Pergamon

European Journal of Cancer Vol. 31A, No. 9, pp. 1498–1505, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00248-0

Original Paper

Inhibition of Mucin Synthesis by Benzyl-α-GalNAc in KATO III Gastric Cancer and Caco-2 Colon Cancer Cells

J.C. Byrd, R. Dahiya, J. Huang and Y.S. Kim

Previous studies from our laboratory have shown that benzyl-α-GalNAc inhibits the glycosylation of mucin in colon cancer cells. In this study, we determined whether benzyl-α-GalNAc inhibits mucin glycosylation in KATO III gastric cancer cells. We also examined its effects on expression of mucin antigens, and compared the mucins made by KATO III with those of a colonic cancer cell line, Caco-2. Results of these experiments suggest that benzyl-α-GalNAc (2 mM) inhibited [^{3}H]glucosamine labelling of mucins by 82% in KATO III and by 70% in Caco-2. For both cell lines, the mucin secreted in the presence of benzyl-α-GalNAc was less acidic. Both cell lines secreted benzyl-oligosaccharides, but those from KATO III (8–9 sugars) were larger than those from Caco-2 (6–7 sugars). In mucins purified from the medium of treated cells, peripheral carbohydrate antigens (sialyl Le^x in KATO III and terminal fucose in Caco-2) were decreased (compared with control), while core carbohydrate antigens (T antigen in both cell lines and sialyl Tn in Caco-2) were increased. Western blots of cell homogenates showed differences between KATO III and Caco-2 in MUC 1 apomucin protein antigens, in sialyl Le^x and in sialyl Tn antigens. We conclude that benzyl-α-GalNAc does inhibit the glycosylation of mucin in KATO III gastric cancer cells as in human colon cancer cells, but that alterations in mucin antigens occur in a cell line-specific manner.

Key words: mucin glycosylation inhibition, benzyl-α-*N*-acetylgalactosamine, mucin synthesis, gastric cancer cells, colon cancer cells

Eur J Cancer, Vol. 31A, No. 9, pp. 1498–1505, 1995

INTRODUCTION

RECENT STUDIES from this laboratory have shown that treatment of colon cancer cells with benzyl-α-GalNAc inhibits the glycosylation of mucins, and can decrease the metastatic capacity of colon cancer cells both *in vitro* and *in vivo* experimental models [1–3]. Benzyl-α-GalNAc was found to be an effective inhibitor of mucin glycosylation in other human colon cancer cell lines [4], and the related inhibitor, phenyl-α-GalNAc, has been shown to inhibit synthesis of a mucin carbohydrate antigen containing *N*-glycolyl-neuraminic acid in MCF-7 breast cancer cells [5]. Benzyl-α-GalNAc has also been shown to block the expression of cell surface sialyl Le^x and sialyl Le^a antigens in human leukaemia, lymphoma, and colon cancer cell lines [6]. However, the effect of benzyl-α-GalNAc gastric cancer cells has not been reported. KATO III gastric cancer cells have been used in several studies of the expression of cancer-associated carbohydrate antigens [7–11], and for studying the cellular binding of *Helicobacter pylori* [12–14]. However, the type and amount of mucin synthesised by KATO III has not been directly studied.

The purpose of this study was to determine whether benzyl-α-GalNAc inhibits mucin synthesis in KATO III (gastric cancer) cells. We also wanted to examine the effects of mucin inhibition on expression of core and peripheral carbohydrate antigens, and to compare KATO III gastric cancer cells to a colon cancer cell line, Caco-2.

MATERIALS AND METHODS

Materials

Benzyl-α-GalNAc was purchased from Sigma Chemical (St Louis, Missouri, U.S.A.). [^{3}H]Glucosamine (30 Ci/mmol) and [^{3}H]threonine (16 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, California, U.S.A.). Leupeptin and pepstatin were from Boehringer Mannheim (Indianapolis, Indiana, U.S.A.). Sepharose CL-4B and DEAE-cellulose were from Sigma. BioGel P-2 was from BioRad (Richmond, California, U.S.A.). Superose-6 was from Pharmacia (Piscataway, New Jersey, U.S.A.).

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Revised 5 Dec. 1994; accepted 15 Dec. 1994.

Succinylated wheat germ agglutinin and *Bandeiraea simplicifolia* agglutinin II, conjugated with horseradish peroxidase, were from EY Labs (San Mateo, California, U.S.A.). Other peroxidase-labelled lectins and rabbit antibodies to lectins were purchased from Sigma Chemical (St Louis, Missouri, U.S.A.). Peroxidase labelled rabbit antimouse IgG+IgA+IgM was from Zymed (South San Francisco, California, U.S.A.). Monoclonal antibody JT1Oe, specific for a sialyl Tn epitope, was prepared in this laboratory [15] using LS174T xenograft mucin [16] as immunogen. Monoclonal antibody 15D3a, specific for Tn epitopes, was prepared in this laboratory [17] using partially deglycosylated LS174T xenograft mucin [18] as immunogen. Monoclonal antibody 19-9, specific for sialyl Le^a, was obtained from Centocor (Malvern, Pennsylvania, U.S.A.). Monoclonal antibody SNH3, specific for sialyl-dimeric Le^x [19], was the kind gift of Dr A. Singhal (Seattle, Washington, U.S.A.). Monoclonal antibody 139H2, specific for MUC1 mucin, was the kind gift of Dr J. Hilkens. KATO III human gastric cancer cells and Caco-2 human colon cancer cells were obtained from the ATCC. Cells were cultured in Dulbeccos's modified Eagles medium (DMEM) containing 10% fetal calf serum.

Radiolabelling of cells

For [^{3}H]glucosamine labelling, subconfluent cells were pre-incubated in low-glucose DMEM containing 10% fetal calf serum for 24 h, then in glucose-free DMEM containing 10% fetal calf serum for 1 h. The cells were labelled for 24 h with 10 μCi/ml [^{3}H]glucosamine in glucose-free DMEM containing 10% fetal calf serum. For [^{3}H]threonine labelling, subconfluent cells were pre-incubated in DMEM with 10% fetal calf serum for 24 h, then for in threonine-free DMEM containing 10% fetal calf serum for 1 h. Cells were then labelled with 83 μCi/ml [^{3}H]threonine in low-threonine DMEM (threonine-free DMEM:DMEM, 9:1) containing 10% fetal calf serum. For benzyl-α-GalNAc-treated cells, 2 mM benzyl-α-GalNAc was included in both the pre-incubation and labelling medium.

After labelling, cells were rinsed twice with phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.4, 150 mM NaCl) containing protease inhibitors (0.5 μ/ml leupeptin, 1 mM EDTA, 0.7 μg/ml pepstatin). The cell washes were added to the medium fraction. Cells were then sonicated in 10 mM Tris pH 8.0 containing 0.5 μg/ml leupeptin, 1 mM EDTA, 0.7 μg/ml pepstatin, and an aliquot was reserved for protein assays [20]. Homogenates were centrifuged (at 4°C) for 60 min at 100 000 ***g*** and the supernatant was designated as the cytosol fraction. The resulting pellet was sonicated in the presence of the same buffer containing 0.1% Triton X-100 and re-centrifuged (at 4°C) for 60 min at 100 000 ***g***. The resulting detergent extract was designated as the membrane fraction.

Chromatographic analyses

For quantitation of the amount of high molecular weight glycoprotein (HMG), aliquots of medium, cytosol, and membrane fractions were analysed on a column (1.0 × 47 cm) of Sepharose CL-4B in 10 mM Tris pH 8.0 at 4°C. For membrane samples, the column eluent also contained 0.1% Triton X-100.

DEAE-cellulose chromatography was performed at 4°C in 10 mM Tris pH 8.0. The [^{3}H]glucosamine-labelled mucins from the void volume of Sepharose CL-4B were applied to columns of DEAE-cellulose (1.0 × 2.5 cm) which were eluted with a step gradient of 50–300 mM NaCl in 10 mM Tris pH 8.0. Fractions of 3 ml were collected, and aliquots were counted for radioactivity.

Oligosaccharides were analysed by gel filtration at room temperature on a column (0.9 × 94 cm) of BioGel P-2, eluted at 25 ml/h with 0.1 M acetic acid. Fractions of 0.64 ml were collected and counted for radioactivity. Internal standards of dextran (to mark the void volume) and of stachyose (tetrasaccharide) were monitored with the phenolsulphuric assay [21]. For analysis of benzyl-oligosaccharides, medium of cells labelled with [^{3}H]glucosamine in the presence and absence of 2 mM benzyl-α-GalNAc was analysed directly on BioGel P-2.

For analysis of the oligosaccharides on secreted mucin, medium of [^{3}H]glucosamine-labelled KATO III cells (4 ml) was lyophilised, then chromatographed at room temperature on Superose 6 in 0.1 M ammonium acetate. The lyophilised void volume fraction was treated with 0.05 M NaOH, 1 M $NaBH_4$ for 16 h at 50°C, then neutralised with acetic acid and per-evaporated from methanol/acetic acid, 200/1. The alkaline borohydride-treated mucins were re-chromatographed on Superose 6, and the included volume fractions, containing the reduced oligosaccharides released from mucin, were lyophilised and analysed on BioGel P-2. The labelling of GalNAc-ol on secreted mucin could not be quantitated because of the presence of degradation products that co-elute with GalNAc-ol.

Electrophoretic analyses

Western blotting was used to analyse antigens in cell homogenates [22]. Homogenates of cells treated for 48 h in the presence or absence of 2 mM benzyl-α-GalNAc were prepared by sonication in 10 mM Tris pH 8.0 containing 0.5 μg/ml leupeptin, 1 mM EDTA, and 0.7 μg/ml pepstatin. Homogenates were assayed for protein [20], then stored at −70°C until use. Samples of the cell homogenates (75 μg/lane) were subjected to electrophoresis in the presence of sodium dodecyl sulphate using a 4% acrylamide stacking gel and a 5% acrylamide separating gel. The samples were transferred to nitrocellulose paper, blocked with 5% bovine serum albumin in PBS, then incubated with antibodies or lectins for 1 h at room temperature. Blots with monoclonal antibodies or lectins were incubated for 1 h at room temperature with rabbit anti-mouse IgG+IgA+IgM or rabbit anti-lectin, then ^{125}I-conjugated protein A (106 cpm/ml in 2% bovine serum albumin in PBS) was applied for 1 h at room temperature. The papers were washed, at each step, with six changes of PBS containing 0.05% Nonidet P-40. Binding of ^{125}I-labelled protein A was visualised by autoradiography at −70°C using an intensifying screen.

Binding of monoclonal antibodies and lectins to purified mucins

Secreted mucins were partially purified from the medium of cells incubated for 48 h in the presence or absence of 2 mM benzyl-α-GalNAc. Preconfluent cells were pretreated for 16 h in DMEM containing 10% fetal calf serum with or without 2 mM benzyl-α-GalNAc. Fresh medium was then added, and collected after 48 h. The medium was dialysed against water, lyophilised, and applied to a column of Sepharose CL-4B (2.5 × 67 cm) which was eluted at 4°C with flow rate of 90 ml/h using 10 mM Tris pH 8.0. The void volume fraction was collected, dialysed, and lyophilised, then assayed for protein [20]. The binding of monoclonal antibodies and lectins was assayed as described previously [16, 23].

RESULTS

Effect of benzyl-α-GalNAc on growth

When KATO III gastric cancer cells were grown in 2 mM benzyl-α-GalNAc (Figure 1), the doubling time was unaffected (142 h versus 126 h in control). There was, however, a decrease in the saturation density with prolonged treatment. Similarly,

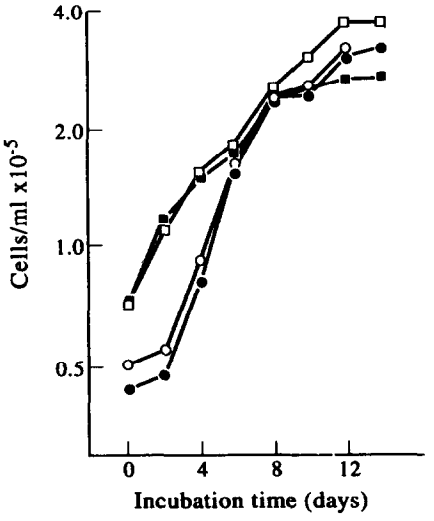

Figure 1. Effect of benzyl-α-GalNAc on growth. KATO III gastric cancer cells (□, ■) and Caco-2 colon cancer cells (○, ●) were grown in 24 well plates in the presence (■, ●) or absence (□, ○) of 2 mM benzyl-α-GalNAc. Cells were harvested and counted every second day as described in Materials and Methods.

there was little effect of 2 mM benzyl-α-GalNAc on the doubling time of Caco-2 colon cancer cells (62 h versus 68 h in control). The doubling time observed here is in close agreement with that previously reported (56 h) for Caco-2 [4]. Viability, as judged by trypan blue exclusion, was unaffected by benzyl-α-GalNAc, and lower concentrations (0.5 mM and 1 mM benzyl-α-GalNAc) gave similar results (data not shown).

Inhibition of mucin labelling in medium, cytosol and membrane fractions

Gel filtration analysis was used to examine the effect of 2 mM benzyl-α-GalNAc on incorporation of [^{3}H]glucosamine into high molecular weight glycoprotein (HMG) in the medium, cytosol, and membrane fractions of KATO III (Figure 2). In all three fractions there was a peak of radioactivity at the void volume of Sepharose CL-4B. In the cytosol fraction (Figure 2a), [^{3}H]glucosamine incorporation was inhibited by 86% by 2 mM benzyl-α-GalNAc (Table 1). Incorporation of [^{3}H]glucosamine into HMG of the membrane fraction accounted for 22% of the total cellular HMG. The labelling of this membrane-bound mucin fraction was inhibited by 74%. Of the total HMG labelled in 24 h, 37% was secreted. Benzyl-α-GalNAc treatment inhibited labelling of the secreted HMG by 79%. Benzyl-α-GalNAc inhibition of the glycosylation of soluble mucin in Caco-2 colon cancer cells has been previously reported [4].

Figure 3 shows the profile of glycoproteins from Caco-2 analysed by gel filtration on Sepharose CL4B. Incorporation of [^{3}H]glucosamine into soluble intracellular HMG was 12-fold less efficient than in KATO III (Table 1). Benzyl-α-GalNAc inhibited incorporation of [^{3}H]glucosamine into cytosol HMG by 78%. Of the total cellular HMG, 32% was in the membrane fraction. Incorporation of [^{3}H]glucosamine into membrane-bound HMG was inhibited 58% by 2 mM benzyl-α-GalNAc. In Caco-2, about 51% of the total HMG labelled in 24 h was secreted and labelling of this secreted HMG was inhibited by 69% by benzyl-α-GalNAc.

It should be noted that, besides the inhibition of HMG labelling, there was also inhibition of [^{3}H]glucosamine incorporation into products that eluted after the void volume on Sepharose CL-4B for the medium, cytosol, and membrane

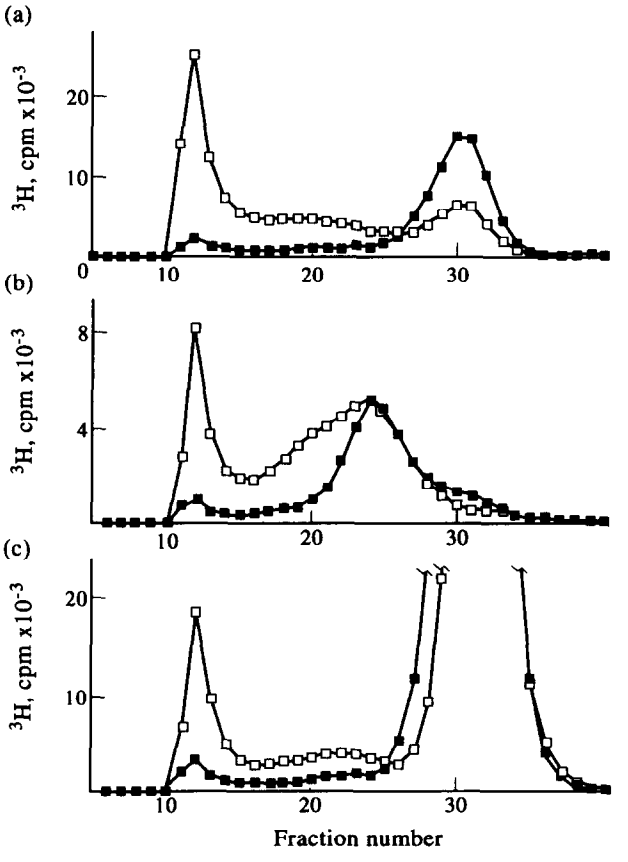

Figure 2. Gel infiltration analysis of products labelled in KATO III cells. [^{3}H]Glucosamine-labelled glycoproteins from untreated (□) and treated (■) cells labelled for 24 h were analysed on Sepharose CL-4B as described in Materials and Methods. (a) Cytosol fraction. (b) Membrane fraction. (c) Medium fraction.

Table 1. *Effect of benzyl-α-GalNAc treatment on metabolic labelling of high molecular glycoprotein*

	KATO III		Caco-2	
	Control	Treated	Control	Treated
	Void volume ^{3}H (cpm/μg protein)			
Medium* [^{3}H]GlcN	1104 ± 87	219 ± 36	169 ± 24	52 ± 2
Cytosol* [^{3}H]GlcN	1374 ± 23	195 ± 23	113 ± 7	25 ± 5
Membrane* [^{3}H]GlcN	390 ± 9	101 ± 24	52 ± 9	22 ± 4
Medium† [^{3}H]Thr	30 (22–38)	32 (27–36)	82 (79–84)	60 (60–61)
Cytosol† [^{3}H]Thr	494 (452–535)	604 (543–666)	216 (186–245)	185 (106–264)

* Mean ± S.E.M. of four experiments; † Mean (range) of two experiments.

fractions of KATO III and Caco-2. This suggests that much of the total O-linked carbohydrate is in low molecular weight non-mucin glycoproteins in these two cell lines. It should also be noted that there was little effect on the incorporation of [^{3}H]threonine into secreted HMG (Table 1). This indicates that benzyl-α-GalNAc treatment does not inhibit the synthesis of apomucin protein.

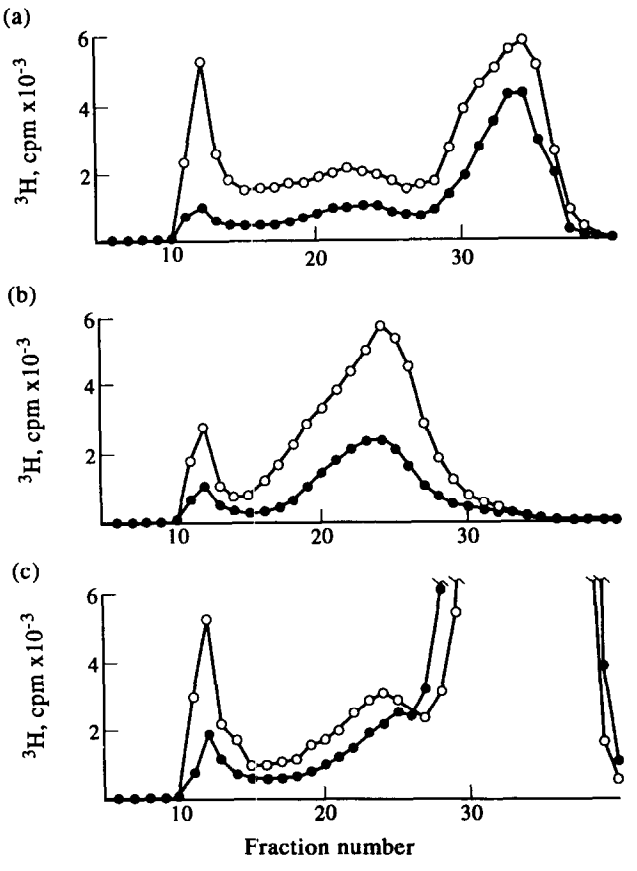

Figure 3. Gel filtration analysis of products labelled in Caco-2 cells. [^{3}H]Glucosamine-labelled glycoproteins from untreated (○) and treated (●) cells labelled for 24 h were analysed on Sepharose CL-4B as described in Materials and Methods. (a) Cytosol fraction. (b) Membrane fraction. (c) Medium fraction.

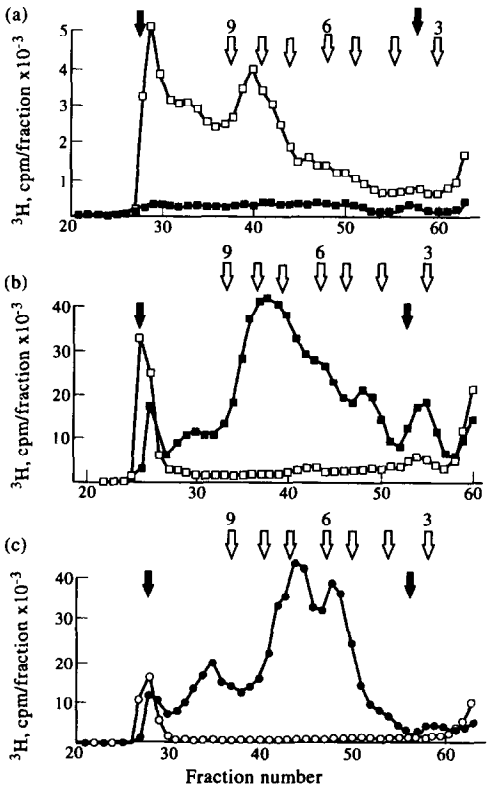

Figure 4. Gel filtration analysis of mucin oligosaccharides. (a) The [^{3}H]glucosamine-labelled mucins from the medium (4 ml) of KATO III cells with (■) or without (□) treatment with 2 mM benzyl-α-GalNAc were subjected to alkaline borohydride treatment, and the released oligosaccharides were analysed on BioGel P-2 as described in Materials and Methods. Solid arrows show the elution positions of dextran (void volume) and of stachyose (tetrasaccharide), and arrowheads show the elution positions of reduced dextran oligosaccharides of trisaccharide to nonsaccharide size. (b) The [^{3}H]glucosamine-labelled medium (0.5 ml) of KATO III cells with (■) or without (□) treatment with 2 mM benzyl-α-GalNAc was directly analysed on BioGel P-2. (c) The [^{3}H]glucosamine-labelled medium (0.5 ml) of Caco-2 cells with (●) or without (○) treatment with 2 mM benzyl-α-GalNAc was directly analysed on BioGel P-2.

Overall, KATO III had 8.4-fold more [^{3}H]glucosamine-labelled HMG than Caco-2. Inhibition was more complete in KATO III (82%) than in Caco-2 (70%). Another difference between the two cell lines is that Caco-2 had less HMG in the cytosol fraction because a greater proportion was secreted or membrane-bound.

Analysis of oligosaccharides

Previous studies have shown that benzyl-oligosaccharides are synthesised and secreted by LS174T cells treated with benzyl-α-GalNAc [1]. It was assumed that the oligosaccharides added to benzyl-α-GalNAc would be similar to those that would otherwise be added to mucin precursors. To test this assumption, we released the oligosaccharides from the mucin secreted by KATO III cells and compared their size to the benzyl-oligosaccharides found in the medium of KATO III cells cultured in the presence of benzyl-α-GalNAc (Figure 4). KATO III had two major peaks of oligosaccharide released from secreted mucin. One was near the void volume of BioGel P-2, with an extrapolated degree of polymerisation of 15.6. The other was of apparent octa- to nona-saccharide size. Labelling of these long oligosaccharides was profoundly inhibited by benzyl-α-GalNAc.

When the [^{3}H]glucosamine-labelled medium from KATO III was analysed directly by gel filtration on BioGel P-2, benzyl-oligosaccharides of about octa-saccharide size were obtained (Figure 4b). Because of the contribution of the benzyl moiety, an exact size comparison of the reduced oligosaccharides from the mucin and the benzyl-oligosaccharides is not possible. However, the benzyl-oligosaccharides roughly correspond in size to one of the major species of oligosaccharide present on the secreted mucins.

Benzyl-oligosaccharides were also detected in the medium of Caco-2 cells treated with 2 mM benzyl-α-GalNAc (Figure 4c). The apparent sizes of these products were 11.5, 7.3 and 5.9 hexose equivalents. Because of the inefficient labelling of Caco-2 mucin (Table 1), the reduced oligosaccharides of Caco-2 mucin were not analysed. However, it should be noted that, in spite of the inefficient labelling of Caco-2 mucin, these cells synthesise benzyloligosaccharides almost as efficiently as KATO III.

DEAE-cellulose chromatography

To determine whether the ion exchange behaviour of secreted mucin was altered by benzyl-α-GalNAc, we examined the mucins purified from media of cells with and without treatment with 2 mM benzyl-α-GalNAc (Figure 5).

In the KATO III control, the major peak (peak III) was eluted by 150 mM NaCl. Labelling of all the DEAE peaks was inhibited by benzyl-α-GalNAc. However, when normalised for total ^{3}H recovered, there was a relative increase in peaks I and VI, and a decrease in peaks III, IV and V (Figure 5).

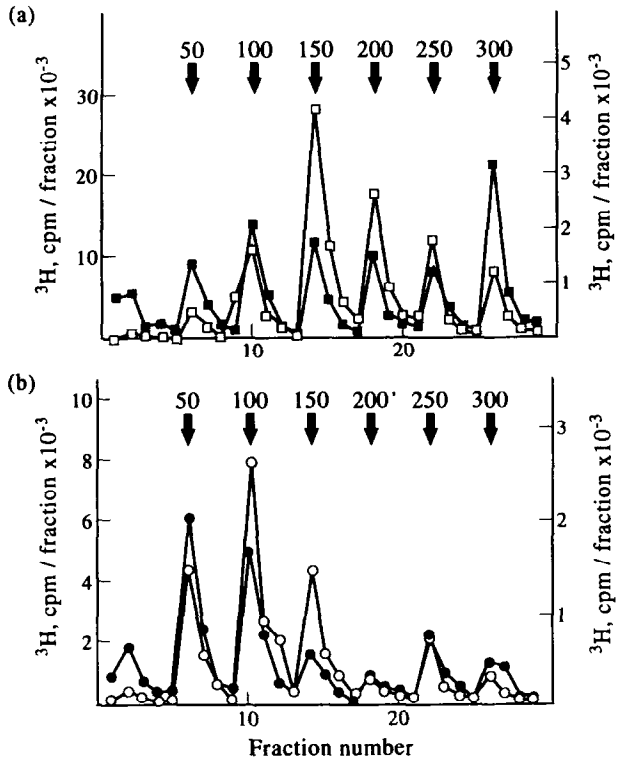

Figure 5. DEAE-cellulose chromatography of [^{3}H]glucosamine-labelled secreted mucins. Mucins purified from medium with (left axis) or without (right axis) 2 mM benzyl-α-GalNAc were analysed on columns of DEAE-cellulose as described in Materials and Methods. Arrows show the concentration of NaCl (in 10 mM Tris pH 8.0) in the step-gradient elution. (a) KATO III with (■) and without (□) benzyl-α-GalNAc. (b) Caco-2 with (●) and without (○) benzyl-α-GalNAc.

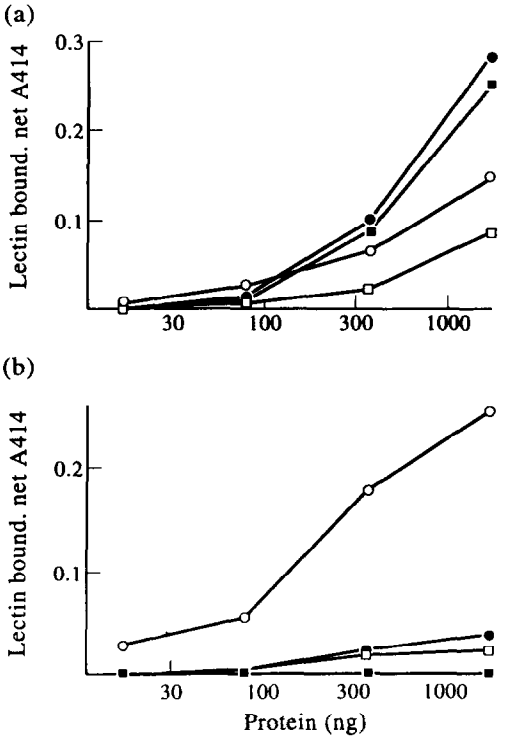

Figure 6. Lectin binding to secreted mucins. Varying concentrations of mucins purified from the media of KATO III (□, ■) and Caco-2 (○, ●) cells incubated for 48 h with (●, ■) or without (○, □) 2 mM benzyl-α-GalNAc were tested for binding of peroxidase-labelled lectins as described in Materials and Methods. (a) Peanut agglutinin. (b) *Ulex europae* agglutinin I.

The mucin secreted by untreated Caco-2 cells was less acidic than that of KATO III, with the major peak (peak II) eluted at 100 mM NaCl. As for KATO III, there was inhibition of labelling of mucin in all peaks. However, when normalised to total ^{3}H, there was a decrease in peaks II and III and an increase in peak I. The shift to less acidic species upon benzyl-α-GalNAc treatment could be due to a preferential inhibition of the labelling of the more acidic peaks. Alternatively, label from the acidic peaks could be shifted to early fractions.

Effect of benzyl-α-GalNAc on antigenicity of mucins

Inhibition of glycosylation of mucins would be expected to decrease the expression of peripheral carbohydrate antigens and might be expected to increase the expression of inner core carbohydrate antigens. To directly test how benzyl-α-GalNAc treatment affects the carbohydrate structures present on secreted mucins, we purified the mucin from spent media of control and treated cells and examined the binding of a variety of lectins and antibodies.

To detect mucins with T antigen, i.e. Galβ–3GalNAc, we used the lectin peanut agglutinin, which has been shown to recognise HMG in KATO III cells [10]. The secreted mucins from KATO III cells were found to bind peanut agglutinin about 3-fold less than those from Caco-2 (Figure 6a). The binding of peanut agglutinin to secreted mucin was greatly increased by benzyl-α-GalNAc treatment (Figure 6a) both for KATO III (approximately 5-fold) and Caco-2 (approximately 3-fold). The lectin *Ulex europaea* agglutinin I was used to monitor the expression of terminal fucose (e.g. H-antigen) on secreted mucins (Figure 6b). KATO III mucin did not bind *Ulex europaea* agglutinin I. Mucin secreted by untreated Caco-2 cells did bind *Ulex europaea* agglutinin I, and expression of this peripheral carbohydrate antigen was profoundly decreased by benzyl-α-GalNAc treatment.

Both serological and immunohistochemical studies have indicated that sialyl Tn antigen may be useful for detection of gastrointestinal cancers [24, 25]. To monitor the effect of benzyl-α-GalNAc on the secretion of sialyl Tn, we used a monoclonal antibody, JT1Oe, prepared against LS174T colon cancer xenograft mucin [18]. The mucin from untreated Caco-2 cells bound to the antibody approximately 8-fold better than that secreted by KATO III cells (Figure 7a). Expression of this core carbohydrate antigen was further increased (approximately 3.5-fold) by benzyl-α-GalNAc treatment of Caco-2 cells. A number of peripheral carbohydrate antigens such as sialyl Le^a and sialyl Le^x are present on gastrointestinal cancer mucins [26]. Inhibition of mucin glycosylation would be expected to decrease the expression of these epitopes in secreted mucin. The expression of sialyl-dimeric Le^x antigen (Siaα3Galβ4 [Fucα3]GlcNAcβ3Galβ4[Fucα3]GlcNAc) was monitored using monoclonal antibody SNH3 (Figure 7b). In this case, KATO III mucin bound to the antibody, but Caco-2 mucin did not. Expression of this peripheral carbohydrate antigen was decreased (approximately 3.5-fold) by benzyl-α-GalNAc treatment of KATO III cells.

Taken together, these results indicate that benzyl-α-GalNAc treatment decreases the expression of peripheral carbohydrate antigens (e.g. H and sialyl Le^x) and increases the expression of

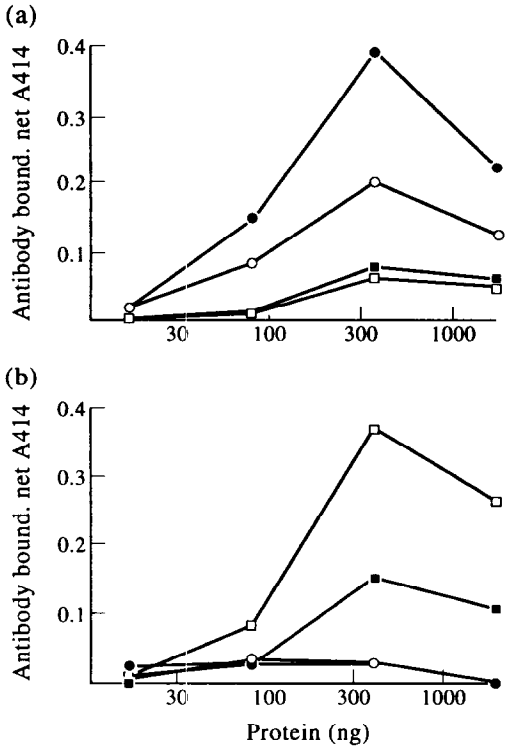

Figure 7. Monoclonal antibody binding to secreted mucins. Varying concentrations of mucins purified from the media of KATO III (□, ■) and Caco-2 (○, ●) cells incubated for 48 h with (●, ■) or without (○, □) 2 mM benzyl-α-GalNAc were tested for antibody binding. (a) Monoclonal antibody JT1Oe, specific for sialyl Tn antigen. (b) Monoclonal antibody SNH3, specific for sialyl Le^x antigen.

core carbohydrate antigens (e.g. T and sialyl Tn) on secreted mucins.

Effects of benzyl-α-GalNAc on mucin antigens in cell homogenates

To assess the effect of benzyl-α-GalNAc treatment on the cellular content of different mucin antigens in KATO III, homogenates from control and treated cells were examined by Western blotting (Figure 8). When homogenates of untreated KATO III cells were subjected to SDS polyacrylamide gel electrophoresis, there were two bands, of 190 kDa and 124 kDa apparent molecular weight, recognised by monoclonal antibody 139H2. These correspond to the MUC 1 mucin precursors that have been previously shown to be present in breast cancer and pancreatic cancer cells [27]. The amount of low molecular weight MUC 1 apomucin was not changed by benzyl-α-GalNAc treatment. However, in treated cells, high molecular weight mucins (extrapolated Mr of 307 kDa and 242 kDa) were recognised by 139H2. The likely explanation for this is that underglycosylation of the high Mr mucins increases the accessibility of the antibody to peptide epitopes in benzyl-α-GalNAc-treated cells. KATO III cells had antigens of about 279 kDa apparent Mr that were recognised by monoclonal antibody 15D3a.

This antibody, prepared against partially deglycosylated LS174T colon cancer mucin, is specific for Tn antigen [17]. Staining was faint in control KATO III, but was greatly increased by benzyl-α-GalNAc treatment.

In control KATO III cells, there was little binding of peanut agglutinin, but the amount of cellular T antigen was increased by benzyl-α-GalNAc treatment. Succinyl wheat germ agglutinin was used to detect GlcNAc-terminal glycoproteins. Both the high Mr glycoproteins (apparent Mr 330 kDa) and a low Mr

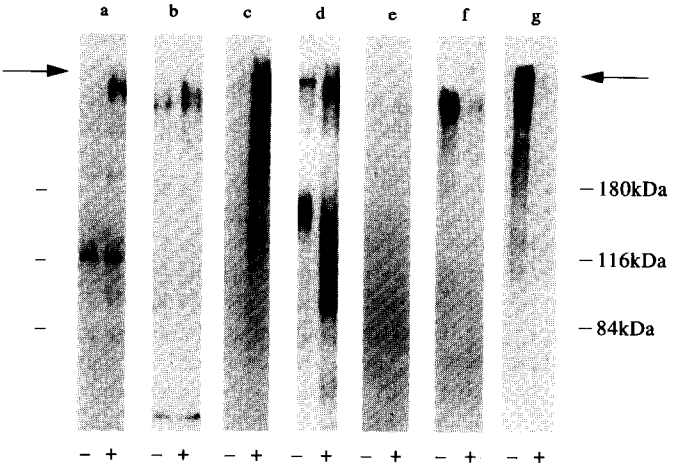

Figure 8. Western blots of KATO III homogenates. Cells incubated for 48 h with (+) or without (−) 2 mM benzyl-α-GalNAc were subjected to electrophoretic analysis as described in Materials and Methods. Arrow heads show the position of the interface between stacking gel and separating gel. Positions of molecular weight markers are shown on the right. (a) Monoclonal antibody 139H2, specific for MUC1 mucin. (b) Monoclonal antibody 15D3a, specific for Tn antigen. (c) Peanut agglutinin, specific for T antigen. (d) Succinylated wheatgerm agglutinin, specific for GlcNAc. (e) Monoclonal antibody JT1Oe, specific for sialyl Tn antigen. (f) Monoclonal antibody 19-9, specific for sialyl Le^a antigen. (g) Monoclonal antibody SNH3, specific for sialyl Le^x antigen.

band (Mr 100 kDa) were increased in intensity by benzyl-α-GalNAc treatment. Another broad band of about 157 kDa apparent Mr, was decreased by the treatment. There was little binding of JT1Oe in either control or treated KATO III cells, consistent with the low level of binding of JT1Oe to secreted mucin.

Sialyl Le^a and sialyl Le^x antigens, recognised by monoclonal antibody 19-9 and SNH3, respectively, were both of high Mr in KATO III. Expression of these antigens was greatly decreased by benzyl-α-GalNAc treatment.

Homogenates of untreated Caco-2 cells had Tn antigen detectable with monoclonal antibody 15D3a (Figure 9). In addition to

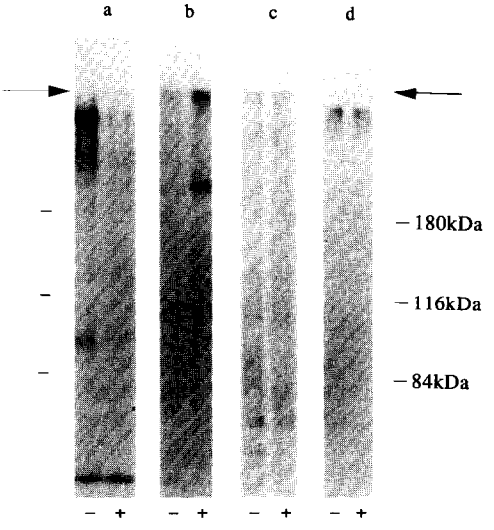

Figure 9. Western blots of Caco-2 homogenates. Cells incubated for 48 h with (+) or without (−) 2 mM benzyl-α-GalNAc were subjected to electrophoretic analysis as for Figure 8. (a) Monoclonal antibody 15D3a. (b) Peanut agglutinin. (c) Succinylated wheatgerm agglutinin. (d) Monoclonal antibody JT1Oe.

high Mr antigen (Mr ~ 291 kDa), there was a strong band of approximately 102 kDa and a broad band of approximately 241 kDa. In marked contrast to KATO III, the Tn antigen in Caco-2 cells was decreased by treatment with benzyl-α-GalNAc. Benzyl-α-GalNAc treatment of Caco-2 increased the cellular content of T antigen, as detected with peanut agglutinin. In treated cells, both high Mr antigen (Mr ~ 320 kDa) and a smaller product (Mr ~ 213 kDa) were detected. In contrast to KATO III, there was little GlcNAc-terminal glycoprotein recognised by succinyl wheatgerm agglutinin in either treated or control Caco-2 cells. Monoclonal antibody JT1Oe, which did not bind to KATO III Western blots, stained high Mr antigen in Caco-2 homogenates. The expression of cellular sialyl Tn appeared to be unaffected by benzyl-α-GalNAc treatment.

Together with the results of ELISA of secreted mucins, these Western blotting results indicate that, in both KATO III and Caco-2, benzyl-α-GalNAc decreases the expression of peripheral carbohydrate antigens, and increases the expression of core carbohydrate antigens.

DISCUSSION

Because of the lack of a general inhibitor of mucin glycosylation, it is important to determine whether mucin synthesis is inhibited in all colon and gastric cell lines by benzyl-α-GalNAc. Prior studies in our laboratory have shown the inhibition of mucin glycosylation in the LS174T cell line [1] and in high-mucin variants of LS174T [2] using aryl-*N*-acetyl-α-D-galactosaminides. We also found that the benzyl-α-GalNAc was effective in Caco-2, HT-29, and T-84 colon cancer cells [4]. Benzyl-α-GalNAc has also been shown to block the expression of cell surface sialyl Le^x and sialyl Le^a antigens in human leukaemia, lymphoma, and colon cancer cell lines [6]. However, the effect of benzyl-α-GalNAc on gastric cancer cells has not been reported. The purpose of this study was to determine whether benzyl-α-GalNAc inhibits mucin synthesis in KATO III (gastric cancer) cells and also to examine the effects of mucin inhibition on expression of core and peripheral carbohydrate antigens, and to compare KATO III gastric cancer cells to a colon cancer cell line, Caco-2.

The results of these experiments suggest that benzyl-α-GalNAc (2 mM) inhibited [^{3}H]glucosamine labelling of mucins by 82% in KATO III and by 70% in Caco-2. For both cell lines, the mucin secreted in the presence of benzyl-α-GalNAc was less acidic. Both cell lines secreted benzyl-oligosaccharides, but those from KATO III (8–9 sugars) were larger than those from Caco-2 (6–7 sugars). Benzyl-α-GalNAc was originally tested for inhibition of mucin glycosylation based on the assumption that it should compete with endogenous Tn antigen as an acceptor for the glycosyltransferases that add galactose, *N*-acetylglucosamine, and sialic acid to the linkage GalNAc of O-linked glycoproteins. If benzyl-α-GalNAc was, indeed, acting solely as a low molecular weight Tn analogue, it would be expected that there should be an accumulation of Tn-antigenic mucins and a decrease in all other mucin carbohydrate antigens. We did find that peripheral carbohydrate antigen levels decreased. We also found, however, that other core carbohydrate antigens besides Tn also accumulated. For example, T antigen (Gal β3GalNAc), recognised by peanut agglutinin, was increased in both secreted and cellular glycoprotein. Sialyl Tn antigen (NeuAcα-6GalNAc), recognised by monoclonal antibody JT1Oe, was increased in the secreted (but not the cellular) glycoproteins of Caco-2. Thus, it is clear that simple competitive inhibition of the glycosyltransferases that act on Tn mucin cannot fully explain the effects of benzyl-α-GalNAc on mucin carbohydrate antigen expression.

In mucins purified from the medium of treated cells, peripheral carbohydrate antigens (sialyl Le^x in KATO III and terminal fucose in Caco-2) were decreased (compared to control), while core carbohydrate antigens (T antigen in both cell lines and sialyl Tn in Caco-2) were increased. Western blots of cell homogenates showed differences between KATO III and Caco-2 in MUC 1 apomucin protein antigens, in sialyl Le^x and in sialyl Tn antigens.

The mucin made by KATO III differs in several respects from that made by Caco-2. First, it appears to be more acidic and more heavily glycosylated, as judged by the ratio of [^{3}H]glucosamine incorporation to [^{3}H]threonine incorporation. This is consistent with the larger benzyl-oligosaccharides made by KATO III cells upon exposure to benzyl-α-GalNAc. Secondly, the two cell lines differ in types of apomucin protein, since KATO III mucin is recognised by monoclonal antibody 139H2, but Caco-2 mucin is not. Preliminary results (R. Dahiya, unpublished observations) indicate that KATO III has mRNA for both MUC1 and MUC3 mucin, but not MUC2 mucin, while Caco-2 has mRNA for only MUC3 mucin [4]. Thirdly, there are clear differences in the carbohydrate antigens present on mucins secreted by the two cell lines. KATO III has much higher levels of sialyl Le^x sialyl Le^a and terminal GlcNAc, while Caco-2 has more terminal fucose and sialyl Tn antigen.

Partly because of the lack of suitable experimental model systems, the biochemistry of human gastric mucins is poorly understood. Since we found that KATO III gastric cancer cells synthesise and secrete relatively large amounts of glycoprotein with the properties expected for mucins, this cell line should be applicable to studies of the biosynthesis and some of the functions of human gastric mucin. However, it is not known how the mucin synthesised by KATO III compares with that in normal stomach. By analogy with human colonic mucins, it can be assumed that the mucin protein should be the same as that in normal tissue while the carbohydrate might be expected to display cancer-associated alterations. We conclude that benzyl-α-GalNAc does inhibit the glycosylation of mucin in KATO III gastric cancer cells as in human colon cancer cells, but that alterations in mucin antigens occur in a cell line-specific manner.

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Acknowledgement—This work was supported by the VA Medical Research Service.