Glycosphingolipid antigens and cancer therapy

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Specific types of glycosphingolipid (GSL), which are chemically detectable in normal cells, are more highly expressed in tumors. The high level of expression on the surfaces of tumor cells causes an antibody response to these GSLs, which can therefore be described as tumor-associated antigens. Some of these GSLs have been shown to be adhesion molecules involved in tumor cell metastasis, and to be modulators of signal transduction controlling tumor cell growth and motility. Tumor-associated GSL antigens have been used in the development of antitumor vaccines. GSLs and sphingolipids involved in adhesion and signaling are therefore targets for cancer therapy.

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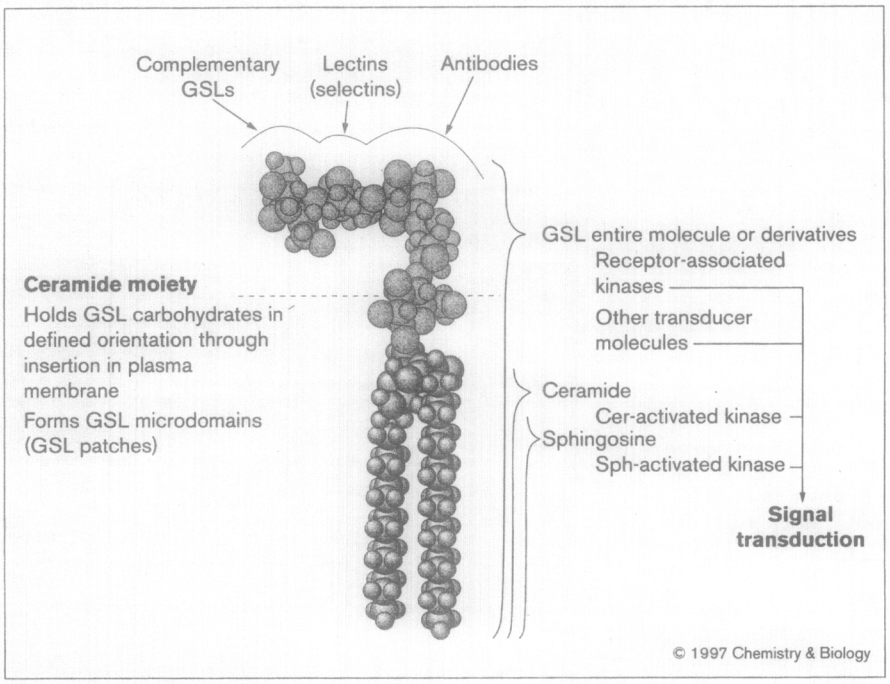
Introduction

The fact that dramatic changes in glycosphingolipid (GSL) composition and metabolism are associated with oncogenic transformation was first shown three decades ago in studies of the *in vitro* transformation of fibroblasts by tumor viruses [1,2]. GSL changes are caused by precursor accumulation resulting from blocked synthesis of complex GSLs or enhanced synthesis of a certain GSL. In either case, changes in glycosyltransferase genes are involved. GSLs are cell-surface antigens, and it was therefore suggested that changes in their composition would result in changes in the antigenicity (ability to bind antibody) and immunogenicity (ability to induce immune response) of the tumor cells expressing them (Fig. 1). The idea of GSLs as tumor-associated antigens is the basis for attempts to utilize GSLs for anticancer vaccine development.

The first clear support for this idea came when rabbit polyclonal antibodies were used to show that the GSL Gg3Cer (gangliotriaosylceramide — GalNAcβ4Galβ4GlcCer) is expressed at high levels only in KiMSV sarcoma cells grown in BALB/c mice, and not in normal cells or tissues [3]. Later, the monoclonal antibody (mAb) approach was introduced in tumor immunology, and many studies focused on identifying the chemical nature of the previously ill-defined 'tumor-associated antigens' that had been identified using polyclonal antibodies. In one set of experiments, mAbs were produced by immunizing mice with human tumors, cloning antibody-producing hybridoma cells, then selecting antibodies that reacted with the tumor cells (but not with normal cells or tissues). These mAbs were claimed to react specifically with the tumor cells. Surprisingly, however, many such mAbs were directed to GSLs, which are expressed in normal cells as well, although the quantity and degree of expression are much lower in normal cells. Typical examples of these antigenic GSLs are shown in Tables 1 and 2. Many of these antigens have since been chemically synthesized [4–13].

In a second set of more critical experiments, rats or mice were immunized with tumors derived from genetically identical (syngeneic) animals. Animals are usually tolerant to their own cells, and cannot raise antibodies to antigens they are tolerant to. Therefore, the antibodies that are raised using this experimental protocol should react only with antigens that are unique to the tumor. Nevertheless, several such antibodies did react with GSLs in the tumor despite the fact that these are present ubiquitously in normal tissues. For example GM3 was identified as the melanoma-associated antigen in mice, hamsters and humans [14], Gb3 (globotriaosylceramide, Gal-α4Galβ4GlcCer) and IV^{3}-αGaInLc$_4$Cer (Galα3Galβ4GlcNAcβ3Galβ4GlcCer)

Figure 1



Conformational structure of globoside, a typical GSL antigen. Note that the axes of the two hydrophobic tails (ceramide, consisting of sphingosine and fatty acid) are oriented perpendicular to the carbohydrate chain. Ceramide holds GSL in the membrane such that carbohydrate chain is presented to ligands (lectins, selectins, antibodies, complementary GSLs). GSLs themselves, ceramide and sphingosine derivatives can all affect transmembrane signaling through activation or inhibition of various kinases involved in the signaling system.

were identified as antigens in fibrosarcoma KMT-17 cells grown in syngeneic WKA rats [15], and extended Le^x, which includes Le^x–Le^x, was similarly identified in F9 teratocarcinoma in mice (Tables 1,2) [16].

These results suggest that although GSLs are present in both tumors and normal tissue, they act as efficient immunogens only when they are present at the surface of tumor cells. In the examples above, GM3, Gb3 and Le^x were chemically detectable in normal tissue, but were not immunogenic and not detectable by an antibody binding assay. There are two possible explanations for the unexpected ability of the rats and mice to mount antibody responses against GSLs. The GSLs found on tumors may be antigenically different, in other words they may react with different antibodies from the GSLs found on normal cells. Alternatively, they may be immunogenically different; this would imply that they are able to elicit an immune response that normal GSLs cannot, and to which the animal is therefore not tolerant.

The difference in the antigenic behavior of tumor GSLs may be explained by the arrangement of GSLs in the membrane. Electron micrographic studies indicated that GSLs are not homogeneously distributed at the outer leaflet of plasma membrane, but that they cluster to form 'GSL patches' or detergent-insoluble 'GSL microdomains' (Fig. 2). The mAb M2590, which reacts specifically with mouse, hamster and human melanoma but not with normal melanocytes or other normal cells or tissues, recognizes GM3 in the membrane only when it is above a threshold density level [14]. Many other tumor-associated GSL antigens, are at high density in tumors and may be organized in microdomains at the tumor cell surface. This leads to the important idea that it is the density of tumor-associated GSLs in tumor cell membranes, above a certain 'threshold' level, that leads to their immunogenicity and antigenicity. Above the threshold value, GSLs cluster to form microdomains at the outer leaflet of tumor cell plasma membrane and either mAbs or the immune system can recognize GSL clusters in which GSL density is above the threshold value. The presence of such microdomains is of fundamental importance for the function of GSLs not only as antigens, but also as adhesion molecules and as modulators or initiators of membrane signaling processes (Fig. 2).

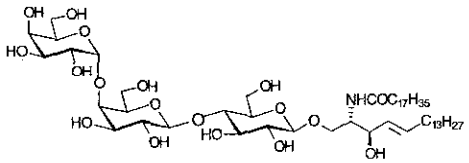
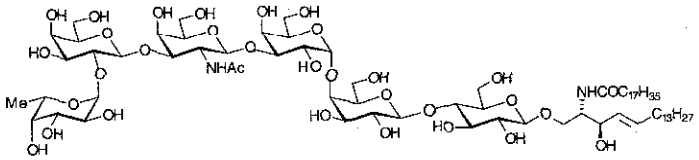
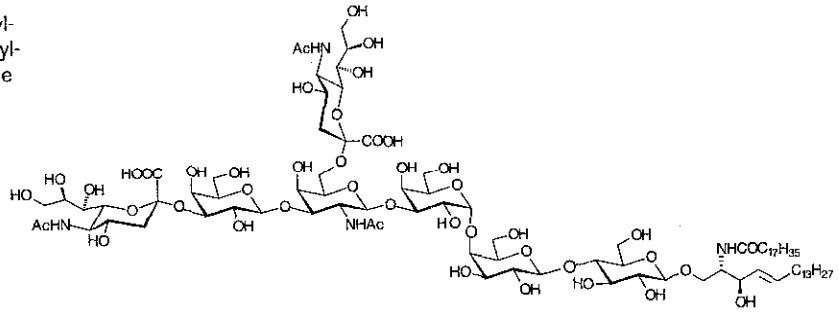
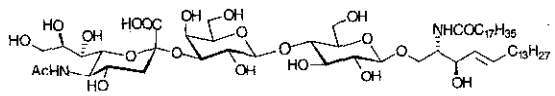
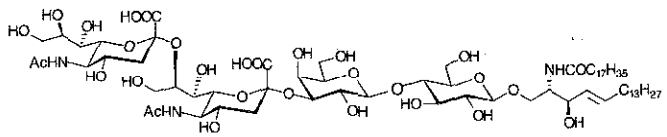
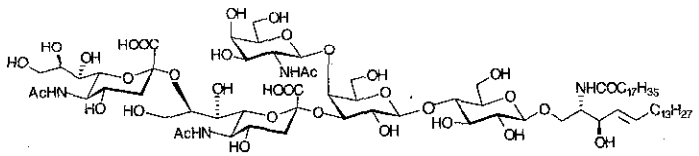
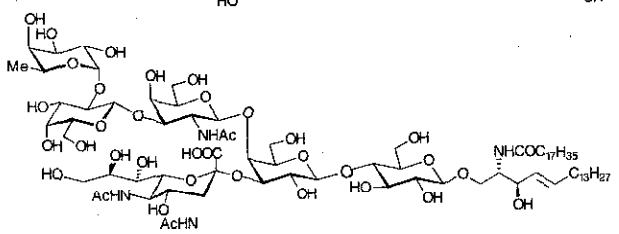
The ability of GSLs at the cell surface to react with antibodies (antigenicity) and/or to elicit a response from the host immune system (immunogenicity) depends not only on the presence of GSL microdomains, but also on the presence of certain other glycoconjugates and cell surface molecules (Fig. 2) [17].

Why do tumors change GSL composition and organization?

Essentially all experimental and human cancers show striking differences in GSL composition and metabolism compared to parental normal cells. They also show changes in N-linked and O-linked glycosylation in glycoproteins, particularly in glycoproteins that are involved in cell

Table 1

Typical tumor-associated GSL antigens.

Glycosphingolipid antigen		Type of cancer where antigen is expressed (reviewed in [43])	Chemical synthesis performed
Globo series			
Gb3		Burkitt lymphoma, ovarian cancer [44]	[4]
Globo H		Breast and ovarian cancer	[5]
Disialosyl-galactosyl-globoside		Renal cell carcinoma [45]	Monosialyl derivative synthesized [6]
Ganglio series			
GM3		Melanoma and many other human and animal cancers	[7]
GD3		Human melanoma	[8]
GD2		Human melanoma and neuroblastoma	[9]
Fucosyl-GM1		Small cell carcinoma of the lung	

Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [46].

adhesion and motility. Abnormal glycosylation in GSLs and glycoproteins is an essential criterion for defining stage, direction and fate of tumor progression. Numerous clinicopathological studies have shown that abnormal glycosylation in a primary tumor is strongly correlated with 5 year or 10 year survival rates of patients [18].

Table 2

Typical tumor-associated GSL antigens.

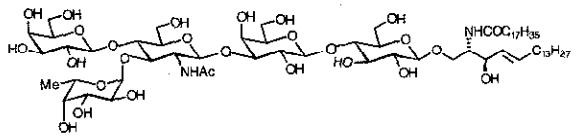
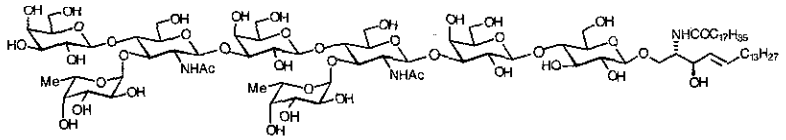
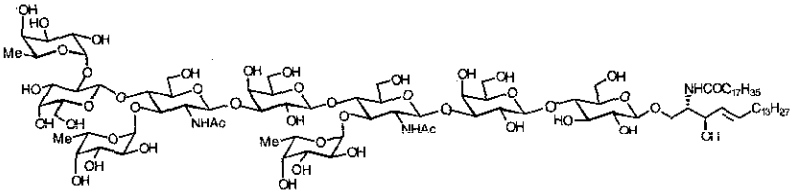
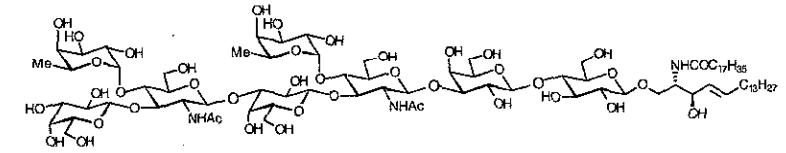
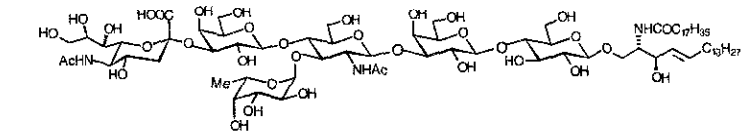
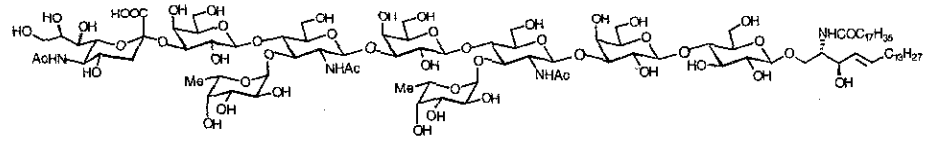
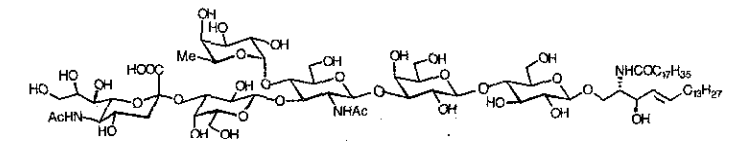
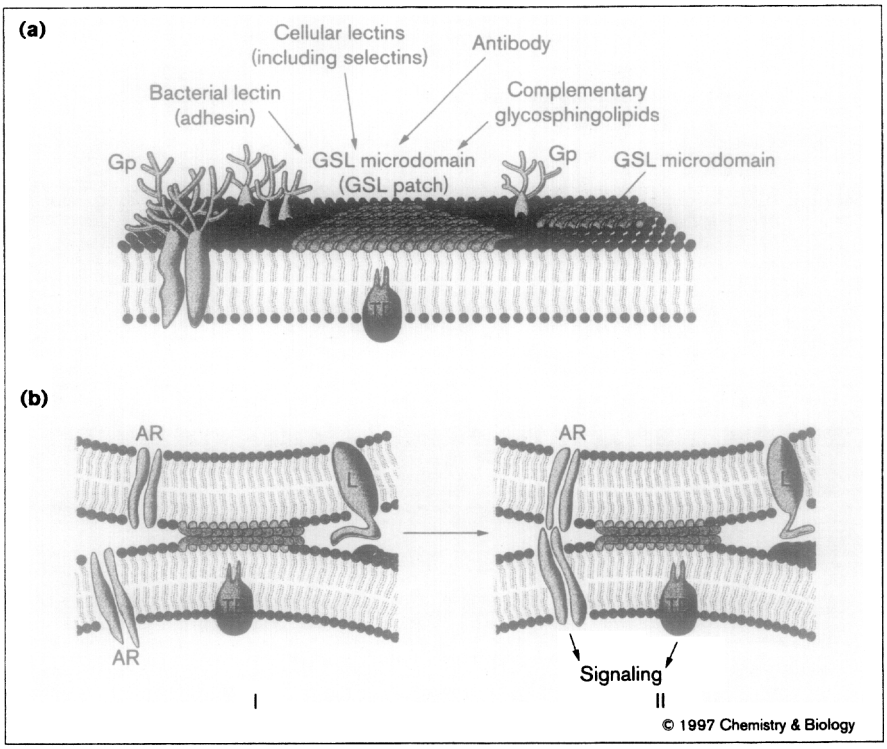
Glycosphingolipid antigen	Type of cancer where antigen is expressed	Chemical synthesis performed
Lacto series		
Le^x 	Gastric, colorectal and breast cancer	[10]
Le^x–Le^x 	Gastric, colorectal and breast cancer	[11]
Le^y–Le^x 	Lung, colorectal and pancreatic cancer	
Le^a–Le^a 	Gastric and colorectal cancer [47]	
SLe^x 	All types of highly malignant cancer	[12]
SLe^x–Le^x 	All types of highly malignant cancer	[13]
SLe^a 	Highly malignant colorectal and pancreatic cancer	

Figure 2



Structure and organization of glycosphingolipid antigens at the cell surface. **(a)** GSLs inserted in the plasma membrane tend to form 'GSL microdomains' when their concentration is above a certain threshold value. GSL 'patches' are observed under electron microscopy with freeze-fracture technique. Glycoproteins (Gp) are arranged in clusters that are separate from GSL microdomains. Recent studies indicate a dynamic interaction between Gp clusters and GSL microdomains, and that transducer (TD) molecules such as Src, Ras, Cak and Rho are sometimes associated with GSL microdomains. The interaction of ligands with GSLs may proceed through the microdomains and affect the function of the transducer molecules. **(b)** Proposed cell adhesion (phase I) initiated through interaction between GSL microdomains, followed by (phase II) adhesion between adhesive receptors (AR) or involvement of lectin (L). Phase I is a quick reaction (complete within a few minutes) that requires only GSL–GSL interaction. Phase II is a slow process involving protein–protein interaction. In phase II, transmembrane signaling may occur through the connection of AR to a cytoplasmic component, or through the connection of TD molecules to the GSL microdomain.

Why is this change in glycosylation so important for the growth of the tumor? It seems clear that aberrant glycosylation in cell-surface receptors modulates their function, controls motility and adhesion, and promotes tumor-cell invasion and progression (see references cited in [18]). The accumulation of large amounts of specific types of aberrantly glycosylated GSLs in specific tumors may thus enhance tumor-cell invasion and metastasis through any or all of four possible mechanisms. First, the GSL itself may be an adhesion molecule which interacts with lectins or selectins (carbohydrate-binding receptors) expressed on the target cell. Second, the GSL may be recognized by another GSL expressed on the metastatic target cell. Third, the GSL may act indirectly, by modulating an adhesion or motility receptor to promote tumor cell invasiveness. Fourth, the GSL and/or its degradation products — including lyso-GSLs, de-N-acetyl compounds, sphingosine and its derivatives and ceramide — may directly trigger transmembrane signaling that enhances tumor-cell motility and invasiveness. Our knowledge of these possible mechanisms is highly fragmentary, however. Only a few cases have been studied, as described below.

GSLs as adhesion molecules and mediators of metastasis

Some GSL antigens that are highly expressed in specific types of human cancer have been identified recently as adhesion molecules that may promote tumor-cell metastasis. A few examples are described below.

GM3 in mouse melanoma B16

The level of GM3 expression is closely correlated with the invasive and metastatic properties of four mouse melanoma B16 variants. B16/BL6 has the highest GM3 expression of these tumor lines, and is also the most invasive and metastatic, followed in descending order by F10, F1 and WA4 (which is non-metastatic and shows minimal GM3 expression) [19]. Recent studies indicate that GM3 can act as an adhesion molecule for two other GSLs, Gg3Cer and LacCer (lactosylceramide (Galβ4GlcCer), which are expressed in non-activated vascular endothelial cells. The metastasis of B16 is thus triggered by the recognition of the GM3 expressed on the melanoma by the Gg3 or LacCer on endothelial cells [19]. This hypothesis is supported by the fact that B16 metastasis is blocked by liposomes containing GM3 or Gg3, or by mAbs raised against GM3 or Gg3 [20].

Disialosyl-galactosylgloboside in renal cell carcinoma

The GSL disialosyl-galactosylgloboside was recently found to be highly expressed in human renal cell carcinoma [21]. The structure is closely related to 'stage-specific embryonic antigen-4' and to GD1α ganglioside (see Table 1). Expression of disialosyl-galactosylgloboside is correlated with the potential of renal cell carcinoma to metastasize to the lung. Cell lines derived from renal cell carcinoma express high levels of disialosyl-galactosylgloboside and adhere strongly to perialveolar lung tissue sections. This adhesion is inhibited by a mAb (RM2) specifically directed

to disialosyl-galactosylgloboside, but not to monosialosyl-galactosylgloboside. A specific (but as yet unidentified) receptor may be present in lung tissue that recognizes renal cell carcinoma [22]. Specific interaction between such a receptor and disialosyl-galactosylgloboside may mediate the metastasis of renal cell carcinoma to the lung.

SLe^x, SLe^a and their analogs as ligands for selectins

The carbohydrate structures SLe^x and SLe^x–Le^x [23], and SLe^a [24] were originally identified as human tumor-associated antigens. Later, they were found to bind to the endothelial cell-surface receptors E-selectin and P-selectin under certain conditions [25–28]. Binding to the endothelium is a necessary step in the process of metastasis, and so this observation explained the fact that tumor cells tend to show increased levels of members of the SLe^x family. Many subsequent extensive studies (e.g. [29,30]) clarified that SLe^x, SLe^a and their analogs are indeed involved in tumor progression in three ways. First, tumor cells that express SLe^x, SLe^a and their analogs, bind to E-selectin in activated endothelial cells [25–29]. These tumor epitopes can be lipid-linked in GSLs or N-linked or O-linked in glycoproteins. Second, leukemic leukocytes show clear binding to P-selectin through a 'scaffold' protein, P-selectin glycoprotein ligand 1 (PSGL-1). Scaffold proteins carrying SLe^x family carbohydrates are important for P-selectin binding of leukemic leukocytes, but not important for P-selectin binding of solid tumors (gastric, breast, colon and lung) [30]. Similarly, many common human cancers of epithelial origin do not bind to P-selectin unless the PSGL-1 gene is also present, even though they may show high levels of expression of SLe^x or SLe^a [30]. Some tumor cells show weak PSGL-1-independent P-selectin binding, however. Third, many tumor cells activate platelets to release an as yet unidentified factor that triggers E-selectin expression in endothelial cells [18].

Any of these three mechanisms or some combination of them would be expected to promote metastasis, primarily by allowing tumor cells to bind to microvascular endothelial cells. Patients with primary tumors expressing SLe^x, SLe^a or their analogs survived for a significantly shorter time than patients whose tumors did not express these epitopes (see [18] for review).

E-selectin and P-selectin were originally identified as adhesion receptors at the surface of activated endothelial cells and platelets. They are involved in rolling/tethering neutrophils, monocytes, and certain lymphocyte subpopulations. This process is regarded as the initial step in the inflammatory response, the body's basic defence mechanism. The real physiological epitope present in neutrophils and other hematopoietic cells was recently characterized as being not simple SLe^x or SLe^a, but rather as long-chain, unbranched poly-N-acetyl-lactosamines (collectively termed 'myeloglycan') having a sialosyl 2→3 substitution at the terminal Gal, and multiple fucosylation at an internal (but not the penultimate) GlcNAc [31]. Rolling/tethering of E-selectin-expressing cells to myeloglycan is more prominent under dynamic flow conditions than rolling/tethering of the same cells to SLe^x [32]. Thus, it is important to distinguish SLe^x and SLe^a from myeloglycan-type epitopes. SLe^x and SLe^a have a primary function in cancer metastasis and invasion, whereas myeloglycan-type epitopes are involved in the inflammatory response under dynamic flow conditions.

GSLs as modulators of tumor cell signaling

GSLs, particularly gangliosides and their degradation products, are also believed to control cell growth, by modulating key molecules involved in signal transduction [33]. Current research trends are focused on the functional roles of ceramide and sphingosine, and their derivatives, as second messengers in signal transduction [34,35].

Our current knowledge of the relationship between GSL composition, metabolism, and their effect on signal transduction in tumor cells is highly fragmentary. GM3 in melanoma binds to LacCer or Gg3Cer through GSL–GSL interactions. The process of interaction, which increases the motility of melanoma cells, must trigger certain signals; it may induce signal transduction [36]. GM3 and other gangliosides that accumulate in tumors may activate specific types of integrin involved in transmembrane signaling, such as $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ (see [18] for review).

GSLs and cancer therapy

Immunotherapy and vaccine development

Antibodies that are directed to GM2, GD2 and other GSLs are detectable in the serum of patients with melanoma. It is not clear whether the antibody production is triggered by the patient's immune response to the tumor, or by environmental factors (e.g. intestinal flora). Many studies indicate that antibiotics present in sera of normal subjects and cancer patients are directed to environmental factors, which directly stimulate an immune response through 'Peyer's patches' on intestinal mucosal epithelia. The antibody level in some (but not all) patients with certain types of cancer is higher than in normal subjects. Therefore the autologous immune response to the tumor's own GSLs is still under debate. It has been suggested that increasing the level of antibodies artificially by active immunization with GM2 or GD2 may suppress melanoma growth. Livingston, Lloyd and coworkers have made extensive studies in this area [37,38]. They were able to show a large increase in antibody titres when the patients were immunized with gangliosides covalently linked to hemocyanin from the keyhole limpet, used as a carrier protein. The double bond of the sphingosine moiety of GSL was oxidized to an aldehyde group, which was covalently coupled to the carrier protein. Immunization of patients with such GSL–protein conjugates significantly enhanced both the IgM and IgG

response to GM2 and GD3. Large-scale clinical trials with melanoma patients are now under way [38].

Another therapeutic approach using antibodies directed to GSLs has recently been developed, (e.g. [39]). In this approach, a gene encoding anti-GD2 antibody is fused to a gene encoding interleukin-2 (IL-2), and used to produce a protein having both anti-GD2 antibody function and IL-2 function. IL-2 activates the $CD8^+$ T cell (or 'killer' cell) response, and the $CD8^+$ cells are therefore directed towards the tumor expressing GD2. A large subcutaneous tumor of cells expressing GD2 (derived from B16 melanoma cells by transfection with the genes for $\beta1\rightarrow4$GalNAc transferase and $\alpha2\rightarrow8$ sialyltransferase) was completely eliminated by infusion of the anti-GD2–IL-2 fusion protein.

Anti-adhesion therapy

Assuming that tumor cell metastasis and invasion are initiated by adhesion of tumor cells to the basement membrane, endothelial cells, platelets and parenchymatous cells in certain organs, blocking of such adhesion may abrogate metastasis and invasion. Integrin function is blocked by the peptides RGDS (a sequence common to various adhesive proteins) and YIGRS (a sequence in laminin), because they are both recognized by integrin receptors. Tumor metastasis, presumably caused by enhanced integrin function, was inhibited by preincubation of tumor cells with these peptides.

Another anti-adhesion approach is based on GM3. The degree of melanoma cell metastasis depends on the level of GM3, and on GM3-dependent adhesion to Gg3Cer and LacCer in microvascular endothelial cells (as described above). Lung metastasis of B16 melanoma can be inhibited not only by preincubation of tumor cells with GM3 or Gg3 liposomes, but also by intravenous administration of these liposomes after primary tumor cells are established [20].

Many tumor-associated GSLs are assumed to be adhesion molecules and to promote metastasis and invasion. Blocking total GSL synthesis may, therefore, suppress these processes. A series of studies by Radin and Inokuchi [40] demonstrated that D-threo-PDMP, an inhibitor of β-glucosylation of Cer, depletes most GSLs and inhibits tumor growth and metastasis.

Ortho-signaling therapy

If tumor cell invasiveness and metastasis depend on signaling leading to expression of E-selectin or P-selectin in endothelial cells or platelets, and on signaling created by integrin receptors, blocking of these signaling processes should inhibit invasion and metastasis. P-selectin expression in microvascular endothelial cells is highly sensitive to *N,N*-dimethyl-sphingosine and *N,N,N*-trimethyl-sphingosine; 1–3 μM concentrations of these compounds produce inhibition. Inhibition in platelets requires slightly higher concentrations (5–10 μM). Selectin expression in general is even more susceptible to lyso-ganglioside derivatives but these derivatives are difficult to obtain in sufficient quantity for *in vivo* and *in vitro* studies. Studies so far on the inhibition of melanoma metastasis by sphingolipids are limited to *N,N,N*-trimethyl-sphingosine. Sphingosine-1-phosphate, a catabolite of sphingosine, strongly inhibits tumor cell motility [41]. Administration of liposomes containing both *N,N,N*-trimethyl-sphingosine and sphingosine-1-phosphate inhibited tumor cell metastasis [42].

A final interesting thought is that there may already be a number of antitumor reagents that block signaling pathways. A combination of such signal blockers may inhibit not only cell proliferation and motility but also the receptor functions involved in adhesion, matrix destruction and infiltration. The molecular mechanism of the effect of specific GSLs and SLs in tumor cell adhesion and signaling processes should be clarified within the next decade. This knowledge will allow the development of synthetic analogs or mimetics with stronger anti-adhesive or ortho-signaling activity than naturally-occurring compounds. This approach will have great practical applications for the prevention and cure of cancer.

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