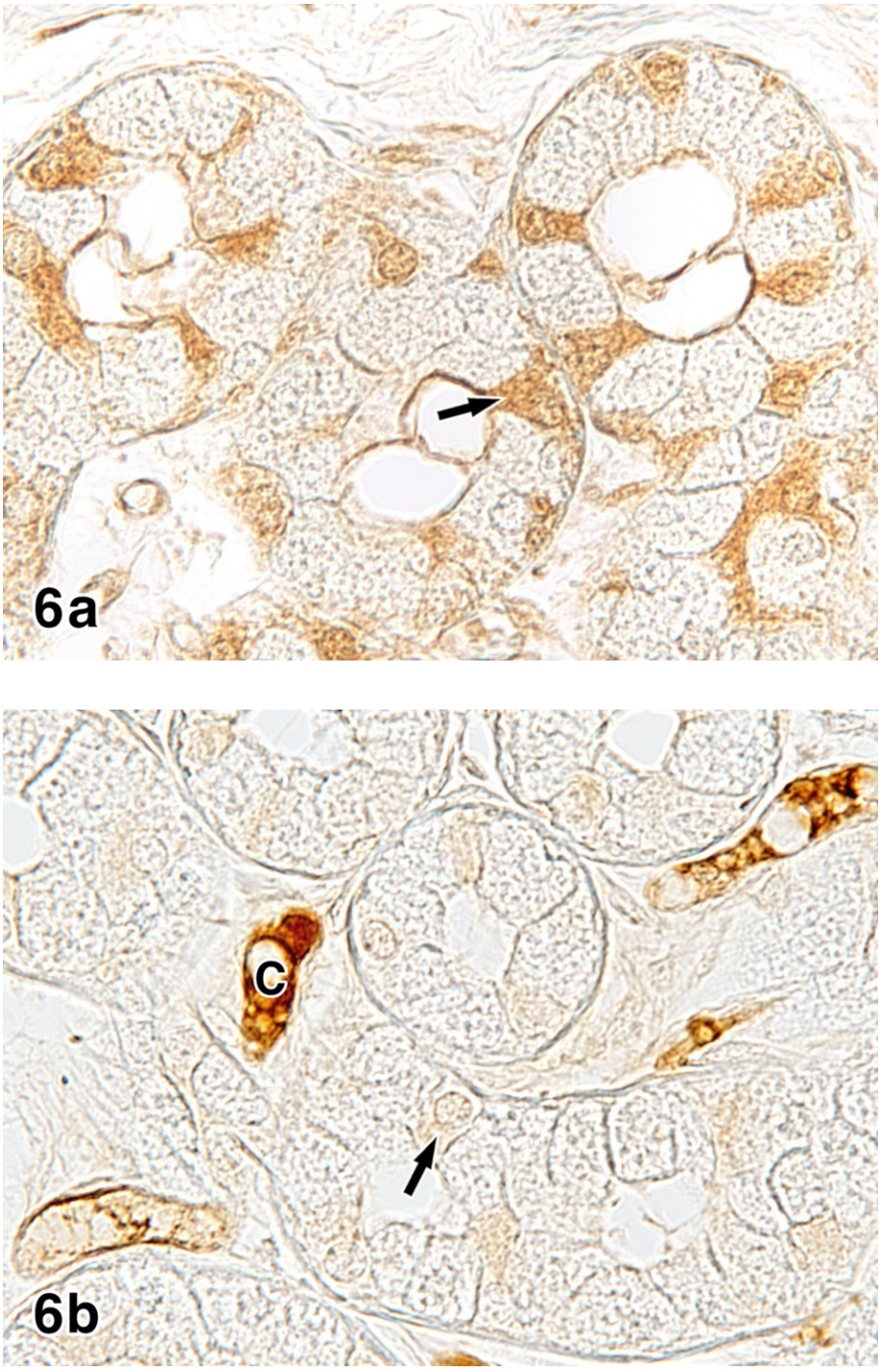


Fig. 6 Immunohistochemical staining in the eccrine glands of the snout skin. a) Lysozyme, ×640, arrow: dark cell; b) IgA, ×640, C: capillary, arrow: dark cell.

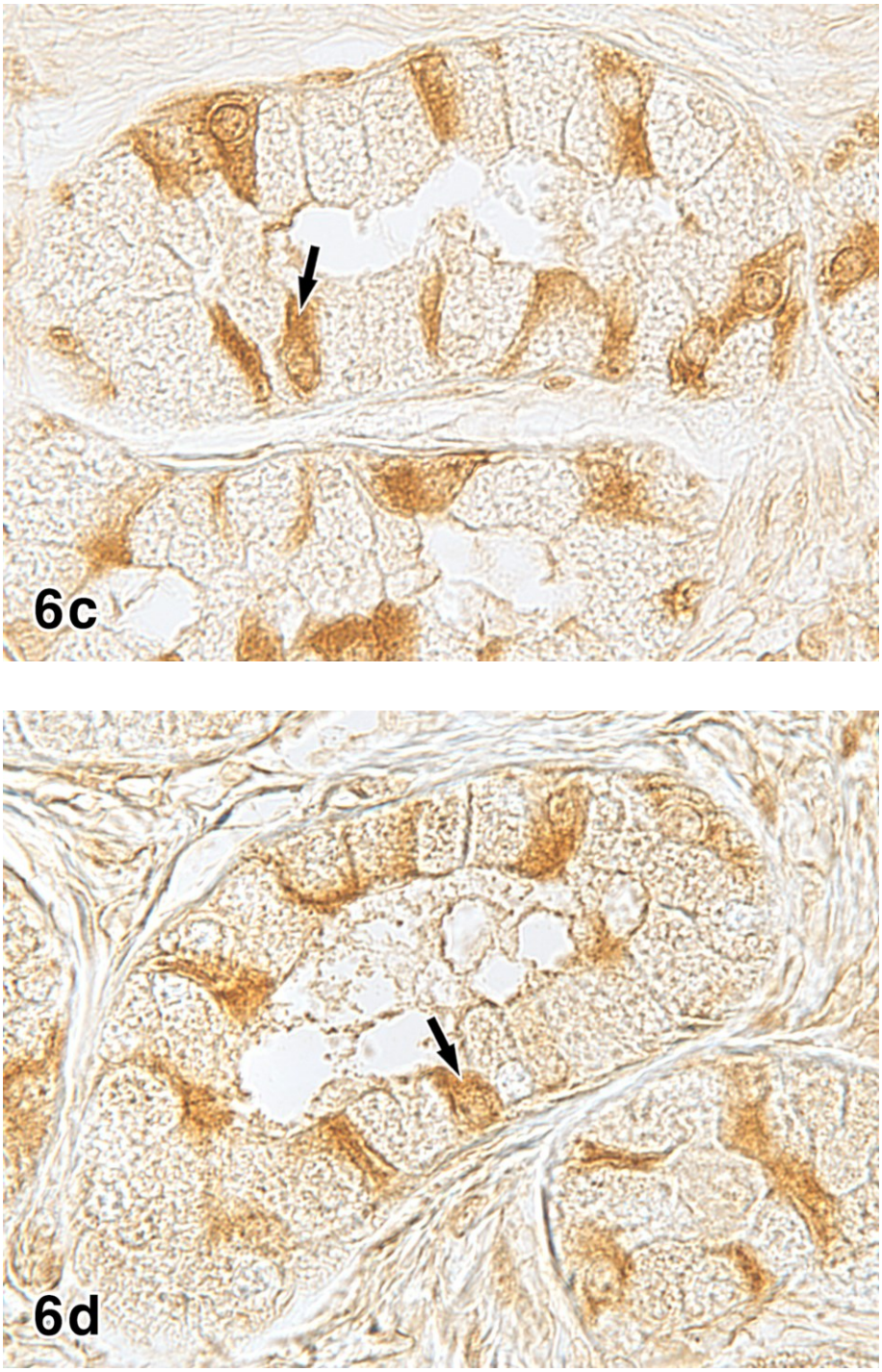


Fig. 6 Immunohistochemical staining in the eccrine glands of the snout skin. c) lactoferrin, ×640, arrow: dark cell; d) β-defensin 2, ×640, arrow: dark cell.

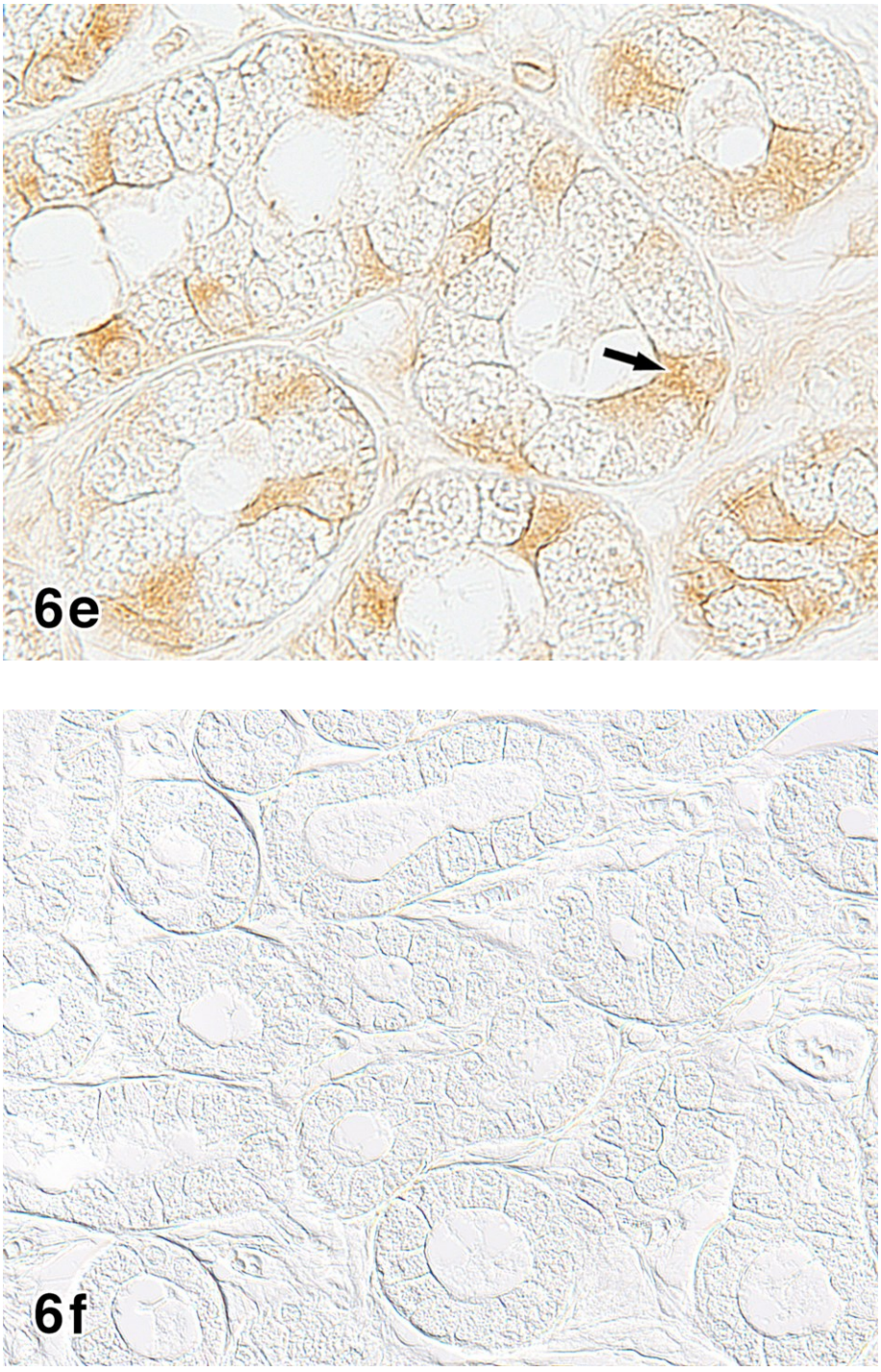


Fig. 6 Immunohistochemical staining in the eccrine glands of the snout skin. e) Rab3D ×640, arrow: dark cell; f) control, ×320.

Table 4. Immunohistochemical reactions of the eccrine glands in porcine snout skin

Substances	Secretory cells		Luminal secretions
	Dark cells	Clear cells	
Lysozyme	1-4*	0-1	2-3
IgA	1-2*	0-1	1
Lactoferrin	1-4*	0-1	2
β-defensin 2	1-4*	1	2
Rab3D	1-3*	0-1	0-1

Reaction intensities: 0: negative, 1: very weak, 2: weak, 3: moderate, 4: strong

* Some dark cells were only stained

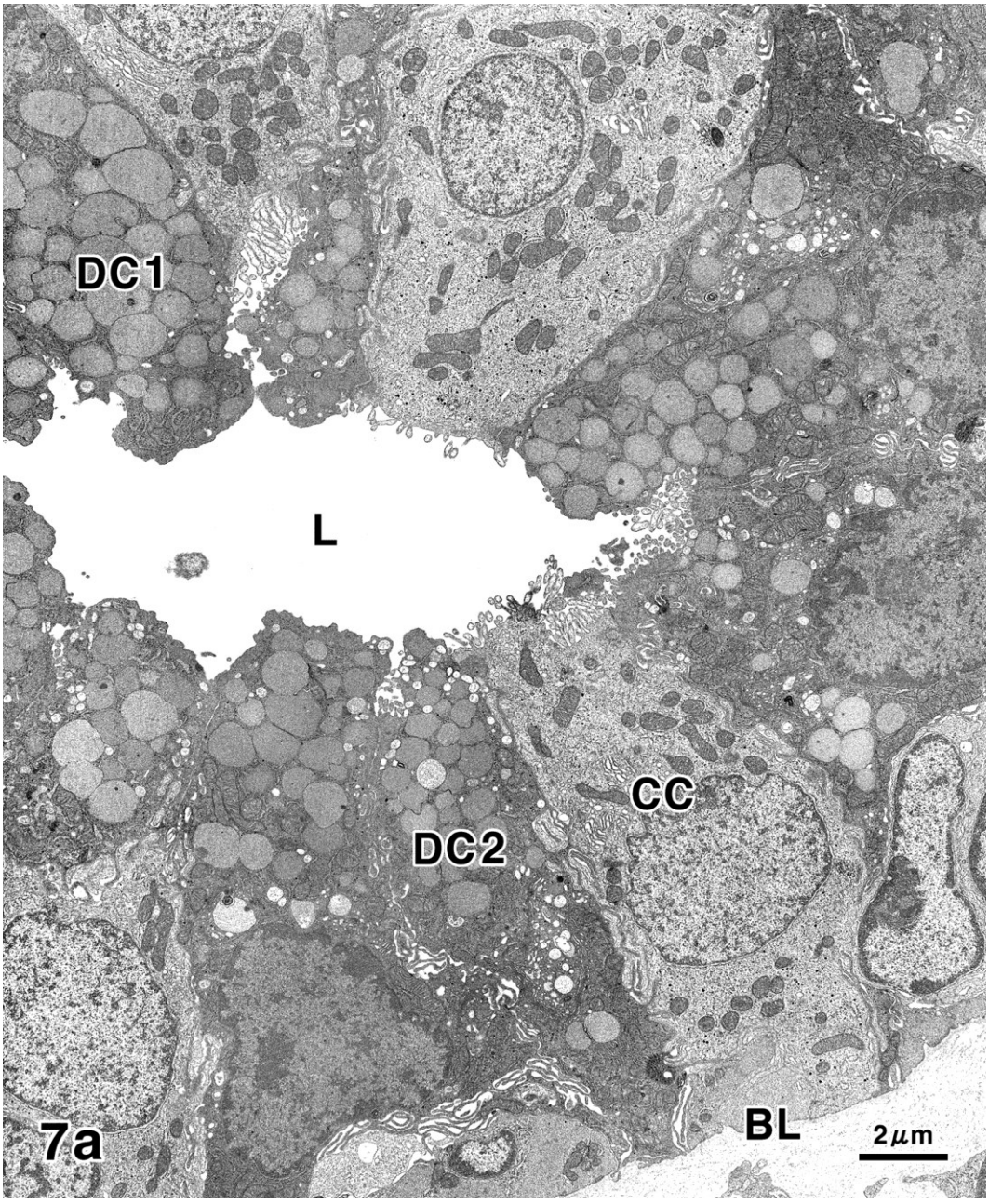


Fig. 7 Ultrastructure of the eccrine glands in the porcine snout skin stained with uranyl acetate and lead citrate. a) The secretory portion consists of type I dark cells, type II dark cells and clear cells. ×6,500, BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen.

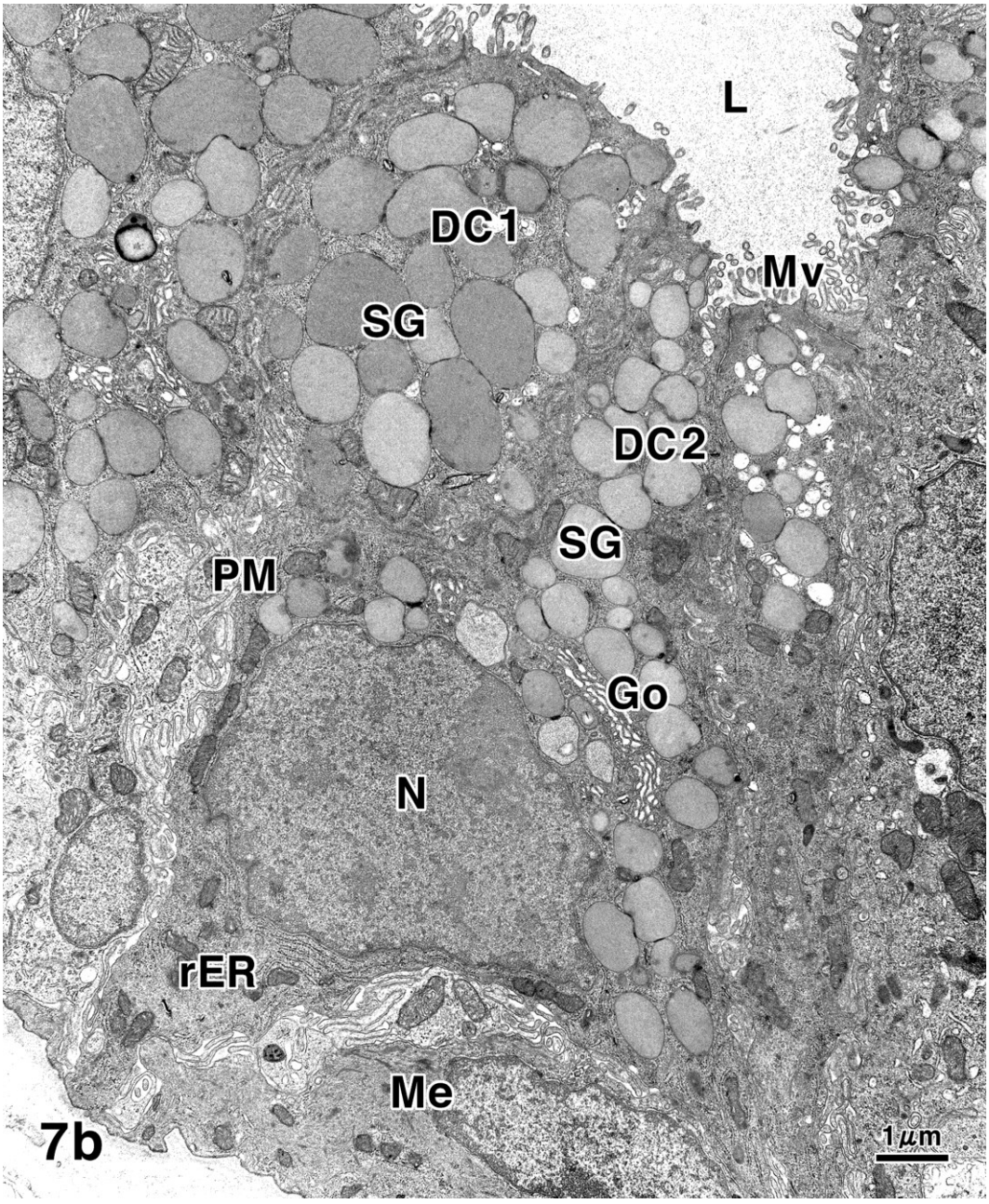


Fig. 7 Ultrastructure of the eccrine glands in the porcine snout skin stained with uranyl acetate and lead citrate. b) Higher magnification of dark cells in the eccrine glandular acini. ×10,500, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, L: lumen, Me: myoepithelial cell, Mv: microvilli, N: nucleus, PM: plasma membrane, rER: rough-surfaced endoplasmic reticulum, SG: secretory granule.

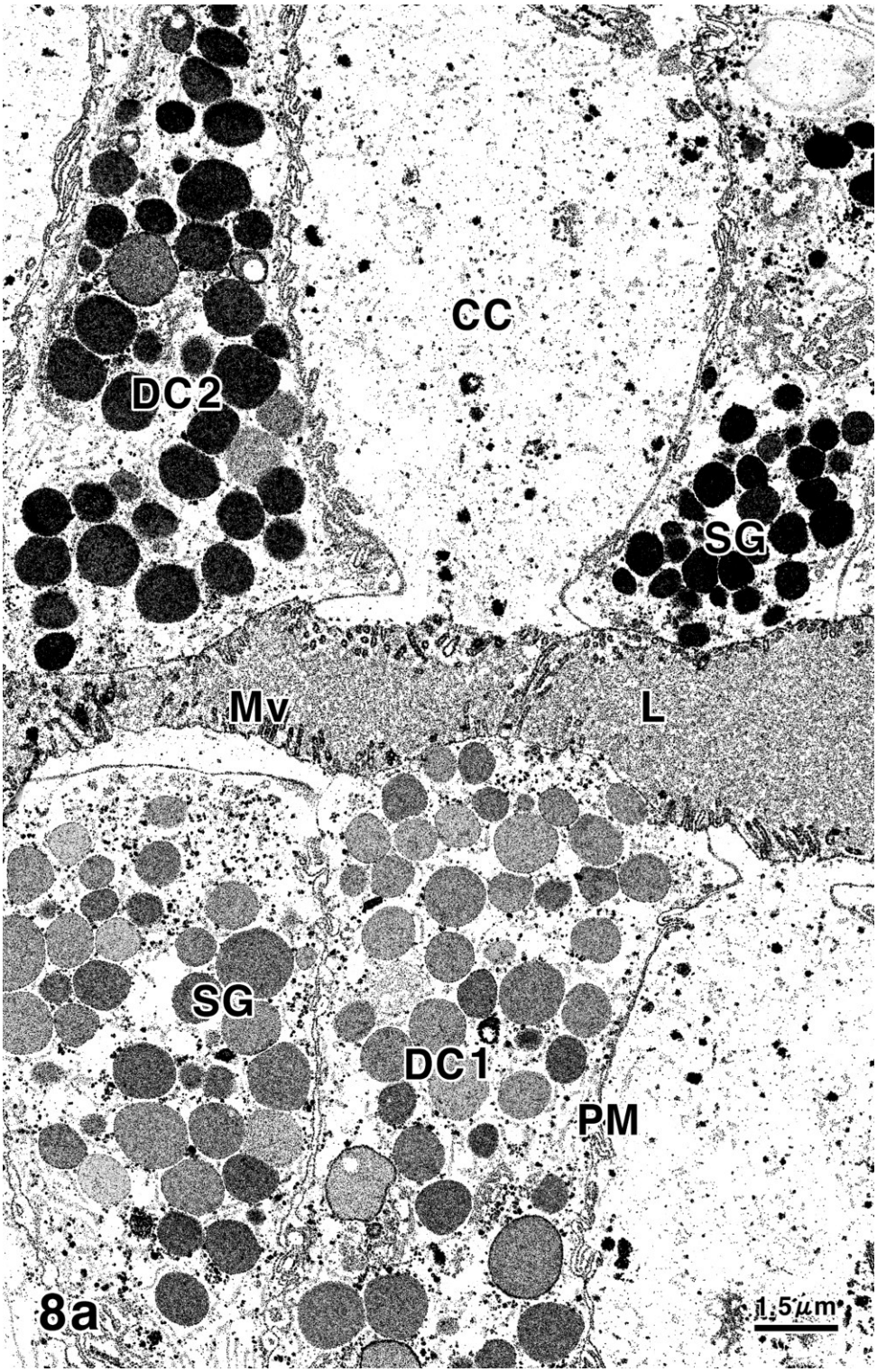


Fig. 8 Cytochemical PA-TCH-SP-PD staining of the eccrine glands in the snout skin. a) Ultrastructures exhibit positive reactions. ×8,000, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, PM: plasma membrane, SG: secretory granule.

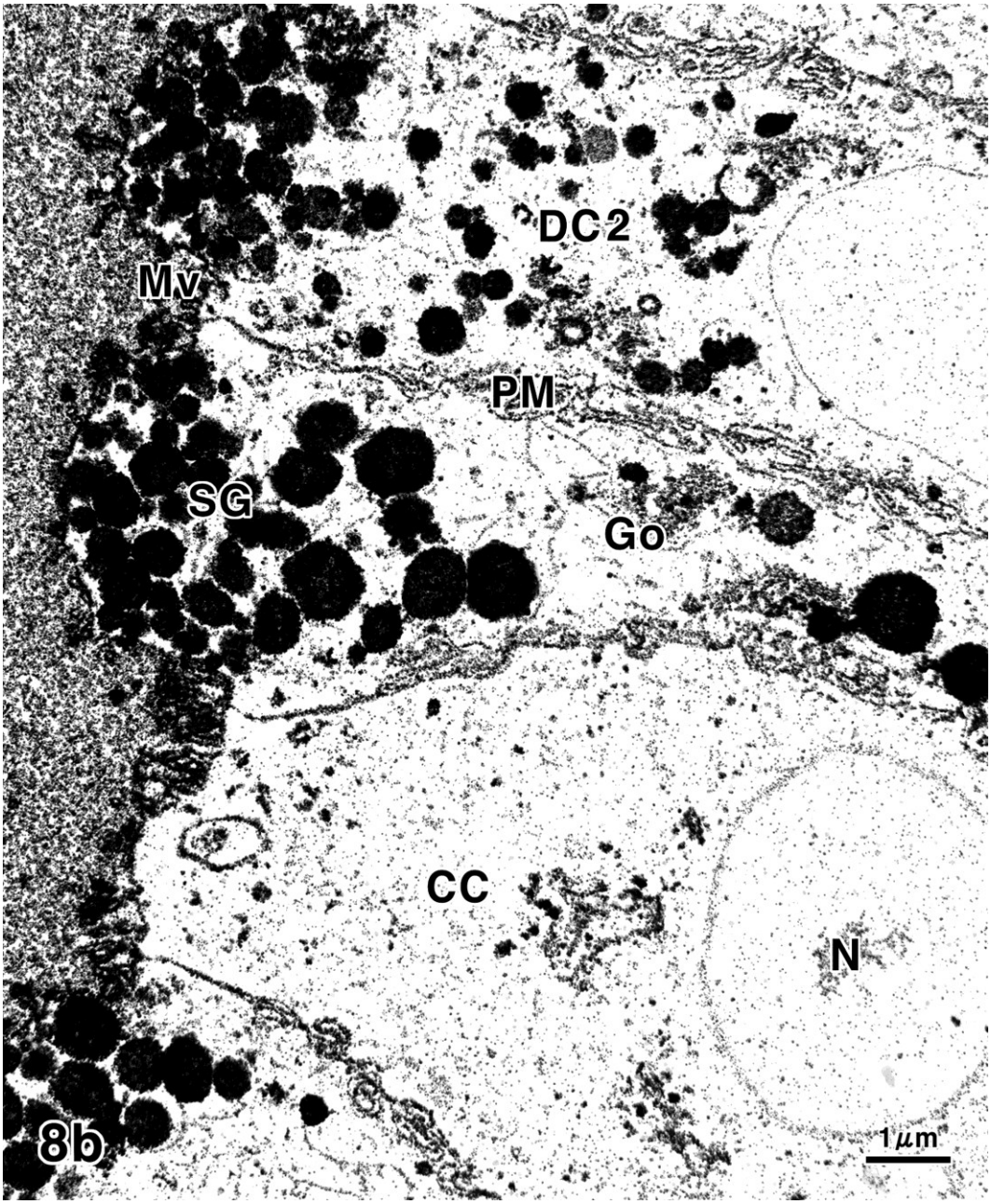


Fig. 8 Cytochemical PA-TCH-SP-PD staining of the eccrine glands in the snout skin. b) Part of the apical cytoplasm of secretory cells. ×12,000, CC: clear cell, DC2: type II dark cell, Go: Golgi apparatus, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

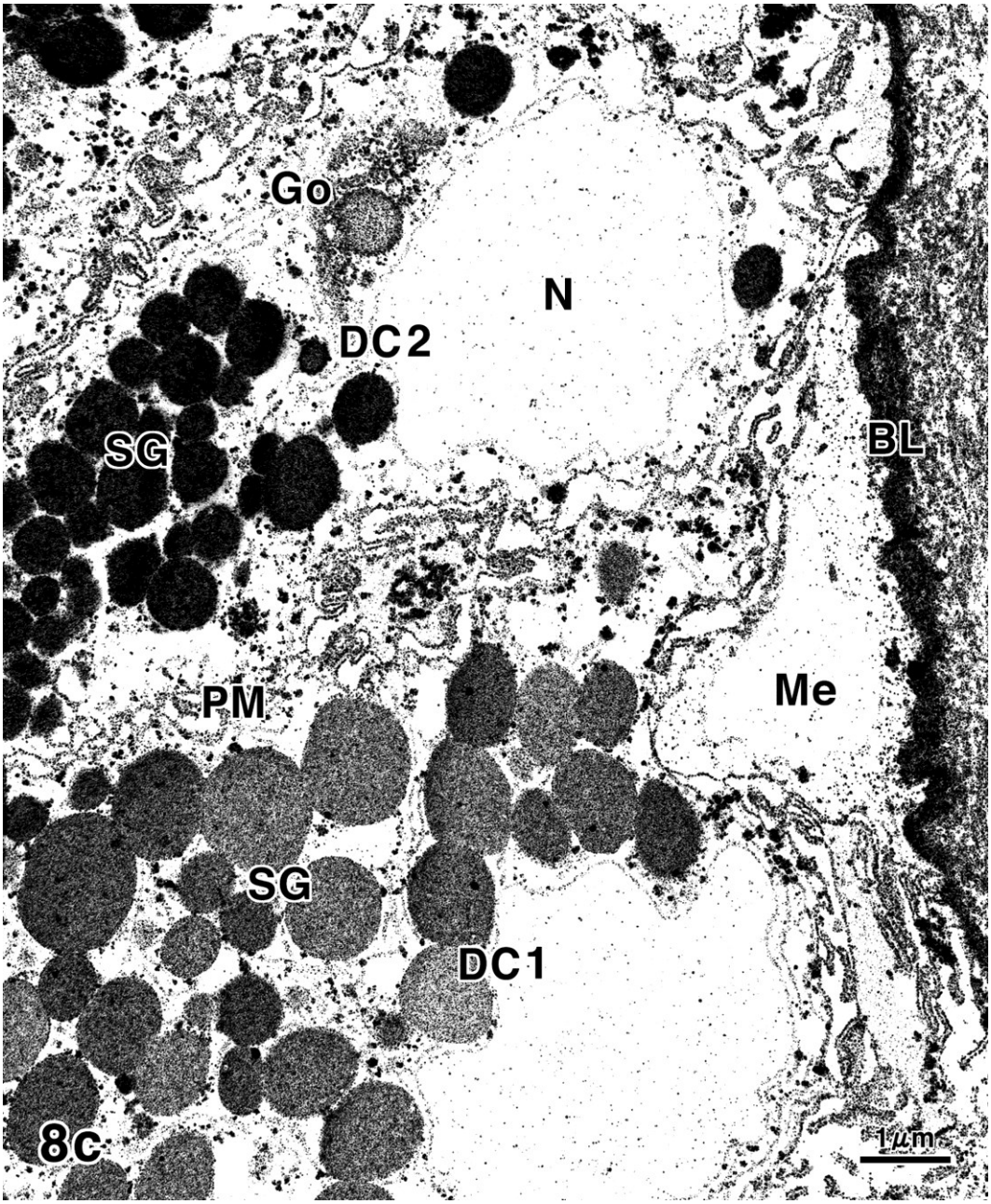


Fig. 8 Cytochemical PA-TCH-SP-PD staining of the eccrine glands in the snout skin. c) Part of the basal cytoplasm of secretory cells. ×13,000, BL: basal lamina, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, Me: myoepithelial cell, N: nucleus, PM: plasma membrane, SG: secretory granule.

2. 4. Discussion

In various mammalian species including humans, the secretory cells of the eccrine glands are generally divided into dark cells and clear cells, which participate in secreting glycoproteins and ion fluid, respectively (Kurosumi et al., 1984; Meyer and Bartels, 1989; Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2004, 2005a). Unlike in other mammalian species, however, the dark cells of the eccrine glands in the porcine snout skin could be classified morphologically and cytochemically as type I cells and type II cells, owing to the ultrastructural features and the results of the PA-TCH-SP-PD procedure. However, it is unclear whether their morphological and morphochemical heterogeneity depends on the different functional stages or maturation stages. Furthermore, our results revealed the presence of sialic acids and various antimicrobial substances in these glands.

Within the saccharide residues, the functional properties of sialic acids appear to be manifold (Schauer, 2004, 2009). These glycoconjugates protect cells and macromolecules against enzymatic and immunological attacks by inhibiting the adherence of different bacteria, and can function as recognition sites for various receptors (Schauer, 2004, 2009 Morrow et al., 2005; Meyer et al., 2007). These functions are also based on the presence of sialic acids with O-acetyl substitution at C-8 and/or C-9, as indicated by the increase of PA-P-TCH-SP-PD reactivity following saponification (Ueda et al., 1998). Moreover, apoptosis was reported to be inhibited by

O-acetylated sialic acids (Varki and Schauer, 2009). In this study, the dark cells contained sialoglycoconjugates that terminated in Siaα2-6Gal/GalNAc, whereas sialic acids linked to α2-3Galβ1-4GlcNAc were only localized in the luminal surface of some of the dark cells. Such distributional patterns of sialoglycoconjugates are clearly different from those in the eccrine glands of other mammals (Meyer and Tsukise, 1995; Illana et al., 1997; Yasui et al., 2004, 2005a, 2010). The great variety of sialic acid residues, in particular, may play an important role in the general defense against pathogenic agents (Suzuki, 2005; Parillo et al., 2009; Schauer, 2009).

Our findings showed that antimicrobial materials are also produced in the eccrine glands of porcine snout skin and released as part of the secretions. The localization of these substances was mainly confined to some of the dark cells in the eccrine glands. In humans, IgA, lactoferrin and β-defensins were immunohistochemically detected in the eccrine glandular cells (Metze et al., 1989; Ali et al., 2001; Park et al., 2011). Although Ezoe and Katsumura (1990) reported that lysozyme was not present in human eccrine glands, another investigation identified the expression of this enzyme (Papini et al., 1982). With regard to this gland type of the feline foot pads, these antimicrobial substances were demonstrated to be immunolocalized in the dark cells (Yasui et al., 2010). Moreover, in hyrax and tenrec, lysozyme and β-defensins are also observed in the eccrine glands of foot pads (Stumpf and

Welsch, 2002; Stumpf et al., 2004).

Lysozyme is a 14.5-KD cationic enzyme with bactericidal activity that catalyzes the hydrolysis of glycosidic bonds in bacterial cell walls (Duszyk, 2001). This enzyme has a close functional relationship to immunoglobulins and lactoferrin (Jollès and Jollès, 1984). The predominant immunoglobulin isotype on most mucosal surfaces and external secretions is secretory IgA composed of polymeric IgA containing J chain and secretory component (Tang et al., 2005; Snoeck et al., 2006). In epithelial defense, IgA contributes to preventing pathogen adhesion to host cells, thereby blocking dissemination and further infection (Corthésy, 2010). IgA as well as J chain is produced by plasma cells and transported through secretory epithelial cells into external secretions (Kaetzel et al., 1991). However, in the present study, the plasma cells were scarcely observed in the connective tissue surrounding the eccrine glandular acini. Furthermore, lactoferrin is a multifunctional glycoprotein of the transferrin family that is widely represented in various secretory fluids. It is one of the components of innate immunity because of its bacteriocidal, fungicidal and antiviral activities (Levay and Viljoen, 1995; Valenti et al., 1998). Additionally, lactoferrin inhibits allergen-induced skin inflammation, which is secondary to its role in regulating the production of cytokines (Ward et al., 2002). In humans and other mammals, defensins are known as some of the main antimicrobial peptides, which attack the cell walls of a wide range of bacterial, fungal and viral pathogens by insertion in the

phospholipid bilayer, leading to disruption and subsequent death of the microorganisms (Ganz, 2003, 2004). This is most important for the integument regarding the multitude of microbes always active on the skin surface (Meyer et al., 2001; Hadaway, 2003).

It is established that Rab proteins function in the tethering or docking of vesicles to their target compartment, leading to membrane fusion (Zerial and McBride, 2001). Rab3D is expressed in secretory granules of exocrine secretory cells, and involved in regulated exocytosis (Millar et al., 2002; Williams et al., 2009). The present study revealed that the secretory granules of the type II dark cells had a higher concentration of glycoproteins than those of the type I dark cells, as supported by the staining ability of the PA-TCH-SP-PD procedure (Yamada, 1993). It is suggested from these findings that sialic acid residues linked to α2-3Galβ1-4GlcNAc and various antimicrobial substances are elaborated by the type II dark cells. Furthermore, the localization of Rab3D seems to be consistent with that of these products. Such features indicate that this regulatory protein plays an important role in the secretory regulation of them.

Summarizing our functional interpretations, the results of this study confirmed the ultrastructures and the distribution of sialoglycoconjugates, antimicrobial substances and Rab3D in porcine snout eccrine glands. Sialoglycoconjugates are involved not only in adsorption of pathogenic agents, but also in viscoelasticity of secretions (Varki and Schauer, 2009). Moreover, the antimicrobial

substances are important in the innate immune response. In conclusion, the eccrine glandular secretions containing these secretory components may be essential for preserving the integrity of porcine snout skin as a sensory organ.

2. 5. Summary

The distribution of sialic acids and antimicrobial products (lysozyme, IgA, lactoferrin, β-defensin 2) as well as Rab3D in the eccrine glands of porcine snout skin was studied by sialoglycoconjugate histochemistry and immunohistochemistry. The secretory epithelium consisted of two types of secretory cells: dark and clear cells. The dark cells exhibited considerable amounts of sialoglycoconjugates, which included O-acetylated sialic acids, whereas sialic acids in the sequence Siaα2-3Galβ1-4GlcNAc were confined to some of the dark cells. All antimicrobial substances and Rab3D were demonstrated to be also mainly present in some of the dark cells. Additionally, in the cytological and cytochemical features, the different characteristics were observed among the dark cells. The results obtained are discussed with regard to the functional significance of the eccrine glands. The secretory products elaborated by this gland type may function as protective agents in order to preserve the skin integrity of the snout region, considering that sialic acids and antimicrobial substances are important in general defense mechanisms.

3 CYTOCHEMISTRY OF SIALOGLYCOCONJUGATES, LYSOZYME AND B-DEFENSIN IN ECCRINE GLANDS OF PORCINE SNOUT SKIN AS STUDIED BY ELECTRON MICROSCOPY

3. 1. Introduction

Regional variations in the structure of the mammalian integument are normally connected with manifold functional properties. The snout of pig is a highly specialized body region that acts as a sensory organ, which has undergone several morphological and functional adaptations (see Halata, 1975; Meyer and Neurand, 1982; Tsukise et al., 1983). In humans, eccrine glands are distributed widely throughout the common integument. In contrast, in most mammals except for humanoid primates, these glands are limited to particular parts of the body, for example, the foot pads of carnivores, the frog of ungulates (*Cuneus ungulae*), the carpus of the pig and the nasolabial region of ruminants and pig (Ellis, 1968; Calhoun and Stinson, 1981; Kurosumi et al., 1984; Hashimoto et al., 1986).

Sialic acids usually occupy the terminal position of glycoconjugates and have various functional properties within the saccharide residues (Schauer 2004; 2009; Varki and Schauer, 2009). On the other hand, diverse antimicrobial substances, which serve as a non-specific defense on the skin surface, may have functional significance in preserving skin integrity (Schröder, 1999; Bos et al., 2001; Yang et al., 2001). In the present study, the glandular acini of

the eccrine glands of porcine snout skin were subjected to cytochemical analyses of sialoglycoconjugates and antimicrobial substances (lysozyme, β-defensin) using electron microscopic methods. We previously reported on the localization of these moieties in this gland type by light microscopy (Fukui et al., 2012a). This work suggested the presence of two types of dark cells showing different staining intensities. However, the precise distribution of sialic acid, lysozyme and β-defensin in this gland type at the cytological level has not been determined. Therefore, our findings may confirm the above assumption that two types of dark cells producing different secretory components exist, and be indispensable to understand the general biological significance of these glands.

3. 2. Materials and Methods

All experiments were performed in accordance with the guidelines for the care and use of laboratory animals at the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University. Five male miniature pigs (potbelly, 1-2 years, 40-50 kg) were deeply anesthetized and then exsanguinated from the common carotid arteries. After bloodletting, the snout skin was removed surgically.

For general structural observation by electron microscopy, the specimens were fixed in 2.5% glutaraldehyde (GA) solution in 0.1 M

phosphate-buffered solution (PB) (pH 7.4) for 2 h at 4˚C. The materials were post-fixed in 2% osmium tetroxide solution for 2 h and embedded in Epon 812 (Luft, 1961). From these tissue blocks, ultrathin sections were cut using an ultramicrotome, mounted on copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

For cytochemical demonstration by electron microscopy, the specimens were fixed in a mixture of 4% paraformaldehyde (PFA) and 0.5% GA in 0.1 M PB (pH 7.4) for 2 h at 4˚C, and embedded in LR-White resin (Newman et al., 1983). From the LR-White-embedded blocks, ultrathin sections were cut as detailed above and placed on nylon or nickel grids. The sections on nylon grids were reacted for periodic acid-thiocarbohydrazide-silver proteinate-physical development procedure (PA-TCH-SP-PD) (Yamada, 1993). For cytochemical identification of glycogen in the cytoplasm, enzyme digestion with α-amylase (from *Bacillus subtilis*, Seikagaku Kogyo Co., 1 mg/ml, at 37°C for 4 h) (Casselman, 1959) was carried out on some sections prior to the PA-TCH-SP-PD procedure. For the localization of sialic acid, the nickel grid-mounted sections were incubated with biotinylated lectins at concentrations of 10-20 μg/ml in 0.05 M phosphate-buffered saline (PBS) (pH 7.2) for 24 h at 4˚C (Roth, 1983, 1996), following preincubation with 1% bovine serum albumin (BSA) (Sigma, MO, USA) in PBS. The lectins used were wheat germ agglutinin (WGA), *Sambucus sieboldiana* agglutinin (SSA) and *Maackia amurensis*

agglutinin (MAM) (Seikagaku Kogyo Co., Tokyo, Japan). Their specific sugar residues and inhibitory sugars are listed in Table 5 (for lectin specificities, see Danguy 1995). After rinsing with PBS, these sections were incubated with 15 nm colloidal gold-labeled streptavidin (British Biocell International, Cardiff, UK) at a dilution of 1:20 in 0.05 M PBS (pH 7.2) for 1 h at room temperature (Roth, 1983). They were then subjected to counterstaining with uranyl acetate and lead citrate. For specificity controls of the cytochemical lectin procedures, ultrathin sections were incubated with the respective lectin solutions to which 0.01 M of an appropriate competing sugar was added or incubated substituting unconjugated lectins for biotinylated lectins.

For immunocytochemisty, ultrathin sections were also placed on nickel grids. Following pretreatment with a solution containing 5% donkey serum albumin (DSA) (Jackson ImmunoResearch Lab., PA, USA) in 0.01 M PBS (pH 7.3), the sections were incubated with primary antibodies for lysozyme (dilution 1:40; polyclonal, anti-human, from rabbit) (Dako, Glostrup, Denmark) and human β-defensin 2 (dilution 1:600, polyclonal, anti-human, from rabbit) (Biolog, Kronshagen, Germany) for 24 h at 4°C. After rinsing with PBS, these ultrathin sections were incubated with biotinylated secondary antibody (anti-rabbit immunoglobulins, from donkey) (Jackson ImmunoResearch Lab.) at a dilution of 1:500 in PBS containing 5% DSA for 60 min, and then with 15 nm colloidal gold-labeled streptavidin at a dilution of 1:20 in PBS for 60 min at

room temperature. They were counterstained with uranyl acetate and lead citrate. Controls for the immunocytochemical procedures were performed by incubation with PBS without primary antibodies or by replacement of the primary antibodies with normal rabbit immunoglobulin diluted to the same extent as the specific antibodies.

Table 5. The lectins used and their sugar-binding specificities and inhibitory sugars

Lectins		Sugar-binding specificity	Inhibitory sugar
WGA	Wheat germ agglutinin	Sia, β-D-GlcNAc	β-D-GlcNAc
SSA	*Sambucus sieboldiana* agglutinin	Siaα2-6Gal/GalNAc	α2-6sialyllactose
MAM	*Maackia amurensis* agglutinin	Siaα2-3Galβ1-4GlcNAc	α2-3sialyllactose

3. 3. Results

Analysis of the eccrine glands of the porcine snout skin revealed that the secretory portion consisted of dark cells and clear cells. The dark cells were equipped with a distinct Golgi apparatus, rough-surfaced endoplasmic reticulum and a varying number of secretory granules within their cytoplasm. In addition, different morphological characteristics were detected among the dark cells. The secretory granules of some dark cells were relatively large, whereas those of other dark cells were smaller. Therefore, we classified the former dark cells as type I and the latter as type II. On the other hand, the clear cells, the cytoplasm of which exhibited lower electron density, contained a Golgi apparatus and glycogen particles. Furthermore, the cytoplasmic matrix of all these cells, especially the clear cells, was studded with many mitochondria of varying morphology (Fig. 9).

In secretory cells stained by the PA-TCH-SP-PD procedure, the relatively large secretory granules of the type I dark cells showed a weak to moderate positive reaction, while a distinct positive reaction was observed in the smaller secretory granules of the type II dark cells (Fig. 10). Other prominent PA-TCH-SP-PD-reactive structures were the surface coat of the plasma membrane and cisternae of the Golgi apparatus in the dark and clear cells (Fig. 10). Although the glycogen particles exhibited a positive reaction in the clear cells, this reactive structure was abolished by digestion with α-amylase.

After the WGA-gold procedure, gold particles were associated

with the surface coat of the plasma membrane in the secretory cells. Additionally, in both cell types of the dark cells, the secretory granules and Golgi apparatus showed positive reactions (Fig. 11a, b). Distinctly concentrated reactive particles were detectable in the secretory granules of the type I and II dark cells after incubation with SSA (Fig. 12a). Furthermore, a number of gold particles for SSA were observed in the Golgi apparatus (Fig. 12b). The MAM-gold procedure resulted in a weak reaction of the free surface of the plasma membrane in the secretory cells. The secretory granules of the type I dark cells were almost negative, whereas those of the type II dark cells showed a distinct positive reaction (Fig. 13a, b). Moreover, clearly concentrated reactive particles for MAM were detected in the Golgi apparatus of the type II dark cells (Fig. 13c). The clear cells reacted negatively with all of the lectin cytochemical procedures except for the surface coat of the plasma membrane.

In the glandular cells incubated with anti-lysozyme, the secretory granules of the type I dark cells were seen to react very weakly or almost negatively, while reactive particles were localized at those of type II dark cells (Fig. 14a, b). A few gold particles for lysozyme were also observed in the Golgi apparatus (Fig. 14b). With regard to immunocytochemical methods for human β-defensin 2, the reaction pattern was similar to that of lysozyme in that reactive particles were visible in the components of the secretory pathway of the type II dark cells, including the secretory granules and Golgi apparatus (Fig. 15a, b). In these immunocytochemical reactions, the

clear cells were almost negative (Fig. 15a).

In the control sections reacted for the lectin cytochemical staining by addition of the appropriate inhibitory sugars to the respective lectin solutions and substitution of the unconjugated lectins, positive reaction staining of all the formerly reactive ultrastructures was greatly suppressed or abolished. Moreover, no glandular structures exhibited any positive reactions in the control ultrathin sections stained using immunocytochemical procedures by incubation with PBS without the primary antibodies and by replacement of the primary antibodies with normal rabbit immunoglobulin.

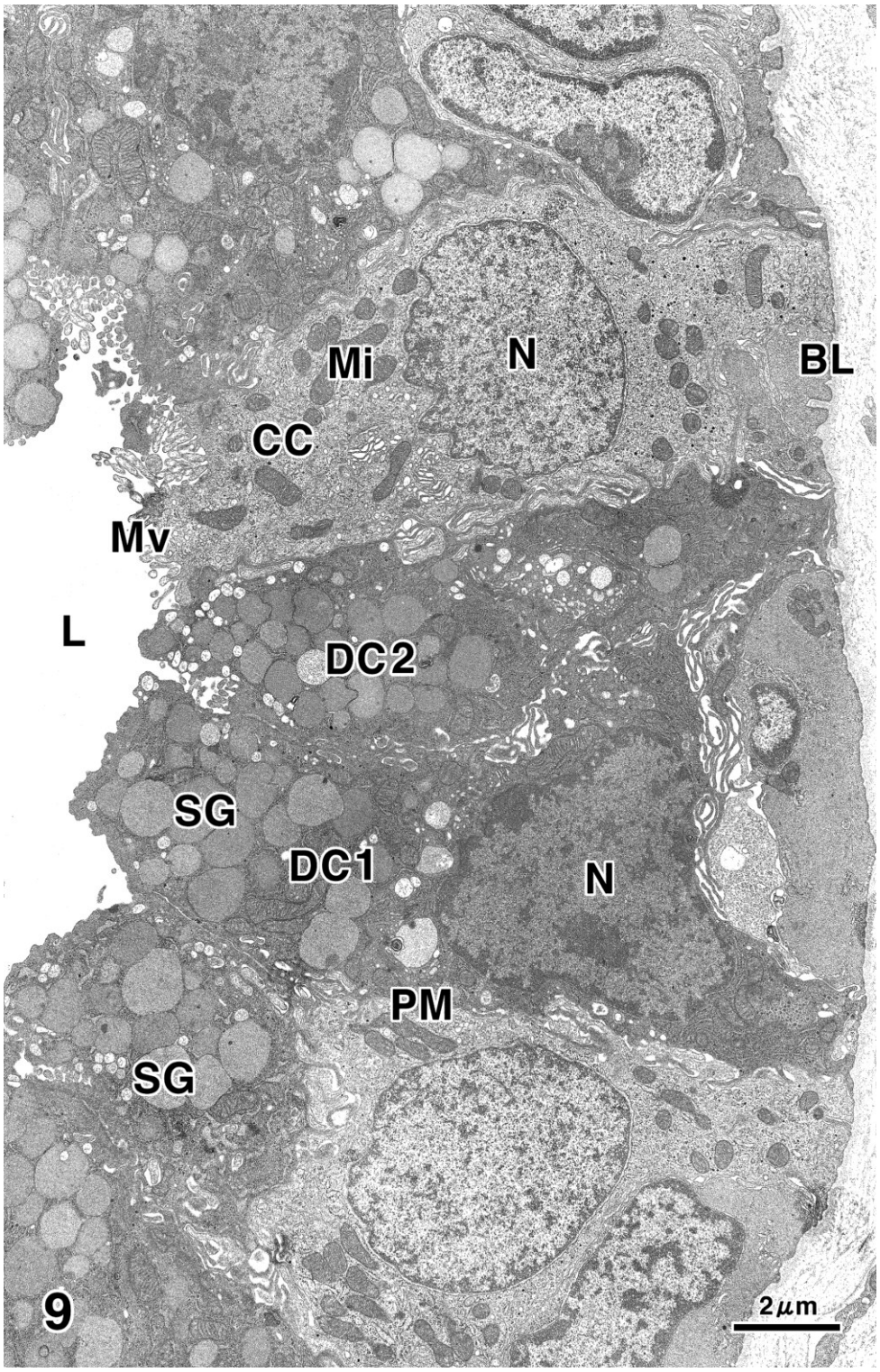


Fig. 9 General ultrastructure of the eccrine glands in the porcine snout skin stained with uranyl acetate and lead citrate. ×8,000, BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mi: mitochondrion, Mv: microvilli, N: nucleus, SG: secretory granule.

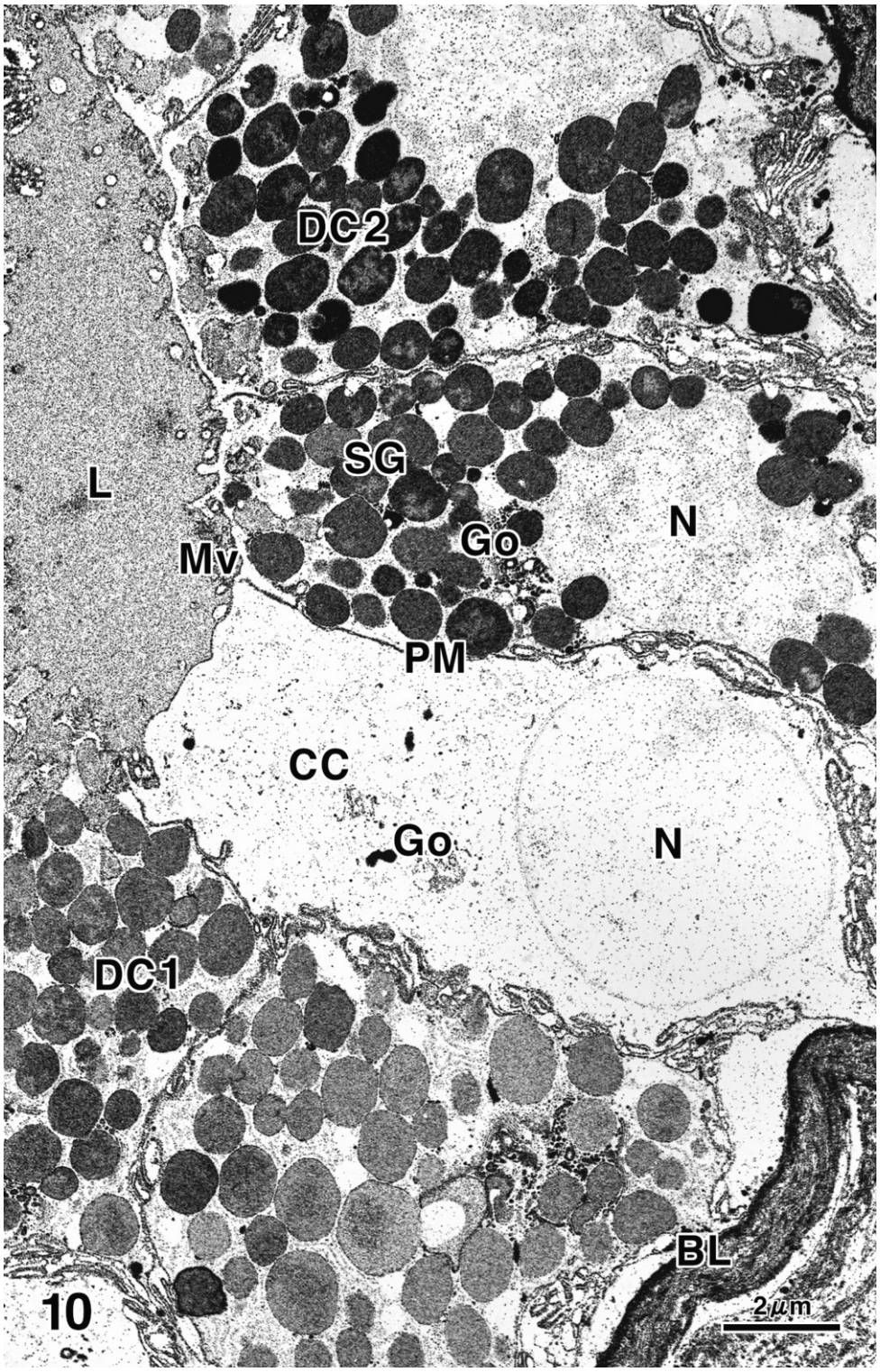


Fig. 10 Cytochemical PA-TCH-SP-PD staining of the eccrine glands in the snout skin. ×8,200, BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

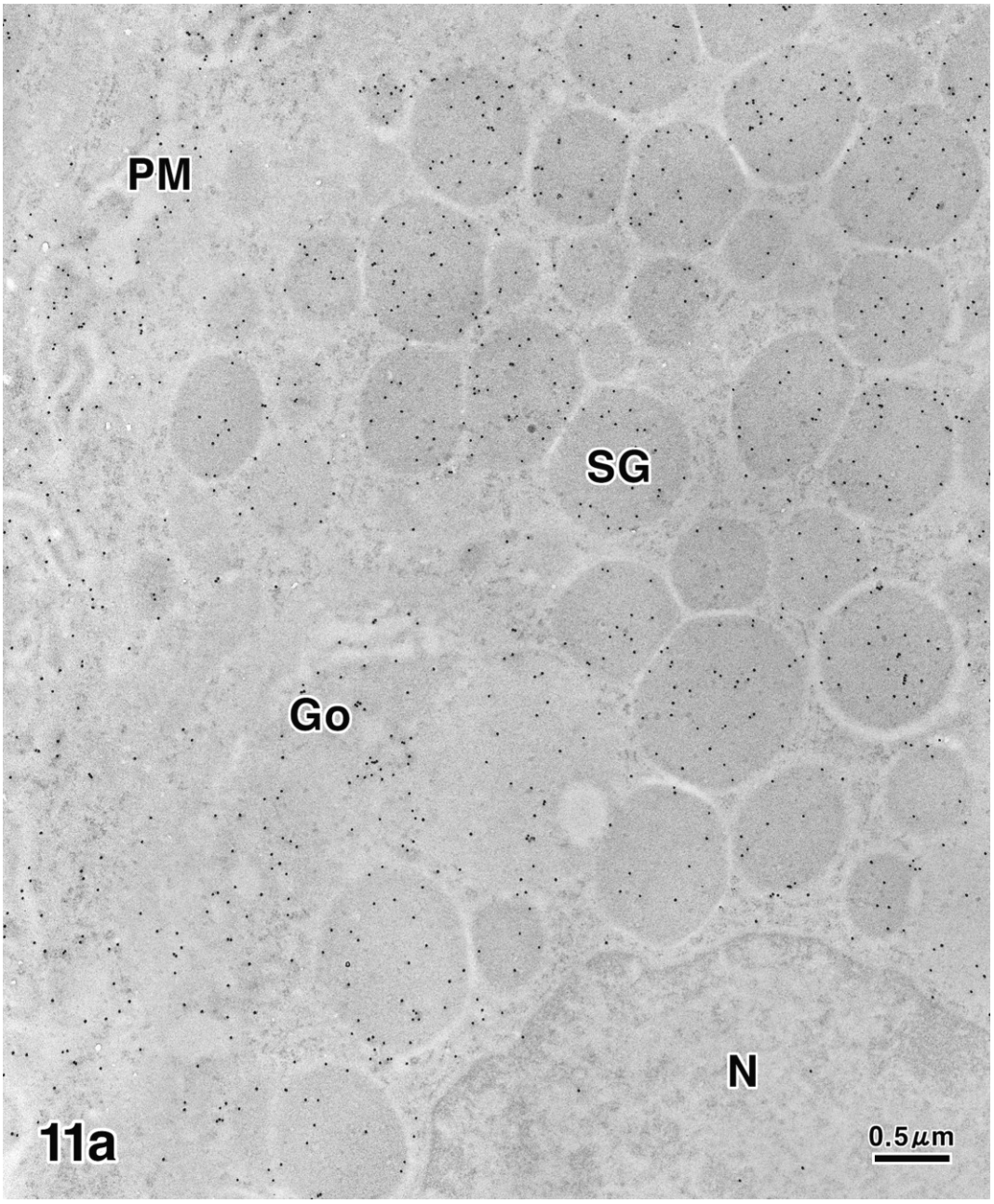


Fig. 11 WGA-gold staining of the eccrine glands in the snout skin. a) Reactions in the surface coat of the plasma membrane, cisternae of the Golgi apparatus and secretory granules of the type I dark cell. ×22,000, Go: Golgi apparatus, N: nucleus, PM: plasma membrane, SG: secretory granule.

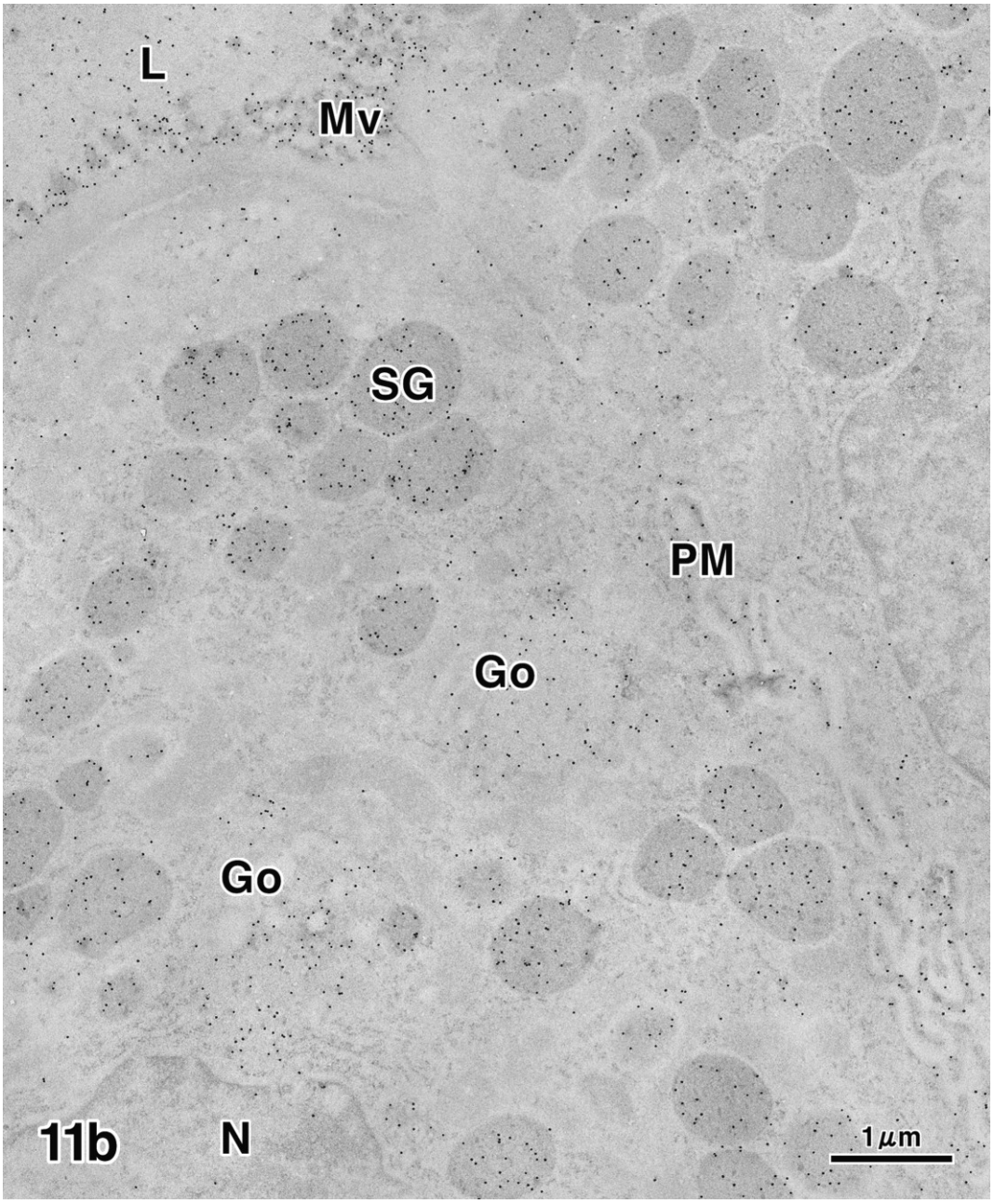


Fig. 11 WGA-gold staining of the eccrine glands in the snout skin. b) Reactions in the surface coat of the plasma membrane, cisternae of the Golgi apparatus and secretory granules of the type II dark cell. ×17,500, Go: Golgi apparatus, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

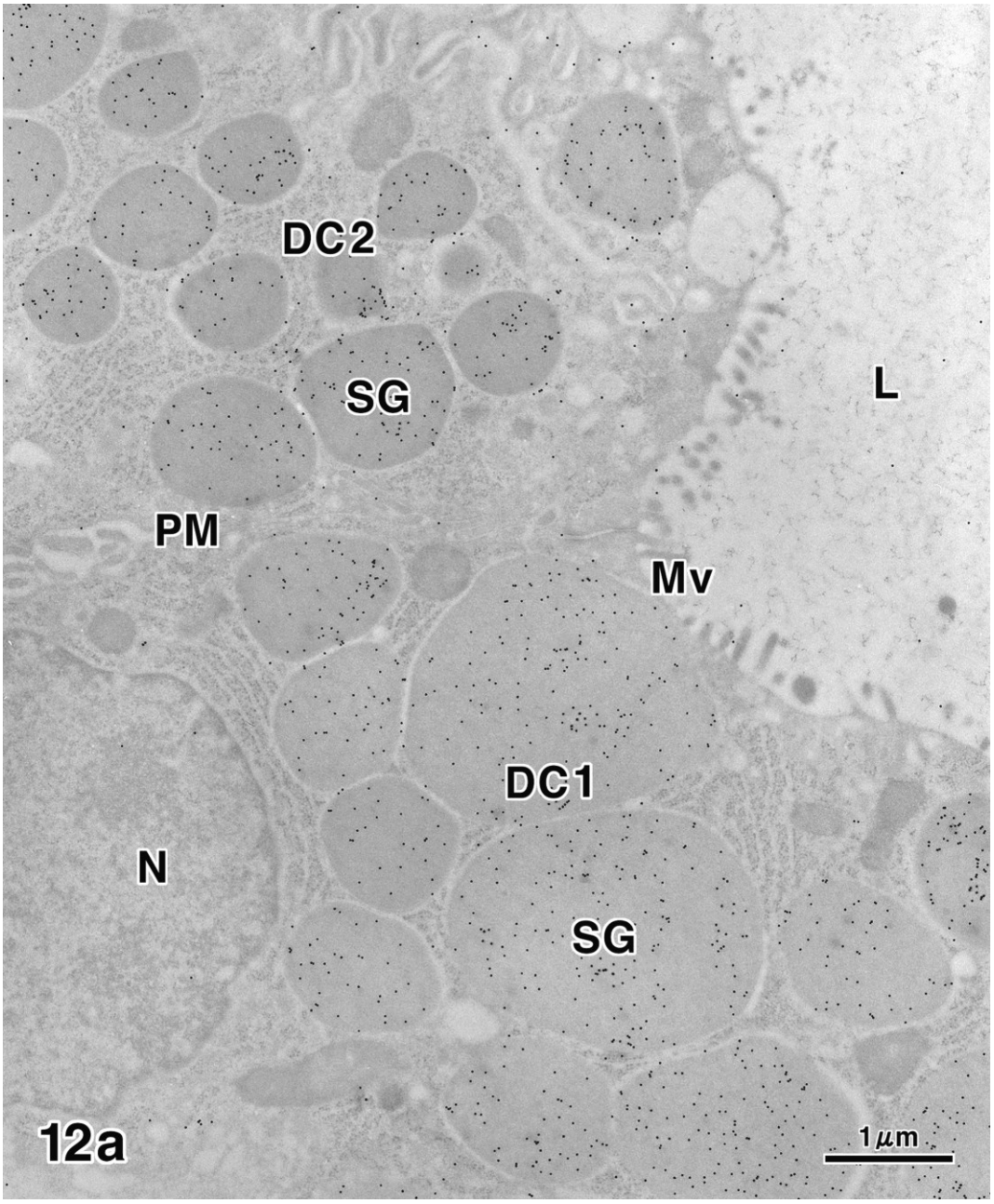


Fig. 12 SSA-gold staining of the eccrine glands in the snout skin. a) Reactions in the surface coat of the plasma membrane and secretory granules of the dark cells. ×18,500, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

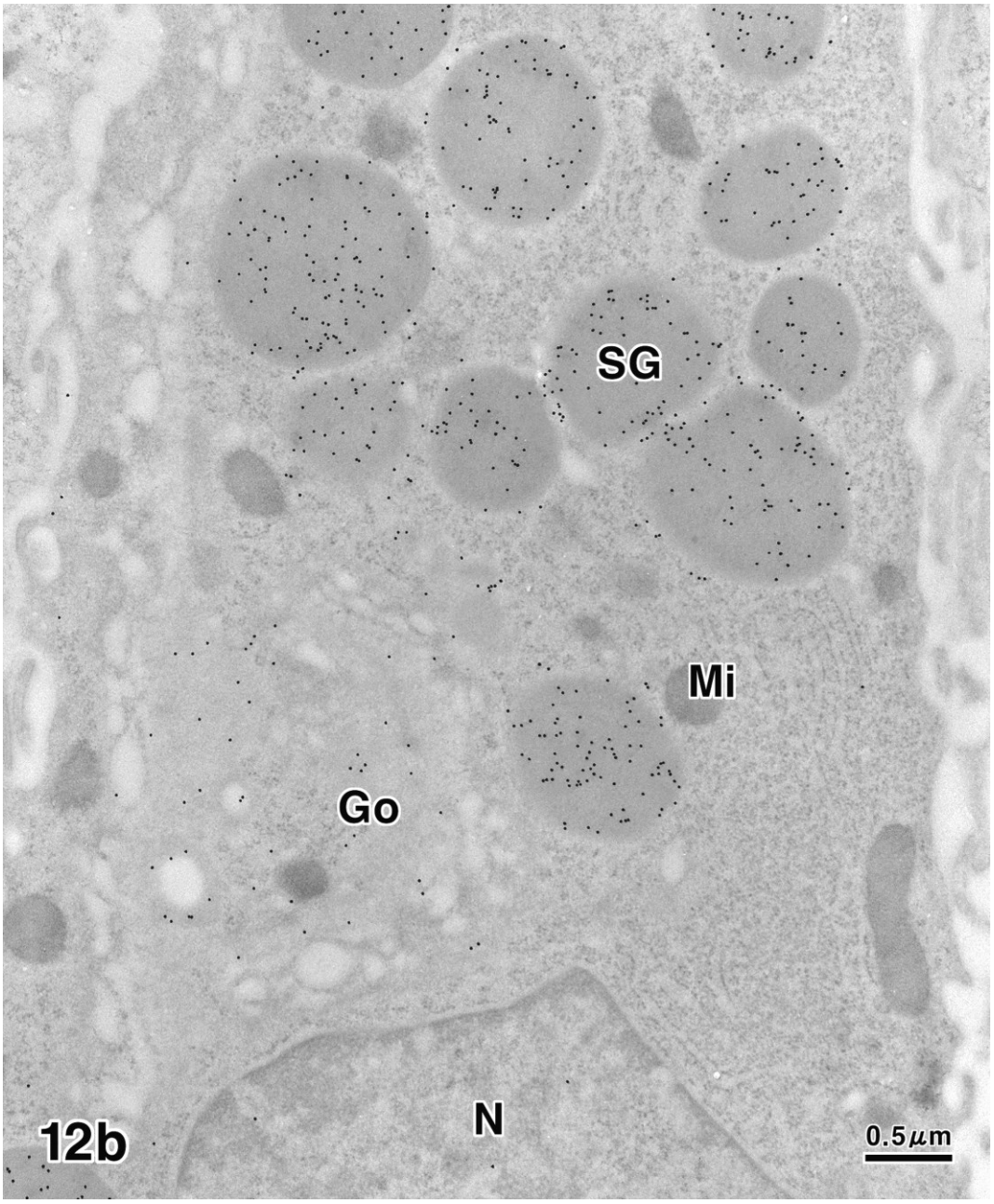


Fig. 12 SSA-gold staining of the eccrine glands in the snout skin. b) Part of the supranuclear cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus and secretory granules. ×25,000, Go: Golgi apparatus, Mi: mitochondrion, N: nucleus, SG: secretory granule.

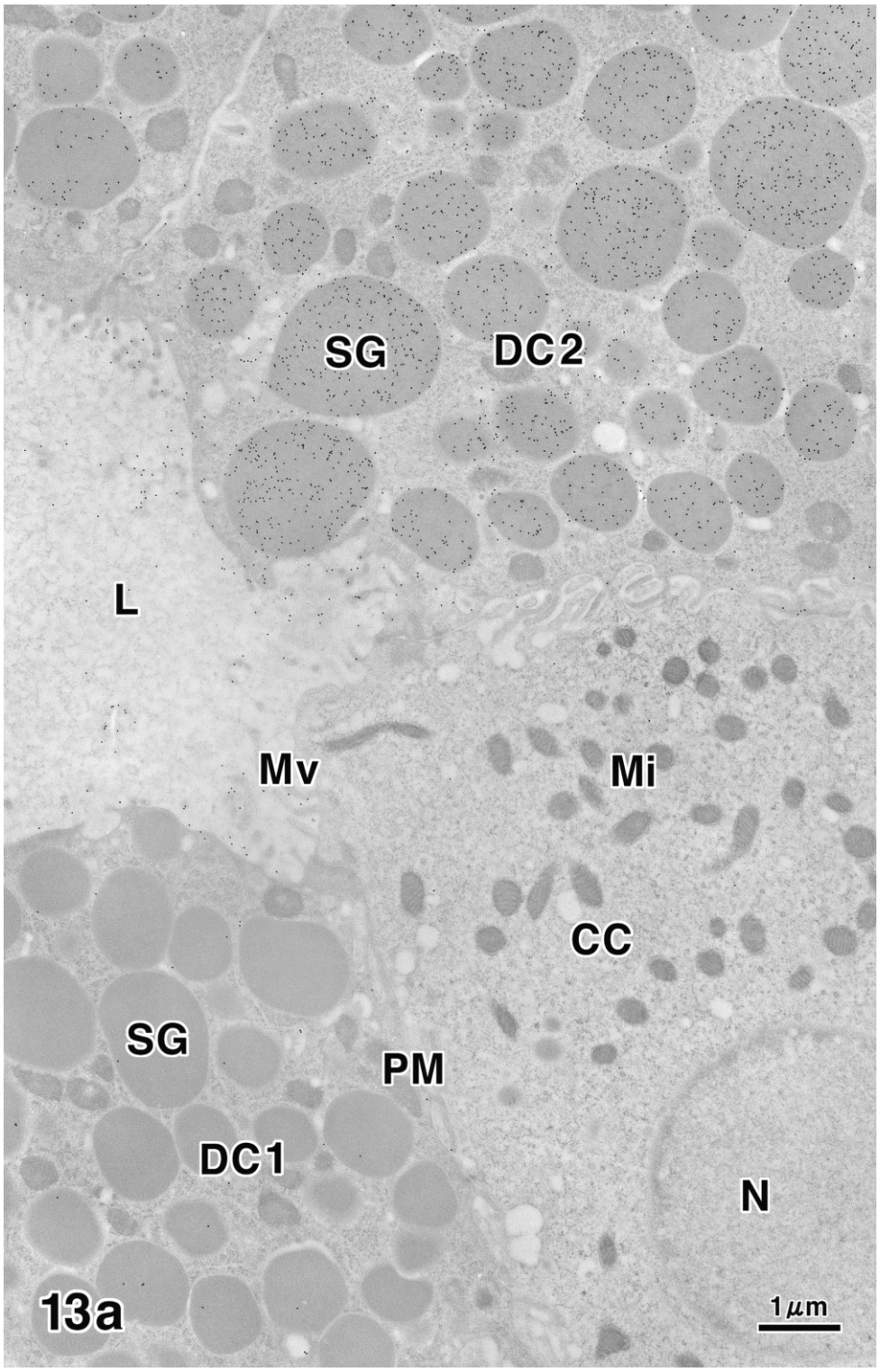


Fig. 13 MAM-gold staining of the eccrine glands in the snout skin. a) Part of the luminal side of the eccrine glandular acini. A distinctly positive reaction is only detectable in the type II dark cells. ×12,000, CC: clear cell, DC1: type I dark cell, DC2: dark cell, L: lumen, Mi: mitochondrion, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

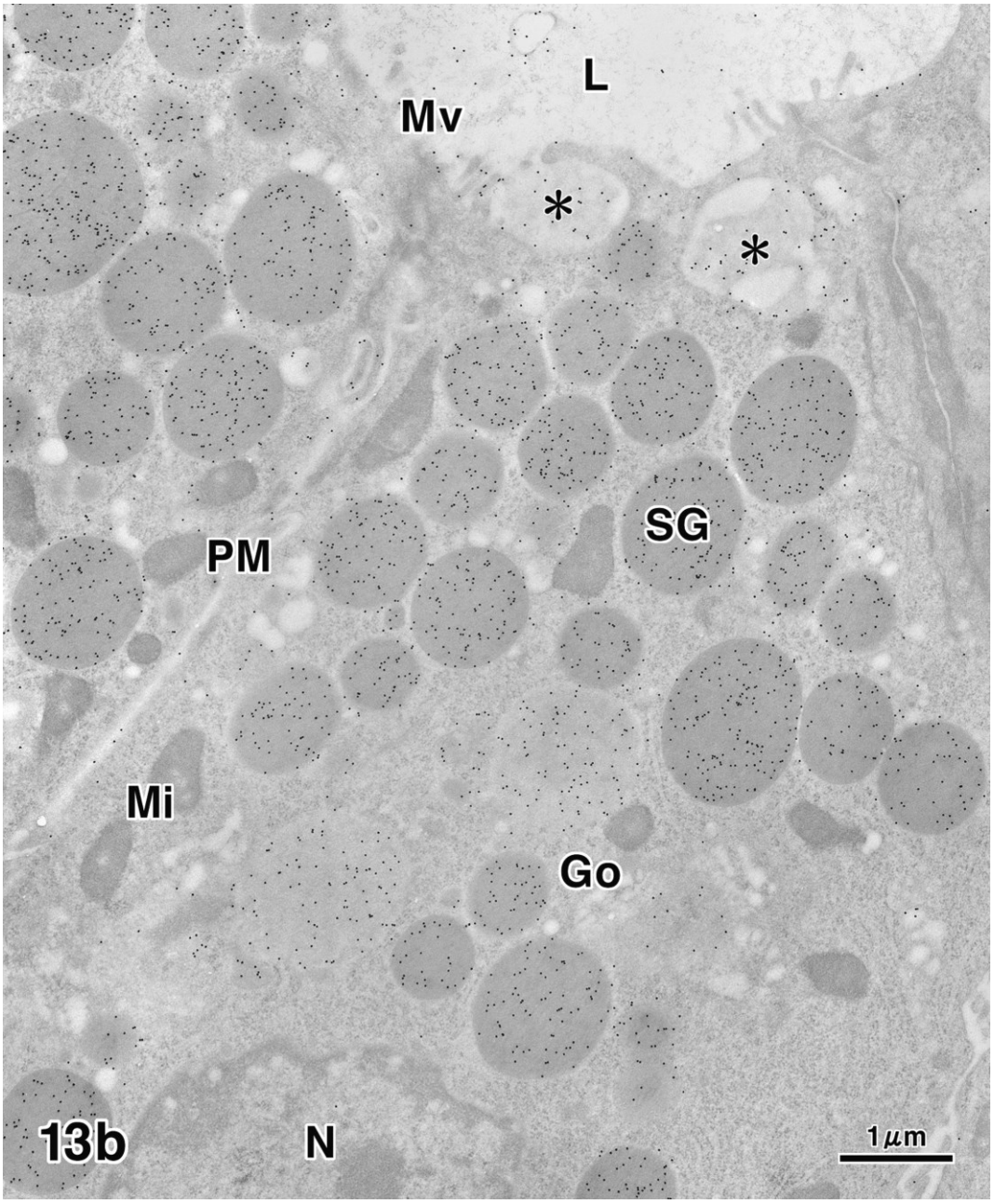


Fig. 13 MAM-gold staining of the eccrine glands in the snout skin. b) Reactions in the surface coat of the plasma membrane, cisternae of the Golgi apparatus, secretory granules and luminal secretions of the type II dark cells. ×16,000, Go: Golgi apparatus, L: lumen, Mi: mitochondrion, Mv: microvilli, cell, N: nucleus, PM: plasma membrane, SG: secretory granule, asterisk: exocytosis.

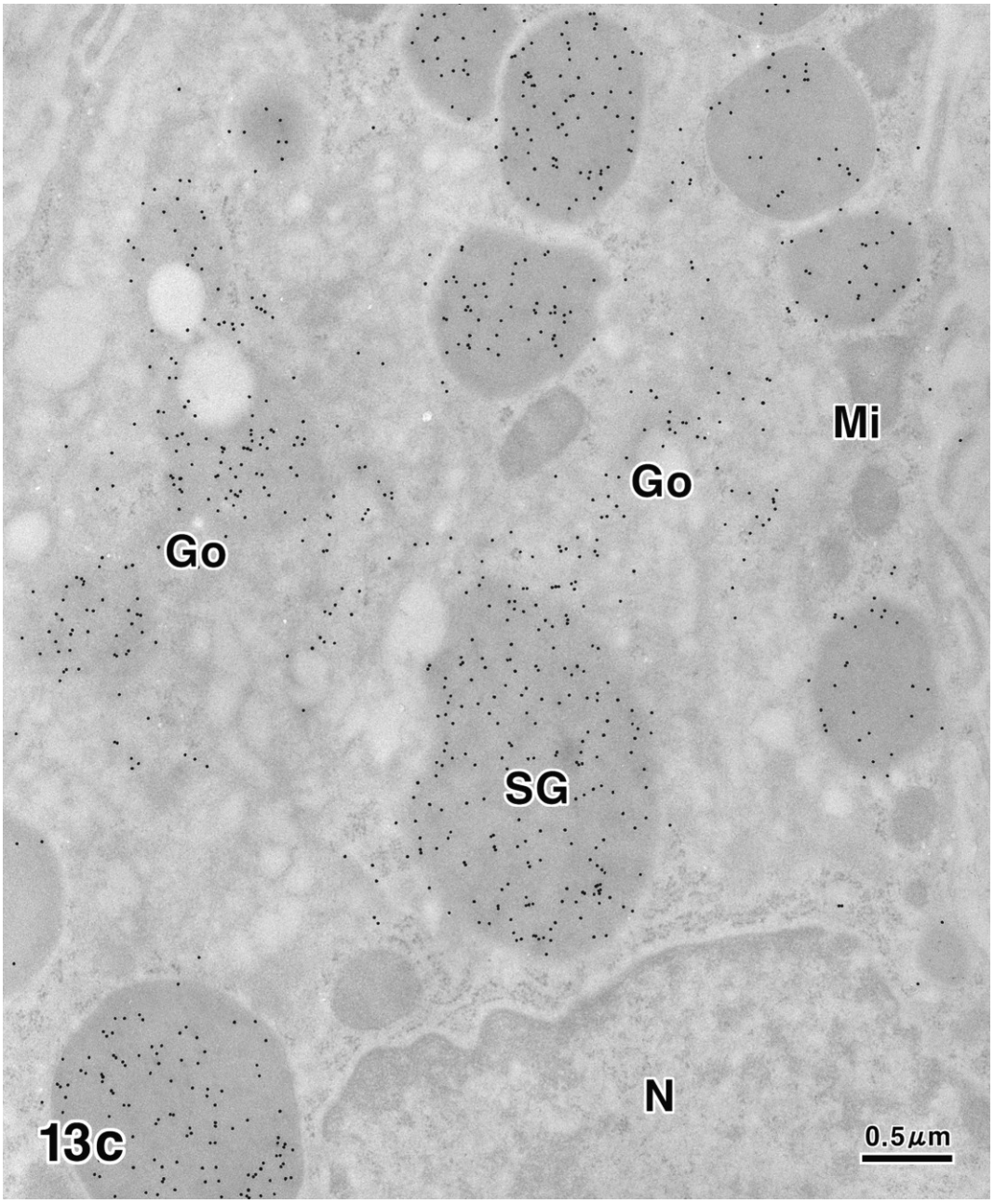


Fig. 13 MAM-gold staining of the eccrine glands in the snout skin. c) Part of the supranuclear cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus and secretory granules. ×26,000, Go: Golgi apparatus, Mi: mitochondrion,, N: nucleus, SG: secretory granule.

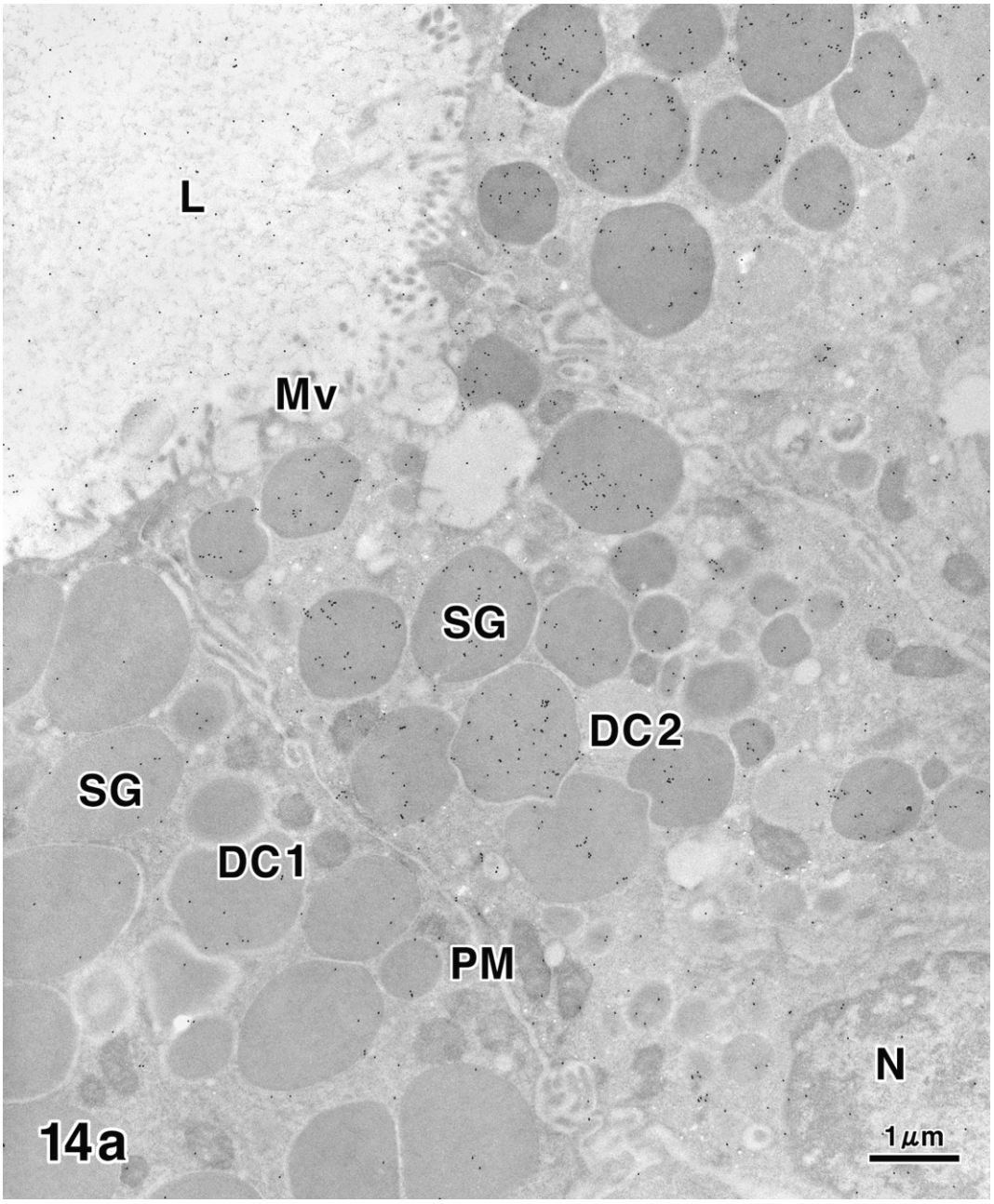


Fig. 14 Immunogold labeling for lysozyme of the eccrine glands in the snout skin. a) Part of the luminal side of the eccrine glandular acini. A clearly positive reaction is detectable in the type II dark cells. ×13,000, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

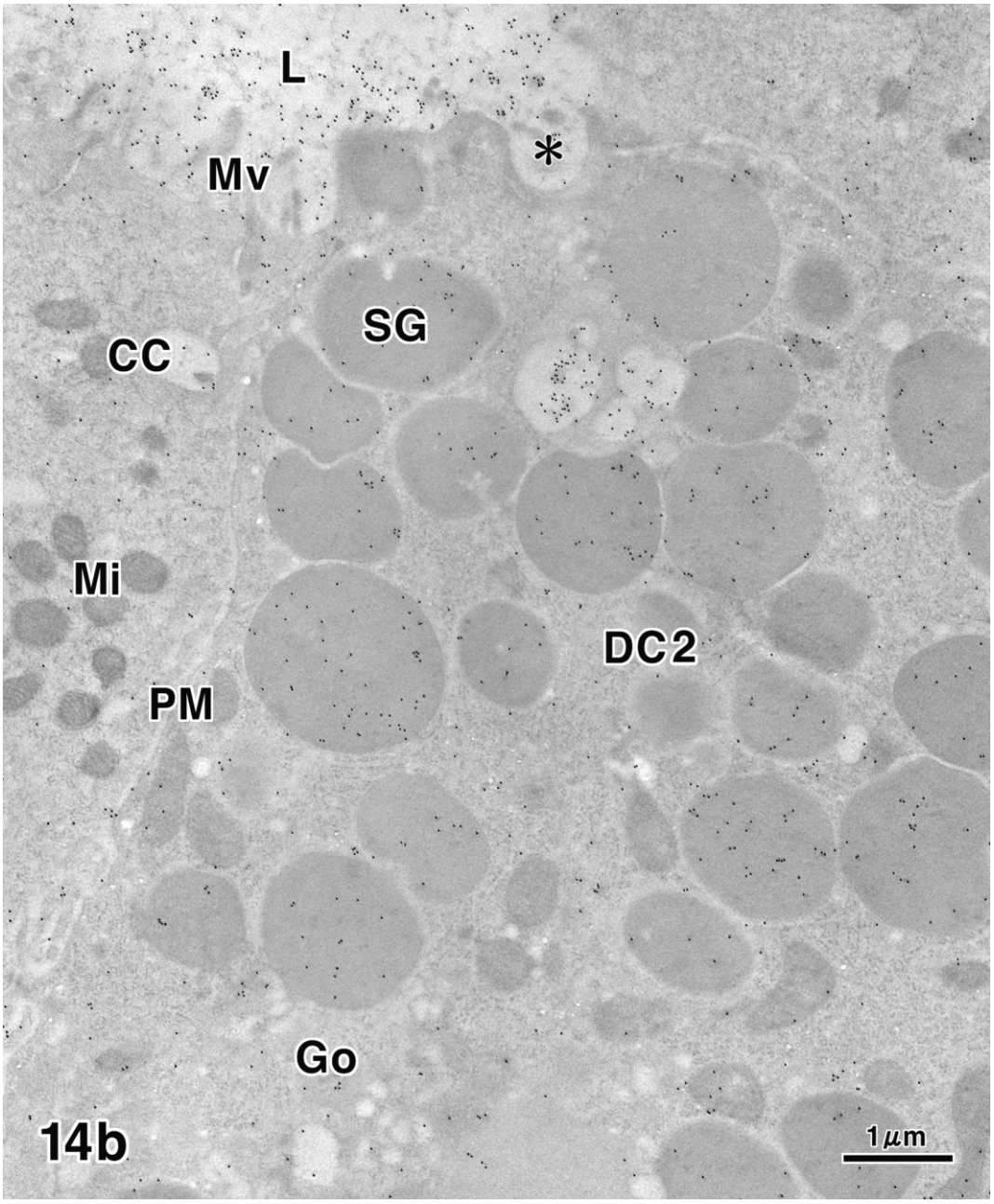


Fig. 14 Immunogold labeling for lysozyme of the eccrine glands in the snout skin. b) Part of the apical cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus, secretory granules and luminal secretions. ×15,500, CC: clear cell, DC2: type II dark cell, Go: Golgi apparatus, L: lumen, Mi: mitochondrion, Mv: microvilli, cell, PM: plasma membrane, SG: secretory granule, asterisk: exocytosis.

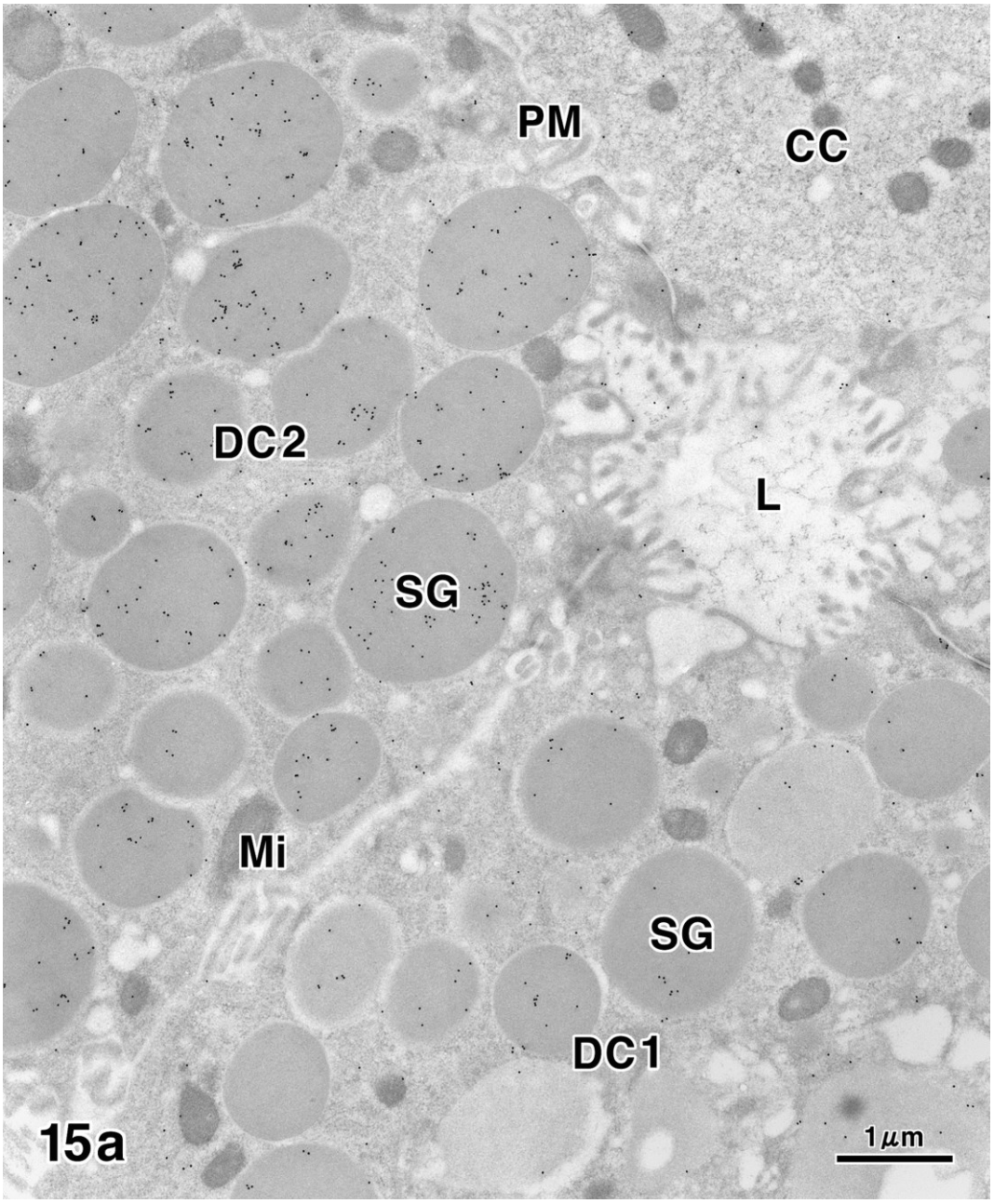


Fig. 15 Immunogold labeling for β-defensin 2 of the eccrine glands in the snout skin. a) Part of the luminal side of the eccrine glandular acini. A clearly positive reaction is detectable in the type II dark cell. ×16,500, CC: clear cell, DC1: type I dark cell, DC2: dark cell, L: lumen, Mi: mitochondrion, PM: plasma membrane, SG: secretory granule.

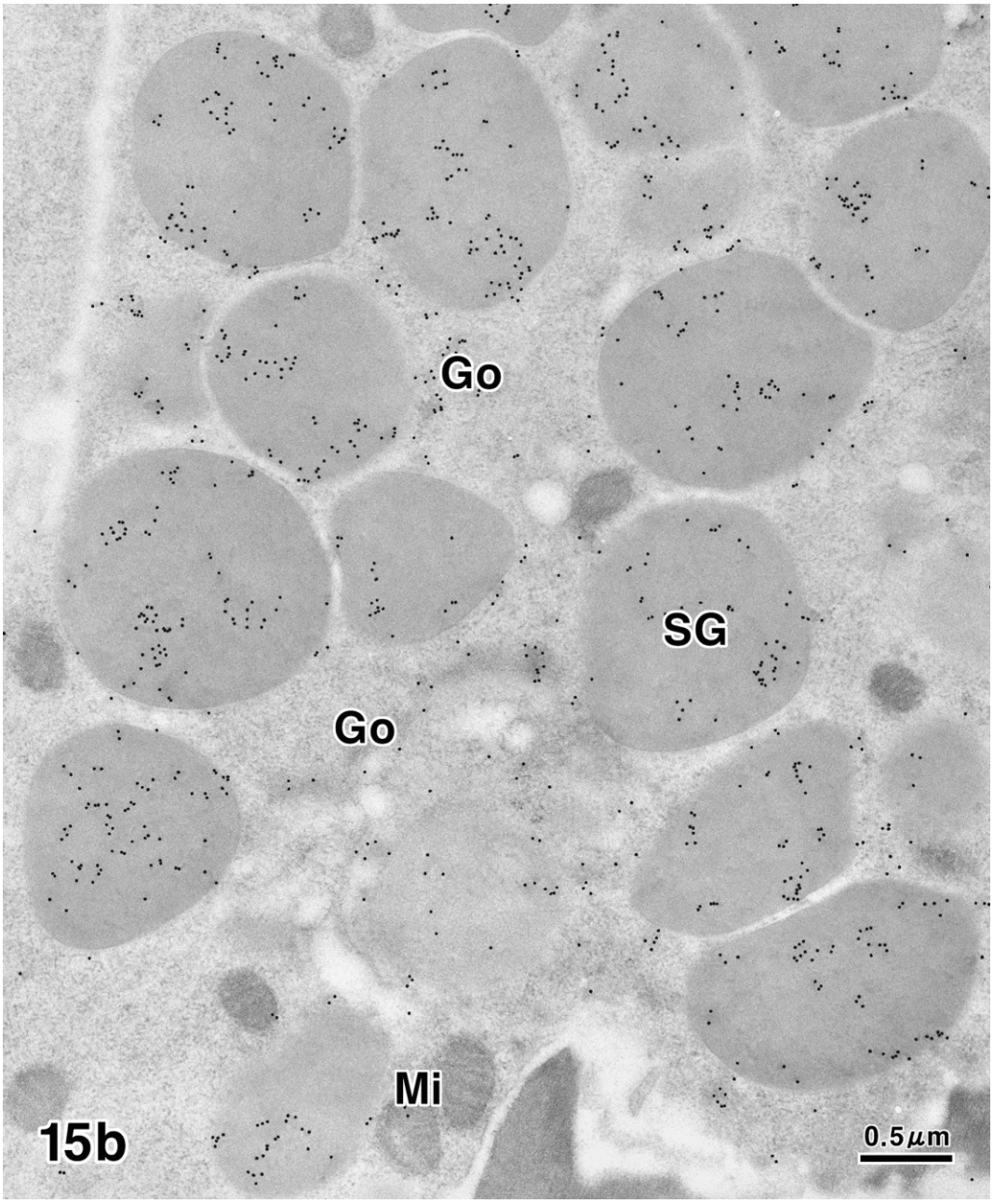


Fig. 15 Immunogold labeling for β-defensin 2 of the eccrine glands in the snout skin. b) Part of the supranuclear cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus and secretory granules. ×26,000, Go: Golgi apparatus, Mi: mitochondrion, SG: secretory granule.

3. 4. Discussion

In mammals, eccrine glands are generally accepted to consist of dark cells and clear cells on the basis of their staining profiles (Kurosumi et al., 1984). Although many histochemical investigations were previously reported in this gland type of mammals, such as in human common integuments (Illana et al., 1997; Sames et al., 1999) and in the foot pads of carnivores (Meyer and Bartels, 1989; Meyer and Tsukise, 1995; Yasui et al., 2004; 2010), different features in terms of the histochemical results were not observed among the dark cells of the eccrine glands. Our previous study light-microscopically demonstrated that the localization of sialic acids linked to α2-3Galβ1-4GlcNAc and antimicrobial substances was confined to a subpopulation of the dark cells of the eccrine glands in the porcine snout skin (Fukui et al., 2012a). In the present study, the general ultrastructural observations of these glands are mostly similar to those in the foot pads of other mammals (Meyer and Bartels, 1989; Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2004; 2005b). However, in our investigation, some slightly different morphological characteristics were detected among the dark cells in that the secretory granules of some dark cells were larger than those of the other dark cells. Additionally, the two types of the dark cells were distinguishable by the results of the PA-TCH-SP-PD procedure, which demonstrates the expression of glycoconjugates with vicinal diol groupings (Yamada, 1993). Therefore, the dark cells of the eccrine glands in the porcine snout skin were classified as type I

cells and type II cells. On the other hand, the clear cells were not equipped with the secretory granules. This cell type may produce mainly the hypertonic or isotonic precursor fluid of sweat (Kurosumi et al., 1984). The basolateral membrane of the clear cells acts as a sodium pump and is the site of energy-consuming active transport of ions (Sato et al., 1989). In this way, the presence of large amounts of glycogen and mitochondria in the clear cells could be closely connected with upregulated their secretory production (Meyer and Bartels, 1989; Sato et al., 1989).

From the results of lectin cytochemical methods, sialic acids were mainly expressed in the surface coat of the plasma membrane, cisternae of the Golgi apparatus and secretory granules of the type I and type II dark cells. However, the localization of sialic acid residues linked to α2-3Galβ1-4GlcNAc was confined to these ultrastructures of the type II dark cells, while both types of dark cells contained sialoglycoconjugates that terminated in Siaα2-6Gal/GalNAc. Sialic acids are involved in binding and transport of ions, stabilizing the conformation of proteins and increasing the viscosity of mucins. Furthermore, they can control the affinity of receptors and are reported to modulate processes involved in transmembrane signaling, growth and differentiation. Sialic acids act not only as recognition sites for various molecules and microorganisms, but also as biological masks that are antirecognition agents by shielding recognition sites (Schauer, 2004; 2009; Varki and Schauer, 2009). In addition to such functions, the

diversity of sialic acid residues arising from variation in the types of linkage and acceptor sugar and their degree of acetylation helps to protect epithelia against harmful substances and pathogens (Suzuki, 2005; Parillo et al., 2009).

The antimicrobial substances are present in various organs, such as salivary glands, genitourinary tract, digestive tract, respiratory epithelium and skin. They are generally part of the innate immune system and of great importance for the skin integrity of various mammalian species by their antibacterial activities. Our findings revealed that lysozyme and β-defensin were principally distributed in the secretory granules and Golgi apparatus of the type II dark cells. Lysozyme is the first antimicrobial peptide found in human skin (Klenha and Krs, 1967; Ogawa et al., 1971). This enzyme has direct bacteriolytic action that hydrolyzes β-(1,4)-glycosidic bonds between N-acetylmuramic acid and N-acetyl-β-D-glucosamine in bacterial cell walls (Jollès and Jollès, 1984; Duszyk, 2001). Mammalian lysozymes are classed as c-type muramidases and capable of both hydrolysis and transglycosylation (Jollès and Jollès, 1984). In mammals, β-defensins are produced by a variety of epithelial cells and are often present at high concentration. These antimicrobial peptides have the ability to insert themselves into the cell membrane, and this leads to the disruption and subsequent death of microorganisms (Ganz, 2003; 2004). A previous study reported the identification and initial characterization of eleven porcine β-defensins including defensin-like molecules.

Moreover, at least, seven porcine β-defensins are expressed in skin (Sang et al., 2006).

Finally, the results of the present study confirm the views that the dark cells of the eccrine glands in porcine snout skin can be classified as type I and type II cells, and sialic acid residues linked to Siaα2-3Galβ1-4GlcNAc and various antimicrobial substances are elaborated by the latter (Fukui et al., 2012a). In humans, lysozyme may be produced by the immature secretory cells of the salivary glands, such as sublingual glands and labial glands (Miyazaki et al., 1998; 2001). Therefore, it is suggested that such heterogeneity concerning the production of these substances depends on the functional stages or maturation stages of the dark cells. It is unclear, however, whether the type II dark cell is mature or immature one. In conclusion, sialoglycoconjugates are associated with the viscosity of secretions and protection against pathogenic agents. Additionally, the antimicrobial substances are important in immune responses. Thus, these eccrine glands may be closely related to effective defense in order to preserve the integrity of porcine snout skin as a sensory organ.

3. 5. Summary

In most mammals except for humanoid primates, eccrine glands are confined to the skin of a series of specific body regions. Sialic acids and antimicrobial substances exhibit various functional

properties and serve as a component of non-specific defense against microorganisms, respectively. In the present study, the distribution of these moieties was studied by electron microscopic cytochemical methods. The eccrine glandular acini consisted of two types of dark cells as well as clear cells. The secretory granules and Golgi apparatus of both types of dark cells contained sialic acid residues linked to α2-6Gal/GalNAc. On the other hand, sialoglycoconjugates with Siaα2-3Galβ1-4GlcNAc sequence were confined to those of the type II dark cells. In addition, lysozyme and β-defensin were mainly detected in the secretory granules of the type II dark cells. These secretory products may create a defensive barrier against microbial invasion and play an essential role in preservation of the integrity of porcine snout skin as a sensory organ.

4 HISTOCHEMICAL DISTRIBUTION OF SIALIC ACIDS AND ANTIMICROBIAL SUBSTANCES IN PORCINE CARPAL GLANDS

4. 1. Introduction

Eccrine glands are normally restricted to specific body regions of mammals except for humanoid primates, for example, the foot pads of carnivores, the frog among ungulates (*Cuneus ungulae*), the carpus of pigs and the nasolabial region of ruminants and pigs (Ellis, 1968; Calhoun and Stinson, 1981; Hashimoto et al., 1986). The secretions elaborated by these glands are known to contain abundant glycoconjugates with various saccharide residues, and appear to play an important part in specific skin functions, such as protection of epidermal surface against bacterial invasion, communication, signaling of sexual activity and water retention (Tsukise et al., 1983; Tsukise et al., 1988a, b; Meyer and Bartels, 1989; Meyer and Tsukise, 1989, 1995; Yasui et al., 2004, 2005a, 2010). Additionally, in several mammalian species, immunohistochemical analyses of different antimicrobial substances in eccrine glands have been reported (Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2010).

Porcine carpal glands are considered to elaborate odorous substances (Hraste and Stojković, 1995; Pedini et al., 1999), although their morphological features are similar to those of human eccrine glands (Kurosumi et al., 1984; Gargiulo et al., 1989). The present

study on the porcine carpal glands investigated the localization of sialic acids and antimicrobial substances using sialoglycoconjugate histochemical and immunohistochemical methods. Sialic acids are known usually to occupy the terminal position of oligosaccharide chains in a variety of glycoconjugates, and their functional properties appear to be manifold (Schauer, 2004, 2009). In addition, antimicrobial substances, which have the ability to disrupt a broad spectrum of microorganisms, are the effective molecules of integumental innate immunity (Schröder, 1999; Bos et al., 2001; Yang et al., 2001). Therefore, our findings may be important for understanding the functional significance of porcine carpal glands, except with regard to their function as odoriferous glands.

4. 2. Materials and Methods

All experiments were performed in accordance with the guidelines for the care and use of laboratory animals at the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University. Five male miniature pigs (potbelly, 1-2 years, 40-50 kg) were deeply anesthetized and then exsanguinated from the common carotid arteries. After bloodletting, the carpal glands were removed surgically (Fig. 16). These skin samples were fixed in Bouin's solution for 24 h at room temperature, then carefully washed and dehydrated through a graded series of ethanol. The materials were embedded in paraffin wax and then sectioned at a

thickness of 5 μm. Sections were stained with hematoxylin-eosin (H-E) and alcian blue (AB) pH 2.5-periodic acid Schiff (PAS) (Mowry, 1963) methods for general histological purposes, as well as with sialoglycoconjugate histochemical and immunohistochemical procedures.

For the localization of sialic acid residues, after deparaffinization, the histochemical staining method employed was periodic acid-phenylhydrazinium chloride-thiocarbohydrazide-silver proteinate-physical development (PA-P-TCH-SP-PD) (Ueda et al., 1995). Selective periodate oxidation (0.4 mM PA in 1 M HCl) of sialic acid residues yields a monoaldehyde at C-7 or C-8, which then condenses with phenylhydrazine to give rise to the corresponding phenylhydrazone. This compound can undergo solvolysis to yield TCH-SP-PD-reactive monoaldehydes. On the other hand, the dialdehydes of neutral sugars oxidized by the selective periodate oxidation condense with phenylhydrazine to result in TCH-SP-PD-unreactive azido or morpholine. In addition, sialic acids with O-acetyl substitutions at C-8 and/or C-9 are undetected by this procedure. Therefore, chemical modification by saponification (KOH) (0.5% potassium hydroxide-70% ethanol) (Spicer, 1960) was performed on some sections, prior to PA-P-TCH-SP-PD staining, since KOH treatment is known to remove the acetyl groups on C-4, C-7, C-8 and C-9. Enzyme digestion with sialidase (from *Arthrobacter ureafaciens*, Nacalai Tesque Inc., Kyoto, Japan; 1.0 U/ml in 0.1 M phosphate

buffer, pH 5.3, at 37°C for 18 h) (Spicer et al., 1967) after saponification was conducted before the PA-P-TCH-SP-PD staining. These physical development procedures for detection of sialic acids and their moieties are summarized in Table 6. Additionally, the sections were incubated with biotinylated lectins at concentrations of 10-20 μg/ml in 0.05 M phosphate-buffered saline (PBS) (pH 7.2) for 60 min at room temperature, following treatment with 0.3% hydrogen peroxide to suppress endogenous peroxidase (PO) activity and then preincubation with 1% bovine serum albumin (BSA) (Sigma, MO, USA) in PBS for 30 min at room temperature. The lectins used were *Sambucus sieboldiana* agglutinin (SSA) and *Maackia amurensis* agglutinin (MAM) (Seikagaku Kogyo Co., Tokyo, Japan). Their specificities and inhibitory sugars are listed in Table 7 (for lectin specificities see Danguy, 1995). After rinsing with PBS, the sections were soaked in PO-labeled streptavidin (Nichirei Co., Tokyo, Japan) for 30 min at room temperature. The activity of the PO employed for labeling was evaluated with a solution containing 0.05 M Tris-HCl buffer (pH 7.6), diaminobenzidine (DAB) and hydrogen peroxide. Lectin controls were prepared by addition of inhibitory sugars at a final concentration of 0.01 M to the respective lectin solutions, by substitution of unconjugated lectins for biotinylated lectin conjugates or by exposure of sections to the PO-DAB system without lectin.

For the immunohistochemical methods, the sections were pretreated with 0.3% hydrogen peroxide for 30 min for the blocking

of endogenous PO activities. Then, they were incubated with primary antibodies diluted in 0.01 M PBS (pH 7.3) against lysozyme (polyclonal, from rabbit) (Dako, Glostrup, Denmark), IgA (polyclonal, from rabbit) (Dako), lactoferrin (polyclonal, from rabbit) (MP Biomedicals, Ohio, USA), human β-defensin 2 (polyclonal, from rabbit) (Biolog, Kronshagen, Germany) and Rab3D (polyclonal, from rabbit) (Proteintech, Illinois, USA) for 24 h at 4°C, following preincubation with 1% BSA in PBS for 30 min at room temperature. The primary antibodies were diluted as follows: lysozyme 1:100, IgA 1:100, lactoferrin 1:100, human β-defensin 2 1:400, Rab3D 1:50. For antigen retrieval, some of the sections were incubated with Proteinase K solution (Dako) for 3 min at room temperature before incubation with the primary antibodies to lysozyme, IgA, lactoferrin and β-defensin 2. With regard to anti-Rab3D, the sections were treated with microwave irradiation (500 W, for 6 min) in 0.01 M citrate buffer at pH 6.0. After rinsing with PBS, these sections were incubated with prediluted dextran-polymer-conjugated secondary antibodies (EnVision+ Dual Link System-HRP, raised goat against mouse and rabbit immunoglobulins, Dako) for 30 min at room temperature. The immunoreaction employed for labeling was revealed by a PO-DAB system, as described above. Controls for immunohistochemical methods were performed by incubation with PBS without primary antibodies or exposure of sections to the PO-DAB system without primary or secondary antibodies.

The labeling reactions of sialoglycoconjugate histochemical

and immunohistochemical methods were assessed using an AxioVision light microscope (Carl Zeiss, Jena, Germary). The labeling intensity was determined by the observation of two slides from each animal, and based on subjective evaluation by three authors.

For electron microscopy, tissue pieces were fixed in 2.5% glutaraldehyde (GA) solution in 0.1 M phosphate-buffered solution (PB) (pH 7.4) for 2 h at 4°C, post-fixed in 2% osmium tetroxide solution and embedded in Epon 812 (Luft, 1968). From these tissue blocks, ultrathin sections were cut on an ultramicrotome, mounted on copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). For the cytochemical demonstration, the tissue specimens were fixed in a mixture of 4% paraformaldehyde (PFA) and 0.5% GA in 0.1 M PB (pH 7.4) for 2 h at 4°C, and embedded in LR-White resin (Newman et al., 1983). For LR-White-embedded materials, ultrathin sections were cut as stated above and placed on nylon grids. The sections on nylon grids were subjected to the periodic acid-thiocarbohydrazide-silver proteinate-physical development procedure (PA-TCH-SP-PD) (Yamada, 1993). These sections were then counterstained with uranyl acetate and lead citrate. For the cytochemical identification of glycogen in the cytoplasm, enzyme digestion with α-amylase (from *Bacillus subtilis*, Seikagaku Kogyo Co., 1 mg/ml in 0.1 M PBS pH 6.0, at 37°C for 4 h) (Casselman, 1959) was carried out on some sections prior to the PA-TCH-SP-PD procedure.

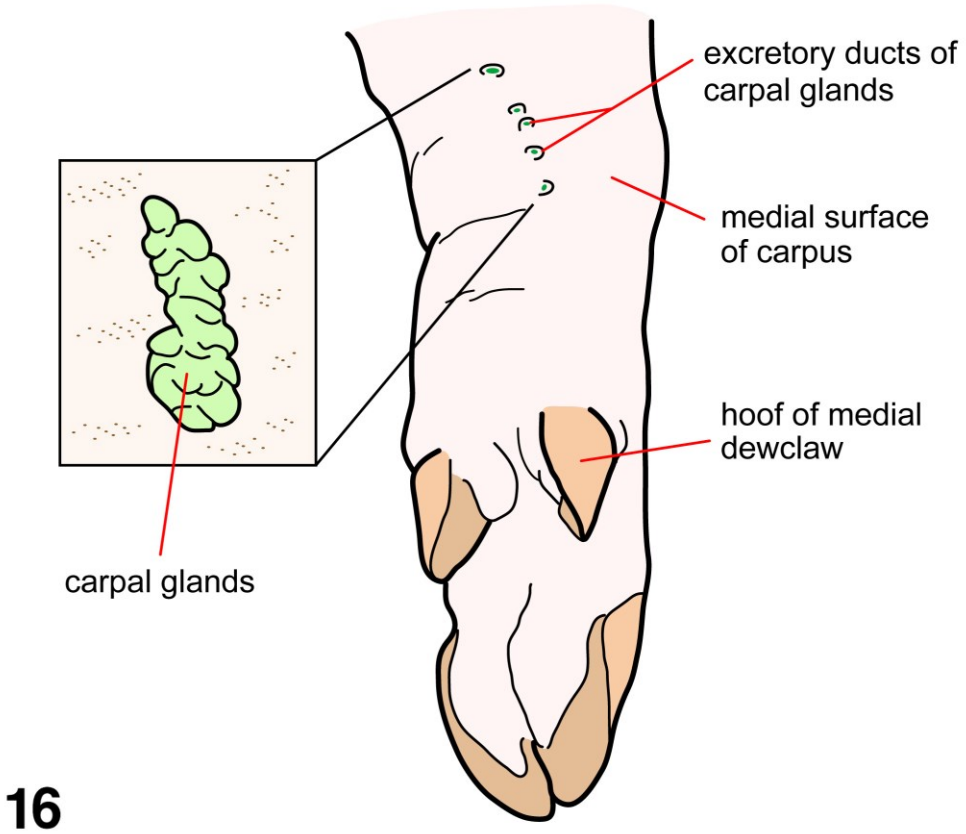


Fig. 16 Schematic illustrations of porcine carpal region (left forefoot, caudomedial view). The inset shows carpal glands under skin surface. Their excretory ducts directly open onto skin surface (Green dots).

Table 6. Physical development procedures for detection of sialic acids and their moieties

Staining method	Moiety detected by the reaction
PA-P-TCH-SP-PD	Sialic acids without side-chain substituent and with O-acetyl substitutions at C-7
KOH-PA-P-TCH-SP-PD	Sialic acids without side-chain substituent and with O-acetyl substitutions at C-7, C-8 and/or C-9
KOH-Sial-PA-P-TCH-SP-PD	Not detected

Table 7. The lectins used and their sugar-binding specificities and inhibitory sugars

	Lectins	Sugar-binding specificity	Inhibitory sugar
SSA	*Sambucus sieboldiana* agglutinin	Siaα2-6Gal/GalNAc	α2-6sialyllactose
MAM	*Maackia amurensis* agglutinin	Siaα2-3Galβ1-4GlcNAc	α2-3sialyllactose

4. 3. Results

In the pig carpal region, the secretory epithelium of the carpal glands, which were observed in the connective tissue of the hypodermis, consisted of dark cells and clear cells. However, a distinct difference in stainability could not be recognized in these secretory cells after H-E staining (Fig. 17). In the sections stained with AB pH 2.5-PAS, the dark cells showed a strong positive reaction, whereas the clear cells exhibited a very weak positive reaction (Fig. 18). The excretory duct penetrated toward the epidermis and directly opened onto the skin surface.

The PA-P-TCH-SP-PD procedure, which demonstrates the localization of sialic acids, produced a moderate positive reaction in the dark cells and a very weak positive reaction in the clear cells (Fig. 19a). Saponification clearly increased the intensity of the PA-P-TCH-SP-PD reaction, in that the reaction intensity of the dark cells was markedly altered (Fig. 19b, c). The PA-P-TCH-SP-PD staining intensity was nearly negative after sialidase digestion following saponification (Fig. 19d). In the sections stained for SSA, the dark cells reacted strongly, while the clear cells were found to exhibit a very weak positive reaction (Fig. 20a). A moderate positive reaction was detectable in the luminal secretions after the application of SSA. The MAM technique resulted in a distinct positive reaction of the luminal surface and apical cytoplasm of some of the dark cells (Fig. 20b). On the other hand, the intensities of the other dark cells, clear cells and luminal secretions were very

weak. The results obtained for the sialoglycoconjugate histochemical procedures are summarized in Table 8.

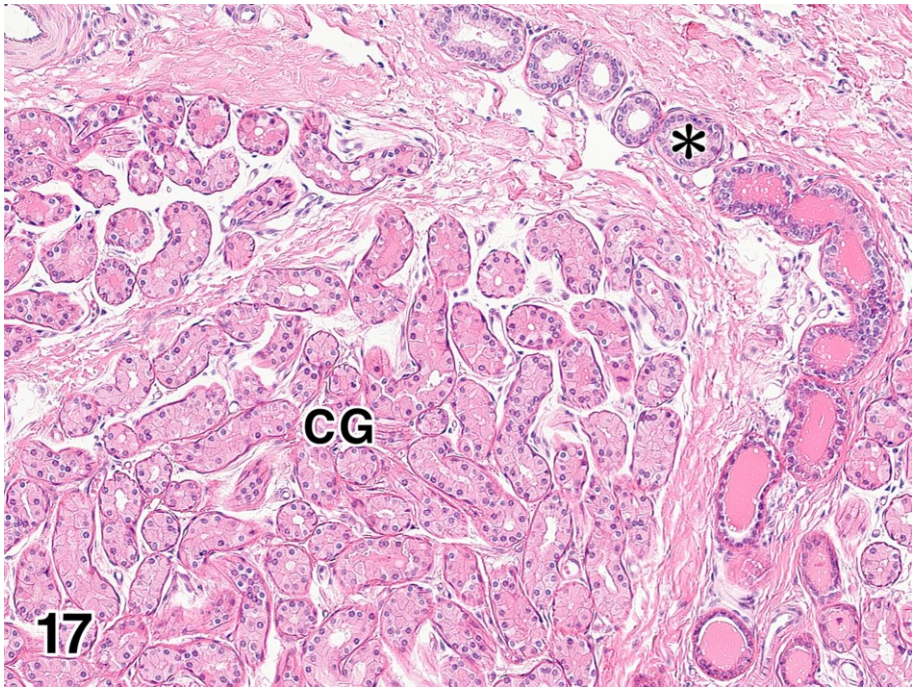
In the carpal glands, a clear positive reaction for lysozyme was detected in some of the dark cells, which showed a strong positive reaction (Fig. 21a). Antibodies to IgA exhibited a weak to moderate positive reaction in some of the dark cells (Fig. 21b, c). Additionally, a positive reaction for IgA was distinctly observed in the lumina of the blood vessels surrounding the secretory portion of the carpal glands. As for these antibodies, the other dark cells and clear cells were seen to exhibit very weak positive reactions. When the sections were incubated with antibodies to lactoferrin, a distinct positive reaction was also confined to some of the dark cells, whereas the other secretory cells were stained rather weakly (Fig. 21d). Incubation with antibodies to β-defensin 2 led to the same staining images as those obtained with antibodies to lactoferrin (Fig. 21e). With regard to these observations, the luminal secretions were generally found to exhibit weak positive reactions. Antibodies to Rab3D gave rise to a moderate positive reaction in some of the dark cells, while other glandular structures reacted very weakly (Fig. 21f). These results obtained from the immunohistochemical methods for antimicrobial substances are summarized in Table 9.

In all of the lectin histochemical and immunohistochemical control experiments, the positive reactions were greatly diminished or nearly abolished in the glandular structures of the carpal glands (Figs. 20c, 21g).

Ultrastructural observation of the carpal glands revealed that the secretory portion consisted of dark cells and clear cells with associated myoepithelial cells. The dark cells contained a well-developed Golgi apparatus, rough-surfaced endoplasmic reticulum and a large number of secretory granules possessing different electron densities. In some dark cells, the secretory granules were relatively large. However, other dark cells were equipped with small secretory granules and their cytoplasm exhibited a high electron density. Therefore, we classified the former dark cells as type I cells and the latter as type II cells (Fig. 22). On the other hand, the clear cells, the cytoplasm of which exhibited a lower electron density, were equipped with a Golgi apparatus and abundant smooth-surfaced endoplasmic reticulum. In addition, distinct numbers of glycogen particles were distributed throughout the cytoplasm. The plasma membrane of the clear cells facing the glandular lumen projected into well-developed microvilli. The cytoplasmic matrix of these cells, especially of the clear cells, was studded with many mitochondria of varying morphology.

In the secretory cells stained with the PA-TCH-SP-PD procedure, the surface coat of the plasma membrane and the basal lamina showed positive reactions (Fig. 23a). The prominent PA-TCH-SP-PD-reactive structures in the dark cells were the secretory granules and cisternae of the Golgi apparatus. In the dark cells, a weak to moderate reaction was observed in the secretory granules of the type I cells, while those of the type II cells exhibited a

distinct positive reaction (Fig. 23a, b). The PA-TCH-SP-PD-reactive ultrastructures in the clear cells were glycogen particles and also cisternae of the Golgi apparatus (Fig. 23a). Digestion with α-amylase abolished the PA-TCH-SP-PD-reactive glycogen particles, found particularly in the cytoplasm of the clear cells.



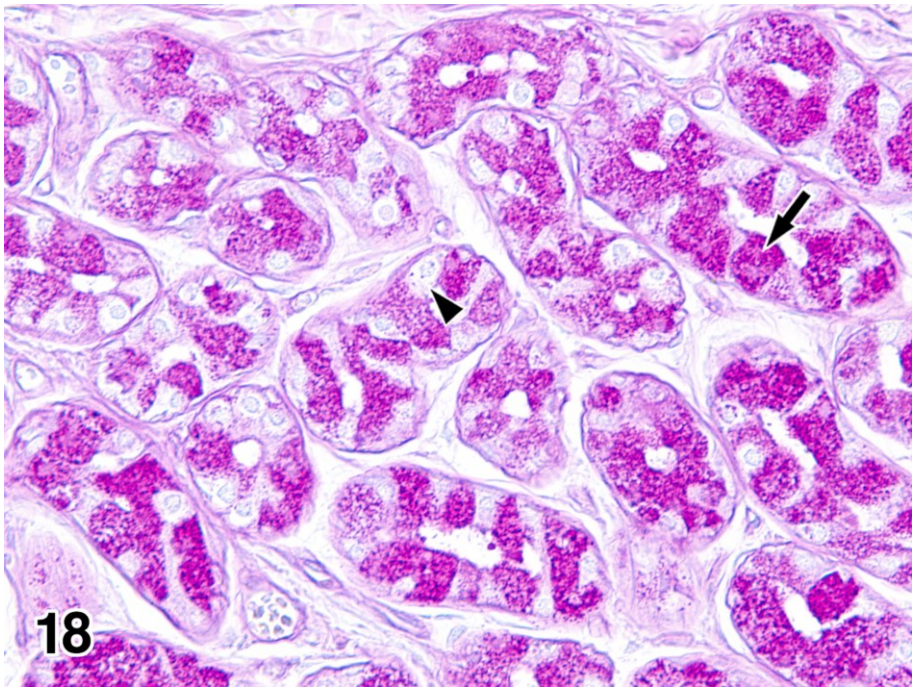


Fig. 17 General structure of the carpal glands in pig. H-E, ×100, CG: carpal gland, asterisk: excretory duct.

Fig. 18 General carbohydrate histochemical appearance of the carpal glands. AB pH 2.5-PAS, ×320, arrow: dark cell, arrowhead: clear cell.

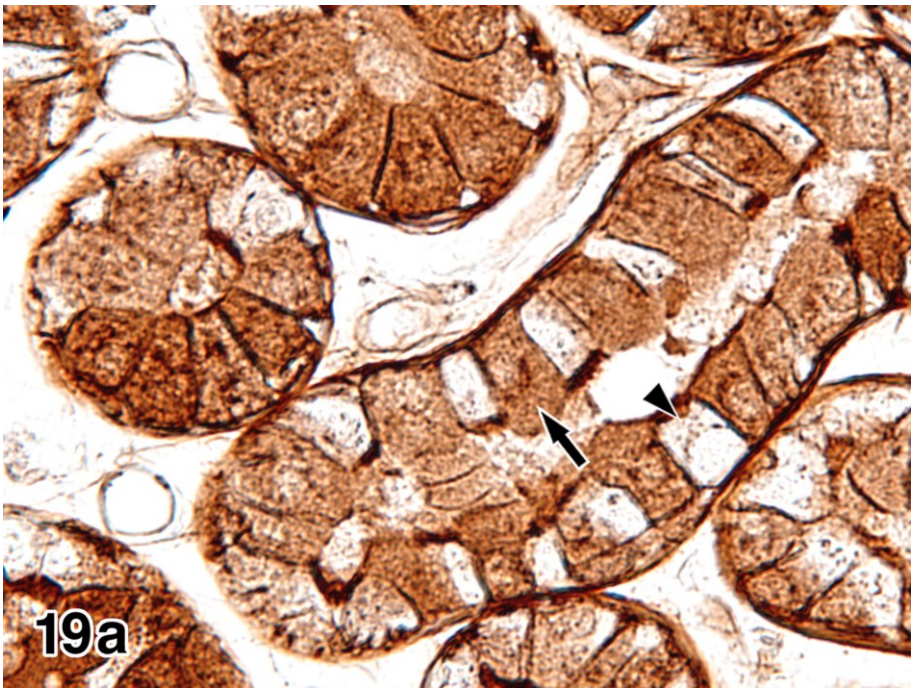
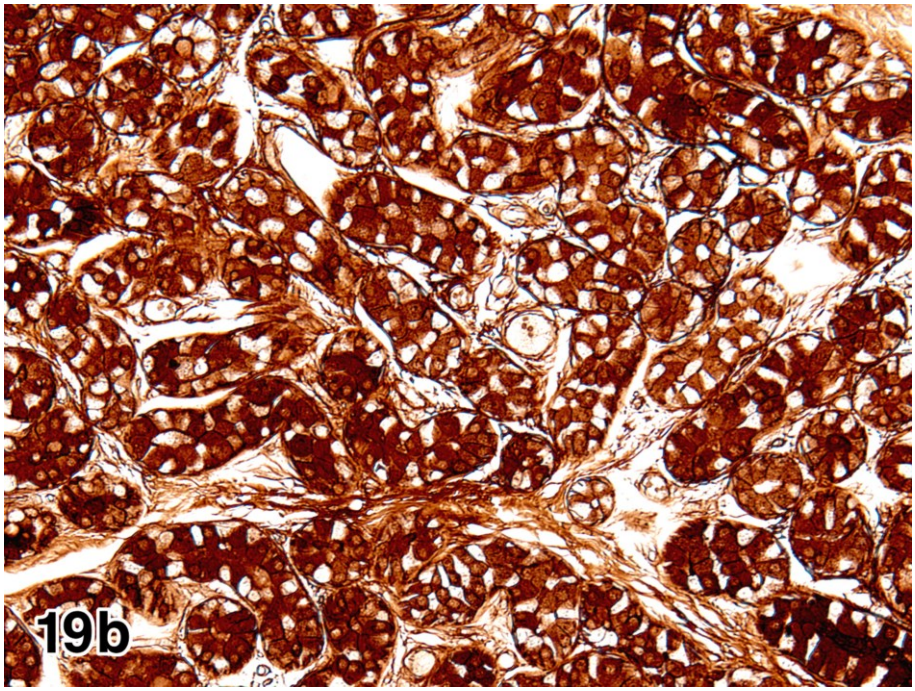


Fig. 19 Histochemical visualization of sialic acids in the carpal glands. a) PA-P-TCH-SP-PD, ×640, arrow: dark cell, arrowhead: clear cell.



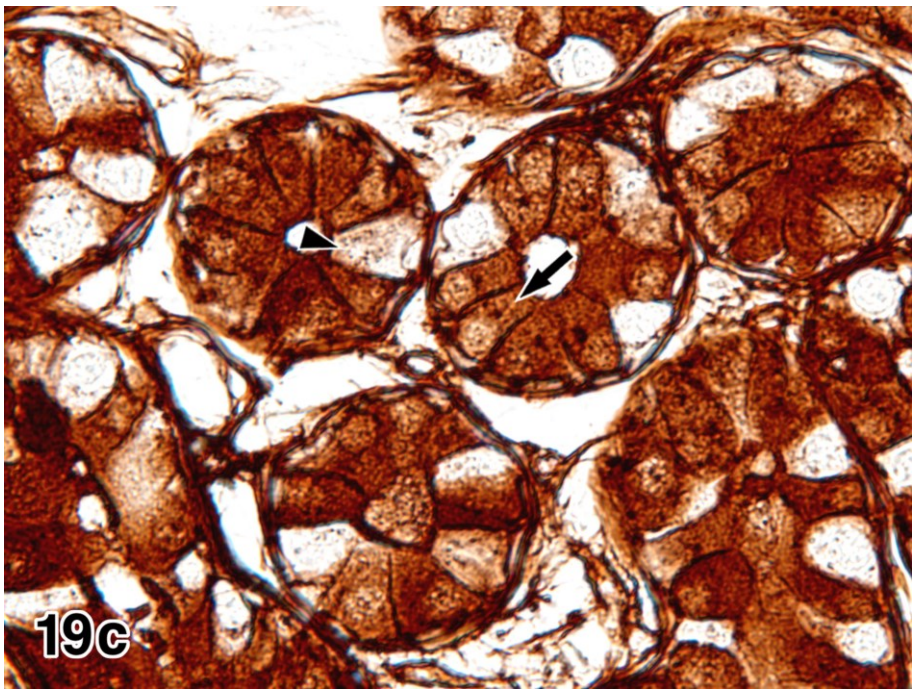


Fig. 19 Histochemical visualization of sialic acids in the carpal glands. b) KOH-PA-P-TCH-SP-PD, ×160; c) KOH-PA-P-TCH-SP-PD, ×640, arrow: dark cell, arrowhead: clear cell.

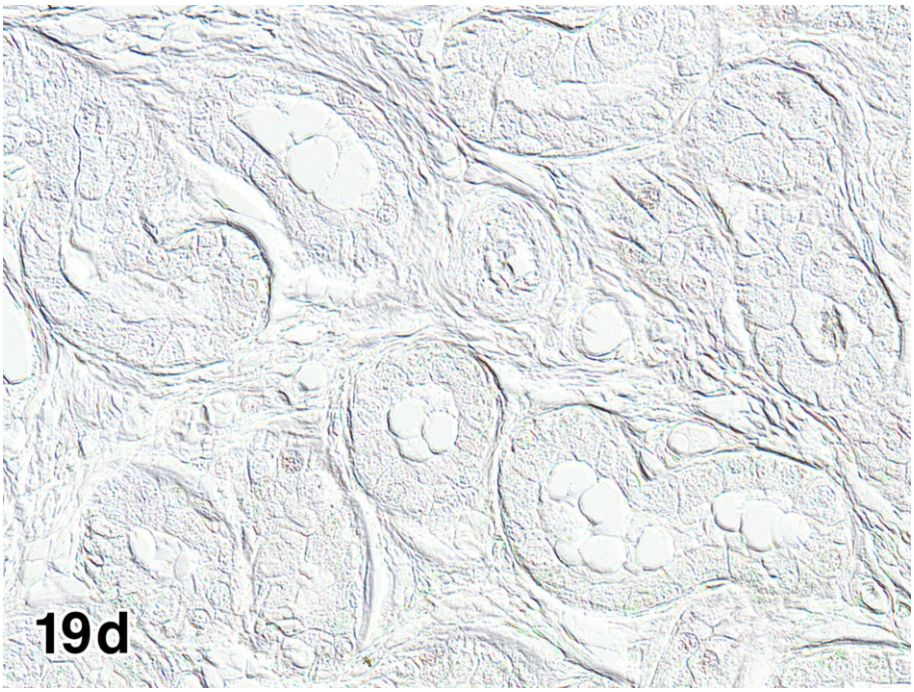


Fig. 19 Histochemical visualization of sialic acids in the carpal glands. d) KOH-Sial-PA-P-TCH-SP- PD, ×320.

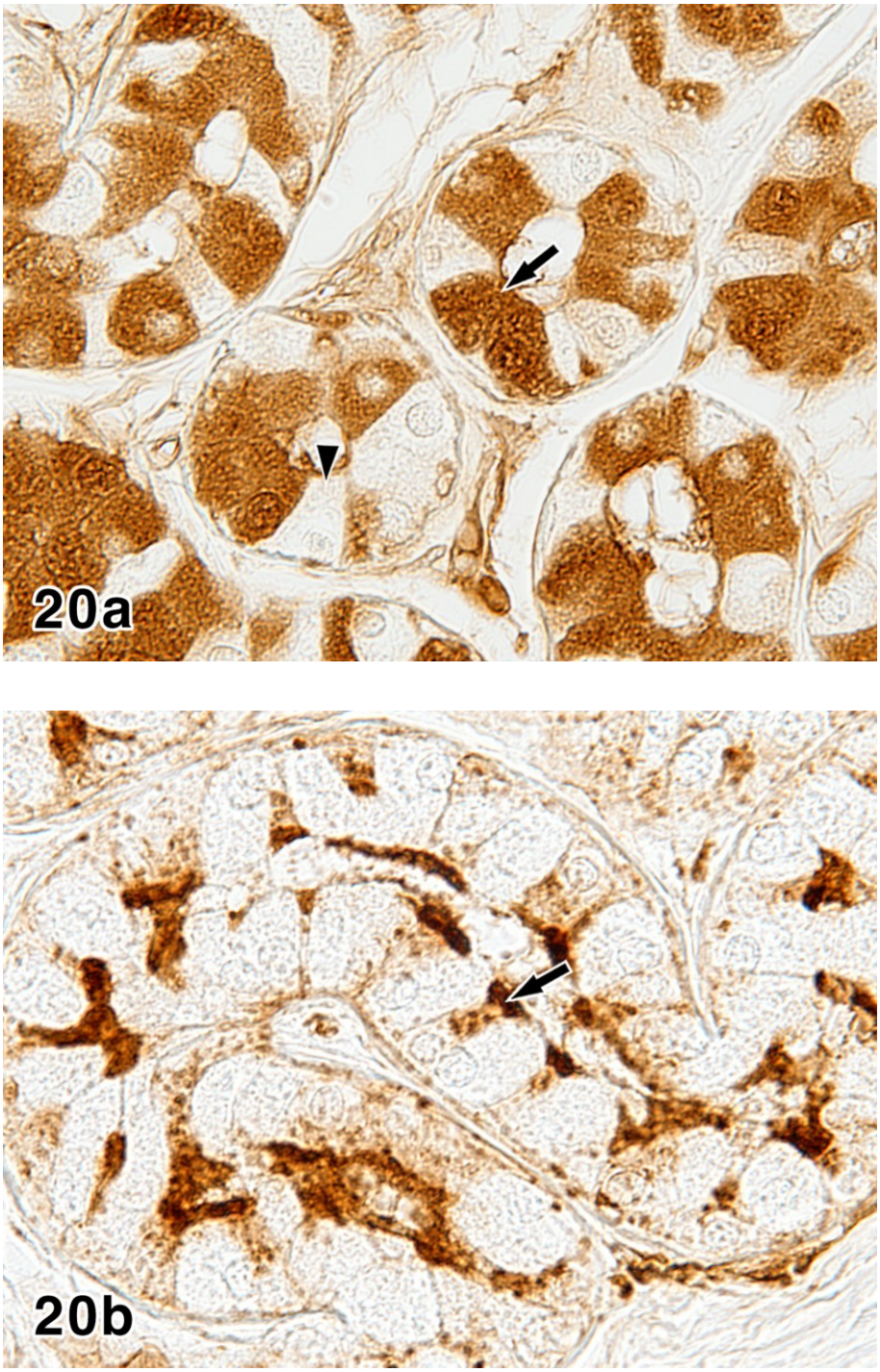


Fig. 20 Lectin histochemical staining in the carpal glands. a) SSA, ×640, arrow: dark cell, arrowhead: clear cell; b) MAM, ×640, arrow: dark cell.

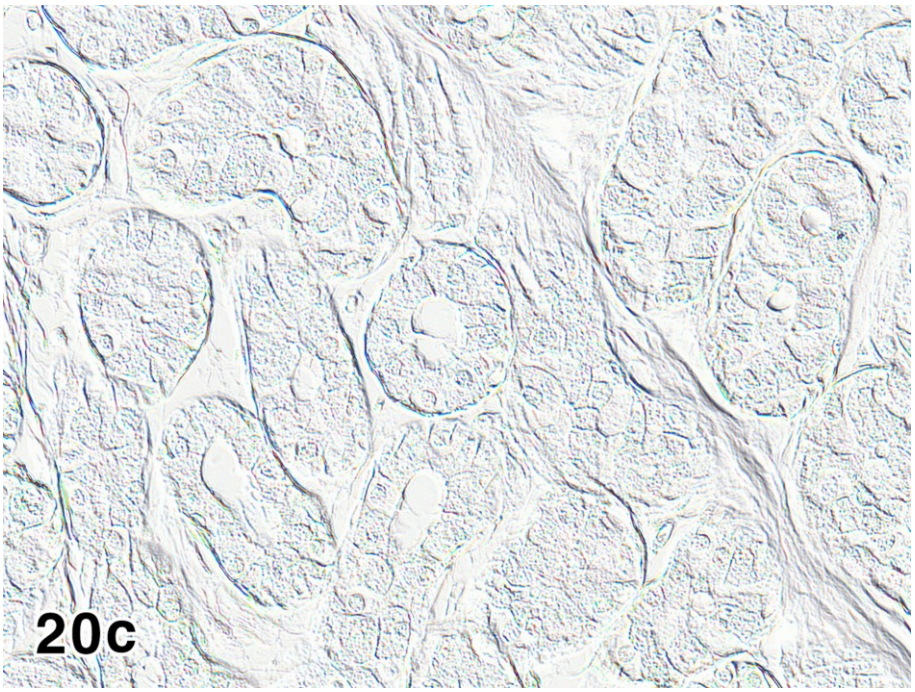


Fig. 20　Lectin histochemical staining in the carpal glands. c) control, ×320.

Table 8. Histochemical reactions of sialoglycoconjugates in porcine carpal glands

Stainings	Secretory cells		Luminal secretions
	Dark cells	Clear cells	
PA-P-TCH-SP-PD	3	0-1	3
KOH-PA-P-TCH-SP-PD	4	0-1	4
KOH-Sial-PA-P-TCH-SP-PD	0	0	0
SSA	4	0-1	3
MAM	1-4*	0-1	1

Reaction intensities: 0: negative, 1: very weak, 2: weak, 3: moderate, 4: strong

* Only a subpopulation of dark cells were stained

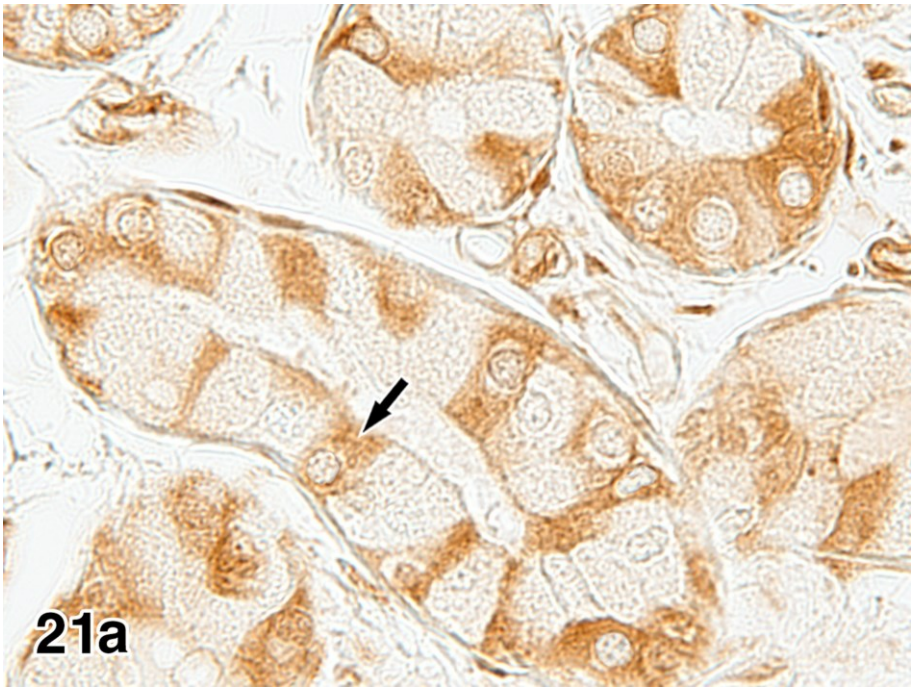


Fig. 21 Immunohistochemical staining in the carpal glands. a) Lysozyme, ×640, arrow: dark cell.

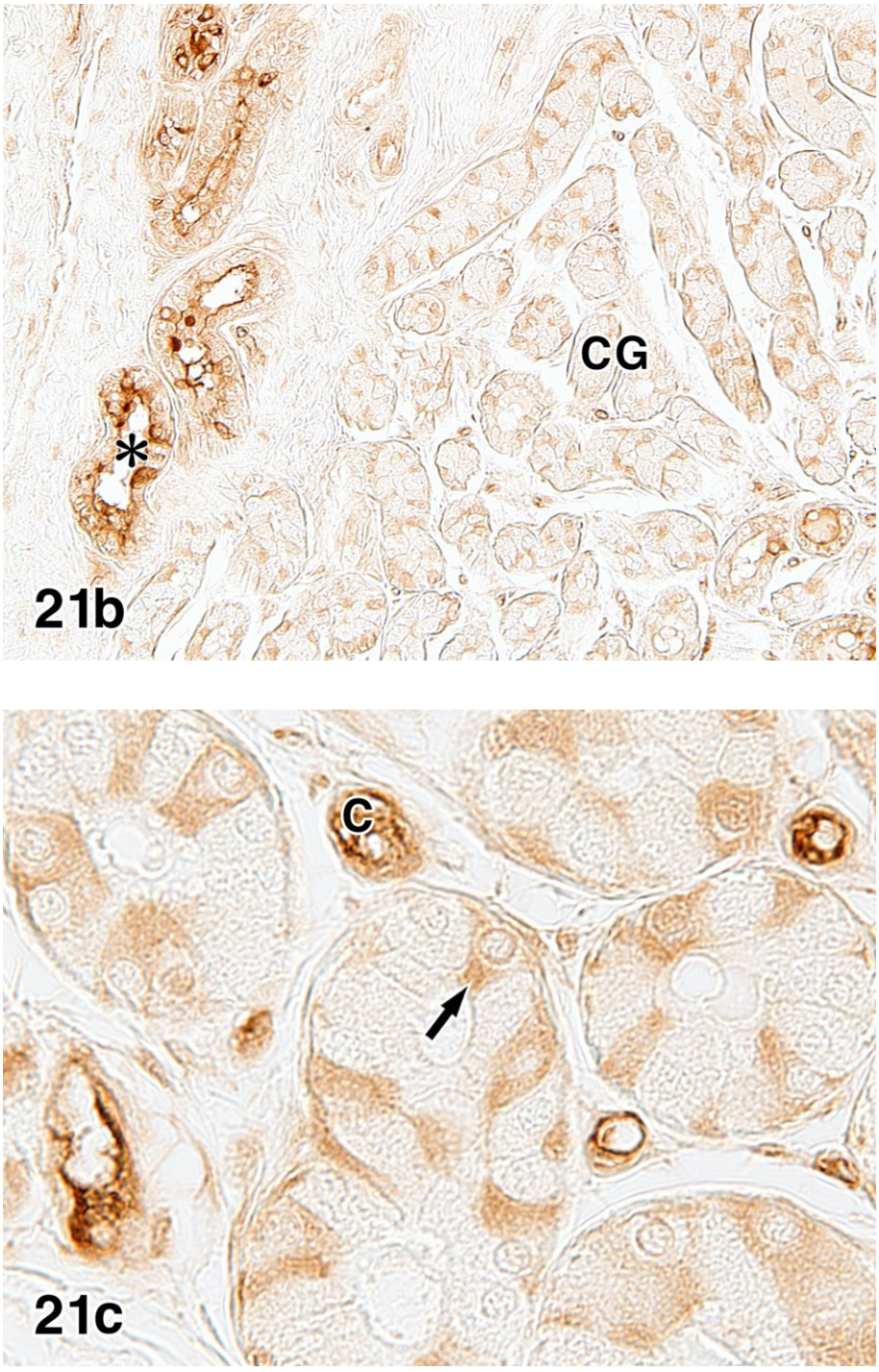


Fig. 21 Immunohistochemical staining in the carpal glands. b) IgA, ×160, CG: carpal gland, asterisk: excretory duct; c) IgA, ×640, C: capillary, arrow: dark cell.

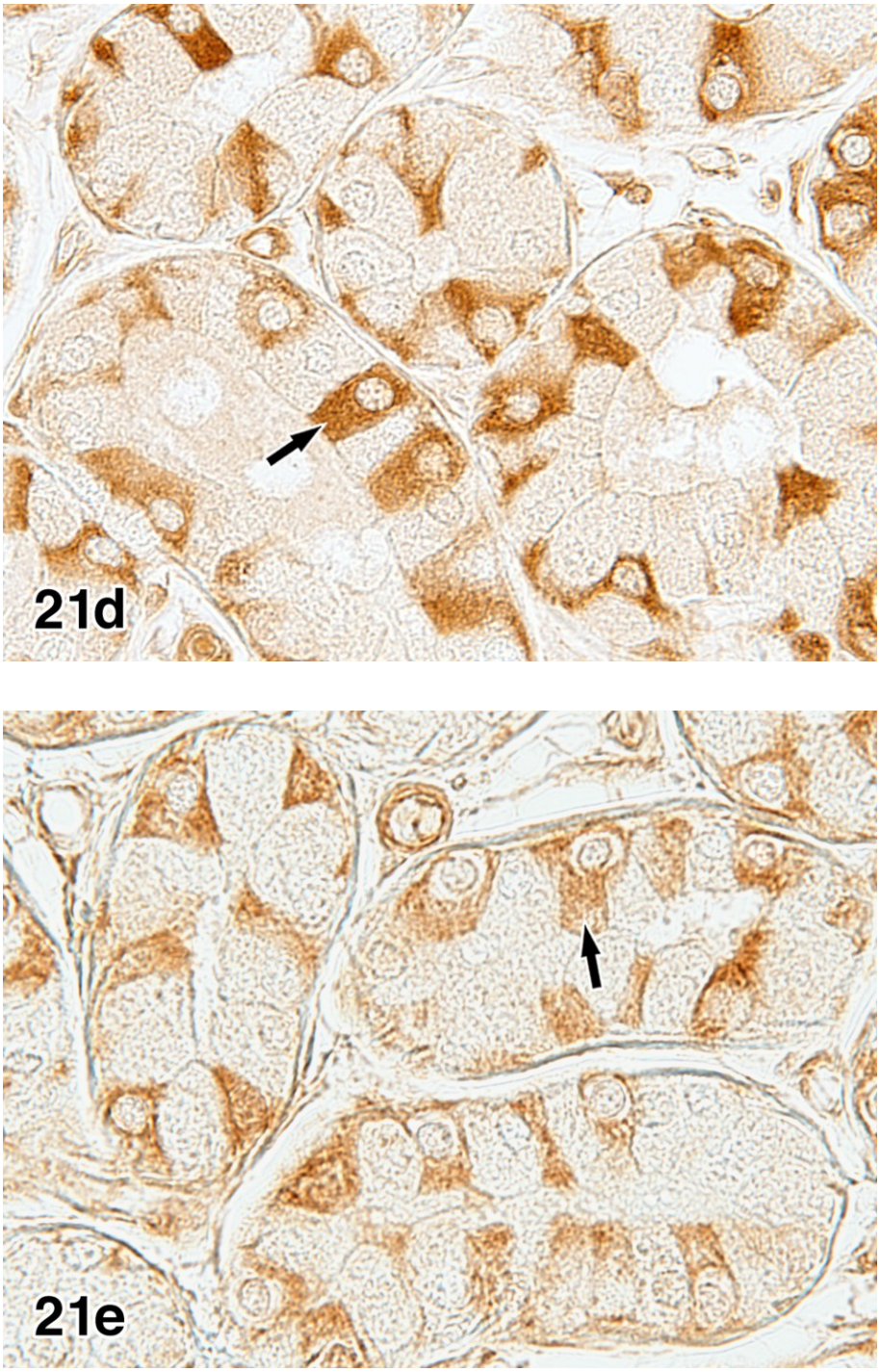
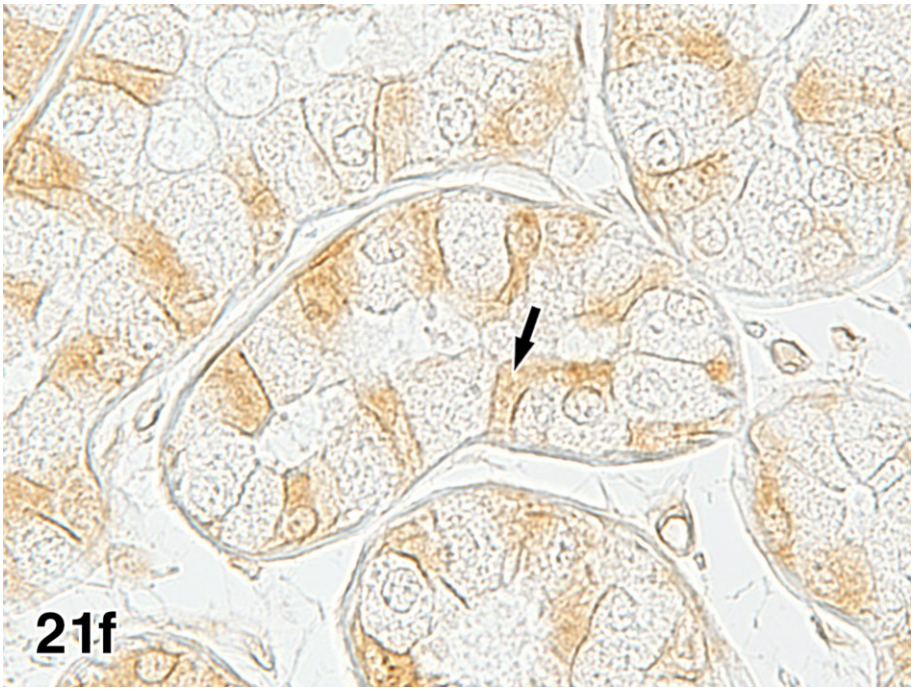


Fig. 21 Immunohistochemical staining in the carpal glands. d) lactoferrin, ×640, arrow: dark cell; e) β-defensin 2, ×640, arrow: dark cell.



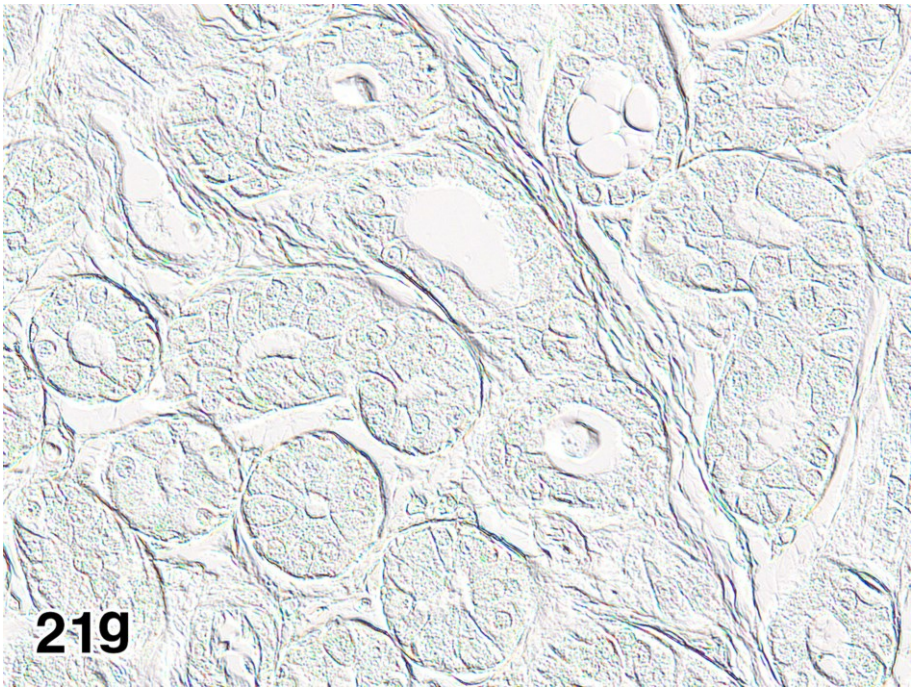


Fig. 21 Immunohistochemical staining in the carpal glands. f) Rab3D ×640, arrow: dark cell; g) control, ×320.

Table 9. Immunohistochemical reactions of porcine carpal glands

Substances	Secretory cells		Luminal secretions
	Dark cells	Clear cells	
Lysozyme	1-4*	0-1	2
IgA	1-3*	0-1	1
Lactoferrin	1-4*	0-1	2
β-defensin 2	1-4*	0-1	2
Rab3D	1-3*	0-1	0-1

Reaction intensities: 0: negative, 1: very weak, 2: weak, 3: moderate, 4: strong

* Only a subpopulation of dark cells were stained

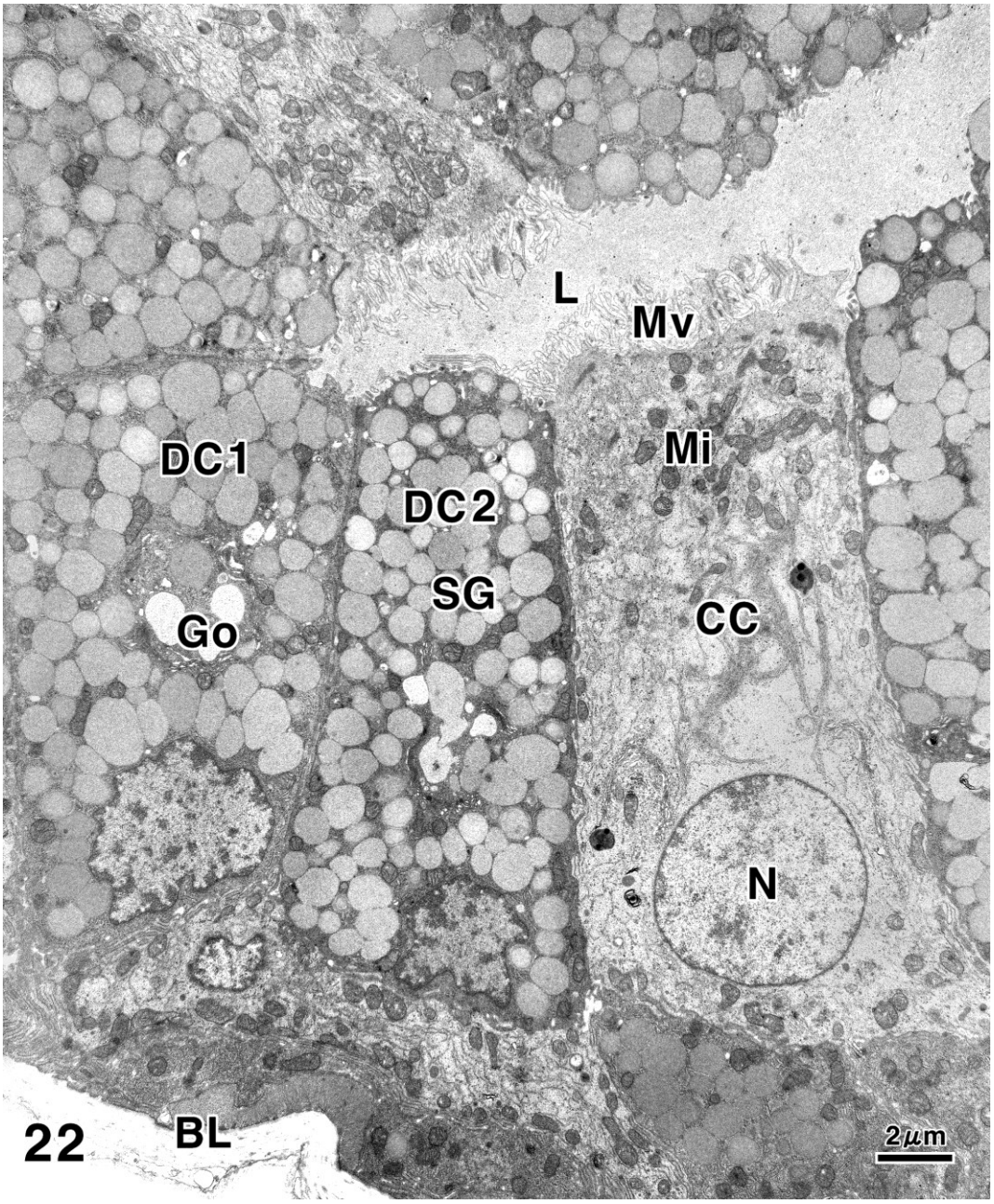


Fig. 22 Ultrastructure of the carpal glands stained with uranyl acetate and lead citrate. The secretory portion consists of type I dark cells, type II dark cells and clear cells. ×5,500, BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, L: lumen, Mi: mitochondrion, Mv: microvilli, N: nucleus, SG: secretory granule.

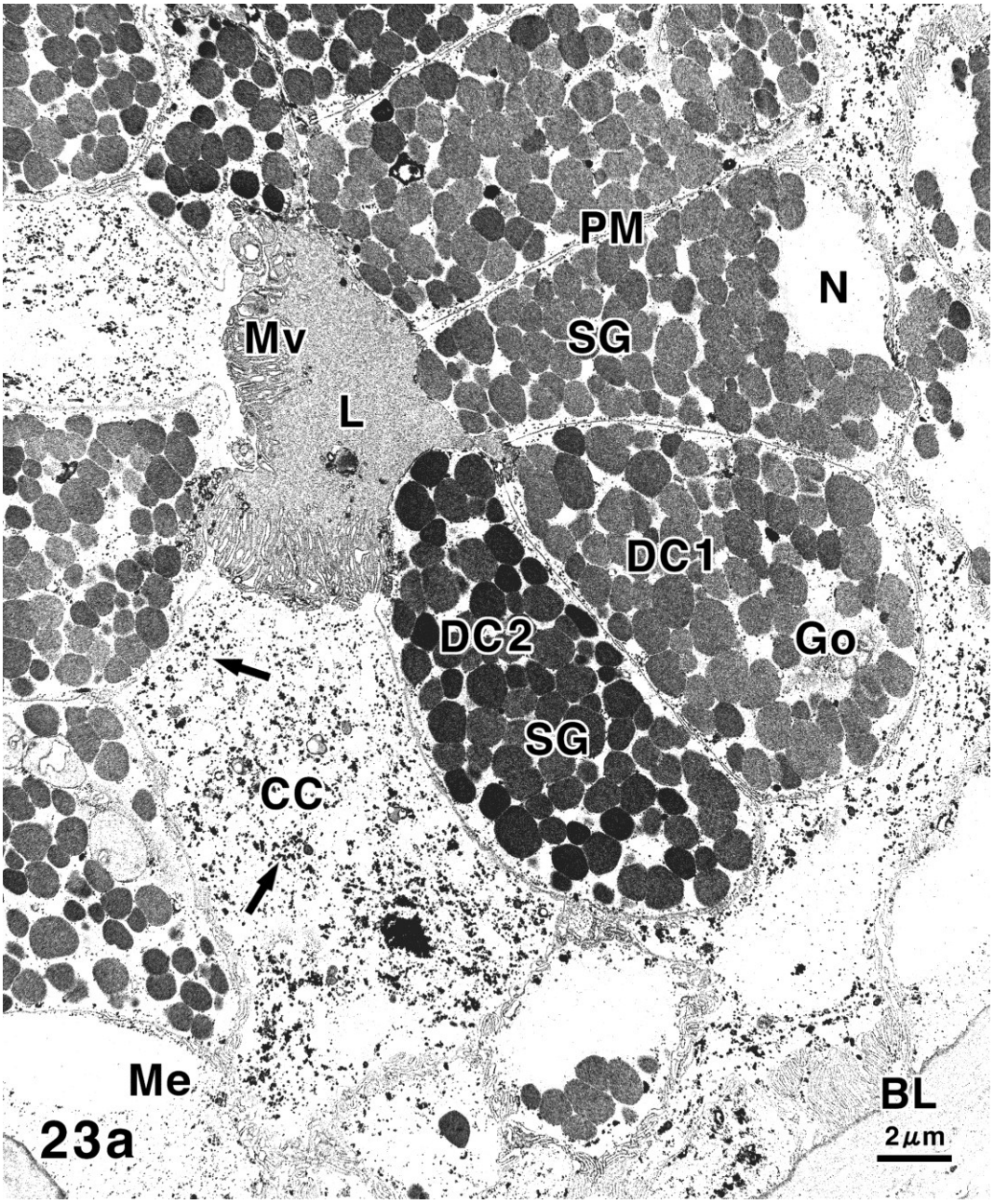


Fig. 23 Cytochemical PA-TCH-SP-PD staining of the carpal glands. a) Ultrastructures exhibit positive reactions. ×5,500, BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, L: lumen, Me: myoepithelial cell, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule, arrows: glycogen particles.

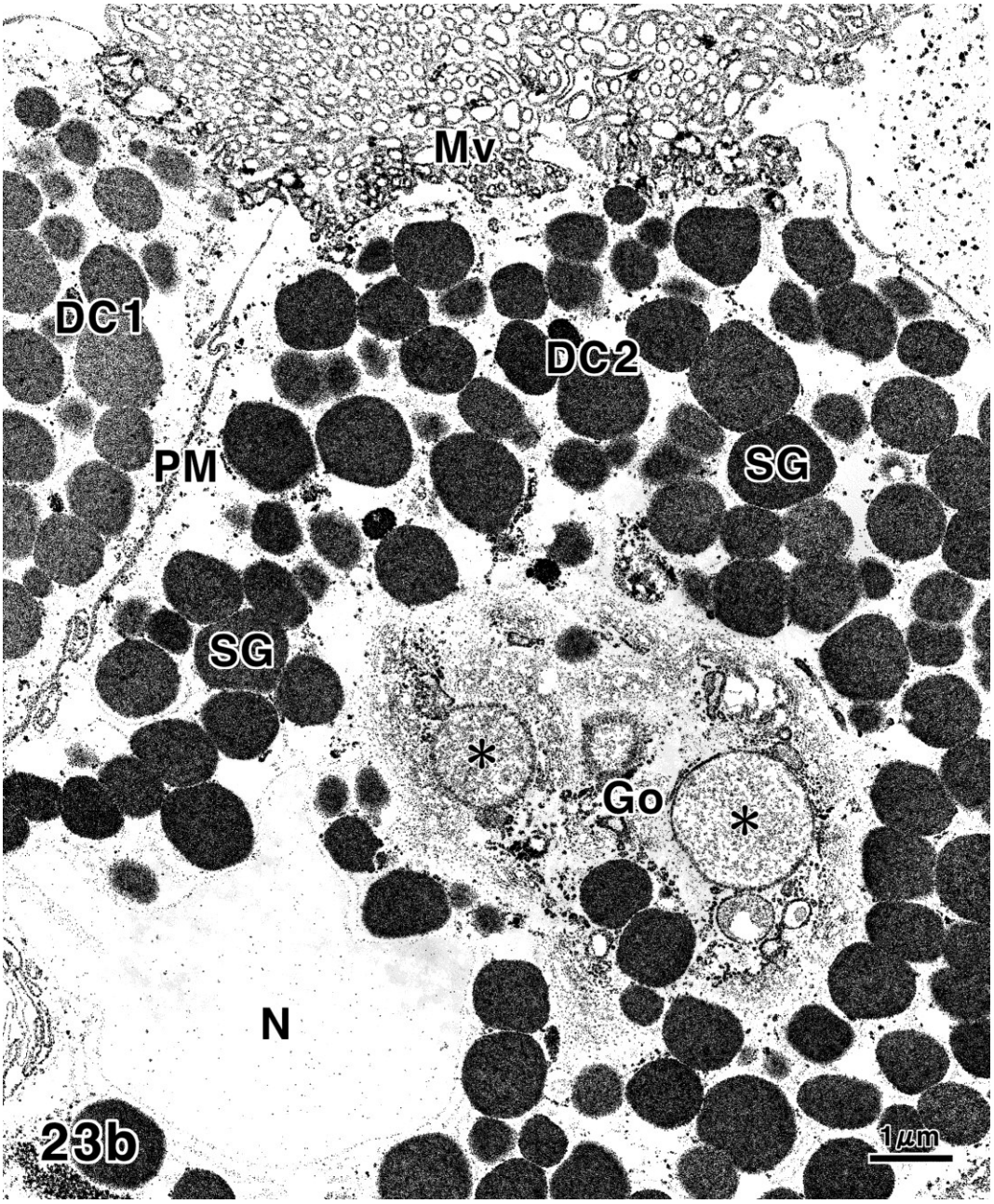


Fig. 23 Cytochemical PA-TCH-SP-PD staining of the carpal glands. b) The type II dark cell. ×12,000, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule, asterisks: immature secretory granules.

4. 4. Discussion

It is widely known that the secretory portion of eccrine glands is generally composed of two cell types, dark cells and clear cells. Our ultrastructural observations are mostly similar to those of this gland type in foot pads of other mammals (Meyer and Bartels, 1989; Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2004) and in common integument of humans (Kurosumi et al., 1984) as well as those of porcine carpal glands (Gargiulo et al., 1989). However, in the present study, the different features were histochemically and cytochemically observed among the dark cells of the porcine carpal glands.

Our investigation revealed the distribution of sialoglycoconjugates and antimicrobial substances in these glands as supported by the PA-P-TCH-SP-PD, the lectin histochemical and immunohistochemical procedures. The presence of sialic acids with O-acetyl substitutions at C-8 and/or C-9 was disclosed by increases in the intensity of the PA-P-TCH-SP-PD reactions following saponification (Ueda et al., 1998). In addition, lectin histochemical results of SSA and MAM indicate the localization of sialic acid residues, linked to α2-6Gal/GalNAc and α2-3Galβ1-4GlcNAc, respectively. Sialic acids are involved in binding and transport of ions, stabilizing the conformation of proteins and enhancing the viscosity of mucins, owing to their negative charge. Moreover, they participate in defending cells against enzymatic and immunological attacks, and can also act as recognition sites for various molecules

and microorganisms (Schauer, 2004, 2009; Varki and Schauer, 2009). Sialoderivatives may play an important role in defense against pathogenic agents because of the fact that the diversity of sialic acid residues arises from variation in their degree of acetylation and the types of linkage and acceptor sugars (Suzuki, 2005; Parillo et al., 2009; Schauer, 2009).

The skin is the first barrier against pathogens. Previous studies revealed, in several mammalian species, that different antimicrobial substances, which are part of the innate immunity system, are distributed in eccrine glands (Papini et al., 1982; Metze et al., 1989; Ali et al., 2001; Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2010). Lysozyme is the first antimicrobial peptide found in human skin (Klenha and Krs, 1967; Ogawa et al., 1971). This enzyme is capable of direct bacteriolytic action because it hydrolyzes β-(1,4)-glycosidic bonds between N-acetylmuramic acid and N-acetyl-β-D-glucosamine, which are commonly present in the peptidoglycan of bacterial cell walls, and has a functional relationship to immunoglobulins (Jollès and Jollès, 1984). The secretory form of IgA is composed of polymeric IgA and a secretory component, which is the extracellular domain of polymeric immunoglobulin receptor (pIgR). Secretory IgA is the predominant immunoglobulin in external secretions of humans and various other mammals and contributes to the prevention of pathogen adhesion to host cells (Tang et al., 2005; Corthésy, 2010). The prevalence of IgA is a result of cooperation between plasma cells producing polymeric

IgA and epithelial cells expressing pIgR (Snoeck et al., 2006). Lactoferrin is widely represented in various secretory fluids. It can be regarded as a key component of the first line of mammalian host defense because of its bacteriostatic and bacteriocidal functions as well as its activities against fungi and viruses (Levay and Viljoen, 1995; Valenti et al., 1998; Ward et al., 2002). Furthermore, lactoferrin inhibits allergen-induced skin inflammation, which is secondary to its role in regulating the production of cytokines (Kimber et al., 2002). Defensins have a role in the innate immune response of the skin against microbial invaders (Bos et al., 2001). They are able to insert themselves into cell membranes. Consequently, most defensins can cause the disruption and subsequent death of microorganisms (Ganz, 2003, 2004). The present study demonstrated that the expression of antimicrobial substances, which are released as secretory products, was mainly confined to a subpopulation of the dark cells in the carpal glands.

In view of the staining selectivity of the PA-TCH-SP-PD procedure (Yamada, 1993), the majority of ultrastructures of the carpal glands clearly contained carbohydrates with vicinal diol groupings. The secretory granules of the type II dark cells had a higher concentration of glycoproteins than those of the type I dark cells. These findings lead to the view that sialic acid residues, linked to α2-3Galβ1-4GlcNAc, and various antimicrobial substances are produced by the type II dark cells. On the other hand, the distributional pattern of Rab3D is consistent with that of these

products. Rab3D is enriched in a number of exocrine cells and is expressed in secretory granules in the cytoplasm of these cells (Valentijin et al., 2007; Williams et al., 2009). Moreover, Rab3D is a key regulator of intracellular vesicle transport during exocytosis because this protein functions in the tethering or docking of vesicles to its target compartment, leading to membrane fusion (Zerial and McBride, 2001; Millar et al., 2002). Thus, this regulatory protein may play an important role in the secretory regulation of sialoglycoproteins with Siaα2-3Gal1-4GlcNAc sequence and antimicrobial substances.

In conclusion, the present study demonstrated the presence of sialic acids, antimicrobial substances and Rab3D in the carpal glands of pig. Sialoglycoconjugates are associated with the viscoelasticity of secretions and adsorption of pathogenic agents. The antimicrobial peptides are important in immune responses. Therefore, in addition to the function as odoriferous glands (Hraste and Stojković, 1995; Pedini et al., 1999), it is suggested from our findings that the glandular secretions elaborated by the porcine carpal glands are closely related to effective defense in order to preserve the skin integrity.

4. 5. Summary

The localization of sialic acids and antimicrobial products (lysozyme, IgA, lactoferrin, β-defensin 2) as well as Rab3D in the

carpal glands of pig was studied by sialoglycoconjugate histochemistry and immunohistochemistry. The secretory epithelium of the carpal glands consisted of dark and clear cells. The dark cells of these glands exhibited high levels of sialoglycoconjugates, including O-acetylated sialic acids, whereas the localization of sialic acids linked to α2-3Galβ1-4GlcNAc was confined to a subpopulation of the dark cells. Furthermore, all antimicrobial substances and Rab3D were mainly detectable in a subpopulation of the dark cells. The results obtained are discussed with regard to the functional significance of these glands. Our findings suggested that Rab3D is involved in the secretory regulation of sialoglycoconjugates and antimicrobial substances. These secretory products may create a defensive barrier against microbial invasion and play an essential role in the preservation of skin integrity.

5 CYTOCHEMISTRY OF SIALOGLYCOCONJUGATES AND B-DEFENSIN IN PORCINE CARPAL GLANDS AS STUDIED BY ELECTRON MICROSCOPY

5. 1. Introduction

In humans, eccrine glands are widely distributed throughout the majority of skin regions of the body. On the other hand, in domestic mammals, this type of gland is found only in a few regions, such as the foot pads of dogs and cats, the frog of ungulates (*Cuneus ungulae*), the carpus of pigs and the nasolabial region of ruminants and pigs (Ellis, 1968; Calhoun and Stinson, 1981; Tsukise et al., 1983, 1988a, b; Meyer and Bartels, 1989; Meyer and Tsukise, 1989, 1995). The glandular secretions of porcine carpal glands contain abundant glycoproteins (e.g. Gargiulo et al., 1989; Pedini et al., 1999). Therefore, it is considered that the main function of these glands is retention of the softness and lubrication of the carpal region. However, previous studies raise the possibility that the carpal glands may represent secondary sexual organs because they elaborate odorous substances (see Simic et al., 1985; Hraste and Stojković, 1995; Pedini et al., 1999).

Some aspects of the histochemisty of sialic acids and various antimicrobial substances have previously been investigated light microscopically in porcine carpal glands (Fukui et al., 2012b). This previous study suggested that their secretory products may create a defensive barrier against microbial invasion and play an essential

role in the preservation of skin integrity. However, precise analyses at the cytological level of sialic acids and antimicrobial substances in the carpal glands have not been available until now.

In the present study, therefore, the glandular acini of the porcine carpal glands were subjected to cytochemical analyses of sialoglycoconjugates and the antimicrobial peptide group of β-defensins. The purpose was to determine which cell compartments are engaged in the secretory pathway of these moieties. The ultrastructural data obtained may be indispensable to understand the general biological function of these glands.

5. 2. Materials and Methods

All experiments were performed in accordance with the guidelines for the care and use of laboratory animals at the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University. Five male miniature pigs (potbelly, 1-2 years, 40-50 kg) were deeply anesthetized and then exsanguinated from the common carotid arteries. After bloodletting, the carpal glands were removed surgically.

For general structural observation by electron microscopy, the specimens were fixed in 2.5% glutaraldehyde (GA) solution in 0.1 M phosphate-buffered solution (PB) (pH 7.4) for 2 h at 4°C. The materials were post-fixed in 2% osmium tetroxide solution for 2 h and embedded in Epon 812 (Luft, 1961). From these tissue blocks,

ultrathin sections were cut using an ultramicrotome, mounted on copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

For glycoconjugate cytochemistry, the tissue specimens were fixed in a mixture of 4% paraformaldehyde (PFA) and 0.5% GA in 0.1 M PB (pH 7.4) for 2 h at 4˚C, and embedded in LR-White resin (Newman et al., 1983). From the LR-White-embedded blocks, ultrathin sections were cut as detailed above and placed on nylon or nickel grids. The sections on nylon grids were reacted for a periodic acid-thiocarbohydrazide-silver proteinate-physical development procedure (PA-TCH-SP-PD) (Yamada, 1993). For cytochemical identification of glycogen in the cytoplasm, enzyme digestion with α-amylase (from *Bacillus subtilis*, Seikagaku Kogyo Co., Tokyo, Japan, 1 mg/ml, at 37°C for 4 h) (Casselman, 1959) was carried out on some sections prior to the PA-TCH-SP-PD procedure. For the lectin cytochemistry, the nickel grid-mounted sections were incubated with biotinylated lectins at concentrations of 10-20 μg/ml in 0.05 M phosphate-buffered saline (PBS) (pH 7.2) for 24 h at 4˚C (Roth, 1983, 1996), following preincubation with 1% bovine serum albumin (BSA) (Sigma, MO, USA) in PBS. The lectins used were *Sambucus sieboldiana* agglutinin (SSA) and *Maackia amurensis* agglutinin (MAM) (Seikagaku Kogyo Co.). Their specific sugar residues and inhibitory sugars are listed in Table 10 (for lectin specificities, see Danguy, 1995). After rinsing with PBS, these sections were incubated with 15 nm colloidal gold-labeled streptavidin (British Biocell

International, Cardiff, UK) at a dilution of 1:20 in PBS for 1 h at room temperature (Roth, 1983). They were then subjected to counterstaining with uranyl acetate and lead citrate. For specificity controls of the cytochemical lectin procedures, ultrathin sections were incubated with the respective lectin solutions to which 0.01 M of an appropriate competing sugar was added or incubated, substituting unconjugated lectins for biotinylated lectins.

For immunocytochemistry, ultrathin sections cut from the LR-White-embedded blocks were also placed on nickel grids. Following pretreatment with a solution containing 5% donkey serum albumin (DSA) (Jackson ImmunoResearch Labs., PA, USA) in 0.01 M PBS (pH 7.3) to prevent non-specific reactions, the sections were incubated with cross-reacting primary antibodies for human β-defensin 2 (dilution 1:600, polyclonal, anti-human, from rabbit) (Biolog, Kronshagen, Germany) for 24 h at 4°C. After rinsing with PBS, these ultrathin sections were incubated with biotinylated secondary antibody (anti-rabbit immunoglobulins, from donkey) (Jackson ImmunoResearch Labs.) at a dilution of 1:500 in PBS containing 5% DSA for 60 min, and then with 15 nm colloidal gold-labeled streptavidin at a dilution of 1:20 in PBS for 60 min. They were counterstained with uranyl acetate and lead citrate. Controls for the immunocytochemical procedures were performed by incubation with PBS without primary antibodies or by replacement of the primary antibodies with normal rabbit immunoglobulin diluted to the same extent as the specific

antibodies.

Table 10. The lectins used and their sugar-binding specificities and inhibitory sugars

	Lectins	Sugar-binding specificity	Inhibitory sugar
SSA	*Sambucus sieboldiana* agglutinin	Siaα2-6Gal/GalNAc	α2-6sialyllactose
MAM	*Maackia amurensis* agglutinin	Siaα2-3Galβ1-4GlcNAc	α2-3sialyllactose

5. 3. Results

Observation of the porcine carpal glands by electron microscopy revealed that the glandular acini consist of dark cells and clear cells with associated myoepithelial cells. Additionally, we classified the dark cells as type I and type II dark cells, depending on their morphological characteristics. The secretory cells and myoepithelial cells rested on a basal lamina (Fig. 24a). Both types of dark cell were equipped with a well-developed Golgi apparatus, rough-surfaced endoplasmic reticulum and a large number of secretory granules. Regarding the morphological difference between the two types of dark cell, the type I dark cells contained relatively large secretory granules. On the other hand, the secretory granules of the type II dark cells were smaller than those of type I dark cells (Fig. 24b). The plasma membrane of the clear cells facing the glandular lumen projected into well-developed microvilli. The cytoplasm of the clear cells showed lower electron density, and secretory granules could not be found. Nevertheless, parts of the Golgi apparatus were frequently observed in the circumnuclear region. Abundant smooth-surfaced endoplasmic reticulum and lysosomes were detectable in this cell type. In addition, large numbers of glycogen particles were distributed in the cytoplasm (Fig. 24c, d). Throughout the cytoplasm of the secretory cells, especially the apical region of the clear cells, many mitochondria of varying morphology were scattered among the ultrastructures mentioned above (Fig. 24c).

In the dark cells, the prominent features exhibiting a positive PA-TCH-SP-PD reaction were the secretory granules and cisternae of the Golgi apparatus (Fig. 25a-c). Although the secretory granules of the type I dark cells showed a weak to moderate positive reaction, a distinct positive reaction was observed in those of the type II dark cells (Fig. 25a, b). On the other hand, in the clear cells, glycogen particles, cisternae of the Golgi apparatus and lysosomes exhibited positive reactions (Fig. 25a, b). Other PA-TCH-SP-PD-reactive structures were the surface coat of the plasma membrane of the dark and clear cells. Digestion with α-amylase abolished the PA-TCH-SP-PD-reactive glycogen particles in the cytoplasm.

The SSA-gold procedure demonstrated reactive particles as concentrated in the secretory granules of the type I and type II dark cells (Fig. 26a). In both types of dark cell, the Golgi apparatus also showed a positive reaction (Fig. 26b). With regard to the MAM-gold technique, in the secretory cells, the free surface coat of the plasma membrane showed a positive reaction. The secretory granules of the type I dark cells were seen to react almost negatively, while distinctly concentrated reactive particles were observed in those of the type II dark cells (Fig. 27a). Furthermore, in the type II dark cells, several gold particles were associated with the Golgi apparatus after incubation with MAM (Fig. 27b). Concerning these lectin cytochemical procedures, the clear cells were almost negative. In control sections reacted for lectin cytochemical staining by addition of the appropriate inhibitory sugars to the respective lectin solutions

and substitution of the unconjugated lectins, positive reaction staining of all the formerly reactive ultrastructures was greatly suppressed or abolished.

As for the immunocytochemical methods using the primary antibody to human β-defensin 2, a few gold particles were detectable in the secretory granules of the type II dark cells. However, those of type I dark cells were very weak or negative, and the clear cells also reacted negatively (Fig. 28a). In addition to these features, reactive particles could be observed in the cisternae of the Golgi apparatus of the type II dark cells (Fig. 28b). With regard to the control ultrathin sections stained by immunocytochemical procedures by incubation with PBS without the primary antibodies or by replacement of the primary antibody with normal rabbit immunoglobulin, no glandular structures exhibited any positive reactions.

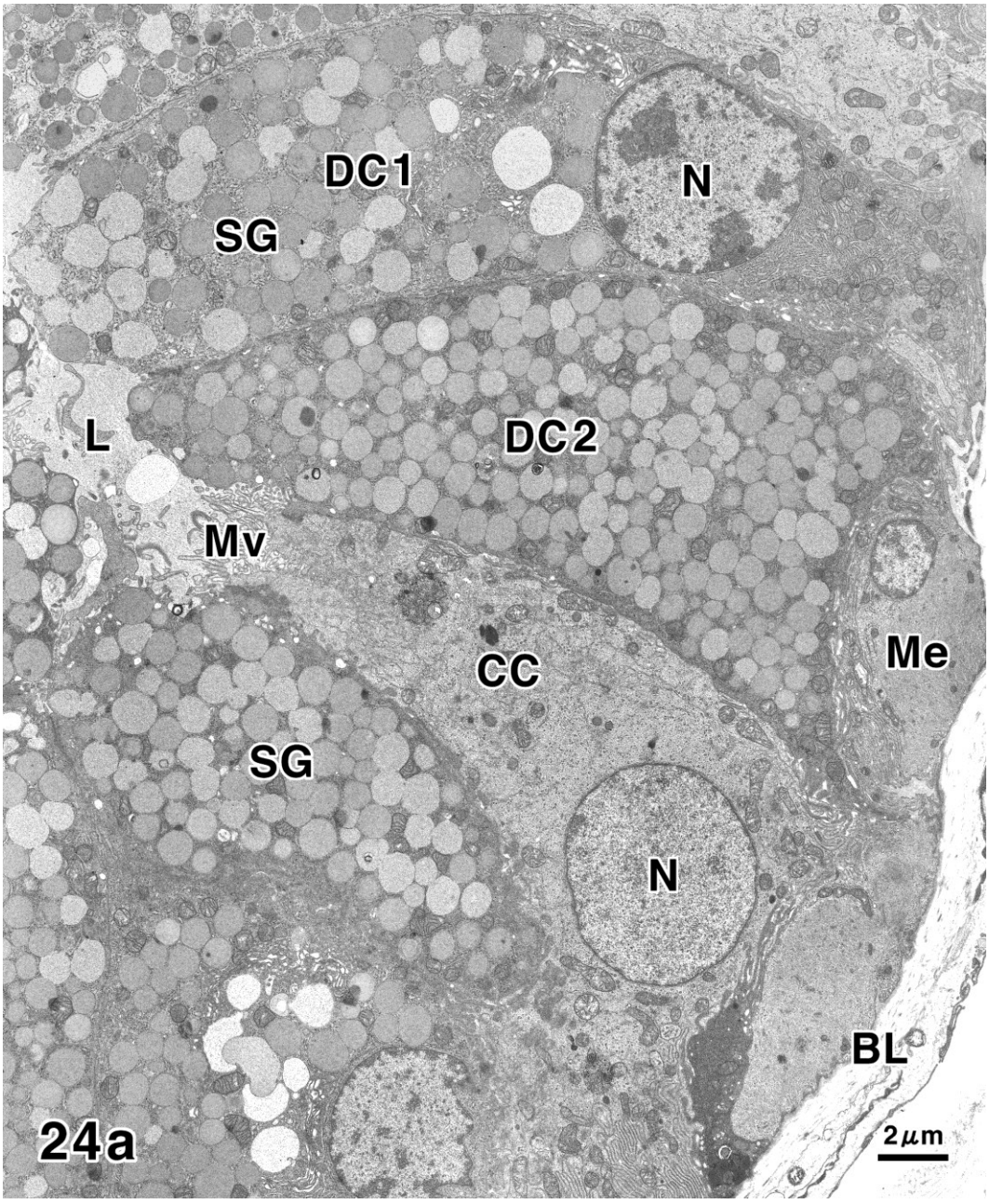


Fig. 24 General ultrastructure of the porcine carpal glands stained with uranyl acetate and lead citrate. a) The secretory portion consists of type I dark cells, type II dark cells and clear cells. ×5,000, BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Me: myoepithelial cell, Mi: mitochondrion, Mv: microvilli, N: nucleus, SG: secretory granule.

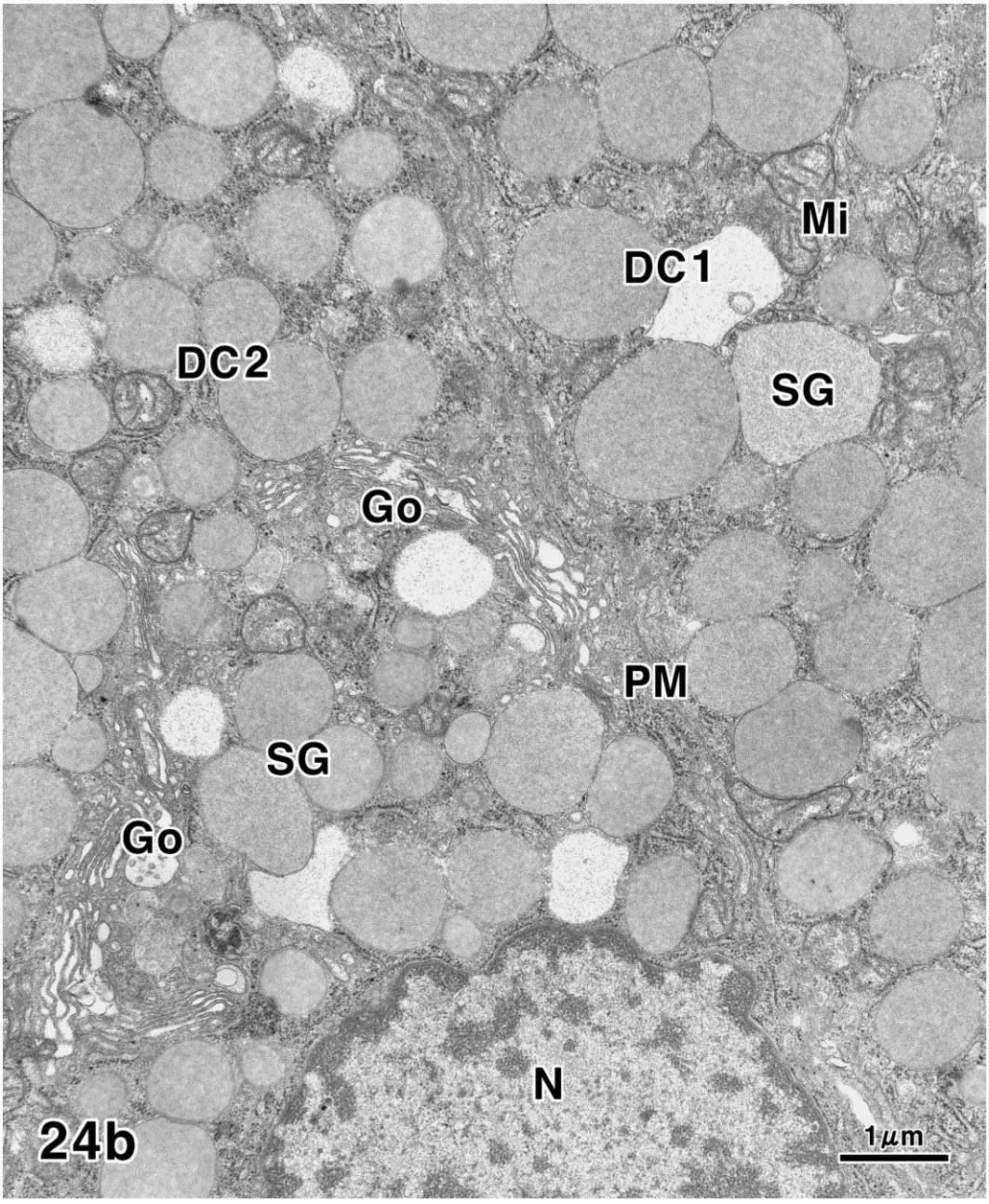


Fig. 24 General ultrastructure of the porcine carpal glands stained with uranyl acetate and lead citrate. b) Higher magnification of the dark cells in the glandular acini. ×15,500, DC1: type I dark cell, DC2: type II dark cell, Go: Golgi apparatus, Mi: mitochondrion, N: nucleus, PM: plasma membrane, SG: secretory granule.

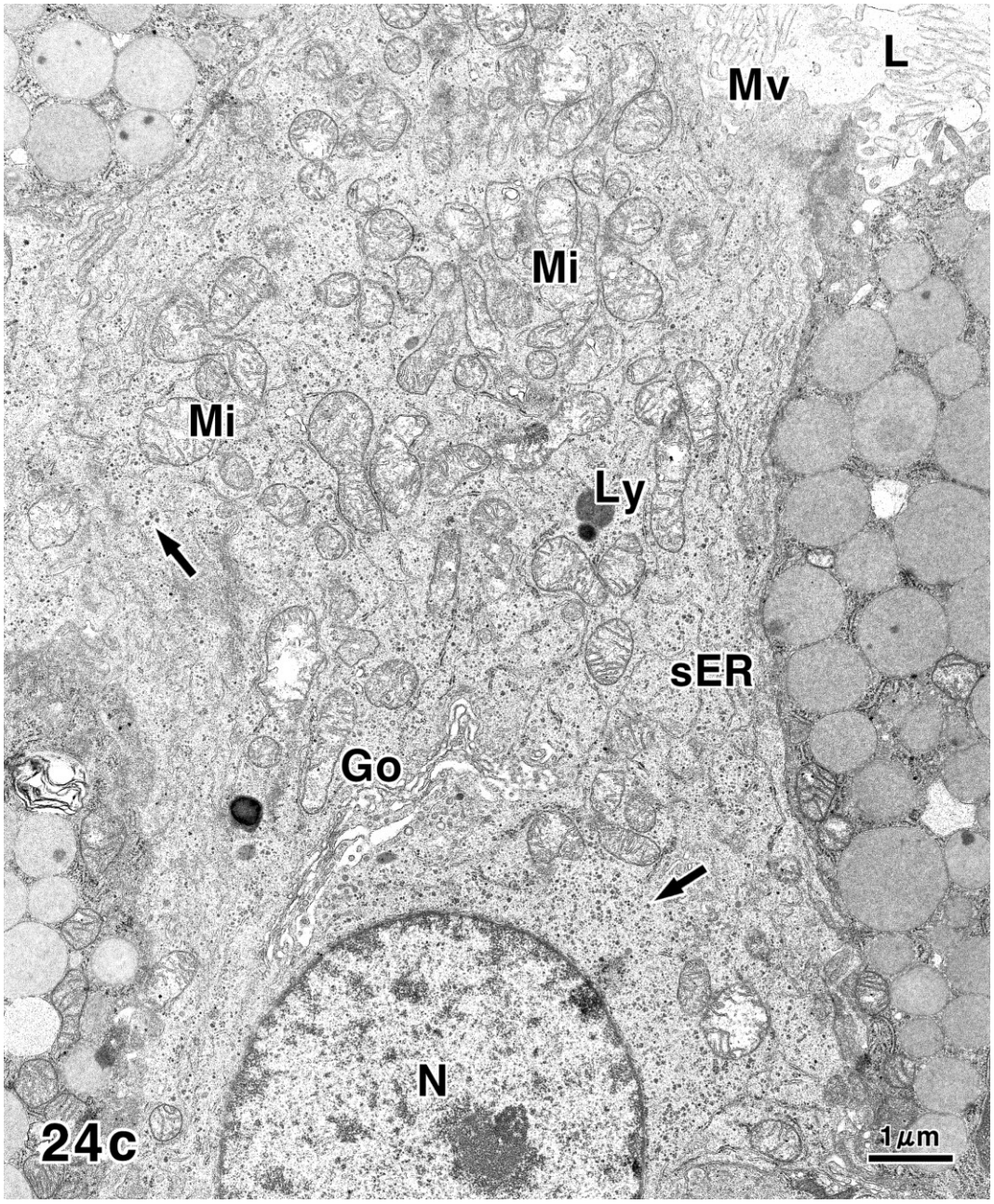


Fig. 24 General ultrastructure of the porcine carpal glands stained with uranyl acetate and lead citrate. c) Higher magnification of the clear cells in the glandular acini. ×12,000, Go: Golgi apparatus, L: lumen, Ly: lysosome, Mi: mitochondrion, Mv: microvilli, N: nucleus, sER: smooth-surfaced endoplasmic reticulum, arrows: glycogen particles.

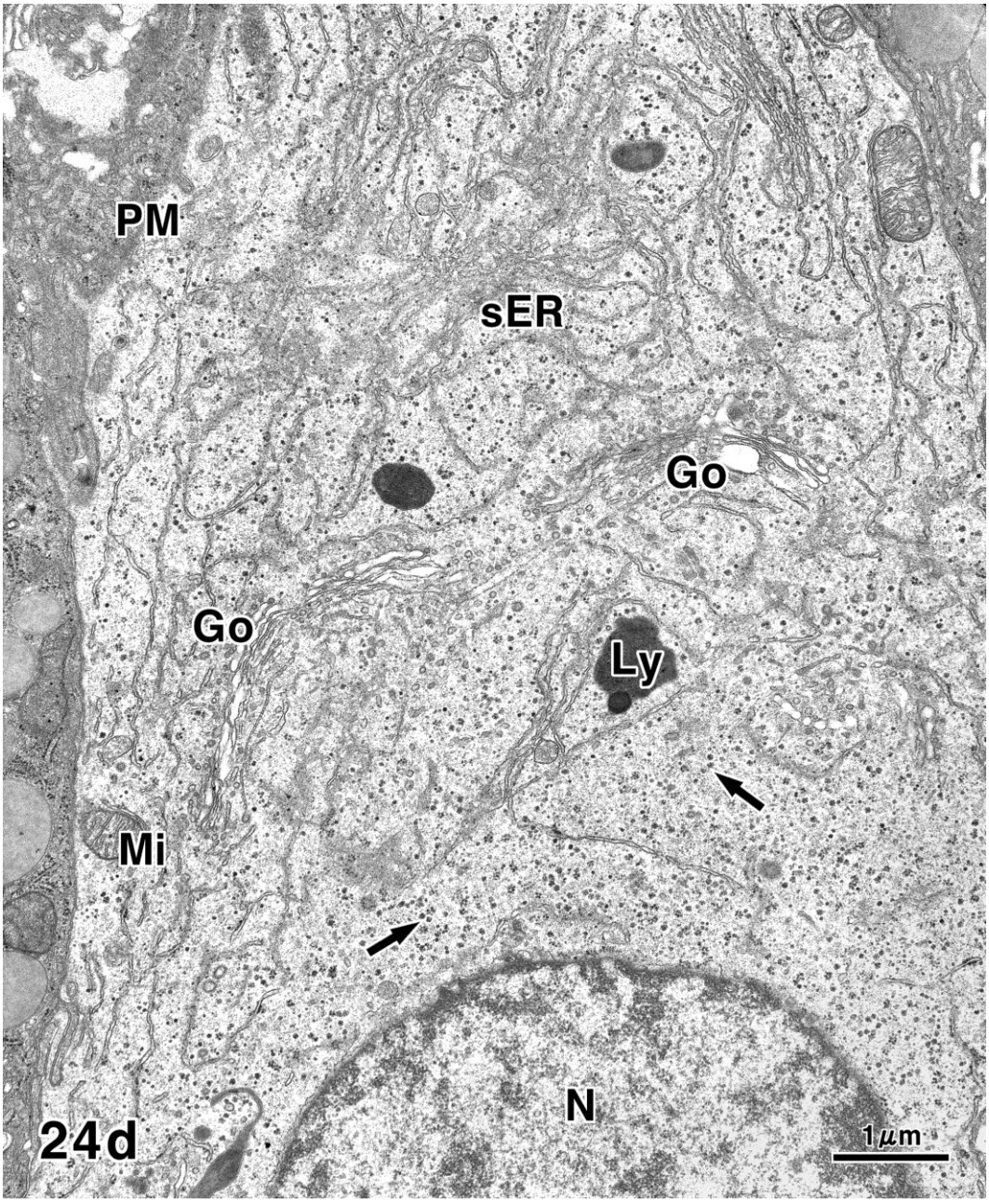


Fig. 24 General ultrastructure of the porcine carpal glands stained with uranyl acetate and lead citrate. d) Part of supranuclear cytoplasm of the clear cells. ×16,500, Go: Golgi apparatus, Ly: lysosome, Mi: mitochondrion, N: nucleus, sER: smooth-surfaced endoplasmic reticulum, arrows: glycogen particles.

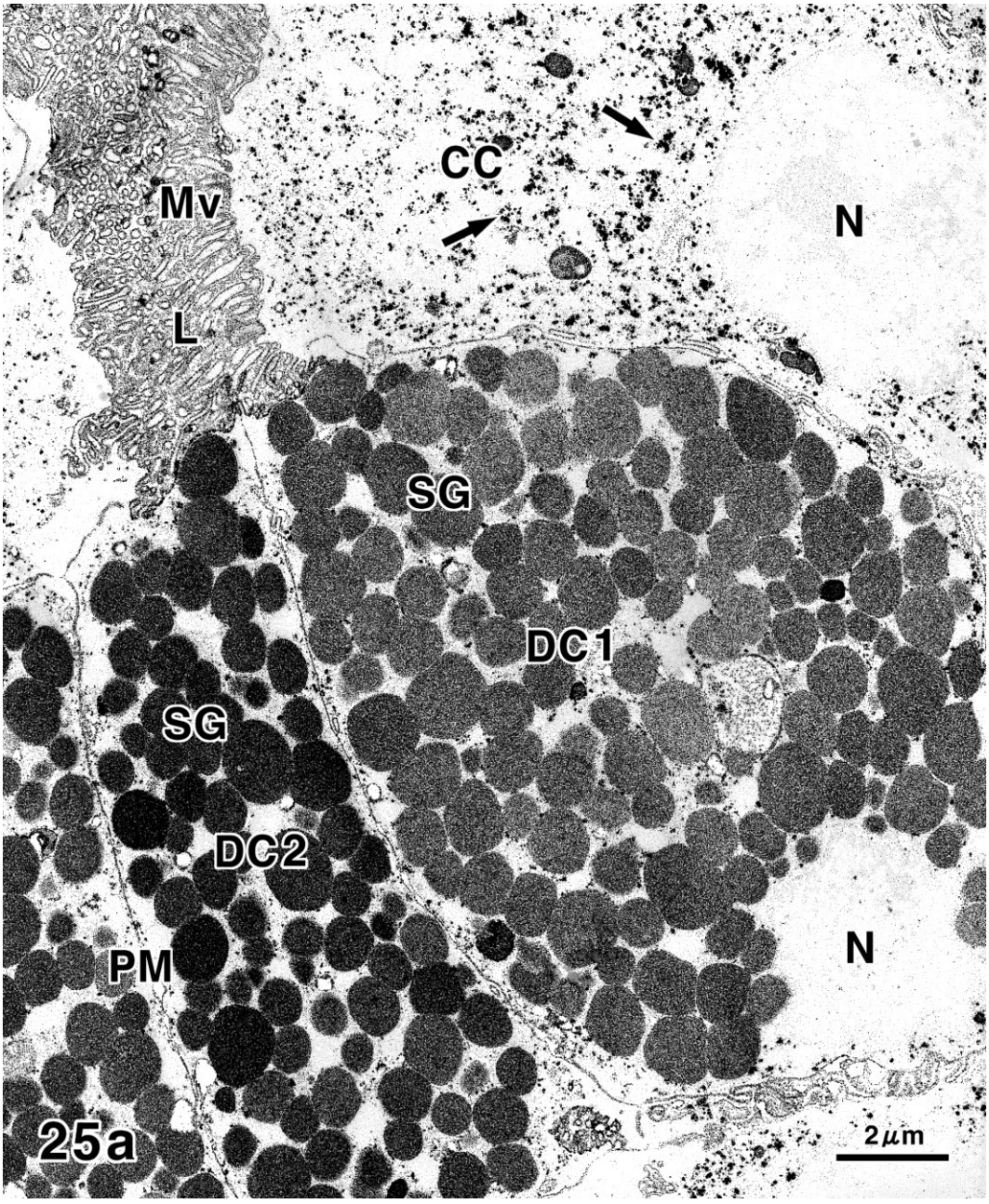


Fig. 25 Cytochemical PA-TCH-SP-PD staining of the carpal glands. a) Ultrastructures exhibit positive reactions. ×8,000, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule, arrows: glycogen particles.

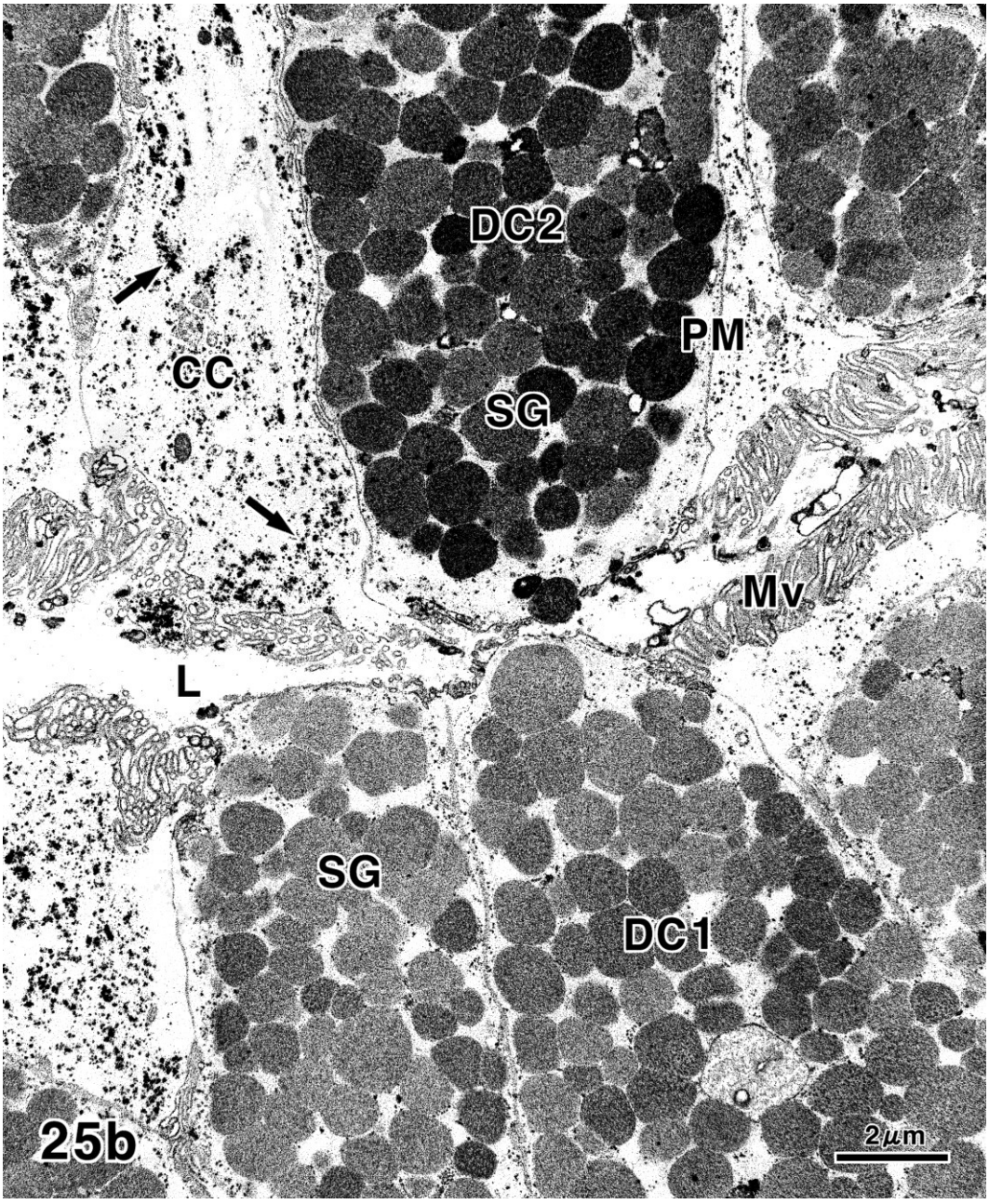


Fig. 25 Cytochemical PA-TCH-SP-PD staining of the carpal glands. b) The luminal side of the glandular acini. ×8,000, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule, arrows: glycogen particles.

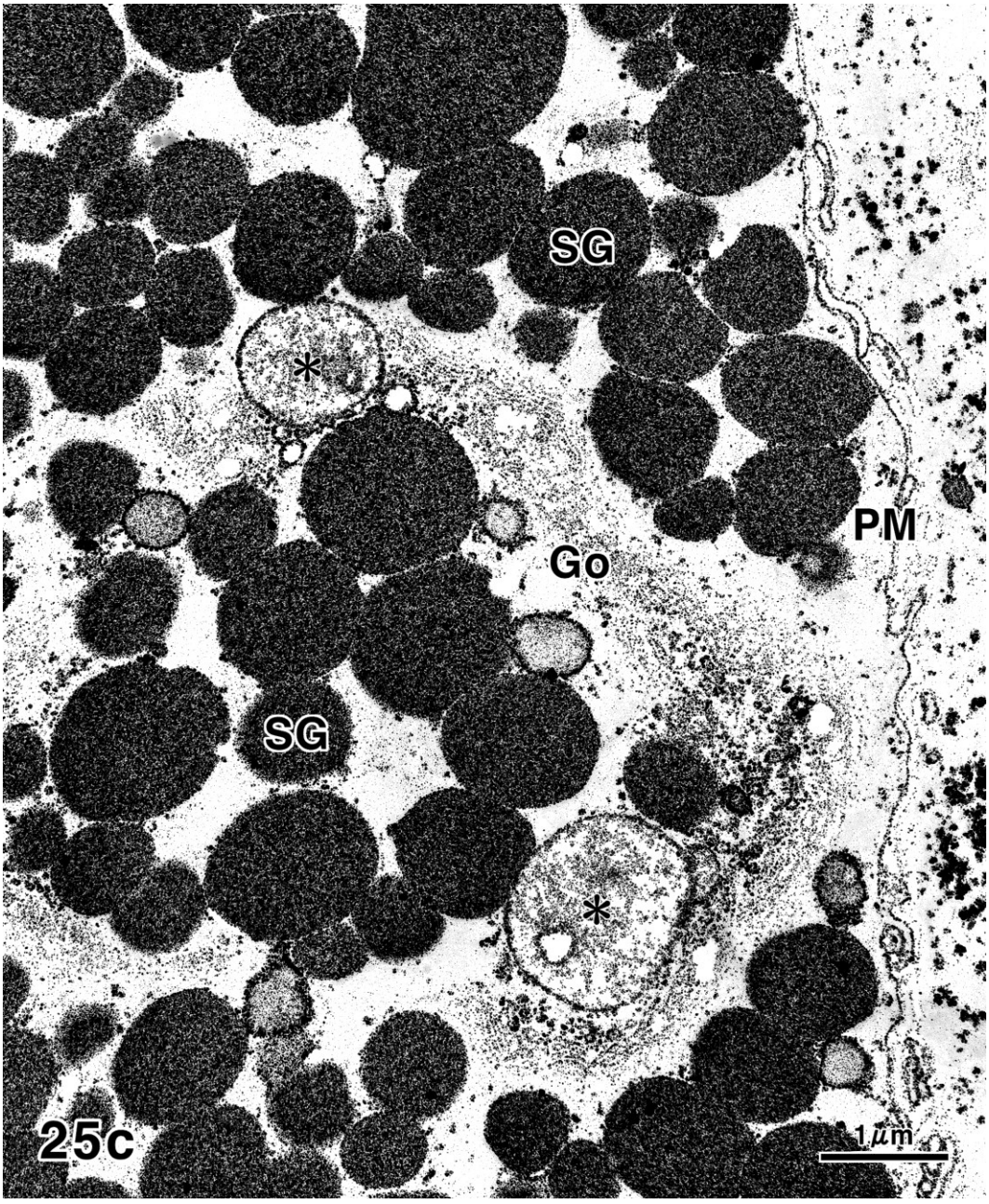


Fig. 25 Cytochemical PA-TCH-SP-PD staining of the carpal glands. c) Part of the supranuclear cytoplasm in the type II dark cell. ×18,500, Go: Golgi apparatus, PM: plasma membrane, SG: secretory granule, asterisks: immature secretory granules.

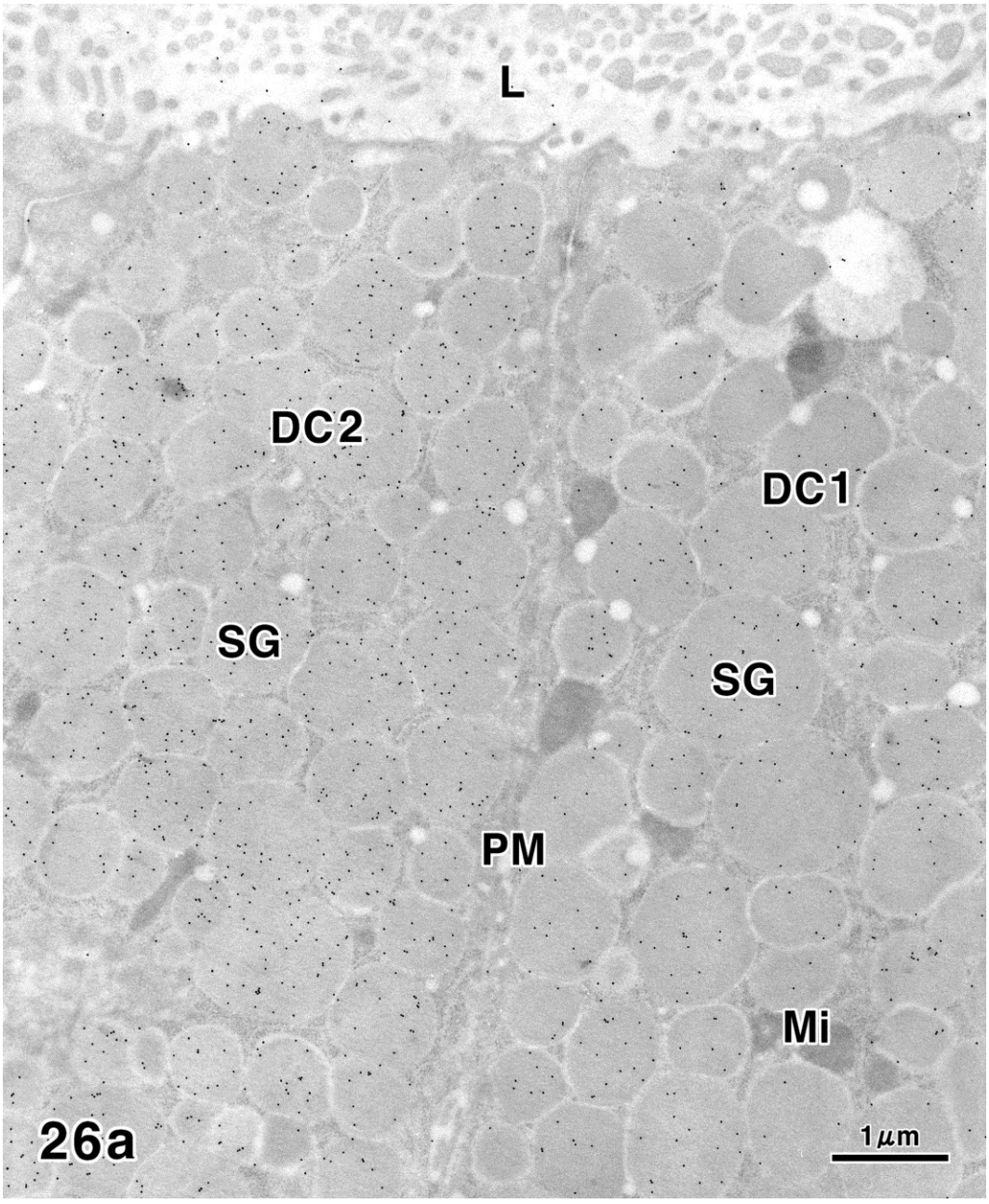


Fig. 26 SSA-gold staining of the carpal glands. a) Part of the apical cytoplasm of the dark cells. Reaction in the secretory granules of the type I and type II dark cells. ×16,500, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mi: mitochondrion, PM: plasma membrane, SG: secretory granule.

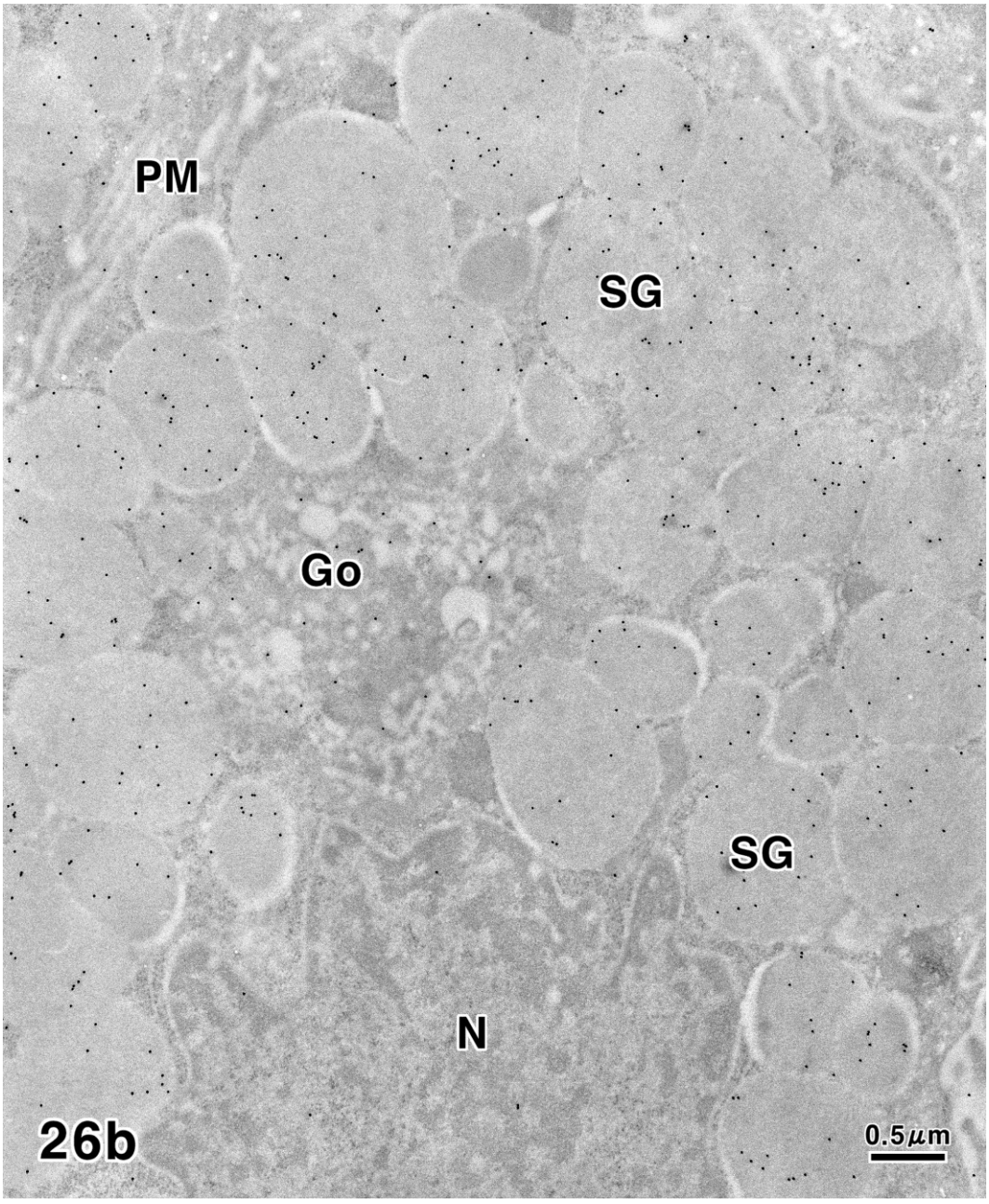


Fig. 26 SSA-gold staining of the carpal glands. b) Part of the supranuclear cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus and secretory granules. ×21,000, Go: Golgi apparatus, N: nucleus, PM: plasma membrane, SG: secretory granule.

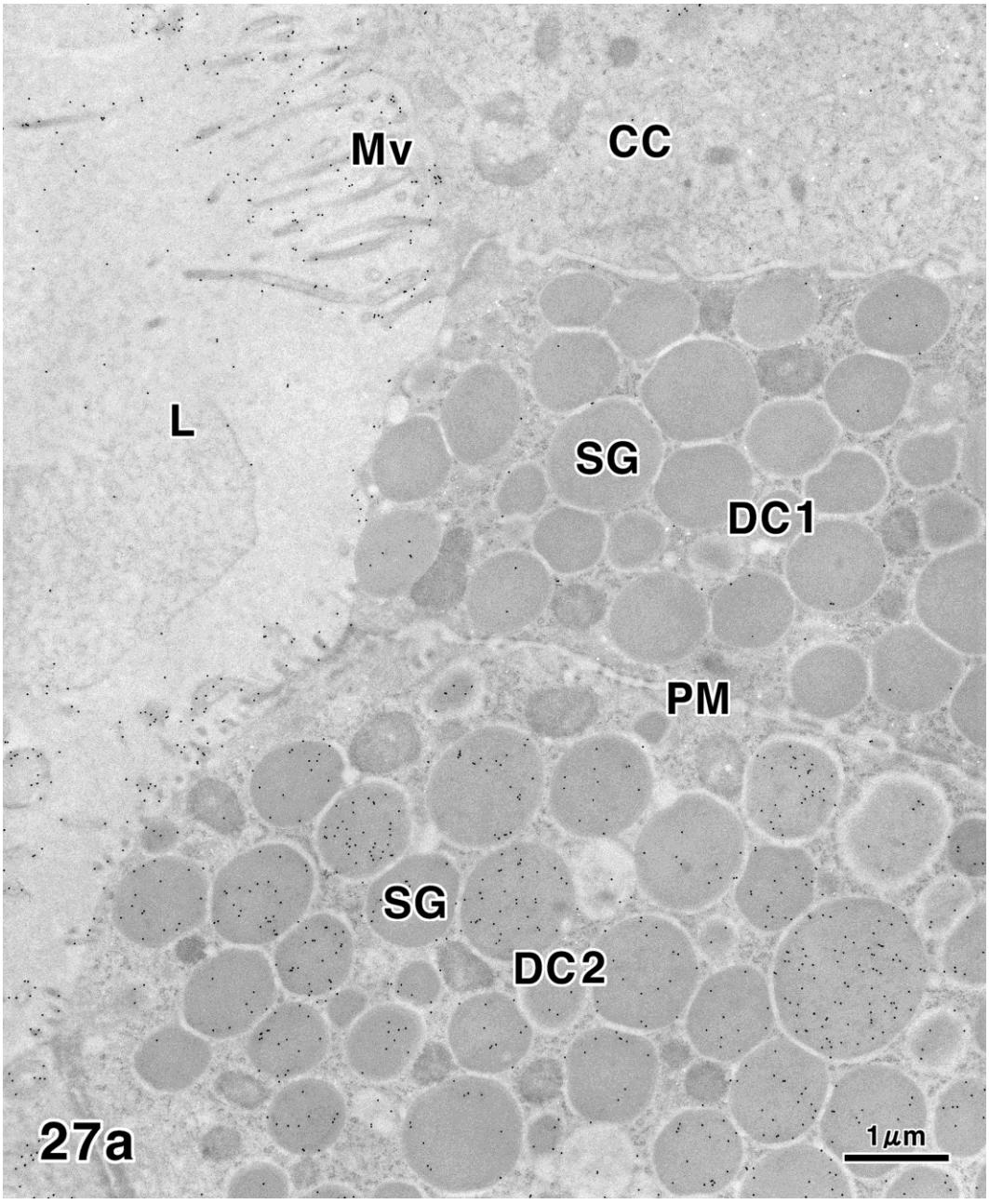


Fig. 27 MAM-gold staining of the carpal glands. a) Part of the luminal side of the glandular acini. Distinctly positive reactions are detectable in the secretory granules of the type II dark cells and in the free surface coat of the plasma membrane of the secretory cells. ×16,000, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, cell, PM: plasma membrane, SG: secretory granule.

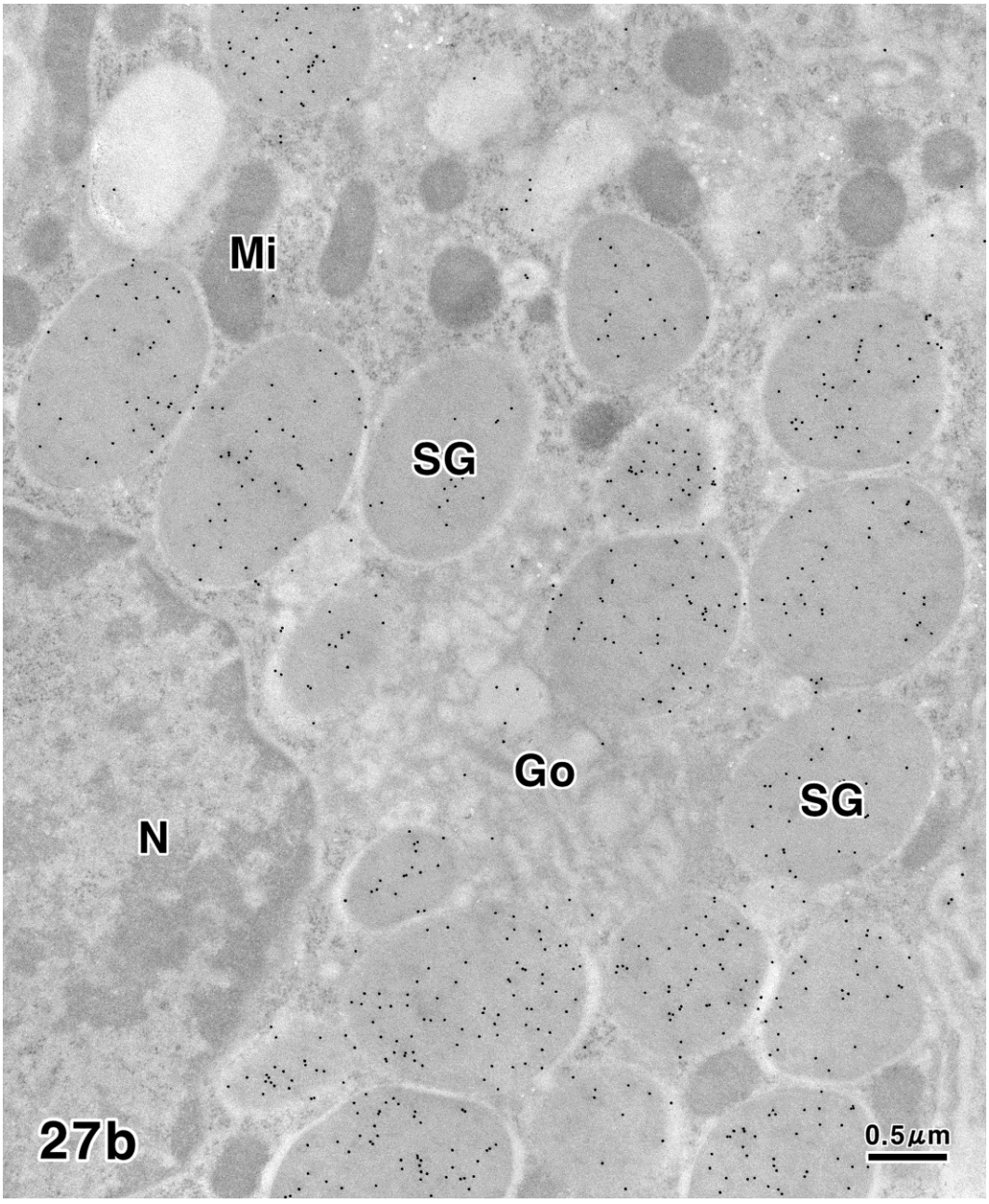


Fig. 27 MAM-gold staining of the carpal glands. b) Part of the supranuclear cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus and secretory granules. ×21,500, Go: Golgi apparatus, Mi: mitochondrion, N: nucleus, SG: secretory granule.

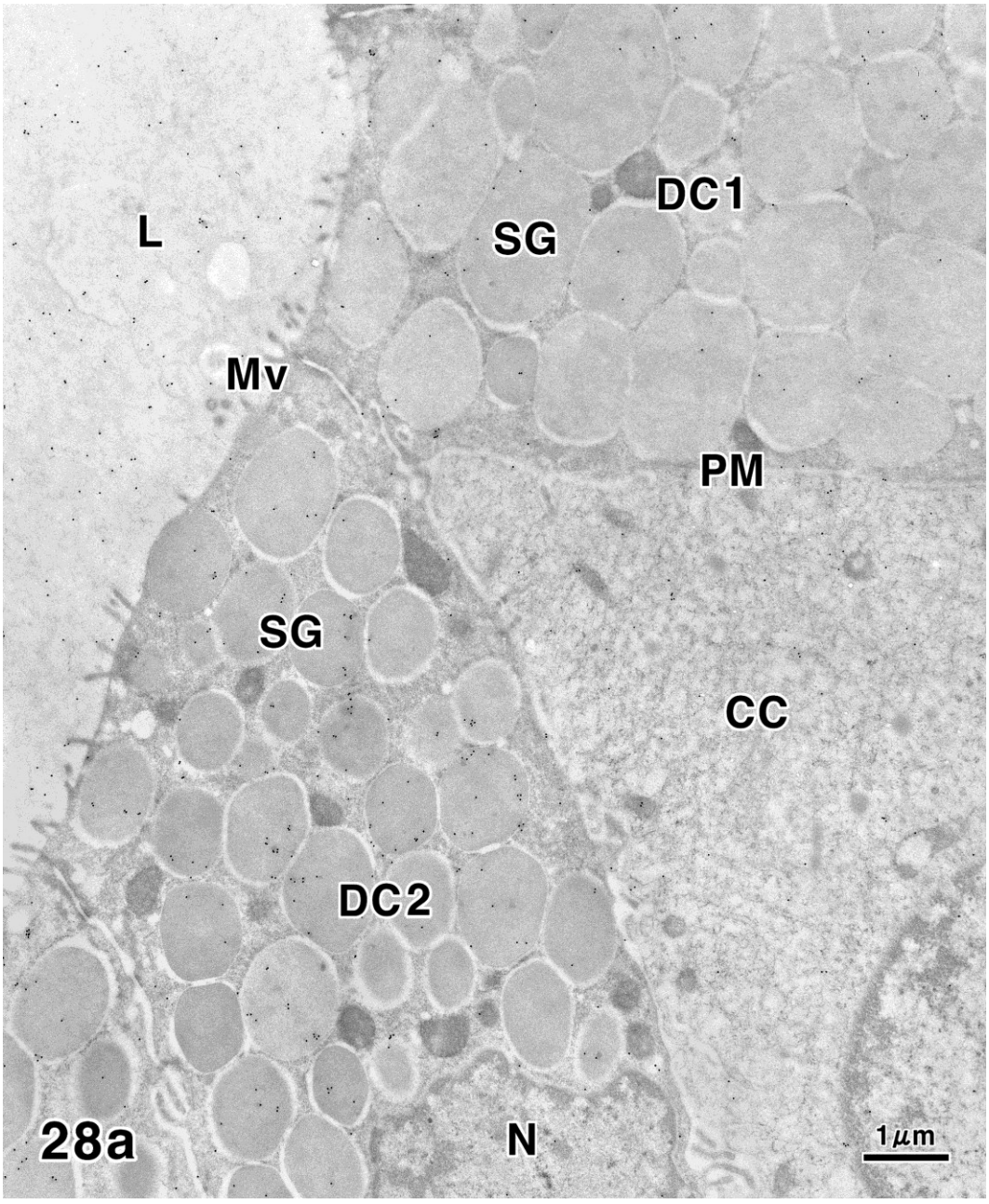


Fig. 28 Immunogold labeling for β-defensin 2 of the carpal glands. a) Part of the luminal side of the glandular acini. A clearly positive reaction is detectable in the secretory granules of the type II dark cell. ×12,000, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule.

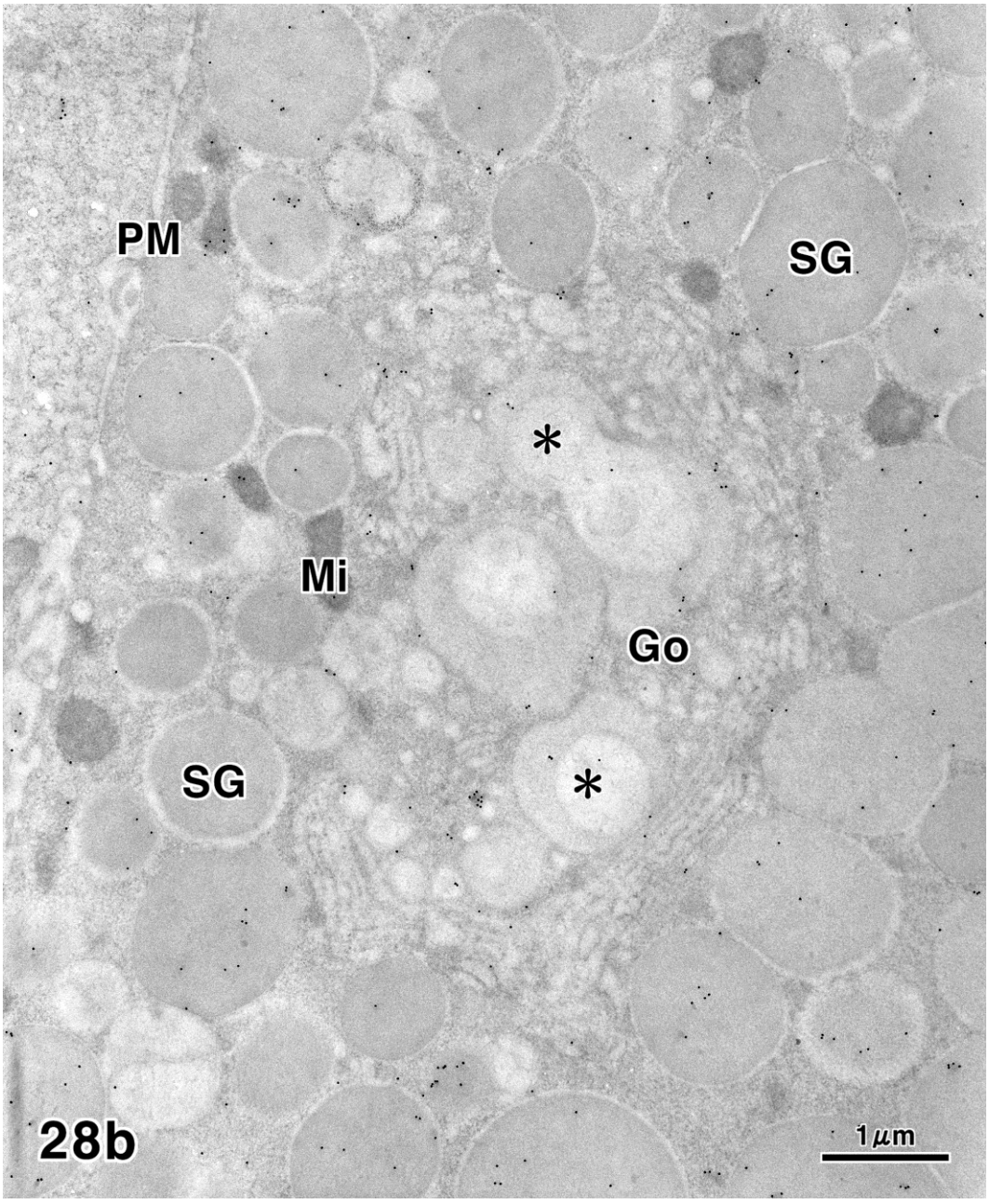


Fig. 28 Immunogold labeling for β-defensin 2 of the carpal glands. b) Part of the supranuclear cytoplasm of the type II dark cell. Reactions in the cisternae of the Golgi apparatus and secretory granules. ×18,000, Go: Golgi apparatus, Mi: mitochondrion, PM: plasma membrane, SG: secretory granule, asterisks: immature secretory granules.

5. 4. Discussion

The glandular acini of mammalian eccrine glands consist of two different cell types, dark cells and clear cells. The secretory granules of the dark cells contain glycoproteins, whereas the clear cells are involved in producing the hypertonic or isotonic precursor fluid of sweat (Kurosumi et al., 1984). In several mammalian species including humans, previous studies disclosed the presence of glycoconjugates with various saccharide residues in the dark cells (Tsukise et al., 1983; Meyer and Bartels, 1989; Meyer and Tsukise, 1995; Sames et al., 1999; Stumpf and Welsch, 2002; Yasui et al., 2004, 2005a). The distinction of cell types of among dark cells has not been reported previously. However, our investigation demonstrated some slight morphological differences among the dark cells. Furthermore, the two types of dark cell were also distinguished by the results of the PA-TCH-SP-PD procedure, which detects the presence of glycoconjugates with vicinal diol groupings (Yamada, 1993). On the other hand, an abundant smooth-surfaced endoplasmic reticulum was observed in the clear cells compared with that in the eccrine glands in porcine snout skin (Fukui et al., 2012a) and in the foot pads of carnivores (Meyer and Bartels, 1989; Yasui et al., 2005b). The presence of smooth-surfaced endoplasmic reticulum is an important feature of clear cells, suggesting their possible involvement in glycogen storage and in ion concentration and transport (see Kurosumi et al., 1982; Gargiulo et al., 1989). Additionally, distinct numbers of glycogen particles and many mitochondria, as

detectable in the clear cells, seem to be related to high energy demands during their production (Meyer and Bartels, 1989; Sato et al., 1989).

From the results of the lectin cytochemical procedures, in the porcine carpal glands, sialic acid residues linked to α2-6Gal/GalNAc were localized in the secretory granules and cisternae of the Golgi apparatus in both types of dark cell. In contrast, the presence of sialoglycoconjugates with a Siaα2-3Galβ1-4GlcNAc sequence and the antimicrobial peptide group of β-defensins was mainly confined to those of the type II dark cells. These results correspond with sialoglycoconjugate histochemical and immunohistochemical observations of the carpal glands by light microscopy (Fukui et al., 2012b). Such features differ from those of the eccrine glands in other mammals (Meyer and Bartels, 1989; Sames et al., 1999; Stumpf and Welsch, 2002; Yasui et al., 2004, 2005a, 2010). It is considered that the morphological and morphochemical characteristics of the dark cells may depend on the different functional stages or maturation stages.

Sialic acids are widely known to occupy the terminal position of oligosaccharide chains in a variety of glycoconjugates and are considered to perform a key role as the most versatile function modulators in cell biology and pathology. They are involved in binding transport of ions, stabilizing the conformation of proteins and enhancing the viscosity of mucins, owing to their negative charge. Sialic acids function not only as biological masks that are antirecognition agents that act by shielding recognition sites, such as

for macrophages, enzymes or galectins, but also as recognition sites for various molecules, for example, hormones, lectins, antibodies and inorganic cations, as well as for microorganisms (Schauer, 2004; 2009; Varki and Schauer, 2009). Furthermore, sialoderivatives may play an important role in the general defense against pathogenic agents via variation in the types of linkage and acceptor sugars and their degree of acetylation because, for example, influenza viruses differ in their ability to recognize Sia-Gal linkages depending on the animal hosts (Suzuki, 2005; Parillo et al., 2009).

In mammals, β-defensins are produced by phagocytes and various epithelial cells, and are often present at high concentration (Ganz, 2003, 2004). Defensins have a role in the innate immune response of the skin against microbial invaders. They have the capability of insertion into cell membranes, depending upon their electrical charge. Consequently, most defensins cause the destruction of microorganisms, that is, bacteria, fungi and viruses (Bos et al., 2001; Yang et al., 2001; Ganz, 2003, 2004). The antimicrobial peptide group of β-defensins is distributed in the eccrine glands of several mammals (Ali et al., 2001; Stumpf and Welsch, 2002; Stumpf et al., 2004; Yasui et al., 2010). A previous study showed the identification and first characterization of eleven porcine β-defensins and the expression of seven porcine β-defensins in skin tissues (Sang et al., 2006).

In conclusion, our cytochemical analyses give a detailed description of the distribution of sialoglycoconjugates and

β-defensin. Additionally, these results confirm that the dark cells of porcine carpal glands can be classified into type I and type II dark cells, and the Siaα2-3Galβ1-4GlcNAc sequence and β-defensin are produced by the type II dark cells (Fukui et al., 2012b). These secretory components may have an important role in effective protection of the skin surface of the carpal region against environmental pathogens. Therefore, the carpal glands are closely involved in effective defense for the preservation of skin integrity, besides their function as odoriferous glands.

5. 5. Summary

The functional properties of sialic acids appear to be manifold. Additionally, antimicrobial peptides are effective molecules for innate immunity. In humans and other mammals, defensins are one of the main antimicrobial peptide families. The present study demonstrated the localization of sialoglycoconjugates and β-defensin in the glandular acini of porcine carpal glands using cytochemical methods for electron microscopy. The secretory portion of the carpal glands was found to be composed of two types of dark cells and clear cells. Sialoglycoconjugates that terminated in Siaα2-6Gal/GalNAc were present predominantly in the secretory granules and Golgi apparatus of both types of dark cell. The presence of sialic acid residues linked to α2-3Galβ1-4GlcNAc was mainly restricted to the type II dark cells. The distributional pattern

of β-defensin was consistent with that of the Siaα2-3Galβ1-4GlcNAc sequence. Their presence and secretion are suggestive of protective effects of these secretory products at the skin surface of the carpal region.

7 CONCLUSION

In domestic mammals, eccrine glands are confined to the skin of a series of specific body regions. These glands are distributed in the dermis and subcutis of the snout skin and carpus in pigs. Sialic acids have different functional properties, such as enhancing the viscosity of mucins, protecting epithelial cells and preventing microbial invasion, and thus serve as versatile function modulators in cell biology and pathology. Additionally, antimicrobial substances are the effector molecules of innate immunity and contribute to non-specific defense on the skin surface.

The secretory portion of the eccrine glands of porcine snout skin consisted of dark cells and clear cells. The dark cells showed high levels of sialoglycoconjugates including O-acetylated sialic acids and Siaα2-6Gal/GalNAc sequence, whereas the localization of sialic acids linked to α2-3Galβ1-4GlcNAc was restricted to some of the dark cells. Antimicrobial substances (lysozyme, IgA, lactoferrin, β-defensin) and Rab3D were also demonstrated to be mainly present in some of the dark cells. By electron microscopy, the dark cells could be classified morphologically and cytochemically as type I cells and type II cells, owing to the ultrastructural features and the results of the PA-TCH-SP-PD procedure. Therefore, it is considered from these findings that sialic acid residues linked to α2-3Galβ1-4GlcNAc and various antimicrobial substances are produced by the type II dark cells. Moreover, Rab3D seems to play

an important role in the secretory regulation of these moieties. For confirmation of the above assumption, the precise distribution of sialic acids, lysozyme and β-defensin in this gland type at the cytological level was investigated using electron microscopic cytochemical methods. The secretory granules and Golgi apparatus of both types of dark cell contained sialic acid residues linked to α2-6Gal/GalNAc. Sialoglycoconjugates that terminated in Siaα2-3Galβ1-4GlcNAc, lysozyme and β-defensin were mainly confined to those of the type II dark cells. Consequently, the results obtained supported the view that two different cell types exist among the dark cells. These secretory components may function as protective agents to preserve the integrity of porcine snout skin as a sensory organ.

The secretory epithelium of the porcine carpal glands was also composed of two types of secretory cell: dark cells and clear cells. The dark cells showed high levels of sialoglycoconjugates, which included O-acetylated sialic acids and Siaα2-6Gal/GalNAc sequence. On the other hand, sialic acids linked to α2-3Galβ1-4GlcNAc were confined to a subpopulation of the dark cells. Furthermore, the distributional pattern of all antimicrobial substances and Rab3D was consistent with that of Siaα2-3Galβ1-4GlcNAc sequence. The general ultrastructures of these glands revealed some slight morphological differences among the dark cells. We classified the different dark cells as type I and type II dark cells. From the results of the cytochemical procedures, the secretory granules of the type II

dark cells had a higher concentration of glycoproteins than those of the type I dark cells. The secretory granules of both types of dark cell exhibited sialoglycoconjugates that terminated in Siaα2-6Gal/GalNAc. Sialic acids linked to α2-3Galβ1-4GlcNAc and β-defensin were principally detected in those of the type II dark cells. Therefore, in addition to the function as odorous glands, the glandular secretions of the carpal glands may create a defensive barrier against microbial invasion, considering that sialic acids and antimicrobial substances are important in defense mechanisms. Moreover, it is suggested that Rab3D is involved in the secretory regulation of these components.

The present histochemical and immunohistochemical analyses demonstrated the detailed localization of sialic acids and antimicrobial substances in the eccrine glands of the snout skin and carpus of pig by light and electron microscopy. It is emphasized, finally, that the secretory products elaborated by these glands may be essential components related to effective protection of the skin. These findings lead to a greater understanding of the functional significance of the skin glands in pig and other mammals.

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本論文の和文要約

哺乳類の皮膚は体表を覆い、外界の種々の刺激から体を保護する最も広く大型の器官であり、毛、皮膚腺など様々な付属器が認められる。また、皮膚は感覚刺激の受容、体温調節、脂肪の貯蔵やビタミンの生成など多様な機能を備え、一部の皮膚では、形態的あるいは機能的特殊化がなされている。ヒトを除く哺乳類皮膚のエックリン腺は、その特定部位にのみ存在し、変形腺として分類される。ブタにおいてこのエックリン腺は、吻鼻平面腺（Gll. plani rostralis）および手根腺（Gll. carpeae）として当該皮膚に分布するが、これらの腺における機能的役割は未だ十分に解明されていない。

一般に、分泌性タンパク質成分を構成するポリペプチドは粗面小胞体に結合しているリボソームで合成された後にゴルジ装置へと送られるが、この過程において、小胞体およびゴルジ装置内でタンパク質の糖鎖付加（グリコシレーション）が起こることが知られている。糖タンパク質に付加される糖鎖は、アスパラギン残基に結合しているN型糖鎖とセリンまたはスレオニン残基に結合しているO型糖鎖とに大別され、複数の糖質が糖鎖の構成に関わるとともに、各糖鎖は特異的なレクチン結合性を有している。特に、糖鎖の外側末端に位置するシアル酸は、その陰性荷電により分子の輸送、タンパク構造の安定化や分泌液への粘性賦与などの機能を有し、また細胞表面において、種々の物質や微生物に対する認識部位としての役割を果たすなど、様々な生理作用が知られている。一方、分泌性タンパク質として細菌、真菌およびウイルスに対する非特異的な免疫機構に関与する抗菌物質は、哺乳類の皮膚において病原微生物に対する感染防御に極めて重要な物質である。

そこで本研究では、ブタの吻鼻平面腺および手根腺におけるシアル酸と抗菌物質の詳細な局在とその性質を、糖質ならびに免疫組織細胞化学的に明らかにし、皮膚機能との関連性について検討した。

本研究は、以下の４章から構成される。第１章では、光顕解析として、ブタの吻鼻平面腺におけるシアル酸と抗菌物質の組織化学的研究を行った。吻鼻平面腺の腺上皮は、暗調細胞と明調細胞から構成され、暗調細胞は増感シアル酸検出法により陽性反応を示し、ケン化処理を施すことでその反応性が増強することが明らか

となった。また、レクチン染色法では、WGA および SSA が中等度から強陽性反応を示したが、MAM は一部の暗調細胞にのみ強い陽性反応を示したことから、暗調細胞には O-アセチル化されたシアル酸が多く含まれ、Siaα2-3Galβ1-4GlcNAc 構造は一部の細胞にのみ局在していることが判明した。免疫組織化学的染色では、一部の暗調細胞において抗菌物質としてリゾチーム、IgA、ラクトフェリンおよびβ-ディフェンシン 2 の発現が認められ、さらに分泌顆粒の輸送関連タンパク質である Rab3D の発現が認められた。以上の結果から、本腺から分泌されるこれらの物質は、感覚器官であるブタ吻鼻平面皮膚における防御機能に深く関わっていることが組織化学的に示唆された。第２章では、電顕レベルで、吻鼻平面腺における暗調細胞の分類とシアル酸、リゾチームならびに β-ディフェンシの細胞化学的研究を行った。暗調細胞はやや大型の分泌顆粒を有するⅠ型と小型の分泌顆粒を有するⅡ型に分類され、中性糖検出反応による分泌顆粒の性状解析において、Ⅰ型暗調細胞では弱から中等度の陽性反応を、Ⅱ型暗調細胞では強陽性反応を示した。電顕レクチン法では、WGA および SSA はⅠ型およびⅡ型両方の暗調細胞の分泌顆粒、ゴルジ装置にそれらの局在が観察されたが、MAM では、Ⅱ型暗調細胞にのみ陽性反応が認められた。リゾチームおよび β-ディフェンシン 2 に対する抗体を用いた免疫電顕法では、Ⅱ型暗調細胞の分泌顆粒やゴルジ装置に陽性反応が認められたが、Ⅰ型暗調細胞ではほとんど認められなかった。以上の結果から、暗調細胞は微細形態的にⅠ型とⅡ型に分類することができ、さらにⅡ型暗調細胞は、Siaα2-6Gal/GalNAc および Siaα2-3Galβ1-4GlcNAc 構造を有するシアル酸とリゾチームおよび β-ディフェンシンといった抗菌物質を産生・分泌することを電顕細胞化学的に明らかにした。第３章では、ブタの皮膚のエックリン腺として吻鼻平面腺と並び特有な構造である手根腺においてシアル酸と抗菌物質の組織化学的研究を行った。手根腺は、縄張りの主張や性臭に関与するニオイ物質の分泌によって種内のコミュニケーション機能に関わるとされているが、本腺分泌物の性状やその機能の詳細については不明な点が多い。手根腺の腺上皮は、光顕レベルで暗調細胞と明調細胞から構成されており、暗調細胞は増感シアル酸検出法により陽性反応を示した。同法にケン化処理を施した場合、その反応性が顕著に増強したことから、O-アセチル化されたシアル酸が局在することが確認された。レクチン染色法によって、暗調細胞には

Siaα2-6Gal/GalNAc 構造が含まれており、また一部の細胞では Siaα2-3Galβ1-4GlcNAc 構造も局在することが明らかとなった。また、免疫組織化学的解析により、一部の暗調細胞において抗菌物質としてリゾチーム、IgA、ラクトフェリンおよびβ-ディフェンシン 2が、分泌顆粒の輸送系タンパク質として Rab3D の発現が認められた。以上の結果から、手根腺上皮の暗調細胞には多様なシアル酸や各種抗菌物質が局在しており、本腺が縄張りの主張やコミュニケーション機能のみならず、手根皮膚の保護機能にも関与していることが示唆された。第 4 章では、手根腺における糖質と β-ディフェンシンの細胞化学的研究を電顕レベルで行った。手根腺上皮の一般形態観察ならびに中性糖検出反応による解析から、暗調細胞は分泌顆粒が中性糖検出反応に弱から中等度の反応を示すⅠ型と、強陽性を示すⅡ型とに分類された。一方、明調細胞では分泌顆粒は見られず、細胞質には、よく発達した滑面小胞体、多数のミトコンドリアおよび豊富なグリコーゲン粒子が観察された。電顕レクチン法による解析では、SSA はⅠ型とⅡ型の両暗調細胞の分泌顆粒やゴルジ装置に、MAM は腺細胞の微絨毛とⅡ型暗調細胞の分泌顆粒およびゴルジ装置に局在が認められた。免疫電顕法による β-ディフェンシン 2 の細胞内局在の解析では、Ⅱ型暗調細胞の分泌顆粒に陽性反応が観察された。以上の結果から、手根腺上皮を構成する暗調細胞はⅠ型とⅡ型とに区別され、Ⅱ型細胞からは結合様式の異なるシアル酸や β-ディフェンシンが分泌されることが明らかとなった。一方、明調細胞の滑面小胞体はグリコーゲンの代謝に、さらに多数のミトコンドリアとグリコーゲンの貯留は電解質や水の輸送・放出に関与していることが示唆された。

本論文は、ブタ皮膚のエックリン腺である吻鼻平面腺と手根腺において、腺上皮を構成する細胞のサブタイプについて光顕および電顕形態学的にそれらの特徴を記載し、かつ、シアル酸および各種抗菌物質の詳細な局在とその性質について組織細胞化学的に明らかにした。新たに見出された暗調細胞におけるⅠ型およびⅡ型の形態学的差異は、細胞の分泌機能活性あるいは細胞または分泌顆粒の成熟度の違いを反映しうる極めて重要な所見であるといえる。本論文で明らかにされた新知見は、ブタ皮膚のエックリン腺である吻鼻平面腺と手根腺における防御機能的重要性を形態化学的に初めて証明した点にあり、これはブタのみならず哺乳類における皮膚腺機能の理解に大きく寄与するものと考えられる。