

# Ultrastructure and immuno-histochemical characterization of proteins concerned with the secretory machinery in goat ceruminous glands

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## Abstract

The expression of soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) proteins in apocrine glands has not been fully elucidated. In addition to performing ultrastructural observation of the ceruminous glands in goats, our study focuses on the demonstration of  $\beta$ -defensins, SNARE proteins and Rab3D in these glands with the use of immunohistochemical methods. The secretory cells were equipped with two types of vesicles, Golgi apparatus and abundant rough endoplasmic reticulum (ER). Additionally, in some of them, the characteristic concentric structures composed of rough ER were observed in their circum- and infranuclear parts. The expression of phosphorylated inositol requiring enzyme 1 $\alpha$  was also detected. These findings may indicate their ability to produce numerous secretory proteins and the maintenance of homeostasis in the glandular cells. Furthermore,  $\beta$ -defensins were demonstrated as products of the ceruminous glands. The present investigation also revealed the presence of SNARE proteins and Rab3D. It is suggested that these proteins are concerned with the secretory machinery of this gland type.

## Introduction

Cerumen commonly known as earwax has been found to contain various components including antimicrobial substances.<sup>1,2</sup> The combined secretions of the ceruminous glands, which are modified apocrine glands, and sebaceous glands form the earwax together with sloughed epidermal cells. In domesticated mammals, cerumen is regarded as a brownish waxy material.<sup>3</sup> The morphological observations of these glands differ among animal species; for example, the wall of the external auditory canal of the

goats contains the prominent ceruminous glands and less-abundant sebaceous glands as compared with that of raccoons and horses. In non-human mammals above mentioned, the ceruminous glands elaborate the secretions containing an abundance of glycoconjugates.<sup>4-6</sup> Additionally, numerous antimicrobial proteins including  $\beta$ -defensins are present in the ceruminous glandular cells of humans.<sup>7</sup> These components may contribute to the protection of the external auditory canal against microbial invasion.

The secretory mechanism of the apocrine glandular cells are considered to be generally a combination of apocrine secretion and exocytosis.<sup>8-10</sup> It is assumed that, in this gland type, antimicrobial substances are released by exocytosis.<sup>7</sup> Soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) proteins play an important role in the machinery for intracellular membrane fusion and exocytotic secretion, and are categorized as vesicle-(v-) SNARE and target-(t-) SNARE.<sup>11-13</sup> Vesicle-associated membrane proteins (VAMPs) known as v-SNAREs are localized at transport vesicles, whereas t-SNAREs such as syntaxins and synaptosomal-associated proteins (SNAPs) are confined to the target membrane. It has been immunohistochemically demonstrated that SNARE proteins are involved in secretory mechanism in several exocrine glandular tissues, such as human submandibular glands.<sup>14</sup> In apocrine gland type, however, the histochemical analysis of SNARE proteins has not been fully explored, because Stoeckelhuber *et al.*<sup>15</sup> is the only study on the apocrine glands, to our knowledge, that the localization of VAMP-8 and syntaxin 2 was reported in human axillae. Rab family of proteins is constituted by more than 60 mammalian members, and Rab proteins serve as important regulators of intracellular vesicle transport.<sup>16</sup> Rab3D is abundant in various non-neuronal exocrine tissues unlike other Rab3 isoforms, and involved in regulating exocytosis.<sup>16,17</sup>

The accumulation of unfolded proteins in the endoplasmic reticulum (ER), so-called ER stress, is caused by perturbation of the homeostatic functions of the ER.<sup>18</sup> ER stress activates the cytoprotective signaling pathway referred to as ER stress response or unfolded protein response (UPR).<sup>19,20</sup> Inositol requiring enzyme 1 (IRE1), known as one of the ER transmembrane proteins, functions as stress sensor.<sup>19</sup> Under ER stress condition, IRE1 undergoes dimerization or oligomerization and trans-autophosphorylation followed by its activation, and initiates the signaling of the

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UPR.<sup>19,21</sup> The present study investigated the detailed ultrastructural features and distribution of  $\beta$ -defensins, SNARE proteins and Rab3D in the well-developed ceruminous glands of the goats. In addition, we attempted to elucidate the relationship between the characteristic ER-derived structures identified and ER stress. Our findings may be important for understanding the secretory machinery of this gland type not only in domesticated ruminants but also in other mammalian groups.

## 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. Skin samples were taken from the external auditory canal of four adult male Japanese miniature (Shiba) goats (1.5-2 years, 20-30 kg), which were deeply anesthetized and then exsanguinated from the common carotid arteries.

## General histology and cytology

For histological purpose, tissue pieces were fixed in Bouin's solution for 24 h at room temperature, then carefully washed and dehydrated in a graded series of ethanol. The specimens were embedded in paraffin wax and cut at a thickness of 5  $\mu$ m. Deparaffinized sections were stained with hematoxylin and eosin (H-E). For general cytological observation, the skin samples were fixed a mixture of 4% paraformaldehyde

hyde and 0.5% glutaraldehyde in 0.1 M phosphate-buffered solution (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. From these tissue blocks, ultrathin sections were cut using an ultramicrotome, mounted on copper grids and routinely stained with uranyl acetate and lead citrate. The ultrathin sections prepared were examined under an electron microscope (JEM1011; JEOL Ltd., Tokyo, Japan).

### Immunohistochemistry

For immunohistochemical analysis, the paraffin sections were prepared in the same way as for histological purpose. The primary antibodies were used as follows:  $\beta$ -defensin 1 (DEF01-S; Biologo, Kronshagen, Germany),  $\beta$ -defensin 2 (DEF02-S; Biologo), VAMP-1 (ab3346; Abcam Plc., Cambridge, UK), VAMP-2 (ab70222; Abcam), VAMP-3 (ABIN675818; Antibodies-online, Inc., Atlanta, GA, USA), VAMP-4 (ab80989; Abcam), VAMP-7 (HPA036733; Atlas antibodies AB, Stockholm, Sweden), VAMP-8 (NBP1-20232; Novus Biologicals, Littleton, CO), syntaxin 2 (ADI-VAP-SV065; Enzo Life Sciences, Inc., Farmingdale, NY, USA), syntaxin 4 (HPA001330; Atlas antibodies), syntaxin 6 (ab56656; Abcam), SNAP-23 (10825-1-AP; Proteintech Group, Inc., Rosemont, IL, USA), Rab3D (12320-1-AP; Proteintech). Additionally, anti-phosphorylated IRE1 $\alpha$  (pIRE1 $\alpha$ ) antibody (NB100-2323; Novus Biologicals) was used for the detection of activated IRE1 $\alpha$ . Deparaffinized sections were pretreated with microwave irradiation in 0.01 M citrate buffer (pH 6.0) for antigen retrieval as described previously,<sup>22</sup> except for anti-pIRE1 $\alpha$  antibody. Then, the sections were immersed in Dako REAL Peroxidase-Blocking solution (Dako, Glostrup, Denmark) for 5 min to suppress endogenous peroxidase activities. Additionally, the slides were washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.3), and incubated with 3% bovine serum albumin (BSA) (Sigma-Aldrich, Inc., St. Louis, MO, USA) in PBS for 30 min at room temperature. Incubation with primary antibodies diluted with PBS containing 3% BSA was also performed for 24 h at 4°C. Their host species and dilutions are listed in Table 1. After rinsing with PBS, these sections were incubated with secondary antibodies using the HRP-labeled polymer technique (EnVision+ Dual Link System-HRP, Dako) for 30 min at room temperature. The immunoreaction was visualized

using a peroxidase substrate kit (SK-4100; Vector laboratories, Burlingame, CA, USA).

Controls for immunohistochemical methods were performed by incubation with normal mouse or rabbit IgG (concentration 1  $\mu$ g/mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) instead of the primary antibodies. Additionally, with regard to polyclonal antibodies, the secretions were incubated the mixture of the diluted antibodies that were pretreated with respective absorbing antigens (concentration 2-20  $\mu$ g/mL) for 12 h at 4°C. Images were acquired with an Axio imager A1 microscope equipped with an AxioCam HRC charged-coupled device camera and digitized using Axiovision software (Carl Zeiss, Jena, Germany).

## Results

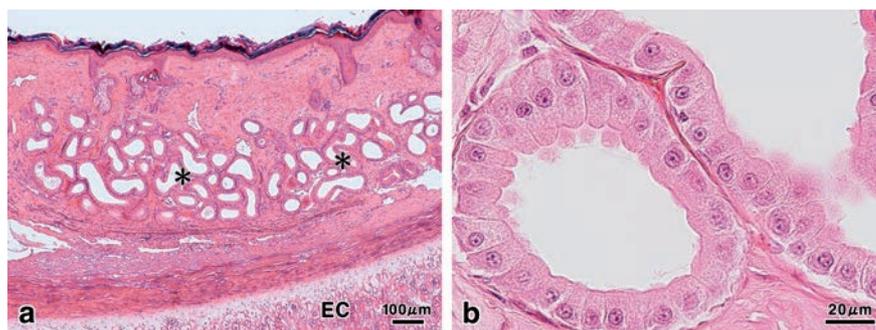
### General histology and cytology

In the goat auditory canal encircled by elastic cartilage, the well-developed ceruminous glands were detected lying below the epidermis (Figure 1a). The height of secretory cells varied from flattened cuboidal to tall columnar (Figure 1b). Additionally, the less-abundant sebaceous glands were observed near the hair follicles.

As for the general cytological observations of the ceruminous glands, it became obvious that the secretory portion consisted of a single layer of the secretory cells with associated myoepithelial cells (Figure 2a). In the supranuclear cytoplasm of the secretory cells, a distinct Golgi apparatus

**Table 1. Primary antibodies used for immunohistochemistry.**

Specificity	Host	Clonality	Dilution
$\beta$ -Defensin 1	Rabbit	Polyclonal	1:800
$\beta$ -Defensin 2	Rabbit	Polyclonal	1:1000
VAMP-1	Rabbit	Polyclonal	1:500
VAMP-2	Rabbit	Polyclonal	1:1200
VAMP-3	Rabbit	Polyclonal	1:400
VAMP-4	Rabbit	Polyclonal	1:5000
VAMP-7	Rabbit	Polyclonal	1:100
VAMP-8	Rabbit	Polyclonal	1:2000
Syntaxin 2	Rabbit	Polyclonal	1:400
Syntaxin 4	Rabbit	Polyclonal	1:200
Syntaxin 6	Mouse	Monoclonal	1:500
SNAP-23	Rabbit	Polyclonal	1:100
Rab3D	Rabbit	Polyclonal	1:50
pIRE1 $\alpha$	Rabbit	Polyclonal	1:2000

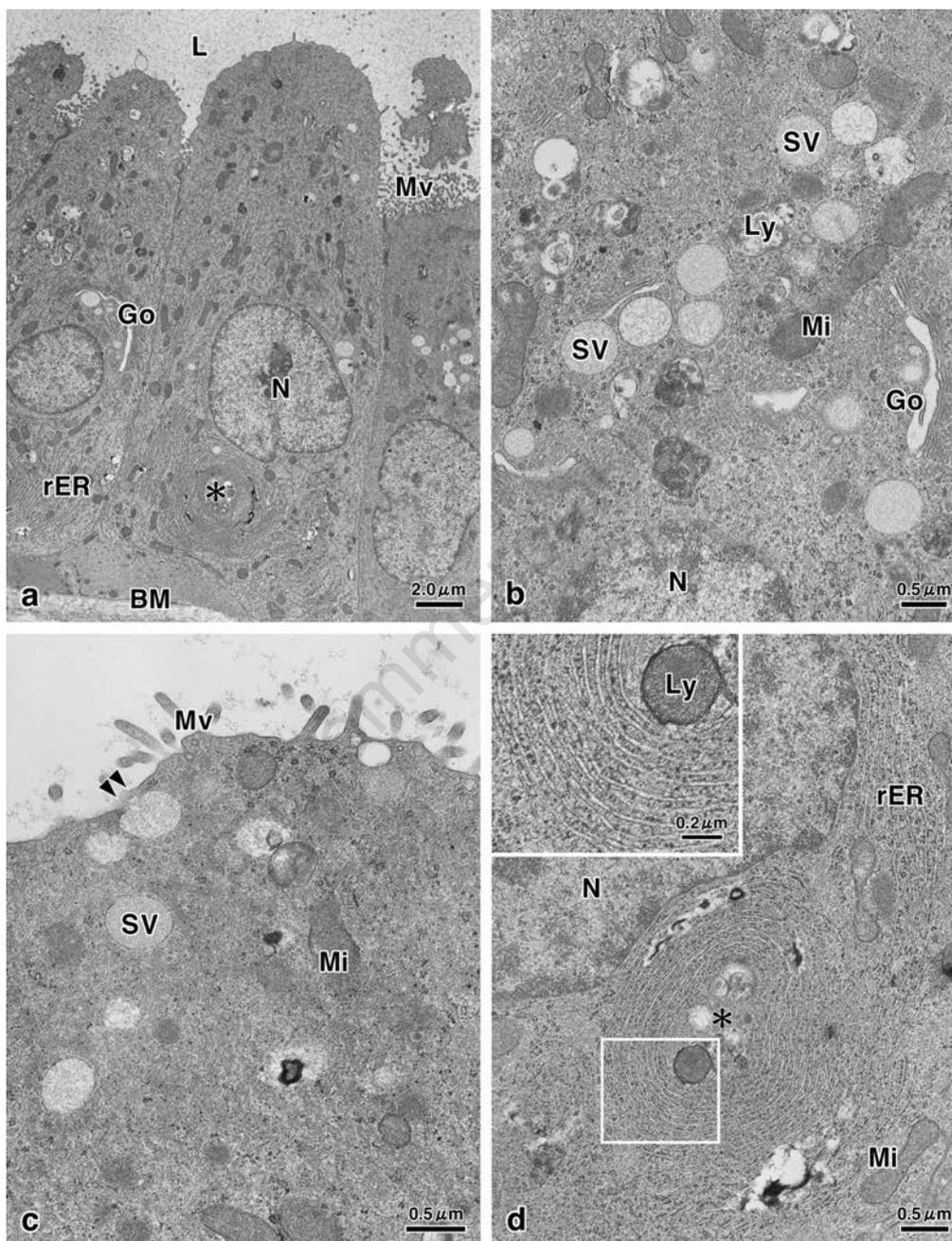


**Figure 1. Light microscopic structure of the ceruminous glands in the goat. a) The well-developed ceruminous glands were detected lying below the epidermis of the external auditory canal, H&E. b) higher magnification of the ceruminous glands, H&E. EC, elastic cartilage; asterisks, ceruminous glands.**

and a variable number of vesicles were detected. Some vesicles, which are regarded as secretory vesicles, possessed relatively low electron density (Figure 2b). They often showed the morphological features suggestive of exocytosis (Figure 2c). Other vesicles were irregularly shaped and contained medium or distinct electron dense substances (Figure 2b). In addition,

several secretory cells appeared to pinch off the apical protrusion according to the apocrine secretion mode. Another feature noted was a prominent rough endoplasmic reticulum (ER) in the circum- and infranuclear cytoplasm (Figure 2a). Furthermore, characteristic concentric structures formed by the elements of the rough ER were observed in some of the secretory cells.

Additionally, cytoplasmic components such as lysosomes were present in the central region of these multilayered structures (Figure 2d). The concentric ER-derived structures were detected in approximately one-third of the tall columnar secretory cells, whereas they were found in about one-fifth of the flattened cuboidal secretory cells.



**Figure 2.** Electron microscopic structure of the ceruminous glands. a) The secretory portion consists of single layer of secretory cells with underlying myoepithelial cells. b) Part of the supranuclear cytoplasm of secretory cells. c) Part of the apical cytoplasm of secretory cells. d) Part of the infranuclear cytoplasm of secretory cells; inset (upper left) shows higher magnification of a concentric structure composed of rough ER (boxed area). Go, Golgi apparatus; L, lumen; Ly, lysosome; M, mitochondria; Mv, microvilli; N, nucleus; rER, rough endoplasmic reticulum; SV, secretory vesicle; asterisk, a concentric structure composed of rough ER; double arrowhead, a morphological feature suggestive of exocytosis.

## Immunohistochemistry

In sections stained according to the immunohistochemical procedures, antibodies to  $\beta$ -defensin 1 produced a weak to moderate positive reaction of the secretory cells. Additionally, in some of the secretory cells, strongly stained granules were observed in the apical and supranuclear cytoplasm (Figure 3a). Incubation with antibodies to  $\beta$ -defensin 2 resulted in a moderate positive reaction of the secretory cells. The apical part of some of the secretory cells was found to exhibit a stronger positive reaction (Figure 3c). A positive reaction for VAMP1 was restricted to the apical and supranuclear cytoplasm of the secretory cells. Strongly stained granules were detectable in these parts of some of them (Figure 4a). The VAMP-2 staining patterns of the secretory cells were similar to the results obtained by the  $\beta$ -defensin 2 staining (Figure 4c). When the sections were incubated with antibodies to VAMP-3, a stronger staining reaction was observed in the basal cytoplasm in some of the secretory cells (Figure 4e). The secretory cells exhibited a moderate to strong positive reaction after the immunohistochemical staining for VAMP-4. In most of secretory cells, strongly stained granules were observed in the apical and supranuclear cytoplasm (Figure 4g). The staining images of VAMP-7 were nearly identical to those obtained with VAMP3 in that a clear positive reaction was confined to the basal cytoplasm in some of the secretory cells (Figure 4i). Antibodies to VAMP-8 gave rise to a moderate positive reaction of the secretory cells (Figure 4k).

A positive reaction for syntaxin 2 was detected in the secretory epithelium. In some of the secretory cells, strongly stained granules were observed in the apical cytoplasm (Figure 5a). The secretory cells were seen to react weakly after the application of anti-syntaxin 4 antibodies (Figure 5c). With regard to the approach for syntaxin 6, the secretory epithelium showed varying reaction intensity that some of the secretory cells exhibited a strong positive reaction (Figure 5e). A distinct reaction intensity of the apical surface of the secretory cells could be obtained after treatment with antibodies to SNAP-23 (Figure 5g). Antibodies to Rab3D caused a moderate positive reaction in the secretory cells (Figure 6a). When incubated with antibodies to pIRE1 $\alpha$ , a positive reaction was detected in their circum- and infranuclear parts in some of the secretory cells (Figure 7a). There was no significant difference in the expression pattern of pIRE1 $\alpha$  between the tall columnar and flattened cuboidal secretory cells. However, the number of pIRE1 $\alpha$ -positive cells in the for-

mer cells was slightly larger than that in the latter cells, subjectively.

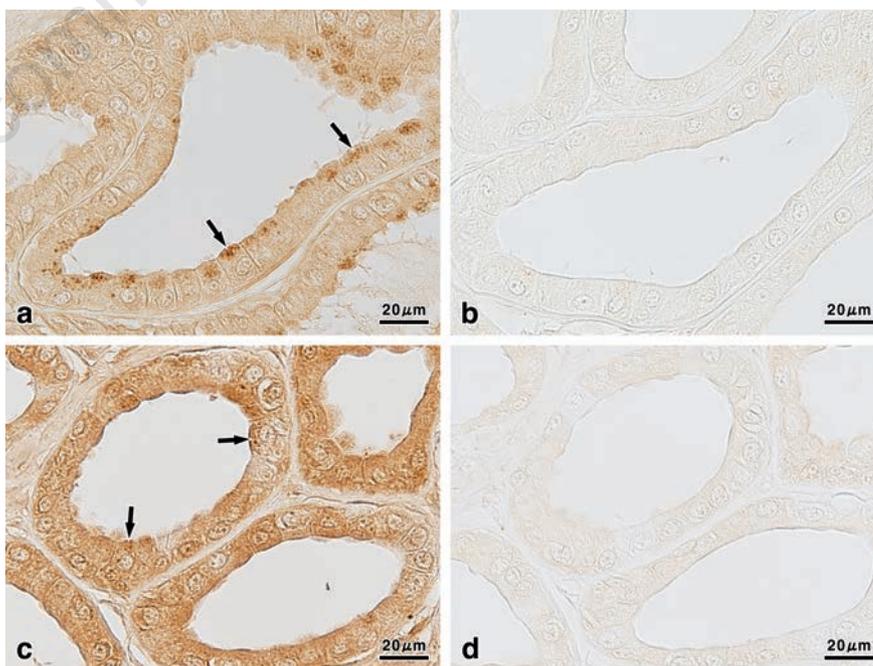
In all of the control experiments for the immunohistochemical staining, the positive reactions were greatly diminished or nearly abolished in the glandular structures of the ceruminous glands (Figures 3-7).

## Discussion

In the external auditory canal, the goat ceruminous glands were well-developed as compared with those of humans, horses and raccoons.<sup>4,6,7</sup> In human ceruminous glands, light vesicles are characterized as secretory vesicles,<sup>7</sup> because they are released by a merocrine secretion mode and rarely found in dry-type glandular cells.<sup>10,23</sup> Such findings correlate with our ultrastructural observations obtained in the goat ceruminous glands, except for the former view that the vesicles containing dense substances are also considered to be of secretory origin.<sup>5</sup> On the other hand, the irregularly shaped vesicles, which contained substances of medium or distinct electron density, are regarded as lysosomes from the viewpoint of the previous investigations.<sup>7,10,23,24</sup> In some of the secretory cells, the distinctive

concentric structures composed of rough ER were detected. They are similar to stress-activated response to  $\text{Ca}^{2+}$  depletion (SARC) bodies which appear specifically in murine differentiating myoblasts under ER  $\text{Ca}^{2+}$  depletion conditions.<sup>25</sup>

The UPR is an intracellular system mitigating ER stress which is caused by the accumulation of unfolded proteins in ER triggered by various environmental or physiological conditions, such as ER  $\text{Ca}^{2+}$  depletion, glucose shortage, viral infection or hypoxia.<sup>18,19,26</sup> IRE1 is the prototype ER stress sensor and highly conserved from yeast to humans and classified into two paralogues in mammals. IRE1 $\alpha$  is ubiquitously expressed, while the expression of IRE1 $\beta$  is principally confined to intestine tissues.<sup>26-28</sup> The activation of the UPR signaling pathways is involved not only in various diseases but also in the differentiation of plasma cells<sup>29</sup> and osteoblasts.<sup>30</sup> Additionally, it has already been known that IRE1 is constitutively activated in the pancreas, which produce considerable amounts of exocrine and endocrine secretory proteins.<sup>31</sup> In this study, the expression of pIRE1 $\alpha$  was observed in some of the ceruminous glandular cells. The distribution patterns of this enzyme were consistent with those of the SARC-like bodies. Thus, it is suggested that

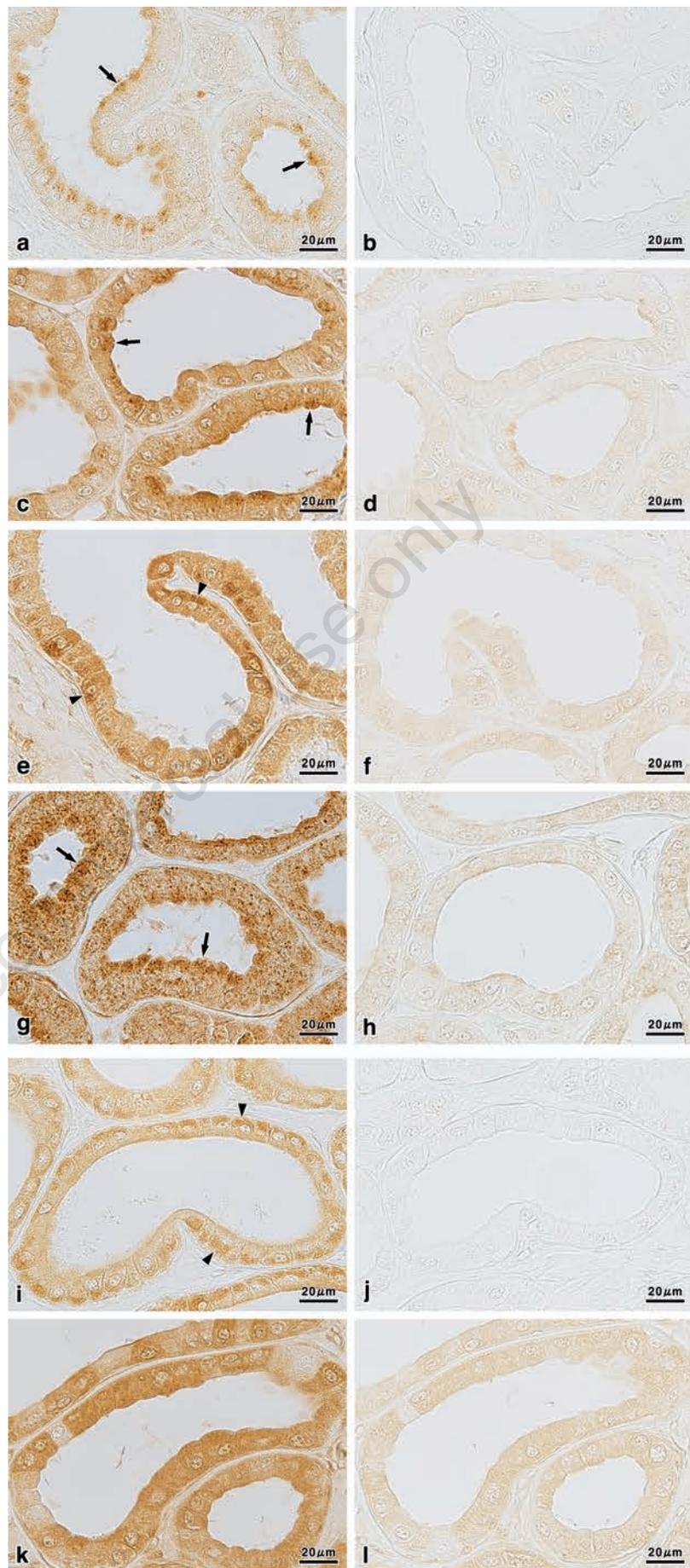


**Figure 3.** Immunohistochemical staining for the detection of  $\beta$ -defensins in the ceruminous glands. a)  $\beta$ -defensin 1. b) Negative control for  $\beta$ -defensin 1 using absorbed antibodies. c)  $\beta$ -defensin 2. d) Negative control for  $\beta$ -defensin 2 using absorbed antibodies. Arrows, positive-stained granules.

the formation of these bodies is caused by ER stress. However, the rings in the SARC bodies of differentiating myoblasts are not interconnected,<sup>25</sup> differing from those of the SARC-like bodies that seem to merge. In hepatic cells, ER stress triggered by Ca<sup>2+</sup> depletion leads to dilatation and degranulation of ER without forming the concentric ER-derived structures.<sup>32</sup> Such structural differences may indicate that the influence of ER stress on ER structures depends on the cell properties. On the other hand, the concentric ER-derived structures, similar to SARC-like body, are detected in the acinar cells of pancreatitis induced by overstimulation of cerulein, a cholecystokinin analogue, in male mouse.<sup>33</sup> These findings support the view that the goat ceruminous glandular cells have highly secretory activity. Furthermore, the activation of IRE1 $\alpha$  may participate in the homeostatic maintenance of these cells.

The present study also demonstrated the distribution of  $\beta$ -defensins in these glands by the immunohistochemical procedures. These antimicrobial peptides, which are widely distributed in various epithelial cells, has been expressed in the apocrine glands of several mammalian species.<sup>6,7,34-38</sup> Defensins are cationic antimicrobial peptides, and hence can insert themselves in phospholipid bilayer of the bacterial and fungal cell walls leading to disruption and subsequent death of the microorganisms.<sup>39-41</sup> Several antimicrobial peptides including  $\beta$ -defensins seem to be component of the light secretory vesicles in human ceruminous glands.<sup>7</sup>

The roles of SNARE proteins in the apocrine glands are not fully elucidated. Therefore, the detection of intracellular localization of SNARE proteins is significant for understanding their biological functions and regulation of membrane trafficking. Although VAMP-1 and -2 are characterized as a neuronal isoform, they are known



**Figure 4.** Immunohistochemical staining for the detection of v-snares in the ceruminous glands. a) VAMP-1. b) negative control for VAMP-1 using absorbed antibodies. c) VAMP-2. d) Negative control for VAMP-2 using absorbed antibodies. e) VAMP-3. f) Negative control for VAMP-3 using absorbed antibodies. g) VAMP-4. h) Negative control for VAMP-4 using absorbed antibodies. i) VAMP-7. j) Negative control for VAMP-7 using absorbed antibodies. k) VAMP-8. l) Negative control for VAMP-8 using absorbed antibodies. Arrows, positive-stained granules; arrowheads, positive reactions in basal cytoplasm.

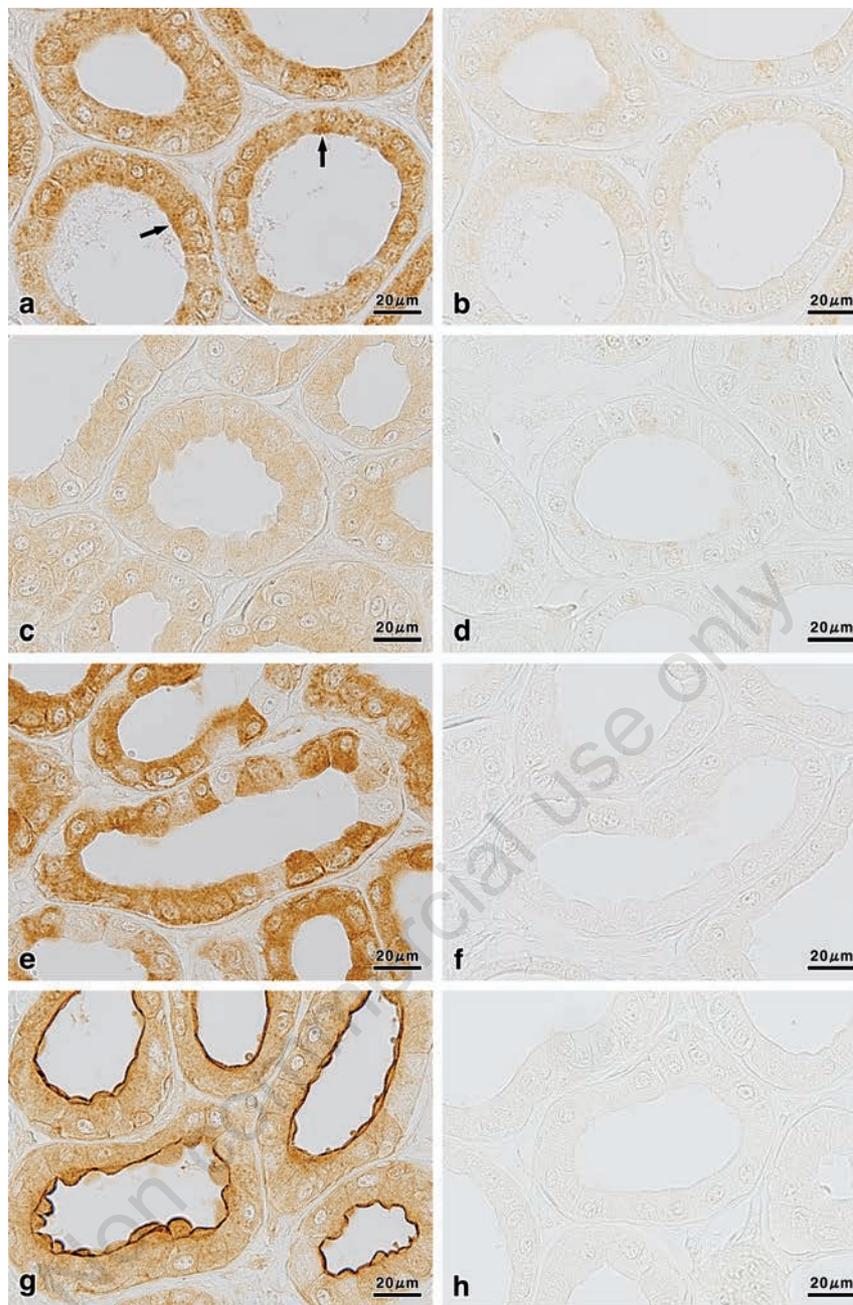


Figure 5. Immunohistochemical staining for the detection of t-snares in the ceruminous glands. a) Syntaxin 2. b) Negative control for syntaxin 2 using absorbed antibodies. c) Syntaxin 4. d) Negative control for syntaxin 4 using absorbed antibodies. e) Syntaxin 6. f) Negative control for syntaxin 6 performed by the incubation with normal mouse IgG. g) SNAP23. h) Negative control for SNAP23 using absorbed antibodies. Arrows, positive-stained granules.

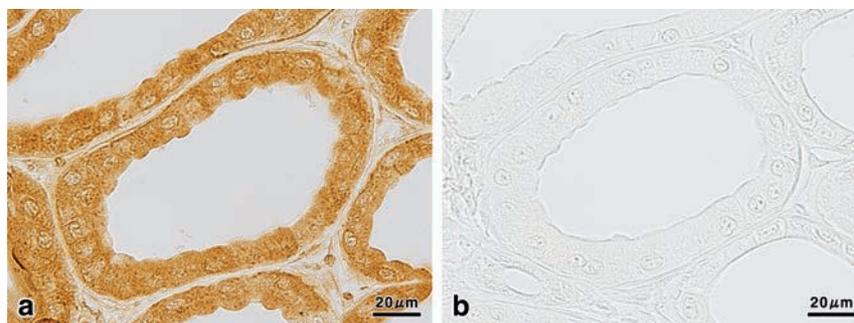
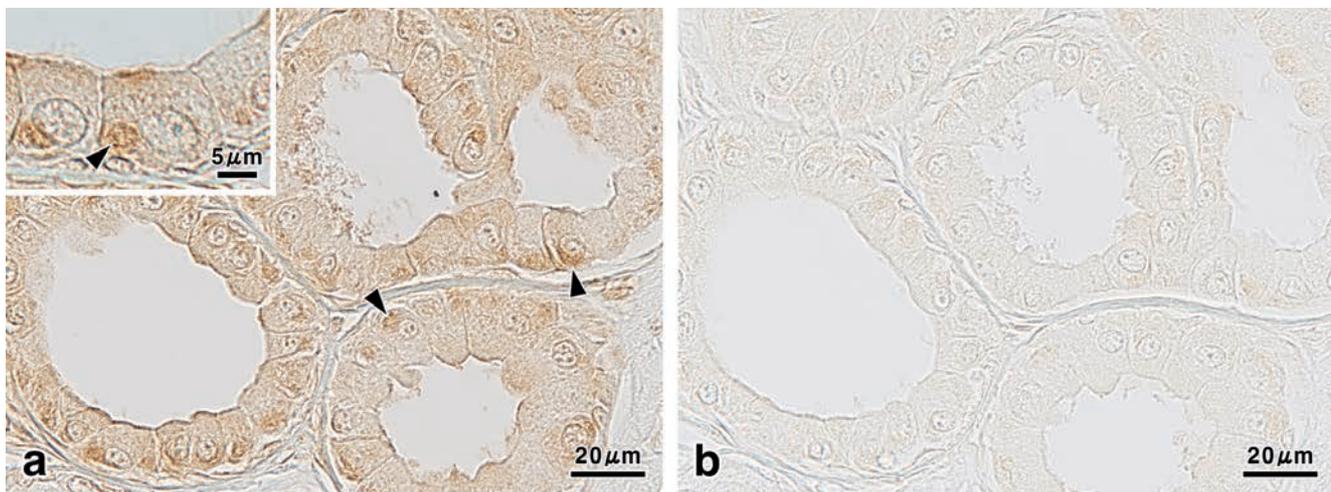


Figure 6. Immunohistochemical staining for Rab3D detection in the ceruminous glands. a) Rab3D. b) Negative control for Rab3D using absorbed antibodies.



**Figure 7. Immunohistochemical staining for the detection of activated IRE1 in the ceruminous glands. a) pIRE1 $\alpha$ ; inset (upper left) shows higher magnification of the secretory cells. b) Negative control for pIRE1 $\alpha$  using absorbed antibodies. Arrowheads, positive reactions in basal cytoplasm.**

to be expressed in endocrine and exocrine cells.<sup>42-44</sup> In rat parotid and pancreatic acinar cells, VAMP-2 is suggested to be involved in the release of amylase and zymogen granules.<sup>45,46</sup> Moreover, VAMP-8 is also detected in the secretory cells of several exocrine tissues, and may be interacted with syntaxin 4 and SNAP-23.<sup>47-49</sup> VAMP-2 may be concerned with constitutive exocytosis, while VAMP-8 is more significant for regulated secretion than for constitutive secretion.<sup>48,49</sup> In mouse mammary glands, VAMP-4 and -8 localized on casein-containing vesicles seem to participate in the formation of an apical SNARE complex for exocytosis. VAMP-4 is suggested to play an important role in the apical transport of these vesicles, whereas VAMP-8 may be directly related to exocytosis of casein together with syntaxin 6 and SNAP-23.<sup>50,51</sup> VAMP-3 and -7 serve as v-SNARE proteins for early and recycling endosomes<sup>52</sup> or late endosome to lysosome transport.<sup>53,54</sup> The possibility that VAMP-1, -2, -4 and -8 are concerned with the exocytotic pathway of the goat ceruminous glands is proposed, because these immunohistochemical features resemble those of  $\beta$ -defensins. In addition, it has been proposed that VAMP-8 and syntaxin 2 are involved in the release of the apocrine protrusion.<sup>15</sup> Rab proteins are known to function in tethering or docking of vesicles to its target compartment, leading to membrane fusion.<sup>55</sup> Rab3D is localized in secretory granules of exocrine glandular cells, and concerned with the regulation of exocytosis.<sup>16,56</sup>

In conclusion, the present study appeared for first time the presence of the

SARC-like bodies formed by rough ER and activated IRE1 $\alpha$  in the apocrine glandular cells, in this case the goat ceruminous glands. Furthermore, we demonstrated the localization of  $\beta$ -defensins, SNARE proteins and Rab3D. The findings indicating ER stress may imply that these glandular cells have the ability to actively produce secretory proteins and to maintain its functions. Moreover, it is suggested from our results that SNARE proteins and Rab3D are related to the secretory mechanism and regulation of these glands.

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