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Regulation of the metastatic cell phenotype by sialylated glycans

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Abstract Tumor cells exhibit striking changes in cell surface glycosylation as a consequence of dysregulated glycosyltransferases and glycosidases. In particular, an increase in the expression of certain sialylated glycans is a prominent feature of many transformed cells. Altered sialylation has long been associated with metastatic cell behaviors including invasion and enhanced cell survival; however, there is limited information regarding the molecular details of how distinct sialylated structures or sialylated carrier proteins regulate cell signaling to control responses such as adhesion/migration or resistance to specific apoptotic pathways. The goal of this review is to highlight selected examples of sialylated glycans for which there is some knowledge of molecular mechanisms linking aberrant sialylation to critical processes involved in metastasis.

Keywords Sialylation · Metastatic cell phenotype · Sialylated glycoproteins · Invasion

1 Introduction

It has been known for decades that glycoconjugates play an important role in cancer development and progression. An alteration in the profile of cell surface glycans was one of the earliest-identified hallmarks of a tumor cell, and many of the anti-tumor antibodies produced by patients are specific for carbohydrate antigens [1–4]. Cancer-associated glycoconjugates in serum and tissue have been used as important biomarkers for disease progression [5–7]. Notably, the changes in glycan structure following tumorigenic transformation are not random. There is a specific subset of oligosaccharides that becomes enriched on the tumor cell surface, implicating a functional contribution to the tumor phenotype, and many of the glycosyltransferases that synthesize these oligosaccharides are upregulated in response to oncogenes such as Ras [3, 8].

Despite these long-standing observations, our understanding of the molecular mechanisms linking altered glycosylation to tumor cell behavior has lagged behind most other areas of cancer research. This is unfortunate in that this dearth of knowledge has left largely unexplored an important category of potential biomarkers or targets for drug discovery and vaccine development. So why has the field of cancer glycobiology progressed so slowly? While many factors are likely involved, one of the challenges encountered is that, unlike the template-driven synthesis of oligonucleotides and proteins, the synthesis of glycans elaborating cell surface molecules is complex and not readily predictable. Technologies for defining glycoconjugate structure are still evolving, and there are limited methods that can be used to determine the position of an oligosaccharide within the three-dimensional structure of a glycan carrier. For example, X-ray crystallography is difficult to perform with glycosylated proteins, therefore the glycans are typically enzymatically removed prior to crystallization. As a consequence, current literature describing conformational analyses of proteins, or identification of protein–protein interaction domains, often excludes information regarding how glycans might alter protein conformational features or peptide-binding interfaces. It is generally assumed that glycans extend into the extracellular space with high mobility (and many do), however there is evidence that at least some glycans are relatively fixed within the larger

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glycoprotein tertiary structure [9, 10], and glycans can also form direct bonds with primary amino acid sequence [11, 12]. Another important factor not always appreciated is that some monosaccharides, such as sialic acid, are negatively charged at physiologic pH, thus the addition of such a sugar (comparable to a phosphate group) has potential to alter protein conformation and/or oligomerization. As well, sialylation is emerging as a major regulator of cell surface retention of various receptors.

In addition to modulating the conformation, clustering and/or surface retention of an individual glycoprotein, glycoconjugates are ligands for numerous glycan-binding proteins such as lectins [13, 14]. While studies of protein–protein interactions have predominated in cancer research, there is increasing recognition that associations between tumor glycans and lectins are of great importance in regulating many aspects of tumor cell behavior. Lectins exist as intracellular, cell surface, or secreted molecules, depending upon the species, and many secreted lectins are incorporated into the extracellular matrix. Hence, glycan-lectin binding partners represent another fundamental class of molecular regulators of cell–cell and cell–matrix interactions. Elucidating the biochemical details of these interactions has proven challenging, however mounting evidence points to a high degree of specificity, comparable to the role of distinct amino acid motifs that drive protein–protein interactions. Indeed, glycans have been referred to as the "third alphabet of molecular biology" (the other two being proteins and nucleic acids) [15]. This capacity to control cell–cell and cell–matrix interactions, in combination with the known effects of glycans on the structure/function of individual glycoproteins, underlies the presumed role of glycans in most metastatic cell behaviors including migration/invasion through matrix, dissemination through the vasculature or lymphatics, evasion from immune surveillance, and resistance to apoptosis.

Given the multiplicity of carbohydrate modifications associated with human cancer, this review will have a restricted focus on one type of glycan modification, protein sialylation. Experimental results gleaned from patient tissue samples, animal cancer models, and cell culture studies, suggest that altered sialylation is a major contributor to the metastatic cell phenotype [16, 17]. The term "sialic acid" refers to a group of approximately 50 different chemical derivatives of neuraminic acid, with the most common variant represented by *N*-acetylneuraminic acid (Neu5Ac, Fig. 1a) [18]. Sialic acids are added onto the termini of either *N*- or *O*-linked glycans of glycoproteins and can also be added onto glycolipids (Fig. 1b). The overall level of cell surface sialylation is regulated by numerous enzymes including: (1) enzymes that control synthesis and availability of the activated sialic acid substrate, CMP-sialic acid, (2) the sialyltransferase family, which adds sialic acid during glycoprotein biosynthesis, and (3) the sialidase (also called neuraminidase) family, which cleaves sialic acid during glycoprotein degradation. These enzymes typically reside within subcellular compartments, with most sialyltransferases localized to the Golgi, and many of the sialidases localized to lysosomes or endosomes. Aberrant activity of both sialyltransferases and sialidases has been observed in cancer; however, the literature overwhelmingly suggests that sialylation levels are higher on tumor cells [3, 19]. Elevated sialylation is thought to be a relatively static tumor cell characteristic, given that sialic acid is added during glycoprotein biosynthesis, but recent studies indicate that some enzymes involved in sialylation can be expressed on the cell surface, or secreted as active soluble enzymes into the extracellular milieu [20–23]. While not a focus of this review, this opens up the fascinating possibility that the sialylation of certain glycoproteins may be dynamically regulated at the cell surface, providing a unique mechanism for transient control of individual glycoprotein structure or glycan/lectin interactions. One of the major barriers in our understanding of tumor cell sialylation is that much of the prior research was directed at correlating total surface sialylation levels with cell responses, with limited regard for the specific type of sialic acid chemical structure or linkage, or the specific glycoprotein carrier of the sialic acid. This lack of mechanistic knowledge has hindered investigative pursuit

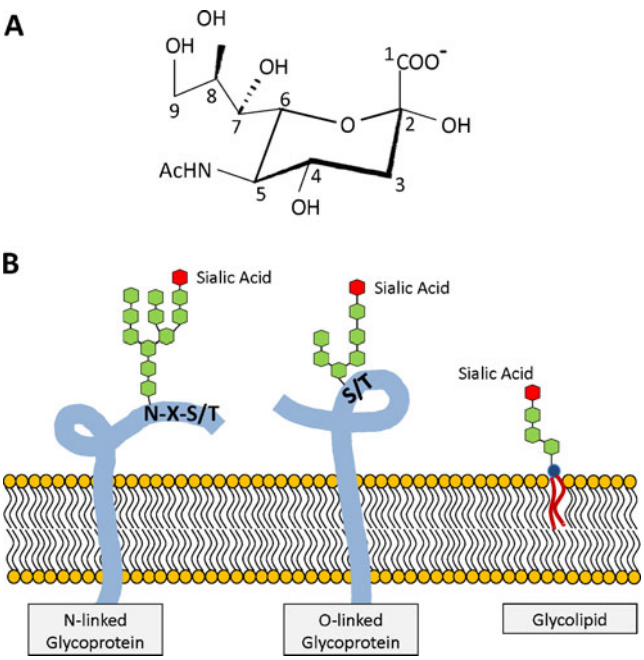

Fig. 1 Types of glycosylation. **a** Structure of the most common sialic acid, Neu5Ac. The negative charge is from the carboxylic acid group on carbon 1. **b** *N*-linked glycans (*left*) are attached to asparagine (N) residues on selected proteins containing the N-X-S/T consensus sequence, while *O*-linked glycans (*center*) are linked to serine (S) or threonine (T) residues. Glycolipids (*right*) are lipids which carry glycan structures

of glycans as clinical targets. The goal of this report is to highlight some of the more prevalent tumor-associated changes in specific types of sialylation, and discuss potential molecular mechanisms by which these modifications influence metastatic progression.

2 Regulation of tumor cell surface sialylation

An upregulation in the expression of selected sialyltransferases is a common event in tumorigenic transformation [16]. Sialyltransferases comprise a family of at least 20 different enzymes that differ in tissue distribution as well as the type of sialic acid linkage elaborated [16, 24]. Some sialyltransferases add sialic acid in an α2-3 linkage to galactose (Gal); whereas others add sialic acid in an α2-6 linkage to either Gal (e.g., the ST6Gal-I and ST6Gal-II sialyltransferases) or *N*-acetylgalactosamine (GalNAc, added by multiple ST6GalNAc sialyltransferases) (Fig. 2). The third type of sialic acid linkage is directed by the polysialyltransferase family, which adds an α2-8 linked sialic acid onto another sialic acid. Cancer-associated

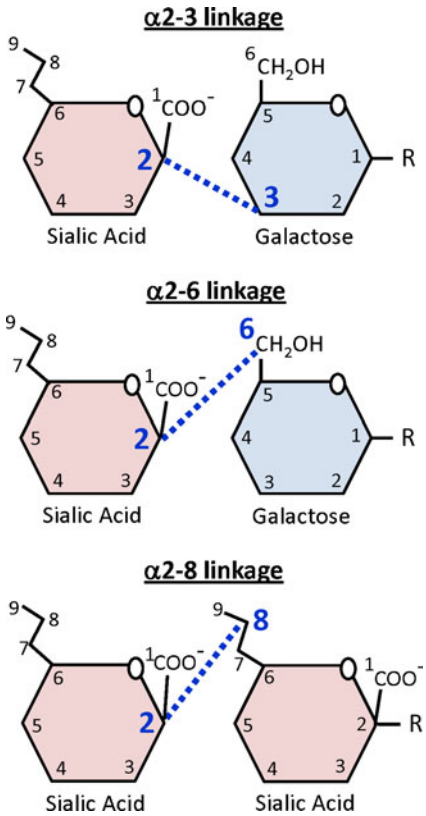

Fig. 2 Sialic acid linkages. Sialic acids are added to the termini of glycans in an α2-3, α2-6, or α2-8 linkage. In the *top two panels*, sialic acid linkage to galactose is depicted, however other sugars, such as GalNAc, can be modified with sialic acid, depending upon the type of linkage. Note that the structures shown in the figure have been simplified (e.g., hydroxyl and acetyl groups are not represented)

dysregulation has been observed for selected members of all three of these sialyltransferase categories. In conjunction with aberrant sialyltransferase expression, certain sialidases are also disrupted in human cancer, although far less is known about the tumorigenic role of this enzyme family. One example is the Neu1 sialidase, which is downregulated in cancer cells, leading to higher levels of cell surface sialylation (due to diminished sialic acid cleavage) [25].

An increase in α2-6-linked sialylation is frequently observed in tumor cells, and is usually attributed to an upregulation in either the ST6Gal-I sialyltransferase [16, 17, 26–28], which primarily sialylates *N*-linked glycans, or members of the ST6GalNAc family, which sialylate either *O*-linked glycans or glycolipids [24, 29]. The selective enrichment of α2-6 sialic acids on tumor cells is significant in that α2-6 sialylation can elicit very distinct biologic outcomes as compared with α2-3 sialylation. One striking example is the effect of α2-6 sialylation on galectin-dependent cell behaviors. Galectins are lectins that bind galactose-containing oligosaccharides [30–32]. Depending upon the galectin species, galectins can be expressed either intracellularly or extracellularly; in the latter case, some galectins are found associated with the extracellular matrix [33–35]. Extensive evidence suggests that α2-6 sialylation of galactose serves as a generic inhibitor of galectin binding [36, 37], unlike α2-3 sialic acids, which have variable effects on binding depending upon the individual galectin (Fig. 3). Accordingly, α2-6 sialylation serves as a key negative regulator of many critical galectin functions. One important activity of cell surface α2-6 sialylation is to block the binding of pro-apoptotic galectins, thereby promoting tumor cell survival [38].

Galectins are not the only glycan-binding proteins influenced by the type of sialylation present on cognate glycoconjugate ligands. Sialic acid linkage, as well as chemical structure (e.g, acetylation), are major determinants in ligand recognition by sialic acid-binding immunoglobulin superfamily lectins (siglecs) [39–41]. Siglecs are mainly expressed by immune cells, and the potential function of siglecs in tumor biology has received minimal attention. One envisions that changes in tumor cell sialylation could affect the activity of siglec-expressing immune cells, and consequently modulate the anti-tumor immune response.

Beyond their pivotal role in regulating interactions with glycan-binding proteins, sialic acids can have direct effects on specific glycoproteins that carry the sialic acid, which is not surprising given the large size and negative charge of this sugar moiety. The effect of sialylation on the structure/function of a given glycoprotein will depend upon the localization of the sialic acid within the larger glycoprotein tertiary structure, which is difficult to determine due to technical challenges. However, it is becoming clear that sialylation can affect glycoprotein activity through many

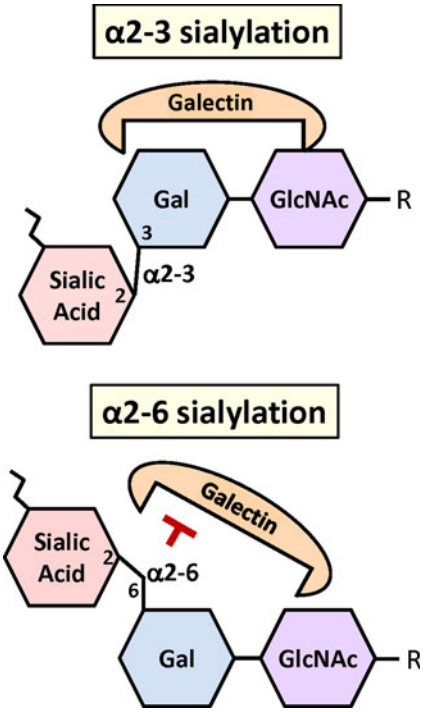

Fig. 3 α2-6 sialylation blocks galectin binding. Galectins require a free hydroxyl group on the 6 carbon of galactose for binding [37], therefore α2-6 sialylation at this site inhibits galectin binding. In contrast, α2-3-linked sialic acids have variable effects on binding, depending upon the specific galectin species

different mechanisms. As examples, sialylation alters: (1) conformation of the β1 integrin [42, 43]; (2) clustering of CD45 [44], EGFR [45], and PECAM [46]; and (3) cell surface retention of PECAM [46] and the Fas death receptor [47]. There is also evidence that sialylation modulates heterotypic associations between two distinct cell surface glycoproteins, as reported for the noncognate interaction between CD8 and MHC class I proteins [48–50]. In addition, many membrane receptors are anchored on the cell surface through a galectin-dependent mechanism that is sensitive to α2-6 sialylation. Extracellular galectins form a multimeric lattice-type structure that binds galactose-containing receptor glycans, and stabilizes glycoprotein surface localization [51–54]. Receptor α2-6 sialylation causes release from the galectin lattice, leading to receptor internalization [20]. Conversely, α2-6 sialylation can facilitate the surface retention of other types of receptors, albeit through pathways that are not generally well-defined. Taken together, the literature indicates that sialylation holds potential to influence tumor cell behavior at many different levels including: regulation of individual glycoprotein conformation, clustering or surface retention, modulation of *cis* or *trans* interactions between two distinct surface receptors, and formation of ligands for glycan-binding proteins that correspondingly control cell–cell and cell–matrix interactions. In the remaining sections of this review, several specific examples of tumor-associated sialoglycans are discussed. These were selected because of the greater knowledge of molecular mechanisms linking these modifications to tumor cell behavior as compared with many of the other prevalent changes in tumor glycosylation.

3 Role of integrin sialylation in tumor cell migration through extracellular matrix

Numerous studies suggest that increased cell surface sialylation contributes to metastasis by stimulating tumor cell movement through the extracellular matrix (ECM). *In vitro* assays performed with many different cancer cell lines indicate a strong positive correlation between migration/invasion and high levels of surface sialylation [55–58]. Likewise, subclones of cell lines selected for enhanced invasiveness often display elevated surface sialylation, including clonal variants from lung [45], colon [59], melanoma [60], and T lymphoma [61] cells. The functional importance of hypersialylation is supported by animal models of metastasis [62, 63]. Intrasplenic injections of two differing populations of 51B colon cells; a heavily α2-6 sialylated population and a poorly sialylated population, resulted in hepatic tumors formed almost exclusively by the highly sialylated cells, indicating selective metastasis [63]. Many other studies have shown that metastasis in murine models can be blocked by pharmacologic inhibitors of sialyltransferase activity [64–66] or sialic acid incorporation [62] or alternately, by pre-treatment of tumor cells with sialidases [63]. Interestingly, certain types of sialic acid linkages may regulate metastatic targeting of selected organs; a breast cancer cell line selected for targeting to bone had higher levels of α2-6-linked sialic acid [67], whereas upregulation of ST6GalNAcV, which adds α2-6 sialic acid to gangliosides, directs breast cancer metastasis to brain [68].

Though the circumstantial evidence linking sialylation to metastasis is extensive, data regarding the specific molecular events driving invasive tumor cell behavior are lacking. The prior experimental use of sialylation inhibitors and sialidases, most of which have low specificity, generally produced widespread ablation of cell surface sialylation, affecting a multitude of glycoproteins and glycolipids. Compounding this issue, such generic approaches are not typically representative of the physiologic changes that occur during metastatic progression, which involve alterations in the expression of specific enzymes. More recent studies applying RNAi technology, or forced overexpression models that better recapitulate the tumor phenotype, are beginning to reveal a more defined view of the role of sialylation in metastasis. Several sialylation-related enzymes have been targeted using this strategy, and such studies are yielding

new insight into how distinct types of sialylation regulate specific receptors to promote tumorigenic cell responses.

Some of the more compelling results implicating a specific sialoprotein in tumor cell migration and invasion have been provided by studies of the integrin family of cell adhesion receptors [69]. Integrin activity is involved in many aspects of tumor metastasis including tumor cell detachment from basement membrane, migration through the stromal matrix, anchorage-independent cell survival in the vasculature, adhesion to endothelium during extravasation, and establishment of metastatic foci in novel ECMs. Glycosylation of integrins has long been known to be required for integrin function [69, 70], and integrins are regulated by several different types of glycan structures [71–73]. Among these, α2-6 sialylation of *N*-glycans is an important modulator of a specific subset of integrins. The β1 integrin subunit (but not β3 or β5 [74]) has been identified as a substrate for the ST6Gal-I sialyltransferase in multiple established cancer cell lines [56, 57, 75, 76]. Furthermore, β1 integrins in human colon cancer tissues display elevated α2-6 sialylation [56], which corresponds to the well-documented overexpression of ST6Gal-I in many different cancers, including colon carcinoma [16, 36]. ST6Gal-I is upregulated in cancer as a consequence of signaling by oncogenic Ras [74, 77–79].

The addition of α2-6-linked sialic acid to the β1 integrin subunit alters the binding activity of several β1-containing heterodimers including receptors for fibronectin (α5β1 [43, 80, 81]), VCAM-1 (α4β1 [42]), laminin (α3β1 [82]), and collagen (α1β1 [74] and α2β1 [75]). Regulation of integrin function by α2-6 sialylation has been confirmed by studies using engineered cell lines, as well as ligand binding assays performed with purified integrin receptors that have been manipulated to express varying levels of sialylation. It has also been shown that, of the ten *N*-linked glycans on the β1 integrin subunit [80], the three *N*-glycans within the β1 I-like domain, a region involved in ligand binding, are essential for heterodimerization of the α and β subunits, and also for ligand-induced cell spreading [83]. These recent results confirm studies performed approximately 20 years ago showing that *N*-glycosylation was indispensible for β1 integrin function [84, 85].

Mechanistic studies of sialylation-related β1 integrin activity are few in number, however experiments using activation-state reporter antibodies suggest that sialylation alters β1 integrin conformation [42, 43], a finding supported by molecular modeling approaches [86]. α2-6 sialylation of collagen-selective integrins increases adhesion to collagen I, enhances coupling of talin to the integrin cytosolic tail, and stimulates cell migration (Fig. 4) [56, 57, 75]. *In vivo* support for sialylation-dependent effects on integrin signaling has been provided by Varki's group, who used the polyomavirus middle T antigen model of spontaneous breast cancer to study ST6Gal-I [87]. Results from this study showed that mammary tumors from ST6Gal-I null mice exhibited a selective alteration in genes associated with focal adhesion signaling, as well as diminished activation of Focal Adhesion Kinase (FAK), a known downstream target of integrin signaling. Tumors from mice lacking ST6Gal-I were also more differentiated, suggesting that the overexpression of ST6Gal-I I that occurs in human carcinoma may contribute to a poorly differentiated tumor phenotype.

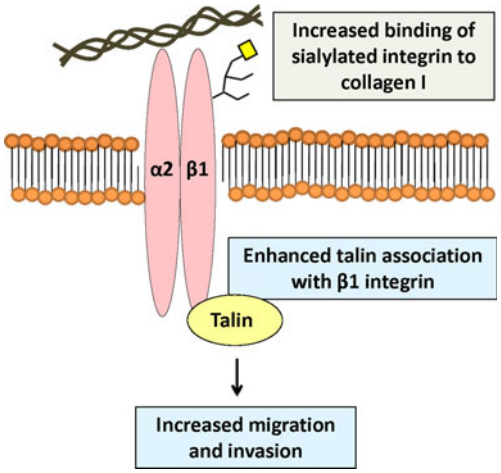

Fig. 4 Regulation of integrins by sialylation. α2-6 sialylation of *N*-linked glycans on the β1 integrin enhances cell adhesion to collagen I and stimulates migration and invasion

ST6Gal-I-directed α2-6 sialylation of the β1 integrin stimulates tumor cell migration and invasion through reconstituted ECM (e.g., Matrigel) [56, 57, 75]. Cells that are null for the β1 integrin do not exhibit differential invasion upon forced ST6Gal-I expression [75], supporting the hypothesis that the effect of upregulated ST6Gal-I is mediated specifically by the β1 integrin. The interaction between integrins and collagen I is thought to be important in metastasis. Microarray studies performed on diverse tumor types identified collagen I as part of a 17-gene signature associated with increased metastasis [88]. The deposition of collagen I in the metastatic microenvironment induces dormant tumor cells to form proliferative metastatic lesions, and this transition is dependent upon β1-integrin signaling [89]. In addition, collagen reorganization at the tumor-stromal interface facilitates local invasion [90] and collagen I fibers provide tracks along which tumor cells migrate during transit to blood vessels [91]. The α2β1 collagen-selective integrin has been suggested as a principal player in metastatic progression; comparative analyses of primary colorectal cancers with corresponding liver and lung metastases suggest that the α2 integrin subunit contributes to liver targeting [92].

Recently a second integrin family member, α6β4 (a laminin-binding receptor), has been reported to be affected by sialylation [25]. In this instance, elevated sialylation of the β4 integrin subunit resulted from decreased expression

of a sialidase rather than increased expression of a sialyltransferase. Downregulation of the Neu1 sialidase, which localizes in part to the plasma membrane [25, 93, 94], was associated with enhanced cell invasiveness and metastatic potential in rat and murine cancer cells [95, 96]. To elucidate the effects of Neu 1 on integrin activity, Neu1 expression was forced in human colon cancer cell lines [25], which led to an accompanying gain in sialylation on the *O*-linked glycans of the β4 subunit. While the effect of sialylation on α6β4 structure has yet to be determined, sialylation clearly influenced α6β4 signaling because the β4 subunit displayed reduced phosphorylation, and α6β4-induced FAK activation was diminished. Importantly, forced Neu1 expression inhibited experimental metastasis to liver [25]. These studies of α6β4, along with β1, point to specific integrin sialoforms (and other glycoforms not discussed herein) as critical mediators of tumor cell migratory and invasive behavior.

4 Sialyl Tn antigen and tumor invasion

The sialyl Thomsen-nouvelle antigen (sialyl Tn) and its unsialylated form, Tn, are well-known tumor-associated carbohydrate antigens, and are highly correlated with cancer invasion and metastasis [97–99]. Sialyl Tn is formed by the sialylation of the Tn antigen: GalNAc linked to serine or threonine (Fig. 5). GalNAc is the first sugar added during *O*-linked glycan synthesis, and this basic unit can be extended to form multiple glycan structures. The sialylation of GalNAc prevents further sugar additions, and effectively truncates the *O*-linked glycan extension [100, 101]. Sialyl Tn is detected in a wide range of cancers including gastric, colorectal, pancreatic, endometrial, breast, and ovarian [102–108], yet sialyl Tn expression is low or absent in normal epithelial cells [109–112]. Sialyl Tn expression is associated with metastatic disease, recurrence, and reduced survival rates in breast cancer [98, 113], and a negative

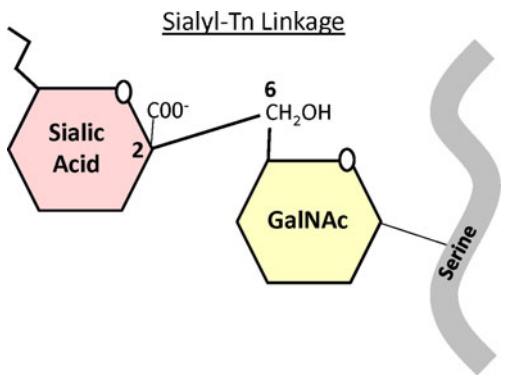

Fig. 5 Sialyl Thomsen-nouvelle antigen. The sialyl Tn antigen is formed by α2-6 sialylation of GalNAc bound to serine or threonine

association between sialyl Tn and survival has been confirmed by many other studies [98, 114–120]; although this is not a universal finding [121, 122]. Approximately 30 % of breast cancers are sialyl Tn positive [123, 124] and over 80 % of all carcinomas express either Tn or sialyl Tn structures [125]. Given that antibodies against sialyl Tn antigen are cancer specific [97, 109], serum sialyl Tn levels are used as a prognostic indicator for cancer aggressiveness and metastatic potential [126]. The relationship between sialyl Tn and cancer progression has been demonstrated experimentally by the forced expression of sialyl Tn structures in cancer cells. Forced sialyl Tn in gastric cancer lines resulted in increased metastasis and decreased survival in nude mice after intraperitoneal injection of tumor cells [127]. This enhanced metastatic capability of sialyl Tn-expressing cells was abrogated by pretreatment with anti-sialyl Tn antibodies. In related studies, various cancer cell lines with forced sialyl Tn expression gained metastatic characteristics such as altered adherence to matrix molecules and increased motility and invasiveness [128–130].

The generation of sialyl Tn is primarily associated with a single sialyltransferase, ST6GalNAc-I, which adds sialic acid in an α2-6 linkage to the Tn antigen [131–133]. Forced expression of ST6GalNAc-I results in sialyl Tn expression in breast and gastric cancer cell lines [127, 129]. Other ST6GalNAc family members may be able to synthesize sialyl Tn; however, while ST6GalNAc-II can create the sialyl Tn structure *in vitro* on peptide-GalNAc substrates [133, 134], to date no other ST6GalNAc has been shown to generate sialyl Tn expression *in vivo*. ST6GalNAc family members have been linked to carcinogenesis: ST6GalNAc-I is upregulated in intestinal metaplasia [132]; ST6GalNAc-II is elevated in colon cancer and is prognostic for patient survival [135], and ST6GalNAc-V is one of four genes upregulated in breast cancer cells with increased metastatic potential to the brain [68]. As an alternate mechanism to ST6GalNAc overexpression, accumulation of sialyl Tn can result from dysregulation of other glycosyltransferases that regulate formation or availability of the Tn substrate. *Cosmc* is a molecular chaperone protein necessary for the activity of T-synthase, an enzyme that adds galactose to GalNAc and therefore competes with ST6GalNAc-I for GalNAc-modified *O*-linked glycan precursors [136, 137]. Cummings' group reported that *Cosmc* disruptions in colon and melanoma cell lines contribute to sialyl Tn expression (due to downregulated T-synthase activity), and further documented two cervical cancer cases with mutations at the *Cosmc* locus, and elevated sialyl Tn expression [125].

Even though there is a strong association between sialyl Tn expression and cancer progression, the specific effects of sialyl Tn on tumor cell behavior remain obscure. Not many studies have been aimed at identifying the carriers of sialyl Tn, or determining the corresponding influence of sialyl Tn

on carrier function. There is also a lack of knowledge regarding physiologically relevant glycan binding proteins that might interact with sialyl Tn in the tumor milieu. However, a few glycoproteins have been determined as carriers of sialyl Tn. CD44 and the mucin, Muc 1, are elaborated with sialyl Tn in breast and gastric cancer cells that have been forced to express ST6GalNAc-I [127, 129]. Mucins are large, densely *O*-glycosylated proteins that act in cell adhesion and signaling [138]. They are upregulated in a variety of cancers and characteristically display truncated *O*-linked glycans as compared with mucins in normal tissue [101, 138, 139]. A general increase in sialylation influences Muc1's role in cell–cell adhesion [140, 141] and elevated levels of Muc 1 with sialyl Tn have been observed in multiple carcinomas [128, 142]. The other sialyl Tn carrier, CD44, is another well-known adhesion protein; this molecule displays a binding specificity for hyaluronan. Certain CD44 splice variants, such as CD44v6, have been associated with breast, lung, colon, and pancreatic carcinomas, among others [143–145]. Two other glycoproteins, β1 integrin [146] and osteopontin [112], are reportedly modified with sialyl Tn in murine cancer cells, although this has not been confirmed in human cells. The β1 integrin was shown to be the primary carrier of sialyl Tn antigen in the non-mucin expressing TS/A murine breast cancer cell line after ST6GalNAc-I forced expression [146]. In this investigation, ST6GalNAc-I expressing cells had reduced mobility and proliferation, suggesting a possible inhibitory effect on the metastatic process. However, in another study which identified Muc1 and CD44 as sialyl Tn carriers in human breast cancer cells, β1 integrin was not found to carry the sialyl Tn antigen [129]. These conflicting reports may be due to differences in mucin expression and competing substrates for ST6GalNAc in the Golgi. Recently, osteopontin was found to carry the sialyl Tn epitope in murine breast cancer cell lines [112]. Osteopontin levels in serum are elevated in a number of human cancers, and increased expression is associated with a negative prognosis [145]. Interestingly, osteopontin serves as a ligand for both CD44 and β1 integrins [145], and it is worth noting that all four proteins identified as sialyl Tn carriers (Muc1, osteopontin, CD44, and β1) are involved in cell adhesion and migration. It is tempting to speculate that the expression of sialyl Tn may subvert the normal function of these proteins to promote an invasive tumor phenotype (Fig. 6).

Sialyl Tn expression may also play an immunologic role in tumor progression. Natural killer cells pre-treated with Muc1 bearing the sialyl Tn antigen, but not Muc1 without sialyl Tn, exhibited diminished capacity for cell-mediated cytotoxicity against K562 leukemia cells [147]. Although mechanistic information is lacking, sialyl Tn structures are capable of binding to siglecs, which are important mediators in immune recognition [148]. The sialyl Tn antigen is the preferred substrate for siglec 6 [149] while some additional siglecs may bind sialyl Tn along with other sialoglycans [148]. It is possible that altered sialyl Tn expression on tumor cells may shift the immune response in ways that promote tumor development through interactions with different siglecs. As well, sialyl Tn-antibody complexes formed from soluble sialyl Tn and antisera stimulated VEGF release from macrophages and granulocytes, leading to increased tumor angiogenesis and invasion [150, 151]. Enhanced blood vessel formation was similarly observed in SCID mice with subcutaneously injected, sialyl Tn expressing breast cancer cells following the introduction of anti-sialyl Tn antibodies into the systemic circulation. It is still unclear whether this effect was separate from the general immune response to tumor antigens or specific for sialyl Tn.

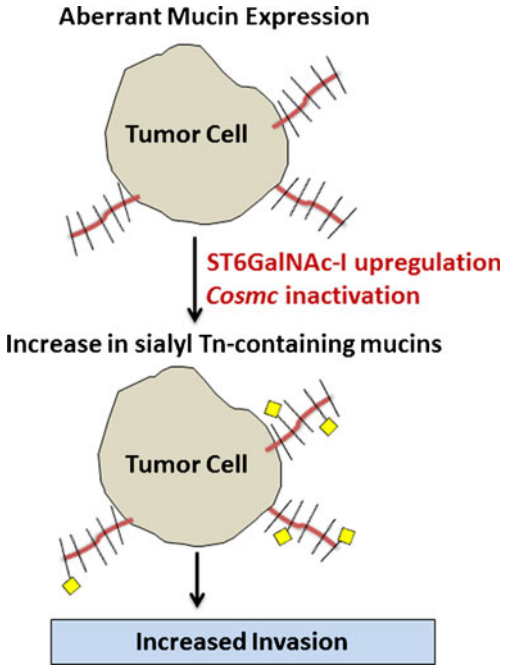

Fig. 6 Sialyl Tn antigen expression correlates to increased invasion. Upregulation of the sialyltransferase, ST6GalNAc-I, or inactivation of the chaperone, *Cosmc*, contributes to an increase in sialyl Tn expression on *O*-linked glycans. Elevated levels of sialyl Tn antigen expressed on tumor cells is correlated to increased invasion; however, the mechanism remains unclear

Theratope® is a cancer vaccine against sialyl Tn conjugated to keyhole limpet hemocyanin, and was initially designed for use in metastatic breast cancer. In a phase II clinical study, patients receiving the vaccine showed a sialyl Tn-specific humoral response and improved overall survival [123]; however, a phase III study concluded that the vaccine did not increase survival in patients with metastatic disease [152]. Independent studies of Theratope in murine models reported detection of sialyl Tn-specific antibodies, and significantly delayed tumor growth [112]. A potential pitfall of

the phase III clinical trial is that the patient population was not evaluated for sialyl Tn expression prior to enrollment, possibly masking any benefit from the vaccine due to heterogeneous sialyl Tn expression between patients. While additional investigation is needed to clarify the discrepant results concerning sialyl Tn, the pursuit of tumor-associated carbohydrate antigens as candidates for vaccine development remains an active area of investigation.

5 Sialyl Lewis structures in tumor dissemination

After entering the systemic circulation, tumor cells must be able to survive within, and then exit, the vasculature in order to metastasize to distant organs. The upregulation of sialyl Lewis (sLe) structures on the tumor cell surface serves as a key mechanism for directing tumor cell adhesion to the endothelium by providing ligands for endothelial selectins [153] (Fig. 7). sLe/selectin interactions also promote the formation of aggregates comprised of tumor cells, platelets and leukocytes, which shields tumor cells from immune attack [154]. sLe glycans are normally present on leukocytes and are critical in leukocyte adhesion and extravasation during an inflammatory response. Conversely, sLe expression is typically low in non-cancerous epithelial cells [19, 155]. sLe structures are tetrasaccharides composed of a GlcNAc-Gal backbone with an α2-3-linked sialic acid attached to Gal, and fucose linked to GlcNAc (Fig. 8). sLe is primarily found on glycolipids or *O*-glycans of glycoproteins [19], and the

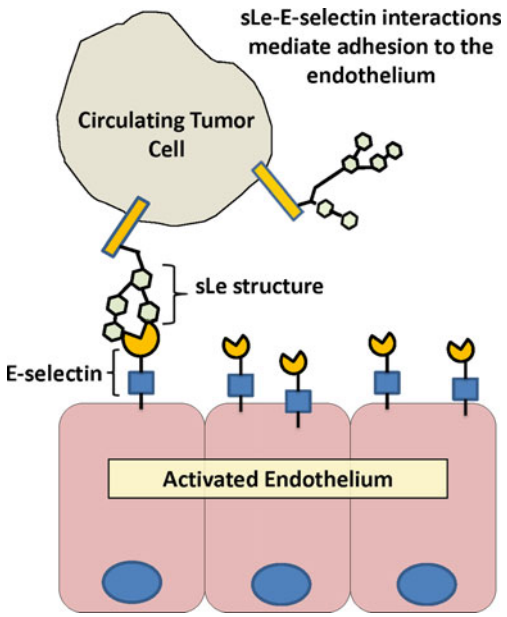

Fig. 7 Sialyl Lewis structures promote tumor dissemination. Sialyl Lewis structures on tumor cell glycoproteins interact with selectins expressed by activated endothelial cells, thereby facilitating tumor cell extravasation

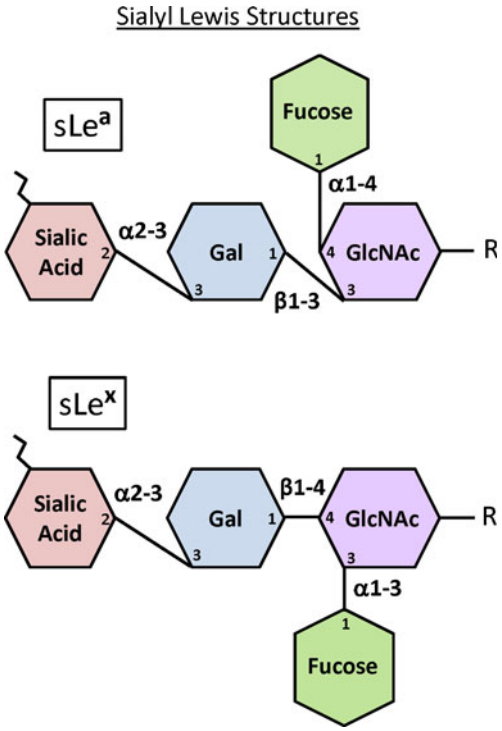

Fig. 8 Sialyl Lewis antigens. Sialyl Lewis (*sLe*) antigens are tetrasaccharide structures composed of a GlcNAc-Gal backbone with fucose linked to GlcNAc and sialic acid α2-3 linked to Gal. sLe^x and sLe^a are different isomers, both of which bind to endothelial selectins

expression of sLe is elevated in many different types of cancer [156–158]. sLe occurs in two isomers, sLe^a and sLe^x (Fig. 8), and cancer cells arising from different organs tend to adhere to endothelium more strongly through one isomer over the other. Colon and pancreatic cancer cells adhere to E-selectin via sLe^a while lung and liver cancer cells adhere through sLe^x [159]. Expression of sLe is increased in patients with metastatic disease and is negatively correlated with patient survival [160–169] although this has been refuted [170]. This association with metastasis is the basis for clinical monitoring of sLe^a structures in cancers of the digestive tract. Screening of serum sLe^a (CA19-9) is part of the standard treatment regimen for colorectal cancer [171–173] and higher preoperative serum CA19-9 levels predict colon cancer recurrence [174]. The CA19-9 antibody is specific for sLe^a and does not recognize the unsialylated Le^a structure [175]. In a cohort of 94 advanced colorectal cancer patients, greater sLe^a expression was positively correlated to hepatic metastasis [176], although this study failed to find a significant association in a similarly sized gastric cancer cohort. A retroactive examination of more than 300 colorectal cancers and their associated metastases found significantly higher sLe^a expression in metastases compared with the primary tumor [171], and a similar finding was reported for breast cancer [162]. In addition to sLe^a,

sLe^x may serve as a prognostic indicator; sLe^x expression predicts outcome in prostate cancer cases after orchiectomy [177] and is also being investigated for use in breast cancer monitoring [178, 179].

A clear clinical correlation continues to prompt investigation of sLe antigens, however the regulation of these structures is far from understood. *O*-linked glycan synthesis requires the coordinated activity of multiple glycosyltransferases, and many of these are altered in carcinogenesis [19, 100]. Glycans are often truncated in cancer cells, due, in part, to incomplete synthesis, contributing to the expression of sLe [100, 173]. Evidence now suggests that the disruption or activation of a single glycosyltransferase may be sufficient to upregulate sLe structures. For instance, epigenetic silencing of ST6GalNAc-VI, as well as experimental reduction of this gene's expression, leads to the accumulation of sLe [173, 180]. ST6GalNAc-VI adds an α2-6-linked sialic acid to GlcNAc, creating the di-sLe (a) structure, which predominates in normal epithelial cells. On the other hand, forced expression of a β1-4 GalNAc transferase reduces sLe expression, and restores a more normal carbohydrate profile [181]. Cells with forced β1-4 GalNAc transferase expression have reduced adhesion to human umbilical cord endothelial cells, and decreased metastasis *in vivo*. Additional factors within the tumor microenvironment likely play a part in sLe expression. Hypoxic conditions stimulate sLe upregulation through HIF-1α signaling [182], while the hormone receptor status of certain cancers appears to influence sLe-E-selectin interactions [183].

Fucosyltransferase activity may also contribute to tumor-associated sLe expression [184–187], although the relative importance of this pathway is debated [173]. Dimitroff's group reported that expression of the Fut-3, Fut-6, or Fut-7 fucosyltransferases in prostate cancer cells was sufficient to stimulate sLe^x production and promote prostate cancer metastasis to bone and liver [185]. Several carriers of sLe structures were identified in this study including CD44, carcinoembryonic antigen, podocalyxin-like protein, and melanoma cell adhesion molecule [185]. Mucins, including Muc1, are also modified with sLe [188, 189]. Antisense strategies directed at Fut-3 reduce sLe expression as well as the number of hepatic metastases observed in mice [190, 191]. sLe structures mediate adhesion to the endothelium through their interactions with selectins on activated endothelial cells [192–195]. The physiologic relevance of tumor cell sLe structures in cancer progression has been confirmed by studies in which sLe/selectin interactions were perturbed. Experimental interventions that blocked sialylation of Lewis structures were effective in inhibiting tumor cell adhesion to both E-selectin-coated plates and endothelial monolayers [196, 197]. Pretreatment of mice with E-selectin peptide agonists decreased the number of metastases in a lung metastasis model [198], and forced liver-specific expression of E-selectin redirected melanoma cell metastasis from the lung to the liver [199]. Finally, E-selectin-deficient SCID mice developed fewer lung metastases in a xenograft colon cancer model [200]. This study also observed a higher number of circulating tumor cells in E-selectin-deficient mice, suggesting metastasis was inhibited at the endothelial binding step of the metastatic cascade.

Despite the established value of sLe as a cancer-associated biomarker, therapeutics targeting these structures have been relatively slow to develop. Cancer vaccines against sLe^a have yielded mixed results [201], even though experimental results support a role for sLe overexpression in stimulating natural killer cell responses [202]. Work by Esko's group demonstrated the utility of disaccharide decoy molecules in reducing overall sialylation and the expression of sLe antigens. Treatment of cells with decoy disaccharides inhibited: sLe^x expression; adhesion to selectin-coated plates; and metastasis to the lung in murine models [203–205], suggesting a possible therapeutic benefit. Additionally, several antibodies against sLe have been shown to be cancer specific and cytotoxic *in vivo*. For example, two monoclonal antibodies developed against sLe^a have demonstrated substantial antitumor effects in an *in vivo* colon cancer model [206]. Although further research is needed, these collective studies highlight the potential for targeting sLe structures in clinical treatment.

6 Tumor cell α2-6 sialylation confers resistance to cell death

Much of the literature regarding tumor cell sialylation has centered on its role in cell adhesion, migration and invasion, but some studies also implicate sialylation in regulating cell death pathways. In particular, α2-6-linked sialic acids may confer an apoptosis-resistant phenotype by modulating the activity of selected receptors and signaling mechanisms. One of the better-characterized functions for α2-6-sialylation is an inhibitory effect on galectin-dependent apoptosis [36]. Many galectins, including gal-1, gal-3, and gal-9, bind to cell surface galactosides and induce cell death [207]. Each galectin exhibits specificity for certain galactosyl structures, and there is evidence that galectins may selectively bind to distinct glycoproteins. The mechanisms underlying galectin selectivity are still under investigation, although some of the documented binding partners for galectins include integrins [38, 208, 209], EGFR [210], CD45 [211], and TRPV5 [20]. Galectins are secreted by immune (and other) cells, therefore α2-6 sialylation on the tumor cell surface may protect tumor cells from the actions of infiltrating immune cells. However, the relationship between galectins and tumor cell α2-6 sialylation is complex.

Many tumor cells overexpress galectins [32], which raises the paradox of why a cancer cell would upregulate an apoptosis-inducing molecule. In fact, galectins have many different tumor-promoting activities; for example, intracellular forms of galectins have anti-apoptotic functions that are independent of cell glycosylation status, and some galectins amplify signaling by the ras oncogene [32, 212]. Thus, tumor cells that coordinately upregulate galectins and α2-6 sialyltransferases would benefit from the pro-tumorigenic activities of intracellular galectins (that are carbohydrate-independent), while simultaneously acquiring resistance to the pro-apoptotic features of secreted galectins (that are carbohydrate-dependent and blocked by α2-6 sialylation).

In tandem with inhibiting galectin-mediated apoptosis, α2-6 sialylation enhances tumor cell survival by regulating the function of individual cell surface receptors. Lee *et al.* reported that treatment of colon tumor cells with ionizing radiation induced increased α2-6 sialylation of the β1 integrin as a secondary consequence of ST6Gal-I upregulation [76, 213]. In this system, α2-6 sialylation of the β1 integrin promoted cell adhesion to fibronectin and contributed to cell survival through the activation of paxillin and AKT [76]. Recently, two members of the TNFR death receptor family, Fas and TNFR1, were also identified as ST6Gal-I substrates, and it was shown that α2-6 sialylation blocked apoptotic signaling by these receptors [47, 214]. Reduced Fas-mediated apoptosis is a well-established factor in tumor cell survival, and Fas expression is downregulated in many different tumor types [215–217]. However, in addition to decreased expression, Fas signaling cascades are disrupted in tumor cells, with Fas activation triggering pro-survival, rather than apoptotic, pathways [218]. These non-apoptotic functions of Fas contribute to tumor-promoting phenotypes [219–222]. Complete knockout of Fas in tumor cell xenografts prevented tumor growth, supporting the hypothesis that Fas is essential for some aspect of tumor cell survival or proliferation [223]. This newer concept is in agreement with many reports that some cancer cells express high levels of Fas, but are yet resistant to Fas-induced apoptosis [224–227].

Several studies have suggested that Fas apoptotic activity is inhibited by sialylation [228–230], although most of these did not define which type of sialic acid linkage is functionally important. In more recent work, forced overexpression or knockdown of ST6Gal-I caused altered α2-6 sialylation of Fas (without affecting α2-3 sialylation) [47]. Elevated α2-6 sialylation of Fas by ST6Gal-I prevented apoptosis stimulated by both Fas-activating antibodies and FasL (the native ligand for Fas). Fas α2-6 sialylation did not interfere with binding of the agonist, but rather inhibited formation of the death-inducing signaling complex, and also restrained Fas receptor internalization (Fig. 9). Intriguingly, there is

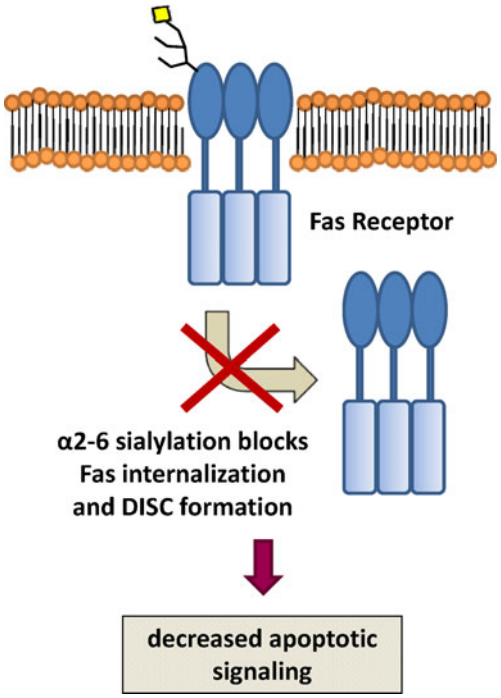

Fig. 9 Inhibition of Fas-mediated apoptosis by α2-6 sialylation. α2-6 sialylation of the Fas receptor blocks apoptosis by preventing receptor internalization and formation of the death-inducing signaling complex (*DISC*)

evidence that plasma membrane-localized Fas receptors may send a pro-survival signal, whereas receptor internalization is important for induction of apoptosis [231]. Hence, the α2-6 sialylation-dependent retention of Fas at the cell surface could serve as a switching mechanism responsible for diverting signaling away from apoptosis and toward survival. It isn't currently known why α2-6 sialylation prevents Fas internalization, but it can be speculated, based on information from other sialylated receptors, that Fas sialylation could regulate: (1) Fas receptor homotrimerization or higher order clustering, (2) tertiary conformation of the receptor, and/or (3) localization of the receptor to lipid raft microdomains.

Similar to Fas, ST6Gal-I-mediated α2-6 sialylation of the TNFR1 death receptor inhibits apoptosis directed by the TNFR1 ligand, TNFα, although at present TNFR1 sialylation has only been evaluated in macrophages [214]. TNFR1 is expressed in epithelial cells, however neither the glycan composition, nor function, of TNFR1 glycans have been characterized in epithelial tumor cells. Nonetheless, the finding that ST6Gal-I-mediated sialylation blocks apoptotic signaling through three major pathways (galectins, TNFR1 and Fas), suggests that upregulated ST6Gal-I may facilitate tumor cell escape from immune surveillance. The ligands for TNFR1 and Fas (TNFα and FasL, respectively) are primarily expressed by immune cells, and immune cells are also a rich source of galectins. Furthermore, as noted

previously, changes in sialylation likely affect tumor cell interactions with siglec-expressing immune cells. These findings underscore the need for elucidating the molecular mechanisms by which distinct tumor-associated sialoglycans or sialoproteins influence the host immune response. The paucity of studies on this topic is noteworthy, particularly given that it has long been believed that surface sialylation shields cancer cells from immune attack. Sialic acids mask antigenic sites on cells, thus weakening immunoreactivity, and sialic acids also protect cells against complement-mediated cell lysis [232]. In addition, loss of sialylation from the cell surface serves as an "eat-me" signal for phagocytes [233] suggesting that high sialylation levels on cancer cells may inhibit phagocytotic targeting by immune cells. These combined observations offer provocative clues that alterations in the profile of tumor cell sialoglycans may be a driving factor in immune escape.

7 Summary

A role for tumor cell sialylation in cancer progression has been presumed for many years, however mechanistic studies of this cell surface modification have been limited when compared with other areas of cancer cell biology. Particularly lacking are studies of: (1) signaling mechanisms that alter transcription or translation of sialylation-related enzymes, (2) sialyltransferase specificity for selected glycoprotein targets, (3) sialylation-dependent changes in glycoprotein structure (e.g., conformation and clustering), and (4) the effects of variant sialylation on the actions of glycan binding proteins. A better understanding of these molecular events is necessary for defining causal relationships between elevated sialylation and metastatic cell behaviors such as invasiveness, hematogenous dissemination, and apoptosis-resistance. The goal of this review was not to comprehensively overview the many reported changes in tumor sialoglycans but rather focus on a select number of examples for which there is substantive information regarding molecular mechanism. The elucidation of sialylation-dependent pathways that control distinct tumor cell responses holds promise for identifying important new diagnostic or prognostic markers, as well as targets for vaccine and drug development.

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