

Histochemical localization of sialic acids and antimicrobial substances in eccrine glands of porcine snout skin

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Abstract

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 Sia α 2-3Gal1-4GlcNAc 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.

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 unguiae*), the carpus of pig and the nasolabial region of ruminants and pig.¹⁻⁷ 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.⁸

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.^{9,10} 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.¹¹⁻¹³ 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.¹⁴⁻¹⁶

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.

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. 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 μ m. Sections were stained with haematoxylin-eosin (H-E), Mallory-Azan and alcian blue (AB) pH 2.5-periodic acid

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Key words: sialic acids, antimicrobial substances, eccrine glands, porcine snout skin, histochemistry.

Received for publication: 5 October 2011.

Accepted for publication: 15 December 2011.

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European Journal of Histochemistry 2012; 56:e6
doi:10.4081/ejh.2012.e6

Schiff (PAS)¹⁷ 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 2.5¹⁸ and periodic acid-phenylhydrazine-silver chloride-thiocarbonyl-silver proteinate-physical development (PA-P-TCH-SP-PD).¹⁹ 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)²⁰ 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)²¹ 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 $\mu\text{g}/\text{mL}$ 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) 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 2.²² 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 in 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 812.²³ From these tissue blocks, ultrathin sections were cut on an ultramicrotome, mounted on copper grids and stained with uranyl acetate²⁴ and lead citrate.²⁵ 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.²⁶ 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.²⁷ 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)²⁸ was carried out on some sections prior to the PA-TCH-SP-PD procedure.

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 (Figure 1 a,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 (Figure 2 a,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 reac-

Table 1. The moieties detected by sialoglycoconjugate histochemical procedures.

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 sialoglycoconjugates
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 receptor sugars and inhibitory sugars.

Lectins	Abbreviation	Sugar binding specificity	Inhibitory sugars
Wheat germ agglutinin	WGA	Sia, β -D-GlcNAc	β -D-GlcNAc
<i>Sambucus sieboldiana</i> agglutinin	SSA	Sia α 2-6Gal/GalNAc	α 2-6sialyllactose
<i>Maackia amurensis</i> agglutinin	MAM	Sia α 2-3Gal1-4GlcNAc	α 2-3sialyllactose

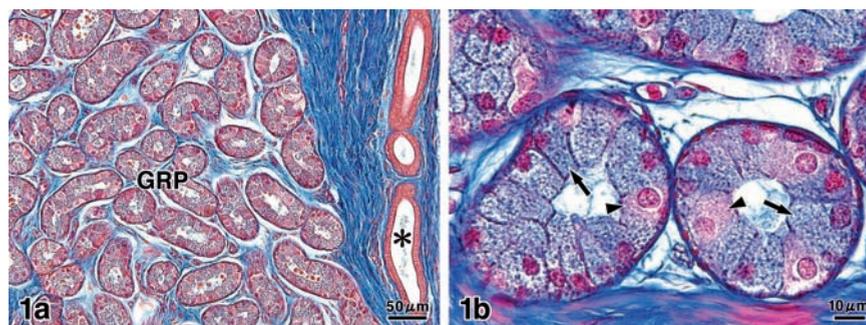


Figure 1. General structure of the eccrine glands in the porcine snout skin. a) Mallory-Azan, GRP: gland of rostral plate, asterisk: excretory duct; b) higher magnification of the eccrine glandular acini, Mallory-Azan, arrows: dark cells, arrowheads: clear cells.

tion of the clear cells (Figure 3a). Enzyme digestion with sialidase following saponification caused a weaker AB pH 2.5 reaction (Figure 3b). 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 (Figure 3c). 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 (Figure 3 d,e). The PA-P-TCH-SP-PD staining was nearly negative after sialidase digestion following saponification (Figure 3f). 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 (Figure 4 a,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 (Figure 4c). In all of the control experiments for the lectin histochemical procedure, positive reactions were greatly diminished or nearly abolished in the eccrine glands (Figure 4d). 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 (Figure 5a). 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 (Figure 5b). 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 (Figure 5 c,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 (Figure 5e).

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

(Figure 5f). 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 morpho-

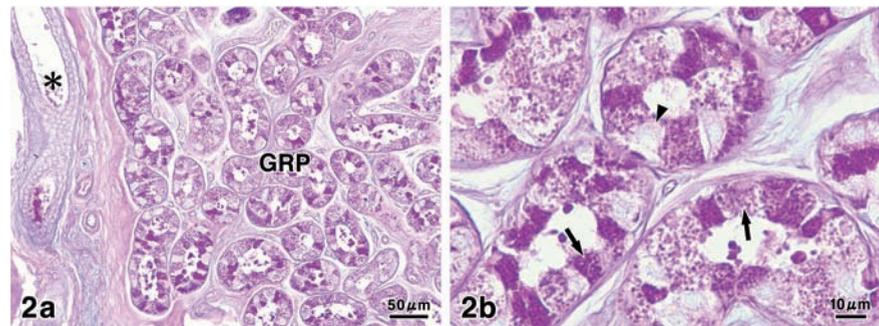


Figure 2. General carbohydrate histochemical appearance of the eccrine glands in the snout skin. a) AB pH 2.5-PAS, GRP: gland of rostral plate, asterisk: excretory duct; b) AB pH 2.5-PAS, arrows: dark cells, arrowhead: clear cell.

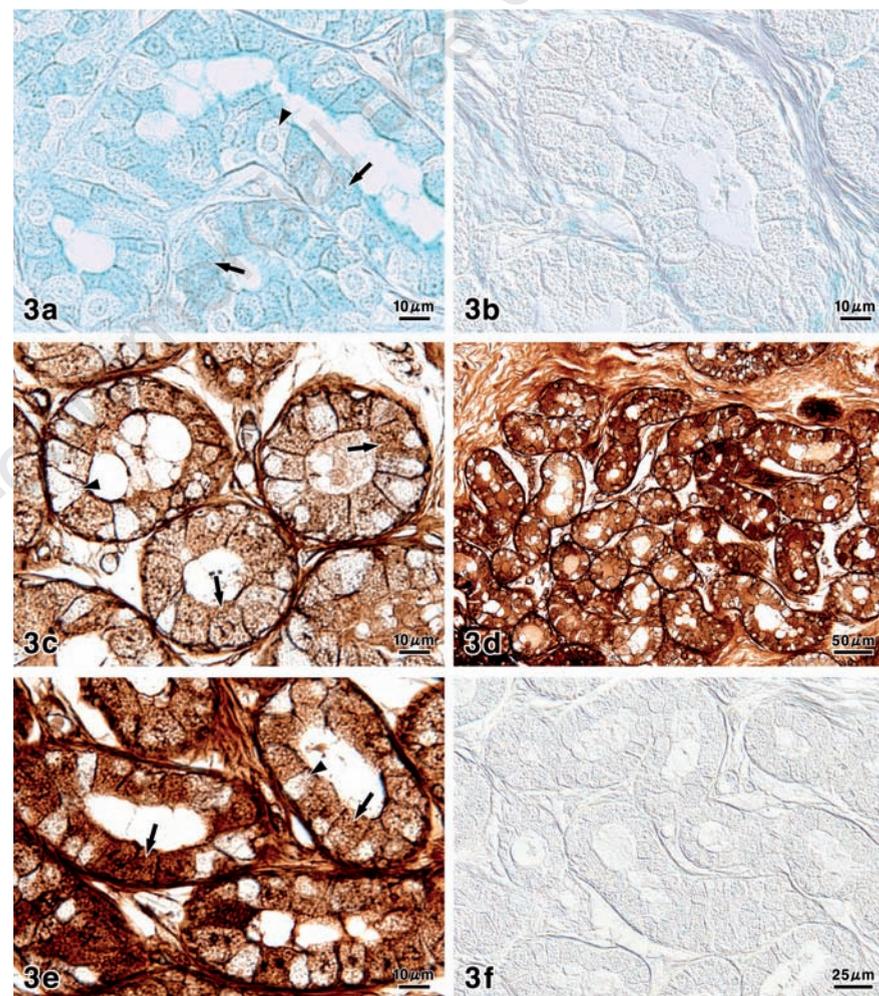


Figure 3. Histochemical visualization of sialic acids in the eccrine glands of the snout skin. a) AB pH 2.5, arrows: dark cells, arrowhead: clear cell; b) KOH-Sial-AB pH 2.5; c) PA-P-TCH-SP-PD, arrows: dark cells, arrowhead: clear cell; d) KOH-PA-P-TCH-SP-PD; e) KOH-PA-P-TCH-SP-PD, arrows: dark cells, arrowhead: clear cell; f) KOH-Sial-PA-P-TCH-SP-PD0.

logical 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 (Figure 6 a,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 (Figure 6b). 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 (Figure 7a-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 (Figure 7a, c). The PA-TCH-SP-PD-reactive ultrastructures in the clear cells were glycogen particles and also cisternae of the Golgi apparatus (Figure 7 a,b). Digestion with α -amylase abolished the PA-TCH-SP-PD-reactive glycogen particles, found particularly in the cytoplasm of the clear cells.

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.^{5,11,12,29-31} 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 func-

tional properties of sialic acids appear to be manifold.^{9,10} These glycoconjugates protect cells and macromolecules against enzymatic and immunological attacks by inhibiting the adherence of different bacteria, and can func-

tion as recognition sites for various receptors.^{9,10,32,33} 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 follow-

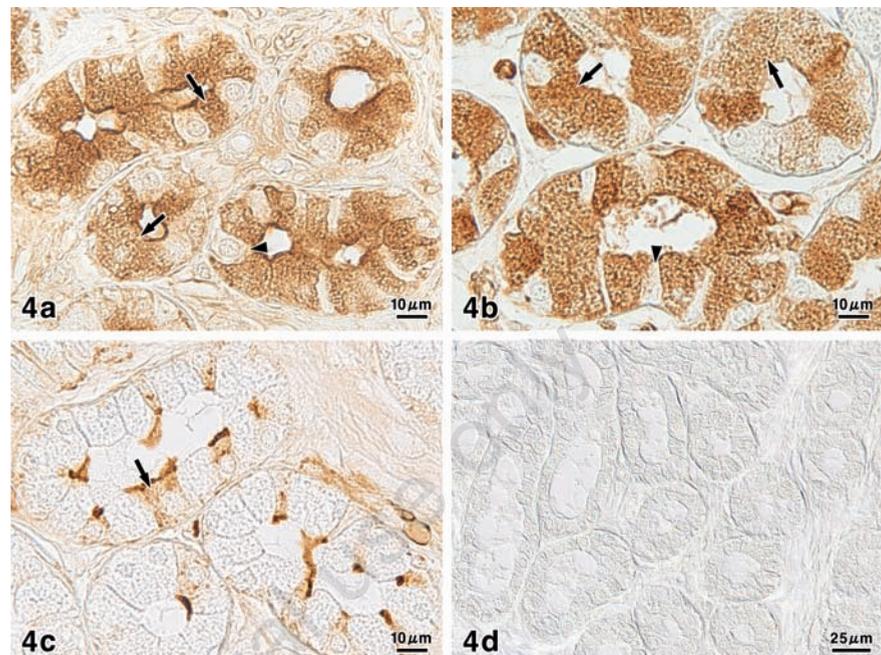


Figure 4. Lectin histochemical staining in the eccrine glands of the snout skin. a) WGA, arrows: dark cells, arrowhead: clear cell; b) SSA, arrows: dark cells, arrowhead: clear cell; c) MAM, arrow: dark cell; d) control.

Table 3. Histochemical reactions of the eccrine glands in pig 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.

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

Stainings	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.

ing saponification.³⁴ Moreover, apoptosis was reported to be inhibited by O-acetylated sialic acids.³⁵ In this study, the dark cells contained sialoglycoconjugates that terminated in Sia α 2-6Gal/GalNAc, whereas sialic acids linked to α 2-3Gal1-4GlcNAc 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.^{7,13,30,31,36} The great variety of sialic acid residues, in particular, may play an important role in the general defense against pathogenic agents.^{10,37,38}

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.³⁹⁻⁴¹ Although Ezo and Katsumura⁴² reported that lysozyme was not present in human eccrine glands, another investigation identified the expression of this enzyme.⁴³ With regard to this gland type of the feline foot pads, these antimicrobial substances were demonstrated to be immunolocalized in the dark cells.¹³ Moreover, in hyrax and tenrec, lysozyme and β -defensins are also observed in the eccrine glands of foot pads.^{11,12}

Lysozyme is a 14.5-KD cationic enzyme with bactericidal activity that catalyzes the hydrolysis of glycosidic bonds in bacterial cell walls.⁴⁴ This enzyme has a close functional relationship to immunoglobulins and lactoferrin.⁴⁵ The predominant immunoglobulin isotype on most mucosal surfaces and external secretions is secretory IgA composed of polymeric IgA containing J chain and secretory component.^{46,47} In epithelial defense, IgA contributes to preventing pathogen adhesion to host cells, thereby blocking dissemination and further infection.⁴⁸ IgA as well as J chain is produced by plasma cells and transported through secretory epithelial cells into external secretions.⁴⁹ 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.^{50,51}

Additionally, lactoferrin inhibits allergen-induced skin inflammation, which is secondary to its role in regulating the production of cytokines.⁵² 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 phospho-

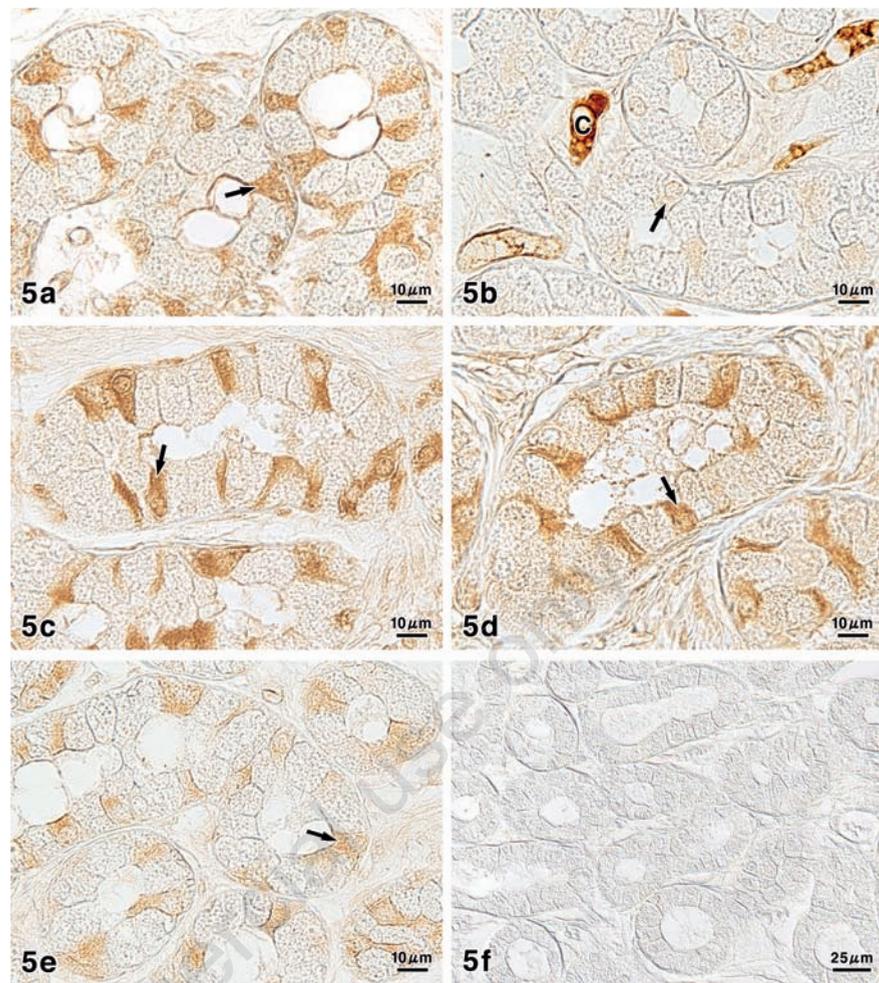


Figure 5. Immunohistochemical staining in the eccrine glands of the snout skin. a) Lysozyme, arrow: dark cell; b) IgA, C: capillary, arrow: dark cell; c) lactoferrin, arrow: dark cell; d) β -defensin 2, arrow: dark cell; e) Rab3D, arrow: dark cell; f) control.

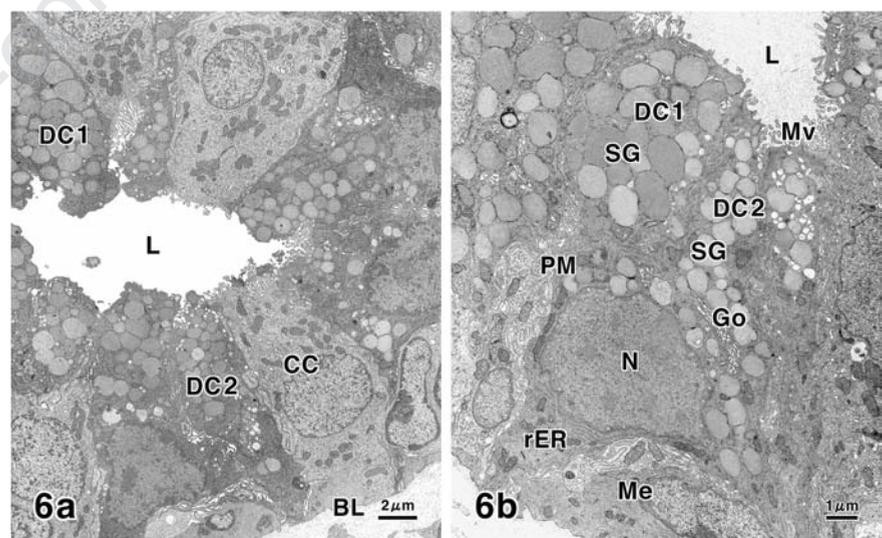


Figure 6. Ultrastructure of the eccrine glands in the porcine snout skin stained with uranyl acetate and lead citrate. a) The secretory portion consists of type I dark cells, type II dark cells and clear cells. BL: basal lamina, CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen; b) Higher magnification of dark cells in the eccrine glandular acini. 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.

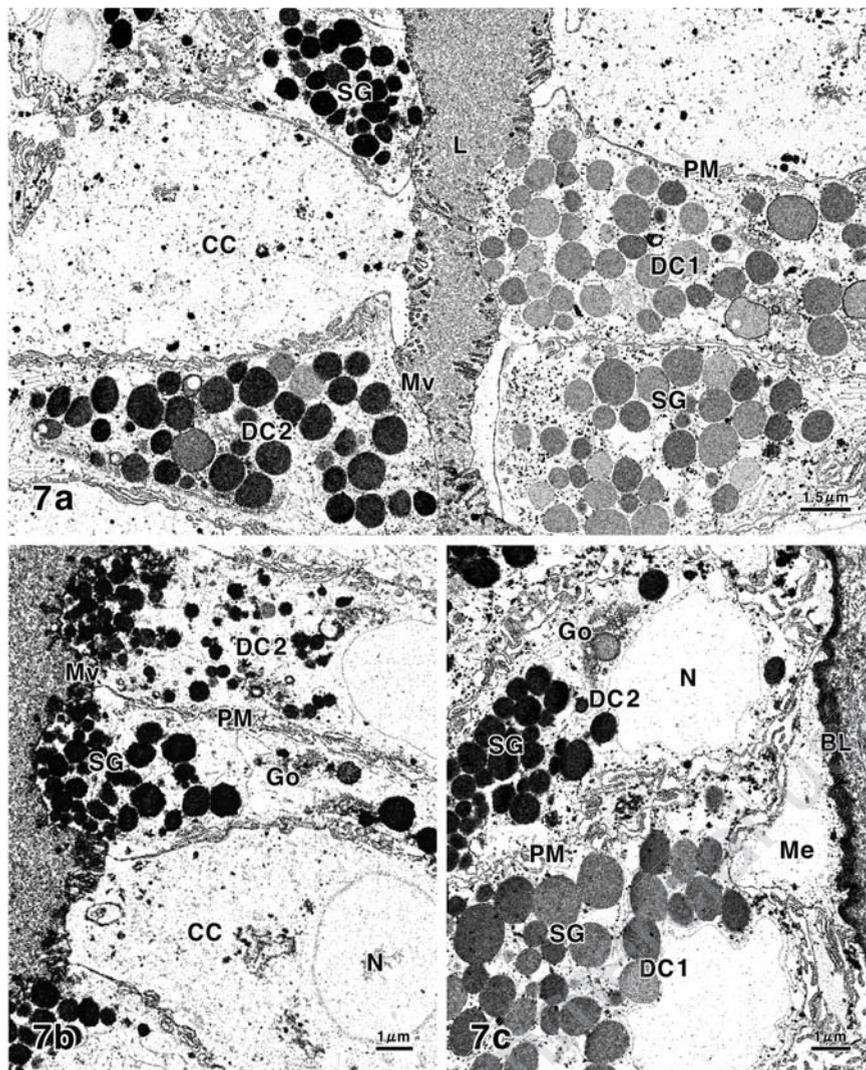


Figure 7. Cytochemical PA-TCH-SP-PD staining of the eccrine glands in the snout skin. a) Ultrastructures exhibit positive reactions. CC: clear cell, DC1: type I dark cell, DC2: type II dark cell, L: lumen, Mv: microvilli, PM: plasma membrane, SG: secretory granule; b) Part of the apical cytoplasm of secretory cells. CC: clear cell, DC2: type II dark cell, Go: Golgi apparatus, Mv: microvilli, N: nucleus, PM: plasma membrane, SG: secretory granule; c) Part of the basal cytoplasm of secretory cells. 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.

lipid bilayer, leading to disruption and subsequent death of the microorganisms.^{53,54} This is most important for the integument regarding the multitude of microbes always active on the skin surface.^{55,56}

It is established that Rab proteins function in the tethering or docking of vesicles to their target compartment, leading to membrane fusion.⁵⁷ Rab3D is expressed in secretory granules of exocrine secretory cells, and involved in regulated exocytosis.^{58,59}

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.²⁷ It

is suggested from these findings that sialic acid residues linked to Sia α 2-3Gal1-4GlcNAc 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.³⁵ 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.

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