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Enzymatic basis for sialyl-Tn expression in human colon cancer cells†

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Sialyl-Tn antigen (SAα2-6 GalNAcα-Ser/Thr) is expressed as a cancer-associated antigen on the surface of cancer cells and its expression correlates with a poor prognosis in patients with colorectal and other adenocarcinomas. To understand the enzymatic basis of sialyl-Tn (STn) antigen expression, we used two clonal cell lines, LSB and LSC, derived from LS174T human colonic cancer cells. LSC cells express only the truncated carbohydrate antigen Tn (GalNAcα-Ser/Thr) and sialyl-Tn on their mucin molecules, whereas LSB cells express elongated oligosaccharide chains. Both cell lines demonstrated similar activities of glycosyltransferases involved in the biosynthesis of elongated and terminal structures of complex O-glycans. However, LSC cells were unable to synthesize core 1 (Galβ1-3GalNAc-) because the ubiquitous enzyme activity of UDP-Gal : GalNAc-R β3-Gal-transferase (core 1 β3-Gal-transferase) was lacking. Core 1 β3-Gal-transferase could not be reactivated in LSC cells by treatment with sodium butyrate or by *in vivo* growth of LSC cells in nude mice. In contrast, LSB cells were able to synthesize and process core 1 and core 2 (GlcNAcβ1-6 (Galβ1-3) GalNAc-). LSC cells represent the first example of a non-hematopoietic cell line which lacks core 1 β3-Gal-transferase activity. The lack of core 1 β3-Gal-transferase in LSC cells explains why they are incapable of forming the common mucin O-glycan core structures and are committed to synthesizing the short Tn and STn oligosaccharides. These findings suggest that the activity of core 1 β3-Gal-transferase is an important determinant of the STn phenotype of colon cancer cells.

***Keywords*: sialyl-Tn, core 1 β3-Gal-transferase, colon cancer**

Introduction

Human colonic mucins carry complex O-glycans [1,2] bearing carbohydrate antigens, and human colonic tissue is rich in the activities of glycosyltransferases that assemble these and other O-glycans [3–5]. Some carbohydrate epitopes, such as T and Tn antigens are often masked in normal tissues, but become exposed in cancer tissues and are therefore considered general carcinoma antigens [6]. Similarly, the sialyl-Tn antigen (STn; SAα2-6 GalNAcα-Ser/Thr) is not usually exposed on normal colonic cells but appears in colon cancer tissues and cell lines [5, 7–9], with intermediate degrees of STn expression in premalignant adenomatous polyps [10]. In patients with longstanding ulcerative colitis, expression of STn antigen in non-dysplastic colonoscopic biopsies is a marker of risk for the subsequent development of colorectal cancer [11]. In addition to colon cancer, a variety of other adenocarcinomas, including those of the pancreas, stomach, breast, ovary, uterus, and gall-bladder also express STn [9, 12–15]. Importantly, the expression of STn and Tn antigens by adenocarcinomas has often been associated with poor clinical outcome and advanced tumor stage, implying a biological role for these antigens in the cancer cell [14–17].

Given the significance of STn as a tumor-associated antigen, it would be important to better understand the mechanisms responsible for its expression and synthesis. In the colon, one mechanism to explain the apparent increase of STn expression in cancers is a loss of *O*-acetyl groups on sialic acid that accompanies malignant transformation. In normal colonic epithelial cells, sialic acid residues are often modified by *O*-acetyl groups, thereby masking the underlying sialic acid epitopes and preventing them from reacting with antibody [18]. However, with malignant transformation, there is a loss of *O*-acetylation so that STn and other sialylated epitopes become available to react with antibodies [18–20]. Another modification of sialic acid is *N*-glycolyl-neuraminic acid which has been found on carbohydrate chains in a number of tumors [21, 22].

While decreases in sialic acid *O*-acetylation can at least partly explain the relative increase in STn expression in

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colon cancer, it does not explain why a subset of colon cancers (approximately 13–15%) still do not express STn. This is an important clinical issue since STn-negative colon cancers are the ones associated with a better survival [16]. Changes in *O*-acetylation also do not explain why STn expression is so prevalent in cancers of other organs such as the stomach, pancreas, and breast, where *O*-acetylation of the normal epithelium is less common. Thus, a more complete understanding of factors that govern STn synthesis is necessary.

The synthesis of STn antigen depends upon the activity and interaction of specific glycosyltransferases responsible for synthesizing O-glycans. The initial step in O-glycan biosynthesis is linkage of the initial GalNAc residue to serine or threonine residues of the apomucin polypeptide under the control of polypeptide: GalNAc transferase(s) [reviewed in 23, 24]. Once synthesized, GalNAc-Ser/Thr can be further processed depending upon the action of several glycosyltransferases. One pathway is for α2,6 sialyltransferase to act on the GalNAc-Ser/Thr precursor to make the STn antigen. Another important enzyme, core 1 β3-Gal-transferase, can act upon the precursor GalNAc to form the core 1 structure (Galβ1-3GalNAc-), or Thomsen-Friedenreich antigen. Also competing for use of the GalNAc residue is the 3 β3-GlcNAc-transferase which forms the core 3 structure (GlcNAcβ1-3GalNAc-). Core 1 and core 3 structures can be further elongated to form complex oligosaccharides through core 2 and core 4 structures, respectively. However, the STn structure does not permit chain elongation or further synthesis of complex O-glycans since glycosyltransferases cannot act on SAα2-6 GalNAc [23, 24].

To study the expression of STn and its role in cancer cell biology, we cloned two cell lines from the LS174T human colon cancer cell line based on their STn expression [25]. The LSC cell line was selected for its strong STn expression, whereas the LSB cell line lacks STn expression. The oligosaccharides of mucin purified from LSC cells contain only GalNAc (Tn) and SAα2-6GalNAc (STn) structures, whereas LSB cells lack Tn and STn expression but have longer oligosaccharide side chains and express core 1 (T antigen) and SLe^a antigens [25]. These cell lines therefore serve as an excellent model to study glycosylation pathways in colon cancer cells because they are clonal populations for which knowledge of their oligosaccharide structures is already available. Thus, although previous studies using tissues or cell lines have provided general insight into glycosyltransferase activities of the colon [reviewed in 24], the presence of a heterogeneous mixture of cells in those studies does not allow one to clearly define the mechanism of STn synthesis in a given cell. We show herein that LSC cells lack the ubiquitous core 1 β3-Gal-transferase, and core 3 β3-GlcNAc-transferase, and are therefore committed to synthesizing only short Tn and STn oligosaccharides. LSB cells, however, contain adequate core 1 β3-Gal-transferase activity and can therefore synthesize complex O-glycans, apparently at the expense of STn antigen. This explains the dramatic difference in Tn and STn expression between these two cell lines and is the first report of a non-hematopoietic cell line that is deficient in core 1 synthesis. Our data suggest that, given sufficient activity of other glycosyltransferases, the absence or presence of core 1 β3-Gal-transferase activity may determine whether or not a colon cancer cell expresses STn antigen.

Materials and methods

Reagents for glycosyltransferase assays

The following compounds were a kind gift from K. Matta, Roswell Park Memorial Institute, Buffalo, NY: 2-*O*-methyl-Galβ1–3GlcNAcβ-Bn, 2-*O*-methylGalβ1–4GlcNAcβ-Bn, GlcNAcβ1–3GalNAcα-Bn, GlcNAcβ1–6 (Galβ1–3) GalNAcα-Bn, and GlcNAcβ1–6 (GlcNAcβ1–3) GalNAcα-Bn. The peptide VTSAPDTRPAPGST from the MUC1 tandem repeat sequence was a kind gift from J. Taylor-Papadimitriou, Imperial Cancer Research Fund, London, UK. Protected peptides $AcPSSSPISTNH_2$, $AcPTSSPISTNH_2$ and glycopeptide AcELA(GalNAcα)TVGPG-NH_2 were donated by H. Paulsen, University Hamburg, Hamburg, Germany. [^{14}C] GlcNAcβ1-6 (GlcNAcβ1-3) Galβ-methyl was prepared as described [26]. Scintillation fluid Readysolv was purchased from Beckman. UDP-[^{14}C]Gal, $PAP^{35}S$, GDP-[^{14}C]Fuc, CMP-[^{14}C]SA, UDP-[^{14}C]GalNAc were purchased from Amersham and were diluted with non-radioactive nucleotide sugars. UDP-N-[1 − ^{14}C] acetylglucosamine was synthesized as described [3]. All other chemicals were of the highest reagent grade and were described previously [4].

Cell lines and preparation of cells for glycosyltransferase assays

The isolation of LSC and LSB cell lines and the characterization of their mucin oligosaccharide and antigenic profiles have been previously described [25]. Cells were harvested at confluency. Glycosyltransferase assays were performed on cultured cells, and in some cases, on *in vivo* tumors derived from these cell lines. *In vivo* tumors were established by injecting 10^6 cells subcutaneously into athymic, nude mice and allowing tumors to grow for approximately ten weeks. In some experiments, cells were treated with 3 mM sodium butyrate *in vivo* for 3 or 7 days before harvesting. Cells were washed three times in 0.9% NaCl followed by centrifugation. Pellets were washed in 0.9% NaCl and centrifuged three times. The pellets were suspended in 0.25 M sucrose (1 ml per 10^8 cells), homogenized with a Potter-Elvehjem homogenizer and stored at −70 °C.

Glycosyltransferase assays

Glycosyltransferase activities were measured in duplicate determinations, repeated at least once. The amount of enzyme

per assay represented that of 1 to 2 million cells. To compare total enzyme activities, the nucleotide sugar and acceptor substrates were present at near saturating concentrations. The conditions for Sep-Pak and HPLC assays were described previously [4]. Assays with substrates containing Bn or p-nitrophenyl groups were run on C18 columns, assays with substrates containing free reducing sugars or methyl groups were run on amine columns, using acetonitrile/water as the mobile phase as described [26]. Rat colon homogenates were used as a positive control for all assays. Background radiation was routinely subtracted from assay values.

UDP-GalNAc: polypeptide α-GalNAc-transferase (polypeptide α-GalNAc-transferase)

Polypeptide α-GalNAc-transferase synthesizing the Tn antigen was assayed using as substrates neutral amino- and carboxy-terminal protected Thr-containing peptides from the MUC2 tandem repeat sequence ($AcPSSSPISTNH_2$ and $AcPTSSPISTNH_2$) and a peptide containing part of the MUC1 tandem repeat sequence (VTSAPDTRPAPGST) as follows: Activities were measured in a total volume of 40 µl containing 0.125 M MES, pH 7, 12.5 mM $MnCl_2$, 0.125% Triton X-100, 2.5 to 4 mM AMP, 1.1 to 1.4 mM UDP-[^{14}C] GalNAc (818 and 1630 dpm nmol^{-1}), 1 mM substrate and 10 µl cell homogenate (0.1 and 0.3 mg protein). Samples were incubated for 1 h at 37 °C, 100 µl cold water were added and mixtures were passed through 0.4 ml AG1 × 8 columns which were then washed twice with 600 µl water. Eluates were mixed with 5 ml scintillation fluid and radioactivity counted in a scintillation counter. For HPLC assays eluates were lyophilized and taken up in 200 µl water.

UDP-GalNAc-R α3/6-GalNAc-transferase (core 5/7 GalNAc-transferase)

GalNAc-transferase activities acting on GalNAc-peptide (core 5/7 GalNAc-transferase) were measured in a total volume of 40 µl containing 0.125 mM MES, pH 7, 12.5 mM $MnCl_2$, 0.125% Triton X-100, 5 mM AMP, 1.8 mM UDP-[^{14}C] GalNAc (818 dpm nmol^{-1}), 2 mM Ac-ELA(GalNAcα)TVGPG-NH_2 glycopeptide substrate and 10 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h at 37 °C and reactions were stopped with 400 µl water. Mixtures were loaded onto C18 Sep-Pak cartridges, equilibrated in water. After washing with water, product was eluted with 5 ml methanol and counted in 10 ml scintillation fluid.

UDP-Gal: GalNAc-β3-Gal-transferase (core 1 β3-Gal-transferase)

Core 1 β3-Gal-transferase activities were measured in a total volume of 40 µl containing 0.125 mM MES, pH 7, 12.5 mM $MnCl_2$, 0.125% Triton X-100, 2.5 to 10 mM AMP, 5 mM γ-galactono-lactone, 0.9 to 1.2 mM UDP-[^{3}H] Gal (6160 dpm nmol^{-1}), 2 or 4 mM GalNAcα-Bn or desialylated ovine submaxillary mucin (DS-OSM) (containing 300 nmol GalNAc) substrate and 10 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h at 37 °C, 100 µl of EDTA/Na-tetraborate solution (pH9) were added and mixtures were passed through 0.4 ml AG1 × 8 columns which were then washed twice with 600 µl water. Eluates were mixed with scintillation fluid and radioactivity counted in a scintillation counter. Product using GalNAcα-Bn substrate was also estimated by HPLC using Galβ1-3GalNAcα-Bn as the product standard as described [26].

UDP-Gal: GlcNAc β4-Gal-transferase (β4-Gal-transferase)

The assay procedure for β4-Gal-transferase was identical to that for core 1 β3-Gal-transferase except that the substrate was 2 to 4 mM GlcNAc instead of GalNAcα-Bn.

UDP-GlcNAc: GalNAc β3-GlcNAc-transferase (core 3 β3-GlcNAc-transferase)

Core 3 β3-GlcNAc-transferase activities were measured in a total volume of 40 µl containing 0.125 M MES, pH 7, 12.5 mM $MnCl_2$, 0.125% Triton X-100, 5 to 10 mM AMP, 5 mM γ-galactono-lactone, 1.4 mM UDP-[^{14}C]GlcNAc (3804 dpm nmol^{-1}), 4 mM GalNAcα-Bn substrate and 10 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h 37 °C and product was estimated by AG1 × 8 and HPLC assays as described above for core 1 β3-Gal-transferase using GlcNAcβ1-3GalNAcα-Bn as the product standard.

UDP-GlcNAc: Galβ1-3GalNAc- (GlcNAc to GalNAc) β6-GlcNAc-transferase (core 2 β6-GlcNAc-transferase)

Core 2 β6-GlcNAc-transferase activities were measured in a total volume of 40 µl containing 0.125 M MES, pH 7, 0.125% Triton X-100, 5 to 10 mM AMP, 0.125 M GlcNAc, 5 mM γ-galactono-lactone, 1.4 mM UDP-[^{14}C]GlcNAc (3804 dpm nmol^{-1}), 4 mM Galβ1-3GalNAcα-Bn substrate and 10 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h at 37 °C and product was estimated as described above by AG1 × 8 and HPLC assays using GlcNAcβ1-6 (Galβ1-3)GalNAcα-Bn as the product standard.

UDP-GlcNAc: GlcNAcβ1-3GalNAc-R (GlcNAc to GalNAc) β6-GlcNAc-transferase (core 4 β6-GlcNAc-transferase)

Core 4 β6-GlcNAc-transferase activities were measured as described for core 2 β6-GlcNAc-transferase with 4 mM GlcNAcβ1-3GalNAcα-Bn or GlcNAcβ1-3GalNAcα-p-nitrophenyl (pnp) substrate and GlcNAcβ1-6 (GlcNAcβ1-3) GalNAcα-Bn as the product standard.

UDP-GlcNAc:Galβ1-4GlcNAc β3-GlcNAc-transferase (i β3-GlcNAc-transferase)

The i β3-GlcNAc-transferase activities were measured as described for core 3 β3-GlcNAc-transferase with 4 mM Galβ1-4GlcNAc substrate. Product was estimated by HPLC assays as described above using GlcNAcβ1-3 Galβ1-4 GlcNAc as the product standard.

UDP-GlcNAc:GlcNAcβ1-3Gal β6-GlcNAc-transferase (I β6-GlcNAc-transferase)

The I β6-GlcNAc-transferase activities were measured as described for core 2 β6-GlcNAc-transferase with 4 mM GlcNAcβ1-3Galβ-Me substrate. Product was estimated by HPLC assays as described above using [^{14}C] GlcNAcβ1-6 (GlcNAcβ1-3) Galβ-Me as the product standard.

CMP-SA:Galβ1-3GalNAc α3-SA-transferase (α3-SA-transferase (O)) and CMP-SA:GalNAc-α6-SA-transferase (α6-SA-transferase (O)):

Sialyltransferase activities were measured in a total volume of 40 µl containing 0.125 mM Tris-HCl, pH 7, 0.5% Triton X-100, 10 mM AMP, 1 mM CMP-[^{14}C] SA (1655 dpm nmol^{-1}), 2 mM Galβ1-3GalNAcα-pnp substrate (for α3-SA-transferase) or DSOSM substrate (containing 150 to 300 nmol GalNAc, for α6-SA-transferase (O)) and 10 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h at 37 °C. 10 µl 20 mM EDTA/2% Na-tetraborate were added and product was applied to Whatman paper and separated by high voltage electrophoresis as described [27].

GDP-Fuc:Galβ α2-Fuc-transferase (α2-Fuc-transferase), GDP-Fuc:Galβ1-4GlcNAc α3-Fuc-transferase (α3-Fuc-transferase) and GDP-Fuc: Galβ1-4/3GlcNAc α3/4-Fuc-transferase (α3/4-Fuc-transferase)

Fuc-transferase activities were measured in a total volume of 40 µl containing 0.125 M MES, pH 7, 12.5 mM $MnCl_2$, 0.125% Triton X-100, 5 mM AMP, 1 mM GDP-[^{3}H] Fuc (2466 dpm nmol^{-1}), 2 mM Galβ-phenyl or 2 mM Galβ1-3GalNAcα-Bn substrate (for α2-Fuc-transferase), 2 mM 2-O-Me-Galβ1-4GlcNAcβ-Bn substrate (for α3-Fuc-transferase, or 2 mM 2-O-Me-Galβ1-3GlcNAcβ-Bn substrate (for α3/4-Fuc-transferase), and 20 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h at 37 °C; 600 µl water were then added and mixtures were separated on AG1 × 8 columns and counted as described above.

Sulfotransferase assays

Sulfotransferase activities were measured in a total volume of 40 µl containing 10 mM Tris-HCl, pH 6.3, 10 mM NaF, 2.5 mM Mg-acetate, 10 mM 2,3-mercaptopropanol, 1% Triton X-100, 2.5 mM ATP, 7 mM PAP^{35}S (2368 dpm pmol^{-1}), 2 mM Galβ1-3GalNAcα-Bn, Galβ1-4GlcNAc or GlcNAcβ1-3Galβ-methyl substrate and 10 µl cell homogenate (0.1 to 0.3 mg protein). Samples were incubated for 1 h at 37 °C. Product was measured by high voltage electrophoresis (HVE) assays as described [27].

Results

Glycosyltransferase activities in LSB and LSC cells

Glycosyltransferase activities of LSB and LSC cells are summarized in Table 1. The first enzyme in the O-glycosylation pathway, polypeptide α-GalNAc-transferase, is typically active using peptide substrates with MUC1 and MUC2 sequences, which have been shown to be substrates for the purified bovine colostrum enzyme [28]. Both LSB and LSC cells exhibited comparable levels of polypeptide:GalNAc transferase activities using a MUC1 tandem repeat peptide substrate. No polypeptide:GalNAc-transferase activity was detectable using the serine-rich PSSSPIST sequence as substrate (not shown).

With respect to glycosyltransferase activities that synthesize the various core structures, LSB and LSC cells demonstrated comparable activities except for one marked difference. LSC cells were markedly deficient in core 1 β3-Gal-transferase activity. This was true using either low molecular weight GalNAcα-Bn or DS-OSM mucin as substrates. LSC cell homogenates showed little or no detectable activity of core 1 β3-Gal-transferase (<0.2 nmol h^{-1} mg^{-1}), measured by direct counting after AG1 × 8 chromatography or by HPLC assays, although in some experiments a small amount of product eluted with the Galβ1-GalNAc-Bn standard. In contrast, in LSB cells, core 1 β3-Gal-transferase activity was present at much higher levels than in LSC cells (Table 1).

The activities of the transferases synthesizing core structures 2 and 4 were not significantly different between the two cell lines. The absence of core 3 β3-GlcNAc-transferase activity in both LSB and LSC is typical of most colon cancer cells [24], and indicates that although core 4 β6-GlcNAc-transferase activity is present in these cells, the core 4 structure is not synthesized because of a lack of synthesis of the precursor core 3 structure. No evidence was obtained in either cell line for novel activities that could synthesize GalNAcα1-3GalNAc (core 5) or GalNAcα1-6 GalNAc (core 7) using a GalNAc-containing glycopeptide.

Both cell lines manifested comparable levels of elongation and branching enzymes β4-Gal-transferase, i β3-GlcNAc-transferase and I β6-GlcNAc-transferase (Table 1). Since the three activities synthesizing core 2 and core 4 (core 4 β6-GlcNAc-transferase) and the I antigen (I β6-GlcNAc-transferase) are present concomitantly, it is the M type of core 2 β6-GlcNAc-transferase that is expressed in both LSB and LSC cells [29]. This enzyme is present in normal and cancerous human colonic mucosa and in a number of colon

Table 1. Glycosyltransferase and sulfotransferase activities in LSB and LSC cell homogenates

Enzyme	*Substrate*	*Activities (nmol h^{-1} mg^{-1})*		
		LSB	*LSC*	*Assay*
Initialization Enzymes:				
ppGA-T	1 mM VTSAPDTRPAPGST	14.7	17.5	AG1 × 8
Core Enzymes:				
core 1 β3-Gal-T	GalNAcα-Bn (4 mM)	3.9	< 0.2	HPLC
	DS-OSM (7.5 mM GalNAc)	14	< 0.2	AG1 × 8
core 2 β6-GlcNAc-T	Galβ1-3GalNAcα-pnp (4 mM)	7.7	2.2	HPLC
core 3 β3-GlcNAc-T	GalNAcα-Bn (4 mM)	ND	ND	HPLC
core 4 β6-GlcNAc-T	GlcNAcβ1-3GalNAcα-pnp (4 mM)	3.3	1.9	HPLC
core 5/7 GalNAc-T	Ac-ELA(GalNAcα)TVGPG-NH2	< 0.2	< 0.2	Sep-Pak
Elongation Enzymes:				
β4-Gal-T	GlcNAc (2 mM)	16.9	23.3	AG1 × 8
i β3-GlcNAc-T	Galβ1-4GlcNAc (4 mM)	0.2	0.2	HPLC
I β6-GlcNAc-T	GlcNAcβ1-3Galβ-CH_3 (4 mM)	0.5	0.7	HPLC
Termination Enzymes:				
α6-SA-T (O)	DS-OSM (7.5 mM GalNAc)	0.3	0.5	HVE
α3-SA-T (O)	Galβ1-3GalNAcα-pnp (2 mM)	0.6	0.7	HVE
		3.7	5.4	
α2-Fuc-T	Gal-phenyl (2 mM)	0.3	0.4	AG1 × 8
	Galβ1-3GalNAc-Bn (2 mM)	1.2	2.3	AG1 × 8
α3-Fuc-T	2-Me-Galβ1-4GlcNAcβ-Bn (2 mM)	4.9	7.6	AG1 × 8
α4-Fuc-T	2-Me-Galβ1-3GlcNAcβ-Bn (2 mM)	8.2	11.1	AG1 × 8
sulfo-T	Galβ1-3GalNAcα-Bn (2 mM)	0.022	0.014	HVE
	Galβ1-4GlcNAc (2 mM)	0.019	0.012	HVE
	GlcNAcβ1-3Galβ-CH_3 (2 mM)	ND	ND	HVE

Assay conditions are those described in Materials and methods assays were done by counting after passing through AG1 × 8 columns (AG1 × 8), by high voltage electrophoresis (HVE), by counting after passing through Sep-Pak columns (Sep-Pak), or by HPLC (HPLC). The variations between duplicate assays in the same experiment were less than 10%. T = transferase; ND, not detectable; ppGA-T, polypeptide α-GalNAc-transferase

cancer lines [30]. This is in contrast to other cancer cell lines and leukocytes which only contain the L type of core 2 β6-GlcNAc-transferase activity. However, we cannot exclude the possibility that both L and M type enzymes are present in LSB and LSC cells.

Of note, the α6-sialyltransferase was expressed at comparable levels in the two cell lines. Thus, the difference in STn expression between the LSB and LSC is not due to different levels of the sialyltransferase responsible for synthesizing STn antigen. Likewise, the α3-sialyltransferase (O) acting on Galβ1-3GalNAc- was comparable between the two cell lines.

The fucosyltransferase activities synthesizing the blood group H, Le^a and Le^x determinants were present at comparable levels in LSB and LSC cells. In addition, both cell lines demonstrated sulfotransferase activities acting on Galβ1-3GalNAcα-Bn and Galβ1-4GlcNAc, but not GlcNAcβ3Galβ-.

Core 1 β3-Gal-transferase product identification

A time course experiment (activity measured up to 24 h) indicated that core 1 β3-Gal-transferase activity in LSB cells was linear up to 4 h of incubation. A large scale preparation of core 1 β3-Gal-transferase enzyme product using GalNAcα-Bn substrate was undertaken by scaling up the assay 50 times using LSB cell homogenate and incubating overnight. After passage of the incubation mixture through AG1 × 8, product was purified on BioGel P4-400 (1.6 × 86 cm), equilibrated in water, followed by HPLC purification on a C18 column (acetonitrile:water, 8:92). The 500 MHz ^{1}H-NMR spectrum of purified enzyme product was identical to that of Galβ1-3GalNAcα-Bn standard [31]. Signals were detected in the product spectrum due to the H-1 of GalNAc at 4.970 ppm ($J_{1,2}$ = 3.5 Hz) and the H-1 of Gal at 4.451 ppm ($J_{1,2}$ = 7.5 Hz). The attachment of Gal to

the 3-position of GalNAc was seen by the chemical shift of GalNAc H-2 (4.305 ppm), H-3 (4.040 ppm) and H-4 (4.254 ppm) while the same values in the substrate, respectively, were 4.12, 3.91 and 3.99 ppm. This confirms that the Gal-transferase activity in LSB cells acting on GalNAcα-Bn was indeed core 1 β3-Gal-transferase.

Regulation of core 1 β3-Gal-transferase

To determine whether core 1 β3-Gal-transferase could be reactivated in LSC cells, LSC cells (and LSB cells as a control) were treated with sodium butyrate for 3 or 7 days. LSC cells harvested after 3 days and 7 days showed no core 1 β3-Gal-transferase activity. β4-Gal-transferase had an activity of 10.3 nmol h^{-1} mg^{-1} before differentiation and the activity remained active after 3 days (18 nmol h^{-1} mg^{-1}) and after 7 days (25 nmol h^{-1} mg^{-1}) of differentiation. In contrast, both β3- and β4-Gal-transferase activities were present in LSB cells at all stages. Thus, the deficient activity in LSC cells could not be reactivated by sodium butyrate treatment.

To further explore whether core 1 β3-Gal-transferase could be upregulated in LSC cells, LSC and LSB cells were injected individually into nude mice and grown as *in vivo* tumors. Cells from the tumor were then harvested and analyzed for enzyme activity. Table 2 shows that core 1 β3-Gal-transferase activity in LSC cells was not reactivated by *in vivo* growth; the activity was barely detectable by HPLC. In addition, core 3 β3-GlcNAc-transferase remained inactive in both cell lines even after *in vivo* tumor growth. The levels of polypeptide GalNAc-transferase were reduced in the *in vivo* growth tumor cells.

To determine whether core 1 β3-Gal-transferase in LSC cells may be inactive due to a factor suppressing its activity, mixing experiments were performed. A number of assays were carried out in which the activities of core 1 β3-Gal-transferase were measured by HPLC, individually in LSC and LSB cells and after mixing the two cell homogenates (Figure 3). No product was detected in LSC cells. Mixing LSC and LSB cell homogenates yielded a 31 to 38% reduction in product yield, compared to the assays with LSB cells alone or mixed with 0.25 M sucrose. These results suggest that the LSC cell extract had an inhibitory effect but the extent of this inhibition could not account for the dramatic reduction of enzyme activity in LSC cells. The inhibitory effect of LSC cells was reduced to about 11% inhibition in duplicate mixing assays when LSC cell homogenate was boiled for 10 min before adding to the assay.

Discussion

The present study has identified a human colon cancer cell line LSC that lacks the ability to synthesize the common O-glycan core structures (cores 1 to 4). The lack of core 1 β3-Gal-transferase in LSC cells prevents the synthesis of

Table 2. Difference in glycosyltransferase activities before and after *in vivo* growth

Enzyme	*Substrate*	*Activities (nmol h^{-1} mg^{-1})*			
				in vivo	
		LSB	*LSC*	*LSB*	*LSC*
β4-Gal-T	GlcNAc (2 mM)	5.7	10.3	3.1	3.9
core 1 β3-Gal-T	GalNAcα-Bn (2 mM)	3.4	<0.2	2.8	<0.2
core 3 β3-GlcNAc-T	GalNAcα-Bn (4 mM)			ND	ND
ppGA-T	VTSAPDTRPAPGST (1 mM)	1.4	2.4	0.3	0.3
	$AcPSSSPISTNH_2$ (1 mM)	ND	ND	ND	ND
	$AcPTSSPISTNH_2$ (1 mM)	6.4	5.9	2.8	2.1

Assay conditions are those described in Materials and methods. With the exception of β4-Gal-transferase assays which were carried out by counting after passage through AG1 × 8, all assays were done by HPLC. In these cell homogenates, VTSAPDTRPAPGST substrate appeared to be degraded during the 1 h assay period. LSC and LSB cells were grown in athymic mice as *in vivo* tumors. T = transferase; ND, not detected; ppGA-T, polypeptide α-GalNAc-transferase.

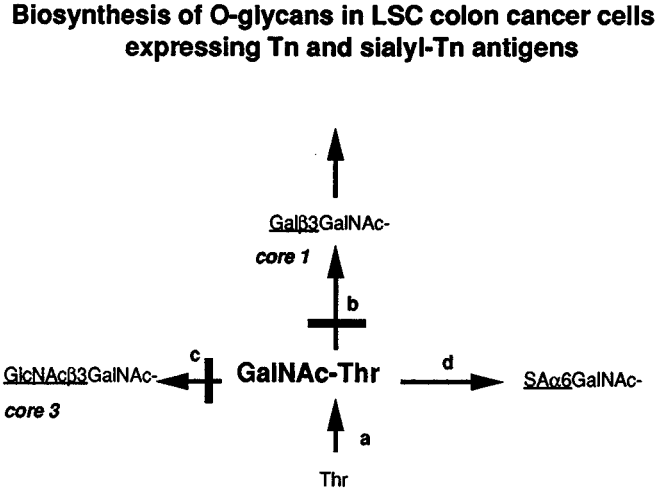

Figure 1. Biosynthesis of O-glycans in LSC human colon cancer cells. The composite pathways show *path a*, polypeptide α-GalNAc-T; *path b*, core 1 β3-Gal-T; *path c*, core 3 β3-GlcNAc-T; and *path d*, α6-SA-T (O). Glycosyl- and sulfotransferase activities were carried out as described in the Methods section. LSC cells lack core 1 β3-Gal-T activity as well as core 3 β3-GlcNAc-T activity. LSC cells therefore cannot make O-glycans with core structures 1 to 4, which leads to the excess formation of GalNAc (Tn antigen) and sialylα2-6GalNAc (sialyl-Tn antigen) structures. T = transferase.

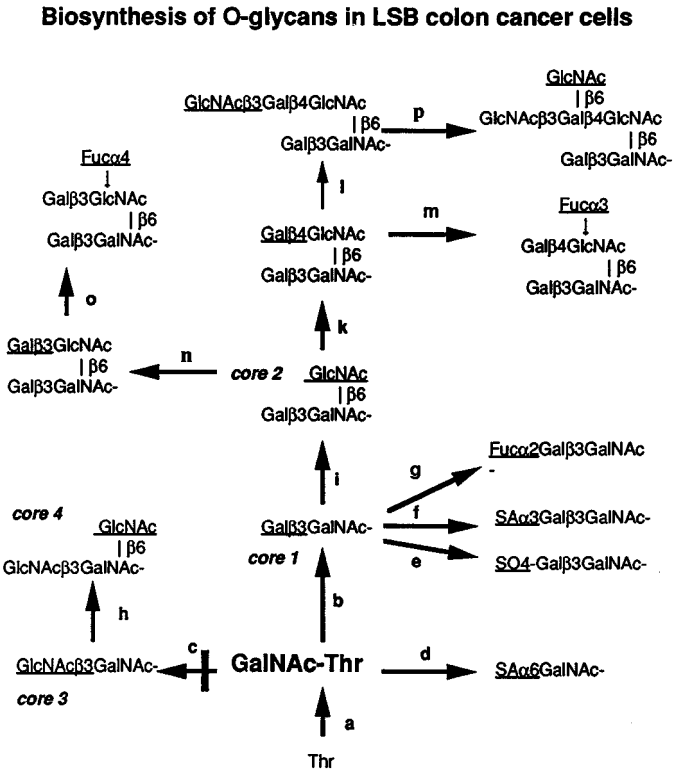

Figure 2. Biosynthesis in human colon cancer cells LSB. The composite pathways show *path a*, polypeptide α-GalNAc-T; *path b*, core 1 β3-Gal-T; *path c*, core 3 β3-GlcNAc-T; *path d*, mucin α6-SA-T (O); *path e*, core 1 3-sulfo-T; *path f*, α3-SA-T (O); *path g*, α2-Fuc-T; *path h*, core 4 β6-GlcNAc-T; *path i*, core 2 β6-GlcNAc-T; *path k*, β4-Gal-T; *path l*, i β3-GlcNAc-T; *path m*, α3-Fuc-T; *path n*, β3-Gal-T; *path o*, α4-Fuc-T; *path p*, I β6-GlcNAc-T. The assays could not distinguish between β3-Gal-T and β4-Gal-T. LSB cells can synthesize O-glycan core 1 and 2 structures, with elongated and branched structures, blood group H and Lewis antigens, sialylated and sulfated structures. The pathways to the synthesis of core 3 and 4 structures are blocked due to the lack of core 3 β3-GlcNAc-T activity.

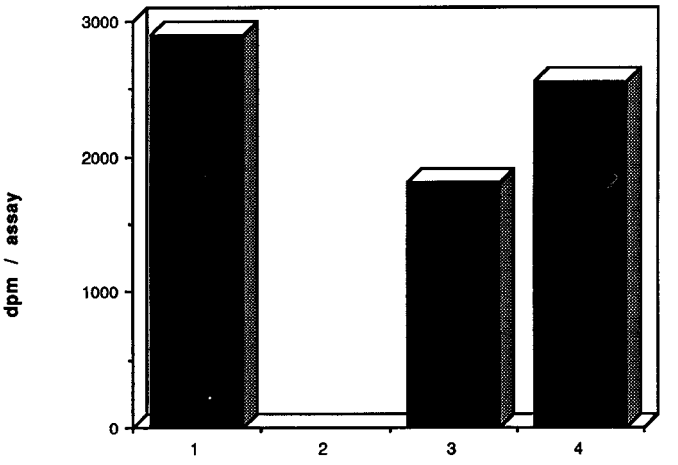

Figure 3. Core 1 β3-Gal-transferase activities in mixing experiments. Assays for core 1 β3-Gal-transferase were carried out in duplicate by HPLC as described in the Methods section. Homogenates from LSB cells (1), LSC cells (2) and LSB plus LSC cells (3) were mixed. The assays in which cells were mixed were repeated with triplicate determinations, yielding similar values. (4) The values shown in bar number 4 were obtained after mixing homogenates from LSB cells and homogenate from LSC cells, boiled for 5 min.

core 1 and its further conversion to core 2 (Figure 1). Likewise, a lack of core 3 β3-GlcNAc-transferase prevents the synthesis of core 3 and its conversion to core 4. Although the enzymes elongating and terminating O-glycans are present in these cells, they cannot act since the common core structures are lacking. As a result, only GalNAc and sialyl-GalNAc are made, explaining the previously observed high Tn and sialyl-Tn expression in LSC cells [25].

In contrast, LSB cells have the biosynthetic ability to synthesize highly complex O-glycans with core structures 1 and 2 (Figure 2). Like the LSC cells, they lack core 3 β3-GlcNAc-transferase activity, so they cannot further convert core 3 to core 4. Curiously, despite the fact that these cells also have the glycosyltransferase machinery to synthesize Tn and sialyl-Tn antigens, they do not highly express these short oligosaccharides [25]. In addition, de-*O*-acetylation of LSB cells does not unmask the STn antigen (unpublished data). This suggests that in the presence of core 1 β3-Gal-transferase, oligosaccharide synthesis is shifted to favor the formation of complex oligosaccharides and away from the synthesis of short structures. This could explain the STn-negative phenotype of some colon cancers.

Normal human colonic mucosa has the enzymes that synthesizes GalNAc-mucin and the four common O-glycan core structures, Galβ1-3GalNAc- (core 1), GlcNAcβ1-6 [Galβ1-3] GalNAc- (core 2), GlcNAcβ1-3GalNAc- (core 3) and GlcNAcβ1-6 [GlcNAcβ1-3] GalNAc- (core 4) [24]. These core structures may be sialylated, sulfated, fucosylated, and elongated to carry various terminal oligosaccharide epitopes. In some ways, LSB cells appear to reflect the situation in normal colonic mucosa in that they contain the enzymes synthesizing core 1, 2, 3 and 4 structures, the STn antigen and α3-linked sialic acid, Fucα2-, Fucα3-, and Fucα4-structures, i and I antigens, and sulfotransferase acting on mucin substrates (Figure 2). Unlike normal colonic mucosa, however, LSB cells lack core 3 β3-GlcNAc-transferase activity and do not synthesize STn. The loss of core 3 synthesis seen in both LSC and LSB cells is typical of cultured human colon cancer cell lines and human colonic tumors although the reason for this is not yet known [4, 26, 30, 32].

The ability of colon cancer cells to synthesize complex oligosaccharides may bear upon their biological behavior. Compared to LSC cells, LSB cells adhere better to E-selectin and activate hepatic endothelial cells *in vitro*, and also appear to be more metastatic *in vivo* [33]. This seems to be due to the expression of the complex carbohydrate antigens sialyl-Lewis^a and sialyl-Lewis^x on LSB cells. Other studies have employed GalNAcα-benzyl, a reagent that prevents

elongation of O-glycans, resulting in an increase in the amount of GalNAc-Ser/Thr (Tn antigen) exposed [34]. Treatment of metastatic LS174T human colon cancer cells with GalNAc-benzyl greatly reduced their liver colonization in athymic mice, thereby implicating O-glycans in the metastatic process [35]. Inhibiting *O*-glycosylation reduces the formation of Lewis-related antigens on cell surface mucins that may play a role in the invasive process of cancer cells by binding to E-selectin on vascular endothelial cells [36]. In Caco-2 human colon cancer cells, GalNAcα-Bn treatment increased STn expression [37]. This may be explained by the fact that GalNAcα-Bn is a substrate for the core 1 β3-Gal-transferase and for the core 3 β3-GlcNAc-transferase but not a substrate for α6-sialyltransferase acting on GalNAc-peptide. Thus, the GalNAc residues on endogenous proteins, exposed upon GalNAcα-Bn treatment can still be converted to SAα2-6 GalNAc- structures.

This is the first report of a non-hematopoietic cell line that lacks the enzyme synthesizing O-glycan core 1, core 1 β3-Gal-transferase. Core 1 β3-Gal-transferase activity in LSC cells could not be induced by treatment with sodium butyrate or growth of cells *in vivo*. In addition, mixing experiments indicated that LSC cell extracts may have an inhibitory substance which may be heat labile. However, an endogenous inhibitor cannot account for the dramatic reduction of core 1 β3-Gal-transferase activity LSC cells. Previously core 1 β3-Gal-transferase was found to be reduced in the human T-lymphocytic Jurkat cell line [38] and in red blood cell membranes from patients with Tn-agglutinability syndrome [39]. Patients with this condition show variable severity of symptoms and may suffer from hemolytic anemia, leucopenia and thrombocytopenia [40]. Core 1 β3-Gal-transferase activity in red blood cells from patients with the Tn syndrome, but not in Jurkat cells, could be reactivated by *in vitro* differentiation with azacytidine and sodium butyrate [40, 41].

In Caco-2 human colonic carcinoma cells, the core 1 β3-Gal-transferase appears to be down-regulated during enterocytic differentiation [26]. In contrast, the activity in LSC cells could not be reactivated by differentiation or *in vivo* tumor growth in athymic mice. The enzyme in LSC cells may therefore be permanently inactivated and not subject to regulation during LSC cell differentiation. We cannot rule out the possibility that core 1 β3-Gal-transferase activity in LSC cells is a novel one requiring unusual assay conditions or substrates. However, since both low molecular weight and mucin substrates were inactive, this possibility is not likely. Alternatively, in LSC cells the core 1 β3-Gal-transferase gene may be mutated leading to reduced expression or inactive enzyme; proof for this must await cloning of the gene for this glycosyltransferase. The fact that a low level of residual activity was observed in some LSC cell preparations suggests that at least some enzyme protein is made. Since most glycosyltransferases represent a family of enzymes, it is also possible that several enzymes with core 1 β3-Gal-transferase activity are normally expressed and one of them is inactivated in LSC cells. Purification and cloning of this important enzyme will help to resolve these questions.

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