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Discrimination of rat Brunner's gland carbohydrate antigens by site-specific monoclonal antibodies

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ABSTRACT

Mucus produced and secreted by gastrointestinal mucosa contains various types of mucins equipped with unique sugar chains considered to play critical roles in protecting mucous membranes; therefore, the identification and verification of mucin sugar chains is important for understanding the underlying mechanisms. In our previous work, we generated three monoclonal antibodies (mAbs), RGM22, RGM26, and RGM42, which recognize sugar chains in rat gastric mucin. Here, we immunohistochemically analyzed the rat gastrointestinal mucosa and found that the antigens recognized by RGM22 and RGM42 were expressed in the rat antrum and Brunner's glands, whereas that recognized by RGM26 was detected in the antrum, but rarely in Brunner's glands. We found that these antibodies reacted with porcine gastric mucin-derived oligosaccharides bearing a common structure: GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-6GalNAc-ol. Moreover, epitope analysis revealed that RGM42 and RGM22 recognized α-linked GalNAc and GalNAcα1-3Gal, respectively, on the GalNAcα1-3(Fucα1-2)Gal structure, whereas RGM26 was specific for GalNAcα1-3(Fucα1-2)Gal. These results indicate that rat Brunner's glands express specific antigens bearing GalNAcα1-3Gal that are recognized by RGM22 and RGM42. Thus, RGM22, RGM26, and RGM42 with their unique antigen specificities could be useful tools for investigation of oligosaccharide diversity among mucins.

1. Introduction

Mucus covering the gastric mucous membrane protects the mucosa from the effects of acid, pepsin, and other damaging agents [1]. Mucin, a highly glycosylated protein with high molecular weight, is a major important component of the mucus layer. Recent studies have shown that mucin types with distinct carbohydrate composition and core protein structures are expressed in different regions of the gastric mucosa. In particular, genetic studies have revealed that in the stomach, mucin bearing the MUC5AC core protein is expressed by surface mucosal cells, whereas MUC6 is expressed by gland mucous cells located in a deeper mucosal layer [2,3]. Histochemical studies have revealed that surface-type mucins have carbohydrate chains different from those of gland-type mucins in the stomach [4,5]. Moreover, among these surface-type mucins, corpus and antral mucins have different carbohydrate chains [6,7].

As gastric mucin is an important factor in the protection of gastric mucosa, it is crucial to determine the specific functional significance of mucin glycoproteins after careful characterization of their biochemical properties. Previous studies of the oligosaccharide structure and function in specific mucins have led to key findings. In particular, gland-type mucin characteristically contains O-linked oligosaccharides with terminal α-1,4-linked *N*-acetylglucosamine (GlcNAc) residues mostly attached to a MUC6 scaffold [8]. Sugar chains with peripheral α-linked GlcNAc residues have antibiotic properties against *Helicobacter pylori* infection [9]. In addition, Karasawa et al. [10] recently revealed that a lack of α-GlcNAc expression in gastric gland-type mucin leads to gastric cancer. These studies suggest that identification of mucin oligosaccharides may help to determine functional roles of mucins.

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In a previous study, we generated three monoclonal antibodies (mAbs), RGM22, RGM26, and RGM42, by immunizing mice with purified rat gastric mucin [6]. These mAbs recognize the carbohydrate portion of mucin and can detect antigenic mucins in the rat antral mucosa; however, the antigen recognized by RGM26 has rarely been detected in rat Brunner's glands. Here, we analyzed epitope structures recognized by these mAbs by characterizing their interactions with oligosaccharides of porcine gastric mucin.

2. Experimental section

2.1. Monoclonal antibodies

Monoclonal Abs RGM22, RGM26, and RGM42 were generated by immunizing mice with purified rat gastric mucin as previously described [6] and provided as hybridoma culture supernatants by Kanto Chemical Co., Inc. (Tokyo, Japan). All mAbs were IgM, κ isotype.

2.2. Immunohistochemical staining of rat gastroduodenal mucosa

Eight-week-old male Sprague Dawley (SD) rats (weighing approximately 250 g; Charles River Laboratories Japan, Inc., Kanagawa, Japan) were sacrificed by CO_2 inhalation, and the gastroduodenal mucosa was excised and cleaned lightly in PBS. After Carnoy's fixation, the tissues were dehydrated with ethanol, cleared in xylene, embedded in paraffin, and cut into 3 μm-thick sections for processing by immunohistochemical staining using a horseradish peroxidase (HRP)-labeled polymer system (Dako, Tokyo, Japan). Briefly, endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 30 min, and the tissue was sequentially incubated with 5% bovine serum albumin (BSA) in PBS for 1 h, each mAb culture supernatant diluted 5 times with PBS for 1 h, and the HRP-labeled EnVision polymer linked to goat anti-rabbit and anti-mouse Igs antibody for 1 h. The tissue sections were then incubated with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan) in 50 mM Tris-HCl, pH 7.6, containing 0.005% H_2O_2 for 4–5 min. The samples were counterstained with Mayer's hematoxylin (Merck KGaA, Darmstadt, Germany).

This study was conducted in accordance with the guidelines of the Animal Research Committee of the Kanagawa Institute of Technology.

2.3. Preparation of oligosaccharides from porcine gastric mucin

Porcine gastric mucin (Sigma-Aldrich, St. Louis, MO, USA) was resuspended in distilled water, and the water-soluble mucin portion was isolated by ethanol precipitation. The fraction precipitated with 33–50% ethanol was collected by centrifugation, and oligosaccharides were isolated by alkaline borohydride treatment (0.05 M NaOH in 1.0 M $NaBH_4$) at 50 °C for 24 h as previously described [11]. The reaction mixture was cooled, acidified by dropwise addition of glacial acetic acid (final pH 5), and applied to a Dowex 50WX8 hydrogen form column (ø 25 mm × 400 mm, Dow Chemical Co., Midland, MI, USA). The eluted fractions were subsequently applied to a Fractogel EMD DEAE (M) column (ø 25 mm × 400 mm, Merck KGaA), and the flow-through fractions were collected. The resulting neutral oligosaccharide fractions were applied to a Bio-Gel P-6 column (ø 15 mm × 1000 mm, Bio-Rad Laboratories, Hercules, CA, USA) and eluted with distilled water. Oligosaccharide elution was monitored by a colorimetric hexose assay using the phenol-sulfuric acid method, as previously described [12].

2.4. HPLC fractionation

A Hitachi L-6000 HPLC system (Hitachi, Tokyo, Japan) equipped with three tandem Mightysil RP-18 columns (ø 4.6 mm × 250 mm × 3 columns, Kanto Chemical) was used for further fractionation of the reduced oligosaccharides. HPLC separation was performed by a gradient elution with distilled water and acetonitrile. The sample was injected into a column that had been equilibrated with distilled water, and it was eluted 30 min later with 10% (v/v) acetonitrile, which was then linearly increased to 20% by 110 min and to 100% by 115 min at a flow rate of 0.65 mL/min. The UV absorption of the eluate was monitored at 215 nm. The purity of the eluted fractions was analyzed using two TSKgel Amide-80 columns (ø 4.6 mm × 250 mm × 2 columns, Toso Co., Ltd., Tokyo, Japan) in tandem under the following isocratic conditions: eluent, 70% acetonitrile; flow rate, 1.0 mL/min; and column temperature, 60 °C.

2.5. Neoglycolipid preparation

Oligosaccharides isolated by HPLC were derivatized to neoglycolipids (NGLs) according to the method of Chai et al. [13] The resulting oligosaccharide alditols (*ca.* 10 μg) were oxidized with sodium periodate in 40 mM imidazole-HCl buffer, pH 6.5, at 0 °C in the dark for 30 min. Excess periodate was removed by incubation with meso-2,3-butanediol (Acros Organics, Geel, Belgium) at 0 °C for 40 min. Next, for Schiff base linkage, the oligosaccharides were reacted with 1,2-dipalmitoyl-3-phosphatidylethanolamine (DPPE, NOF Corp., Tokyo, Japan) in a chloroform-methanol mixture (1:1, v/v) at 60 °C for 1 h and then reduced with sodium cyanoborohydride at 60 °C for 16 h. NGLs derived from standard oligosaccharides (GalNAcα1-3Gal, GalNAcα1-3Galβ1-4Glc, GalNAcα1-3(Fucα1-2)Gal, and GalNAcα1-3(Fucα1-2)Galβ1-4Glc purchased from Dextra Laboratories Ltd, Reading, UK) were prepared in the same manner without oxidation by periodate. The resulting NGLs were subjected to dot-blot analysis to identify the oligosaccharides that reacted with the mAbs.

2.6. Dot-blot assay

NGLs derived from porcine gastric mucin oligosaccharides or standard oligosaccharides were spotted on nitrocellulose membranes; NGLs derived from 10 ng of oligosaccharides [0.5 μL in a chloroform-methanol mixture (1:1, v/v)] were applied to each spot. After blocking with 5% BSA in PBS for 1 h, the membranes were incubated for 1 h with each mAb culture supernatant diluted 5 times with 1% BSA in PBS, and then for 1 h with HRP-conjugated goat anti-mouse Igs (DAKO) diluted 2000 times with PBS. The reaction was developed by incubation in 0.1% DAB (Dojindo Laboratories) in 50 mM Tris-HCl buffer, pH 7.6, containing 0.001% H_2O_2 for a few minutes. The membranes were washed three times with PBS between steps. Each step was performed at room temperature (RT).

2.7. ELISA and competitive ELISA

ELISA was performed as described previously [7]. Briefly, the wells of microtiter plates were coated with 50 ng of porcine gastric mucin (in a volume of 50 μL) that had been partially purified by ethanol precipitation in 50 mM carbonate buffer, pH 9.6. The wells were then incubated overnight at 4 °C, followed by blocking with 400 μL of 2% (w/v) skim milk in PBS at RT. After washing, the wells were incubated at RT for 1 h with 50 μL of each mAb culture supernatant diluted 10 times with PBS, and then for 1 h with 50 μL of HRP-conjugated goat anti-mouse Igs (DAKO) diluted 20,000 times with PBS. The wells were finally incubated with 50 μL of 2,2'-azino-