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Alternating laminated array of two types of mucin in the human gastric surface mucous layer

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Summary

Attempts have been made to develop a procedure for preserving and analysing the surface mucous layer of the human stomach in paraffin sections. Histologically normal gastric mucosae were obtained from 20 surgically removed stomachs. Of the different fixatives tested, Carnoy's solution gave rise to the most satisfactory results. In Haematoxylin–Eosin stained sections, the surface mucous layer appeared as a thick eosinophilic layer coating the gastric mucosal surface and measured 55.4 ± 2.5 μm in the fundus and 21.8 ± 1.0 μm in the pylorus respectively. A dual staining method consisting of galactose oxidase–cold thionine Schiff and paradoxical concanavalin A staining was applied to the surface mucous layer in order to reveal the distribution pattern of mucins secreted by two types of mucous cell in the gastric mucosa: surface mucous cells and gland mucous cells. As a result of this staining, an alternating laminated layer was visualized which consisted of the particular two types of mucin. In five cases, the surface mucous layer was examined in unfixed frozen sections. This layer was only partially preserved but revealed the same laminated structure. These results indicated that gland mucous cell mucins contribute to form the surface mucous layer.

Introduction

The mucosal surface of the stomach is coated with a continuous mucous layer which occurs in two forms, i.e., water-insoluble mucus gel adhering to the mucosal surface, and soluble mucus mixed with the luminal juice (Allen & Carroll, 1985). The surface mucus gel layer has been thought to play an important role in protecting the mucous membrane from noxious agents (Allen *et al.*, 1986; Allen *et al.*, 1989; Allen, 1989). Both the histological visualization and histochemical analysis of the surface mucous gel layer, however, have been hampered by the difficulty of preserving it in tissue preparations, and little or no extracellular mucus is visible on tissue sections routinely fixed and stained for light microscopy (Morris *et al.*, 1984). One of the most successful attempts to examine the surface mucosal gel layer was made by Kerss *et al.*, who prepared unfixed, thick gastric tissue sections from the rat and human by using parallel razor blades and observed them with an inverse microscope (Kerss *et al.*, 1982). According to this method, the surface mucus layer was recognized as a dark, opaque, continuous layer.

However, the internal structures of the surface mucous layer and the distribution of different mucins within it remained to be clarified. In the present study, attempts have been made to explore means for preserving the gastric mucous layer in paraffin sections and to reveal the pattern in which different mucins derived from the gastric mucosa could contribute to the formation of the layer. The results obtained have shown that the surface mucous layer exhibited a characteristic structure and consisted of alternating laminated layers of two types of mucins, surface mucous cell mucins and gland mucous cell mucins, the latter including mucins of cardiac gland, mucous neck and pyloric gland cells. The present techniques for preserving the surface mucus layer in paraffin sections and the dual staining method employed would be useful for investigations into its pathophysiological role.

Materials and methods

The surface mucous layer of human stomachs, surgically removed due to gastric carcinoma, was studied by following two procedures. In both procedures we took extreme care not to affect the mucosa mechanically.

1. Five cases were examined by this procedure. Immediately after the removal, macroscopically normal portions measuring approximately 1×0.5 cm were resected from the fundus and antrum. Without any rinsing procedures, the tissue specimens were laid in plastic cases (Cryomold, Miles Laboratories, Naperville, IL, USA) and snap-frozen in OCT embedding medium (Miles Laboratories, Naperville, IL, USA) by submersion

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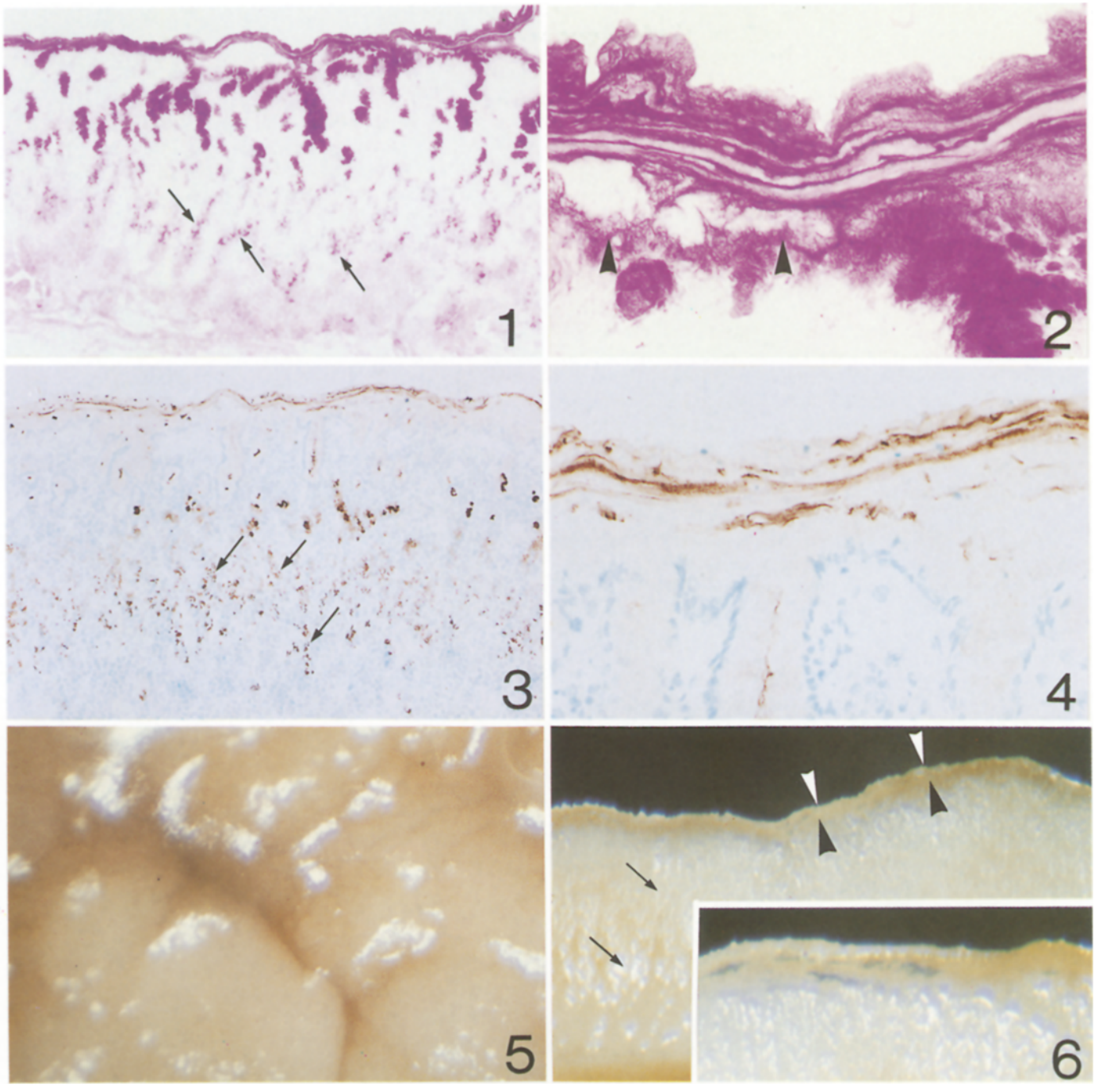


Fig. 1. Fundic mucosa. The mucosal surface is coated by PAS-positive mucous layer showing lamination. Surface mucous cells are more intensely stained than mucous neck cells (arrows). Amylase digestion-PAS. ×45.

Fig. 2. Fundic mucosa. Higher magnification of the surface mucous layer. Lamellar arrays showing intense or weak reaction are evident. The surface mucous layer is detached from the mucosal surface (arrow heads) and the luminal surface of the surface mucous layer is ragged. Amylase digestion–PAS. ×225.

Fig. 3. Fundic mucosa. Mucous neck cells reveal intense GSA-II reactivity (arrows). The mucosal surface is coated by the mucous layer showing lamination. GSA-II-HRP staining. ×45.

Fig. 4. Fundic mucosa. Higher magnification of the surface mucous layer. Mucous neck cell mucins form dark brown layers, whereas surface mucous cell mucins remain almost unstained. The stratified arrangement composed of these two types of mucins is evident. GSA-II-HRP staining. ×225.

Fig. 5. Carnoy-fixed material viewed from above under a dissecting microscope. A translucent coat covering the mucosal surface is evident. ×54.

Fig. 6. A Carnoy-fixed tissue block viewed transversely under a dissecting microscope. The mucosal surface is covered with continuous semitransparent layer (between arrowheads). The gastric glands show a striated pattern (arrows) on the section surface. ×30. *Inset*: Higher magnification of the surface mucous layer. ×90.

Figs 1 to 4 were prepared from frozen sections; Figs 5 and 6 were prepared from Carnoy-fixed materials.

in an isopentane bath cooled in liquid nitrogen. Serial sections of approximately 5 μm thickness of each material were cut on a cryostat and mounted on poly-L-lysine-coated slides (Muto Pure Chemicals, Tokyo, Japan). These frozen sections were dried at room temperature, coated with 0.2% celloidin in 1 : 1 v/v-ethanol–diethyl ether and stained by amylase digestion-PAS reaction or a lectin, *Griffonia simplicifolia* agglutinin-II (GSA-II) labelled with horseradish peroxidase (HRP) (E.Y. Laboratories, San Mateo, CA, USA). GSA-II is known to bind selectively with gastric gland mucous cells (Ihida *et al.*, 1988). Although the paradoxical concanavalin A staining surpasses the GSA-II-HRP staining in its specificity for the gland mucous cell mucins, the latter staining was employed to avoid damage of the surface mucous layer caused by the reduction step in the paradoxical concanavalin A staining. The staining procedure of GSA-II-HRP followed the method described previously (Katsuyama *et al.*, 1985).

2. Twenty cases were examined by this procedure. Immediately after removal, normal-appearing gastric portions of approximately 2 × 2 cm were excised from the fundus and antrum of the resected stomachs. Without any rinsing procedures, the tissue specimens thus obtained were laid flat with the mucosal surface up and pinned on a supporting board. The luminal gastric fluid was almost completely lost during this processing. Then, these specimens were immersed in one of a variety of fixatives such as phosphate-buffered 10% formalin (pH 7.4), 100% ethyl alcohol, Carnoy's solution, Bouin's solution and a buffered $HgCl_2$–glutaraldehyde solution (containing 6% $HgCl_2$, 1% sodium acetate and 0.1% glutaraldehyde; Schulte & Spicer, 1983) for 2 h at 4° C. To substantiate the preservation of the surface mucus layer, the fixed specimens were observed under a dissecting microscope. They were then dehydrated through a graded ethyl alcohol series (starting from 100% ethyl alcohol for materials fixed in 100% ethyl alcohol or Carnoy's solution), cleared in xylene and embedded in paraffin. For histological and histochemical examinations, 3-μm paraffin sections were prepared, affixed to glass slides, dewaxed, dehydrated and stained with Haematoxylin–Eosin (H&E) or Alcian Blue pH 2.5-PAS (AB-PAS). In addition, a dual staining was performed, in which tissue sections were stained first by galactose oxidase–cold thionine Schiff (GOCTS) (Ota *et al.*, 1991) and then with paradoxical concanavalin A staining (PCS) (Katsuyama & Spicer, 1978). As reported previously (Ota *et al.*, 1991), such a dual staining provides a useful means of differentiating two types of mucins produced by human gastric mucosa; one type is secreted by surface mucous cells and the other by gland mucous cells. The former type of mucin shows an intense galactose oxidase–Schiff (GOS) reactivity (Katsuyama *et al.*, 1985), whereas the latter reveals a class III concanavalin A reactivity with PCS and will be referred to as class III mucin in the present paper. As stated above, the Schiff reaction of the GOS sequence was replaced by the blue cold thionine Schiff technique (Ota *et al.*, 1991) in the present study, since thionine bound to galactose oxidase-engendered aldehydes is resistant to the ensuing periodate–borohydride sequence of paradoxical concanavalin A staining (Ota *et al.*, 1991).

The thickness of the mucous layer was measured at 10 randomly selected points on the top of the area gastricae with an ocular scale. Thickness was defined as the distance from the outermost surface of the continuous mucous layer to the luminal surface of the surface mucous cells. The data were expressed as mean ± SEM.

To determine whether tissue processing following the fixation in Carnoy's solution would alter the thickness of the surface mucous layer, we measured it in the fundic mucosa of two cases in photographs of the lateral view of tissue blocks resected from Carnoy-fixed materials. The results thus obtained compared with those obtained by measuring the thickness in H&E preparations of the same tissue blocks.

Results

Histologically normal portions of the gastric mucosa were used for microscopic observation on the surface mucous layer.

In frozen sections of two cases examined, approximately one-third to two-thirds of the mucosal surface of the fundic mucosa was covered by the continuous mucous layer, although it was frequently frayed and detached from the luminal surface of the surface mucous cells (Figs 1 and 2). The luminal surface of the surface mucous layer, moreover, was more or less irregular and ragged. Its mean thickness was 43.8 ± 15.4 μm, minimum thickness was 10.2 μm and maximum thickness was 76.8 μm. The amylase digestion–PAS sequence disclosed a laminated structure consisting of alternating intensely reactive and weakly reactive layers (Figs 1 and 2). With the GSA-II-HRP staining, the mucous layer also showed alternating lamellar layers of intensely positive or faintly positive mucus (Figs 3 and 4). In the remaining cases, the mucous layer was mostly lost in frozen sections.

Among paraffin-embedded materials, only Carnoy-fixed ones yielded satisfactory results. In Carnoy's solution, the transparent mucous layer turned to a milky whitish translucent coat in 10 to 20 s. After 2 h in Carnoy's solution, the surface mucous layer was seen under a dissecting microscope as a translucent coat covering the gastric mucosa without any fissures but occasionally containing air bubbles (Fig. 5). On the side view of the tissue block, the luminal surface of the surface mucous layer was smooth (Fig. 6). In H&E preparations, the particular layer was well preserved in all cases and appeared as an eosinophilic thick band containing cellular debris (Figs 7 and 8). The mean thickness of the surface mucous layer in the fundic mucosa was estimated to be 55.4 ± 2.5 μm, minimum 7.7 μm and maximum 204.8 μm, and in the pyloric mucosa the mean thickness was 21.8 ± 1.0 μm, minimum thickness was 2.6 μm, and maximum thickness was 66.6 μm.

Tissue-preparing procedures reduced the thickness of the surface mucous layer approximately 5% (mean 93.9 ± 1.5 μm, minimum 65.3 μm, and maximum 117.5 μm) compared with that of Carnoy-fixed sections (mean 98.3 ± 11.3 μm, minimum 66.3 μm, and maximum 136.8 μm).

In the AB-PAS stained preparations, the surface mucous layer exhibited almost no alcianophilia and appeared as a PAS-reactive thick band with fine linear striped patterns (Figs 9 and 10). The GOCTS-PCS

procedure revealed a consistent alternating laminated array consisting of two types of mucin, one from the surface mucous cells and the other from the gland mucous cells (Figs 11–14). In the surface mucous layer of the fundic mucosa, the sheets of class III mucins were in general smaller in thickness than those of surface mucous cell-type mucins (Fig. 12). In the pyloric mucosa, on the other hand, class III mucins predominated and occupied substantial portions of the surface mucous layer (Fig. 14). Class III mucins which were released extended upward through pits and joined the bulk flow of the surface mucous layer (Fig. 14).

In gastric tissue preparations from materials fixed either in formalin or 100% ethyl alcohol, the surface mucous layer was largely lost. Likewise, the tissue specimens fixed in the buffered $HgCl_2$–glutaraldehyde or Bouin's solutions underwent both the marked distortion and shrinkage of histological structures, even though the surface mucous layer was preserved in tissue preparations and its laminated structure was also noted.

Discussion

The present results have revealed that the surface mucous layer consisted of two types of mucins derived from the gastric mucosa and exhibited a laminated structure. Among the two procedures and various fixatives examined, materials which were fixed in the Carnoy's solution and embedded in paraffin provided the most satisfactory results. Essentially the same results were obtained by the frozen section method. By this method, the preservation of the surface mucous layer, however, was not consistent, and the layer had deteriorated markedly.

Histological observation of the surface mucous layer of the stomach has been attempted by several authors (Iida, 1976; P.L. 1984: Morris *et al.*, 1984). Morris *et al.* (1984) studied the surface mucus layer of the rat stomach in specimens fixed in a phosphate-buffered 2.5% glutaraldehyde solution, postfixed in buffered 1% osmium tetroxide and embedded in epon–araldite. In thick epon–araldite sections, one-third to two-thirds of the surface area of the fundic mucosa was not covered by mucus. They stated that the absence of a complete coat was not likely to have resulted from removal of mucus during processing of control specimens. The luminal surface of the gastric epithelium, however, is unquestionably covered by a continuous layer of insoluble mucus gel, as demonstrated by observing unfixed thick sections of the mucosa (Kerss *et al.*, 1982; Sandzén *et al.*, 1988). Morris *et al.* (1984) also noted a lamellar appearance with dark-staining strands intermingled with a lightly-stained amorphous matrix in the mucous layer. In figures appearing in their paper, orderly lamellar segregation of mucus is not conspicuous, and they did not refer to the laminated structure in histochemical preparations using Alcian Blue pH 2.5–PAS or Alcian Blue pH 1.0. Unlike our observation, they noted that there was no obvious contribution to the mucous neck cells to the extracellular mucus in unstressed mucosae. Iida (1976) also attempted to observe this layer in human stomachs. They fixed surgically removed human stomach in a formal-alcohol solution and observed a PAS-positive extracellular mucus in part of the mucosal surface, but did not mention the laminated structure.

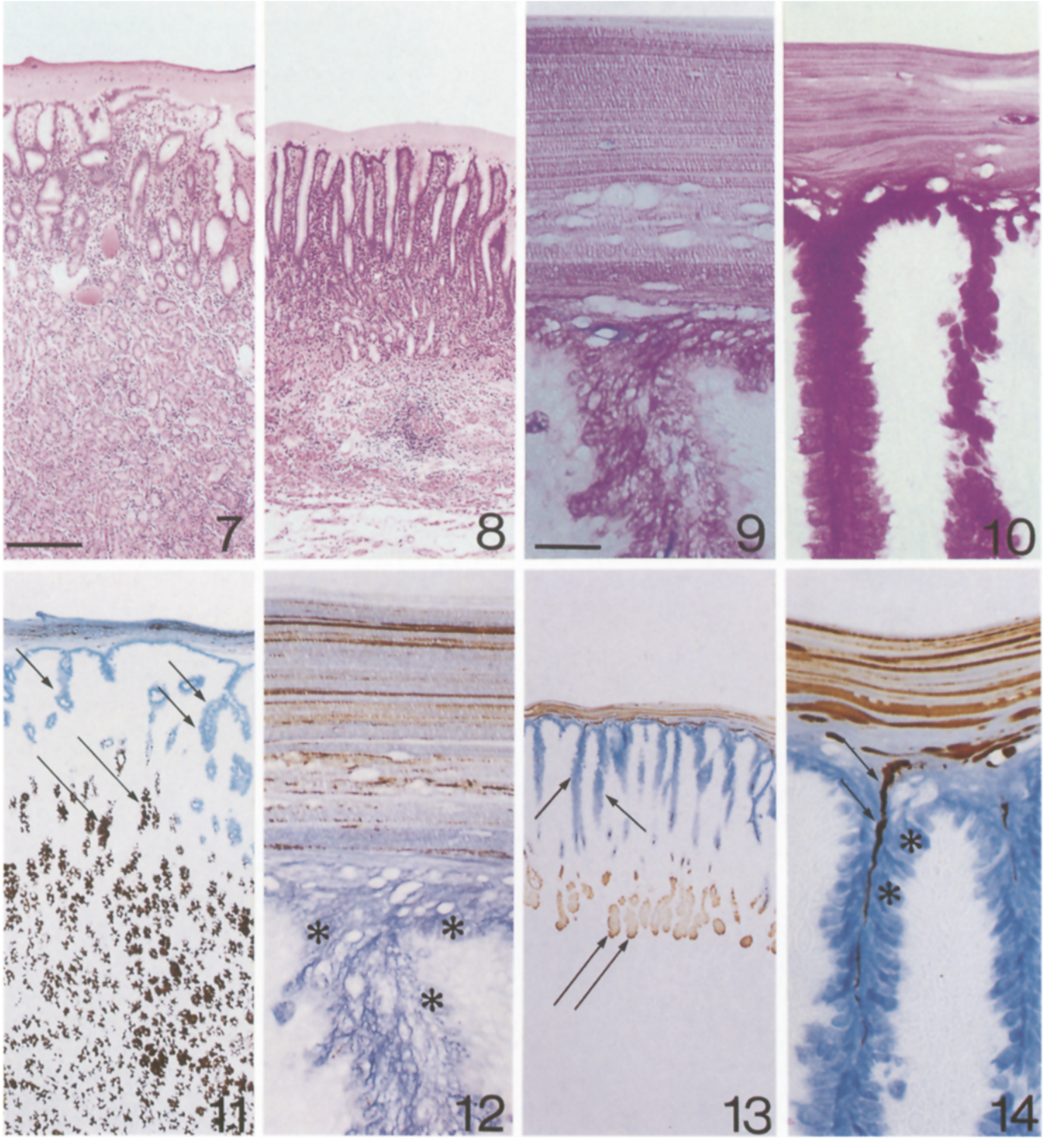
The surface mucous layer has also been studied using unfixed tissue sections (Kerss *et al.*, 1982, Sandzén *et al.*, 1988). These studies were performed with a view to measuring the thickness of the surface mucous layer and to observing its alteration under various conditions. These tissue preparations, however, were not appropriate to the investigation of the internal structure of the surface mucous layer. Kerss *et al.* (1982) measured the thickness of the surface mucous gel layer in the pyloric mucosa from unfixed human gastric tissue specimens and in the rat stomach, recording 192 ± 7 μm for the former. In our preparations, the surface mucous layer has obviously been recognized as a relatively condensed one, as compared with that described by Kerss *et al.* (1982), and this considerable difference could be explained by different tissue handling and processing procedures. Kerss *et al.* separated the outer muscle layer from the gastric wall, and this handling would distend the mucosa and might have influences on the thickness of the mucous layer, as pointed out by Sandzén *et al.* (1988). In the present study extreme care was taken not to affect the mucous layer mechanically.

In small experimental animals, the surface mucous layer of the intestine was observed using special techniques such as freeze substitution following vapor fixation (Sakata & Englehart, 1981) or stabilization with antibodies (Rozee *et al.*, 1982). In tissue blocks of the human gastric mucosa processed by freeze substitution, however, the surface mucous layer became extremely fragile and was almost completely lost during paraffin embedding (Ota & Katsuyama, unpublished observation).

It remains a problem whether the surface mucous layer observed in this study corresponds to both of the soluble and insoluble mucus layers or only the insoluble mucus layer.

Gastric mucus has been classified into two groups: soluble mucus and visible (or insoluble) mucus (Babkin, 1950; Glass, 1953; Allen & Carroll, 1985). The soluble mucus is the degradation product of the insoluble mucus (Allen & Caroll, 1985). In the present study, soluble mucus was mostly lost while tissues were incised and pinned on the flat board. It seems likely that insoluble mucus takes part mainly in the formation of the surface mucous layer fixed in Carnoy's solution.

Both in frozen and paraffin sections, an alternating laminated array consisting of two types of mucin was observed on the mucosal surface, indicating that this structure in the surface mucous layer is not an artifactual product of procedures employed. In frozen sections stained by PAS, the surface mucous layer consisted of intensely positive layers and weakly positive layers. Since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the surface mucous cell mucins reveal more intense PAS reactivity than the gland mucous cell mucins (Katusyama *et al.*, 1991), the former layers most probably contain the surface mucous cell mucins and the latter the gland mucous cell mucins. GSA-II, which has an affinity for gland mucous cell mucins (Ihida *et al.*, 1988), also discloses the presence of the laminated structure in the surface mucous layer.

Contrary to our expectations that two different mucins mixed homogeneously in the surface mucous layer, they remained unmixed in the superficial layer of the surface mucous layer. Such a phenomenon appears to reflect different physicochemical properties of the two types of mucins secreted from the two types of mucous cells, the surface mucous cells and the gland mucous cells. Various histochemical techniques have already revealed that the two types of mucin are considerably different in their histochemical properties, suggesting varying chemical structures of saccharide moieties with each mucin involved (Katsuyama *et al.*, 1985; Ota *et al.*, 1991; Katsuyama *et al.*, 1991).

The thickness and composition of the surface mucous layer differed between the fundic and pyloric mucosae. Thus, the surface mucous layer of the fundic mucosa was thicker than that of the pyloric mucosae. Moreover, class III mucins predominated in the pyloric mucosa. The latter finding could be explained by higher secretory activity of the pyloric gland cells as compared with the mucous neck cells.

Throughout amphibians, reptiles and mammals, two types of mucous cell are known to occur in the gastric mucosa; the surface mucous and gland mucous cells, the latter including cardiac gland, mucous neck and pyloric gland cells (Suganuma *et al.*, 1981; Katsuyama *et al.*, 1991). In contrast, fishes lack the gland mucous cells (Suganuma *et al.*, 1981). The biological significance of the acquisition of gland mucous cells first in amphibians has not yet been elucidated.

With no knowledge of the laminated structures, the surface mucous layer has hitherto been explored by means of rheological, biochemical and physiological methods (Bell *et al.*, 1982; Allen *et al.*, 1984; Takeuchi *et al.*, 1983). For better recognition of the pathophysiological functions and structures of the surface mucous layer and of how these two types of mucins could cooperate to form the surface mucous layer, the chemical properties of the two different mucins should be studied separately. The laminated structure of the surface mucous layer could play an important role in retarding the diffusion of harmful agents from the gastric lumen to the apical surface of the surface mucous cell.

Further studies are required to elucidate the mechanism by which non-mucinous secretions, such as pepsinogen and hydrogen chloride, penetrate the mucous gel layer in the stomach. Allen (1989) explained that hydraulic pressure from the volume of secretion in the gland forces the acid and pepsin into the lumen through channels or bubbles in the overlying mucus gel. However, such channels were not found in this study.

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Fig. 7. An upper half of the fundic mucosa. The mucosal surface is covered with a thick continuous surface mucous layer. H&E.×65. Bar = 200 μm.

Fig. 8. Pyloric mucosa. The mucosal surface is covered with a thick continuous surface mucous layer. H&E.×65.

Fig. 9. A higher magnification of the surface mucous layer in the fundic mucosa. The surface mucous layer exhibits intense PAS reactivity and appears as a thick layer with fine striped patterns. AB–PAS.×380. Bar = 30 μm.

Fig. 10. A higher magnification of the surface mucous layer in the pyloric mucosa. The surface mucous layer resembles that in the fundic mucosa; however, it is thinner than the latter. AB–PAS.×380.

Fig. 11. An upper half of the fundic mucosa. Surface mucous cells reveal galactose oxidase–cold thionine Schiff (GOCTS) reactivity (short arrows), whereas mucous neck cells exhibit class III concanavalin A reactivity (long arrows). GOCTS–PCS. Prepared from a serial section of that used for Fig. 2.×65.

Fig. 12. A higher magnification of the surface mucous layer in the fundic mucosa. An alternating laminated structure is evident, which consists of thick layers of surface mucous cell mucins (coloured faintly blue) and thin layers of mucous neck cell mucins (coloured brown). Asterisks indicate mucins in the apical cytoplasm of the surface mucous cells. GOCTS–PCS.×380.

Fig. 13. Pyloric mucosa. The surface mucous cells reveal GOCTS reactivity (short arrows), whereas pyloric gland cells exhibit class III concanavalin A reactivity (long arrows). GOCTS–PCS. Prepared from a serial section of that used for Fig. 3.×65.

Fig. 14. A higher magnification of the surface mucous layer in the pyloric mucosa. An alternating laminated structure is evident which consists of layers of surface mucous cell mucins (coloured faintly blue) and layers of pyloric gland cell mucins (coloured brown). Asterisks indicate mucins in the apical cytoplasm of surface mucous cells. Class III mucins released from the gastric pits are seen to join the bulk flow (arrows). GOCTS–PCS.×380.

Figs 7 to 14 were prepared from Carnoy-fixed materials.

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