Cytochemical characterization of glycoconjugates in the apocrine glands of the equine scrotal skin

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Summary. Cytochemistry of glycoconjugates in the apocrine glands in the scrotal skin of the horse was studied using cytochemical methods for electron microscopy, particularly lectin cytochemistry. The secretory cells possessed a variable number of secretory vesicles, a well-developed Golgi apparatus, and abundant cisternae of the rough endoplasmic reticulum. Additionally, the basolateral plasma membrane formed numerous interdigitating folds. Glycoconjugates with vicinal diol groupings were present predominantly in the secretory vesicles, the Golgi apparatus, the surface coat of the plasma membrane, and the majority of the intracellular membranes. With lectin cytochemistry, the secretory vesicles of the glandular cells exhibited glycoproteins with different terminal sugars (α -D-mannose, β -D-galactose β -N-acetyl-D-glucosamine, and sialic acid). Several sugars were distinctly prominent in the surface coat of the plasma membrane of the secretory cells. The cytochemical properties of the complex glycoconjugates found are discussed in relation to the specific functions of the glandular secretions. These glands may have an important role in not only thermoregulation but protection of the scrotal skin, a specific body region.

Introduction

It is well recognized that the scrotum is one of the most specialized parts of the mammalian integuments, since it covers the primary sex organs (testes) and is provided with abundant smooth muscle cells in the hypodermis (Calhoun and Stinson, 1981). The apocrine skin glands of this region are concerned to some extent with thermoregulation in regard to testicular function (see e.g. Waites and Voglmayr, 1962; Robertshaw and Vercoe, 1980; Dhingra and Sharma, 1981). In addition, previous light microscopic studies have demonstrated that the secretory products of these glands contain various saccharide residues indicating the presence of complex glycoconjugates that have a broad functional significance as related to the signaling of sexual activities, water retention on the skin surface, and suppression of the proliferation of microorganisms (Tsukise and Yamada, 1981; Tsukise and Meyer, 1982, 1987; Tsukise *et al.*, 1985; Meyer *et al.*, 1987; ; Meyer and Tsukise, 1989).

The apocrine glands of the horse are the best developed, most numerous among various domesticated mammals, and quite active (Calhoun and Stinson, 1981; Kurosumi *et al.*, 1984). These glands observed in the equine scrotal skin are rather large and strongly coiled, and are associated with the primary hair follicles (PH) that form a sparse hair coat of about 100 PH/cm², whereby wool hairs are missing (Meyer, 2002). Although the general localization and properties of complex glycoconjugates in the secretion of these glands have already been revealed histochemically (Tsukise and Meyer, 1987), data are still lacking concerning a precise analysis of such carbohydrates at the cytological level. Therefore, the present study examined the localization of complex glycoconjugates in more detail using electron microscopic cytochemical methods. Such ultrastructural data may

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be indispensable for understanding the biological characteristics of these particular glands and their products in the scrotal skin.

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 scrotum of three adult horses (male, 4–5 years old, Thoroughbred), which were deeply anesthetized and then exsanguinated from the common carotid arteries.

General morphology

For general cytological observation, the skin specimens were fixed in a mixture of 4% paraformaldehyde (PFA) and 2% glutaraldehyde (GA) in 0.1 M phosphate-buffered saline (PBS) (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 routinely stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

Carbohydrate cytochemistry

For the cytochemical distribution of glycoconjugates, the specimens were fixed in a mixture of 4% PFA and 0.5% GA solution in 0.1 M PBS (pH 7.4) for 2 h at 4°C and embedded in LR-White resin (Newman *et al.*, 1983). Ultrathin sections were cut as described above and placed on nylon or nickel grids. The nylon grid-mounted sections were subjected to the periodic acid-thiocarbohydrazide-

silver proteinate-physical development procedure (PATCH-SP-PD) for the demonstration of vicinal diols of carbohydrates (Kitamura *et al.*, 1988; Yamada, 1993).

The sections on the nickel grids were incubated with different biotinylated lectins (Seikagaku Kogyo Co., Tokyo) at concentrations of 5–10 µg/ml in 0.05 M PBS (pH 7.2) for 12 h at 4°C (Roth 1983, 1996). The lectins used, their specific sugar residues and inhibitory sugars are listed in Table 1 (for lectin specificities see Debray *et al.*, 1981; Spicer and Schulte, 1992; Danguy, 1995). After rinsing with PBS, the 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). The ultrathin sections were 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 monosaccharide was added, or incubated while substituting unconjugated lectins for biotinylated lectins.

Results

General morphology

Ultrathin sections of the apocrine glands of the equine scrotal skin showed that the secretory epithelium consisted of a single layer of columnar glandular cells surrounded by an outer layer of myoepithelial cells (Fig. 1a). All the glandular cells rested on a basement membrane, and the oval nuclei of the secretory cells were located in the central or basal regions of the cytoplasm. The plasma membrane of the secretory cells facing the glandular lumen projected into well-developed microvilli. Additionally, the basolateral plasma membrane formed

Table 1. The lectins used and their sugar binding specificities

	Lectins	Sugar binding specificity	Inhibitory sugar
Con A	Concanavalin A	α -D-Man, α -D-Glc	α -D-Man, α -methyl-D-glucoside
RCA-120	<i>Ricinus communis</i> agglutinin-120	Gal β 1-4GlcNAc	β -D-Gal
WGA	Wheat germ agglutinin	β -D-GlcNAc, Sia	β -D-GlcNAc
SSA	<i>Sambucus sieboldiana</i> agglutinin	Sia α 2-6Gal/GalNAc	α 2-6sialyllactose
MAM (MAL)	<i>Maackia amurensis</i> agglutinin	Sia α 2-3Gal β 1-4GlcNAc	α 2-3sialyllactose

Fig. 1. General ultrastructure of the apocrine glands in the horse scrotal skin, uranyl acetate and lead citrate staining. **a:** The secretory portion consists of a single layer of glandular cells with underlying myoepithelial cells. $\times 4,300$. **b:** Part of the supranuclear cytoplasm of a secretory cell. $\times 11,400$

Abbreviations for figures. BM: basement membrane, Go: Golgi apparatus, Mi: mitochondria, Mv: microvilli, N: nucleus, PM: plasma membrane, rER: rough endoplasmic reticulum, SV: secretory vesicle, arrow: interdigitation, arrowhead: glycogen particle, double arrowhead: cis-side of Golgi apparatus

numerous interdigitating folds. In the supranuclear cytoplasm of the secretory cells, a distinct Golgi apparatus and a variable number of secretory vesicles were detected. These vesicles possessed a relatively low electron density and/or contained particles (Fig. 1b). Another feature noted was a prominent rough endoplasmic reticulum in the circum- and infranuclear cytoplasm. Throughout the cytoplasm of the glandular cells, mitochondria of varying morphologies were abundantly scattered among the above-mentioned ultrastructures.

Carbohydrate cytochemistry

In the glandular cells stained with the PA-TCH-SP-PD technique, the prominent features exhibiting positive

reactions were the surface coat of the plasma membrane, the secretory vesicles, and the cisternae of the Golgi apparatus. Other PA-TCH-SP-PD reactive structures included the glycogen particles and the majority of the intracellular membranes, such as elements of the rough endoplasmic reticulum and the nuclear envelope (Fig. 2a, b).

After the Con A-gold procedure, the secretory vesicles and glycogen particles showed a positive reaction, and reactive particles were also observed in the intracellular membrane of the elements of the rough endoplasmic reticulum (Fig. 3a, b). The RCA-120-gold procedure demonstrated reactive particles clearly concentrated in the surface coat of the plasma membrane and in secretory vesicles (Fig. 4a). Additionally, the Golgi apparatus

Figs. 2 and 3. Legends on the opposite page.

Fig. 4. RCA-120-gold staining of the apocrine glands in the scrotal skin. **a:** Part of the apical cytoplasm of secretory cells. Reactions in the surface coat of the plasma membrane and secretory vesicles. $\times 12,400$. **b:** Part of the supranuclear cytoplasm of a secretory cell. Reactions in secretory cells and the Golgi apparatus. $\times 15,200$

was found to exhibit a positive reaction (Fig. 4b). The WGA-gold procedure resulted in the same staining as that obtained with the RCA-120-gold procedure (Fig. 5). The free surface coat of the plasma membrane exhibited distinctly positive reactions after incubation with SSA, as did the secretory vesicles and the Golgi apparatus (Fig. 6). A few reactive particles for MAM were detected in the secretory vesicles, and the surface coat of the plasma

membrane and the Golgi apparatus also showed positive reactions (Fig. 7).

In the control sections stained with lectin cytochemical procedures by adding the appropriate inhibitory monosaccharides to the respective lectin solutions, and by substituting unconjugated lectins, the positive lectin reactions were greatly diminished or nearly abolished (Fig. 8).

Fig. 2. Cytochemical PA-TCH-SP-PD staining of the apocrine glands in the scrotal skin. **a:** A series of ultrastructures exhibits positive reactions. $\times 3,800$. **b:** Part of the supranuclear cytoplasm of a secretory cell with reactions in secretory vesicles, cisternae of the Golgi apparatus, and elements of the rough endoplasmic reticulum. $\times 11,400$

Fig. 3. Con A-gold staining of the apocrine glands in the scrotal skin. **a:** Part of the supranuclear cytoplasm of a secretory cell. Reactions in secretory vesicles. $\times 15,200$. **b:** Part of the infranuclear cytoplasm of a secretory cell. Reactions in the elements of the rough endoplasmic reticulum. $\times 19,000$

Fig. 5. WGA-gold staining of the apocrine glands in the scrotal skin. Part of the apical cytoplasm of a secretory cell. Reactions in the surface coat of the plasma membrane and secretory vesicles. $\times 13,300$

Fig. 6. SSA-gold staining of the apocrine glands in the scrotal skin. Part of the apical cytoplasm of secretory cells. Reactions in the surface coat of the plasma membrane and secretory vesicles. $\times 15,200$

Discussion

The apocrine skin glands found in the scrotal region are known to produce more sweat than elaborated by same gland type in the common integument in order to maintain low scrotal temperature, closely related to testicular temperature (Robertshaw and Vercoe, 1980). Although these glands of the horse have the morphological characteristics of the apocrine gland type in the common integument (Meyer and Neurand, 1987; Meyer, 2002), their functions seem to be similar to those of the eccrine glands in the human skin (Kurosumi *et al.*, 1984). According to previous investigations, the prominent features noted in the secretory cells of the apocrine glands in the equine common integument are a large number of secretory vacuoles seen as empty or very clear, as well

as the well-developed rough endoplasmic reticulum and Golgi apparatus. Additionally, many long microvilli protrude from the luminal surface of the secretory cells (Kurosumi *et al.*, 1963; Sørensen and Prasad, 1973; Meyer and Neurand, 1987). Such findings are consistent with our ultrastructural observations obtained in the apocrine glands in the scrotal skin, except that the secretory vesicles were of low density and/or contained particles. Moreover, the presence of many mitochondria and of distinct basolateral interdigitations may be connected with a considerable capacity of transepithelial ion-related fluid transport (Stoeckelhuber *et al.*, 2003).

In view of the staining selectivity of the PA-TCH-SP-PD procedure (Yamada, 1993), the majority of the ultrastructures of the apocrine glands in the scrotum, such as the surface coat of the plasma membrane, secretory vesicles, glycogen particles, and intracellular membranes

Fig. 7. MAM-gold staining of the apocrine glands in the scrotal skin. Part of the apical cytoplasm of secretory cells. Reactions in the surface coat of the plasma membrane and secretory vesicles. $\times 15,200$

Fig. 8. Control experiment for lectin cytochemical procedures of the apocrine glands in the scrotal skin. Part of the apical cytoplasm of secretory cells. $\times 11,400$

of the rough endoplasmic reticulum and the nuclear envelope obviously contained carbohydrates with vicinal diol groupings. Additionally, the presence of glycoproteins with various saccharide residues became evident by the more sensitive lectin cytochemical procedures. In the secretory epithelial cells, β -D-galactose, β -N-acetyl-D-glucosamine, and sialyl residues were detected in the surface coat of the plasma membrane, particularly at the luminal surface, defining the specific cellular glycocalyx (Roth, 1996; Pavelka and Roth, 2005). In this case, the same free sugar residues were found, for example, as in the surface coat of the secretory cells of the eccrine glands of digital pads (Yasui *et al.*, 2005). Furthermore, the secretory vesicles obviously contained considerable amounts of α -D-mannose, β -D-galactose, β -N-acetyl-D-glucosamine, and sialyl residues.

Equine sweat elaborated by apocrine glands has an

unusually high concentration of protein as compared with that of other mammalian species (Jenkinson *et al.*, 1974; McCutcheon and Geor, 1998). Additionally, it also contains glycoproteins with neutral carbohydrate and sialyl residues. Their surfactant-like quality has been considered to facilitate a more even distribution of sweat over the skin surface so as to improve sweat evaporation (Eckersall *et al.*, 1984). In this connection, all the terminal sugars found in the secretory vesicles of the apocrine glandular cells in the equine scrotum have been shown to exert certain antimicrobial functions on the skin surface, because they have the ability to prevent the adherence of different bacteria and fungi to epidermal cells, following liberation from their glycoprotein bases by microbial activities (Meyer *et al.*, 2000, 2001). The inclusion of β -D-galactose and sialic acid, moreover, indicates the presence of mucin-type glycoproteins (Strous and Dekker,

1992; Campbell, 1999). Secreted mucin can form a barrier that shields epithelial cells and selects substances for binding and uptake by epithelia (Strous and Dekker, 1992). In this case, sialic acid could be a regulatory factor participating in the determination of mucus viscosity (Majima *et al.*, 1999). All of these glycoconjugates may be of general importance for the rheological properties of water containing mucus on the skin surface (Yasui *et al.*, 2003). Sialoglycoconjugates seem also to protect cells from enzymatic and immunological attacks and can function as recognition sites for various receptors (Kelm and Schauer, 1997; Schauer, 2004). The presence of glycogen in the secretory cells agrees with the observations of other authors (Evans *et al.*, 1957; Tsukise and Meyer, 1987) and seems to be related to high energy demands during secretion production.

In conclusion, our study revealed in detail the substructural localization of glycoproteins with different saccharide residues in the secretory cells of the apocrine glands in the equine scrotal skin, a finding difficult to be obtained from the results of the histochemical investigation at the light microscopic level. The complex glycoconjugates produced by these secretory cells may be involved in further important functions, such as improving evaporation of sweat and preventing microbial attacks to the epidermis and secretory cells themselves. Thus, these glands seem to participate in the evaporative cooling and protect the integrity of the scrotal skin concerned with the preservation of testicular functions. Our results corroborate the findings of a previous study on this gland type in the horse (Tsukise and Meyer, 1987).

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