Histol Histopath (1993) 8: 279-284

Histology and Histopathology

Histochemical localization of glycosaminoglycans in the omasal papillae of sheep

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Summary. Histochemical localization of the glycosaminoglycans in the omasal laminae were examined at light and electron microscopic levels. The core region of the omasal papillae was stained strongly with alcian blue at pH 2.5 and 1.0. The staining was degraded moderately and completely when tissue sections were pretreated with mild and active methylation, respectively. Alcianophilia was moderately decreased with saponification. Furthermore, enzymatic digestion procedures showed that these alcianophilic substances contained a large amount of hyaluronic acid and chondroitin sulphate. Ultrastructurally, a spider web-like structure was widely distributed among the spindle-shaped fibroblast-like cells and fibrous networks of collagen and elastin. These results suggest that the core region of the omasal papillae not only acts as a physical buffer resisting the local pressure from the lumen, but may also influence material transport through the omasal mucosa.

Key words: Sheep, Forestomach, Omasum, Glycosaminoglycans, Enzyme digestion, Ruthenium red

Introduction

The omasum is the third compartment of the ruminant forestomach, lined with nonglandular keratinized epithelium. Many parallel folds named omasal laminae arise from the large curvature of the organ and project into the lumen. The surface of the laminae is studded with many omasal papillae. Physiologically, the omasal epithelium has different characteristics from the ruminal epithelium, including a higher electrical potential and short circuit current (Harrison, 1971; Martens and Gäbel, 1988). The omasum secretes chloride ion and absorbs water, short chained-fatty acids and some electrolytes (Engelhardt and Hauffe, 1975). After examination of the morphological characteristics of the surface structure, the subepithelial capillary networks and the subepithelial connective tissue of sheep omasal mucosa by scanning electron microscopy (Yamamoto et al., 1991, 1993a,b), it has been suggested that omasal papillae is highly adapted to the omasal function including absorption and/or secretion.

Glycosaminoglycans are amorphous intercellular substances, are highly hydrophilic and have a high negative charge density. They play an important role in the resisting local mechanical forces and in regulating the diffusion of small molecules and ions (Comper and Laurent, 1978). It has been reported that the core of the omasal papillae stain with cationic dyes such as alcian blue (Blownlee and Elliot, 1960; Krölling and Grau, 1960; Habel, 1963). However, a detailed histological description of its histochemical staining properties is still lacking.

Thus, the present study was performed in order to clarify the histochemical localization of the glycosaminoglycans in the omasal papillae at light and electron microscopic levels.

Materials and methods

Nine adult sheep of both sexes were used. The animals were killed by exsanguination in the University Slaughterhouse. The specimens were cut from oral (near the reticulo-omasal orifice), middle and aboral (near the omaso-abomasal orifice) regions of the omasal laminae and fixed with 10% formalin containing 2% calcium acetate for two days at 4 °C. Specimens from rumen and reticulum, the first and second compartments of the forestomach respectively, were also used for comparison. Tissues were embedded in paraffin, sectioned at 5 μm and stained with haematoxylin-eosin, the periodic acid-Schiff (PAS) reaction, Watanabe's silver impregnation and the elastica van Gieson method for general observation.

Histochemically, the sections were stained with PAS to demonstrate glycoconjugates containing vicinal diols,

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and with alcial blue 8GX (AB; I.C.I. Dyestuffs, Blacklay) at pH 2.5 for all acidic functional groups and at pH 1.0 to demonstrate sulphated groups (Pearse, 1968). Some sections were treated with mild and active methylation prior to AB (pH 2.5 and 1.0) staining (Spicer, 1960). Other sections were treated with mild or active methylation-saponification-AB (pH 2.5) staining sequences (Spicer, 1960).

Further information concerning the presence of specific glycosaminoglycans was provided by enzymatic digestion performed prior to AB staining. Enzymes used were hyaluronidase from *Streptomyces hyalurolyticus* (Seikagaku Kogyo Co., Tokyo) and bovine testis (Type VI-S; Sigma, St. Louis), chondroitinase ABC from *Proteus vulgaris* (protease free, Seikagaku Kogyo Co.), chondroitinase B and heparitinase from *Flavobacterium heparinum* (Seikagaku Kogyo Co.). Details of the use of these enzymes and their substrate specificity are provided in Table 1.

For ultrastructural investigation, the omasal papillae from three animals were fixed for 2 hours at 4 °C in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) containing 1500 ppm ruthenium red solution purified as described by Luft (1971a). Postfixation were carried out for 1.5 hours at room temperature in 1% osmium tetroxide in the same buffer in the presence of ruthenium red. After dehydration, the specimens were embedded in Spurr's resin and cut by an ultramicrotome. These sections were observed in the transmission electron microscope (H-600, Hitachi, Tokyo) without counterstaining.

Results

The core of the omasal papillae was composed of loose connective tissue (Figs. 1, 2). This area was not

Table1. Enzyme digestion procedure.

ENZYME	CONCENTRATION	BUFFER	pH	TIME	TEMPERATURE	SUBSTRATES	REFERENCES
Hyaluronidase *(Streptomyces hyalurolyticus)*	50 TRU/ml	0.1M PB	6.0	4 h	37 °C	HA	Yamada (1973), Yamada and Hirano (1973)
Hyaluronidase (Bovine testis)	750 unit/ml	0.1M PB	5.5	4 h	37 °C	HA, C, CS	Leppi and Stoward (1965), Yamada (1973)
Chondroitinase ABC *(Proteus vulgaris)*	1 unit/ml	0.1M Tris-HCl	8.0	4 h	37 °C	HA, C, CS, DS	Yamada (1974), Yamada et al. (1982)
Chondroitinase B *(Flavobacterium heparinum)*	1.5 unit/ml	0.1M Tris-HCl	8.0	4 h	30 °C	DS	Michelacci and Dietrich (1975)
Heparitinase *(Flavobacterium heparinum)*	0.5 unit/ml	0.1M Na-Acetate	7.0	3 h	37 °C	HS	Silverberg et al. (1985)

PB, phosphate buffer; Tris-HCl, Tris-HCl buffer; Na-Acetate, sodium-acetate buffer; HA, hyaluronic acid; C, Chondroitin; CS, chondroitin sulphate; DS, dermatan sulphate; HS, heparan sulphate.

Fig. 1. Omasal papilla from the oral region. Haematoxylin-Eoxin. x 75

Fig. 2. Higher magnification of the core region of Fig. 1. Loose connective tissue with fibroblast-like cells are seen. x 300

Fig. 3. The omasal papilla stains with AB (pH 2.5). The core region of the papilla is strongly stained. x 40

Fig. 4. The omasal papilla stains with AB (pH 1.0). The core region is also stained. x 40

Figs. 5-8. Serial sections of the omasal papilla. x 50

Fig. 5. The omasal papilla stains with AB (pH 2.5); the control section for Figs. 6-8.

Fig. 6. Mild methylation performed prior to AB (pH 2.5) staining. The stainability of the core region is moderately weakened.

Fig. 7. Active methylation performed prior to AB (pH 2.5) staining. The stainability of the core region has totally disappeared.

Fig. 8. Active methylation-saponification-AB (pH 2.5) sequence. The stainability of the core region of the omasal papilla has been partially restored.

Figs. 9-12. Semi-serial sections of the omasal papilla. x 50

Fig. 9. The alcianophilic region of the core region of the omasal papilla shows moderately weaker reaction. Streptomyces hyaluronidase-AB (pH 2.5).

Fig. 10. The alcianophilic region of the core region of the omasal papilla shows total abolition of reaction. Testicular hyaluronidase digestion-AB (pH 2.5).

Fig. 11. The alcianophilic region of the core region of the omasal papillae shows disappearance of reaction. Testicular hyaluronidase digestion-AB (pH 1.0).

Fig. 12. The alcianophilic region of the core region of the omasal papillae shows abolition of reaction. Chondroitinase ABC digestion-AB (pH 1.0).

bordered by any discrete structure and merged gradually into the surrounding connective tissue continuous to the inter-papillary spaces. There were a few spindle-shaped fibroblast-like cells, vascular components and rich ground substances with a large quantity of elastic fibres. Similar structures with a relatively small amount of the elastic fibres were also present in the reticular papillae but not in the rumen. Reticular fibres were not observed in the core of the reticulum nor the omasal papillae.

Results of histochemical stainings, including

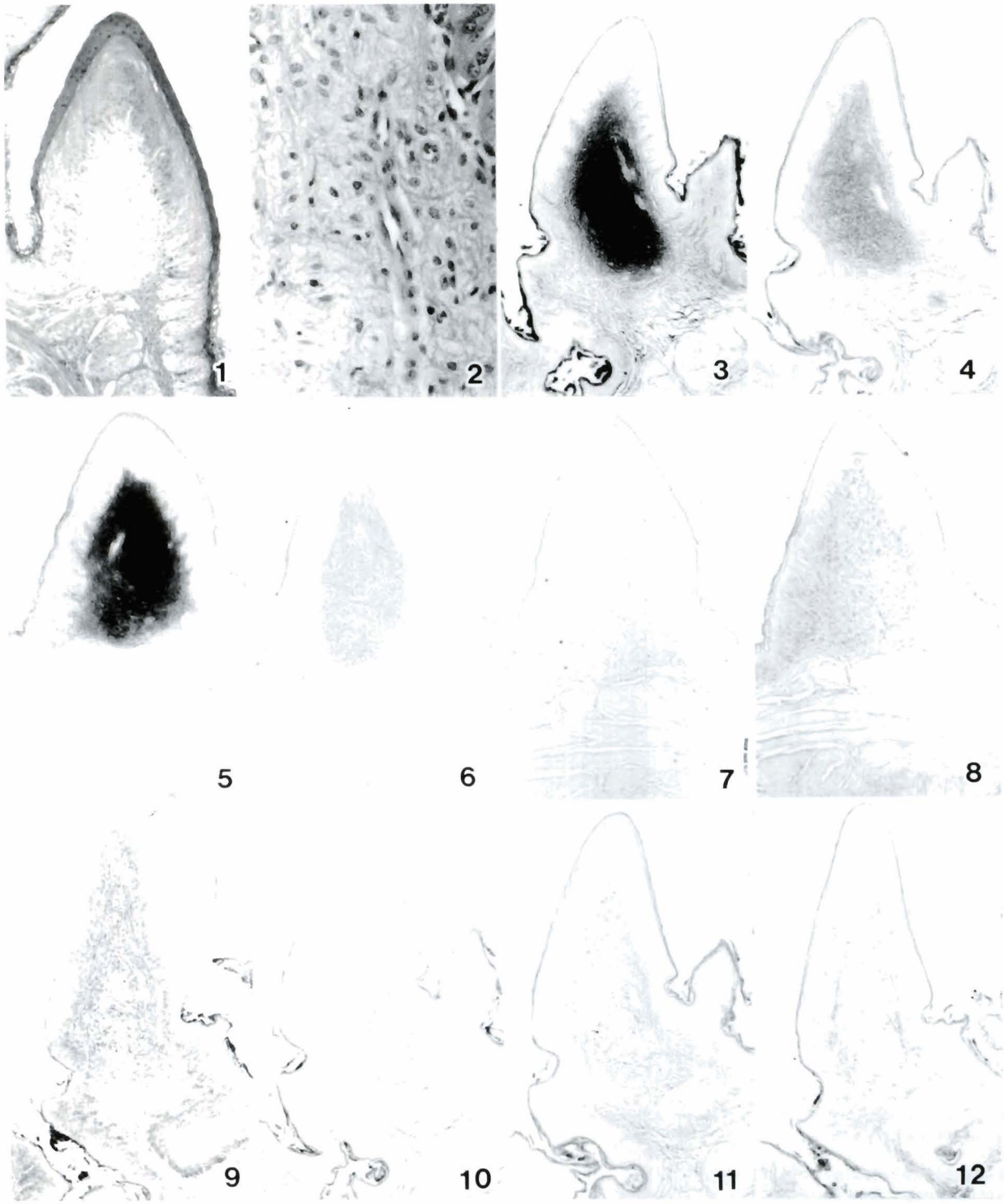
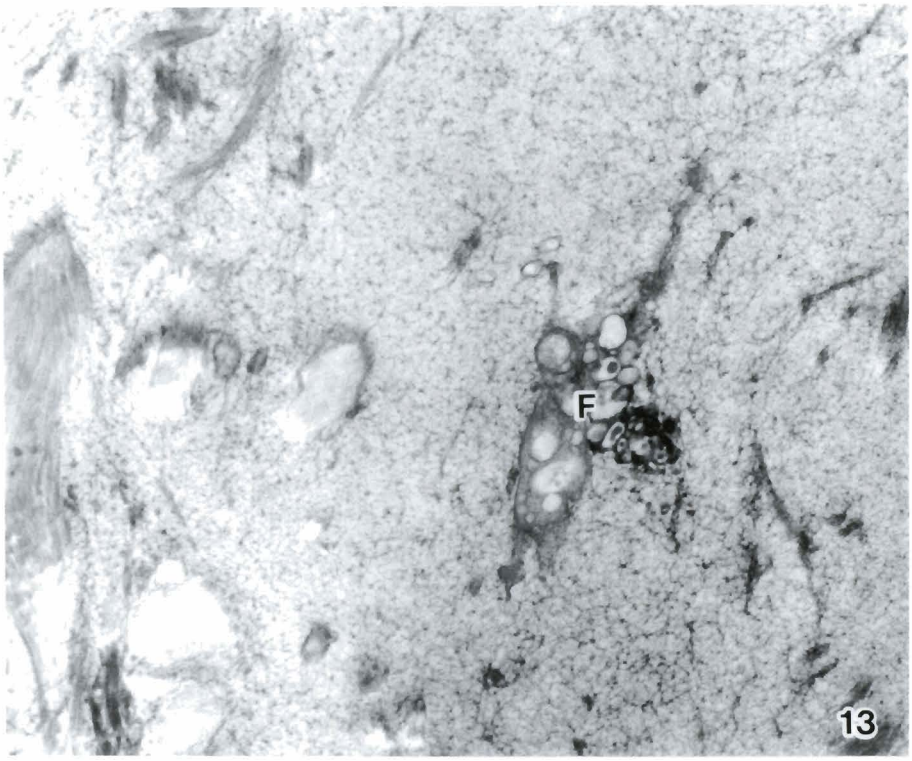


Table 2. Histochemistry of acid carbohydrate in the sheep omasum.

	ICS	BASAL CELLS	BASAL LAMINA	CORE OF PAPILLA	MAST CELL	NUCLEI
AB (pH 2.5)	++	±	±	++	++	+
AB (pH 1.0)	++	+	+	++	++	-
Chemical modification						
M-MET-AB (pH 2.5)	++	-	±	+	++	-
M-MET-AB (pH 1.0)	++	-	±	+	++	-
A-MET-AB (pH 2.5)	-	-	-	-	-	-
A-MET-AB (pH 1.0)	-	-	-	-	-	-
M-MET-SAP-AB (pH 2.5)	+	-	±	+	±	±
A-MET-SAP-AB (pH 2.5)	+	-	-	+	-	±
Enzyme digestion						
SHase-AB (pH 2.5)	++	±	±	+	++	+
THase-AB (pH 2.5)	++	±	±	±	++	+
THase-AB (pH 1.0)	++	+	+	±	++	-
CaseABC-AB (pH 2.5)	++	±	±	±	++	+
CaseABC-AB (pH 1.0)	++	+	+	±	++	-
CaseB-AB (pH 1.0)	++	+	+	+	++	-
Hase-AB (pH 1.0)	++	±	±	+	++	-

AB, alcian blue; ICS, inter-cellular spaces of the keratinized epithelial cells; M-MET, mild methylation; A-MET, active methylation; SAP, saponification; THase, testicular hyaluronidase; SHase, streptomyces hyaluronidase; CaseABC, chondroitinase ABC; CaseB, chondroitinase B; Hase, heparinitase; ++, strong; +, moderate; ±, weak; -, negative.



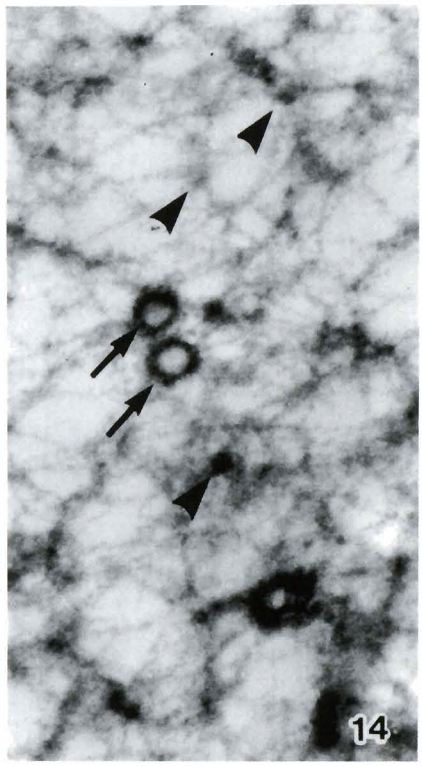


Fig. 13. An electron micrograph of the core of the omasal papilla stained with ruthenium red. A ruthenium red-positive web-like network is found among the fibroblast-like cells (F) and fibrous component. x 9,000

Fig. 14. A higher magnification of Fig. 13. Ruthenium red-positive particles (arrowheads) with filamentous components found among them. Peripheral regions of the collagen fibrils (arrows) are also stained with ruthenium red. x 100,000

chemical modification and enzymatic digestion procedures, were generally similar in the omasal papillae from the three regions. Furthermore, the staining pattern of the core of the reticulum papillae was similar to that of the omasal papillae. The findings on the omasum are summarized in Table 2.

The core of the omasal papillae was intensely stained with alcian blue both at pH 2.5 and 1.0 (Figs. 3, 4). Mast cells and intercellular substances in the keratinized epithelial layer were also intensely stained at both pH values. The surface of epithelial cells of the spinous and basal layers were weakly stained at pH 1.0. AB staining at pH 1.0 and 2.5 changed markedly in the papillary core when the chemical modifications and the enzymatic digestion procedures were performed (Figs. 5-12). AB staining at pH 2.5 was abolished by active methylation and decreased by mild methylation (Figs. 6, 7). When the active methylation was followed by the saponification, some alcianophilia (pH 2.5) was recovered (Fig. 8). Streptomyces hyaluronidase digestion moderately reduced stainability with AB (pH 2.5) (Fig. 9). Digestion with testicular hyaluronidase or chondroitinase ABC almost completely abolished the staining with alcian blue at pH 1.0 and 2.5 (Figs. 10-12). Chondroitinase B and heparitinase digestion promoted only a small reduction in staining intensity with AB (pH 1.0). The other structures which reacted with alcian blue were not affected by these enzymatic digestions except for some effect of heparitinase on the basal lamina of the epithelium.

Ultrastructurally, there were ruthenium red-reactive particles (about 15-30 nm in diameter) and thin threads (about 3-5 nm in diameter) in the extracellular space in the core of the omasal papillae (Figs. 13, 14). These threads formed spider web-like networks. The particles were arranged randomly and at the junctions of the threads. The networks were found among fibroblast-like cells, collagen fibrils and elastic fibres. The peripheral region of the elastic fibres and basal lamina also reacted positively.

Discussion

The histological characteristics of the core of the omasal papillae of sheep revealed in the present study were similar to those reported by Basu et al. (1957), Blownlee and Elliot (1960), Krölling and Grau (1960) and Habel (1963). The alcianophilic regions in the omasal papillae seem to be common in the adult animals of the domestic ruminant species.

The present study revealed further details concerning the histochemical nature of the core tissue of ovine omasal papillae. Mild methylation degrades carboxyl groups, whereas active methylation hydrolyzes both carboxyl groups and sulphate-esters of the mild methylation (Spicer, 1960). On the other hand, saponification restores the affinity for cationic dyes of the carboxyl groups which were lost following methylation (Spicer, 1960). Thus, the present results indicate that the alcianophilic regions in the omasal papillae contain a large amount of carboxyl groups as well as some sulphated groups. Furthermore, the results of the enzymatic digestion suggest that this tissue contained at least some hyaluronic acid and chondroitin sulphate.

The ultrastructural observations of the extracellular matrices have been described in dermis and cartilage (Luft, 1971b; Myers et al., 1973). The spider web-like network found in the present observation is similar to the case of dermis. It probably interacts between the cells and the fibrous networks as Junqueira and Montes (1983) described for various tissue.

The present results help to explain the significance of the omasum from a functional point of view. The omasal epithelium has different characteristics from the ruminal epithelium on the basis of ion transport and has a higher electrical potential and short circuit current (Harrison, 1971; Martens and Gäbel, 1988). The omasum secretes chloride ions and absorbs water, short chained-fatty acids and some electrolytes (Engelhardt and Hauffe, 1975). On the other hand, the carboxylated and sulphated glycosaminoglycans have a large amount of fixed negative charge. They participate in the diffusion of water and small molecules *in vitro* (Comper and Laurent, 1978; Comper et al., 1990). For example, articular cartilage has ion-exchange properties (Maroudus, 1968). The glycosaminoglycans in the omasal papillae may affect diffusion and transport of small molecules within the mucosa, especially of charged molecules and ions. This may contribute to the different physiological properties of omasum as compared to the rumen, which lacks any concentration of alcianophilic tissue. Moreover, proteoglycans containing glycosaminoglycans should be resistant to local mechanical changes and may help to maintain tissue organization due to their hydrophilic properties and in cooperation with the collagen and/or elastin networks, as in various other tissues; for review, see Comper and Laurent (1978) and Junqueira and Montes (1983).

In conclusion, the histochemical characteristics of the omasal papillae seem to be suitable for helping to regulate absorption and/or secretion. The sulphated and non-sulphated glycosaminoglycans in the core of the omasal papillae are likely to affect the movement of molecules within the mucosa and to resist local mechanical changes. In addition to the morphological characteristics of the surface structure, the subepithelial capillary networks and the subepithelial connective tissue (Yamamoto et al., 1991, 1993a,b), the glycosaminoglycan content of the omasal papillae is one of its distinctive characteristics, and may be related to the function of the omasum in processes such as absorption and/or secretion.

Acknowledgements. This work was partly supported by a Sasakawa scientific grant (3-076) from the Japanese Scientific Society.

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Accepted November 20, 1992