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review

Mucin-type *O*-glycans in human colon and breast cancer: glycodynamics and functions

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The glycoproteins of tumour cells are often abnormal, both in structure and in quantity. In particular, the mucin-type *O*-glycans have several cancer-associated structures, including the T and Tn antigens, and certain Lewis antigens. These structural changes can alter the function of the cell, and its antigenic and adhesive properties, as well as its potential to invade and metastasize. Cancer-associated mucin antigens can be exploited in diagnosis and prognosis, and in the development of cancer vaccines. The activities and Golgi localization of glycosyltransferases are the basis for the glycodynamics of cancer cells, and determine the ranges and amounts of specific *O*-glycans produced. This review focuses on the glycosyltransferases of colon and breast cancer cells that determine the pathways of mucin-type *O*-glycosylation, and the proposed functional and pathological consequences of altered *O*-glycans.

Keywords: breast cancer cell; colon cancer cell; glycoprotein; glycosyltransferase; *O*-glycosylation

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Introduction

Cell-surface-bound and secreted mucin glycoproteins from epithelial and other mucin-producing cells contain a range of *N*-acetylgalactosamine (GalNAc)-Ser/Thr *O*-linked oligosaccharides (*O*-glycans) that comprise more than 50% of the mucin molecule by weight. Mucins have distinct peptide domains of functional importance (Hollingsworth & Swanson, 2004), and the Ser/Thr/Pro-rich variable number of tandem repeat (VNTR) domains of mucins are heavily *O*-glycosylated. The secreted mucins function as a protective layer over the epithelium. The *O*-glycans of cell-surface-bound mucins control antigenicity as well as interactions with the environment and bind to mammalian lectins. Mucins also participate in the control of the immune system (Ogata *et al*, 1992; Blottiere *et al*, 1992; Onami *et al*, 2002; Crocker, 2005; Gerloni *et al*, 2005; Varki & Angata, 2006). Depending on the structures of their *O*-glycan chains, mucins can be involved in cell adhesion or can be anti-adhesive (Hollingsworth & Swanson, 2004). Four main *O*-glycan core structures (1–4) are found in colonic mucins (Table 1), whereas mammary mucins have only core structures 1 and 2. These cores are usually extended and substituted with other sugars and sulphate esters, resulting in many different structures. Some of the structural alterations found in cancer glycoproteins are listed in Table 1.

About 20 genes encode mucin protein backbones. The cell-surface mucin MUC1 has been the focus of considerable interest owing to its changing expression levels and modification by *O*-glycans in many types of cancer (Burchell *et al*, 2001). Both the peptide backbone and the carbohydrate chains of MUC1 and other mucins show heterogeneity and altered levels in cancer, and the mucins often have new and unusual carbohydrate and peptide epitopes. This property can be useful for the diagnosis, prognosis and monitoring of disease progression, and has been exploited to develop experimental therapies and immunization approaches to cancer (Magnani, 1983; Fung *et al*, 1990; Hollingsworth & Swanson, 2004; Stepensky *et al*, 2006). Compared with *O*-glycans from normal mucins, cancer-associated *O*-glycans can be highly sialylated and less sulphated (Brockhausen & Kuhns, 1997; Brockhausen, 1999, 2003a,b); they are often truncated, and commonly contain the Tn (GalNAc-) and T (Galβ1-3GalNAc-) antigens, and their sialylated versions (Table 1). Both the Tn and T antigens can trigger immune responses, and have been exploited to develop cancer vaccines (Singhal *et al*, 1991; Springer *et al*, 1995).

The members of a large family of polypeptide GalNAc-transferases (ppGalNAc-T) synthesize the Tn antigen (Ten Hagen *et al*, 2003). Glycosyltransferases then add sugar residues through many pathways that lead to a complex range of *O*-glycans (Brockhausen 1999, 2003b; Figs 1–3). The four main *O*-glycan core structures (1–4; Table 1) can be synthesized, extended, branched and terminated by many different families of glycosyltransferases.

Biological roles of altered *O*-glycans in cancer cells

Alterations in mucin structures in cancer have many biological and pathological consequences, because potential ligands responsible for interactions between cancer cells and their microenvironment are changed. This influences the growth and survival of the cell, its ability to invade and metastasize, and its interactions with lectins and cell-surface receptors or cells of the immune system. Mucins interact with cell-adhesion molecules, and can block cell–cell adhesion mediated by integrins and E-cadherin. Mucins can also inhibit

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Table 1 | Mucin-type *O*-glycans and alterations in cancer

O-glycan	Structure	Increased/ decreased in cancer*
Tn antigen	GalNAcα-Ser/Thr	↑
STn antigen	Sialylα2-6GalNAcα-Ser/Thr	↑
Core 1, T antigen	Galβ1-3GalNAcα-Ser/Thr	↑
Sialyl-T antigens	Sialylα2-3Galβ1-3GalNAcα-Ser/Thr Sialylα2-6(Galβ1-3)GalNAcα-Ser/Thr	↑ ↑
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcα-Ser/Thr	↑↓
Core 3	GlcNAcβ1-3GalNAcα-Ser/Thr	↓
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-Ser/Thr	↓
Type 1 chain	$[GlcNAc\beta1\text{-}3\ Gal\beta1\text{-}3]_n$	↓
Type 2 chain	$[GlcNAc\beta1\text{-}3\ Gal\beta1\text{-}4]_n$ poly-*N*-acetylactosamines	↑
Sialyl-Lewis[a]	Sialylα2-3Galβ1-3 (Fucα1-4)GlcNAcβ1-3Gal-	↑
SLe[x]	Sialylα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑
Sialyl-dimeric Lewis[x]	Sialylα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1-3 Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑

*The symbol ↑ denotes an increase in cancer, whereas the symbol ↓ denotes a decrease in cancer. Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; sialyl, sialic acid; SLe[x], Sialyl-Lewis[x]; STn, Sialyl-Tn.

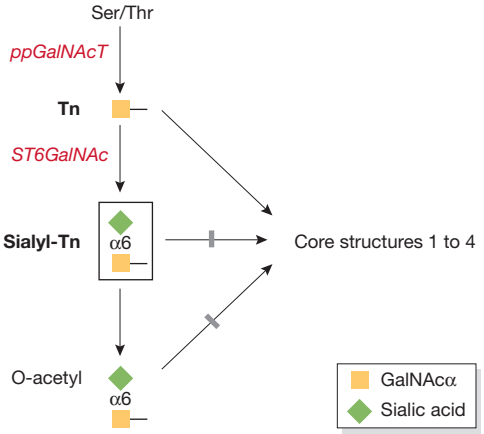

Fig 1 | Biosynthesis of the sialyl-Tn antigen. In the biosynthetic pathway that forms the sialyl-Tn (STn) antigen, a family of polypeptide *N*-acetylgalactosamine (GalNAc)-transferases (ppGalNAcT) initially adds a GalNAc residue to Ser or Thr of the peptide. This is followed by the addition of sialic acid in α2-6 linkage to GalNAc, catalysed by a family of α6-sialyltransferases (ST6GalNAc), and produces the STn antigen. Although GalNAc can be further converted to core structures, STn can only be a substrate for an *O*-acetylation reaction to produce *O*-acetyl-STn that is not recognized by anti-STn antibodies. Bars across the arrows indicate a block in the biosynthetic pathway.

cell lysis by natural killer cells (Ogata *et al*, 1992) and interactions with cytotoxic lymphocytes (van de Wiel-van Kemenade *et al*, 1993). A block of *O*-glycan-extension reactions with inhibitors such as GalNAc-benzyl can increase E-cadherin-mediated cell adhesion by decreasing the inhibitory action of the *O*-glycosylated protein dysadherin, or by affecting mucins that normally have anti-adhesive effects (Tsuji *et al*, 2003).

Sialylated *O*-glycans have been shown to be associated with an enhanced growth rate of mammary carcinoma cells in MUC1 transgenic mice (Mungul *et al*, 2004). Sialic acid has also been implicated in the metastatic process (Bresalier *et al*, 1996). Therefore, removal of sialic acid can reduce the metastatic potential of cancer cells. There is an association of sialyl-Tn (STn) with a poor prognosis in cancer patients. The role of STn has been investigated using T47D breast cancer cells. Clones expressing STn as well as the enzyme that synthesizes STn, α6-sialyltransferase (ST6GalNAc-I), showed slower cell growth but increased cell migration. The adhesion of ST6GalNAc-I-transfected cells displaying STn to several types of matrix protein was decreased (Julien *et al*, 2005). These properties might allow STn[+] cells to metastasize.

The occurrence of the sialyl-Lewis[x] (SLe[x]) epitope in colon cancer patients is also associated with poor survival (Nakamori *et al*, 1993). Compared with primary tumours, metastatic cancer cells often have increased amounts of sialyl-Lewis[a] and SLe[x], as well as sialyl-dimeric Lewis[x] (Hanski *et al*, 1996; Ito *et al*, 1997; Nishihara *et al*, 1999; Petretti *et al*, 2000; Table 1). These sialyl-Lewis structures are ligands for selectins that normally participate in the attachment of leukocytes to the endothelium. Treatment of colon cancer cells with GalNAc-benzyl reduces the extension of mucin-type *O*-glycans, as well as their attachment to E-selectin and endothelial cells expressing E-selectin. Therefore, cancer cells might use the SLe[x]-selectin-binding mechanism during tumour invasion and metastasis (Kojima *et al*, 1992).

Adhesive carbohydrate structures of colon cancer cells

Cancer tissues often have aberrant terminal sugar structures of *O*-glycan chains, such as Lewis antigens, as well as sialic acids, which represent ligands for adhesive interactions with lectins and antibodies from the external environment (Crocker, 2005). Glycan branches and extensions are important backbone structures for the attachment of these terminal epitopes (Table 1). The appearance of SLe[x] antigens is controlled by enzymes that synthesize SLe[x] (Fig 3), as well as by enzymes that build backbone structures. On leukocytes, the core 2 branch in particular contains these selectin ligands (Table 1; Nakamura *et al*, 1998; Lowe, 2003).

In normal colonic mucosa, type 1 and type 2 chain extensions are formed, but only the terminal type 2 chain-repeat unit is the precursor for the SLe[x] antigen. In adenocarcinomas, and especially in high-grade and advanced human colorectal cancers, type 2 chains are produced; the activity of β4-Gal-transferase involved in their synthesis is upregulated, whereas specific members of the β3-Gal-transferase family are downregulated. Increased activities of α3-sialyltransferases can also contribute to increased amounts of SLe[x] in colorectal carcinomas and invasion of lymph vessels. In particular, the sialyl-dimeric Lewis[x] is related to a poor prognosis and is controlled by α3-Fuc-transferase IV, which is significantly elevated in colorectal carcinoma (Kudo *et al*, 1998).

Colon cancer cells simplify *O*-glycan biosynthesis

Normal colonic mucins have a large range of *O*-glycan structures, and colonic tissues and cells are rich in enzyme activities that synthesize the common core structures 1–4 (Table 1; Fig 2). The expression of several ppGalNAcTs that initiate mucin-type *O*-glycosylation and synthesize the Tn antigen is increased in colon and other carcinomas. Shibao *et al* (2002) correlated the expression of ppGalNAc-T3 with histological tumour differentiation and the likelihood of 5-year

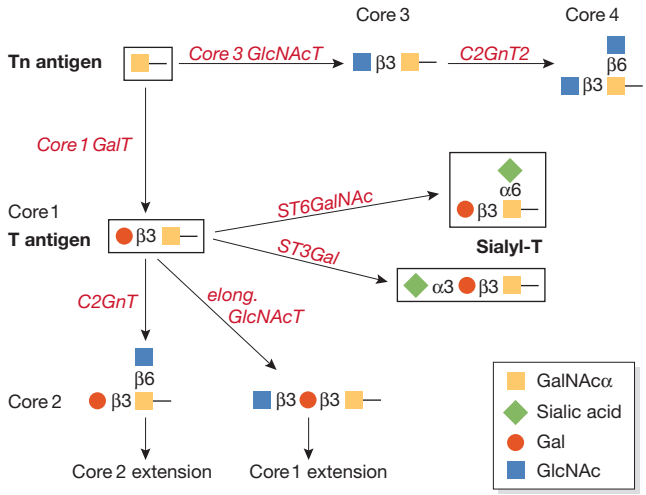

Fig 2 | Biosynthesis of the T antigens. *N*-acetylgalactosamine (GalNAc; Tn antigen) can be converted to core 1 (T antigen) by core 1 β3-Gal-transferase (core 1 GalT) or to core 3 structures by core 3 β3-*N*-acetylglucosamine (GlcNAc)-transferase (core 3 GlcNAcT), which can be branched by C2GnT2 to form core 4. Colonic mucosum expresses the enzymes that synthesize cores 1–4. In mammary cells, cores 1 and 2, but not cores 3 and 4, are synthesized. Core 1 can be converted to sialyl-T antigens by α3-sialyltransferases (ST3Gal) or by α6-sialyltransferases (ST6GalNAc). Alternatively, core 1 can be elongated by a β3-GlcNAc-transferase (elong. GlcNAcT) or can be branched by C2GnT to form core 2. All core structures can be further extended to form complex *O*-glycans.

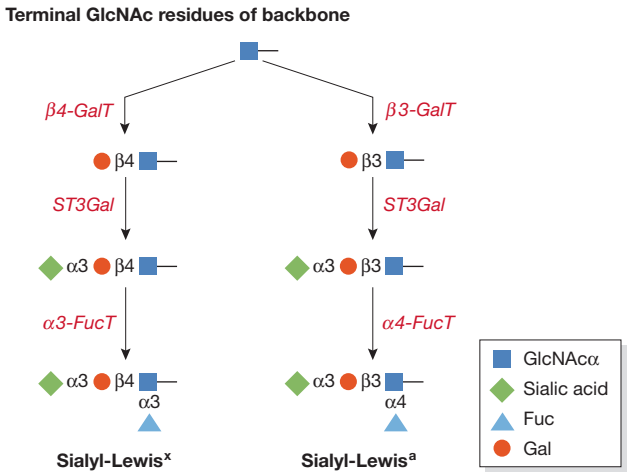

Fig 3 | Assembly of Lewis antigens. Terminal *N*-acetylglucosamine (GlcNAc) residues, and especially those of core 2 structures, can be used as the basis for the attachment of Lewis antigens. The sialyl-Lewis^x (SLe^x) structure is synthesized by the addition of a β4Gal to GlcNAc (type 2 chain) by β4-Gal-transferase (β4-GalT), followed by the addition of α3-sialic acid to Gal by α3-sialyltransferase (ST3Gal) and of α3-Fuc to GlcNAc by α3-Fuc-transferase (α3-FucT). Type 1 chains are synthesized by β3-Gal-transferase (β3-GalT). ST3Gal and α4-Fuc-transferase (α4-FucT) then assemble the sialyl-Lewis^a antigen.

survival. Both the Tn and STn antigens are markers for poorly differentiated adenocarcinomas and mucinous carcinomas, and their increased occurrence is associated with advanced cancer, invasive and highly proliferative tumours, metastasis and a poor clinical outcome (Itzkowitz *et al*, 1990). The STn antigen also correlates with the progression of intestinal disease to malignancy (Ogata *et al*, 1998).

The T antigen (core 1) is prevalent in colon cancer, whereas normal colonic mucins display *O*-glycans with mainly core 3 structures (Table 1; Fig 2). Core 3 β3-*N*-acetylglucosamine (GlcNAc)-transferase, which is the enzyme that synthesizes core 3, is reduced in colon cancer tissues and is below detectable levels in cultured colon cancer cells (Brockhausen, 1999). As both of the enzymes that synthesize cores 1 and 3 use GalNAc-peptide substrates, the absence of core 3 synthesis might contribute to the prevalence of the T antigen in colon cancer. The messenger RNA (mRNA) levels of ST6GalNAc-II, which modifies the T antigen (Fig 2), are increased in cases of colorectal cancer metastases to lymph nodes, and correlate with shorter patient survival (Schneider *et al*, 2001).

The T antigen is converted to the core 2 structure by core 2 β6-GlcNAc-transferases, C2GnT1 (leukocyte-type L-enzyme) and C2GnT2 (mucin-type M-enzyme). Normal colon tissues contain high M-enzyme activity. Several human colon cancer cell lines, including tumorigenic cells derived from human adenoma cells, lack M-enzyme activity, whereas HT29 and other colon cancer cell lines maintain a high level of C2GnT2 (Vavasseur *et al*, 1994, 1995; Schwientek *et al*, 1999). It seems that C2GnT1 is upregulated in most colon cancer tissues, relative to C2GnT2. This is expected to result in a decrease of core 4 structures, and a relative increase of core 2 structures, which are the main carriers of SLe^x. C2GnT1 expression has been correlated with the extent of tumours and with vessel invasion (Shimodaira *et al*, 1997), as well as lymph-node metastasis and disease progression, supporting the idea that tumours use their selectin ligands to invade through the endothelium.

Synthesis of sialylated *O*-glycans in breast cancer cells

Mucins produced by normal mammary epithelial cells contain a mixture of *O*-glycans, many of which have extended core 2 structures. In cultured breast cancer cells, however, *O*-glycans contain less total carbohydrate, and sialylated core 1 is prevalent. An increase in the expression of α3-sialyltransferase ST3Gal-I, which acts on core 1, is characteristic of breast cancer cells and tissues (Brockhausen *et al*, 1995; Burchell *et al*, 1999). This is associated with the exposure of mucin peptide epitopes that are masked in the normal mucins.

Although the amount of core 2 structure scaffolds might be decreased in breast cancer, the levels of the SLe^x antigen are increased. This has been correlated with the expression of α3-Fuc-transferase VI (Matsuura *et al*, 1998), which seems to be an important regulator of the SLe^x antigen in breast tumours.

The occurrence of the tumour-specific antigen STn is associated with an unfavourable prognosis and formation of metastatic cancer. In breast tumours, the appearance of the STn antigen has been correlated with the expression of α6-sialyl-transferase I (ST6GalNAc-I). Both ST6GalNAc-I and core 1 β3-Gal-transferase use GalNAc-peptide substrates. Studies in breast cancer T47D cells have shown that ST6GalNAc-I successfully competes with core 1 β3-Gal-transferase (Fig 4), owing to the broad Golgi localization of ST6GalNAc-I (Sewell *et al*, 2006).

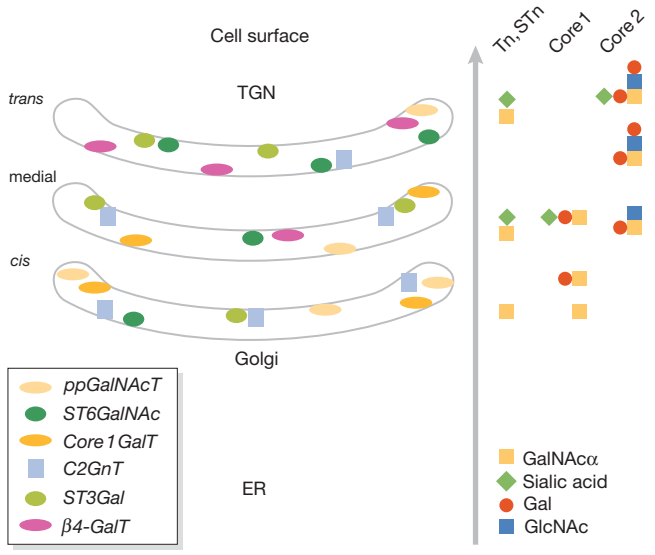

Fig 4 | Distribution of glycosyltransferases in the Golgi. The diagram shows the localization of several membrane-bound glycosyltransferases that assemble *O*-glycans in the Golgi, on the basis of studies of several different cell types. β4GalT, β4-Gal-transferase; C2GnT, core 2 β6-*N*-acetylglucosamine (GlcNAc)-transferase 1; Core 1GalT, core 1 β3-Gal-transferase synthesizing core 1, the T antigen; ppGalNAcT, polypeptide *N*-acetylgalactosamine (GalNAc)-transferase; ST3Gal, α3-sialyltransferase I acting on core 1; ST6GalNAc, α6-sialyltransferase I acting on GalNAc. Due to the broad localization of ppGalNAcT in HeLa cells, GalNAc can be added to Ser/Thr throughout the Golgi. Core 1 and 2 synthesis starts in the *cis*-Golgi, and chains are completed in the medial Golgi and *trans*-Golgi. C2GnT competes with ST3Gal for the core 1 substrate. Most of the elongation reactions involving Galβ1-4 residues (that is, the synthesis of type 2 chains and SLe^x) occur in the *trans*-Golgi. The arrow shows the transport of glycoproteins from the endoplasmic reticulum (ER) to the cell surface. Some of the glycosyltransferases might form protein complexes of as yet unknown composition. TGN, *trans*-Golgi network.

Mechanisms controlling *O*-glycan biosynthesis

Glycodynamics—that is, the dynamically changing pathways of *O*-glycan biosynthesis and their relevant control mechanisms—are complex and poorly understood. Many tumour cells have altered biosynthetic pathways of *O*-glycans, and aberrant mRNA levels and activities of glycosyltransferases (Brockhausen, 1999). A large family of polypeptide GalNAc-transferases (ppGalNAcT) in the Golgi catalyses the first step of *O*-glycan synthesis. These enzymes are similar but distinct, and are expressed in a cell-type-specific fashion (Mandel *et al*, 1999; Berois *et al*, 2006). ppGalNAcT proteins have a lectin-like domain that seems to enable them to recognize GalNAc residues added to the peptide. Therefore, *O*-glycosylation is regulated by the specificities of ppGalNAcT isoenzymes towards peptide and glycopeptide substrates. As proteins are fully folded in the Golgi, only those Ser/Thr residues that have accessible side chains will be glycosylated.

The arrangement of biosynthetic enzymes in the assembly line in the Golgi (Fig 4) is an important factor controlling *O*-glycan biosynthesis and can vary between cell types. Immunocytochemical studies localized ppGalNAcT to early *cis*-Golgi compartments in submaxillary glands (Roth *et al*, 1994). However, in HeLa cervical cancer cells, ppGalNAcT1, ppGalNAcT2 and ppGalNAcT3 are present throughout the *cis*-Golgi, medial Golgi and *trans*-Golgi compartments (Röttger *et al*, 1998). Other glycosyltransferases have a more distinct Golgi localization. The enzymes that synthesize cores 1 and 2 are mainly in the *cis*-Golgi, whereas terminally acting enzymes are mainly in the *trans*-Golgi. If the first sugar (GalNAc) is added in a later compartment than the one in which the extension enzymes reside, then the glycoprotein cannot be a substrate for extension reactions. Therefore, a broad Golgi localization of ppGalNAcT is consistent with only some of the GalNAc residues being fully processed, and with the presence of partly processed and structurally heterogeneous *O*-glycans. Altered Golgi localization of enzymes in cancer cells (Egea *et al*, 1993) might contribute to a disturbance in the assembly line and to the synthesis of truncated or aberrant glycans.

Specific sugar modifications can mask glycan epitopes. For example, the STn antigen is a terminal structure that cannot be used as a substrate for glycosylation reactions. However, the sialic-acid moiety can be *O*-acetylated, preventing the recognition of STn by anti-STn antibodies. In colon cancer, the masking of sialic acid by *O*-acetylation is reduced, which contributes to the increased occurrence of sialylated antigens (Jass & Walsh, 2001; Fig 1).

A loss of core-synthesizing enzymes in cancer cells might also increase the amount of STn of mucins, and produces the STn^+ phenotype. For example, LSC colon cancer cells cannot synthesize the normal colonic core structures 1–4 and, therefore, biosynthetic pathways cannot continue beyond the Tn and STn antigens (Brockhausen *et al*, 1998a). In addition, a high activity of α6-sialyltransferase acting on GalNAc-mucin, relative to the enzyme activities that synthesize core 1 and 2 structures, can result in an STn^+ phenotype (Brockhausen *et al*, 2001; Fig 1).

Other extension and termination reactions in the *O*-glycosylation pathways known to be abnormal in cancer include the addition of blood group and tissue antigens, sialic acid, fucose (Fuc) and sulphate residues (Brockhausen & Kuhns, 1997; Brockhausen *et al*, 1998b, Brockhausen, 2003a; Fig 3). These reactions involve several glycosyltransferase and sulphotransferase families, in which one or more enzymes have altered mRNAs or activity levels in cancer cells. Moreover, the blood groups of cancer glycoproteins can be incompatible with those of the non-cancerous cells of the body (David *et al*, 1993) and might elicit an immune response.

Most intermediates in the biosynthetic pathways form substrates for several competing reactions. The relative activities of competing enzymes that co-localize with the substrate in the Golgi determine the amounts of individual enzyme products synthesized (Fig 4). For example, core 1 can be converted to the branched core 2 structure by C2GnT or, alternatively, to the sialylated core 1 structure by α3-sialyltransferase (ST3Gal-I; Fig 2). The localization of these two enzymes partly overlaps in breast cancer cells, allowing them to compete effectively for the core 1 substrate. After core 1 is α3-sialylated, it cannot be converted to core 2 owing to the specificity of C2GnT, which does not recognize the sialylated core 1 structure as an acceptor substrate. Therefore, the relative expression and activities of ST3Gal-I and C2GnT1 determine the ratio between sialylated core 1 chains and more complex core 2 chains, as well as the exposure of mucin peptide epitopes (Burchell *et al*, 2001).

Another mechanism that controls *O*-glycan structures in cancer is based on the ability of some glycosyltransferases to distinguish between different glycosylation sites on a peptide backbone. The enzymes that synthesize core structures 1–3, as well as the β4-Gal-transferase that extends the core structures, act in a site-directed manner (Brockhausen *et al*, 1990, 2006). The relative expression of mucins with different peptide sequences is often altered in cancer owing to either upregulation or downregulation of mucin gene expression. As the synthesis of individual *O*-glycans depends, in part, on the peptide backbone of mucin substrates, it is conceivable that the structures of *O*-glycans show quantitative changes in tumours expressing abnormal amounts of mucins.

The activation of specific signalling pathways induced by cytokines, growth factors, bacterial lipopolysaccharides or agents that affect cellular growth, differentiation and cell death, have all been shown to affect the mRNA or activity levels of glycosyltransferases (Brockhausen & Kuhns, 1997; Brockhausen *et al*, 1998b; Delmotte *et al*, 2002; Yang *et al*, 2004). Factors that control directly the activities of glycosyltransferases include those that affect protein folding, a change in metal ion concentrations and pH of the Golgi, post-translational modifications of the enzyme and the formation of protein complexes. The interactions of glycosyltransferases with components of the Golgi membranes could be crucial for the regulation of enzyme activities, but need to be more clearly defined. The pathology of cancer is often associated with alterations in these factors, which then contribute to altered glycodynamics.

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