Protein Glycosylation in Cancer

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Abstract

Neoplastic transformation results in a wide variety of cellular alterations that impact the growth, survival, and general behavior of affected tissue. Although genetic alterations underpin the development of neoplastic disease, epigenetic changes can exert an equally significant effect on neoplastic transformation. Among neoplasia-associated epigenetic alterations, changes in cellular glycosylation have recently received attention as a key component of neoplastic progression. Alterations in glycosylation appear to not only directly impact cell growth and survival but also facilitate tumor-induced immunomodulation and eventual metastasis. Many of these changes may support neoplastic progression, and unique alterations in tumor-associated glycosylation may also serve as a distinct feature of cancer cells and therefore provide novel diagnostic and even therapeutic targets.

Keywords

glycoprotein; glycosylation; glycans; oligosaccharides; cancer; transformation; biomarkers; immunohistochemistry

INTRODUCTION

As neoplastic disease continues to be one of the most formidable challenges in modern medicine, accurate diagnosis and treatment of neoplasia remain a fundamental focus of biomedical research. Although early studies recognized that cells can accumulate abundant morphological changes following neoplastic transformation, it was not until the genetic basis for neoplastic disease became apparent that the molecular mechanisms of neoplastic progression began to be known (1). As a variety of mutations appear to drive the progression of neoplasia in different types of tissue, understanding the impact of DNA mutations continues to be a central goal in the study of neoplastic disease (1). Although these studies continue to provide significant insight into the genetic parameters that may govern neoplastic progression, epigenetic alterations may exert an equally powerful effect on the

outcome of neoplastic disease (2). Indeed, some of the earliest studies seeking to understand potential differences in neoplastic and normal cells recognized that a variety of metabolic and other changes commonly occur within neoplastic lesions (3). Although many of these alterations likely reflect downstream consequences of genetic mutations, epigenetic changes in and of themselves may not be readily recognized as the outcome of a single genetic mutation but instead may reflect the integrated consequence of a variety of genetic and nongenetic changes that facilitate the development of neoplastic transformation.

One of the classic examples of epigenetic changes that occur during neoplastic transformation is posttranslational glycosylation (2). Glycosylation reflects the coordinated effort of a complex array of enzymes, organelles, and other factors that are needed to successfully generate carbohydrate-associated posttranslational modifications. These modifications of proteins by covalent addition of organic and inorganic moieties can occur on virtually all known proteins in mammalian cells, and they represent an orchestrated and fascinating mechanism to expand the linear information of DNA into a grand panorama of three-dimensional structures. More specifically, glycosylation represents a unique set of protein modifications that follow enzymatic additions of sugar and may involve the linkage of monosaccharides, or even whole oligosaccharides (glycans), in a preformed fashion to specific amino acids within glycoproteins. At least 9 of the 20 amino acids can be modified by a variety of carbohydrates, ranging from a single monosaccharide to glycan chains containing hundreds of monosaccharides. In mammals the major glycans comprise 10 monosaccharide building blocks—glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose (Fuc), mannose (Man), xylose (Xyl), glucuronic acid (GlcA), iduronic acid (IdoA), and 5-*N*-acetylneuraminic acid (Neu5Ac, or sialic acid)—all derivable from glucose in every cell (Figure 1) (4); thus, glycosylation is comparable to phosphorylation in terms of the range of modified sites (5) and certainly exceeds phosphorylation in terms of the complexity of modifications. Note that glycosylation is different from glycation, the nonenzymatic modification of lysine residues on proteins with glucose or other monosaccharides—a prominent problem in diabetes (6, 7).

Given the complexity of protein glycosylation and its fundamental impact on a diverse range of biological processes (8, 9), it is not surprising that seemingly minor alterations in carbohydrate structure can significantly impact the biology of a cell. As changes in cellular behavior and alterations in protein glycosylation accompany neoplastic transformation (10), understanding the mechanisms and consequences of variations in glycosylation associated with neoplastic disease will provide important insight into neoplastic progression (2). This review focuses on changes in glycoprotein glycans accompanying neoplastic transformation and the implications of these alterations in the progression of neoplastic disease. In addition, we examine potential opportunities to utilize these changes as diagnostic markers and novel therapeutic targets.

GENERAL ASPECTS OF PROTEIN GLYCOSYLATION

Before launching into biosynthetic mechanisms of protein glycosylation, we first briefly consider the history and major advances through the years that have brought protein glycosylation and glycosciences in general into the forefront of modern biomedical research.

The key developments in this area began with the finding by Landsteiner that blood group antigens are distinguishing features of blood cells and could be used to accurately predict the outcome of a blood transfusion (11, 12), followed by the subsequent structural identification of these antigens as carbohydrates (Table 1) (13). Parallel developments in chemistry and biology led to the realization that glycans and glycoconjugates not only serve as key barriers to transfusion and transplantation but also perform fundamental roles in a wide variety of biological processes. Cell surface glycans constitute the major portion of the membrane (glycocalyx) and of secreted molecules from all cells (14). Not only do changes in the expression and structures of glycans accompany development and differentiation, but factors that regulate glycosylation and glycoconjugate metabolism also play a central role in the pathogenesis of a host of lysosomal storage diseases (Tay–Sachs, Fabry, etc.) and many other congenital disorders of glycosylation (15–17). Efforts to understand the role of glycosylation in each of these diseases also uncovered a multitude of glycan-binding proteins (GBPs) with the ability to regulate a wide variety of biological processes, ranging from leukocyte trafficking to innate immunity (18, 19). As a result, a variety of studies within glycoscience demonstrate that carbohydrate-based posttranslational modifications and the GBPs that recognize them can significantly impact fundamental biological processes (19). As neoplastic progression typically reflects alterations not only in neoplastic cell survival and growth but also in cellular migration, metastasis, and host antitumor immunity, it is not surprising that alterations in glycosylation impact a wide spectrum of key biological processes needed for the development and progression of neoplastic disease (Table 1) (2, 4, 10, 20, 21).

Protein glycosylation ultimately results in the modification of many different protein products within a given cell. Indeed, membrane and secreted proteins are nearly all glycosylated, with only rare exceptions of nonglycosylated proteins in the secretome, such as small peptide hormones, insulin, glucagon, and human serum albumin (22–24). The elaboration of complex glycans on glycoproteins and glycolipids is a major function of the Golgi apparatus (Figure 1). However, the initiation of many types of protein glycosylation, such as *N*-glycosylation, *O*-mannosylation, and glycosylphosphatidylinositol (GPI) anchor addition, occurs in the endoplasmic reticulum (ER). Such protein modifications are typically posttranslational but occasionally cotranslational and are irreversible, with one known exception, *O*-GlcNAc, which occurs on proteins in the cytoplasm/nucleus as discussed below. The glycosylation of secreted and membrane glycoproteins requires the specific actions of an assortment of glycosyltransferases; over 200 gene-encoded enzymes are known (4). The molecular mechanisms of protein substrate recognition and modification are very poorly understood, especially in regard to the organization and topology of the Golgi apparatus. The residence time of glycoconjugates in the Golgi apparatus is likely very short, seconds to minutes, yet the efficiency of glycosylation in this tiny gatekeeping organelle is astonishingly high. ER/Golgi transport of glycoproteins and localization of glycosylating enzymes in the secretory pathway are required for normal protein glycosylation, and these processes may be altered, as discussed below, in tumor cells.

A common misconception is that protein glycosylation is relatively random and characterized by high degrees of heterogeneity. In reality, glycosylation is typically site

specific, and specific classes of glycans are found on restricted subsets of glycoproteins (25). Indeed, at any single site on a protein, a relatively limited number of major structures (microheterogeneity) or even a single major structure (homogeneity) is typically present. For example, polysialic acid, linear sequences of Neu5Ac in α2–8 linkage, occurs primarily on NCAM (26) and a few other glycoproteins (27); the polysialic acid on NCAM is restricted to *N*-glycans in specific immunoglobulin domains (28), and that on neuropilin-2 occurs on *O*-glycans within a single cluster of Ser/Thr residues (29). *O*-Mannosylation in mammalian cells is relatively restricted to α-dystroglycan (30), but certainly not exclusively, and extended *O*-mannose-containing glycans on α-dystroglycan can also contain the novel mannose 6-phosphate linker (31) to which repeating units of xylose and glucuronate are added (32), again in a protein-specific manner. The most famous protein-specific glycosylation occurs on lysosomal hydrolases, where high-mannose-type *N*-glycans are modified by a unique pathway involving a protein-specific GlcNAc 1-phosphate transferase that adds GlcNAc 1-phosphate to the C-6 of mannose, and the mannose 6-phosphate signal is subsequently exposed by removal of GlcNAc, all within the Golgi (33).

Thus, the primary and secondary structures of proteins generally define the types of posttranslational glycosylation to which they are subjected. *N*-Glycosylation requires –Asn-Xaa-Ser/Thr– and rarely utilizes –Asn-Xaa-Cys–(where Xaa ≠Pro) (34); *O*-xylosylation generally occurs on Ser within –Ser-Gly-Xaa-Gly–(where Xaa ≠Pro) (35); and *C*-mannosylation (36) requires the C-terminal –Trp-Ser-Xaa-Trp or internal motif –Trp-Ser-Xaa-Trp-Ser–(37). GPI anchor addition within the ER, such as to the cellular prion protein (38)—which, like many other membrane glycoproteins, including acetylcholinesterase, also has *N*-glycans (39)—requires a unique propeptide, a cleavable C-terminal sequence (40). Such GPI anchor sites can now be more readily predicted using the database tool PredGPI (38). By contrast, we know little about the protein sequence or structure dictating initiation for most of the other glycosylation pathways, many of which occur in the Golgi (or in the cytoplasm/nucleus, as for *O*-GlcNAc on many different glycoproteins and *O*-Glc to initiate glycogen). For example, *O*-mannosylation occurs in the ER either co- or post-translationally using dolichol-phosphomannose as the donor and appears to prefer Ser/Thr-rich regions within specific types of structural determinants (41). Addition of *O*-Fuc and *O*-Glc occurs on Ser/Thr residues within specific consensus sequences of epidermal growth factor (EGF) repeats, as in Notch (42) and a few other glycoproteins; these repeats are small, cysteine-rich motifs with six conserved cysteines and three disulfide bonds and are found in many different secreted and cell surface glycoproteins (43, 44). Predictions of *O*-GalNAc addition to specific Ser/Thr residues can be made on the basis of known databases and primary and secondary structures of proteins using the tool NetOGlyc 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/) (24). Recent studies on *O*-GalNAc addition indicate that >85% of all proteins with a signal sequence in the secretory pathway have a least one Ser/Thr site that is modified with *O*-GalNAc-type *O*-glycans (24). Collagen and collagen-like molecules containing hydroxylated Lys (HyK) within the Gly-Xaa-HyK glycosylation motifs (45) may acquire the disaccharide Glcα1-2Galβ1 (46) through the action of two collagen β-galactosyltransferases that specifically recognize HyK residues (47).

A fascinating modification of intracellular cytoplasmic, nuclear, and mitochondrial proteins was discovered in 1984: the enzymatic addition of a single sugar GlcNAc (*O*-β-GlcNAc) donated by UDP-GlcNAc to specific Ser/Thr residues of proteins by the enzyme *O*-GlcNAc transferase (OGT) (48). This modification occurs on hundreds of nuclear and cytoplasmic proteins and represents the only normally reversible glycosylation in animals, as the *O*-β-GlcNAc may be removed by an *O*-GlcNAcase in these compartments and then re-added by the OGT. This modification can be competitive with phosphorylation but can also have its own unique sites. *O*-β-GlcNAc additions are probably at least as common as Ser/Thr phosphorylation and represent a method of regulating protein functions that is independent from phosphorylation (49). A form of OGT occurs in the mitochondria (50), and a number of key proteins in oxidative phosphorylation and the tricarboxylic acid cycle are modified (51). The site specificity and function of the single enzyme OGT are regulated by its association with a large number of accessory proteins that direct its interactions with specific protein sites and substrates. Interestingly, a novel OGT termed EOGT occurs in the lumen of the ER and can add *O*-GlcNAc to specific EGF-like domains (52). Evidence is accumulating that *O*-GlcNAcylation may contribute to altered signaling and gene expression in tumor cells (53). In vertebrates, the only other glycosylation demonstrated in the cytoplasm is glycogen, which contains Glcα1–4(Glcα1–4/6)$_n$Glcα1–Tyr, in which the initiating Tyr residue within glycogen is self-glucosylated (54) and then further modified by glycogen synthase using UDP-Glc as the donor (55). Overall, in addition to the protein substrates themselves, there are many other factors that can regulate glycosylation in cells and contribute to biosynthesis of the cellular glycome and glycoproteome (see sidebar, Some Major Factors That Affect Protein Glycosylation in Tumor Cells).

It should be noted that the addition of glycans can result in significant alterations to the overall three-dimensional structure of a modified protein. Unlike the primary amino acid sequence of a protein, which dictates its tertiary structure and often results in many amino acids being buried within the protein's core, glycan modifications reside on the surface, often extending as large molecular masses away from the attached protein. In this way, heavily glycosylated proteins may be viewed as a tree, with glycan branches extending away from a core protein trunk but often having intramolecular associations with the trunk or other branches. Given the significant role glycans play in the molecular composition of a glycoprotein, alterations in glycosylation can significantly impact overall glycoprotein charge and conformation and therefore readily alter its biological activity (4, 17). Furthermore, as each branch may be modified by a particular glycosyltransferase and a given glycosyltransferase may be responsible for impacting the glycosylation of many different glycoproteins, relatively simple changes in one glycosyltransferase can impact the biology of many different proteins in meaningful ways, thus enabling rather subtle genetic changes to induce highly pleiotropic effects on cancer cell survival and overall progression (2, 4).

TYPES OF CHANGES IN PROTEIN GLYCOSYLATION IN CANCER

There have been many excellent recent reviews on the overall nature of changes in glycosylation and glycan-mediated processes in tumor cell biology as well as other disease processes (2, 17, 56–60). Here we focus on protein glycosylation as a posttranslational

modification involved in cancer biology, both as a biomarker and as a contributor to the development and progression of cancer. The major types of changes in protein glycosylation associated with cellular transformation include changes in *O*-glycans (GalNAc-Ser/Thr) and *N*-glycans (Figure 2), which may occur both early and late in cancer progression and metastasis (Figure 3). These changes in glycosylation can be characterized by specific changes in *O*- and *N*-glycan core structures. In addition, alterations in glycosyltransferase expression, defined as production of transcripts and enzyme activity, may not only significantly impact the generation of different core glycans but also govern the degree of core glycan branching (61, 62), which in turn can alter overall glycan structure and function.

In addition to alterations in core glycans, each of these carbohydrates can be further modified to generate unique terminal glycan motifs that may also undergo specific changes following neoplastic transformation. For example, highly fucosylated glycans, such as Lewis antigens [Lewis$^{a/b}$ (Le$^{a/b}$) and Lewis$^{x/y}$ (Le$^{x/y}$)], can become enriched on the cell surface following neoplastic transformation (63–65). Similarly, sialylation, a common terminal glycan modification, can also undergo significant changes during neoplastic progression (66). Similar changes in the expression of long polymers of lactosamine (polyLacNAc) can also become enriched on neoplastic cells (Figure 4) (67). In each of these situations, the types of cell surface glycans present on a given glycoprotein are dictated in part by the expression, localization, and activity of the glycosyltransferases in a given cell. As a result, slight alterations in glycosyltransferase expression or function can significantly impact the types of glycan modifications in a variety of ways, each with the potential to impact the biological activity of a cell (4). With modern methods and expansions in our understanding of the vast repertoire of glycan classes in cells, changes in other types of protein glycosylation are increasingly observed.

DETECTION OF CHANGES IN PROTEIN GLYCOSYLATION: CHALLENGES AND FUTURE DIRECTIONS

In an effort to understand the impact of changes in glycosylation following neoplastic progression, numerous studies have sought to characterize and catalog the types of alterations in carbohydrate modifications that occur following neoplastic transformation. Some of the earliest histochemical stains were used to highlight distinct molecular features of cancer-targeted glycosylation (68). These early studies strongly suggested that tissue transformation into neoplastic lesions resulted in significant changes in glycosylation (69), but the potential impact of these changes and the specific nature of the alterations within neoplastic lesions remained unknown. Indeed, it was not until the advent of monoclonal antibodies that more definitive studies could be done to describe the expression and localization of any antigenic determinant, including glycans (69, 70). As a result, it is not surprising that the vast majority of studies that have sought to examine protein glycosylation in neoplastic lesions have employed monoclonal antibodies, followed by less specific polyclonal antibodies and even GBPs such as plant lectins. These studies have provided significant insight into changes that may occur following neoplastic transformation and suggest that alterations in glycan epitopes nearly always accompany neoplastic transformation.

Although antibody-based approaches to examining posttranslational modifications continue to provide important insight into changes in glycosylation following neoplastic transformation, glycans pose unique challenges. As most epitopes recognized by monoclonal antibodies reflect amino acid sequences, these distinct epitopes can often be mapped out with reasonable certainty, allowing the specificity of a given antibody to be accurately determined. In contrast, as glycosylation often reflects posttranslational modifications of many different proteins, defining the exact carbohydrate epitope recognized by a particular antibody can prove difficult (14). As a result, although many studies continue to attempt to describe changes in glycosylation following neoplastic transformation, using these data to define the expression of an individual epitope within tissue sections using traditional immunohistochemical approaches can be challenging.

Although genomic approaches can lend themselves to detection of changes in neoplastic lesions and could be considered when examining alterations in glycosyltransferase expression, the end product of glycosylation reflects the coordinated effort of many different enzymes, whose expression, localization, and posttranslational modifications can significantly impact enzyme activity and therefore affect the final glycosylation product on the cell surface (14). As a result, very specific and defined reagents that directly interact with the glycan product itself are needed in order to use potential changes in glycosylation as an effective diagnostic, prognostic, and even therapeutic target in routine clinical practice. In an effort to overcome challenges associated with using inadequately defined monoclonal antibodies or other reagents to define the expression and localization of specific glycan epitopes, several investigators developed novel tools that completely revolutionized the process of defining the carbohydrate specificity of a particular detection reagent (71). These tools comprise a series of glycan microarrays, characterized by the use of a variety of highly defined glycans obtained from chemoenzymatic synthesis, natural sources, or a combination of both, to generate robust and diverse libraries of the carbohydrate determinants that are needed to accurately define the binding specificity of a carbohydrate-reactive testing agent (72–77). Although most of the anti-carbohydrate antibodies used previously to examine tissue expression of distinct carbohydrate epitopes have not been examined on glycan microarrays, these types of studies are ongoing. Such studies will likely provide significant insight into the glycan epitopes that a particular anti-carbohydrate reagent recognizes and thus will significantly aid in the interpretation of past and future studies seeking to explore glycophenotypes of neoplastic lesions using traditional immunohistochemical approaches.

In addition to the advent of novel tools to define the specificity of previously developed anti-carbohydrate detecting agents, unique approaches to generating highly specific anti-carbohydrate antibodies may also significantly aid in the development of the repertoire of highly specific yet diverse reagents—capable of recognizing a variety of glycan epitopes—that is needed to accurately define potential changes in glycosylation within a neoplastic lesion. As some anti-carbohydrate antibodies are IgM (78), such reagents tend to be low affinity, which directly impacts their ability to specifically engage a particular glycan epitope. Furthermore, as the mammals that often serve as the vehicle to develop monoclonal reagents share common features of human glycosylation, immunological tolerance to glycan epitopes may further limit the immune responses needed to develop reagents with high affinity and specificity toward carbohydrate antigens. Recent studies suggest that jawless

vertebrates, such as lampreys, can make highly specific antibodies through a completely different recombination program secondary to the unique evolution of their adaptive immune system. Given the evolutionary distance between humans and jawless vertebrates (79), recent studies have attempted to make highly specific reagents against carbohydrate antigens in these model systems (80). These studies demonstrated that very specific antibodies against blood group antigens could be developed (81), strongly suggesting that a similar approach may be used to generate additional anti-carbohydrate antibodies relevant to examining tumor-associated changes in glycosylation.

It should be noted that although the use of antibodies or similar reagents provides a practical approach that could be incorporated into the workflow of many immunohistochemistry laboratories, the accessibility of individual carbohydrate epitopes can be heavily influenced by the glycoprotein to which they are attached. Similarly, other unique structural features within the glycocalyx and beyond might sterically impede interactions. Thus, lack of detection of a particular glycan epitope should be interpreted with caution, even when standard approaches, such as positive controls, are used in parallel. Mass spectrometry may overcome these and other limitations. Indeed, technological breakthroughs in mass spectrometric analysis of intact tissue for specific glycan epitopes will likely provide a more objective approach to examining potential changes in neoplastic glycosylation (82). Similar to genomic approaches, successful mass spectrometric analysis will likely require the neoplastic cells within the analyzed sample to display a sufficient degree of alteration in glycosylation, as mixtures of commonly occurring glycosylation patterns associated with normal cells in the sample may obfuscate real tumor-associated signals. Thus, although the examination of changes in glycosylation in cancer lesions poses unique challenges, recent developments in glycomics offer promising solutions and may reveal specific associations between altered glycosylation and neoplastic progression.

Despite current limitations in the accurate detection of specific tumor-associated alterations in glycosylation, early studies using partially defined reagents provide some intriguing suggestions about the expression and consequences of a variety glycan epitopes within neoplastic lesions. For example, several studies using reagents thought to be specific to Tn and STn antigens indicated that these antigens appear to be preferentially expressed in poorly differentiated adenocarcinoma of the colon (83), hinting at a potential role for these antigens in the pathophysiology of colon cancer. Additional studies suggested a similar association in individuals with gastric cancer: Increased expression of STn appears to be associated with a significantly poorer prognosis (84, 85). A similarly reduced prognosis was reported in patients with increased T antigen expression in bladder cancer (86). Changes in terminal glycan epitopes also appear to convey poor prognosis in individuals with neoplastic disease. For example, increased expression of the glycan epitope sialyl Lewis[a] antigen (SLe[a]) appears to correlate with increased metastatic disease and poorer overall survival in patients with colon cancer (Figure 4) (87). Similar results were obtained from analysis of a related glycan antigen, SLe[x], in colorectal, prostate, and breast cancer and a combination of SLe[a], SLe[x], and Le[y] antigens in patients with non–small cell lung cancer (88–91). Although these results may simply reflect correlative changes, studies examining the biological consequences of these antigens strongly suggest that these altered glycoforms directly impact the metastatic potential of neoplastic disease (92–98).

Whereas several glycan signatures appear to convey a poorer prognosis, the expression of some other glycan epitopes may be associated with a more favorable outcome. In blood group–positive individuals, for example, retention rather than loss of blood group A expression on non–small cell lung cancer correlates with a fairer prognosis (99). Similar studies demonstrated that bladder or oral cancer patients who lost blood group antigen expression exhibited poorer survival (100, 101). Although blood group antigen expression itself may reduce cellular proliferation and metastasis, the truncated and/or altered glycosylation resulting from the loss of blood group antigen expression, such as the development of STn or Tn antigens (84, 85), may actually be responsible, at least in part, for the altered biology observed in these individuals (102).

SERUM GLYCOPROTEIN BIOMARKERS FOR HUMAN CANCER

Many currently employed antibodies that recognize commonly occurring alterations in neoplastic cell antigens actually target glycoproteins or directly recognize altered tumor-associated carbohydrate antigens. Importantly, these antigenic targets were often identified after isolation of monoclonal antibodies developed following exposure of animals to neoplastic cells, reinforcing the unique nature of some of these carbohydrate alterations. Several of these glycoproteins and glycan-related biomarkers (glycobiomarkers) are currently being used for screening, diagnosis, and/or management of human cancer (Table 2; see 103 for a recent review). A few of the more commonly employed antigens are outlined below.

Cancer antigen (CA) 19-9 corresponds to a carbohydrate structure initially found in glycolipids and now known to also occur on glycoproteins, such as mucins, that contain the SLe^a antigen (104). It is expressed primarily in pancreatic and biliary tract cancers but may also be present in patients with other malignancies, such as ovarian cancer and pancreatitis. CA 19-9 was first characterized by a monoclonal antibody, 1116-NS19-9, generated by immunizing mice with a human colorectal cancer cell line (105). Its sensitivity and specificity as a serum biomarker for pancreatic cancer are 79–81% and 82–90%, respectively (106).

The CA 125 antigen was discovered in 1981 with the mouse monoclonal antibody OC125.1, generated by immunizing mice with an ovarian cancer cell line (107). The CA 125 antigen is on MUC16 (108), which is a highly *N*- and *O*-glycosylated type I membrane glycoprotein with a long extracellular domain. The extracellular domain of MUC16 is prominently detected in serum, yet the molecular mechanism(s) of its release from the cell surface is unknown. Elevated serum CA 125 antigen is often used as a diagnostic biomarker for ∼85% of patients with ovarian cancer; however, this marker exhibits a high false-positive rate because many different conditions can cause an increase in CA 125, including other cancers, such as endometrial and peritoneal, in addition to other non-neoplastic conditions, including uterine fibroids, endometriosis, pelvic inflammatory disease, cirrhosis, and pregnancy (109).

Carcinoembryonic antigen (CEA) is a group of GPI-anchored glycoproteins involved in cell adhesion (110). CEA is normally produced in intestinal tissue during fetal development, and its levels drop significantly just before birth. Therefore, it is normally present at very low

levels in the blood of healthy adults. In colorectal carcinoma, CEA expression resumes its high levels and CEA levels in the blood are consequently elevated. CEA measurement is mainly used as a tumor marker to stage malignancy, monitor colorectal carcinoma treatment, and identify recurrences after surgical resection. Serial CEA measurements can detect recurrent colorectal cancer with a sensitivity of ∼80% and a specificity of ∼70% (111). Notably, CEA levels may also be raised in other carcinomas, including gastric, pancreatic, lung, breast, and medullary, as well as in some non-neoplastic conditions.

Prostate-specific antigen (PSA) is a glycoprotein of the kallikrein protease family (specifically, human kallikrein 3) primarily produced by the prostate. Its level in the serum of healthy men is very low; however, it increases in association with different pathological states of the prostate, such as benign tumor, prostatitis, and prostate cancer. PSA has been employed extensively for prostate cancer screening and is one of the most widely used tumor markers (112). However, not all prostate tumors cause increased levels of serum PSA. Indeed, studies using PSA as a biomarker for prostate cancer have shown dramatic variation in its specificity and sensitivity (113). However, recent studies have indicated that altered PSA glycosylation patterns may provide an additional discriminatory marker and thus provide a more specific method to differentiate significant and insignificant increases in PSA levels when screening for prostate cancer (114).

α-Fetoprotein (AFP) was one of the first so-called oncofetal antigens to be described (115). Normally secreted by fetal liver and present in fetal serum, AFP is associated with hepatocellular carcinoma and nonseminomatous germ cell tumors when present in adult serum. Similar to PSA, unique glycoforms of AFP, such as forms with altered sialylation, may be used to distinguish elevated AFP levels associated with hepatocarcinoma from the general increases in serum AFP that can result from other benign liver diseases. The core-fucosylated form of AFP was reported to be more specific to hepatocellular carcinoma (116), but another study showed that high levels of AFP and fucosylated AFP (AFP-L3) could be found in patients with pancreatic acinar cell carcinoma (117).

HER2 (also known as ErbB2 or HER2/neu) is a member of the EGF receptor (EGFR) family and is a tyrosine kinase. HER2 is an *N*-glycosylated glycoprotein (118) that is overexpressed in several malignancies, especially breast cancer. It is the therapeutic target of the monoclonal antibody trastuzumab as well as a series of inhibitors designed to target its tyrosine kinase activity (119).

Human chorionic gonadotropin (hCG) belongs to a group of glycoprotein hormones that includes luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. All of these hormones are composed of two subunits: an α subunit common to all three and a β subunit specific to each. hCG is normally produced by the placenta, and serum levels of hCG are commonly used to monitor pregnancy and pregnancy disorders. Recent studies have shown that the synthesis of hCG is a characteristic feature of a wide variety of malignant and nonmalignant tumors. High levels of hCG are associated with trophoblastic disease and nonseminomatous germ tumors. Elevated levels of hyperglycosylated hCG that contains more complex glycan structures appear to be more specific to patients with malignancies (120).

CA 15-3 and CA 27–29 are different epitopes on the same protein, mucin 1 (MUC1) (121, 122). Upregulated MUC1 expression is associated with breast cancer, although the CA 27–29 epitope appears to have enhanced sensitivity and specificity when compared with CA 15-3. For example, CA 27–29 is elevated in 30% of patients with low-stage disease and in 60–70% of patients with advanced-stage breast cancer. However, when combined with CEA, CA 15-3 raises the specificity for breast cancer up to 95%. MUC1 may also be elevated in patients with other tumors and diseases.

POTENTIAL GLYCOBIOMARKERS FOR HUMAN CANCER

Many different glycoproteins and tumor-associated carbohydrate antigens continue to be used in the clinical management of patients with neoplastic disease, and several recent studies using highly defined carbohydrate recognition reagents suggest that additional glycan markers may provide highly specific tags for recognizing and potentially targeting neoplastic lesions. For example, recent results suggest that the Tn and STn carbohydrate antigens may be the most commonly altered *O*-glycansongly on glycoproteins (Figure 2),such as mucins, within neoplastic lesions.Tnand/or STn are highly expressed by many types of tumors, such as colon, breast, pancreatic, lung, cervix, and ovarian. Studies suggest their expression is associated with tumor progression and metastasis. Therefore, Tn and STn may serve not only as a prognostic marker but also as a therapeutic target, as recently outlined in several reviews (e.g., 123, 124). Furthermore, combination of Tn or STn with other glycobiomarkers, especially mucin biomarkers, may improve the diagnostic specificity of cancer.

Human tumor-associated glycoprotein 72 (TAG-72) is a carcinoma mucin expressed in colon, breast, pancreatic, ovarian, lung, and gastric cancers. Epitopes of TAG-72 recognized by the monoclonal antibodies B72.3 and CC49 are the STn and sialyl T [ST; Galβ1–3(NeuAcα2–6)GalNAcα1–Ser/Thr] antigens, respectively (125, 126). A humanized version of CC49 has been generated and is under clinical trial for use in radioimmunoguided surgery (127). Because STn antigen recognized by B72.3 is expressed strictly on tumors, whereas T and ST antigens are normal *O*-glycans seen in hematopoietic cells, STn would be predicted to be a more attractive target for human cancer. Efforts have been made to make STn-KLH and other forms of STn antigens as vaccine immunotherapeutics for breast cancer.

GPI-anchored glycoproteins contain a GPI anchor rather than a transmembrane domain. The GPI anchor is a glycan and lipid posttranslational modification added to proteins in the ER (Figure 1). GPI-anchored glycoproteins were recently found to be elevated in the plasma of patients with many types of cancers, including breast, ovarian, kidney, liver, lung, colon, and brain (128, 129). Its potential diagnostic and screening value is under evaluation.

N-Glycolylneuraminic acid (Neu5Gc)-containing glycans have recently been identified in human tissues. Human cells normally can synthesize Neu5Ac but not Neu5Gc due to an evolutionary inactivation the CMP-Neu5Ac hydroxylase that converts CMP-Neu5Ac to CMP-Neu5Gc in lower species such as bovines, chickens, and rodents (130). Human carcinomas can metabolically incorporate and present the dietary nonhuman sialic acid, Neu5Gc, which differs from the human sialic acid Neu5Ac by one oxygen atom. Normally,

there are low levels of anti-Neu5Gc antibodies in circulation. In the presence of carcinomas, however, these antibody levels are elevated. As a result, increases in polyclonal anti-Neu5Gc antibodies may also serve as a potential cancer biomarker (131).

It is worth noting that many of these glycobiomarkers were established by generating monoclonal antibodies through immunization of mice with tumor cells. Many of the tumor-specific antibodies developed using this approach were later discovered to recognize glycan or glycoprotein epitopes. As a result, this strategy—combined with the use of jawless vertebrates as hosts for antibody production and glycan microarrays to define specificity—may continue to prove useful in the discovery of additional carbohydrate-based biomarkers for diagnosing and potentially treating neoplastic disease.

GENETIC AND BIOCHEMICAL MECHANISMS FOR CHANGES IN PROTEIN GLYCOSYLATION

Protein glycosylation is regulated by complex mechanisms, including the expression and localization of glycosyltransferases and the ratio of glycosyltransferase activity to donor substrate availability. In transformed cells, altered glycosylation often results from altered expression, altered activity, or mislocalization of glycosyltransferases and related proteins, such as chaperones, that regulate their activity.

Genetic Mutations

Tumor cells carry many gene mutations, yet documented mutations of genes encoding glycosyltransferases are relatively uncommon in tumor cells. However, there are a few exceptions. Expression of Tn and STn antigens on *O*-glycoproteins and mucins is associated with pathological situations such as Tn syndrome and human tumors such as cervical cancer (132). Studies have shown that mutation or deletion of the X-linked *Cosmc* gene is one of the major mechanisms by which the key enzyme T-synthase is inactivated; without the assistance of the functional chaperone Cosmc, T-synthase is misfolded, leaving it unable to modify the Tn antigen on glycoproteins (123). Additionally, in colon cancer, a mutation in *ppGalNAcT12* was identified (133), but its consequences remain elusive.

Misregulated Expression of Glycosyltransferase and Chaperone Genes

Like that of other genes, the expression of genes encoding glycosyltransferases is regulated through both transcription factors and epigenetic mechanisms. As a result, each tissue or cell type has a unique set of glycosyltransferases that generate specific types of glycan structures on their mucins and other glycoproteins, both secreted and membrane bound. In transformed cells, the expression of glycosyltransferases is often misregulated. In normal mammary gland, for example, GnT-V is expressed either not at all or at very low levels (Figure 4). However, in breast cancer, it is upregulated by the transcription factor Ets through the HER2 pathway, resulting in highly branched *N*-glycan structures on tumor cells (134). Higher expression levels of FuT8 in tumor cells compared with the surrounding normal hepatocytes results in elevated core-fucosylated AFP, which can thus serve as a relatively specific biomarker for hepatocellular carcinoma (135). Examples of such upregulated expression also include enzymes responsible for the synthesis of Le^x, SLe^x, Le^a, and SLe^a antigens in

many types of tumors (63–65). Upregulation of β3GnT8 results in increased levels of polylactosamine structures in colorectal carcinoma (67), and similar alterations in GnT-III increase bisected *N*-glycans in liver cancer (61, 62).

Polypeptide GalNAc transferases (ppGalNAcTs) are the enzymes responsible for the initiation of mucin-type *O*-glycosylation. Overexpression of the ppGalNAcT gene *GALNT6* was detected in breast cancer and may contribute to mammary carcinogenesis through aberrant glycosylation and stabilization of MUC1 (136). Its expression has also been observed in gastric cancer and is associated with the presence of venous invasion (137). In addition, ppGalNAcT-14 is overexpressed in colorectal carcinoma and pancreatic cancer and is associated with altered sensitivity to TRAIL-induced apoptosis through modulation of the *O*-glycosylation of death receptors on these tumor cells (138). *ST6GalNAc-I*, whose product is primarily responsible for the sialylation of the Tn antigen to form the STn antigen on *O*-glycoproteins and mucins, is not normally expressed in any human tissues. In many types of human tumors, however, the STn antigen is detected at high levels, presumably due to the upregulation of *ST6GalNAc-I* in tumor cells, such as in breast cancer (139). However, how these glycosyltransferases are upregulated in tumors is not fully understood.

Expression of the Tn antigen in human metastatic pancreatic cancers has been associated with epigenetic silencing of the *Cosmc* gene by hypermethylation, as determined by exome sequencing of many glycosyltransferase genes and the *Cosmc* gene in primary and metastatic specimens (140). Furthermore, directed deletion of *Cosmc* in cell lines induces oncogenic features including altered cell growth and invasion (140). This is also interesting in light of prior studies showing that hypermethylation of the *Cosmc* gene occurs in Tn4 cells, an immortalized B cell line from a male patient with a Tn-syndrome-like phenotype (141). In that case, hypermethylation is associated with expression of Tn antigen and loss of T-synthase in a reversible fashion as treatment of cells with 5-*aza*-2′-deoxycytidine, which reverses methylation, causes restoration of Cosmc transcripts.

Conversely, overexpression of Cosmc and/or T-synthase and expression of the T antigen has been associated with malignant behavior of cells (142–144). The T-synthase (C1GALT1) has been reported to be overexpressed in hepatocellular carcinoma and expression is associated with advanced disease and survival (145). Mechanistically, it was demonstrated that overexpression altered β_1 integrin, leading to changes in signaling and adhesion. Interestingly, overexpression of T-synthase led to enhanced metastasis of hepatocellular carcinoma in NOD/SCID mice, whereas knockdown of T-synthase led to decreased metastasis. Although it is not clear how or whether overexpression of Cosmc and/or T-synthase actually leads to general enhanced T antigen expression and altered protein glycosylation in many types of tumors, such studies suggest that altered expression of these proteins may somehow be associated with malignant phenotypes.

ABO blood group glycosyltransferases are the enzymes responsible for the synthesis of ABO blood groups, mainly on erythrocytes but also on squamous and gastrointestinal tract epithelial cells (146). However, they are silenced in some oral squamous cell carcinomas due to hypermethylation (147). Deletion or reduction of A and B epitopes in other solid tumors, such as gastric and bladder carcinoma, has also been observed, yet the mechanisms

for altered expression of A and B glycosyltransferases are not fully understood (146). There are also examples of glycosyltransferase expression that is decreased or suppressed in tumor cells compared with their normal counterparts, including loss of expression of *C3GnT* (148) and low expression of *β4GalNAcT2* (149) in colorectal carcinoma and decreased expression of *αGnT* in gastric carcinomas (150). However, the mechanisms by which the expression of these genes is decreased also remain unclear.

Mislocalization of Glycosyltransferases

Glycan structures are built in a sequential fashion by a set of glycosyltransferases localized in the ER and Golgi apparatus (Figures 1 and 4). Even within the Golgi apparatus, glycosyltransferases are not evenly distributed, but rather specifically reside in the *cis*, medial, and *trans* Golgi cisternae and the *trans* Golgi network through complicated and not fully characterized mechanisms (151). Correct localization of glycosyltransferases also relies on the integrity of Golgi structures. Furthermore, the structures of the Golgi are dynamic rather than in a steady state. Therefore, it is easy to imagine that altered glycan structures may arise from the mislocalization of glycosyltransferases and altered Golgi architecture. For example, highly active Src kinase can relocate the normally *cis*-Golgi enzyme ppGalNAcT2 back to the ER through a COP-I-dependent pathway, resulting in the expression of Tn antigen on glycoproteins in the ER, which in turn probably alters sites of *O*-glycosylation (152). The Golgi structure is often altered and its pH often increased in tumor cells, which may also contribute to the mislocalization of glycosyltransferases and/or their enzyme activities in tumor cells.

In summary, the detailed mechanisms that produce all the altered glycan structures in tumor cells await full investigation. Much more work needs to be done to elucidate the mechanisms responsible for regulating the expression and localization of glycosyltransferases and to forge a comprehensive understanding of the detailed dynamics of Golgi structures.

BIOLOGICAL ROLES OF PROTEIN GLYCOSYLATION IN NORMAL AND CANCER CELLS

Given their location on numerous cell surface glycoproteins, glycans can impact a wide variety of cellular functions, ranging from glycoprotein trafficking to cellular signaling. As a result, alterations in glycosylation can significantly impact the localization and stability of cell surface receptors and their sensitivity to a broad range of signaling molecules, with obvious implications in cellular division, differentiation, and localization within tissue. Thus, not only do alterations in glycosylation appear to correlate with changes in neoplastic cell behavior, with implications for an individual patient's prognosis, but these changes likely reflect fundamental alterations in the biology of the neoplastic cell that may be critical in the spread of disease.

Although cell surface receptors signal through a variety of mechanisms following ligand engagement, ligand-induced oligomerization of cell surface receptors reflects a common theme among receptors involved in regulating cell growth (153). Recent studies demonstrate

that alterations in glycosylation may significantly impact the intrinsic ability of cell surface receptors to undergo appropriate oligomerization, thereby directly influencing the sensitivity of these receptor systems to stimulation. For example, inhibition of complex *O*-glycan formation, a common feature of neoplastic cells, results in impaired sensitivity of DR4 and DR5, apoptosis-inducing receptors that relay signaling by TRAIL (138). As TRAIL normally induces death in neoplastic cells through engagement of DR4 and DR5 (154), cancer-associated alterations in *O*-glycosylation of these receptors likely reduces the cells' sensitivity to this antineoplastic pathway in vivo, thereby conferring a selective advantage with regard to the TRAIL surveillance program (154). Additional complex *O*-glycan formation, including core 2 *O*-glycan generation, appears to directly correlate with cancer invasion (155). Similarly, expression of STn antigen appears to inhibit cell adhesion, likewise increasing cell spread and the potential for metastasis (156).

Just as alterations in complex *O*-glycosylation appear to influence a variety of signaling pathways that may be directly or indirectly related to cancer (138, 157), alterations in *N*-glycans also likely impact the survival and progression of neoplastic cells. For example, increased expression of GnT-V, which can be driven by *ras* oncogenes and generates a β1–6 GlcNAc *N*-glycan branch commonly found in tetraantennary *N*-glycans (158, 159), results in impaired epithelial contact inhibition and significantly increased cellular motility, key features of cells undergoing neoplastic transformation (Figure 4) (160). Similarly, increased expression of GnT-V-dependent *N*-glycan modifications appears to enhance the invasiveness of glioma, colon cancer, and gastric cancer cell lines (161, 162), possibly through loss of inhibition of collagenase activity, which in turn enhances the ability of cells to percolate through normal extracellular matrix barriers (162, 163). In contrast, increased expression of GnT-V may actually enhance cellular sensitivity to apoptosis through unknown mechanisms (164), once again demonstrating the pleiotropic and occasionally opposing activities of altered glycosylation on neoplastic cell survival. Similarly, overexpression of GnT-III, which adds a β1–4 bisecting branch to *N*-glycans, appears to inhibit EGFR sensitivity to EGF (Figure 4) (165), thereby reducing cellular sensitivity to the proliferative effects of EGF on sustained cell growth. Additional studies demonstrate that critical *N*-glycans serve to inhibit autodimerization and therefore autonomous activation of other growth receptors, such as ErbB3 (166), providing another pivotal checkpoint whereby *N*-glycans may regulate key cellular processes involved in cell proliferation and potential progression to neoplastic transformation.

In addition to alterations in core *O*- or *N*-glycans, changes in terminal glycan structures may likewise induce changes in cellular behavior that may enhance the growth and spread of neoplastic disease. Whereas decreases in sialylation may enhance integrin-mediated cellular adhesion (167), increases in sialylation appear to inhibit integrin interactions with extracellular constituents, such as fibronectin (168, 169), thereby potentially facilitating cancer spread and eventual metastasis. In contrast, enhancing the sialylation and fucosylation of *N*-glycans on the EGFR of various lung cancer cell lines appears to inhibit EGFR dimerization (170), thus inhibiting this important process in the continued survival and progression of some types of lung cancer (171). Consistent with this, transfection of cells with the sialidase gene decreases EGFR sialylation and activity in vitro (172).

Furthermore, altered expression of α2–6 sialylation appears to enhance growth of various glioblastoma cell lines through a glycoprotein-independent mechanism (173), suggesting that alterations in the sialylation of various cell surface molecules can significantly impact cellular viability. Alterations in cellular fucosylation through upregulation of FuT8 also appear to regulate cellular proliferation, as knockdown of FuT8 in cell lines derived from non–small cell lung cancer results in a significant reduction in cellular proliferation rate in vitro (174).

In addition to intrinsically altering the location of cell surface receptors and their sensitivity to key ligands required for cell growth and invasion, alterations in glycosylation also appear to affect the ability of neoplastic cells to engage and differentially impact the activity of infiltrating immune cells normally responsible for immunosurveillance. For example, truncated *O*-glycans in the form of *O*-GalNAc can interact with macrophage galactose-type lectin (MGL) (175); this engagement may enhance the uptake of unique tumor-specific glycoproteins (176). This lectin normally plays an important role in responses to helminthes; thus, neoplastic cell engagement of MGL appears to deviate the immune response away from a potentially productive Th1 cell–mediated immune response toward a more tolerogenic phenotype that likely plays a critical role in the establishment of immunological tolerance to a neoplastic lesion (177). Similar engagement of natural killer (NK) cells by STn may inhibit NK cell–mediated antitumor immunity (178). The utilization of dendritic cell C-type lectins to escape or otherwise favorably influence immunity does not appear to be limited to MGL; several other lectins that likely engage other commonly occurring cancer-associated glycan signatures, such as Le[a] and Le[b], may also inadvertently play a role in facilitating the type of immunoprivileged environment that favors neoplastic cell survival in vivo (179).

Alterations in glycosylation can clearly impact cellular survival and engagement of immune effector cells through a variety of immune cell GBPs. In addition, cancer cells appear to possess the capacity to secrete their own GBPs, which can likewise impact neoplastic growth and survival. Galectins, which are soluble GBPs overexpressed in many types of cancer, represent a classic and well-studied example (20). Galectins can affect cell proliferation and survival through intercellular interactions with key players in cell growth and viability (20, 21, 180), but similar to C-type lectins, they likely evolved as innate immune proteins with the capacity to significantly impact immune cell viability and function (181–183). As a result, it seems that the secretion of various galectin family members by neoplastic cells can significantly modulate immune function. For example, galectin-1, the first family member described, likely engages dendritic cells and T cells, inducing alterations in cytokine production that ultimately prevent the effective elimination of neoplastic cells, thus providing a mechanism whereby these GBPs might enhance neoplastic cell survival (184, 185). Several studies suggest that, in addition to regulating immune function (186–188), galectins may directly facilitate neoplastic cell adhesion to the extracellular matrix and enhance endothelial cell–mediated extravasation (189). Given that these proteins also appear to directly regulate angiogenesis, they may influence neoplastic cell survival in a variety of ways (190–192).

LESSONS FROM ANIMAL MODELS OF CANCER

Whereas in vitro studies provide compelling insights into the potential impacts of altered glycosylation on cell-intrinsic behavior, including cell growth and viability, examination of cancer cells in vivo can illuminate the potential consequences of altered glycosylation for the interaction of neoplastic cells with environmental factors that may be critical for their survival, growth, and metastasis. Consistent with the ability of GnT-V overexpression to enhance neoplastic cell survival in culture, early studies suggested that simple inhibition of total *N*-glycans could significantly reduce neoplastic cell growth in vivo (Figure 4) (193). Increased GnT-V activity secondary to overexpression of H-ras likewise resulted in increased metastatic potential in several cell lines in vivo (159). More recent studies also suggested a central role for GnT-V in this process, as GnT-V-knockout mice display significantly reduced polyomavirus-induced tumor formation, which appears to reflect, in part, altered phosphatidylinositol 3-kinase–dependent cell proliferation (194). Variations in GnT-V expression may not only impact cell growth and survival but also facilitate metastasis. Tumors in GnT-V-knockout mice appear to display not only decreased growth but also impaired metastasis (194); the latter may simply be indicative of the former, but it also may reflect direct inhibition of metastatic spread. In contrast, expression of GnT-III (Figure 4) appears to inhibit metastatic spread of melanoma (195), providing an additional mechanism whereby GnT-III may negatively regulate cancer cell survival and progression. Similarly, enhanced α2–6 sialylation secondary to overexpression of an α2–6 sialyltransferase (ST6Gal1) (Figure 4) also inhibits cell growth and metastasis, suggesting that terminal glycan modifications can likewise impact cell growth and survival in vivo (173, 196).

Although altered glycosylation likely influences a variety of cellular features that contribute to an enhanced or reduced propensity for metastatic spread, early studies in leukocyte biology demonstrated that cell surface carbohydrates can play a key role in leukocyte extravasation. As neoplastic cells may utilize similar pathways during hematogenous spread, studies on neoplastic cell metastasis began to examine whether cancer cells may co-opt a similar pathway during intravascular dissemination. Indeed, neoplastic cell expression of SLe[x], a potential ligand for the E- and P-selectin vascular adhesion molecules, portends a poor prognosis for individuals with a variety of different neoplastic diseases. Metastatic tumors express higher levels of SLe[a] or SLe[x] compared with primary tumors (88, 197), once again suggesting that expression of these ligands may directly convey metastatic potential. Subsequent studies demonstrated that inhibition of complex *O*-glycans bearing selectin ligands reduces attachment to endothelial cells (198). Similarly, increased expression of SLe[a] and SLe[x] secondary to altered glycosyltransferase expression enhances neoplastic cell binding to P- and E-selectins (92–96). Tumor carbohydrate-mediated interactions with vascular adhesion molecules not only facilitate endothelial attachment but also appear to directly mediate vascular spread of cancer cells (97, 98). Whereas some of these altered glycoforms may facilitate metastasis, the same changes may serve as targets for NK cell–mediated immunosurveillance (199), suggesting that evolution may have selected these changes as potentially poor prognosticators in tumor growth.

Several studies suggest that, in addition to selectin-mediated extravasation, other endothelial lectins may also participate in cancer cell metastasis. For example, in addition to reports documenting a role for galectins in leukocyte extravasation (200), a variety of papers suggest that cancer cells may express galectin ligands (201), which in turn facilitate cancer cell metastasis. In contrast to selectin-mediated cancer cell extravasation, galectin-mediated homotypic interactions appear to facilitate the formation of tumor emboli that may directly enhance tumor lodging in distal capillaries, which may in turn enhance subsequent extravasation and metastasis (202). Consistent with this, galectin-3 expression in various cell lines correlates with metastatic potential in a murine model, and overexpression of galectin-3 in galectin-3-negative cell lines results in the acquisition of a more aggressive phenotype in vivo. Addition of factors thought to inhibit galectin binding in vivo, such as modified citrus pectin, likewise inhibits tumor growth and metastatic progression in vivo (203), strongly suggesting that galectin-3 or related galectin family members may play a key role in this process. Although the ability of galectin family members to modulate cell-cell and cell-matrix interactions likely accounts for a significant share of their impact, galectins also possess intracellular roles that can directly impact cell growth and survival (204). Consistent with the role galectins may play in favorably modulating immunity in tumor microenvironments, cancer cells expressing galectin-1 can inhibit T cell function and induce a tolerogenic phenotype in surrounding dendritic cells (184). Furthermore, galectin-1 also appears to facilitate tumor angiogenesis (186, 205), possibly through a mechanism that conveys resistance to vascular endothelial growth factor inhibition (186). As a result, just as alterations in glycosylation can potentially affect the function of cancer cells and the progression of cancer, GBPs not only decode these complex carbohydrates but in doing so regulate a wide variety of biological processes relevant to neoplastic cell growth and survival.

Besides their direct impact on cancer progression, GBP-carbohydrate interactions can also influence many of the sequelae associated with metastatic disease. For example, patients with Trousseau syndrome develop migratory microthrombi that are characteristically resistant to warfarin anticoagulation, suggesting a thrombin-independent mechanism of generation. In a recent model of Trousseau syndrome, mucin isolated from glycoproteins could induce significant platelet-rich microthrombi that remained sensitive to heparin-induced inhibition, similar to what occurs clinically (206). Consistent with previous reports suggesting that heparin may engage P- and L-selectin, heparin failed to inhibit microthrombus formation in P- and L-selectin-deficient recipients (206). Furthermore, P- and L-selectin-knockout recipients displayed significantly attenuated microthrombus formation following mucin challenge, strongly suggesting that these carbohydrate-binding proteins may be directly involved in this process (206). Indeed, subsequent studies demonstrated that neutrophil selectin, PSGL-1, and platelet P-selectin work in concert to facilitate microthrombus development following engagement of adenocarcinoma-derived mucin (207). Thus, GBP-carbohydrate interactions appear to directly engage a variety of biological phenomena that impact direct and indirect consequences of neoplastic disease.

Whereas neoplasia-associated alterations appear to impact progression and disease, cell surface carbohydrates on non-neoplastic cells may also inadvertently facilitate the development of neoplastic lesions. Although humans can generate de novo many of the

monosaccharide substrates required for complex glycan formation, some monosaccharides found in nature, but not synthesized by our own cells, can be absorbed and utilized by the human glycoprotein synthesis machinery. A classic example of this is the "nonhuman" sialic acid, Neu5Gc, which, as stated above, can be incorporated into human glycoconjugates through dietary sources such as red meat. As this alternative form of sialic acid is not present normally, immunological tolerance fails to develop. As a result, when Neu5Gc incorporation into glycoconjugates occurs, "autoantibodies" to this sugar develop, which have been proposed to enhance inflammatory pathways associated with cancer initiation (208). In a similar manner, exposure to microbes that express carbohydrate antigens that look like us not only may enhance the probability of autoimmunity but also may likewise facilitate neoplastic progression secondary to autoimmune-mediated inflammatory changes that set the stage for neoplastic transformation (209).

TARGETED THERAPIES BASED ON CHANGES IN PROTEIN GLYCOSYLATION

Current cancer-specific therapies target either the unique products of chromosomal rearrangements that typically result in the generation of specific chimeric gain-of-function protein products, as occurs in chronic myelogenous leukemia (210), or the overexpression of a particular target, as occurs in HER2-positive breast cancer (211). Although these alterations represent fundamental changes critical in the progression of the corresponding types of neoplastic disease, each of these mutations occurs in only a select number of cancers (210, 211), thus limiting the therapeutic potential of targeting these alterations to a relatively narrow spectrum of neoplastic disease. In contrast, as changes in posttranslational modifications, such as glycosylation, may occur on a wide variety of cell types, specific changes in neoplasia-related glycosylation may be found on multiple types of cancer, providing potentially novel, specific, and therefore unique therapeutic targets for a broad range of neoplastic lesions (69).

In addition to their potential to impact a wide variety of cells, the very nature of posttranslational modifications allows a single genetic defect in an enzyme to exert a fundamental impact on the phenotype of a cell (14). For example, as a single glycosyltransferase can modify many different glycoproteins, and may even modify an individual glycoprotein at multiple locations, alterations in the expression or function of a particular glycosyltransferase can alter the glycosylation of a variety of glycoproteins at multiple sites per glycoprotein, ultimately inducing significant, dense, and specific changes in the carbohydrate profile of a cell (14, 69). In this way, these types of posttranslational modifications provide a unique opportunity to translate a single genetic lesion or alteration in the expression of a glycosyltransferase to a highly amplified and unique marker that may be used to specifically target neoplastic cells (69).

In addition to being present at multiple copies per glycoprotein and on multiple glycoproteins per cell, complex carbohydrate modifications occur overwhelmingly on proteins that reside on the cell surface, where antibody-based therapeutics can directly engage them and thereby direct endogenous immune effector cells to neoplastic lesions (14). Similarly, antibody-based therapeutics designed to specifically target neoplasia-associated

changes in glycosylation would enable significant increases in the effective concentration of conjugated toxic antineoplastic therapeutics and therefore enhance the specificity of chemotherapeutic approaches.In addition, vaccine approaches using neoplasia-specific carbohydrates possess the same capacity to induce anti-carbohydrate antibodies that may enhance immunological memory against commonly occurring neoplasia-specific glycosylation and therefore enhance immunological surveillance (212). Consistent with this, several vaccine strategies appear to induce specific neoplastic anti-carbohydrate antibodies that show significant promise in the prevention of neoplastic disease (213, 214). However, it should be noted that, although vaccination approaches might indeed induce significant immunity to a cancer-related carbohydrate antigen, such immunity may produce selective pressures that enable neoplastic lesions to develop independent of a particular carbohydrate antigen (215). As a result, development of therapeutics that target existing cancer carbohydrate antigens may provide an equally compelling approach to treat neoplastic disease.

A classic example of a genetic change yielding a useful target cancer neoantigen is the formation of the Tn and STn antigens, which, as stated above, reflects the loss of the X-linked chaperone Cosmc and thus results in the loss of T-synthase activity, the rate-limiting enzyme necessary for complex *O*-glycan formation (Figure 5) (216). Whereas ppGalNAcTs do not need T-synthase for their enzymatic activity, T-synthase requires the product of ppGalNAcTs, which is a GalNAcα1–Ser/Thr or the Tn antigen, to form a core 1 *O*-glycan (otherwise known as the T antigen) (Figure 4). As a result, ppGalNAcTs continue to generate significant Tn antigen in the absence of T-synthase (132, 216). As *O*-glycans can occur on over 80% of cell surface glycans, this results in significant accumulation of the Tn antigen on the cell (217). The Tn antigen is normally completely modified to the T antigen by T-synthase; thus, accumulation of Tn antigen on many different glycoproteins on the cell surface results in the formation of antigenic determinants not normally present on the cell (102) and can therefore alter the conformation and function of glycoproteins (Figure 2) (157). As a result, the Tn antigen provides an unprecedented example of a truly neoplasia-specific antigen that does not appreciably occur normally but can be expressed on numerous targets on the cell surface following the loss of a single enzyme (102). Although the extent of Tn expression within neoplastic lesions, as detected using highly defined agents, remains to be fully elucidated, preliminary studies using very specific reagents suggest that this antigen may be present on nearly 50% of all neoplastic lesions from diverse sources of tissue, including breast, colon, ovary, lung, and endometrium (S.R. Stowell, T. Ju & R.D. Cummings, unpublished results).

In addition to the loss of glycosyltransferase function, aberrant expression of a particular enzyme may inadvertently truncate or otherwise modify a cell surface glycoprotein and therefore likewise generate a unique cancer-associated glycan antigen. For example, whereas expression of the STn antigen likely occurs in neoplastic cells as a result of the loss of Cosmc function and subsequent generation of a substrate for the α2–6 sialyltransferase capable of modifying the Tn antigen to form STn (102), several studies suggest that overexpression of the α2–6 sialyltransferase may outcompete the T-synthase and therefore generate the STn antigen directly (218, 219), effectively preventing T-synthase from

generating normally occurring core 1 *O*-glycans. Although overexpression of α2–6 sialyltransferase would be predicted to generate an aberrantly truncated and therefore potentially specific neoplastic alteration, some neoplastic lesions appear to exhibit glycosylation that significantly deviates from the tissue of origin but that may exist in similar structural form in other tissues. For example, other alterations in glycosyltrans-ferase function may result in significant increases in the formation of the T antigen, Le[a], Le[b], Le[y], Le[x], GM2, GD3, and others (69). Although some of these antigens exhibit expression on other cells, their overexpression on neoplastic cells may provide enhanced targets for neoplasia-related drugs, allowing increased specificity for cells bearing enriched levels of these antigens compared with normal tissue (69).

In addition to changes in glycosylation associated with genetic or even epigenetic changes, altered expression of glycosyltransferases and baseline changes in metabolism (220, 221) may cause neoplastic cells to preferentially incorporate sugar analogs that can be used to metabolically tag a neoplastic cell, thereby providing another unique cancer target. For example, early work demonstrated that sugar analogs can be equipped with unique chemical modifications that do not appear to affect incorporation into a cell's surface carbohydrate repertoire but that can, owing to their unique engineered chemistry, be readily detected and therefore targeted to specifically engage a modified cell with an antibody- or chemical-based probe (59, 221–224). Incorporation of antineoplastic agents into these probes could offer another strategy to specifically target neoplastic lesions that may preferentially incorporate a particular carbohydrate analog (59, 224).

Whereas specifically targeting neoplasia-associated changes in glycosylation provides an attractive opportunity for novel therapeutic approaches, the expression of altered glycosylation also offers an opportunity to inhibit fundamental biological processes that neoplastic cells rely on for progression. For example, as altered expression of cell surface glycans may directly facilitate neoplastic cell spreading and eventual metastasis, inhibition of selectin- or galectin-mediated cancer cell extravasation may also inhibit the pathological sequelae of neoplastic disease by directly blocking the functional consequences of altered glycosylation (20, 185, 225, 226). Similarly, as overexpression or altered expression of GBPs, such as galectins, may directly promote the development of an immunoprivileged environment or facilitate nutrient delivery by favorably altering vascularization, inhibiting these GBPs may also inhibit neoplastic disease progression through critical glycosylation-dependent pathways required for overall neoplastic cell survival (20, 185, 205). As a result, cancer glycans and the proteins that recognize them may not only serve as unique targets for direct toxic compounds designed to specifically eliminate neoplastic lesions; they may also enable the manipulation of key biological pathways critical for the progression of neoplastic disease.

CONCLUSIONS

Modern studies have now shown that glycoproteins are found on all animal cells and that their glycan structures are commonly altered upon cellular transformation. Such changes in glycosylation provide new directions for understanding the molecular nature of cancer and cellular transformation and offer new opportunities for identifying biomarkers of disease and

developing interventional strategies for treatment. Because glycans are abundant on the surface of tumor cells, and because specific glycan structures, such as the Tn and STn antigens, may be uniquely expressed on many types of tumors, these may serve as novel targets for diagnosing disease, characterizing prognosis, and delivering drugs. Increasingly, biochemical, molecular, and genetic studies are showing that alterations in protein glycosylation can be a major contributor to the transformation process and that alterations in the glycocalyx of cells can change the tumor microenvironment and metastatic process with significant effects on the progression of neoplastic disease. Future studies will undoubtedly uncover previously unrecognized roles for glycosylation in the pathogenesis of neoplasia and provide important and novel targets in the treatment of this formidable disease.

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SOME MAJOR FACTORS THAT AFFECT PROTEIN GLYCOSYLATION IN TUMOR CELLS

- Levels of expression of specific glycosyltransferases

- Localization of glycosyltransferases in the secretory compartments and other cellular compartments, such as the nucleus and mitochondria

- Expression of specific molecular chaperones that regulate protein folding and quality control of glycoproteins and glycosyltransferases

- Site- and protein-specific nature of glycosyltransferases

- Levels of expression of specific glycosidases in the processing pathway

- Levels of expression of lysosomal hydrolases in lysosomes and cellular secretions

- Availability of protein substrates

- Availability and levels of nucleotide sugars and 3′-phosphoadenosine-5′-phosphosulfate (PAPS)

- Activity of nucleotide sugar and monosaccharide transporters

- Glycoprotein turnover kinetics

- pH of the ER and Golgi

- Competition reactions between glycosyltransferases for similar glycan acceptors

SUMMARY POINTS

1. The membranes and secretions of animal and human cells contain glycoproteins, including membrane receptors, transporters, hormones, and signaling molecules, that incorporate an assortment of carbohydrate structures linked to specific amino acids.

2. Common linkages to membrane and secreted glycoproteins include Asn-linked oligosac-charides (*N*-glycans) and Ser/Thr-linked oligosaccharides (*O*-glycans), and changes in these glycans are commonly found in tumor cells.

3. Changes in glycosylation arise from altered expression of glycosyltransferases, enzymes that modify glycans in the ER and Golgi apparatus during their biosynthesis.

4. Alterations in protein glycosylation can perturb the structure and function of glycoproteins by changing their oligomerization, turnover, conformation, and interactions with other molecules.

5. Changes in protein glycosylation can contribute directly to tumor progression and metastasis.

6. Altered glycan structures can alter the adhesion of tumor cells and their interactions with the microenvironment.

7. Novel glycan structures on tumor cells, such as the Tn and STn antigens, represent new targets for the diagnosis and treatment of neoplastic disease.

8. Much mechanistic work remains to be done to define the specific roles of protein glycans as drivers or passengers in neoplastic transformation.
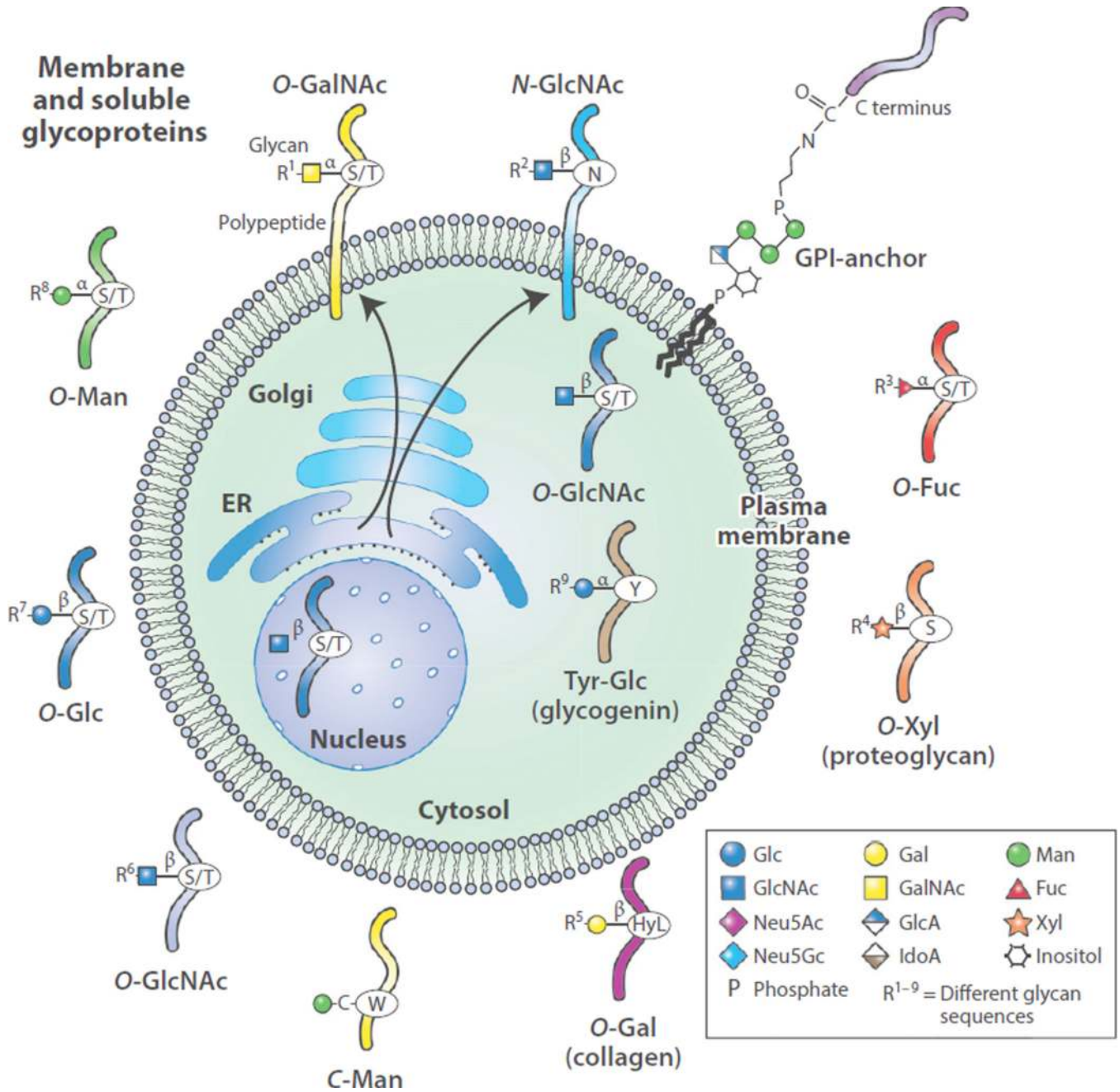
Figure 1.

Animal cells synthesize a wide assortment of glycoproteins in which different amino acids may be modified to contain specific glycan structures. Biosynthesis of such glycoproteins is initiated in the secretory pathway comprising the endoplasmic reticulum and Golgi apparatus of cells and can lead to membrane localization and secretion of glycoproteins. In addition, *O*-GlcNAc may be added to glycoproteins in the cytoplasm, nucleus, and mitochondria. This single GlcNAc residue is not extended but can be reversibly added and removed. The multiple other types of glycan linkages to proteins can be extended (R groups indicated) with many additional sugar molecules to form oligosaccharides or

polysaccharides, all termed glycans. The 10 common monosaccharides that make up animal glycans are indicated: *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), 5-*N*-acetylneuraminic acid (Neu5Ac, or sialic acid), fucose (Fuc), galactose (Gal), glucose (Glc), glucuronic acid (GlcA); iduronic acid (IdoA); mannose (Man), and xylose (Xyl). 5-*N*-Glycolylneuraminic acid (Neu5Gc), a non-natural sugar, is also shown. Other abbreviations: ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; HyL, hydroxylated lysine.
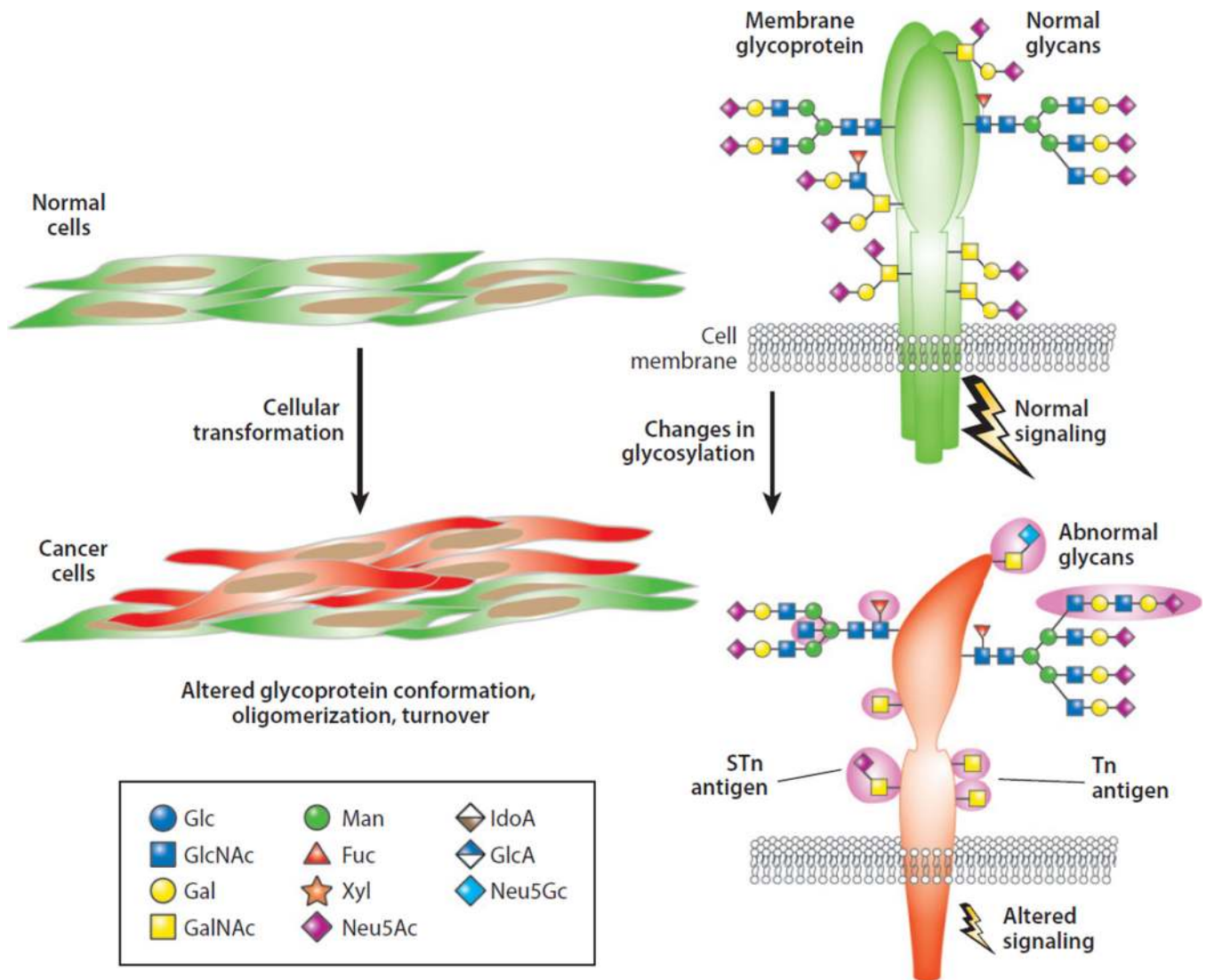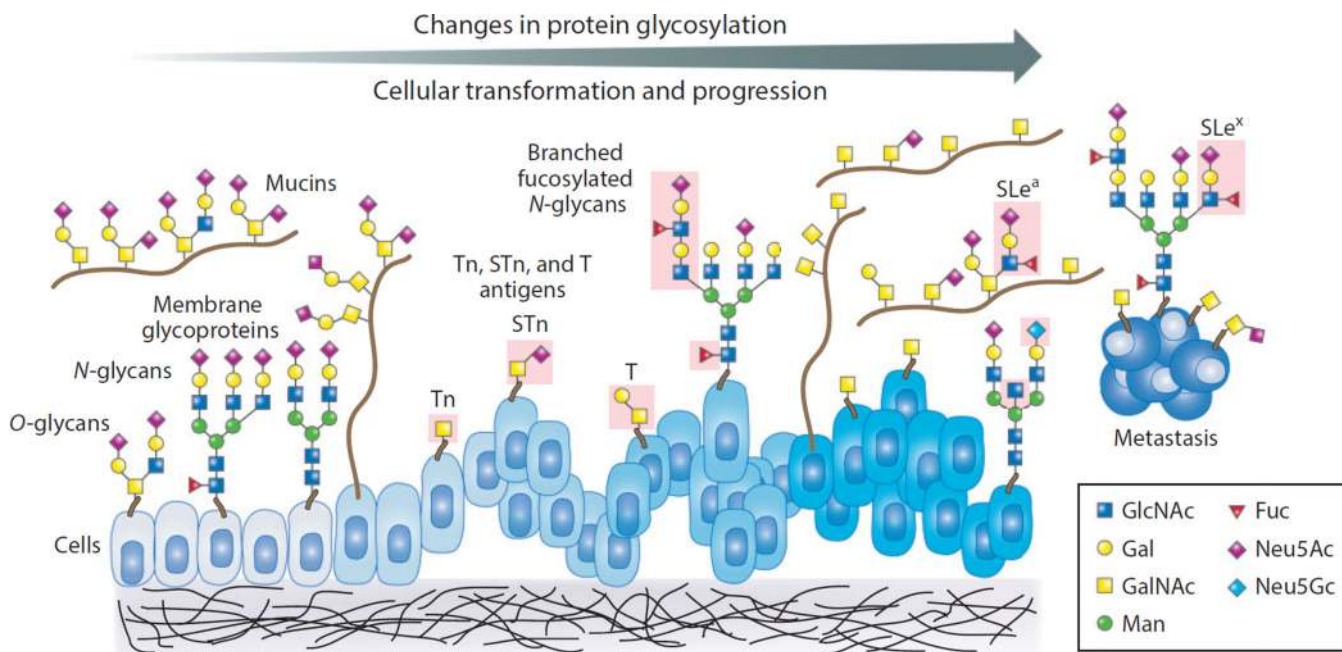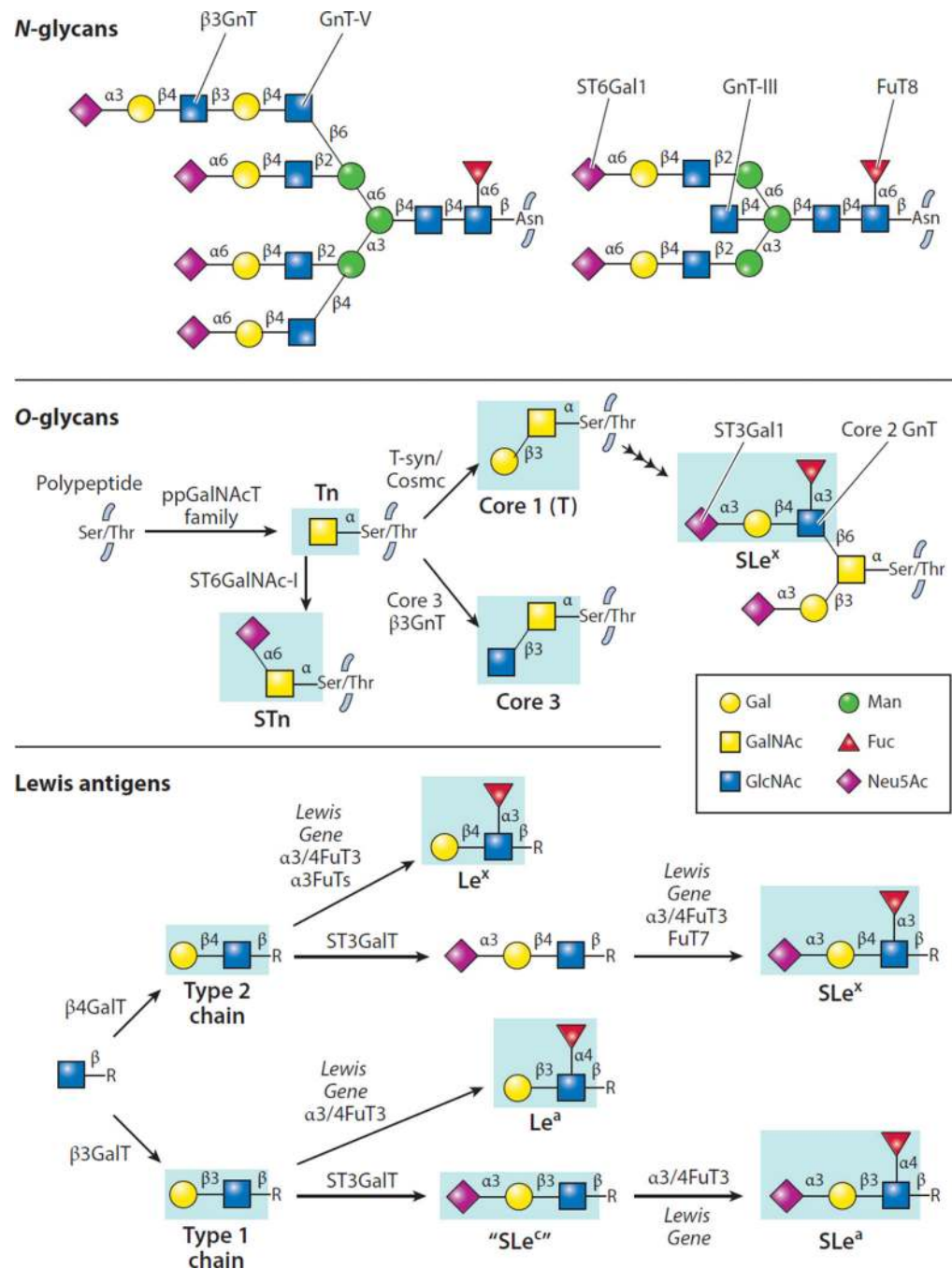
Figure 2.
Cellular transformation is typically accompanied by changes in protein glycosylation on multiple types of glycans; the most commonly studied are *N*-glycans and *O*-glycans. Changes in protein glycosylation can result in altered glycoprotein conformation, oligomerization, and turnover and can also be associated with altered cell signaling pathways. Frequently observed altered *O*-glycans include the Tn and STn antigens. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; IdoA, iduronic acid; Man, mannose; Neu5Ac, 5-*N*-acetylneuraminic acid (sialic acid); Neu5Gc, 5-*N*-glycolylneuraminic acid; STn, sialyl Tn; Xyl, xylose.

Figure 3.
During cellular transformation, changes in protein glycosylation on membrane and soluble glycoproteins, such as mucins, are typical and may occur early and/or late in cancer progression, but this phenomenon is not well understood. Different types of changes are shown in the pink-boxed areas, highlighting changes in *O*-glycans (T, Tn, and STn antigens) and altered expression of branched and fucosylated *N*- and *O*-glycans, including changes in Lewis antigens (SLe[x] and SLe[a]). Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, 5-*N*-acetylneuraminic acid (sialic acid); Neu5Gc, 5-*N*-glycolylneuraminic acid; SLe, sialyl Lewis; STn, sialyl Tn.

Figure 4.
Specific structures of *N*- and *O*-glycans and Lewis antigens along with enzymes responsible for addition of specific sugar residues. Each glycosyltransferase indicated requires a nucleotide sugar donor and acts to add a sugar in a specific anomeric linkage (α or β) to a specific acceptor glycan. Antigens indicated in blue boxes represent the major determinants recognized by monoclonal antibodies. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; IdoA, iduronic acid; Le, Lewis; Man, mannose; Neu5Ac, 5-*N*-acetylneuraminic acid (sialic
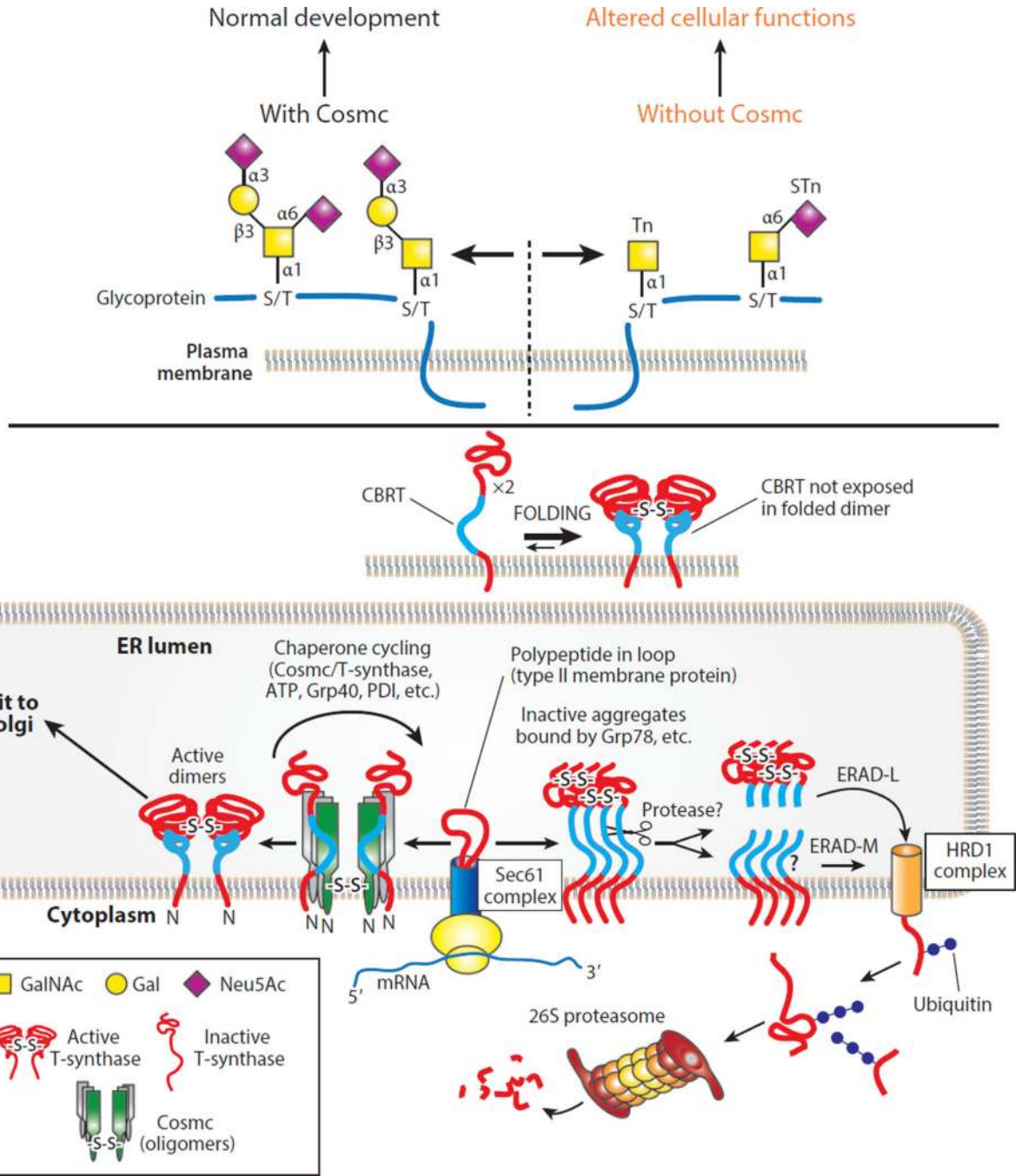
acid); Neu5Gc, 5-*N*-glycolylneuraminic acid; SLe, sialyl Lewis; STn, sialyl Tn; Xyl, xylose. The Lewis gene encodes the fucosyltransferase responsible for Lewis antigen synthesis.

Figure 5.
The expression of the Tn and/or STn antigens can occur in cells lacking the molecular chaperone Cosmc. Cosmc is present in the ER of animal cells and has a single client protein, the T-synthase, to which it binds cotranslationally in the ER to prevent oligomerization and destruction in the proteasome. Successful binding of Cosmc to the T-synthase requires the presence of a novel CBRT (297), which is exposed in non-native T-synthase but becomes buried and inaccessible in the folded T-synthase. Once folded properly, the T-synthase moves to the Golgi apparatus, where it acts quantitatively on the products of the

ppGalNAcTs to generate normal *O*-glycans (102). Abbreviations: CBRT, Cosmc-binding region within T-synthase; ER, endoplasmic reticulum; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Neu5Ac, 5-*N*-acetylneuraminic acid (sialic acid); ppGalNAcT, polypeptide GalNAc transferase; STn, sialyl Tn.

Table 1

Milestones in studies on glycosylation and cancer

Year(s)	Milestone	Reference(s)
General functions of cell surface glycans		
1950s–1960s	Structural definition of carbohydrate-based ABO(H) blood group antigens	13, 227–229
1960s–1970s	Discovery that plant lectins (PHA, ConA) are mitogenic for peripheral lymphocytes and recognize specific glycopeptides	230, 231
1964	Finding that glycosidase treatment of cells alters cellular interactions in vivo	232
1966–1974	Identification of a hepatic receptor for asialoglycoproteins	233–235
Surface glycans in cancer		
1949	Demonstration of decreased expression of human blood group A antigen in gastric cancer	236
1962	Finding that ascites tumor cells express unusual sialylated glycans	237, 238
1963–1969	Description of altered agglutination (by WGA and ConA) of virally transformed cells	239–246
1968	Demonstration of altered glycolipids in virally transformed cells	247
1969–1971	Demonstration of altered glycopeptide sizes upon cellular transformation	248–251
Monoclonal antibodies identifying specific glycan-based biomarkers of cancer		
1968	Demonstration of antigenicity of glycoprotein glycans	252
1970s–1980s	Discovery of T, Tn, and sialyl Tn (STn) antigens as tumor biomarkers	253–256
1977	Discovery of Forssman antigen in human gastrointestinal tumors	257
1978	Discovery of stage-specific embryonic antigen and oncofetal antigen SSEA-1	258, 259
1979–1983	Identification of CA 19-9 as a circulating cancer-associated antigen, sialyl Lewis a (SLe[a])	105, 260–262
1983	Demonstrated expression of CA 125 (MUC16) in epithelial cancer and serum	263, 264
1988	Development of monoclonal antibodies to Tn antigen	265
1988	Altered fucosylation of serum α-fetoprotein (AFP) used as biomarker for hepatocellular carcinoma	266
1981–1992	Development of monoclonal antibody to the STn antigen	267–269

Table 2

Carbohydrate tumor markers

Marker[a]	Cancer type	Reference(s)
AFP	Hepatocellular	270
β-hCG	Testicular, ovarian	271, 272
CA 15-3	Breast, lung, prostate	273, 274
CA 19-9	Gastrointestinal (pancreatic)	275–277
CA 27.29	Breast, lung, prostate	278
CA 125	Ovarian	279, 280
CA 549	Ovarian	281
CEA	Colorectal	282, 283
CEACAMs	Colorectal, pancreatic	284, 285
HER2	Breast	286, 287
onfFN	Thyroid	288
PLAP	Testicular, muscle	289
PSA	Prostate	290–293
sTn antigen	Colon, other	83, 294
TAG-72	Ovarian, other	295
TG	Thyroid	288
Tn antigen	Colon, breast, cervical, other	83, 132, 296

[a]Common abbreviated marker names are shown: AFP, α-fetoprotein; β-HCG, β human chorionic gonadotropin; CA, cancer antigen; CEA, carcinoembryonic antigen; CEACAMs, carcinoembryonic antigen–related cell adhesion molecules; onfFN, oncofetal fibronectin; PLAP, placental alkaline phosphatase; PSA, prostate-specific antigen; STn antigen, sialyl Tn antigen; TAG-72, tumor-associated glycoprotein 72; TG, thyroglobulin.