Direct Demonstration of Increased Expression of Thomsen-Friedenreich (TF) Antigen in Colonic Adenocarcinoma and Ulcerative Colitis Mucin and Its Concealment in Normal Mucin

Barry J. Campbell, Ian A. Finnie, Elizabeth F. Hounsell,* and Jonathan M. Rhodes
Glycobiology Unit, Department of Medicine, University of Liverpool, Liverpool L69 3B; and *Glycoprotein Structure/Function Group, Department of Biochemistry and Molecular Biology, University College London, London WC1E 6BT, United Kingdom

Abstract

Increased binding of the lectin peanut agglutinin is a common feature in epithelial malignancy and hyperplasia. This may have considerable functional importance in the intestine by allowing interaction between the epithelium and mitogenic lectins of dietary or microbially origin. Peanut agglutinin binds the disaccharide Thomsen-Friedenreich (TF,T or core 1) blood group antigen, Galβ(1-3)GalNAcα-1, but is not totally specific for this site. Consequently, there has been controversy about the presence of this structure in colon cancer; studies with anti-TF monoclonal antibodies have failed to detect it. We have examined the presence of TF antigen in colonic mucus glycoprotein (mucin) using endo-α-N-acetylgalactosaminidase (O-Glycanase®, which specifically catalyzes the hydrolysis of TF antigen from glycopolymers. Samples of adenocarcinoma, inflammatory bowel disease (ulcerative colitis), and normal mucin were treated with O-glycanase, the liberated disaccharide was separated from the glycoprotein and analyzed using dual CarboPac PA-100 column high performance anion-exchange chromatography coupled with pulsed amperometric detection. O-Glycanase treatment released increased amounts of TF antigen from both colonic adenocarcinoma (8.0±3.9 ng/μg protein, n=11; P<0.0001 ANOVA) and ulcerative colitis mucin (3.3±0.3 ng/μg protein, n=5; P=0.04) compared with mucin samples from histologically normal mucosa distant from carcinoma (1.5±1.1 ng/μg protein, n = 9). However, after mild acid treatment to remove sialic acids and fucose, releasable TF antigen was increased in all nine of these histologically normal mucin samples (5.5±2.6 ng/μg protein, P<0.0002). We conclude that TF antigen is an oncofetal antigen which is expressed in colon cancer, but is concealed by further glycosylation (sialylation and/or fucosylation) in the normal colonic mucosa. (J. Clin. Invest. 1995. 95:571–576.) Key words: carbohydrate oncofetal antigen • colorectal cancer • mucoprotein • ulcerative colitis • peanut agglutinin binding site

Introduction

Increased binding of the lectin peanut agglutinin (PNA) is a common feature in epithelial malignancy and hyperplasia, occurring in colorectal tumors (1–3), both within the cells and in secreted material (4, 5), in adenomatous and metastatic polyps (2, 3, 6), and in the mucosa of patients with inflammatory bowel disease (7, 8). PNA binding is not detectable in normal colonic mucosa (3, 6, 9), unless sensitivity is increased by avidin-biotin amplification (2, 6).

PNA has been shown previously to be mitogenic for normal human colonic epithelium (10), colorectal cancer cell lines (10, 11), and for colonic polyp and inflammatory bowel disease mucosa (12). PNA binds to the disaccharide Thomsen-Friedenreich (TF) blood group antigen (Galβ1–3GalNAcα–) which is a core carbohydrate structure of O-linked oligosaccharides on glycoproteins (13). It has been suggested that this disaccharide behaves as an oncofetal antigen in many epithelial tissues being expressed in hyperplasia and malignancy; for example, TF is expressed in neonatal colon and meconium (14, 15) but in normal adult epithelium it is thought to be masked by further glycosylation and/or sialylation (16, 17). The hypothesis has been proposed (10) that increased expression of TF antigen by hyperplastic and malignant epithelia may be an important determinant of the altered proliferation, by allowing interaction with luminal Galβ1–3GalNAc-binding lectins of dietary and microbial origin.

However, there has been controversy about the presence of TFα antigen in malignant tissues. PNA is not totally specific for TFα (Galβ1–3GalNAcα–, on glycoproteins), possessing a high degree of affinity for both TFβ (Galβ1–3Galβ1–3GalNAcβ–, on glycolipids) and some affinity for type 1 (Galβ1–3Galβ1–3GalNAcβ1–3), chains (18, 19). This uncertainty has been increased by some studies which have failed to demonstrate TFα in colonic malignant tissue using carbohydrate-specific monoclonal antibodies (20, 21). In addition, a rigorous analysis of the oligosaccharides in normal adult colonic mucin failed to detect Galβ1–3GalNAc as a core structure (22). However, a study using α-galactose oxidase/Schiff’s reagent (23) has suggested the presence of Galβ1–3GalNAc in malignant tissues.

We have sought to clarify this confusion by specific quantification of TFα antigen released from histologically normal...
ulcerative colitis and carcinomatous colonic mucin, using high-pH anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) after specific enzymatic release with endo-α-N-acetylgalactosaminidase (O-glycanase), which hydrolyzes glycosidic bonds formed by N-acetylgalactosamine and the hydroxyl group of either serine or threonine only when in the sequence Galβ1-3GalNAc-α-O-Ser/Thr (24, 25).

**Methods**

**Materials**

Galβ1-3GalNAc standard was purchased from Russel Fine Chemicals (Chester, United Kingdom); collagenase [EC 3.4.24.8], hyaluronidase [EC 3.2.1.35], and protease-free chondroitin ABC lyase [EC 4.2.2.4] from Boehringer Mannheim (Lewes, Sussex, United Kingdom); endo-α-N-acetylgalactosaminidase (O-Glycanase); from Streptococcus [Diplococcus] pneumoniae) [EC 3.2.1.97] and bovine testis β-galactosidase [EC 3.2.1.23] from Oxford Glycosystems Ltd. (Abingdon, Oxfordshire, United Kingdom). CAM17.1 monoclonal anti-mucin antibody was generously donated by Dr. Carol A. Makin (Imperial Cancer Research Fund, London, United Kingdom). The Antarctic fish (Trematomus borghrevinki) antifreeze glycopeptides AFGP-1 (antifreeze glycopeptides 1–5; BD-87–1-I) and AFGP-XV (antifreeze glycopeptides 7 and 8; CC-86–2–XV) were kind gifts from Professor Arthur L. DeVries (University of Illinois, Urbana, IL). Asialofetuin (type 1), α-L(-)-fu-

sine, N-acetyl-d-galactosamine, and d-galactose were all purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). d(+)-melibiose (6-O-d-galactopyranosyl-d-glucopyranoside) was purchased from BDH Chemicals Ltd. (Poole, Dorset, United Kingdom).

**Tissue specimens**

Surgical resection specimens were obtained from 11 patients with malignant colorectal adenocarcinoma, from 9 of which adjacent histologically normal colonic tissue (at least 10 cm away from carcinoma) was available. Five active ulcerative colitis colectomy specimens were also obtained. Tissue was stored at −80°C before treatment.

**Purification of colonic mucus glycoproteins (mucin)**

Each surgical resection specimen was diced frozen and homogenized (100 mg tissue/ml) using a Polytron® (Kinematica GmbH, Kriens-Luzern, Switzerland), in phosphate-buffered saline (PBS), pH 7.4, containing a cocktail of protease inhibitors (2 μM aprotinin, 10 μM leupeptin, 5 mM benzamidine hydrochloride, and 0.01% wt/vol thimerosal). Each homogenate was ultrasonicated and centrifuged using conditions described previously (26), and supernatants were desalted on PD10-Sephadex GM25 (5 × 1.5 cm) columns (Pharmacia AB, Uppsala, Sweden). Contaminating proteoglycans were removed by successive incubation with collagenase (1 mg/ml in PBS, pH 7.4), hyaluronidase (1 mg/ml in 20 mM phosphate buffer, pH 5.8), and protease-free chondroitin ABC lyase (0.02 U/ml in 0.01 M Tris-HCl, pH 8.0), each for 6 h, at 37°C.

Mucin was purified from homogenates by Sepharose CL-2B mini-columns (5 × 1.5 cm) eluted with PBS, pH 7.4, after which the void volume was subject to high performance gel filtration using Superose 6 as described previously (26). Purified mucin was identified by ELISA using the CAM 17.1 anti-mucin monoclonal antibody (see reference 26) and assayed for protein using a bichinchoninic acid assay (kit BCA-1; Sigma Chemical Co.), expressed relative to a standard of bovine serum albumin (BSA).

**Hydrolysis of Galβ1-3GalNAc using O-glycanase**

O-Glycanase was supplied in 10 mM citrate/phosphate buffer, pH 6, containing 25% vol/vol glycerol. The glycerol interfered with HPAEC analysis and was removed by two passages through Bio-Gel P30 polyacrylamide gel columns (Bio-spin®; Bio-Rad Laboratories, Hercules, CA).

Lyophilized samples of purified colonic mucin (100 μg protein) were incubated with 0.6 mU deglyceroalted O-glycanase in 100 μl 100 mM citrate/phosphate buffer, pH 6, containing 100 μg/ml BSA and 0.02% wt/vol sodium azide, for 18 h at 37°C. Enzymatic release was confirmed using the antifreeze glycopeptides AFGP-I and AFGP-XV (0–100 μg) and asialofetuin (0–200 μg) as control TF-expressing substrates (27, 28). Appropriate control samples, incubated in the absence of O-glycanase and incubated with O-glycanase after pretreatment with bovine testis β-galactosidase, were studied.

O-Glycanase–liberated disaccharide was separated from intact glycoprotein and enzyme by sequential chromatography on Dowex 50X-12. Enzymatic release was monitored at an OD of 280 nm and using a phenol-sulfuric acid assay for neutral sugars (see later). Fractions containing neutral sugar (6.5–10.5 ml) were pooled, lyophilized, redissolved in 500 μl HPLC water (Rathburn Chemicals, Walkerburn, United Kingdom), and filtered through 0.2-μm (pore-size) Anotop IC® filters (Whatman, Maidstone, Kent, United Kingdom).

**Mild acid hydrolysis of mucin**

Lyophilized colonic mucin (100 μg protein) was subjected to mild acid hydrolysis (0.05 M sulfuric acid, 80°C, for 2 h) to release sialic acid and fucose (15, 29), followed by resolution of products using PD10-Sephadex GM25. The hydrolyzed mucin was then subjected to O-glycanase treatment.

**Preparation of degalactosylated glycopeptides**

Lyophilized samples of colonic mucin (100 μg protein), asialofetuin (0–200 μg), and antifreeze glycopeptides AFGP-I and AFGP-XV (0–100 μg) were incubated with 10 μl bovine testis β-galactosidase in 100 mM citrate/phosphate buffer, pH 4, for 6 h at 37°C (final volume, 20 μl). Treated glycopeptides, resolved using PD10-Sephadex GM25, were subjected to O-glycanase treatment.

**Phenol-sulfuric acid assay for neutral sugars**

A scaled down version of the method of Dubois et al. (30) was used. 10-μl samples were placed into ELISA microtiter plates (Dynatech Laboratories Inc., Chantilly, VA), agitated with 100 μl 4% wt/vol phenol in distilled water, and left to stand for 5 min. 150 μl of concentrated sulfuric acid was added rapidly (with care) from a fast-delivery multichannel pipette and mixed immediately (five aspirations) to generate a high temperature. Plates were allowed to cool for 20 min and were read at 490 nm. A water blank and standards of 0.5–20 μg galactose (in triplicate) were included in each assay. The between assay coefficient of variation for standards was 7.7% (n = 8).

**HPAEC**

Sample analysis was performed using a HPLC system coupled with PAD (Dionex UK, Camberley, Surrey, United Kingdom).

**Oligosaccharide analysis**

Galβ1-3GalNAc was separated using conditions described previously for neutral oligosaccharide aldithiols (31). 100-μl aliquots of O-glycanase reaction products, containing 320 ng of melibiose as internal standard, were injected onto the dual CarboPac PA-100 anion-exchange columns, calibrated with standard Galβ1-3GalNAc. The calibration curve for Galβ1-3GalNAc (0.0625–1.0 μg), recovered from PD10-Sephadex GM25 (percent analytical recovery, 96.7 ± 2.3 [mean ± SD]; n = 8), was represented by the equation y = 188.77 x − 0.69 (r = 0.99) using peak height and internal standard ratio quantification analysis. The within assay coefficient of variation was 9.3%, the between assay coefficient of variation was 10.1% (n = 8), and the lower limit of detection for Galβ1-3GalNAc (1 μA output) was 0.02 μg.

In addition, O-glycanase–liberated products were reduced to oligosaccharide aldithiols using alkaline-borohydride as described previously (31) and reanalyzed using HPAEC-PAD. Samples were compared with reduced standard Galβ1-3GalNAc and with the alditol R1-ol (Galβ1-3GalNAc-ol) derived from human meconium glycoprotein (15).
Monosaccharide analysis. The monosaccharide composition of O-glycanase products was determined after digestion with β-galactosidase. 200-μl aliquots of colonic mucin O-glycanase reaction mixtures were lyophilized and incubated with 10 mU of β-galactosidase in 20 μl 100 mM citrate/phosphate buffer, pH 4, for 6 h at 37°C. O-Glycanase-treated antifreeze glycopeptides (100 μl), asialofetuin (200 μl), and 10 μg standard Galβ1–3GalNAc were identically treated. Aliquots (1–10 μl) of the β-galactosidase reaction mixtures were diluted to 100 μl with HPLC grade water containing 100 ng L-fucose as an internal standard, injected directly onto a single CarboPac PA-100 column, and eluted isocratically with 15 mM sodium hydroxide for 20 min, at 0.8 ml/min. 300 mM sodium hydroxide solution (post-column reagent) was added to the column effluent at 0.6 ml/min to facilitate detection. PAD conditions were as described previously (31). Between each analysis the column was regenerated for 5 min with 20% 500 mM sodium acetate/15 mM sodium hydroxide and then reequilibrated for 30 min with 15 mM sodium hydroxide.

Statistical analysis
Sample groups were analyzed using Kruskall-Wallis nonparametric ANOVA (Arcus Pro-Star®, Medical Computing, Aughton, Lancashire, United Kingdom). Differences were considered significant when P < 0.05.

Results
Release of Galβ1–3GalNAc (TF antigen) from mucin using O-glycanase. The treatment of colonic adenocarcinoma mucin with O-glycanase released a saccharide structure with a compatible retention time (using dual column HPAEC) to that of both standard Galβ1–3GalNAc and Galβ1–3GalNAc released by O-glycanase from the control TF antigen—expressing substrate AGFP-I (Fig. 1). Subsequent monosaccharide composition analysis of the O-glycanase released saccharide from adenocarcinoma mucin and AGFP-I, after treatment with bovine testis β-galactosidase, produced two monosaccharide peaks consistent with galactose and N-acetyl-galactosamine (Fig. 2). Likewise, alkaline-borohydride reduction of O-glycanase released products from adenocarcinoma mucin and the antifreeze glycopeptides resulted in a disaccharide alditol with a retention time comparable with R1-ol (Galβ1–3GalNAc-ol, from human meconium glycoprotein) and to that of reduced standard Galβ1–3GalNAc (data not presented).

O-Glycanase treatment released Galβ1–3GalNAc from all three of the control TF antigen—expressing substrates AGFP-I (39±32 ng/μg glycoprotein [GP]), AGFP-XV (227±26 ng/μg GP), and asialofetuin (42±4 ng/μg GP) (mean±SD, n = 6; 100-μg sample). Pretreatment with β-galactosidase reduced the release of Galβ1–3GalNAc by O-glycanase to 36±11, 33±4, and 25±4% of control levels, respectively.

Tissue expression of Galβ1–3GalNAc. O-Glycanase treatment liberated Galβ1–3GalNAc from 10 out of 11 colonic adenocarcinoma mucus glycoprotein samples (8.0±3.9 ng/μg protein [mean±SD], 95% confidence intervals [CI] 5.2–8.8; n = 11) and from all 5 ulcerative colitis mucin samples (3.3±0.3 ng/μg protein, 95% CI 2.2–4.4; n = 5). Release of Galβ1–3GalNAc from O-glycanase-treated histologically normal colonic mucin was, however, significantly lower (1.5±1.1 ng/μg protein, 95% CI 0.5–2.5; n = 9) in comparison with adenocarcinoma (P < 0.0001 ANOVA) and colitic mucin (P = 0.04 ANOVA). Prior treatment of adenocarcinoma mucin with bovine testis β-galactosidase markedly reduced the release of Galβ1–3GalNAc by O-glycanase (1.7±0.8 ng/μg protein, n = 11) when compared with untreated samples (8.0±3.9 ng/μg protein; P < 0.0001).

The total content of neutral O-glycans in both colonic adenocarcinoma (0.68±0.2 μg/μg protein; n = 11, P < 0.0001 ANOVA) and ulcerative colitis mucins (0.85±0.2 μg/μg protein; n = 5, P = 0.002) was significantly lower when compared with levels in histologically normal colonic mucin (1.24±0.2 μg/μg protein; n = 9). TF antigen, expressed as a proportion
Figure 2. Monosaccharide composition analysis of β-galactosidase-treated O-glycanase reaction products using single CarboPac PA-100 column HPAEC. All samples contained 100 ng L-fucose (1) as internal standard (retention time, 5.27 min). (a) β-Galactosidase alone; (b) standard N-acetylgalactosamine (2) and galactose (3) (both 300 ng); (c) β-galactosidase–treated standard Galβ1–3GalNAc; (d–f) β-galactosidase–treated O-glycanase–liberated disaccharide from antifreeze glycopeptide AFGP-I, colonic adenocarcinoma mucin, and mild acid–treated normal colonic mucin, respectively. PAD output was 1 μA.

Figure 3. Galβ1–3GalNAc, expressed as nanograms per microgram of mucin neutral carbohydrate, liberated by O-glycanase from mucin purified from colonic adenocarcinoma (n = 11), adjacent histologically normal (n = 9), and ulcerative colitis (n = 5) resection specimens. TF antigen was significantly higher in adenocarcinoma and colitic mucin samples when compared with normals. ***P < 0.0001 and **P = 0.0018, respectively; ANOVA.

of the neutral sugar content, was markedly increased in adenocarcinoma (12.3±2.1 ng Galβ1–3GalNAc/μg neutral carbohydrate, 95% CI 7.8–16.9; n = 11, P < 0.0001 ANOVA) and colitic mucin (4.2±0.7 ng/μg neutral carbohydrate, 95% CI 2.3–6.2; n = 5, P = 0.0018) compared with the histologically normal mucin samples (1.2±0.3 ng/μg neutral carbohydrate, 95% CI 0.48–1.9; n = 9) (Fig. 3).

After mild acid treatment of histologically normal colonic mucin to remove sialic acids and fucose, levels of Galβ1–3GalNAc released by O-glycanase treatment were substantially increased in all nine samples (5.5±2.6 ng/μg protein, 95% CI 3.5–7.6; P = 0.0002 ANOVA) when compared with untreated normal mucin.

Discussion

This study provides the first conclusive demonstration of the presence of the Thomsen-Friedenreich (TF) carbohydrate antigen, β-D-galactoseβ(1 → 3)α-N-acetylgalactosamine (in α-anomeric configuration), in adult human intestinal mucin. The method uses the specificity of streptococcal endo-α-N-acetylgalactosaminidase (24, 25) which eliminates any possibility of detection of TFβ antigen released from glycolipids. Moreover, monosaccharide analysis has shown that the released disaccharide has the same composition as the disaccharide released by...
O-glycanase from TFα antigen–expressing antifreeze glycopeptide, which is glycosylated in the form of repeating diglycosyltripeptide units, Galβ1–3GalNAcα-O-(Thr-Ala-Ala)₃ (27). This study also confirms the increased expression of Galβ1–3GalNAc in premalignant and malignant colonic mucosal disease and its relative lack of expression in histologically normal mucosa, which had previously been demonstrated indirectly by PNA histochemistry (1–9) and the α-galactose oxidase/Schiff’s reactivity test (23).

In addition, we have shown an overall reduction in neutral O-glycans of 45% in human adenocarcinoma mucin when compared with histologically normal mucin, a fact consistent with data from a previous study of malignant colonic mucin (32). There was also a reduction in neutral carbohydrate content seen in ulcerative colitic mucin (32%). Decreased mucin oligosaccharide side-chain length has been observed in active colitis and Crohn’s disease (33) and oligosaccharide changes have also been demonstrated by alteration in the binding of anticallose antibodies and lectins (9, 34, 35). It is interesting that so far no change in mucin structure has been found in inflammatory bowel disease that has not also been found in colon cancer and in hyperplastic or adenomatous colonic polyps. Although these changes in carbohydrate expression seem likely to be secondary to the disease process, they are likely to have very important functional consequences for mucosal protection (36), perhaps making the mucin more susceptible to degradation by bacterial mucus-degrading glycosidases which are secreted into the colonic lumen (37), and provide a good explanation for the marked thinning of the surface mucus layer seen in ulcerative colitis (38).

The increased availability of Galβ1–3GalNAc for PNA binding in malignancy could be explained either by reduced sialylation (16, 17) or reduced fucosylation (39) of Galβ1–3GalNAc, or by a loss of the more common core 3 structure GlcNAcβ1–3GalNAc (22), allowing preferential synthesis of core 1, Galβ1–3GalNAc. Evidence from the present study, that mild acid hydrolysis reveals TF antigen in normal mucin, would suggest reduced sialylation or fucosylation as the mechanism, but a recent study has reported reduced β1–3GalNAc → GalNAc transferase but unchanged β1–3GalNAc transferase in colorectal carcinoma cells (40). It is quite likely that different mechanisms account for TF expression in different situations. Little is currently known about the mechanisms which regulate the relevant glycosyltransferases but ras oncogene transformation has been shown to result in decreased α2–3 → Galβ1–3GalNAc sialyltransferase activity (41).

Mucins are the predominant glycoproteins secreted by colonic epithelium and unlike most membrane-bound glycoconjugates they can be isolated in sufficient quantity to permit biochemical analysis of their carbohydrate. However, histoch- eological studies suggest that changes in O-glycosylation of epithelial mucin are often paralleled by similar changes in the O-glycosylation of epithelial cell–surface glycoconjugates (3). We have recently proposed that increased TF antigen expression on hyperplastic and malignant epithelial cell–surface glycoconjugates may have functional significance by allowing interaction with Galβ1–3GalNAc–binding lectins of dietary and microbial origin. These lectins can have marked effects on proliferation and mucosal metabolism. PNA, which resists digestion in the mammalian intestine, stimulates proliferation and mucus synthesis in vitro (10–12). Likewise, anti-TF monoclonal antibodies have been shown to produce similar marked effects on colorectal cancer cell line proliferation (42). Moreover, in a recent study we have shown that ingestion of peanuts for 1 wk resulted in an increased rectal mitotic index of 40% in the one third of subjects (mainly irritable bowel syndrome) who had identifiable expression of PNA receptor by the histologically normal colonic mucosa (43). Other Gal-GalNAc–binding lectins, such as edible mushroom (Agaricus bisporus) and jack fruit (jacalin) lectins, cause noncytotoxic inhibition of proliferation of colorectal cell lines (44, 45). Therefore, it seems likely that the interaction of luminal dietary and microbial lectins with TF-expressing epithelial cells could be quantitatively as important as interaction with growth factors in determining the increased proliferation that occurs in hyperplasia and as a prelude to malignant change. Further studies are required to determine those glycosyltransferase changes which account for the increased expression of TF antigen.

Acknowledgments

The authors would like to thank Mr. M. J. Hershman (Department of Surgery, Royal Liverpool University Hospital) for his cooperation in obtaining the resection tissue specimens.

Ethical committee permission for this study was granted by the Royal Liverpool University Hospital Ethics committee (Liverpool, United Kingdom). B. J. Campbell was funded by the Medical Research Council and I. A. Finnie was funded by the Mersey Regional Health Authority.

References