Mucus glycoproteins from pig gastric mucosa: identification of different mucin populations from the surface epithelium

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Pig gastric mucins were isolated from the surface epithelium of the cardiac region, corpus and antrum using density-gradient centrifugation after extraction in 6 M guanidinium chloride. In CsCl/0.5 M guanidinium chloride, mucins solubilized from the cardiac region appeared as a broad unimodal band at 1.52 g/ml whereas those from the corpus and antrum occurred as high- and low-density populations at 1.50 and 1.45 g/ml respectively. High-iron diamine reacted more strongly with the cardiac mucins and the high-density populations from corpus and antrum than with the two low-density ones. In keeping with this, approx. 60 % of the oligosaccharides from the former mucins and 20 % from the latter contained sulphate. All surface epithelial cells of the cardiac region stained with high-iron diamine, whereas in the corpus only the epithelium in the bottom of the pits reacted, suggesting that the high-density population from this region

originates from these cells. Mucins from all regions were composed of subunits, each containing highly glycosylated domains. The mucins from the cardiac region were larger than those from the corpus and antrum, and reduced subunits as well as high-molecular-mass glycopeptides from the cardiac mucins were larger than the corresponding fragments from the other regions. Ion-exchange HPLC showed that reduced subunits from the cardiac mucins and the high-density populations from the corpus and antrum were more 'acidic' than reduced subunits from the two low-density ones. All mucins contained a 'neutral' fraction, in particular those from the antrum. Pig gastric mucus thus contains a number of distinctly different mucin populations varying in buoyant density, size, 'acidity', glycosylation, sulphation and tissue origin.

INTRODUCTION

Gastric mucus is a highly hydrated viscoelastic gel that protects the mucosa from mechanical stress as well as from erosion by pepsin and HCl [1]. The polymer matrix of the gel is provided by large secreted glycoproteins referred to as mucus glycoproteins or mucins. Histochemical studies suggest that mucins are produced by all cells in the surface mucosa, as well as by certain cells in the gastric glands, and that differences in the structure between surface and gland mucins between the different regions of the stomach and between species are likely to occur (see for example [2,3]; for a review, see [4]). It has been suggested that the secretions from the various cell populations do not blend but form a laminated structure over the epithelium [5]. The physiological significance of this organization of the gel is not known.

Gastric mucins are oligomeric glycoproteins composed of subunits joined by disulphide bonds as first described by Allen and co-workers [6]. The oligomeric nature of the macromolecules is critical for the mechanical properties of mucus since reduction of disulphide bonds, or proteolytic cleavage, inhibits gel formation and dissolves a preformed gel [7]. Protease digestion of subunits from cervical, gastric and respiratory mucins gives rise to high-molecular-mass glycopeptides corresponding to highly glycosylated domains within the macromolecules [8–11]. For example, human cervical mucin subunits contain, on average, four such domains rich in serine and threonine that are flanked by protease-sensitive stretches of the apoprotein [12]. Regional differences in the structure of pig gastric mucins have been observed but no distinct mucin populations were identified [13]. However, biochemical studies of pig gastric mucins have identified both neutral and acidic species [14].

Early immunohistochemical studies suggested that the so-

called M1 mucin antigen is confined to the surface epithelium of human gastric mucosa and thus that surface and gland mucins may have different apoproteins [15]. More recently, *in situ* hybridization and immunohistochemistry have been used to localize the MUC5AC and the MUC6 mucins to the human gastric surface epithelium and glands respectively [16–19]. A clone (HGM cDNA), obtained from a human stomach library using antibodies against deglycosylated human gastric mucins, may correspond to part of the MUC5AC mucin [20]. Sequencing of two partial cDNA clones from pig gastric mucosa has revealed a mucin that contains a serine/threonine-rich domain composed of tandemly repeated motifs and a short stretch of unique sequence [21]. Northern-blot analysis suggests that this is a large mucin, and *in situ* hybridization and immunohistochemistry show that this mucin originates from the surface mucosa.

Here we have isolated mucus glycoproteins from the surface epithelium of the cardiac region, corpus and antrum of pig gastric mucosa and identified a number of distinct populations differing in extractability, buoyant density, size, charge density, glycosylation and sulphation. Staining with high-iron diamine (HID) of the various mucin populations blotted on to nitrocellulose membranes correlated well with the histochemical appearance of the cognate tissue, providing an approach for assessing structural differences between mucins with different histochemical staining properties.

MATERIALS AND METHODS

Materials

Trypsin (EC 3.4.21.4; type XIII, Tos-Phe-CH₂Cl-treated), DNA (type I from calf thymus), guanidinium chloride and iodoaceta-

Abbreviations used: DFP, di-isopropyl phosphorofluoridate; DTT, dithiothreitol; HID, high-iron diamine; PAS, periodic acid/Schiff.

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mide were obtained from Sigma. Stock solutions (approx. 8 M) of guanidinium chloride were treated with charcoal and filtered through an Amicon PM-10 ultrafiltration membrane (nominal cut-off 10 kDa) before use. CHAPS was purchased from Boehringer-Mannheim, di-isopropyl phosphorofluoridate (DFP) from Fluka and dithiothreitol (DTT) from Merck. Sepharose CL-2B, Sephadex G-10, DEAE-Sephadex and Mono Q HR5/5 columns were from Pharmacia. AG 50W-X8 was from Bio-Rad. Lacto-*N*-difucohexaose I was obtained from Oxford GlycoSystems.

Analytical methods

Density measurements were performed using a Carlsberg pipette as a pycnometer. Carbohydrate was determined by the anthrone method using manual [22] and automated [23] procedures for fractions from density-gradient centrifugation and chromatography respectively. As performed, the anthrone procedure gives approx. 30 % higher colour yield with fucose than with galactose and shows little reactivity with hexosamine and sialic acid. Sialic acid was determined by the method of Jourdain et al. [24] using an automated procedure [25]. In the original paper [24], it was shown that the conditions used for the periodic acid oxidation step, as well as O-acetylation, affect the sensitivity and that free and glycosidically bound sialic acids react differently. In our hands, DNA and an Le^b-reactive oligosaccharide (lacto-*N*-difucohexaose I; containing Fuc, Gal, GlcNAc and Glc) give, on a weight basis, approx. 1 % and less than 0.5 % respectively of the colour yield obtained by glycosidically bound sialic acid. Carbohydrate was also assayed by staining aliquots of samples blotted on to nitrocellulose membranes by the periodic acid/Schiff (PAS) procedure [26]. Sulphated mucins were detected by the same approach using the HID procedure [27]. In both cases, the colour yield was quantified with a Hoefer GS 300 densitometer in the reflectance mode, and results were within the linear range of response as determined using a dilution series of purified mucins.

Isolation of mucins

Pig stomachs were obtained from the local abattoir where scraping of the mucosa was performed. The surface mucosae from the cardiac region, corpus and antrum were removed by gently scraping the epithelium with a glass microscope slide. Samples were frozen as small aliquots in liquid N₂ and stored at -70 °C. Mucins were isolated from four different pools of mucosa as well as from the mucosa from a single animal. The data presented here are from preparations obtained from one pool of mucosa (five animals) and other preparations are referred to in the text when relevant.

Frozen material (2 g) was thawed in the presence of DFP (1 ml of a 0.1 M solution in propanol) for 5 min before the addition of 50 ml of extraction buffer (ice-cold 6 M guanidinium chloride/5 mM disodium EDTA/5 mM *N*-ethylmaleimide/10 mM sodium phosphate buffer, pH 6.5). The mucosa was dispersed with a Dounce homogenizer (four strokes; loose pestle) and slowly stirred overnight at 4 °C. Insoluble material was removed by centrifugation [23 000 *g* (17 000 rev./min); Beckman JA-20 rotor; 50 min; 4 °C] and re-extracted twice with extraction buffer (25 ml). The final extraction residues were reduced and alkylated as described below, centrifuged as above, and the supernatants subjected to isopycnic density-gradient centrifugation in the same way as the 'soluble' material.

CsCl and 5 mM disodium EDTA/10 mM sodium phosphate buffer, pH 6.5, were added to an initial density of 1.39 g/ml in 4 M guanidinium chloride, and centrifugation was performed as

described in the legend to Figure 1. Fractions were collected from the bottom of the tubes and analysed for *A*₂₈₀, sialic acid, hexose/fucose and density. The mucin-containing fractions were pooled, dialysed into buffer consisting of 0.5 M guanidinium chloride, 5 mM disodium EDTA and 10 mM sodium phosphate, pH 6.5, and subjected to a second isopycnic density-gradient centrifugation step in CsCl with an initial density of 1.50 g/ml. Fractions were analysed as above, as well as for sulphate by the HID procedure. After the second gradient step, mucins from the corpus and antrum were pooled into high- (HD) and low- (LD) density populations, dialysed into 4 M guanidinium chloride, pH 7.0, and stored at 4 °C. The amounts obtained were assessed gravimetrically after dialysis against water and freeze-drying.

Reduction/alkylation and trypsin digestion

Reduction of whole mucins was performed with 10 mM DTT in buffer consisting of 6 M guanidinium chloride, 5 mM disodium EDTA and 0.1 M Tris/HCl, pH 8, for 5 h at 37 °C. Subsequently, samples were alkylated with 25 mM iodoacetamide overnight. High-molecular-mass glycopeptides were obtained after trypsin digestion of subunits in 0.1 M NH₄HCO₃, pH 8, for 5 h at 37 °C using approx. 10 µg of trypsin/mg of subunit.

Release and analysis of oligosaccharides

Reduced and trypsin-digested mucins (9–15 mg) were incubated in 1 M NaBH₄/0.05 M KOH (1 ml/mg of glycopeptide) for 45 h at 45 °C. The reaction was stopped by neutralization with acetic acid, and the oligosaccharide alditols released were desalted using an AG 50W-X8 resin (1.5 ml resin/ml of 1 M NaBH₄) eluted with 5 ml of water/ml of resin. Water was removed by vacuum rotary evaporation at 40 °C. Methanol and a few drops of acetic acid were added and subsequently removed by vacuum rotary evaporation (repeated four times). The oligosaccharide alditol mixtures were separated into fractions containing the neutral, sialylated and sulphated species as described [28] using columns containing 0.5 ml of DEAE-Sephadex A-25 (acetate form)/mg of mucin subunits. The neutral oligosaccharide alditols were eluted with 10 ml of dry methanol/ml of resin. The sialylated oligosaccharide alditols were derivatized to methyl esters by adding 0.6 ml of DMSO/iodomethane (5:1)/ml of DEAE-Sephadex in three portions, incubating the column for 5 min after each addition, and eluting the column with 5 ml of dried methanol/ml of DEAE-Sephadex. Finally, the sulphated oligosaccharides were eluted with 1.0 M pyridinium acetate, pH 5.4 (5 ml/ml of DEAE-Sephadex). All fractions were concentrated using vacuum rotary evaporation and lyophilized. The sialylated glycans were converted into methyl amides by adding 1 ml of dried methanol and 25 % methylamine and stirring the samples at room temperature for 10 min. The sulphated fractions were desalted using either AG 50W-X8 or Sephadex G-10 columns.

Sugar analyses were performed as described [29]. Aliquots (1–2 % of each fraction) were hydrolysed with 4.0 M trifluoroacetic acid for 4 h at 100 °C. After the sample had been dried and the amino sugars re-*N*-acetylated, the monosaccharides were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection using a Carbpac MA-1 analytical column (4 mm × 250 mm; Dionex Corp., Sunnyvale, CA, U.S.A.) with a guard column (4 mm × 50 mm). The sugars were separated using a linear gradient from 0.4 to 0.55 M NaOH for 20 min at a flow rate of 0.6 ml/min and detected by a pulsed amperometric detector (Dionex PAD-II). The sulphated fractions were analysed for sulphate after hydrolysis of 2 % of each sample in 0.52 M HCl at 100 °C for 4 h. Sulphate was separated from

other components by capillary electrophoresis (77 cm) in a chromate buffer (pH 8.0) and detected by absorption at 254 nm (negative peaks).

Laser-light scattering

The intensity of light scattered at angles between 20° and 90° was determined using a Malvern Molecular Analyzer System 4300 equipped with a 35 mW He/Ne laser. Samples were analysed at 20 °C in 6 M guanidinium chloride, data were plotted as described by Zimm [30] and the radius of gyration and molecular mass determined as described by Tanford [31]. Mucins were studied in the concentration range 15–150 µg/ml, and a refractive index increment of 0.104 ml/g was used.

Rate-zonal centrifugation

Whole mucins and reduced subunits were subjected to rate-zonal centrifugation in a gradient (6–8 M) of guanidinium chloride as described [32]. Samples (100 µl) were layered on top of the gradients and centrifugation was performed at 200 000 g (40 000 rev./min) for 2 h 45 min at 20 °C using a Beckman SW-41 rotor. Tubes were emptied from the top and aliquots of the fractions blotted on to nitrocellulose membranes and analysed by the PAS and HID procedures.

Gel chromatography of mucin fragments

Gel chromatography was performed on a column (1.6 cm × 90 cm) of Sepharose CL-2B. The column was eluted with either 4 M guanidinium chloride, pH 7 (reduced subunits) or 0.1 M ammonium acetate, pH 7 (high-molecular-mass glycopeptides) at a flow rate of 6 ml/h. Fractions (2 ml) were collected and analysed by the anthrone method (high-molecular-mass glycopeptides) or blotted on to nitrocellulose membranes and stained using the PAS procedure (reduced subunits).

Ion-exchange HPLC of reduced subunits

Reduced subunits were subjected to ion-exchange HPLC on a Pharmacia Mono Q HR 5/5 column with a 2150 LKB titanium-head pump connected to a 2152 LKB controller, a 2040-203 LKB mixing valve and a Pharmacia V-7 injector. Gradients were formed on the low-pressure side and the column was eluted (0.5 ml/min) with starting buffer [6 M urea, 0.1 % (w/v) CHAPS, 10 mM piperazine/perchlorate buffer, pH 5.0] for 10 min followed by a linear gradient (60 min) to a final concentration of 0.25 M LiClO₄ in starting buffer. Fractions (0.5 ml) were collected and analysed using the anthrone method.

Histochemistry

Specimens of pig stomach were fixed for about 48 h in 10 % neutral buffered formol saline, dehydrated and embedded in paraffin. Sections (approx. 4 µm) were stained using the PAS [33] and HID procedures.

RESULTS

Isolation of mucins

The surface mucosae from the cardiac region, corpus and antrum of pig stomach were quickly collected by scraping with a microscope slide and frozen immediately to minimize degradation. Histological examination of the remaining tissue showed that the mucosa had been removed to a level slightly above the

bottom of the gastric pits (results not shown). Mucins from the epithelium in the bottom of the pits might thus be under-represented in the preparations, particularly in the antrum where the gastric pits are deeper than in the corpus and the cardiac region. The 'soluble' mucins were obtained after repeated extractions of the tissue in 6 M guanidinium chloride using gentle dispersion with a Dounce homogenizer, slow stirring and high-speed centrifugation. Most of the residue remaining after three extractions was solubilized by reduction with DTT.

Mucins from the extracts and reduced residues were purified from protein, lipids and DNA by isopycnic density-gradient centrifugation in CsCl/guanidinium chloride [34]. In CsCl/4 M guanidinium chloride, mucins and nucleic acids appeared at a density of 1.4–1.5 g/ml close to the bottom of the tubes, well-separated from material at the top of the gradient (Figures 1a–1c). In the cardiac region, most of the mucins appeared at the same density as DNA (Figure 1a) whereas mucins from the corpus and antrum were partially separated from this component (Figures 1b and 1c).

The mucin-containing fractions were pooled and subjected to a second density-gradient centrifugation step in CsCl/0.5 M guanidinium chloride to separate mucins from DNA. Mucins from the cardiac region appeared as a broad unimodal band with a peak density of 1.52 g/ml (Figure 1d) whereas those from the corpus and antrum separated partially into high-density (HD) and low-density (LD) populations at approx. 1.50 and 1.45 g/ml respectively (Figures 1e and 1f). The two components from the corpus (corpus-HD and corpus-LD) and antrum (antrum-HD and antrum-LD) differed in their reactivities in the anthrone and sialic acid assays, the low-density component reacting more strongly with the former method. The high-density populations stained more intensely with HID than the low-density ones when blotted on to nitrocellulose membranes, suggesting a higher sulphate content. In the antrum, the relative yield of the high-density component varied significantly between preparations. The high- and low-density populations in corpus and antrum were also found in mucin preparations from a single animal (results not shown), indicating that the presence of these populations in mucosal samples pooled from several animals is not merely due to interindividual variation.

Material that was insoluble after extraction with guanidinium chloride was solubilized by reduction, and mucin subunits were subjected to isopycnic density-gradient centrifugation as described for the soluble species. This material showed the same distribution as the cognate soluble mucins (results not shown) with a single broad population of mucins from the cardiac region and high- and low-density populations in the samples from corpus and antrum. The amount of mucin that resisted extraction unless reduction was used was determined gravimetrically after extensive dialysis against water and freeze-drying aliquots of the various fractions. In the cardiac region, about 30 % (w/w) of the total was solubilized with 6 M guanidinium chloride alone, whereas only approx. 15–20 % of the high- and low-density components from the corpus and antrum resisted extraction with this solvent.

Oligosaccharide analysis

The mucin populations were pooled as shown in Figures 1(d)–1(f) to give samples referred to as cardia, corpus-HD, corpus-LD, antrum-HD and antrum-LD. After release by alkaline borohydride treatment, the oligosaccharides from the various populations were fractionated into neutral, sialic acid- and sulphate-containing species. The relative yields of the three types of oligosaccharide, estimated from the amount of GalNAcol, are

Figure 1 Isopycnic density-gradient centrifugation of pig gastric mucins in CsCl/guanidinium chloride

Mucosal extracts were subjected to centrifugation in CsCl/4 M guanidinium chloride, initial density 1.39 g/ml (a–c). Mucin-containing fractions were pooled and subjected to a second density-gradient centrifugation step in CsCl/0.5 M guanidinium chloride with an initial density of 1.50 g/ml (d–f). (a, d) Mucins from the cardiac region; (b, e) mucins from the corpus; (c, f) mucins from the antrum. After centrifugation (Beckman L8 ultracentrifuge; 50.2 Ti rotor (36 000 rev./min); 90 h; 15 °C), fractions were collected from the bottom of the tubes and analysed for A_{280} (—), sialic acid (●), hexose/fucose (▲), HID reactivity (○) and density (■). Fractions were pooled as shown by the bars.

Table 1 Distribution of neutral, sialic acid- and sulphate-containing oligosaccharides from pig gastric mucins

Oligosaccharides were released by alkaline borohydride treatment, and the neutral, sialic acid- and sulphate-containing species were isolated using ion-exchange chromatography. The numbers represent the distribution of GalNAcol between the fractions.

Type of oligosaccharide	Yield (%)				
	Cardia	Corpus-HD	Corpus-LD	Antrum-HD	Antrum-LD
Neutral	36	38	78	38	80
Sialylated	1	< 0.1	3	3	1
Sulphated	63	62	19	59	19

shown in Table 1. In cardia and the high-density population from corpus and antrum, neutral oligosaccharides comprise approx. 40% of the total, whereas the low-density populations from corpus and antrum contain about 80% neutral glycans. The relative amount of sulphate-containing oligosaccharides is the same (approx. 60%) in cardia, corpus-HD and antrum-HD, whereas the level is lower in corpus-LD and antrum-LD. The presence of a high proportion of sulphated oligosaccharides in the high-density mucins is consistent with their higher reactivity with HID (Figures 1d–1f). The proportion of sialylated oligosaccharides was low, although all mucins reacted in the sialic acid assay used to monitor fractions from the density gradients. By using a mucin glycopeptide with a known content of sialic acid as a standard, the sialic acid content of the cardiac mucins was

estimated to be about 4% by weight, whereas all other populations contained less than 1%. These low amounts also explain why the DNA band in the gradients gives a rather high response relative to the mucins in the sialic acid assay, although the colour yield relative to glycosidically bound sialic acid is only about 1% (see the Materials and methods section).

The profiles of monosaccharide composition of the various oligosaccharide fractions were typical of those of mucin oligosaccharides (Table 2). Because of the low levels of sialylated oligosaccharides, results are only presented for the neutral and sulphated fractions. In addition to GalNAcol (representing the link GalNAc), GalNAc, GlcNAc, Gal, Fuc and Man were found whereas no sialic acid could be detected. The presence of Man in some of the oligosaccharide fractions is likely to reflect the presence of *N*-linked oligosaccharides known to be present in mucins. The amounts are likely to be overestimated here because of the difficulties of separating and quantifying this monosaccharide by the method used [29]. Since *N*-linked oligosaccharides may contain Gal, GlcNAc and possibly also Fuc, their presence may cause a slight overestimation of the average lengths given here for the mucin-type oligosaccharide but otherwise should not affect any of the conclusions drawn.

The composition analyses suggest that the glycosylation of the various mucin populations is not the same. For instance, the relative amount of Fuc is higher in the neutral oligosaccharides from corpus-HD and antrum-HD than in the sulphated ones, whereas the opposite is true for the low-density mucins. The mean length of the various oligosaccharide fractions is approximately ten residues, in agreement with observations made by other investigators [13]. Sulphation of the gastric mucins appears

Table 2 Monosaccharide composition of oligosaccharides from pig gastric mucins

The oligosaccharides were released using alkaline borohydride treatment and the monosaccharide compositions determined using HPLC after acid hydrolysis. The amounts are expressed as molar ratios relative to GalNAcol. Man was excluded when estimating the average lengths of the glycans because this monosaccharide is not present in *O*-linked mucin-type oligosaccharides. ND, not detected.

Mucin	Type of oligosaccharide	GalNAcol	GalNAc	GlcNAc	Gal	Fuc	Man	Mean length	SO ₄
Cardia	Neutral	1	1.1	2.8	2.2	3.4	0.8	11	
	Sulphated	1	1.6	4.4	5.4	3.4	0.7	16	0.9
Corpus-HD	Neutral	1	0.4	3.0	3.4	2.5	0.6	10	
	Sulphated	1	0.8	4.4	4.2	1.6	ND	12	0.8
Corpus-LD	Neutral	1	0.8	4.1	5.8	2.2	0.2	14	
	Sulphated	1	2.2	8.5	9.0	3.4	1.8	24	1.9
Antrum-HD	Neutral	1	ND	3.2	4.1	1.5	ND	10	
	Sulphated	1	0.4	3.6	3.4	1.0	ND	9	1.2
Antrum-LD	Neutral	1	0.3	2.7	3.9	0.6	0.1	9	
	Sulphated	1	0.4	6.1	6.2	1.1	ND	15	1

Table 3 Summary of physical data for pig gastric mucins

Total intensity light scattering was used to determine molecular mass and radius of gyration (R_g). All measurements were performed at 20 °C in 6 M guanidinium chloride.

Mucin	Molecular mass (MDa)	R_g (nm)
Cardia	18	340
Corpus-HD	8.4	260
Corpus-LD	10	280
Antrum-HD	13	310
Antrum-LD	14	270

to be limited to one or two sites per sulphated chain, and, since the level of sialic acid is low in all populations, it is concluded that differences in the negative charge of the various pig gastric mucin populations is mainly related to the degree of sulphation. The structure of the oligosaccharides is studied in greater detail in the following paper [35].

Size and subunit composition

The molecular masses of the various mucin populations were determined by laser-light scattering (Table 3). The value for the cardiac mucins (18 MDa) is significantly higher than those obtained for the other populations which are in the range 8–14 MDa. The relationship between R_g and molecular mass is similar to that observed previously for human cervical and respiratory mucins [8,11] and is in keeping with large expanded structures. To investigate the molecular-mass distribution, the various mucin populations were subjected to rate-zonal centrifugation. All mucins were distributed over the entire gradient indicating a polydispersity in molecular size (Figures 2a–2e). The distribution of the cardiac mucins was skewed towards the bottom of the tube (Figure 2a), reflecting the higher molecular mass of this population. Staining with HID followed that of PAS, although in some populations the largest species are apparently relatively more highly sulphated (Figures 2a and 2b). After reduction of the whole mucins, subunits appeared as

Figure 2 Rate-zonal centrifugation of whole mucins and reduced subunits

Mucin samples (100 μ l) in 5 M guanidinium chloride were layered on top of a gradient (6–8 M guanidinium chloride). Whole mucins (a–e) and reduced subunits (f–j) were centrifuged for 2 h 45 min at 20 °C using a Beckman SW-41 rotor. Fractions were collected from the top of the tubes and analysed for carbohydrate using PAS (●) and for sulphate using HID (○) after aliquots had been blotted on to nitrocellulose membranes. Mucins from the cardiac region (a, f), corpus-HD (b, g), corpus-LD (c, h), antrum-HD (d, i) and antrum-LD (e, j) are shown.

narrow bands close to the top of the gradient (Figures 2f–2j), indicating that the whole mucins are oligomeric and composed of subunits. Since the subunits are relatively uniform in size, it is likely that the polydispersity of the oligomeric mucins is mainly due to a varying number of subunits. The larger size of subunits from the cardiac mucins may explain why these glycoproteins are larger than those from the other regions.

Subunit structure

Reduced subunits and trypsin-resistant high-molecular-mass glycopeptides from all mucin populations were chromatographed on Sepharose CL-2B (Figures 3a–3e). Subunits from the cardiac mucins (Figure 3a) eluted before those from the high- and low-density mucins from corpus (Figures 3b and 3c) and antrum (Figure 3d and 3e), suggesting that these subunits are larger than those from the other regions, in keeping with their behaviour on

Figure 3 Gel chromatography on Sepharose CL-2B of reduced subunits and tryptic fragments thereof

The column was eluted with either 4 M guanidinium chloride, pH 7 (reduced subunits) or 0.1 M ammonium acetate (high-molecular-mass glycopeptides) at a flow rate of 6 ml/h and fractions (2 ml) were collected. Reduced subunits were analysed for PAS reactivity after slot-blotting on to nitrocellulose membranes and high-molecular-mass glycopeptides by the anthrone method. Mucins from the cardiac region (a), corpus-HD (b), corpus-LD (c), antrum-HD (d), antrum-LD (e) are shown. ●, Reduced subunits; ○, high-molecular-mass glycopeptides obtained after trypsin digestion of subunits. V_0 , void volume; V_t , total volume.

rate-zonal centrifugation (Figure 2). In all cases, high-molecular-mass glycopeptides obtained after trypsin digestion of reduced subunits were substantially smaller than the cognate reduced subunits (Figures 3a–3e) and, in addition, glycosylated fragments smaller than the major population were present in all preparations. High-molecular-mass glycopeptides from the cardiac region showed a distinct shoulder on the larger side of the distribution suggesting the presence of two large glycopeptide populations. Mucins from the insoluble residue from the cardiac region were relatively enriched in the larger of these two populations (results not shown).

Subunit heterogeneity

Reduced subunits from the various mucin populations were

Figure 4 Ion-exchange HPLC on Mono Q of reduced subunits of pig gastric mucins

Mucins from the cardiac region (a), corpus-HD and corpus-LD (b) and antrum-HD and antrum-LD (c) are shown; ●, high-density and ○, low-density populations. Reduced subunits were chromatographed on a Mono Q column eluted with a linear gradient. Solvent A: 6 M urea, 0.1% (w/v) CHAPS, 10 mM piperazine/HClO₄ buffer, pH 5; solvent B: 0.25 M LiClO₄ in solvent A. Samples were eluted with solvent A for 10 min followed by a 0–100% gradient of solvent B (—) for 60 min (flow rate 0.5 ml/min). Fractions were analysed by the anthrone method.

subjected to ion-exchange HPLC to investigate their charge densities. In the cardiac region, most of the molecules eluted as a single broad population late in the gradient, suggesting the presence of a relatively acidic population (Figure 4a). In addition, some material was not retained by the column. Reduced subunits from the high-density mucins from corpus and antrum eluted as two partially separated 'acidic' populations (Figures 4b and 4c), and again some material not retained by the column was present. In subunit preparations from the low-density mucins from corpus and antrum, a larger proportion of the material was not retained by the column or eluted early in the gradient, indicating a low charge density (Figures 4b and 4c). The major subunit population in these samples eluted before those from both the cardiac mucins and the high-density populations. The behaviour of the subunits from the various mucin populations correlates well with the relative proportions of neutral and sulphated oligosaccharides

Figure 5 Light microscopy of sections from pig gastric mucosa

Sections from the cardiac region (**a, b**), corpus (**c, d**) and antrum (**e, f**) of pig gastric mucosa were stained with PAS (**a, c, e**) or HID (**b, d, f**). Nomarski optics were used to enhance the mucosal architecture. The bar indicates 100 μm .

(Table 1). Tryptic fragments of subunits showed the same elution pattern as the subunits (results not shown).

Histology

Tissue sections from the cardiac region, corpus and antrum were stained with either PAS or HID. All cells in the surface epithelium of the three regions reacted strongly with PAS (Figures 5a, 5c, 5e). In the cardiac region, all surface mucosal cells stained intensely with HID (Figure 5b) whereas in the corpus, only the cells in the bottom of the gastric pits showed strong reactivity (Figure 5d). Occasionally cells in the surface epithelium of corpus reacted weakly with HID whereas the surface epithelium in antrum did not stain at all (Figure 5f). In contrast, some cells in the antral glands showed strong reactivity (results not shown).

DISCUSSION

The gel layer overlying the gastric epithelium is composed of mucus which originates from both the surface mucosa and the glands. By removing the surface epithelium from the glandular mucosa it was possible to concentrate surface-derived mucins; however, a selective loss of mucins from the epithelial cells in the

bottom of the gastric pits may have occurred, particularly in the antrum. Extractions were performed by mild homogenization of the tissue in 6 M guanidinium chloride supplemented with proteinase inhibitors to minimize mechanical and enzymic degradation of the macromolecules [8]. With this procedure, mucins from the three major histological regions in the stomach have different solubility in 6 M guanidinium chloride. In the corpus and antrum, most of the mucins were solubilized by this solvent whereas most of the cardiac mucins were insoluble. This behaviour may be explained by their larger size, but the possibility that these mucins are more extensively cross-linked cannot be ruled out. Most human and rat intestinal mucins also occur as a glycoprotein complex that resists extraction with guanidinium chloride [19,36].

Mucins from the cardiac region comprised a unimodal poly-disperse population when subjected to isopycnic density-gradient centrifugation whereas two partially separated populations were present in both corpus and antrum. All mucin populations are oligomeric and composed of subunits linked by disulphide bonds. Whole mucins from the cardiac region are larger than the high- and low-density mucins from both corpus and antrum and this is also true for the corresponding subunits. In all populations, trypsin digestion of reduced subunits gave rise to high-molecular-

mass glycopeptides, indicating that highly and sparsely glycosylated regions alternate along the protein core. This fragmentation pattern is similar to that observed for human cervical [8,12] and tracheobronchial mucins [10,11] and suggests that these mucins have a similar macromolecular structure. Allen and collaborators [6] showed early on that pig gastric mucins from the cardiac region are composed of subunits; however, these subunits were not as extensively fragmented by proteolysis as those studied here [37]. Other investigators did not obtain significantly smaller fragments after reduction of pig gastric mucins [13], probably because of degradation during extraction and purification of the glycoproteins.

The cardiac mucins and the high-density populations from the corpus and antrum all showed reactivity with HID suggesting the presence of sulphate groups. This interpretation is corroborated by the finding that all three populations are relatively enriched in sulphated oligosaccharides. The low-density populations from corpus and antrum showed weaker staining with HID and eluted earlier on ion-exchange HPLC, in agreement with the much higher proportions of neutral oligosaccharides in these mucins. Since the level of sialylated oligosaccharides is low, it is concluded that the differences in charge density observed using ion-exchange HPLC are largely due to the presence of sulphate groups.

In order to determine the cellular origins of the HID-reactive mucin populations, tissue sections were stained using this procedure. In the cardiac region, all surface epithelial cells stained with HID, in keeping with the fact that all mucins from this region react with HID and are retarded on the ion-exchange column. In the corpus, where two populations with different HID reactivities are present, only the cells in the bottom of the gastric pits stained with HID, suggesting that the high-density population originates from these cells. In the antrum, the surface epithelial cells did not stain at all with HID; however, a subpopulation of the cells in the glands did, suggesting that the high-density population may originate from the glands rather than from the surface epithelium. This is in keeping with the observation that the amount of high-density mucins in this region varied between preparations.

cDNA cloning has identified a large mucin in pig stomach [21], and *in situ* hybridization and immunohistochemistry show that this component is present in all cells in the surface mucosa. This finding suggests that the high- and low-density mucins from the corpus may have the same apoprotein and that these populations are thus different glycoforms. However, the cardiac region was not studied, but these mucins with their larger subunits may contain a different core structure.

In summary, several different mucin populations were identified in pig gastric mucosa varying in extractability, buoyant density, size, charge density, glycosylation and tissue localization. Differences in 'acidity' were mainly ascribed to differences in sulphation. All 'soluble' populations have a similar macromolecular structure. The oligosaccharide structures are investigated in more detail in the following paper [35].

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