Structures of the Neutral Oligosaccharides Isolated from A-active Human Gastric Mucin*

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Alkaline borohydride reductive cleavage of the mucin, purified from gastric aspirates of the secretors with blood group A, resulted in a heterogeneous population of neutral (79.7%) and acidic (20.3%) oligosaccharide alditols. Nine oligosaccharides (I–IX), ranging from 6 to 15 sugar units, have been purified from the neutral oligosaccharide fraction. Based on the results of immunological assays, sugar composition, degradation with specific exoglycosidases, and methylation analyses, we propose the following structures for these oligosaccharides:

I \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4(Fucal \rightarrow 4)GlcNAc\beta 1\)

II \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

III \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

IV \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

V \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

VI \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

VII \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

VIII \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

IX \(GalNAc \beta 1 \rightarrow 3(Fucal \rightarrow 2)Gal\beta 1 \rightarrow 3/4GlcNAc\beta 1\)

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active secretion or by passive transudation. This viscous layer consists of proteins, glycoproteins, and lipids imbibed with water and electrolytes (1-5). The integrity of mucus gel, essential for its physiological role as a lubricative and protective layer, depends primarily on its mucin component which is secreted by mucous cells of gastric epithelium (1, 3, 5-9). The mucin of human and pig stomach is a large (2 x 10^6), highly glycosylated polymer composed of four subunits of equal size joined together by disulfide bridges at their nonglycosylated regions. These naked nonglycosylated protein regions are susceptible to proteolysis, and under physiological conditions, the mucin is broken down to glycoprotein subunits (5 x 10^5) by pepsin (3, 10, 11). The glycosylated region of each glycoprotein subunit contains numerous carbohydrate side chains O-glycosidically linked to the hydroxyl groups of seryl and threonyl residues of the protein core (1, 3, 12). The size and structure of the carbohydrate chains in mucins from different regions of the gastrointestinal tract vary in the same species as well as between species (13-22). Furthermore, considerable heterogeneity exists in the structure of carbohydrate chains of mucin within the same tissue (15, 18-23).

As part of the project directed towards establishing the role of carbohydrate chains of mucin in gastric mucosal protection (9, 24), we have begun a study on the structure of carbohydrate moiety in human gastric mucin. In a previous report, we have described the structure of neutral oligosaccharides of H-active mucin (18). Here, we present the structural characterization of nine oligosaccharides derived from A-active gastric mucin. These oligosaccharides range in size from 6 to 15 sugar units in length and represent 78% of the neutral carbohydrate chains of this mucin.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

A common characteristic of mucin-type glycoproteins from various organs and species is the heterogeneous nature of their carbohydrate chains (16, 18-22, 43, 44). These chains vary in length from a single N-acetylgalactosamine residue to elongated complex structures containing as many as 20 sugar units (13-22, 31, 44-46). Although the physiological importance of variations in carbohydrate chains of mucins is far from being well understood, it is generally accepted that this feature allows for each mucin an infinite number of distinct biological functions. To determine how these variations affect the functional properties of mucins, it is necessary to establish the structures of individual carbohydrate chains.

Recently, we have shown that H-active human gastric mucin contains at least 10 different neutral carbohydrate chains (18). The data presented here indicate that a high degree of structural heterogeneity also exists in the carbohydrate chains of A-active human gastric mucin. We have found that the carbohydrate chains released from this mucin by alkaline borohydride treatment consist of neutral (79.7%) and acidic (20.3%) oligosaccharides. Nine oligosaccharides, representing 62% of the total carbohydrate chain of mucin, were purified from the neutral oligosaccharide fraction. These compounds accounted for 78.2% of the neutral oligosaccharide fraction and ranged from 6 to 15 sugar units in length. The results of hemagglutination inhibition assays indicated that all oligosaccharides bore the A antigenic determinant. Furthermore, oligosaccharides II, III, IV, V, and VI exhibited H antigenic activity, while oligosaccharides I, V, and IX had the ability to inhibit hemagglutination in the I anti-I system. The presence in each oligosaccharide of carbohydrate sequences conforming its antigenic properties was confirmed with the aid of specific glycosidases and permethylanalysis. The data revealed that two of the isolated oligosaccharides (oligosaccharides IV and VIII) contained the unbranched core with N-acetylgalactosaminol substituted at C-3, while in the remaining compounds, this sugar residue was C-3,6 disubstituted. The C-6 substituent consisted of Galβ1→GlcNAc disaccharide in oligosaccharides I, V, and IX, Fucα1→2Galβ1→3GlcNAc trisaccharide in oligosaccharide II, and galactose residue in oligosaccharides III, VI, and VII. In all oligosaccharides, C-3 on N-acetylgalactosaminol was substituted with galactose. The results of enzymatic degradation suggested that this galactose residue served as a branching point for chains bearing A and H determinants in oligosaccharides V and VI. The side chains bearing A determinant in the oligosaccharides I and II and A and H determinants in the oligosaccharides III and IV were shown to be β(1→3/6) linked to the Galβ1→3GlcNAcα1→3Galβ1→4GalNAc-ol tetrasaccharide. Based on these data, the following structures are proposed for the isolated oligosaccharides (Structures I-IX).

The results presented in this report, together with our previous findings (18), indicate that the neutral carbohydrate chains which predominate in human gastric mucin constitute a spectrum of oligosaccharides composed from 4 to 15 sugar units arranged in linear or branched fashion. The majority of the chains are highly branched and contain up to three antennae. The antennae bearing A or H antigenic determinants contain two types of carbohydrate chains, type 1 (Galβ1→3GlcNAc) and type 2 (Galβ1→4GlcNAc), with the former being predominant. The predominance of type 1 carbohydrate chains in mucin-type glycoproteins in man has been reported previously (15, 16), and type 2 chains were found in mucins of pig, horse, dog, and sheep stomach (16, 17, 20, 22). The core portion of the neutral carbohydrate chains of human gastric mucin appears to consist of either Galβ1→3GlcNAc disaccharide or Galβ1→3GlcNAcα1→3Galβ1→4GalNAc tetrasaccharide. In simple oligosaccharides from H-active mucin, the distal galactose of this core is substituted at C-2 by α-L-fucose and in A-active oligosaccharides by GalNAcα1→3(Fucα1→2). In more complex structures, the core distal galactose gives rise to additional branches by β(1→3/6) substitution with N-acetylgalcosamine. As in oligosaccharides isolated from gastric mucins of other species (16, 17, 20, 22), further branching in carbohydrate chains of human gastric mucin occurs C-6 of the N-acetylgalactosamine residue involved in the O-glycosyl linkage to serine/threonine of the protein core. Our data indicate that in the neutral carbohydrate chains of human gastric mucin, this C-6 substituent may be a single residue of galactose or N-acetylgalactosamine, or it may consist of blood group H or I antigenic determinant.

Since up to 30% of the carbohydrate chains in human gastric mucin are represented by the sulfated and/or sialylated chains, it remains to be determined whether these chains possess the structures homologous to neutral oligosaccharides described here and elsewhere (18). Only then will we be able to fully ascertain how the structural heterogeneity in the carbohydrate chains of gastric mucin is translated into its biological functions to which this mucin is destined.
**Oligosaccharides of Human Gastric Mucin**

| Structure | Oligosaccharide Structure |
|-----------|---------------------------|
| I         | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| II        | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| III       | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| IV        | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| V         | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| VI        | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| VII       | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| VIII      | GalNAcβ1→3Galβ1→3GlcNAcβ1 |
| IX        | GalNAcβ1→3Galβ1→3GlcNAcβ1 |

**Structures I-IX**
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Oligosaccharides of Human Gastric Mucin

EXPERIMENTAL PROCEDURES

Material. - The study was performed on gastric secretion of patients undergoing medical gastrectomical examination for upper abdominal complaints. Samples of gastric secretion were obtained from six patients with blood-group A whose gastric juice showed no hemagglutinational characteristics (25). The pooled collections were dialyzed against distilled water (three changes), lyophilized from aqueous solution, and enzymatic degradation (in the case of mucin X) was performed by pepsin (0.25%). Pooled materials were then dried under high vacuums and lyophilized from aqueous solution. The following reagents were used: 3-aminomethyl thymidine, silyldiethyl reagents, 3-aminomethyl thymidine, and 3-aminomethyl thymidine. The compounds were used in the final concentration of 1:10,000.

Chromatography. - The oligosaccharides were separated in 80% methanol on a Bio-Gel P-30 column connected in duplicate with a gradient of methanol-water (100:0, 90:10, 80:20, 70:30, 60:40, and 50:50). The flow rate was maintained at 0.5 ml/min, and the effluent was monitored by paper chromatography on Whatman No. 1 paper with an alkaline developer. The resulting fractions were collected and analyzed for their carbohydrate content.

Electrophoresis. - The oligosaccharides were separated by agarose gel electrophoresis in 0.1M phosphate buffer, pH 7.0, with a constant current of 2mA per well. The gels were stained with Coomassie blue R-250 and destained with a solution of 10% acetic acid in water.

Table I. Chemical composition of the active human mucin glycoprotein isolated from human gastric secretion.

| Component | relative weight (%) |
|-----------|---------------------|
| Fucose    | 13.0                |
| Galactose | 24.6                |
| Glucose   | 20.5                |
| N-acetylglucosamine | 21.7           |
| Sialate   | 3.4                 |
| Sulfate   | 7.1                 |

*Expressed as N-acetylneuraminic acid.

Preparation of Neutral Oligosaccharides. - Reagents of the oligosaccharides with neutral glycopeptides were isolated by gel filtration on a Sepharose CL-2B column (25) using 0.1M sodium acetate buffer, pH 5.0, and 0.1M sodium acetate buffer, pH 6.0.

Fig. 1. Cell density profile centrifugation of pooled human gastric mucin at a starting density of 1.35p (dotted line). Mucin, eluted from the column at a density of 1.35p and centrifuged at 40,000 rpm for 18h. Fractions of II were collected and assayed for neutral sugar (phenol-sulfuric method, mg) and protein (absorbance at 280nm).

**Fig. 2.** Thin layer chromatography of the isolated oligosaccharides purified from human gastric mucin, A, O-linked sialyl oligosaccharide; B, N-linked sialyl oligosaccharide; C, N-acetylglucosamine; D, sialyl oligosaccharide; E, sialyl oligosaccharide; F, sialyl oligosaccharide; G, sialyl oligosaccharide; H, sialyl oligosaccharide; I, sialyl oligosaccharide; J, sialyl oligosaccharide. The isolated compounds were purified by high performance liquid chromatography followed by thin layer chromatography using BuOH:HAc:AcOH:water (5:5:5:2) and hexane:AcOH:water (5:5:2).

**Fig. 3.** Thin layer chromatography of sialyl oligosaccharides after alkaline hydrolysis and purification of glycopeptides. The isolated compounds were purified by high performance liquid chromatography followed by thin layer chromatography using BuOH:HAc:AcOH:water (5:5:5:2). The isolated compounds were purified by high performance liquid chromatography followed by thin layer chromatography using BuOH:HAc:AcOH:water (5:5:5:2) and hexane:AcOH:water (5:5:2) as described above. The isolated compounds were purified by high performance liquid chromatography followed by thin layer chromatography using BuOH:HAc:AcOH:water (5:5:5:2) and hexane:AcOH:water (5:5:2) as described above. The isolated compounds were purified by high performance liquid chromatography followed by thin layer chromatography using BuOH:HAc:AcOH:water (5:5:5:2) and hexane:AcOH:water (5:5:2) as described above.
Oligosaccharides of Human Gastric Mucin

The purified oligosaccharides ranged from six to fifteen sugar units in length and were composed of fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and glucose. In the neutral oligosaccharides (Table II), the molar ratios of these carbohydrates were 3.0:4.9:1. Among the neutral oligosaccharides, the most abundant were those containing fucose and galactose. There were also four compounds of similar composition with lesser amounts of glucose.

In hemagglutination-inhibition assays, all nine oligosaccharides were active substrates of N-acetyl-D-mannosamine endemic lectin and all with anti-H activity. Furthermore, oligosaccharides 1, 4, and 5 were also active in the lectin system. The results of hemagglutination-inhibition assays are presented in Table III. The molar ratio of the alditol acetates found in the hydrolysates of permethylated intact oligosaccharides are summarized in Table IV. The table shows that all oligosaccharides bear GlcNAc(GlcNAc)3GalNAc and 2-O-methylgalactose. The major activity is not observed by oligosaccharides 1, 11, 14, and 15. The presence of these compounds of H(1,3,4,6)-GalNAc-GalNAc reduces the activity by 10% with the activity of 6-O-methyl-N-acetylglucosamine. The activity is expressed relative to 1.0, 1.0, 1.0, and 1.0. The activity by 1.0, 1.0, 1.0, and 1.0.

The molar ratio of the components derived from oligosaccharides is expressed relative to Fuc-2GalE(1-3)Gal. The molar ratio of the components derived from oligosaccharides is expressed relative to Fuc-2GalE(1-3)Gal. The molar ratio of the components derived from oligosaccharides is expressed relative to Fuc-2GalE(1-3)Gal. The molar ratio of the components derived from oligosaccharides is expressed relative to Fuc-2GalE(1-3)Gal.
The oligosaccharide II exhibited α and β antigenic activity, and was susceptible to the action of α-L-fucosidase and α-N-acetylgalactosaminidase (Table III). After α-L-fucosidase treatment, one residue of N-acetylgalactosamine was released from the isolated oligosaccharide II. On the other hand, treatment with α-N-acetylgalactosaminidase resulted in the release of one residue of N-acetylgalactosamine, and two residues of N-acetylglucosamine from the isolated oligosaccharide II. The results indicated that the isolated oligosaccharide II contained a glycan structure linked by a α(1-3)-C-6 substituted N-acetylgalactosaminyl bond to the core structure. The isolation of the isolated oligosaccharide II was confirmed by a single method of isolation from the intact compound (Table IV).

The results of sequential degradation of oligosaccharides III and IV are presented in Table VII. Both compounds exhibited α and β antigenic activity, and were susceptible to the action of α-L-fucosidase and α-N-acetylgalactosaminidase. In addition, oligosaccharide III was found to be susceptible to α-galactosidase (Table VII, experiment 1). The results indicated that the oligosaccharide III contained a glycan structure linked by a α(1-3)-C-6 substituted N-acetylgalactosaminyl bond to the core structure. The isolation of the isolated oligosaccharide III was confirmed by a single method of isolation from the intact compound (Table IV). The results indicated that the isolated oligosaccharide III contained a glycan structure linked by a α(1-3)-C-6 substituted N-acetylgalactosaminyl bond to the core structure. The isolation of the isolated oligosaccharide III was confirmed by a single method of isolation from the intact compound (Table IV).