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Perspectives on the significance of altered glycosylation of glycoproteins in cancer

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***Abbreviations*: Sia, sialic acid, type unspecified; Tn antigen, GalNAcα1-O-Ser/Thr; T antigen, Galβ1-3GalNAcα-O-Ser/Thr; Sialyl Lewis^x, Siaα2-3Galβ1-4(Fucα1-3)GlcNAc; Sialyl Lewis^a, Siaα2-3Galβ1-3(Fucα1-4)GlcNAc; Sialyl-Tn antigen, Siaα2-6GalNAcα1-O-Ser/Thr; FucT, fucosyltransferase; ST, sialyltransferase.**

Introduction

A common phenotypic change in malignancy is a dramatic transformation of cellular glycosylation [1–9]. Indeed, altered glycosylation can be considered an universal feature of cancer cells. Interest in the pathophysiological role of glycosylation in cancer arose from early findings that certain oligosaccharides are common markers of tumor progression. In particular, malignant transformation was associated with increased size of membrane glycopeptides [10, 11], and monoclonal antibodies raised against various types of carcinomas frequently recognized novel or truncated oligosaccharides which were noted to be oncofetal antigens [1–3, 12]. Significant correlational patterns between altered glycosylation and clinical prognosis further fueled interest in their potential biological importance [6, 13, 14]. Some *in vitro* molecular and cellular assays have further supported the notion that these changes may indeed be critical to aspects of tumor cell behavior. This topic has been the subject of many detailed reviews, including several recent ones [7–9]. We do not intend to duplicate these efforts. Rather, we will attempt to summarize some of these observations and to synthesize mechanistic hypotheses that may provide agendas for future research. The focus of this discussion is primarily on N- and O-linked glycosylation of glycoproteins, and not on glycosphingolipids. It is assumed that the reader is familiar with the pathways of N- and O-linked glycosylation in mammals [15], with the major families of mammalian lectins, including selectins [16–19], galectins [20] and I-type lectins [21, 22], and with basic concepts of tumor biology, particularly invasion and metastasis [23–26].

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Problems with elucidating the biological consequences of altered glycosylation in cancer cells

There is general agreement that most of the early events in the emergence of neoplasia involve genetic alterations, and it seems unlikely that altered glycosylation *per se* plays a major role at this stage. Rather, changes in glycosylation are more likely to have functional consequences in the later events of invasion and metastasis. Unfortunately, despite the clonal origins of neoplasia, the intrinsic mutability of transformed cells precludes a stable genetic state of advanced tumors. Thus, cellular heterogeneity is very common during the evolution of neoplasias. As a result, functional studies of a specific oligosaccharide structure in cancer studies are difficult to interpret conclusively, and exceptions to every rule are likely to be found. Microheterogeneity of glycosylation also presents additional problems with correlating the presence of a specific glycan to the biology of a given tumor. Additionally, glycoconjugates can constitute complex structural information that cannot always be reduced to a simple biomolecular 'fit' model. Finally, glycans are likely to be only one of many molecular components that play a variety of roles in the behavior of cancer cells. Thus, simple and focused experiments may not easily tease out critical pathophysiological functions. For all these reasons, mechanistic details regarding the significance of most tumor associated glycosylation changes are still lacking.

Ways in which altered glycosylation could affect the biology of cancers

Their cellular location predicates that cell surface glycoconjugates could be either adhesion or antiadhesion molecules, contributing to the complex array of intercellular interactions among the tumor cells themselves, and with many of

the normal cells they encounter during the invasive and metastatic cascade. Among these interactions, the more specific adhesion events must be mediated by endogenous lectins. On the other hand, glycoconjugates secreted by a tumor often mirror the same glycosylation changes as those seen on the cell surface. Such glycosylated molecules secreted by a tumor could be interacting with other cell types to induce events that facilitate its own progression. Furthermore, glycosylation of other adhesion molecules can modulate their intrinsic functional characteristics (see below).

Only a limited subset of biosynthetic pathways are frequently altered in cancer

Out of the many known types of biosynthetic reactions in the N- and O-linked glycosylation pathways [15], only a few structural changes have been frequently correlated with tumor progression [1–9]. Most are either truncated versions of normally expressed oligosaccharides (*eg* Tn antigen) or relatively unusual types of outer/terminal oligosaccharide sequences (*eg* Lewis$^{x/a}$ structures). Such structures arise from upregulation of some glycosyltransferases, downregulation of competing glycosyltransferases, or changes in the elongation of the core oligosaccharide structures that create favored acceptors for the capping glycosyltransferases [4, 27]. Table 1 lists the commonest of the changes in glycosylation reported in tumors, indicates any biosynthetic mechanisms known to be involved, and suggests possible consequences. Some of the structural changes listed are not mutually exclusive. Immunohistochemical studies on tumor specimens show that Lewis$^{x/a}$ structures, Tn/sialyl-Tn/T antigens, and β1,6 GlcNAc branching of N-linked core structures [13, 14, 28–32, 32–35] are increased in expression in advanced cancers. The association between β1-6 branching and tumor progression [36–38] explains previous reports of increased size of tumor membrane glycopeptides [10, 11], and the biological effects have been attributed by some to the resulting increase of polylactosamine chains, rather than to the actual branching event itself. A general increase in sialylation is a common feature of tumors [39–41], with specific increase in α2-6 linked sialic acids noted in certain situations [42, 43]. For unknown reasons, the loss of normal AB blood group expression (and the related increase in expression of H and Le^y structures) is associated with a poor prognosis [44–48], while the loss of sulfation [49, 50] or sialic acid O-acetylation [31, 51, 52] is associated with advanced tumor grade (although increased O-acetylation of sialic acids on gangliosides is seen in melanomas) [53, 54].

Experimental evidence that altered glycosylation can affect the biology of cancers

In some of the examples mentioned above, direct or indirect experimental evidence indicates that the altered glycosylation can affect the biology of cancer. Most convincing are the studies where transfection of GlcNAc transferase V has been shown to induce tumorigenic behavior in non-tumorigenic cells [55]. Others have metabolically altered N- or O-linked glycan expression with compounds such as swainsonine (which prevents complete processing of N-linked chains) or with benzyl-α-GalNAc (which metabolically inhibits the expression of mature O-linked chains on mucins), and modified the cellular behavior [56–58]. Consistent clinical correlations between metastasis and the expression of various O-linked glycans such as sialyl-Tn and Lewis structures in different carcinoma types indicate clonal expansion of cells with such specific phenotypes, and thereby strongly suggest functional roles for these structures (Table 1). *In vitro* studies using cell culture systems for breast and colon carcinomas that mimic tumor progression showed increased sialylation and decreased core 3 structures on mucins, corroborating the clinical correlation between sialyl-Tn expression and poor prognosis. These expression changes corresponded with changes in the activities of appropriate glycosyltransferases [59–61]. These studies involved variant cell lines of common clonal origins arising from chemical or viral induction, thus avoiding the inherent artifacts involved in comparing cells of different origin. In other studies, soluble oligosaccharides like sialyl Lewis^x (or antibodies to oligosaccharides) have prevented tumor cell invasion through gel matrix assays, or have decreased the colonization of metastatic sites [62]. All these data strongly support the notion that the altered oligosaccharides are themselves a primary molecular phenotype with functional significance in cancer, and not just secondary effects of changes in the proteins to which they are attached.

Mucins and carcinomas

It is clear that mucins are major carriers of altered glycosylation in most carcinomas, and it is thought that their molecular interactions define some of the cellular phenotypes of metastatic tumors [8, 63–65]. Indeed, except for the GNT-V product, all of these glycosylation changes have been found on mucins. Mucins are large glycoproteins which have the majority of their mass derived from O-linked oligosaccharides. The presence of many glycosylated serines and threonines and an over-representation of prolines and glycines in tandem repeats contribute to the 'rodlike' conformation of mucins [66–68]. Most mucin polypeptides belong to the MUC family, but other glycoconjugates with similar structural elements [8, 69–71] are well known. Reported associations of some specific mucin polypeptides with various carcinomas are discussed in detail elsewhere [8]. Since the apomucins themselves can independently correlate with tumor progression, separating the effects of the polypeptide and the carbohydrate can be experimentally difficult [72, 73]. Furthermore, the same

Table 1. Changes commonly seen in N- and O-linked oligosaccharides of carcinoma cells.

Structural change	*Polypeptide carrier(s)*	*Biosynthetic basis of structural change*	*Structural consequences*	*Potential endogenous lectin partners*	*Prognostic and clinical correlations*
Increased β1-6 branching (N-linked)	Many, including LAMPs	Increased GNT-V gene expression and activity	Increased size, branching, polylactosamines, and Lewis^x structures	Galectins Selectins	Correlated with grade, invasion, and metastasis
Increased Lewis^x and Lewis^a antigens	Many, including mucins	Increased/altered expression of FucT enzymes	Increased sialyl Lewis^x and Lewis^a	Selectins	Correlated with poor prognosis
Loss of A/B and/or H/Lewis^y structures	Many, including mucins	Decreased/altered expression of A/B or FucT enzymes	Loss of A or B antigens. Accumulation of H and Lewis^y antigens.	Unknown	Loss correlated with poorer prognosis
General increase in sialylation	Many	Unknown in most cases (see below)	Increased surface negative charge Masking of other molecules?	Selectins, H protein CD22, CD33, Sialoadhesin	Correlated with grade, invasion, and metastasis
Increased α2-6 Sialylation	Unknown	Increase in ST6Gal I expression	Increased Siaα2-6 on Galβ1-4GlcNAc Decrease inα2-3Sia	H protein CD22	Unknown
Increased Sialyl-Tn expression	Mucins	Loss of *O*-acetylation? Other?	Truncation of O-linked chains?	H protein CD22	Correlated with poor prognosis
Increased Tn antigens	Mucins	?Altered ST activity ?Altered Golgi function	Loss of negative charge. Exposure of mucin polypeptide	Gal receptors Galectins	Correlated with poor prognosis
Increased T antigens (Core 1 structure)	Mucins	?Decreased ST activity ?Altered Golgi function	Loss of negative charge, exposure of mucin polypeptide	Gal receptors Galectins	Unknown
Loss of oligosaccharide sulfation	Mucins	Unknown	Allows increased sialylation?	E-selectin Loss of L- or P-selectin ligands?	Correlated with grade and invasion
Loss of Sia *O*-acetylation	Mucins	Unknown	Increased availability of Sia side chain for recognition	H protein CD22, CD33, Sialoadhesin	Correlated with grade and invasion

tumor associated glycans can also be attached to non-mucin aglycones on the same cells.

Regardless of these complexities, the critical pathophysiological role of mucins is supported by studies on the metastatic potential of various colon carcinoma cell lines in athymic mice [58, 74], and by incubations with benzyl-α-GalNAc or sialidase prior to intravenous injection, both of which decreased the metastatic rate [75, 76]. What is the explanation for these effects? Apart from specific lectin-based interactions of the oligosaccharides (see below), the rodlike structures of the mucins and their negative charge can repel intercellular interaction and sterically prevent cell adhesion molecules such as E-cadhedrin and integrins from achieving intermolecular distances necessary for effective interactions [77]. Mucins could also act as antiadhesins to promote displacement of a tumor cell from the primary mass during the onset of the metastatic process [64, 78]. In addition, they could prevent adhesion between blood borne carcinoma cells and the host's immune cells such as NK cells and cytotoxic T lymphocytes (CTLs), blocking a lytic response [79, 80]. Indeed, mucin gene transfer experiments prevented tumor cell lysis [81].

It should be noted that apart from the antiadhesive properties of mucins, other glycans, as well as mucins, can also modulate protein–protein interactions and alter cellular adhesion/de-adhesion. For example, experimental gene modulation of GlcNAc transferase III and V altered E-cadherin dependent homotypic tumor cell adhesions [82]. Likewise, integrin interactions among cancer cells can be modulated by glycosylation [77, 83–85].

Lectin-mucin interactions

Several endogenous lectins have been identified that could potentially recognize tumor associated glycan changes. Before dealing with the individual lectins, some general principles must be mentioned. Single lectin domains usually bind to specific glycans with weak affinity under static conditions. Thus, glycan–lectin interactions usually require clustering of lectin domains to generate avidity between the corresponding components [16]. Selectins may be an exception, wherein a single lectin domain can bind to its ligands with nanomolar affinity [86–88]. The alternate explanation is the clustering of selectin molecules at the surface of cells, which has yet to be demonstrated [18]. Regardless of whether affinity or avidity explains the functional interactions, the molecular recognition required is conferred by the specificity of the individual components, namely the glycoconjugate and the lectin. However, since the adhesive interactions frequently occur in the microenvironment of the bloodstream, the influence of flow must also be considered. Indeed, in an assay mimicking the physiological situation of P-selectin binding to its mucin ligand, PSGL-1, the strength of the interaction was, in fact, due to resistance to flow [89]. In this regard, it is worth noting that the vasculature is an extremely inhospitable environment for cancer cells that must not only survive the host immune system, but also the turbulence of the blood stream [24]. In fact, it has been estimated that less than 0.01% of hematogenously disseminated cells survive. Since mucin–lectin interactions can survive such flow conditions, their potential role in the biology of the metastatic cascade becomes very relevant. Of course, it can also be speculated that appearance of new structures blocks and/or inhibits the adhesion between non-tumor associated oligosaccharides and their appropriate lectins.

Lectins that can potentially recognize the altered glycosylation of cancer cells

Regardless of whether 'flow' or 'fit' model is the biophysical basis of interaction, the lectins that can actually recognize the tumor associated glycoconjugates under the appropriate conditions *in vivo* must be identified. For the most part, such data are wanting. In the sections below, the current status of several of the known endogenous lectins is considered with regard to their potential for interactions with tumor glycans (see also Table 1). Figure 1 summarizes these complex possibilities in a composite schematic.

E-selectin

Soon after sialyl-$Le^{x/a}$ structures were shown to be specifically recognized by E-selectin, the presence of calcium-dependent E-selectin ligands on carcinoma cells was directly shown, using various tissue and cell lines, and competition with oligosaccharides and antibodies to E-selectin [90–94]. In many of these cases, E-selectin recognized mucins. Indeed, the presence of mucin cancer antigens binding to E-selectin was directly shown in the blood of colon carcinoma patients [95–98]. Various other types of carcinoma tissues also stained well with soluble E-selectin probes. Furthermore, a correlation was demonstrated between the metastatic potential of colon carcinoma cell lines in nude mice and their expression of sLe^x [99]. Others showed similar correlations in related systems [100, 101]. Overexpression of E-selectin in the transgenic mouse liver induced redirection of the metastatic patterns of syngeneic carcinomas that normally colonize the lung [102]. Although final proof is lacking, these studies indicate that interactions between mucins and E-selectin molecules may play a critical role in the metastatic cascade of some carcinoma cells.

P-selection

Carcinoma cells also express potential P-selectin ligands. The recognition by P-selectin was originally attributed to sulfatides secreted by the cells [103]. However, *O*-sialoglycoprotease sialidase sensitive mucins expressed by colon carcinoma cells can also bind P-selectin [104]. Since P-selectin is expressed on activated endothelium, it could

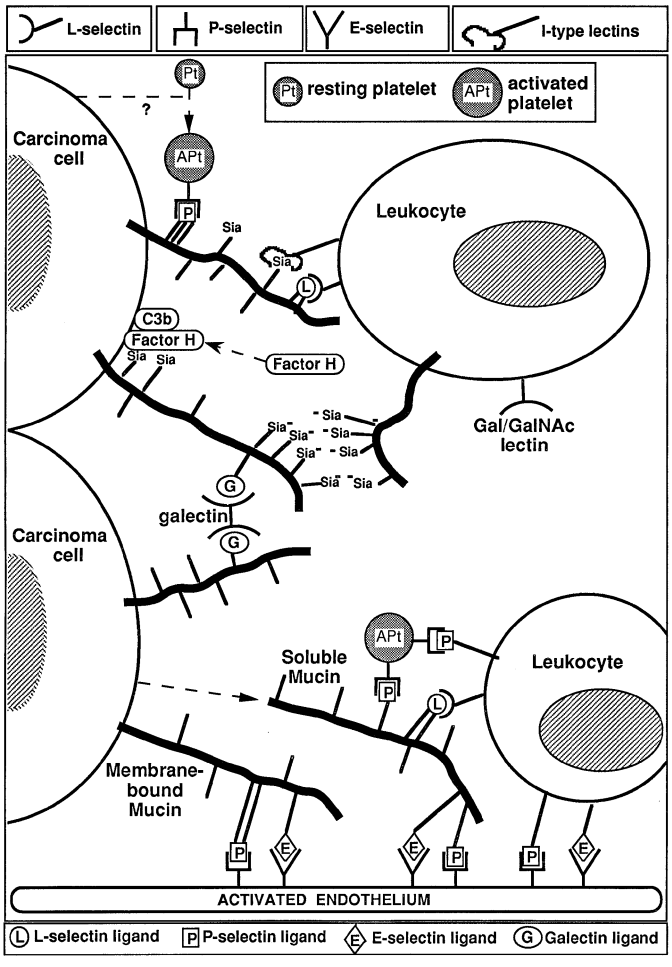

Figure 1. Composite schematic showing the many types of interactions that could potentially occur between carcinoma cell glycoproteins (particularly mucins) and endogenous lectins expressed in the plasma or on leukocytes, platelets or endothelium. All of these interactions may not occur at the same time. However, multiple interactions could potentially occur, in various combinations and additional endogenous lectins may be as yet unidentified. The leukocyte shown could be a granulocyte, lymphocyte or a monocyte/macrophage.

also act as the receptor for the extravasation of carcinoma cells in conjunction with E-selectin. Carcinoma cells can also bind to activated platelets that express P-selectin [104]. Since lymphocytes covered with platelets can roll on endothelium [105], this interaction could also theoretically facilitate the extravasation of tumor cells. Mechanistically, cancer cells entering the bloodstream may form complex thromboemboli with platelets and leukocytes – these complexes are thought to facilitate transport of the cancer cells to ectopic sites and to help in their evasion of the host immune system [106–109]. Indeed, induction of thrombocytopenia in the mouse reduced the rate of metastasis of syngeneic carcinoma. Whether this interaction is due to P-selectin is unclear. In this regard, it is also interesting that cancer patients sometimes develop thromboemboli and hypercoagulable states that may be associated with cancer cell-platelet interactions [106–109].

L-selectin

The tissue homing of leukemic cell lines can be affected by their own expression of L-selectin [110]. Antibodies against L-selectin enhanced primary tumor growth by preventing CTL sensitization in the lymph node draining the tumor area [111] and loss of vascular L-selectin ligand expression has been shown to correlate inversely with lymphocytic infiltration into evolving tumors [112]. On the other hand, L-selectin can recognize certain cancer mucins [104, 113] and the potential for direct interaction between L-selectin on normal leukocytes and the mucins on carcinoma is less well studied. Having L-selectin ligands should theoretically be a disadvantage for cellular survival of a carcinoma since this could attract unwanted attention from lytic leukocytes. However, an advantageous scenario can occur whereby the tumor cell-leukocyte interaction can enhance the survival in the bloodstream. Tumor emboli have been found to have leukocytes associated with the tumor cells, which may physically shield the carcinoma form the other components of the immune system. Conversely, the secretion of an L-selectin binding mucin could theoretically have a protective effect as a soluble inhibitor (see below).

Galectins and other galactose binding lectins

Expression of galectins (especially galectin-3) has also been associated with tumor progression [20, 114, 115]. The molecular mechanism of this correlation has been proposed to be the interactions of galectins with poly-*N*-acetyllactosamines on laminin, aiding cellular invasion. Since polylactosamines are also expressed on cancer mucins, this molecular interaction could mediate homotypic adhesion of carcinoma cells as well [116]. Galectins have also been recently shown to recognize mucins, independent of polylactosamine content [117]. Galectin recognition may also explain the findings of a study in which adding cell surface Gal to tumor mutants lacking the Golgi UDP-Gal transporter enhanced metastasis [118]. Overall, it remains to be seen exactly how galectin-carcinoma glycoconjugate interactions alter the biology of cancer. Another Gal/GalNAc recognizing molecule of note is the recently cloned C-type lectin from macrophages [119], that selectively recognizes the Tn and T antigens.

The H protein

As mentioned earlier, the association between overexpression of sialic acids and tumor progression was one of the earliest findings in this field [41]. In this regard, it is interesting that Factor H is a sialic acid binding lectin that blocks activation of the alternate complement pathway on normal homologous cell surfaces, by recognizing the non-substituted exocyclic side chain of the sialic acid [120, 121].

Table 2. Potential interactions of selectins with cell surface bound and secreted or shed carcinoma mucins

	Potential interactions of selectin:	
	With secreted mucins or mucins attached to shed plasma membrane vesicles	*With membrane-bound mucins*
E-selectin	Block extravasation of leukocytes into tumor Induce expression of other adhesion molecules	Aid in extravasation of tumor cells into bloodstream
P-selectin	Block extravasation of leukocytes into tumor Induce expression of other adhesion molecules Aggregate platelets and initiate coagulation cascade	Aid in extravasation of tumor cells into bloodstream Aggregate platelets with tumor cells Help to form cellular thromboembolus Induction of other adhesion molecules on leukocytes
L-selectin	Block leukocyte aggregation at extravasation sites? Activate/desensitize leukocytes by ligating L-selectin?	Enhance leukocyte aggregation to tumor cells Induction of other adhesion molecules on leukocytes

Thus, high levels of sialylation on circulating tumor cells could potentially confer the same protective biological effect.

I-type sialic acid binding lectins

Recently, endogenous sialic acid binding lectins other than selectins and the H protein have been recognized. The I-type lectins are members of the immunoglobulin superfamily, and include several members that can specifically recognize and differentiate between α2-6 and α2-3 linkages of sialic acids [12, 22, 88]. CD22 (which is restricted to immature B-cells) is specific for α2-6 linkages, which sialoadhesin and CD33 (which are expressed on macrophages) binds to α2-3 linked sialic acids [21]. Furthermore, *O*-acetylation of the 9-carbon position of the sialic acid abrogates recognition by these lectins [122]. Since mucins express both α2-6 and α2-3 linkages of sialic acids, it is tempting to speculate that such vascular I-type lectins do actually recognize some of these cancer associated glycoconjugates. This could be of relevance in modulating the humoral immune response (via CD22) or recognition by macrophages (via CD33 or sialoadhesin). However, these issues have not yet been subjected to direct experimentation.

Secreted vs bound forms of mucins

As described above, mucins from many sources can be recognized by selectins. However, the simultaneous expression of two different topographic forms of mucins (membrane-bound and secreted) confounds any easy prediction of their pathophysiological roles. Even if a specific mucin is only bound to the cell surface, the shedding of tumor plasma membrane vesicles may render the mucin functionally 'soluble'. Table 2 lists the some of the potential effects of selectin-mucin interactions in carcinomas. It can be seen that in the context of tumor biology, the two forms of mucins could actually have opposing effects. Thus, both the secreted and the bound form of a mucin must be accounted for in any experimental or mechanistic proposal, and their relative importance determined.

Future challenges

It will be a truly arduous task to establish precise mechanistic functions for each of the types of aberrant glycosylation seen in the glycoproteins of cancer cells. Not only are the molecular interactions difficult to study *in vivo*, but many additional players (*eg* other endogenous lectins) may be yet undiscovered. However, the process of tumor progression involves the survival of the fittest cells *in vivo*, and it is reasonable to conclude that these highly selective changes in tumor cell glycosylation are not random accidents. Furthermore, it is invasion and metastasis that finally kill most patients with cancer, not simple tumor growth. Thus, the immediacy of the potential clinical consequences demands perseverance in this difficult area of research. One promising approach to these complex situations may come from the increasing availability of mice with germline genetic disruption of specific endogenous lectins or glycosyltransferases [123]. Comparing the biological behavior of tumors in these 'knockout' animals to that seen in their normal littermates may help to delineate specific roles for each of the lectins and structures in question. If the precise molecular basis for the functions of these aberrant glycans becomes clear, the pursuit of oligosaccharides or synthetic mimics as glycan interaction inhibitors may become worthwhile.

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