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Mechanisms of cancer-associated glycosylation changes

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1. ABSTRACT

Cell membrane glycoconjugates undergo characteristic changes as a consequence of neoplastic transformation. The cancer-associated carbohydrate structures play key roles in cancer progression by altering the cell-cell and cell-environment interactions. In this review, we will discuss some of the most relevant cancer-associated carbohydrate structures, including the β1,6-branching of N-linked chains, the sialyl Lewis antigens, the α2,6-sialylated lactosamine, the Thomsen-Friedenreich-related antigens and gangliosides. We will describe the mechanisms leading to the expression of these structures and their interactions with sugar binding molecules, such as selectins and galectins. Finally, we will discuss how the glycosylation machinery of the cell is controlled by signal transduction pathways, epigenetic mechanisms and responds to hypoxia.

2. INTRODUCTION

The surface of eukaryotic cells is covered by a sugar coat, known as glycocalix. The carbohydrate molecules forming the glycocalix are either linked to proteins or to lipids. The vast majority of cell membrane or secreted proteins are decorated by sugar chains and are consequently referred to as glycoproteins. The sugar chains linked to glycoproteins are classified in two main types: those linked to the amidic nitrogen of asparagine (referred to as N-linked chains) (1) and those linked to the hydroxyl group of serine or threonine (referred to as *O*-linked chains) (2). The sugar chains of glycoproteins can play highly specific roles, including the receptor function for microorganisms and toxins and the modulation of the cell-cell and cell-microenvironment interactions (3). In cancer tissues, glycosylation is profoundly altered, leading to the expression of cancer-associated antigens which, in some

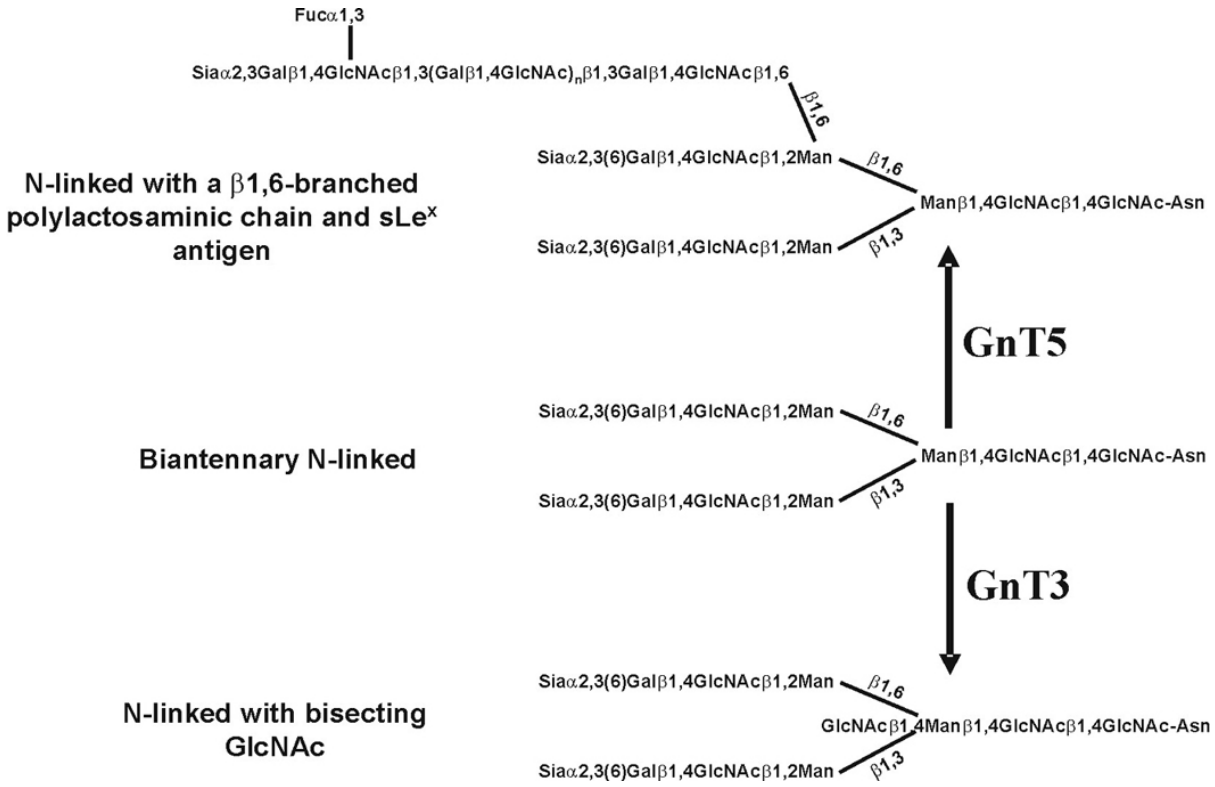

Figure 1. Structures of *N*-linked chains. A typical biantennary *N*-linked chain (middle) can be transformed in a β1,6-branched structure (upper) by the action of GnT5. The β1,6-linked structure is frequently elongated by polylactosaminic chains, which are often terminated by complex structures, such as the sialyl Le^x antigen. The action of GnT3 leads to the addition of a bisecting GlcNAc (lower), which inhibits the formation of the β1,6-branched structure.

cases, recapitulate the antigens expressed during fetal life (4, 5). These structures may play fundamental roles in cancer progression through different mechanisms. In this review we will discuss the molecular mechanisms which are at the basis of the expression of some cancer-related carbohydrate structures and the mechanisms through which these structures exert their biological effects. Many of these biological effects are mediated by interactions with animal lectins, in particular selectins, which recognize sialylated Lewis antigens (6-8) and galectins which bind structures terminated by galactose and whose importance in cancer is increasingly recognized (9-15). The identification of the mechanisms of cancer-associated glycosylation changes is crucial in the perspective of therapeutic interventions aimed at the normalization of the glycosylation pattern of cancer cells. Cancer-associated glycosylation changes are in some cases tissue-specific, while in other cases are broadly expressed. In the next section we will focus on some of the broadly expressed cancer-associated carbohydrate structures.

3. SURVEY OF CANCER-ASSOCIATED GLYCOSYLATION CHANGES

3.1. β1,6 branching.

The β1,6 branching of *N*-linked chains consists in the addition of an antenna whose first GlcNAc is β1,6-linked to a core mannose residue (Figure 1). This antenna, which can be detected by the leukoagglutinin from *Phaseolus vulgaris* (L-PHA), is preferentially elongated by polylactosaminic sequences and is frequently terminated by antigens of the Lewis type (Figure 1). Although the association of β1,6-branching with metastasis has long been known (16), the conclusive evidence about the causative role played by these structure in metastasis formation came from studies in mice in which the enzyme responsible for this modification, β1,6N-acetylglucosaminyltransferase V, (GnT5 product of the Mgat5 gene), was knocked down ($Mgat5^{-/-}$). Mice expressing the polyomavirus middle T antigen (PyMT) from a transgene in mammary epithelium, spontaneously develop mammary tumors. When these mice were crossed with $Mgat5^{-/-}$ mice, the tumors grew slower than in the PyMT-transgenic littermate expressing Mgat5 and metastasis formation was almost completely inhibited (17). Cells derived from $Mgat5^{-/-}$ mice exhibited increased contact inhibition and substratum adhesion than Mgat5-expressing cells (18).

The relationship between β1,6-branching and increased growth and metastasis is probably due to more than one mechanism (19). The sugar chains elaborated by GnT5 decorate various cell surface molecules, including growth-promoting receptors (such as PDGFR and EGFR) and receptors with arrest/morphogenic activity (such as TGF-βR and CTLA-4). β1,6-branched, polylactosaminic

chains are a preferred ligand for galectin-3 which, consequently, forms a lattice which stabilizes the receptors on the cell surface (19). However, growth-promoting receptors express an average higher number of N-linked glycans (high-n receptors) than receptors with arrest/morphogenic activity (low n-receptors) (20). As a consequence, the galectin-3-mediated stabilization of membrane receptors favors highly-branched, growth promoting receptors. Interestingly, the switch from growth to arrest can be regulated by the nutrient flux. In fact, glucose is converted to glucosamine and UDP N-acetylglucosamine, which is the donor substrate of GlcNAc-transferases. An increase of UDP-GlcNAc levels results in a little change of the glycosylation of high n-receptors but in a dramatic increase of glycosylation (and galectin binding capacity) of low-n receptors. This change mediates the switch from a growth to an arrest condition. Moreover, galectin-3 binding to β1,6-branched glycans regulates tumor cell motility by stimulating focal adhesion remodeling, FAK and PI3K activation, local F-actin instability, and α5β1 integrin translocation to fibrillar adhesions (21).

Another mechanisms proposed to explain the relationship between β1,6-branching and metastasis involves matriptase, an activator of both urokinase-type plasminogen activator and hepatocyte growth factor. This molecule, when glycosylated by GnT5, acquires resistance to degradation and increased activity (22, 23). A direct effect of galectin-3 on VEGF- and bFGF-mediated angiogenesis has been shown by a recent paper showing that this effect is due to the binding of galectin-3 to the β1,6-branched chains of αvβ3 integrin and to the activation of focal adhesion kinase signaling (24). As discussed in detail in section 5.1, MGAT5 expression is regulated by the Ras pathway, thus explaining its close association with cancer. In many circumstances, GnT5 activity is counteracted by that of a competing enzyme, GnT3 (Figure 1) (section 4.4), which synthesizes bisecting *N*-linked glycans.

3.2. Sialyl Lewis antigens

Lewis a and Lewis b antigens are originated by the mono- or di- fucosyl substitution of type 1 chains while Lewis x and Lewis y derive from the mono- or di-fucosyl-substitution of type 2 chains (Figure 2). The mono-fucosyl substitution of the α2,3-sialylated type 1 or type 2 chains leads to the formation of sialyl Lewis^a (sLe^a) and sialyl Lewis^x (sLe^x) antigens, respectively (25). These structures are usually present at the terminal non-reducing ends of polylactosaminic chains, preferentially mounted on the β1,6-branching of *N*-linked chains (Figure 1) or of *O*-linked chains (26), but also on glycolipids (27). An aberrant expression of Lewis-type antigens appears to be a general cancer-associated phenomenon, reported in carcinomas of the lung (28), colon (27, 29, 30), stomach (31) and kidney (32). The sLe^a tetrasaccharide is the epitope of CA19-9 antigen, a cancer-associated marker widely used in the clinical practice. The interest in the expression of sialyl Lewis antigens in cancer increased enormously after the discovery that sLe^x and sLe^a acted as ligands for E- and P-selectin cell adhesion molecules expressed on activated endothelial cells (6, 33-35). The physiological role of E- and P-selectins is to mediate leukocyte extravasation at sites of tissue damage or injury (36). However, these molecules may also regulate the metastatic cascade by forming emboli of cancer cells and platelets and favoring their arrest on endothelia (7, 37-39). In some cell lines, the major glycoproteins carrying sialyl Lewis antigens have been identified as the hyaluronate receptor CD44 (37, 40, 41), mucin 1 (MUC1) (42-44) and lysosomal membrane glycoproteins 1 and 2 (LAMP-1 and LAMP-2) (44). The relationship between expression of sialylated Lewis antigens and hematogenous metastasis is demonstrated by many clinical and experimental studies (45). In colon cancer patients, increased expression of sLe^x and sLe^a antigens correlated with metastasis and poor survival (46-49). sLe^x correlated with malignancy also in renal cell carcinoma (50) and breast cancer (51, 52), although in the latter the survival did not appear to be related with sLe^x expression (53).

Several studies have reported that down-regulation of sialyl Lewis antigen expression by knock-down of key glycosyltransferases in cancer cell lines resulted in reduced selectin binding and reduced metastatic ability (54-57), while cancer cells forced to express sialyl Lewis antigens by gene transfer exhibited increased adhesion to selectins *in vitro* and increased metastatic ability *in vivo* (58). Consistently, populations of cancer cells selected for their increased metastatic potential often displayed increased expression of sialyl Lewis antigens (59, 60). The role of selectins in the metastatic process was confirmed by the findings that the formation of experimental pulmonary metastases could be inhibited by the use of peptides mimicking sLe^a and were inhibited in E-selectin- knock-out mice (61).

Apart from the role as selectin ligands, sialyl Lewis antigens can play a role in cancer progression in at least two other key steps of invasion: angiogenesis and immune recognition of cancer cells. The role of sLe^x in angiogenesis is supported by the finding that when epidermoid cancer cells were co-cultured with endothelial cells, the former produced nests of growing cells surrounded by tube-like networks consisting of endothelial cells. These phenomena could be reproduced *in vivo* and could be inhibited by antibodies against sLe^x (62). The ability of sLe^x-expressing cancer cells to promote angiogenesis was confirmed by the fact that inhibition of sLe^x biosynthesis in hepatocarcinoma HepG2 cells resulted an impairment of their ability to induce angiogenesis (63). The role of sLe^x in the recognition of cancer cells by natural killer (NK) cells stemmed from the unexpected observation that melanoma cells expressing high sLe^x levels were less metastatic than cells expressing moderate levels of the antigen (64, 65). This striking behavior was explained by the finding that high sLe^x-expressing cells were a better target of NK cells than cells expressing moderate levels of the antigen (65). Altogether, these findings indicate that sialyl Lewis antigens are important in mediating key steps of the metastatic process, in particular the adhesion of emboli of cancer cells to endothelia and

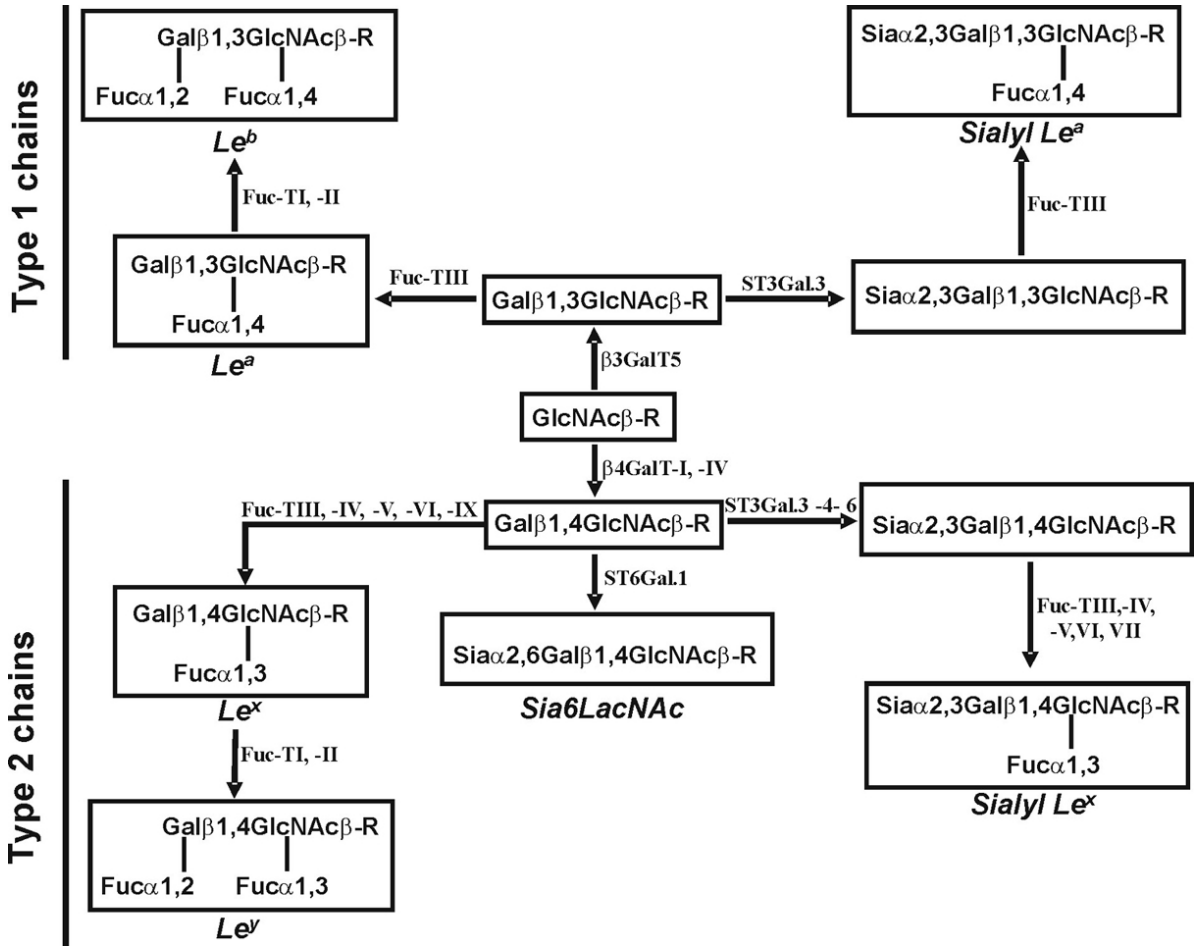

Figure 2. Biosynthesis and structures of Lewis-type antigens. Substitution of GlcNAc by a β1,3-linked galactose leads to the basic unit of type 1 chain, while substitution with a β1,4-linked galactose leads to lactosamine, the basic unit of type 2 chains. The addition of a fucose linked either *via* α1,4 or α1,3 to N-acetylglucosamine in type 1 or 2 chains respectively, leads to the formation of Le^a and Le^x, respectively. These antigens can be further elongated by a α1,2 fucose, leading to the formation of Le^b and Le^y antigens, respectively. The α2,3-sialylation of type 1 or 2 chains, followed by the addition of α1,4- or α1,3-linked fucose, respectively, leads to the biosynthesis of sialyl Le^a and sialyl Le^x antigens, respectively. The α2,6-sialylation of type 2 chains leads to the formation of Sia6LacNAc.

neoangiogenesis. Nevertheless, very high expression levels of sLe^x can trigger a strong NK-mediated tumor rejection.

The terminal steps of the biosynthesis of sialyl Lewis antigens proceeds from the α1,3/4 fucosylation of α2,3-sialylated type 1 (sLe^a) or type 2 (sLe^x) chains. On this basis the role of α1,3/4 fucosyltransferases and α2,3 sialyltransferases in the cancer-related over-expression of sialylated Lewis antigens has been the focus of intense investigation. It should be noted that the forced expression or down-regulation of α2,3 sialyltransferases (58, 66) or of α1,3 fucosyltransferases (54, 56) or of core 2 β1,6 N-acetylglucosaminyltransferase (C2GnT, Figure 3) (67) could modulate the expression of sialyl Lewis antigens in experimental systems. However, this does not necessarily imply the regulatory role of each mentioned glycosyltransferase *in vivo*. There are at least five enzymes which can mediate the addition of fucose in α1,3 linkage to an α2,3-sialylated type 2 chain: fucosyltransferases III, IV, V, VI and VII (Fuc-TIII-Fuc-TVII, products of genes FUT3-FUT7), while only one (Fuc-TIII) can add fucose in α1,4-linkage to an α2,3-sialylated type 1 chain. The expression of sLe^x appears to be regulated mainly by Fuc-TVI in breast tumors (68), while in lung tumors it is regulated by a coordinate up-regulation of Fuc-TIII and Fuc-TVI (69). On the contrary, in gastrointestinal tumors, such as pancreatic cancer, the over-expression of sialyl Lewis antigens did not correlate with any single glycosyltransferase gene (70). In colon cancer, the molecular basis of the over-expression of sialyl Lewis antigens are particularly complex. An investigation on the level of activity of the fucosyltransferases synthesizing sLe^a or sLe^x concluded that an altered activity of fucosyltransferases could not explain the increased expression of sLe^a/sLe^x antigens in colon cancer tumors (71). Consistently, other investigations reported that the

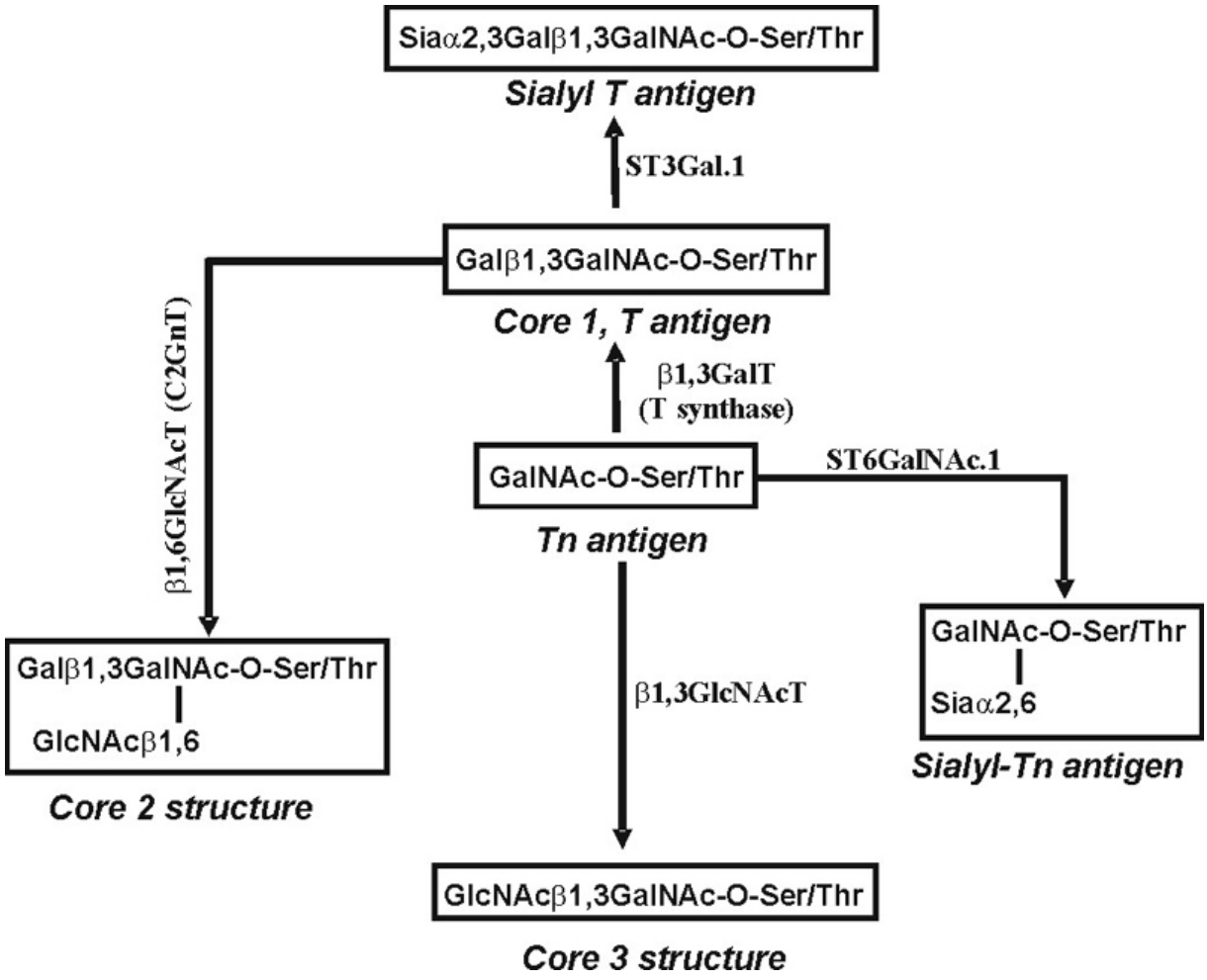

Figure 3. Biosynthesis and structures of short *O*-linked chains. Elongation of the Ser/Thr-linked GalNAc residue (also known as Tn antigen) by β1,3-linked galactose leads to the formation of core 1 structure, also known as T antigen, while the addition of α2,6-linked sialic acid to GalNAc, mediated by ST6GalNAc.1, leads to the biosynthesis of sialyl-Tn antigen. The addition of a β1,3-linked GlcNAc forms the core 3 structure. The addition to the core 1 structure of a sialic acid α2,3-linked to galactose forms the sialyl-T antigen, while the addition of a GlcNAc β1,6-linked to GalNAc forms the core 2 structure. This β1,6-linked branch can be elongated by polylactosaminic chains and is frequently terminated by sialyl Lewis antigens.

mRNA level of different fucosyltransferases and sialyltransferases involved in the biosynthesis of sialylated Lewis antigens could not explain their increased expression in colon cancer tissues (72, 73). In a recent paper, we have shown that Fuc-TVI is the major, if not the only, sLe^x synthase in colorectal cancer tissues and cell lines (74). The activity of this enzyme showed a significant relationship with sLe^x in cancer tissues, suggesting that terminal α1,3 fucosylation is a limiting step in sLe^x biosynthesis in colon cancer tissues. However, in agreement with previous studies, we found that Fuc-TVI was not over-expressed in cancer. Thus, the problem of sLe^x over-expression in cancer remains open. A likely explanation is based on a competition between Fuc-TVI and enzymes synthesizing alternative structures, such as the Sd^a antigen (75, 76) or the sialyl 6-sulfo $Lewis^x$ antigen (77) (Figure 4, section 4.4). However, the biosynthesis of sialyl Lewis antigens is a complex process involving the coordinate expression of several glycosyltransferases, which might be different depending on the nature of the glycoconjugate (*N*- or *O*-linked chains of glycoproteins or glycolipids) carrying the antigen. In fact, the expression of both sLe^x and sLe^a antigens expressed by glycolipids in colon cancer tissues has been related to the activation of a β1,3GlcNAc transferase which synthesizes a sugar chain which is a precursor for both type 1 and 2 Lewis structures (78). Interestingly, this enzyme is activated by *Helicobacter pylori* infection, leading in stomach cells to increased expression of sLe^x ,which is a ligand for *H. pylori* adhesin SabA (79). On the other hand, the expression of sLe^x/sLe^a antigens on *O*-linked chains of glycoproteins is strongly dependent on C2GnT (26). The relative abundance of type 1 and type 2 chains is an important factor in determining the relative level of expression of sLe^x/sLe^a antigens. An up regulation of lactosaminic chains (80) and of their biosynthetic enzymes β1,4-galactosyltransferase I (81) and

-IV (82) and a down-regulation of the β1,3-galactosyltransferase which synthesizes type 1 chains in epithelia (β3GalT5) (83, 84), has been reported in colon cancer (85-87), indicating a switch towards the synthesis of type 2 chains in the transformation of colonic tissues. The key role of β3GalT5 in the regulation of the balance between type-1 and -2 chains was also indicated by the finding that suppression by anti-sense DNA of β3GalT5 resulted in down-regulation of sLe^a and up-regulation of sLe^x and of lactosaminic chains in the pancreatic cancer cell line BxPC3 (88). The down-regulation of the biosynthesis of type 1 chains in colon cancer tissues leaves unanswered the question on the origin and the nature of the circulating sLe^a antigen (CA 19.9) present in the blood of several patients affected by various cancers of digestive organs. Recent data identified glycolipids associated with bile globular membrane as another CA 19.9 carrier, other than mucins, in the sera of pancreatic cancer patients (89).

3.3. α2,6-sialylated lactosamine (Sia6LacNAc).

Lactosaminic chains expressed by *N*- or *O*-linked chains of the polylactosaminic type are frequently terminated by sialic acid linked either through an α2,3- or an α2,6 bond (Figure 2). α2,6-sialylated lactosamine (Sia6LacNAc) is the product of β-galactoside α2,6-sialyltransferase (ST6Gal.1) (90, 91). Although a second enzyme able to mediate the α2,6-sialylation of lactosaminic chains, ST6Gal.2, was cloned (92, 93), its strict substrate specificity for oligosaccharides and its narrow tissue distribution leaves ST6Gal.1 as the major, if not the only enzyme responsible of the biosynthesis of Sia6LacNAc. This structure can be detected by the α2,6-sialyl-specific lectin from *Sambucus nigra* (SNA) (94) and, although widely expressed by normal tissues, shows a dramatic increase in several cancers (95, 96). We (97) and successively others (98-102) reported that ST6Gal.1 was increased in colon cancer tissues compared with normal mucosa. Other malignancies, including acute myeloid leukemia (103), choriocarcinoma (104), cervical carcinoma (105) gastric cancer (106) and some types of brain tumors (107) show an elevation of ST6Gal.1 activity or of the ST6Gal.1 transcript. As a consequence of the enhanced ST6Gal.1 expression, the vast majority of colon cancer specimens expresses an increased level of α2,6-sialylation of lactosaminic chains, as detected by SNA (108-110), although its level does not always correlate with that of ST6Gal.1 (110). A clinical study has indicated that high SNA reactivity is an independent predictive marker of poor prognosis (111). The CDw75 antigen is a peculiar form of α2,6-sialylated lactosamine, formerly identified in lymphocytes (112). This antigen is somehow different from that recognized by SNA in that colon cancer cells transfected with ST6Gal.1 exhibited SNA reactivity but not anti CDw75 reactivity (113). A recent study (98) has indicated that CDw75 is elevated in colorectal cancer, although its expression does not correlate with that of ST6Gal.1. In colon cancer, ST6Gal.1 over-expression leads also to the expression of an α1,2-fucosylated variant of Sia6LacNAc: the STH2 antigen (Fucα1-2(NeuAcα2-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) (114). In human hepatocarcinomas, only a minority of the patients exhibits increased ST6Gal.1 expression (115), while ST6Gal.1 and α2,6-sialylated glycans show an altered distribution (116, 117). In breast cancer, high ST6Gal.1 is associated with poor prognosis markers, such as high grade and absence of progesterone receptor (118). Among brain tumors, ST6Gal.1 and Sia6LacNAc are expressed only by those of non-neuroectodermal origin (107). In general, among brain tumors, a more aggressive behavior appears to be related with reduced, rather than increased, expression of ST6Gal.1 and SNA reactivity (119).

Mice transgenic for the SV40 large T antigen under the control of a liver-specific promoter spontaneously develop well-differentiated hepatocellular carcinomas. In these animals, an elevated α2,6-sialylation of plasma and liver glycoproteins, as well as an increase of liver and serum ST6Gal.1 activity closely followed tumor progression (120). To reconcile these findings with the fact that only a minority of liver cancer cases displays increased ST6Gal.1 and α2,6-sialylation (115) we hypothesized that only a few of the multiple mechanisms of cell transformation operating in human hepatocarcinomas led to ST6Gal.1 activation.

Owing to the fact that ST6Gal.1 is transcriptionally regulated by the Ras pathway (discussed in section 5.1), it is not clear whether the obvious association of ST6Gal.1 activation with neoplastic transformation merely reflects the activation of the Ras pathway or is causally related to a growth advantage provided by Sia6LacNAc structures. This latter possibility was suggested by the observation that human colon cancer cell lines grown as nude mice xenografts, expressed increased levels of ST6Gal.1 and of Sia6LacNAc than cell lines grown in the usual *in vitro* conditions (121). To get insights into the causal role of ST6Gal.1 and of the cognate Sia6LacNAc structures in cancer progression, we (122) and others (123-125) stably inserted the ST6Gal.1 cDNA in different cell types. The analysis of these transfectants consistently indicated an increased adhesion of ST6Gal.1-expressing cells to extracellular matrix substrates, such as collagen, fibronectin and laminin in both colon cancer (124, 126) and breast cancer cell lines (123). Unexpectedly, in the colon cancer cell line SW948, ST6Gal.1 expression appeared to reduce the tumorigenic potential in nude mice and the ability to grow as a multilayer *in vitro* (126). Reduced invasive properties upon ST6Gal.1 transfection were described also in glioma cells (125, 127). Altogether, these data indicate that the relationship between expression of Sia6LacNAc termini and invasive growth is complex and probably strongly tissue dependent. An important clue on the role of α2,6-sialylation in tumor growth has been provided by a study showing that breast cancer tumors developed by PyMT mice (see section 3.1) displayed increased differentiation when developed in a *ST6GAL1*-null background (128). However, ST6Gal.1-null tumors displayed similar growth properties when compared with tumors developed by $ST6Gal.1^{+/+}$ mice, indicating that at least in this mouse model of breast cancer, Sia6LacNAc termini play a role in tumor differentiation but not in tumor growth.

Several lines of evidence indicate that β1-integrins are crucial substrates of ST6Gal.1. α2,6-sialylation exerts opposite effect on of β1-integrin binding to extracellular substrates in colon cancer and in myeloid cells that is, it increases the adhesion (124, 126, 129, 130) and the expression of β1-integrins on the surface (126) of colon cancer cells while it decreases adhesion in myeloid cells (131, 132). A recent study (133) has provided the thermodynamic basis for the increased binding to fibronectin of desialylated β1-integrins. However, it is not clear how to reconcile these data with the observed increased binding of sialylated fibronectin in colon and breast cancer cells. A stronger binding of α2,6-sialylated β1-integrins to extracellular substrates can reinforce integrin-based signal transduction, as suggested by its increased binding to talin (124). In addition, α2,6-sialylation of β1-integrins can play a major role in cancer biology by reducing the binding of galectin-3 (134) a lectin which, in some circumstances, can exert a pro-apoptotic effect (135). Thus, the reduced binding of galectin-3 to ST6Gal.1-expressing cells would prevent their apoptotic death, resulting in increased malignancy. It is interesting to note that breast tumors developed by *ST6GAL1*-null mice (128) exhibited altered expression of genes associated with focal adhesion signaling and had decreased phosphorylation of focal adhesion kinase, a downstream target of β1-integrins.

It has been shown that exposure to ionizing radiations results in increased expression of ST6Gal.1 in both animals and cultured cell lines (136, 137). A causal relationship between high ST6Gal.1 expression and radiation resistance was indicated by the finding that transfection of ST6Gal.1 cDNA in colon cancer cell lines resulted in radiation resistance (137). Increased signaling through α2,6-sialylated β1-integrins is at the basis of this phenomenon (138), because of a stronger activation of paxillin and AKT signaling (139). It is known that the activation of these molecules leads to cell survival and to the activation of radiation-resistance pathways (140). These data depict a scenario in which increased expression of ST6Gal.1, by activation of the Ras pathway as discussed in section 5.1 or by other means leads to α2,6-sialylation of key membrane receptors, including β1 integrins, which convey activation and survival signal to cancer cells.

3.4. T, Tn and sialyl-Tn antigens

These low molecular weight sugar antigens derive from an incomplete synthesis of *O*-linked chains (25, 141, 142) (Figure 3). The Tn antigen is formed by a GalNAc linked to Serine or Threonine. This sugar can be substituted by α2,6-linked sialic acid, leading to the formation of sialyl-Tn antigen, or by a β1,3-linked galactose, forming the Thomsen-Friedenreich (T) antigen, or by a β1,3-linked GlcNAc, forming the core 3 structure. The β1,3-galactosyltransferase which mediates the formation of the T antigen (T-synthase) is peculiar because it requires the presence of a molecular chaperone, the product of the gene Cosmc (143) which, in the endoplasmic reticulum, binds to T synthase preventing its ubiquitin-mediated proteosomal degradation (144). While the presence of core-3 based glycans prevented colitis and colorectal cancer in a murine model (145), the expression of Tn, sTn and T antigens has often been correlated with cancer progression (146). During neoplastic transformation of breast epithelium, mucin glycosylation undergoes a characteristic switch from the expression of core 2 structures to accumulation of T (147-150) and sialyl-Tn structures (150, 151), (reviewed in (152)). In normal colonic tissues, T antigen is not expressed (153-155) because it is masked by sialylation (156). On the contrary, it is expressed by the majority of colon carcinoma specimens (153, 155) and by an even higher percentage of liver metastases (157). A molecular basis for the relationship between T-antigen expression and metastasis was provided by the observation that the interaction between this carbohydrate structure and galectin-3 could mediate both the homotypic aggregation of cancer cells (158, 159) and the docking of tumor cells to endothelial cells (160, 161). The homotypic aggregation protects cancer cells from the apoptosis induced by the lack of adhesion to extracellular substrates (anoikis) (159). The interactions between galectin-3 and the T antigen can be specifically inhibited by peptides (162, 163) which are able to inhibit both homo- and heterotypic cell adhesion and metastasis. In addition, the presence of cancer cells expressing the T antigen has been shown to induce the expression of galectin-3 by endothelial cells (164). Altogether, these data point to the interaction between galectin-3 and T antigen as an important determinant of cell malignancy (165). The exposure of the T antigen by colonic cells might be *per se* at the origin of proliferative signals. In fact, the binding to the T antigen of mitogenic dietary lectins (such as peanut lectin) results in increased cell proliferation (166) through stimulation of c-Met and MAPK (167); a similar stimulatory effect could be obtained by anti T antibodies (168). T antigen appears to be a possible target for cell-mediated anti cancer immunity. In fact, its expression increased NK susceptibility of cancer cells (169), while peptides containing the T antigen were able to elicit a specific and MHC class-I-restricted anti-tumor CTL response (170, 171). Moreover, anti-T antibodies were able to inhibit lung metastasis formation by breast cancer cells (172).

Despite the fact that breast cancer tissues often accumulate T antigen, ST3Gal.1 the enzyme which synthesizes sialyl T antigen, is usually elevated in breast cancer (173). The relationship between over-expression of ST3Gal.1 and breast cancer progression has recently been studied in a murine breast cancer model over-expressing ST3Gal.1 under the control of the MUC1 promoter (174). In ST3Gal.1 over-expressing mice, tumors developed with a shorter latency. However, this effect did not appear to be mediated by the accumulation of the sialyl-T antigen but, rather, by the mere over-expression of the sialyltransferase, suggesting the possibility that the enzyme acts as a tumor promoter (174). Interestingly, an elevation of ST3Gal.1 mRNA was reported also in bladder cancer specimens (175). The role of this modification in the biology of bladder cancer remains to be established.

Sialyl-Tn antigen is expressed by many malignancies, including stomach (176), liver (177), pancreas (178). In particular, in breast cancer its expression

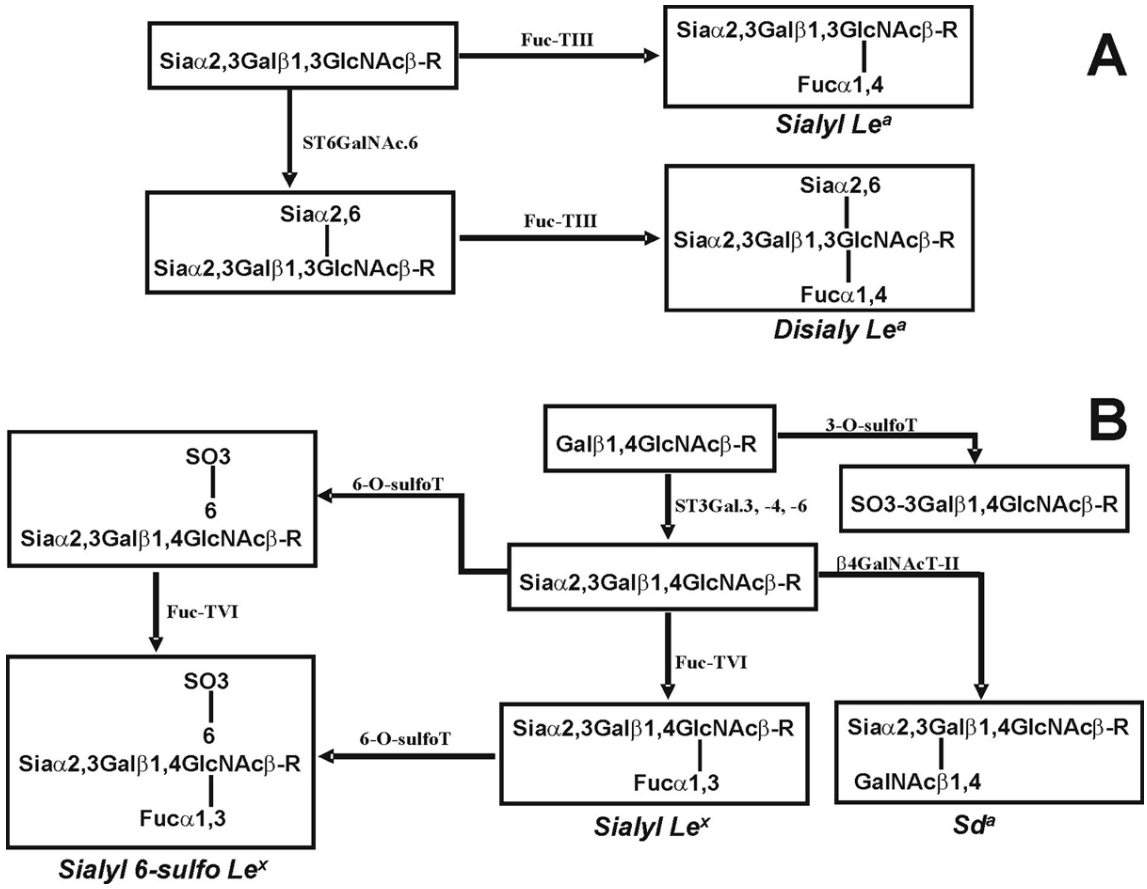

Figure 4. Alternative biosynthetic pathways in colonic tissues. A, type 1 chains: normal colonic mucosa expresses mainly disialyl Le^a antigen, while in colon cancer sialyl Le^a predominates (264). The biosynthesis of the former antigen proceeds from α2,6-sialylation mediated by ST6GalNAc.6, followed by α1,4-fucosylation, mediated by Fuc-TIII. In colon cancer, the expression of ST6GalNAc.6 is down-regulated, thus only sialyl Le^a can be synthesized. B, type 2 chains: the sialyl Le^x antigen is expressed at a much higher level in colon cancer than in normal colonic mucosa. On the contrary, the Sd^a (76), the sialyl 6-sulfo Le^x (77) and the 3-sulfo Le^x (266) antigens are strongly expressed by normal colon and poorly expressed in cancer. 3-O sulfation prevents the formation of α2,3-sialylated lactosamine, which is the precursor of the Sd^a, sialyl 6-sulfo Le^x and of sialyl Le^x antigens The down-regulation of β4GalNAcT-II and of 6-O-sulfotransferase contributes to the increased expression of sialyl Le^x.

correlates with a poorly differentiated state (179) and resistance to adjuvant therapy in node-positive patients (180), while in colon cancer, sTn antigen is expressed by most primary tumors and metastasis and correlates with a worse prognosis (153, 157, 181, 182). The significance of sTn antigen as a tumor marker and its association with increased malignancy (183) suggested its use as a cancer vaccine (184, 185). Theratope is the commercial name given a conjugate formed by the sialyl-Tn disaccharide chemically linked to a highly immunogenic protein carrier. When administered to metastatic breast cancer patients, the conjugate induced an humoral as well as a cellular anti-cancer response. The protective effect of Theratope has been confirmed in a murine model of breast cancer (186).

The biological effects of sTn over-expression have been studied in cells over-expressing sialyltransferase ST6GalNAc.1, which is the major sTn synthase (187). In murine carcinoma cells, ST6GalNAc.1 over-expression led to sTn expression on β1-integrins, to major morphological changes and to reduced ability to migrate on fibronectin and hyaluronic acid (188). On the contrary, ST6GalNAc.1 over-expression in human breast cancer cell lines resulted in the expression of sTn antigen on MUC1 and other high molecular weight glycoproteins; this was associated with reduced cell adhesion and increased cell migration (189, 190).

T and sialyl Tn antigens are carried mainly by a high molecular weight splice variant of CD44 (191) and MUC1 (192-194) in colon cancer, by MUC2 in gastric cancer (176) and by MUC1 in breast cancer (194).

A general mechanism which has been proposed to be at the basis of the over-expression of Tn and sTn antigens in cancer is based on the somatic inactivation of the gene Cosmc which, in colon cancer and melanoma cell lines is associated with the expression of Tn and sTn antigens (143). These data confirm a previous observation (195) reporting that the down regulation of a carbohydrate

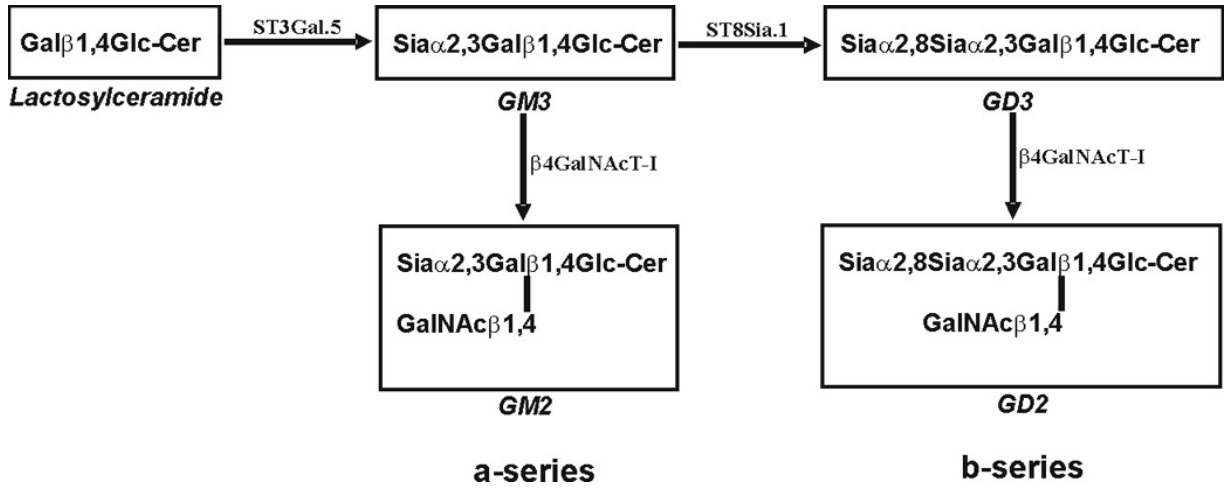

Figure 5. Simplified representation of ganglioside structure and biosynthesis. Sialylation of lactosylceramide, mediated by ST3Gal.5, leads to the formation of GM3, which is the founder of a-series gangliosides. The addition of a second, α2-8-linked sialic acid to GM3 by ST8Sia.1, yields GD3, which is the first member of b-series gangliosides. The addition of GalNAc on either GM3 or GD3 yields GM2 and GD2, respectively and is mediated by the same enzyme: β4GalNAcT-I (GM2/GD2 synthase). The structure and biosynthesis of higher gangliosides and of c-series gangliosides are omitted for simplicity.

structure can deviate the glycan biosynthesis towards alternative structures.

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3.5. Gangliosides

Gangliosides are sialic acid-containing glycolipids, whose expression is often deranged in cancer cells (4). In particular, accumulation of ganglioside GD3 is characteristic of melanoma (196-198), while accumulation of GD2 characterizes neuroblastoma (199). Gangliosides may play a role in cancer biology not only as cell surface structures (200), but also as molecules shed by the tumor, which can exert an immunosuppressive effect by sensitizing T lymphocytes to apoptosis (201). On the cell membrane, gangliosides are organized into complex structures known as glycosynapses, regions of the cell membrane where glycoconjugates are clustered with growth factor receptors and adhesion receptor (200, 202). The relevance of gangliosides as cancer-associated molecules has suggested their use as target for anti-cancer immunotherapy (203).

Key steps in ganglioside biosynthesis (Figure 5) are represented by the α2-3-sialylation of lactosylceramide, catalyzed by ST3Gal.5 (GM3 synthase), which results in GM3 synthesis, and by the α2-8 sialylation of GM3 by ST8Sia.1 (GD3 synthase), which yields GD3. GM3 and GD3 are the founders of the a- and b-series gangliosides respectively and are transformed in GM2 and GD2 respectively by the action of the same enzyme: β1,4GalNAcT-I (GM2/GD2 synthase). As a general rule, it appears that malignancy is positively associated with the expression of GD3 (25, 204-206) and negatively with that of GM3 (207-210). Expression of GD3 enhances *in vivo* growth and metastasis formation (211-215) through mechanisms involving interactions with both, receptors for growth factors such as c-Met (216) and receptors for the extracellular matrix, such as integrins (217). Interestingly, this interaction takes place between the carbohydrate portion of GD3 and the sugar chains of integrins (217). ST8Sia.1 transfection of a breast cancer cell line resulted in the activation of c-Met in the absence of its ligand, the hepatocyte growth factor (also known as scatter factor). This, in turns, resulted in the activation of both the PI3/AKT and MAPK signaling pathways (216). GD3 expression is necessary for VEGF signaling (215), and results in the activation of signaling molecules, such as focal adhesion kinase, paxillin and p130Cas (218, 219) and eventually of the Ras/MEK/ERK pathway (220). The over-expression of the tumor suppressor molecule caveolin-1 displaces GD3 from lipid rafts and inhibits paxillin and p130Cas activation, resulting in an attenuation of the malignant phenotype (221). The promoter region of GD3 synthase (ST8Sia.1) contains putative binding sites for transcription factors c-Ets-1, CREB, AP-1 and NF-kB (222), but appears to be regulated mainly by NF-kB (223).

The negative effect of GM3 on cell growth and malignancy is mediated through different mechanisms. It down-regulates EGF signaling (224) by interacting with its carbohydrate portions (225, 226). Moreover, GM3 inhibits VEGF (227), and PDGF (228) signaling, it associates with ErbB2 (229) and stimulates the expression of the cell cycle inhibitors p21(WAF1) and p27(kip1) through the tumor suppressor phosphatase PTEN (230). In addition, fibroblasts from ST3Gal.5-KO mice, lacking GM3 and a-series gangliosides, display a highly activated state of the MAPK pathway (231). However, these cells lack also GD3 and other b-series gangliosides; this indicates that the mere absence of GM3 in the absence of GD3 is sufficient to derange the MAPK pathway. GM3 induces apoptotic death in neuronal cells (232) and, in association with tetraspanin CD82, is able to inhibit c-Met signaling and its cross-talk with integrins, resulting in reduced cell motility (233, 234). In considering the relative contribution of GD3 and GM3 to the neoplastic phenotype, it should be kept in mind that a

near complete ganglioside depletion in oncogene-transformed cells resulted in a dramatic inhibition of *in vivo* growth in syngeneic animals (235). Altogether, these results suggest that either the absence of GM3 or the over expression of GD3 are sufficient to exacerbate the neoplastic phenotype of cancer cells.

Beside the many studies indicating a role of GM3 in the attenuation of the neoplastic phenotype and of GD3 in its exacerbation, a few studies report that in given condition the opposite may happens (236-238). Very recently, it has been shown that over-expression of sialyltransferase ST6GalNAc.5 in glioma cells led to deranged expression of complex gangliosides, including increased expression of GM3 (239); these changes were associated with reduced malignancy.

4. MECHANISMS LEADING TO ALTERED GLYCAN STRUCTURES

Different mechanisms can account for the cancer-associated altered glycosylation pattern. These include the derangement of biosynthetic enzymes (e.g. glycosyltransferases, sugar nucleotide transporters) or of sugar degrading enzymes (e.g. glycosidases) and the masking of sugar epitopes by substituent groups. Examples of deranged glycosyltransferase expression have been provided in the preceding sections. In this chapter we will discuss other mechanisms.

4.1. Altered glycosidase expression

The best example of altered glycosidase activities in cancer is provided by Neu sialidases, a group of four enzymes (Neu1, lysosomal; Neu2, cytosolic; Neu3, cell membrane and Neu4, mitochondrial) showing marked alterations in cancer (240). Neu1, which is specific for oligosaccharides and glycopeptides, shows marked down-regulation in cancer, promoting anchorage-independent growth and metastatic ability. Over-expression of this enzyme in murine melanoma cells led to reversion of the malignant phenotype (241). Over-expression of cytosolic Neu2 also led to reduced invasion of cancer cells and to a concomitant reduction of sialylated molecules, such as GM3 and sLe^x (242). The ganglioside-specific, cell membrane-associated Neu3 displayed up-regulation in cancer. The mechanism through which Neu3 promotes cancer growth is related to inhibition of apoptosis through increased Bcl-2 and decreased caspase expression (243-245). The signal leading to apoptosis inhibition is originated at the plasma membrane and involves a potentiation of the EGFR signaling, resulting in the activation of the Ras cascade (243).

4.2. Masking of sugar structures by substituent groups

Cancer-associated sugar structures, such as sLe^x/sLe^a and sTn might be expressed also by normal tissues but their recognition by monoclonal antibodies can be hindered by substituent groups. In normal colon, the recognition of the sTn antigen is hindered by the O-acetylation of sialic acids. In fact, after de-acetylation of the samples by alkali treatment, the antigen appeared to be expressed at similar level by normal mucosa and colon cancer (246). However, this treatment rarely increased sTn expression in normal and neoplastic gastric and pancreatic tissues, suggesting that different mechanisms might be at the basis of the increased sTn expression in different tissues. O-acetylation of sialic acid plays a relevant role also in masking sLe^x in normal colonic tissues, in that after alkali treatment the expression of sLe^x carried by mucins in normal mucosa equaled that of cancer tissues (247). Our recent data (74, 76) confirms the expression of sLe^x in normal mucosa after de-acetylation of the samples. However, in cancer tissues the expression of sLe^x remained higher than in normal mucosa even after removal of O-acetyl groups (74).

Another example of the masking of a cancer-associated carbohydrate antigen is represented by the addition of a sulfate group linked to the 6 position of the GlcNAc residue of sLe^x in normal colonic mucosa, generating the sialyl 6-sulfo $Lewis^x$ antigen (Figure 4) (77). The presence of this antigen, which is highly expressed in non malignant colonic mucosa but poorly or not expressed by colon cancer (77), contributes to explain the low sLe^x expression in normal colon.

4.3. Altered expression of sugar and sugar nucleotide transporters

During the biosynthesis of the glycoconjugates, the addition of the more distal sugars (sialic acid, fucose, galactose) in the Golgi apparatus by the respective glycosyltransferases requires the availability of the appropriate sugar nucleotide donors inside the Golgi cisternae. These compounds are actively transported from the cytoplasmic side to the luminal side of the Golgi membranes by specific sugar nucleotide transporters. An involvement of a UDP-galactose transporter in the regulation of the expression of the cancer associated antigens T, sLe^a and sLe^x was indicated by a study showing that the mRNA of this transporter was up-regulated in colon cancer tissues, compared with normal mucosa (248). Transfection of this cDNA in SW1083 colon cancer cells resulted in elevation of T and sLe^a but not of sLe^x antigen, whereas transfection in SW480 cells resulted in little or no changes in the expression of T and sLe^a antigens but in strong elevation of sLe^x. This cell line-specific effect on sugar antigen biosynthesis of the UDP-galactose transporter suggests that it might be a limiting factor in the sugar antigen biosynthesis in some cell lines but not in others. Other examples of the influence of transporters on the biosynthesis of cancer-related sugar antigens are provided by the sialic acid transporter sialin (249) (discussed in section 5.2) and by the sulfate transporter DTDST (250), which is necessary for the biosynthesis of the sialyl 6-sulfo $Lewis^x$ antigen (section 4.2 and Figure 4). The down-regulation of this gene in colon cancer tissues (250) provides a molecular basis for the reduced expression of the sialyl 6-sulfo $Lewis^x$ antigen in colon cancer and for the concomitant over-expression of sLe^x (77).

4.4. Competition between normal and cancer-associated carbohydrate structures

The expression of a given carbohydrate antigen can be regulated by the level of expression of enzymes

synthesizing alternative structures. In this chapter we will discuss some examples of this mechanism. A first example is provided by the competition between GnT5 and GnT3 in the biosynthesis of the *N*-linked chains (reviewed in (251)) (Figure 1). The addition of the bisecting GlcNAc inhibits the addition of the β1,6-branched chain (252, 253) and consequently, the elaboration of the polylactosaminic chains and of the terminal carbohydrate antigens (such as sLe^x). The main substrates of GnT3 are integrins (254), EGFR (255) and E-cadherin (256, 257). A negative effect of bisecting GlcNAc on cancer growth is suggested by several studies. For example, the addition of a bisecting GlcNAc on E-cadherin led to a down-regulation of tyrosine phosphorylation and to an altered localization of β-catenin after EGF stimulation (258), while the expression of GnT3 suppressed lung metastases of melanoma cells (259). Conversely, down-regulation of Wnt/β-catenin signaling led to reduced GnT3 expression and down-regulation of bisecting GlcNAc on β1-integrins (260). However, it has also been reported that under some circumstances bisecting GlcNAc can promote cancer growth. For example, in B16 melanoma cells expression of GnT3 led to the formation of bisecting structures on CD44 (261) which, in turns, led to increased adhesion to hyaluronate and increased tumor growth and metastasis. Moreover, circulating glycoproteins bearing bisecting GlcNAc promote hepatocyte proliferation (262). Nevertheless, the bulk of data supports the view that cell surface receptors modified by bisecting GlcNAc exert an inhibitory effect on cancer cell growth, in part by inhibition of the β1,6-branching.

Another example of competition between normal and cancer-associated structures is provided by the alternative presence of disialyl $Lewis^a$ or sLe^a antigens in normal and cancer colon, respectively (Figure 4) (263). The final steps of disialyl $Lewis^a$ biosynthesis, which is expressed mainly by normal mucosa and serves as ligand for the sialic acid binding inhibitory receptor Siglec-7 expressed by lymphoid cells (264), is mediated by the coordinate action of sialyltransferase ST6GalNAc.6 and fucosyltransferase 3 (Fuc-TIII) (265). Owing to the fact that sLe^a is not a substrate of ST6GalNAc.6, the biosynthesis of disialyl Le^a can proceed only through the α2,6-sialylation of the GlcNAc residue of NeuAcα2,3Galβ1,3GlcNAc, followed by the Fuc-TIII-mediated addition of an α1,4-linked fucose (Figure 4). Thus, ST6GalNAc.6 and FucT-III contribute to the biosynthesis of this antigen in a manner that is at the same time cooperative and competitive. In fact, even though the contribution of both enzymes is necessary for the elaboration of this antigen, when the activity of ST6GalNAc.6 is not adequate (as occurs in colon cancer), only sLe^a antigen is synthesized.

Many of the studies on tissue expression of carbohydrate antigens utilized monoclonal antibodies. One of the few chemical analysis of the carbohydrate structure of mucins from normal and cancer colon reported the prevalence of a 3-sulfo $Lewis^x$ structure in which the α2,3 linked sialic acid of sLe^x was replaced by a sulfate group (266). In cancer mucin, this 3-sulfo Le^x structure was down-regulated and sLe^x became predominant (266).

Another "normal" carbohydrate antigen whose down-regulation in cancer might be responsible for the expression of cancer-associated structures is the Sd^a antigen. This antigen is formed by a GalNAc β1,4-linked to the galactose residue of α2,3-sialylated lactosamine (Figure 4). The addition of this GalNAc residue is mediated by β4GalNAcT-II (also known as CT GalNAc transferase) (267), product of the B4GALNT2 gene. The enzyme is expressed at a very high level by normal colonic mucosa but is dramatically down-regulated in colon cancer (76, 268, 269). *In vitro* studies have shown that forced expression of this enzyme in colon and stomach cancer cell lines expressing the sLe^x or sLe^a antigens, resulted in the expression of the Sd^a antigen and in a dramatic down-regulation of sLe^a/sLe^x antigens (75, 76). This change was reported to be associated with a complete (stomach cells) or near complete (colon cells) loss of the metastatic potential (75). Moreover, structural studies have shown that the Sd^a or the sLe^x antigens can be expressed by colonic mucins in a mutually exclusive manner (270), in that the structures containing the β1,4-linked GalNAc on galactose did not contain the α1,3/4 fucose on GlcNAc. Altogether, these data strongly suggest that the cancer-associated down-regulation of β4GalNAcT-II plays a role in the expression of sLe^x/sLe^a antigens by cancer tissues.

Other glycosyltransferases have shown the potential to down-regulate sLe^x expression upon transfection in cancer cells. An example is provided by α1,2 fucosyltransferase I (Fuc-TI, product of FUT1 gene), whose expression has been reported to inhibit, through a competitive mechanism, the biosynthesis of sLe^x and the binding to E-selectin, without affecting the biosynthesis of sLe^a and P-selectin binding (271, 272). In HepG2 cells, this modification resulted in inhibition of vasculogenesis and tumor growth (63).

Another example is provided by the competition between ST3Gal.1, which synthesizes the sialyl-T antigen and core 2 GlcNAcT-1 (C2GnT1), which synthesizes core-2 branching (Figure 3) (273). The distribution of these enzymes along the Golgi apparatus displays a certain degree of overlapping, with C2GnT1 more proximal and ST3Gal.1 more distal. Transfection experiments have indicated that when ST3Gal.1 was increased, as occurs in breast cancer, the *O*-glycans of MUC1 became dominated by core 1 structures, even in the presence of C2GnT1 expression (273). Consistently, in the human colon cancer cell line SW480, C2GNT1 expression led to down-regulation of T antigen expression (274). In rat colon cancer cells, the expression of sTn appears to be controlled by the balance between the α2,6-sialyltransferase which synthesizes sTn and the GlcNAcTs which synthesize core 2 structures (275).

5. MECHANISMS OF REGULATION OF GLYCOGENES

Genes whose products are involved in the biosynthesis, degradation or recognition of carbohydrate chains can be referred to as "glycogenes". In this section

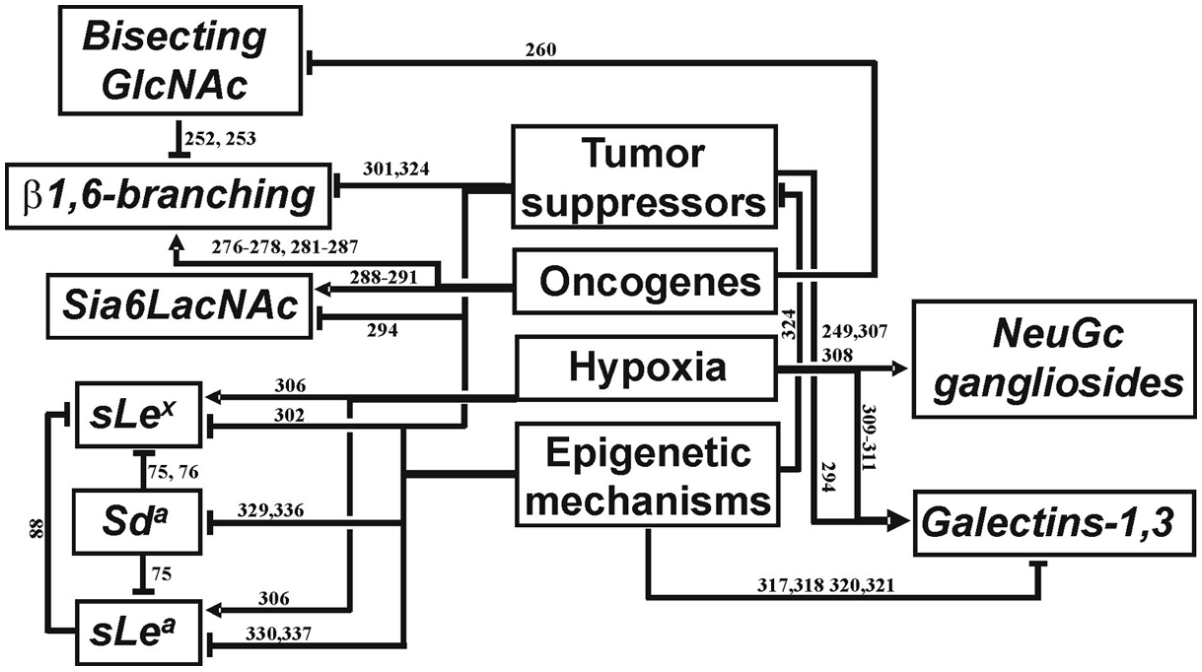

Figure 6. Control of glycosylation by the mechanisms regulating cell growth. Centrifugal relationships link oncogenes, tumor suppressor genes and hypoxia, sometimes through epigenetic mechanisms, to cell membrane structures, such as sugar antigens and galectins (italicized). Frequently, these relationships are mediated through glycosyltransferase expression. Numbers indicate the most relevant references supporting the indicated relationships.

we will discuss the mechanisms of regulation of these genes, with focus on glycosyltransferases and galectins.

5.1. Regulation of glycogenes by oncogenes and tumor suppressor genes

From the early papers published in the '80s, it turned out that transfected (276-278) or virally expressed (279, 280) oncogenes induced an increased size of the *N*-linked chains due mainly to increased β1,6 branching and increased expression of polylactosaminic chains. Among the oncogenes able to induce this effect were Ras (276-278), vfps/fes but not myc (278), while among viruses were polyoma (280) and Rous sarcoma viruses (279). The relationship between altered glycosylation and altered signal transduction is bidirectional. In fact, on the one hand the cancer-associated alterations of the signal transduction pathways frequently lead to increased expression of specific glycosyltransferases, resulting in altered glycosylation pattern (centrifugal relationship) (Figure 6). On the other hand, cancer-associated glycans expressed on cell membrane receptors can modify the cell signaling, resulting in the modulation of the basic properties of cancer cells (centripetal relationship, Figure 7). In the first case the alteration of the glycosylation pattern is the consequence of altered signaling, while in the second is the cause. Examples of the first relationship are mainly provided by MGAT5 and ST6GAL.1 genes, whose transcription is under the control of the ras/MAPK/Ets-1 pathway. MGAT5 is up-regulated by src (281), ErbB2 (282), v-sis (283) and Ras (284, 285) oncogenes, through Ets-1 (286, 287). Also sialyltransferase ST6Gal.1 is regulated by both N-ras and H-ras through RafGEF signaling (288-291). Other glycosyltransferases involved in increased branching of *N*-linked chains, such as galactosyltransferase-1 (292) and galactosyltransferase-5 (293) are under the control of Ets family members of transcription factors. Altogether, these data suggest that different upstream agents, signaling through the Ras pathway, cooperate in determining an increased size of *N*-linked chains.

In the pancreatic cancer cell line Capan1, expression of the tumor suppressor $p16^{INK4a}$, which encodes an inhibitor of cyclin D CDK4/6 complex, restored the cell susceptibility to anoikis, and a profound alteration of the glycosylation machinery (294). $p16^{INK4a}$ induced increased expression of the fibronectin receptor integrin α5β1, altered expression of galactosyltransferase genes, down-regulation of α2,3-sialylation of *O*-linked chains and of α2,6-sialylation of *N*-linked chains. Decreased cell sialylation was accompanied by increased expression (and binding) of the pro-anoikis galectin-1, which interacts with the sugar chains of α5β1 integrin (295), while the expression of the anti-anoikis galectin-3 was decreased (296). Both, galectin-1 (297) and galectin-3 reinforce Ras-signaling (298) by directly interacting with Ras proteins. Through potentiation of the Ras signaling, cancer-derived galectin-1 facilitates cancer growth by stimulating the pro-angiogenic activity of endothelial cells (299). In addition, galectin-3 activity is regulated by c-abl-mediated phosphorylation of specific sites (300).

The metastasis-suppressor gene nm23-H1 has been reported to down regulate several glycosyltransferases involved in the biosynthesis of metastasis-associated

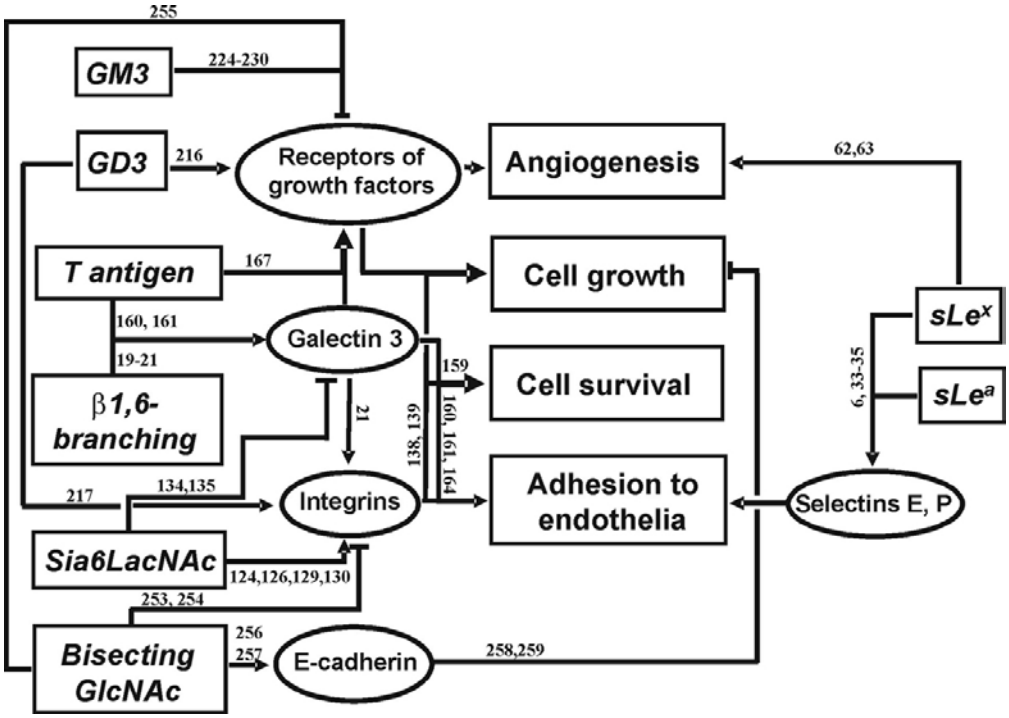

Figure 7. Control of the cancer cell phenotype by glycosylation. Centripetal relationships link cancer-associated carbohydrate structures and prominent features of the cancer cell phenotype. Signals originated by the cancer-associated sugar structures (italicized) on the cell membrane converge on a few "hubs"(ovals) which convey signals inside the cell, resulting in increased cell growth and survival, angiogenesis and adhesion to endothelia. Numbers indicate the most relevant references supporting the indicated relationships.

structures, including GnT5 (301) as well as fucosyltransferases and sialyltransferases involved in sLe^x biosynthesis (302) and to inhibit integrin glycosylation resulting in reduced cell surface expression of β1 integrins (303). These effects are likely to play a role in the nm23-H1-induced reduction of the metastatic ability of cancer cells.

5.2. Glycosylation changes and hypoxia

Owing to the irregular and insufficient blood supply, large parts of a growing tumor can undergo hypoxia (insufficient oxygen supply). Cells respond to hypoxia through the hypoxia inducible factor (HIF), a dimeric transcription factor responsible for the transcription of several genes whose products compensate for the hypoxic conditions (304). The regulatory subunit of HIF is known as HIF-1α. The genes positively regulated by HIF-1α include those involved in angiogenesis, anaerobic metabolism, erythropoiesis and cell motility. In addition, recent findings have shown that also some glycosylation-related genes are under the control of HIF-1α (305). Colon cancer cell lines kept either in hypoxic conditions or in the presence of the hypoxia-mimic drug desferioxamine exhibited an increased expression of the selectin ligands sLe^x and sLe^a (306). The transcription of the mRNA of two glycosyltransferases potentially involved in selectin ligand biosynthesis, ST3Gal.1 and Fuc-TVII, was found to be under the control of HIF-1α and was increased in colon cancer tissues (306), suggesting that this increase is responsible for the augmented selectin ligand biosynthesis. However, the level of the FucT-VII transcript in colonic tissues is extremely low (72-74), consequently the contribution of this enzyme to sLe^x biosynthesis in colonic tissues is at least uncertain. Another gene stimulated by hypoxic conditions is that encoding for sialin, a sialic acid transporter (249). The over-expression of sialin resulted in increased expression of gangliosides containing the non-human sialic acid N-glycolyl-neuraminic acid (NeuGc). The presence of this non-human sugar in human cancer tissues and cell lines is due to the uptake from the diet or from bovine serum, respectively (307). These studies suggest that hypoxic conditions of tumor growth enhance incorporation of non-human sialic acid in gangliosides (308). Hypoxic conditions influence also the expression of sugar binding molecules such as galectin-1 (309). HIF-induced expression of galectin-1 in head and neck squamous cell carcinomas led to a reduced presence of tumor infiltrating lymphocytes, probably because of the known ability of galectin-1 to induce apoptosis of T lymphocytes (310). In colorectal cancer cell lines, HIF-dependent expression of galectin-1 is responsible for increased migration and invasion (311), indicating that this lectin is an important mediator of the effects of tumor hypoxia on cancer growth.

5.3. Epigenetic regulation

The best known examples of epigenetic regulation of gene expression include DNA methylation of

CpG islands in gene promoter regions, chromatin alterations (i.e. histone acetylation, methylation, and ubiquitylation) and the expression of non-coding RNAs (312-314). The relevance of these modifications in cancer progression is increasingly recognized and the mutual interplay among these mechanisms is emerging as a novel paradigm of gene regulation (315). Many glycogenes appear to be aberrantly regulated in cancer because of epigenetic mechanisms (305, 316), including galectins (317-321), enzymes involved in the biosynthesis of sugar nucleotides (322, 323), transporters (250) and glycosyltransferases (264, 324-333). However, many of the cited studies were restricted to the investigation of the methylation status of the promoter region, usually through the use of the demethylating agent 5-aza-2-deoxycytidine (5-aza-dC). However, recent studies on hypermethylated tumor-suppressor promoters have reported only partial reactivation upon treatment, because they maintain several repressive histone modification marks (334, 335). Interestingly, a CpG island nearby the putative promoter region of the B4GALNT2 gene was found to be heavily methylated in colon cancer tissues (329), while treatment of colon cancer cell lines with 5-aza-dC resulted only in a partial recovery of enzyme expression (336). A similar behavior is displayed by the native promoter of β3GalT5, which lies in the context of two CpG islands (337). In fact, the activity of this promoter inversely correlated with the methylation status of the CpG islands in different cell lines, but 5-aza-dC treatment resulted in little or no effect on gene expression (Caretti, Dall'Olio, Trinchera, unpublished results). These data suggest that other glycogenes might be under the control of epigenetic mechanisms even if poorly responsive to 5-aza-dC. Consequently, the contribution of epigenetics to the regulation of glycosylation could be more relevant, as hypothesized (338).

6. PERSPECTIVE

The availability of genetically manipulated cell lines as well as of transgenic and knock-out mouse strains has allowed to establish unequivocally the causal role played by the cancer-associated glycosylation changes in cancer biology and to establish the multiple links between the cell glycosylation machinery and the signal transduction mechanisms. As depicted in Figure 6, the basic mechanisms controlling cell behavior affect the expression of cell surface carbohydrate structures and carbohydrate binding molecules, through "centrifugal" relationships. However, as depicted in Figure 7, carbohydrate structures on the cell membrane are able to affect the basic properties of cancer cells through "centripetal" relationships". These interactions appear to be integrated by a few types of molecules (including receptors of growth factors, integrins, galectin-3, E-cadherin), acting as "hubs". Glycosylation, like other post-translational modifications, has the potential to "fine tune" the interactions between cells and molecules. The full elucidation of these interactions, which are at the basis of the healthy development of complex organisms and are profoundly altered in cancer, is a major challenge of the post-genomic era and will provide the conceptual basis for therapeutic interventions aimed at the normalization of the cell surface of cancer cells.

7. ACKNOWLEDGEMENTS

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