[CANCER RESEARCH 56, 5309–5318, December 1, 1996]

Perspectives in Cancer Research

Tumor Malignancy Defined by Aberrant Glycosylation and Sphingo(glyco)lipid Metabolism[1]

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Abstract

Aberrant glycosylation expressed in glycosphingolipids and glycoproteins in tumor cells has been implicated as an essential mechanism in defining stage, direction, and fate of tumor progression. This general concept is supported by results from three lines of study:

(*a*) Numerous clinicopathological studies have shown a clear correlation between aberrant glycosylation status of primary tumor and invasive/metastatic potential of human cancer as reflected by 5- or 10-year survival rates of patients.

(*b*) Carbohydrates expressed in tumor cells are either adhesion molecules *per se* or modulate adhesion receptor function. Some are directly involved in cell adhesion. They are recognized by selectins or other carbohydrate-binding proteins or by complementary carbohydrates (through carbohydrate-carbohydrate interaction). *N*- or *O*-glycosylation of functionally important membrane components may alter tumor cell adhesion or motility in a direction that either promotes or inhibits invasion and metastasis. Examples of such receptors are E-cadherin, integrins, immunoglobulin family receptors (*e.g.*, CD44), and lysosome-associated membrane protein.

(*c*) Gangliosides and sphingolipids modulate transmembrane signaling essential for tumor cell growth, invasion, and metastasis. The transducer molecules susceptible to gangliosides and sphingolipids include integrin receptors, tyrosine kinase-linked growth factor receptors, protein kinase C, and G-protein-linked receptor affecting protein kinase A. Some glycosphingolipids (*e.g.*, Gb3Cer, Le^y), ceramide, and sphingosine induce tumor cell differentiation and subsequent apoptosis. Shedded gangliosides may block immunogenicity of tumor cells, providing conditions favorable for "escape" from immunological suppression of tumor growth by the host.

Various reagents that block carbohydrate-mediated tumor cell adhesion or block glycosylation processing have been shown to inhibit tumor cell metastasis. This provides the basis for further development of "anti-adhesion therapy." Ganglioside analogues and sphingolipid analogues that inhibit protein kinase C and receptor-associated tyrosine kinase have been applied for inhibition of metastasis. A crucial mechanism for inhibition of metastasis by these reagents may involve blocking of transmembrane signaling for expression of P- and E-selectin. This provides the basis for development of "ortho-signaling therapy."

Introduction

Two types of cellular function, cell social and housekeeping functions, are distinguishable (although each is affected by the other). These two functions are regulated by two biochemical mechanisms, phosphorylation and glycosylation. In general, cell social function is predominantly affected by glycosylation, whereas housekeeping functions depend mainly on phosphorylation (Fig. 1). Specific carbohydrate epitopes *per se* are adhesion molecules directly involved in cell recognition on one hand, and on the other hand, glycosylation modulates various receptors essential for cell social function. The function of essentially all receptors involved in cell growth, adhesion, signal transduction, motility, and others are modulated by both glycosylation and phosphorylation. Housekeeping mechanisms involved in metabolism, cell replication, gene expression, cytoskeletal function, and cell motility are controlled mainly by phosphorylation. However, many of these housekeeping mechanisms are also modulated by sphingolipids and GSLs.[3] Malignancy, in terms of invasion and metastatic potential, depends more on aberrant cell social function than on aberrant housekeeping function (which merely leads to tumor formation).

The most common types of aberrant glycosylation observed in experimental and human cancers are: (*a*) a β1→6GlcNAc antenna in *N*-linked structures (1) resulting from enhanced or induced expression of GlcNAc transferase-V (2); (*b*) Tn (3, 4) and STn (5, 6) antigens caused by simplification of *O*-linked structures (reviewed in Refs. 7 and 8); (*c*) promiscuous *O*-glycosylation and resulting peptide conformational changes (9); (*d*) overexpression of lacto-series type 1 and type 2 structures (often in the form of poly-LacNAc) with a variety of fucosylation and sialylation (reviewed in Refs. 7 and 8); and (*e*) precursor accumulation of ganglio- and globo-series structures (reviewed in Ref. 7). Processes *a–c* occur in glycoproteins, process *d* occurs in both glycoproteins and glycolipids, and process *e* occurs only in glycolipids (ceramide-linked). Some of the structures accumulated in tumors are immunogenic in humans (10). Poly-LacNAc with sialosyl or fucosyl substitution in glycoproteins is often expressed at the β1→6GlcNAc side chain of *N*-linked structure or at the β1→6GlcNAc-linked side chain of "Core 2" *O*-linked structure (reviewed in Ref. 11).

GSLs are altered in carbohydrate structure as well as Cer composition in experimental and human cancers (12, 13). Aberrant accumulation of specific GSLs in specific types of cancer can be correlated with altered cell-cell or cell-substratum interaction. It may also reflect aberrant cell motility and transmembrane signaling.

Received 9/9/96; accepted 9/27/96.

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[1] The author's own studies cited in this paper were supported by National Cancer Institute Outstanding Investigator Grant CA42505.

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[3] The abbreviations used are: AMF, autocrine motility factor; BM, basement membrane; Cer, ceramide; D-PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; dimeric Le^x, Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→R); DMS, *N,N*-dimethyl-sphingosine; EC, endothelial cell; ECM, extracellular matrix; Gb3Cer, Galα1→4Galβ1→4GlcB1→1Cer; G_{D3}, NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1Cer; GlcNAc, *N*-acetylglucosamine; globoside, GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer; G_{M3}, NeuAcα2→3Galβ1→4Glcβ1→1Cer; GSL, glycosphingolipid; H, Fucα1→2Galβ1→3/4GlcNAcβ1→R; ICAM, intercellular adhesion molecule; L-PHA, *Phaseolus vulgaris* leucoagglutinin; LAMP, Lysosome-associated membrane protein; Le^a, Galβ1→3[Fucα1→ 4]GlcNAcβ1→3Galβ1→R; Le^a/Le^x, Galβ1→3[Fucα1→4]GlcNAcβ1→3Galβ1→4 [Fucα1→3]GlcNAcβ1→3Galβ1→R; Le^x, Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→R; Le^y, Fucα1→2Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→R; NeuAc, *N*-acetyl neuraminic acid; NF, nuclear factor; PKC, protein kinase C; poly-LacNAc, poly-*N*-acetyllactosamine; RCC, renal cell carcinoma; SLe^a, sialosyl-Le^x (NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAcβ1→3Galβ1→R); SLe^x, sialosyl-Le^x (NeuAcα2→3Galβ1→4[Fucα1→3] GlcNAcβ1→3Galβ1→R); Sph, sphingosine; Sph- 1-P, sphingosine-1-phosphate; STn, sialosyl-Tn (NeuAcα2→6GalNAcα1→O-Ser/Thr in clustered *O*-linkage); TACA, tumor-associated carbohydrate antigen; TMS, *N,N,N*-trimethyl-sphingosine; Tn, GalNAcα1→O-Ser/Thr in clustered *O*-linkage.

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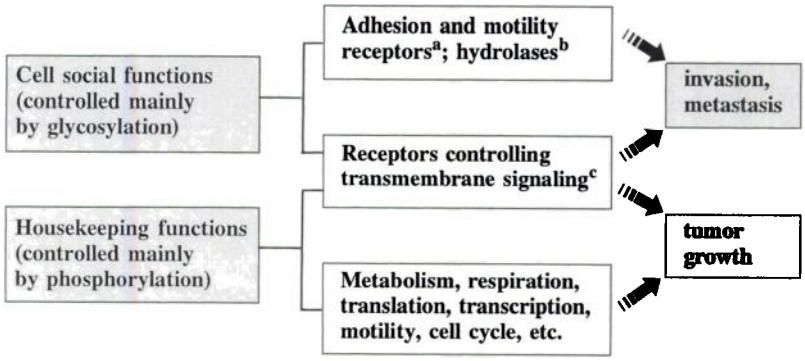

Fig. 1. Cell social and housekeeping functions and their supporting mechanisms. The ability of cells to adapt their function to their environment, including cell-cell or cell-matrix interaction, is collectively called cell social function. This function is maintained by receptors for adhesion/recognition (*a*), cell surface hydrolases (*b*), and partially by receptors for growth factors and hormones (*c*). Receptor types a and b are controlled mainly by glycosylation. Receptor type c is affected by GSLs and SLs. Tumor invasion/metastasis is greatly influenced by aberrant expression of all of these receptor types. In contrast, a number of other cellular functions controlling metabolism, protein synthesis, DNA replication, ATP synthesis through respiration, ATP consumption for metabolic turnover, and others do not directly affect invasion/metastasis but only affect tumor growth. *a:* (i) integrin receptors recognizing fibronectin, laminin, collagen, ICAMs; (ii) immunoglobulin family receptors (*e.g.*, CD44), ICAMs, and sialoadhesins; (iii) selectins that recognize SLe^x, SLe^a, myeloglycans, and sulfated glycans; (iv) cadherins; and (v) GSLs that recognize GSLs. Functions i–iv are affected by *N*- or *O*-glycosylation or by GSLs. *b:* cell surface expression of proteases and endoglycosidases. This process may be controlled by LAMP and its glycosylation. *c:* (i) tyrosine kinase-linked receptors; (ii) G-protein-linked receptors (affecting protein kinase A); (iii) PKC; and (iv) sphingomyelinase-linked receptors. Functions i, ii, and iii are affected by GSLs or sphingolipids.

This article will provide an overview of: (*a*) how aberrant glycosylation and sphingo(glyco)lipid metabolism lead to aberrant cell social behavior in tumor cells; (*b*) known or suspected molecular mechanisms underlying this phenomenon; and (*c*) current status and future perspectives of "anti-adhesion" and "ortho-signaling" therapy based on this phenomenon.

Carbohydrate Epitopes Closely Associated with Malignancy of Human Cancer and Defining Survival Rates of Patients with Cancer

Among the numerous types of aberrant glycosylation observed in human cancers, the structures listed in Table 1 have been claimed to be correlated with invasive/metastatic properties of tumors in terms of 5- or 10-year patient survival rates. Six examples of distinctive differences in Kaplan-Meier survival curves depending on presence versus absence of specific TACAs in specific types of cancer are shown in Fig. 2. The first item in the table, $\beta1\rightarrow6$GlcNAc antenna in *N*-linked multi-antennary structures, appears to be the most universal form of aberrant glycosylation in a large variety of experimental and human cancers. Clinicopathological and immunohistological studies using L-PHA as a probe showed a correlation between expression of this structure and metastatic potential (14, 15). However, $\beta1\rightarrow6$GlcNAc antenna is expressed in various normal glycoproteins, and its "enhanced" expression in tumors is merely relative and highly variable. Furthermore, the detection method, based solely on L-PHA, could be improved if GlcNAc-T V mRNA expression is quantitated. The second item on the list, deletion *versus* persistence of histo-blood groups A and B epitopes has been studied for many years (reviewed in Refs. 7, 8, and 16). This item and the third item (H/Le^y expression resulting from precursor accumulation) are: (*a*) the most reproducible in many independent studies; and (*b*) observable in a wide variety of cancers, regardless of origin (17–26, 28, 47). Therefore, these two types of glycosylation changes are of good prognostic value in a variety of human cancers.

Expression of SLe^x (29, 30), sialosyl-Le^a (SLe^a) (31, 32), and STn (33, 34, 36) has been correlated with patient survival for certain types of cancers, and the results are reproducible. However, the effect of SLe^x on survival of patients with RCC was unclear (27), presumably because SLe^x level in RCC is low. On the other hand, RCC expresses high levels of disialosylgalactosylgloboside, a new adhesion molecule that plays a major role in defining RCC metastasis (44). The effect of STn on survival of patients with cervical cancer is also unclear (35). There is a possibility that STn positivity in tissue sections is influenced by 9-*O*-acetylation (48), which varies depending on tissue type. The effects of Tn, Le^x, dimeric Le^x, and Le^a antigens on metastatic and invasive properties are more variable than the effects of ABH, Le^y, STn, and SLe^x antigens.

In general, correlation of a particular type of aberrant glycosylation with patient survival rate is more obvious in early stages than in later stages of human cancer (21, 33, 36). Many factors may be involved in later stages, whereas glycosylation may be the dominant factor in early stages of cancer development.

Functional Role and Mechanism of Aberrant Glycosylation in Defining Malignancy

Invasive and metastatic potential of tumor cells are highly complex yet well-coordinated processes defined by a series of mechanisms as follows (reviewed for Refs. 49–51): (*a*) release of tumor cells from primary tumor mass; (*b*) adhesion of tumor cells to ECM or BM; (*c*) hydrolytic activity of tumor cells for destruction of ECM and BM, possibly associated with cell surface proteases and endoglycosidases; (*d*) migration of tumor cells through the degraded matrix and into the blood or lymph circulation; (*e*) access of tumor cells to activate platelets and ECs; (*f*) signaling to express selectin, ICAMs and other adhesion receptors; (*g*) selectin and ICAM-dependent tumor cell adhesion to ECs followed by extravasation; and (*h*) formation of metastatic deposits and interaction with parenchymatous organ cells (Fig. 3).

Effect of Glycosylation on Adhesion between Tumor Cells (Step 1 in Fig. 3). Cell-cell adhesion in epithelial tissues is mediated by E-cadherin in combination with α- and β-catenin and other cytoplasmic components, which link E-cadherin to the cytoskeleton. The same mechanism presumably applies to adhesion among tumor cells within a primary tumor tissue mass, which reduces release of cells from the mass and therefore reduces the probability of metastasis (49). Inversely, a decreased intertumor cell adhesion results in increased motility of tumor cells on collagen and laminin and increased metastatic potential (52).

The type of *N*-linked glycosylation to E-cadherin has been recently implicated in the modulation of cadherin-dependent tumor cell adhesion and release of tumor cells from tumor mass (53). An increase of bisecting $\beta1\rightarrow4$GlcNAc to the mannose core induced by GlcNAc-T

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Table 1 *Specific glycosylation that defines survival rate of patients with cancer*

Structure; notes	Promotes malignancy	References
β1→6GlcNAc antenna in *N*-linked multi-antennary structures[a]	Yes	1, 2, 14, 15
Deletion *vs.* persistence of histo-blood group A/B epitopes[b]	Deletion promotes; persistence inhibits	Reviews: 7,16; recent studies: 17–23
H/Le^y expression[c]	Yes	24–26, 28
SLe^{xd}	Yes and/or no	27, 29, 30
SLe^{ae}	Yes	31, 32
STn[f]	Yes and/or no	33–36
Tn and *Helix pomatia* antigen[g]	Yes and/or no	37–40
Le^x, dimeric Le^x, Le^a, and their analogues[h]	Yes and/or no	41–43
Disialosylgalactosylgloboside in renal cell carcinoma[i]	Yes	44
Galactosylgloboside in seminoma[i]	No; inhibits	45

[a] Due to enhanced GlcNAc transferase V, competitive to GlcNAc transferase III; correlated with metastatic potential in many animal studies (2); relatively small number of human clinicopathological studies (14, 15).

[b] Ref 21: 5-year survival of patients with primary lung cancer; stage I A (+) 80% (n = 18), A (−) 15% (n = 9); stage II A (+) 50% (n = 10), A (−) 18% (n = 8). Median survival time of patients with A (−) tumors (n = 28) *versus* patients with A (+) tumors (n = 43) was 15 *versus* 71 months. Ref 22: overall 5-year survival A (+) 80% (n = 37), A (−) 55% (n = 42); B (+) 75% (n = 19), B (−) 35% (n = 9). A/B deletion or reduction, due to suppressed mRNA encoding A and B transferases: reduction in benign or less malignant tumors; complete deletion in highly malignant tumors (23). A determinant may inhibit H/Le^y-dependent cell motility or adhesion to endothelial cells (47).

[c] Ref 25: overall average 5-year survival of patients with primary lung cancer MIA 15-5 (H/Le^y) (−) 60% (n = 58), MIA 15-5 (H/Le^y) (+, ++) 18% (n = 91). Ref 28: 5-year survival of patients with bladder cancer. H/Le^y expression defined by *Lotus tetragonolobus* lectin. H/Le^y (−) 70% (n = 22), H/Le^y (+) 20% (n = 49). H/Le^y correlates tumor cell motility; different anti-H/Le^y antibodies (MIA 15-5 and BR96) inhibit tumor cell motility (24, 26).

[d] Ref 30: overall 5-year survival of patients with colorectal cancer. SLe^x (−) 93% (n = 75), SLe^x (+) 58.3% (n = 58). Such a trend is not found for renal cell carcinoma (27).

[e] Ref 31: overall 5-year survival of patients with colorectal cancer. SLe^a (−) 64% (n = 19), SLe^a (+) 31% (n = 26). Ref 32: 5-year survival of patients with advanced colorectal cancer after curative surgery SLe^a (−) 92% (n = 235), SLe^a (+, ++) 72% (n = 70).

[f] STn in colorectocarcinoma (33) and in gastric cancer (34), but not in cervical cancer (35), correlates well with cancer prognosis. Ref 33: 5-year survival of patients with Duke's stage B/C colorectal cancer. STn (TKH2) (−) 100% (n = 13), STn (+) in Duke's B patients 80% (n = 72), in Duke's C patients 59% (n = 33). Ref 36: STn levels in sera of patients with ovarian cancer correlate well with their 5-year survival rate. STn serum levels: >50 units/ml 5%, <50 units/ml 55%.

[g] Expression of Tn in breast cancer, defined by the *Helix pomatia* antigen, was claimed to be correlated with invasive/metastatic potential (37). In two independent follow-up studies, no significant correlation was found between *Helix pomatia* antigen expression in breast cancer invasiveness/metastasis (38, 39). Tn defined by *Vicia villosa* lectin is claimed to be correlated with survival of patients with ovarian cancer. Ref 40: 5-year survival in: Tn (−) parametrial spread (−) 95% (n = 51), Tn (−) parametrial spread (+) 90% (n = 10), Tn (+) parametrial spread (+) 48% (n = 19).

[h] Esophageal cancer expression Le^x had high lymph node metastasis (41); a comparison of Le^x and GalNAc (probed by *Dolichos biflorus* lectin) in lung cancer showed that Le^x−/GalNAc+ tumors had lower metastasis than Le^x+/GalNAc+ or Le^x+/GalNAc− tumors (42). Expression of hybrid epitope Le^a/Le^x (defined by monoclonal antibody 43-9F) in squamous cell lung carcinoma inversely correlated with patient survival rate (43).

[i] RCC expressing disialosylgalactosylgloboside, defined by mAb RM2 (46), showed a preferential metastasis to lung and adhesion to lung tissue sections (44); seminoma expressing galactosylgloboside did not show metastasis (45).

III gene transfection reduces β1→6GlcNAc antenna (54), resulting in overall structural change, enhanced E-cadherin activity, and reduced malignancy. The opposite effect, *i.e.*, enhanced GlcNAc-T V gene activity resulting in increased β1→6GlcNAc antenna to form multi-antennary structure without bisecting GlcNAc, decreases cadherin activity. This reduces adhesion between tumor cells and increases release of tumor cells from tumor tissue mass, thereby promoting metastasis (53).

Effect of Glycosylation on Integrin Receptors and CD44, Which Control Matrix-dependent Adhesion and Motility (Step 2 in Fig. 3). An essential step in tumor progression is the interaction of tumor cells with ECM and BM, leading to destruction of these components. Integrin receptors play a major role in this process (reviewed in Ref. 50). Transformed cells express higher levels of integrin receptors than their progenitor cells (55, 56). The function of integrin receptors is, in general, highly controlled by *N*-glycosylation (57–59). De-*N*-glycosylation of α5β1 results in dissociation of α5 and β1 subunits and loss of binding to fibronectin (59). The same mechanism may be applied to other integrin functions, but it remains to be studied.

Gangliosides also affect integrin receptor function. For example, fibronectin binding activity of α5β1 is strongly enhanced at certain concentrations of G_{M3} but inhibited at higher concentrations. Other gangliosides and GSLs have no effect on α5β1 receptor function (60). The function of αvβ3 (vitronectin receptor), recognizing the RGDS sequence in human melanoma cells, depends on G_{D3} ganglioside (61). Systematic studies are needed to evaluate the functional dependence of other integrins on gangliosides. Some tumor cells, *e.g.*, human and mouse melanoma, are characterized by high accumulation of G_{M3} and G_{D3}. It is possible that this accumulation results in enhancement of integrin-dependent adhesion and motility.

Another membrane receptor involved in matrix-dependent cell adhesion and motility is CD44. This is a family of immunoglobulin adhesion receptors claimed to bind to hyaluronic acid and appears to be involved in tumor progression. Expression of a splicing variant (CD44-E) in rat tumors was found to be strongly correlated with metastatic potential (62). A subsequent series of studies confirmed

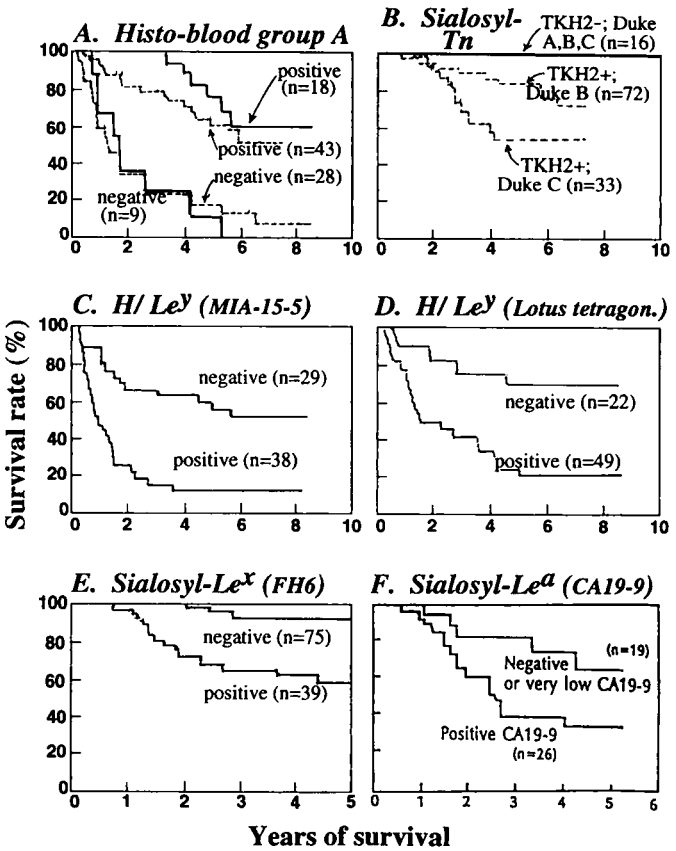

Fig. 2. Kaplan-Meier survival curves of patients with primary carcinoma showing expression of specific TACAs. See Table 1 for explanation of cases. *A: solid line*, patients with stage I lung carcinoma. *Dashed line*, overall survival of lung carcinoma patients. Histo-blood group A positive and negative cases are indicated (adapted from Ref. 21). *B: solid line* (coincides with 100% line of ordinate), mAb TKH2-negative cases of colorectal carcinoma regardless of Duke stage A, B, or C. *Two dashed lines*, TKH2-positive cases with Duke stage B and C, respectively (adapted from Ref. 33). *C*, squamous cell lung carcinoma cases stained by mAb MIA-15-5, which defines H/Le^y expression (adapted from Ref. 25). *D*, bladder transitional carcinoma cases stained by *Lotus tetragonolobus* lectin, which reacts preferentially with H/Le^y (adapted from Ref. 28). *E*, colorectal carcinoma cases stained by mAb FH6, which reacts preferentially with extended sialosyl-Le^x or sialosyl dimeric Le^x (adapted from ref. 30). *F*, colorectal carcinoma cases stained by mAb CA19-9, which defines sialosyl-Le^a (adapted from Ref. 31). As exemplified in *A* and *B*, correlation of expression of a specific TACA with patient survival rate is more obvious for early-stage than for later-stage tumors.

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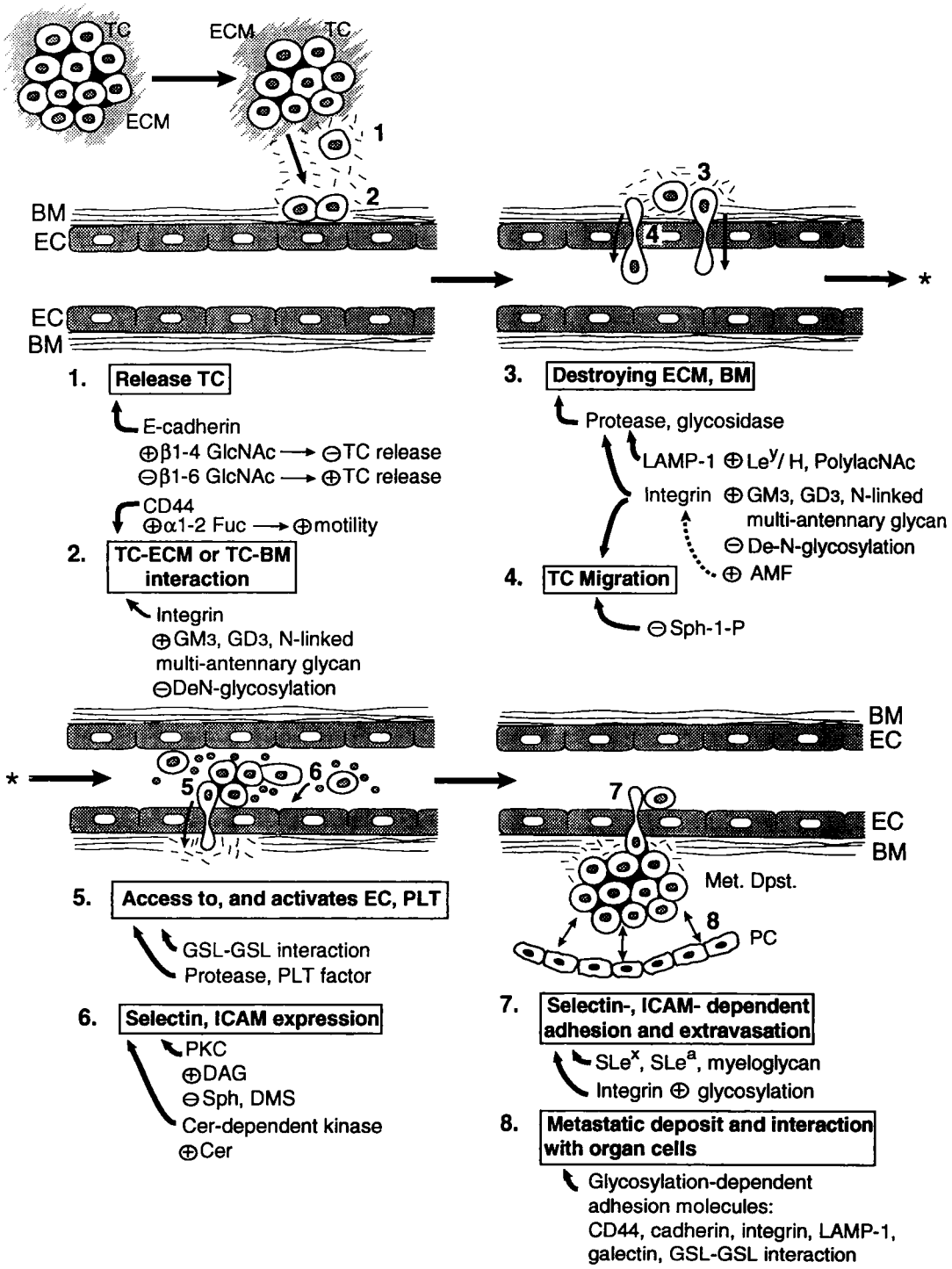

Fig. 3. Proposed scheme for involvement of glycosylation in tumor cell metastasis. *TC,* tumor cell; *PLT,* platelet; *DAG,* diacylglycerol; *PC,* parenchymatous cells of organs. The process of clonal proliferation of TCs to form a TC mass, leading eventually to metastatic deposit (*Met. Dpst.*), is arbitrarily divided here into eight steps. The mechanism at each step is greatly influenced by glycosylation or by sphingolipid breakdown products. *Step 1:* release of TCs from TC mass depends on E-cadherin. Decreased E-cadherin activity results in increased TC release, and *vice versa.* E-cadherin activity is decreased by β1→6GlcNAc antenna in the *N*-linked structure and increased by the β1→4GlcNAc bisecting structure through competitive decrease of β1→6GlcNAc antenna formation. *Step 2:* TC-ECM or TC-BM interaction (prerequisite for steps 3 and 4) depends on two TC membrane receptors, integrin and CD44. Integrin activity is promoted by G_{M3}, G_{D3}, and *N*-linked multi-antennary structures and abolished by de-*N*-glycosylation. CD44 receptor interacts with ECM and BM glycans, and its activity is affected by glycosylation, particularly α1→2 fucosylation. Without this glycosylation-dependent process, destructive and invasive properties of TCs are not activated. *Step 3:* destruction of ECM and BM by TCs. Following integrin- and CD44-dependent adhesion of TCs to the ECM and BM, proteases and possibly endoglycosidases destroy the ECM and BM. LAMP-1 and integrin play major roles in this process (see text). LAMP-1 activity is promoted by poly-LacNAc with H/Le^y glycosylation. Integrin activity depends on glycosylation as described in step 2. *Step 4:* transendothelial migration and invasion. This process depends on TC motility, in close association with step 3, and is controlled by glycosylation-dependent integrin function and possibly AMF signaling. TC motility is strongly inhibited by Sph-1-P (at nM concentration). *Step 5:* initial contact with and activation of ECs or PLTs by TCs. Initial TC-EC contact takes place in some cases through interaction of complementary GSLs expressed on the two cells (see text). Next, TCs activate PLTs, and TCs in the presence of activated PLTs activate ECs (see text and Fig. 3). Glycosylation may not be involved in this step. *Step 6:* expression of selectin, ICAM, and other adhesion molecules upon TC-dependent activation of EC and PLT. Two major signaling pathways are involved that are PKC dependent and Cer dependent. The former is activated by DAG and inhibited by Sph and DMS. The latter is mediated by Cer and leads to activation of NF-κB. *Step 7:* selectin- and ICAM-dependent adhesion and extravasation of TCs. This process depends on expression of SLe^x and SLe^a in TCs of epithelial origin or expression of myeloglycans in leukemic cells. It also depends on glycosylation-dependent integrin function. *Step 8:* metastatic deposition and interaction with target organ cells. This process depends on various glycosylation-dependent adhesion molecules and carbohydrate-binding molecules, *e.g.,* CD44, cadherin, integrin, LAMP-1, galectin, and GSL.

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and extended the correlation between expression of splicing epitopes in various human tumors and metastatic/invasive properties of the tumors (Refs. 63 and 64; reviewed in Ref. 65). For example, expression of exons V7 and V8 is correlated with invasive properties of cervical cancer (63). These findings pose two major questions: (*a*) what are the glycoconjugates that interact with CD44 splicing variants expressed in tumors, *i.e.*, are any glycoconjugates other than hyaluronic acid involved in CD44 binding? and (*b*) are there any effects of glycosylation on CD44 variant functions that define matrix-dependent motility and adhesion? Recently, clear evidence for the effect of glycosylation on CD44 function was provided. H glycosylation of CD44, induced by transfection of $\alpha 1 \rightarrow 2$ fucosyltransferase gene, greatly enhanced tumor cell motility and tumorigenicity in rat colon carcinoma cells (132).

Possible Effect of Glycosylation on Matrix-destructive Properties of Tumor Cells and Motility (Steps 3 and 4 in Fig. 3). Invasive properties of tumor cells are based on enhanced hydrolytic properties (presumably caused by proteases and endoglycosidases expressed at the cell surface) and enhanced motility (possibly mediated by growth factors and AMF; Refs. 66 and 67). Recent studies indicate that type IV collagenase expression is modulated by integrin receptors $\alpha v \beta 3$ and $\alpha 5 \beta 1$ (68). Function of these receptors, and therefore collagenase expression, are highly susceptible to *N*-glycosylation and coexisting gangliosides (60, 61).

AMF was recently identified as a signaling molecule for integrin ($\alpha IIb \beta 3$ and $\alpha 5 \beta 1$)-mediated tumor cell adhesion and invasion (67). Thus, a common signaling pathway is shared between AMF-dependent and integrin-dependent processes. It should be noted that chemotactic and haptotactic motility of certain tumor cells, but not normal cells, was inhibited by Sph-1-P at nM concentration levels (69).

LAMP-1, the major carrier of polylactosamine, plays an essential role in trafficking of membrane components between plasma and lysosomal membranes (reviewed in Ref. 70). Its higher expression in tumor cells than in normal cells may be associated with high expression of lysosomal enzymes at the surface of aggressive tumors. The relationship between aberrant glycosylation (*e.g.*, excessive expression of Le^y in breast cancer LAMP-1; Ref. 26) and enhanced trafficking of LAMP-1 in tumors is an important topic for future investigation.

Tumor Cell Activation of ECs and Platelets, Expression of Selectins and ICAMs, and Subsequent Interaction of Tumor Cells with Selectins and ICAMs (Steps 5, 6, and 7 in Fig. 3). Tumor cell-induced activation of platelets has long been regarded as an important step in the metastatic process, although the mechanism involved still remains unclear (reviewed in Refs. 51 and 71). Since discovery of the expression of P-selectin in activated platelets and of both P- and E-selectins in activated ECs in addition to expression of ICAMs, many studies have focused on a possible correlation between selectin expression and the adhesion of tumor cells to ECs and platelets. This trend of study is based on several major assumptions or findings: (*a*) SLe^x, SLe^a, and their analogues have been claimed to be the epitopes for both E- and P-selectins, although this remains very uncertain (72)[4]; (*b*) both SLe^x and SLe^a are well-established tumor-associated antigens (reviewed in Ref. 7); (*c*) tumor cells may activate platelets to express P-selectin and form platelet-tumor cell aggregates, which may induce microembolisms or lead to adhesion of tumor cell aggregates to ECs, which in turn initiates metastatic deposition; and (*d*) tumor cells may have the ability to activate and elicit P- and E-selectin expression on ECs.

Addressing these assumptions, we performed a series of experiments, with the following results: (*a*) many human and mouse tumor cell lines are capable activating platelets, although none of them are capable of binding to P-selectin (73); (*b*) many gastric, colonic, and lung cancer cell lines that express SLe^x or SLe^a are unable to bind to P-selectin, although they are all capable of binding E-selectin (73). Adhesion of P-selectin to its ligand requires a specific core peptide, PSGL-1. Transfection of the *PSGL-1* gene into P-selectin nonadherent, SLe^x/SLe^a-expressing tumor cells induces P-selectin-dependent adhesion (73); and (*c*) human and mouse tumor cell lines tested do not secrete detectable levels of tumor necrosis factor-α or transforming growth factor-β, which are believed to activate ECs (74). However, tumor cells gain the ability to activate ECs when coincubated with a physiological concentration (10^8/ml) of platelets, through an unknown platelet factor. This leads to the formation of large aggregates of tumor cells adhered to ECs (74). The role of P-selectin in tumor progression is, therefore, obscure or more complex than that of E-selectin. On the other hand, P-selectin plays a well-documented central role in the recruitment of neutrophils in acute inflammatory processes (75).

Based on these findings, we propose a model of the interrelationship between platelet and EC activation, selectin expression, tumor cell adhesion, and initiation of metastasis, as illustrated in Fig. 4.

Galectins and Other Lectins. A family of galactose-, lactose-, and LacNAc-binding lectins present in a large variety of cells have been structurally characterized as galectin-1 through galectin-9 (76). Most galectins are located intracellularly, and their functional role is still ambiguous. Galectin-1 and galectin-3 are expressed in 21 human colonic cancer cell lines studied. Galectin-3 is capable of binding to carcinoembryonic antigen and LAMP-1, which are involved in cell adhesion. Surface expression of galectin-3 appears to be correlated with tumor cell invasion (77). In contrast, the expression of galectin-1 on ECs may provide the basis for adhesion of tumor cells to ECs (78). C-type lectin, recognizing *O*-linked *N*-acetylgalactosamine (Tn-antigen) was found to be expressed in macrophages (79), the expression of which may correlate with metastatic lesions in mouse lung (80).

TACAs Recognized by Complementary Carbohydrates. A specific TACA highly expressed in a defined type of experimental or human cancer is recognized by a specific carbohydrate expressed in target cells, through carbohydrate-carbohydrate (usually GSL-GSL) interaction (reviewed in Ref. 81). Le^x-Le^x interaction (82, 83) could be the basis for autoaggregation of Le^x-expressing tumor cells, which may lead to microembolisms by tumor cell mass and may trigger metastasis. Interaction of melanoma cells (highly expressing G_{M3}) with ECs (that express LacCer or Gg3Cer) leads to adhesion of melanoma cells on ECs; this also triggers metastasis (84, 85). GSL-GSL interaction occurs more rapidly than (but in synergy with) integrin-dependent adhesion and, therefore, plays an important role in adhesion of tumor cells to ECs under dynamic flow conditions (85, 86). H antigen is highly expressed in specific regions of human vascular ECs (87), and H-Le^y interaction has been clearly observed (47). Adhesion of human tumor cells to non-activated ECs based on H-Le^y interaction is, therefore, considered an initial event in metastasis of H/Le^y-expressing tumor cells (47).

[4] SLe^x accumulated in various human cancers is chemically well characterized (7, 16). SLe^x binds to E- and P-selectin under certain conditions (72). However, SLe^x is present in small quantity or virtually absent in neutrophils and other leukocytes, and the real physiological epitopes of E-selectin have been identified as "myeloglycans" and their analogues, *i.e.*, poly-LacNAc gangliosides lacking SLe^x but having $\alpha 2 \rightarrow 3$NeuAc at terminal Gal and $\alpha 1 \rightarrow 3$Fuc at internal (but not penultimate) GlcNAc (131). The physiological carbohydrate epitope of P-selectin presented by PSGL-1 is yet unidentified. Therefore, expression of SLe^x and SLe^a is high in various human cancers (7) but restricted or absent in human leukocytes and other cell types. Thus, the role of these epitopes as tumor-associated antigens, and in promoting malignancy through interaction with E-selectin, are increasingly obvious.

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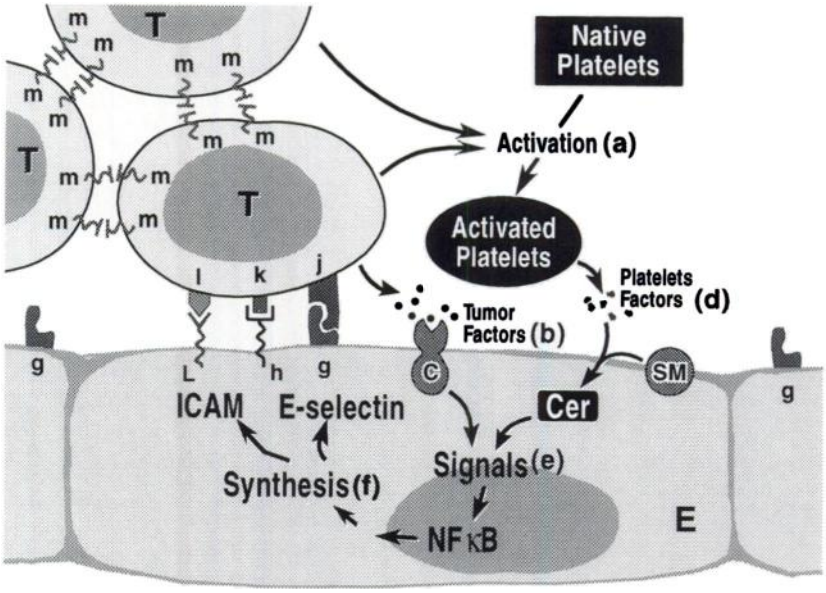

Fig. 4. Proposed role of platelet activation and selectin expression in tumor cell adhesion to microvascular endothelial cells. Tumor cells are capable of activating platelets, which then release platelet factors (*d*). These factors, in combination with tumor factor (*b*; low levels of tumor necrosis factor-α and transforming growth factor-β), open the signal pathway (*e*) through Cer production from sphingomyelin (SM). One example of signaling is activation of NF-κB to elicit E-selectin expression. Initial tumor cell tethering through interaction between tumor cell carbohydrate (*j*) and nonactivated endothelial cell carbohydrate (*g*) accelerates this process. Firm binding of tumor cells to activated endothelial cells (*E*), which elicits E-selectin (*h*) and ICAM (*L*), takes place through their respective ligands: SLe^x/SLe^a (*k*) and integrin receptor (*l*). The role of P-selectin in this process is unknown. Tumor cell-tumor cell interaction takes place through homotypic aggregation receptors, such as carcinoembryonic antigen (*m*).

Structural and Metabolic Features of GSLs Accumulated in Tumor Cells and Functional Implications

Aberrant Glycosylation in Combination with Aberrant Ceramide Profile. High accumulations of: (*a*) Le^x GSLs containing phytosphingosine (3-hydroxy-sphingosine) in metastatic deposits of various types of human cancer (88); (*b*) a series of dimeric Le^x (Le^x-Le^x) GSLs with highly hydroxylated Cer structure in colonic cancer (12); and (*c*) gangliosides with highly hydroxylated Cer in neuroblastoma cells (13) have been reported. GSLs with hydroxylated Cer (relative to those with nonhydroxylated Cer) showed higher antigenicity (89, 90) and higher ability to undergo GSL-GSL interaction (91). Accumulation of G_{D3} and G_{D2} in human melanoma, G_{M3} in mouse melanoma, G_{D2} in neuroblastoma, Gg3 in mouse lymphoma and human Hodgkin's lymphoma, fucosyl-G_{M1} in small cell lung carcinoma, globo-H in breast and ovarian carcinoma, and Gb3 in Burkitt lymphoma are examples of high accumulation of specific GSLs in specific types of cancer (reviewed in Ref. 7). The structural profile of Cer in accumulated GSLs may be altered, although it was not closely studied except in the cases mentioned above.

Aberrant Cell Social Behavior Mediated by Accumulated GSLs. Each type of tumor is characterized by accumulation of specific types of GSLs (7). Our knowledge of the functional significance of this phenomenon is highly fragmentary, except that the GSLs have been identified as tumor-associated antigens (7, 10). A few possibilities have been suggested:

(*a*) Accumulated GSLs in tumors may: (*i*) be involved in selectin- or galectin-dependent adhesion; (*ii*) mediate initial tumor cell adhesion to ECs prior to their activation (47, 85); and (*iii*) cause tumor cell to tumor cell adhesion, as typically observed with Le^x-Le^x interaction (82).

(*b*) Some GSLs at the tumor cell surface are anti-adhesive (repellent) to each other and may induce the release of tumor cells from tumor cell mass, thus promoting tumor cell metastasis. For example, G_{M3}-G_{M3} (84) and Le^y-Le^y[5] are highly repellent.

(*c*) Gb3Cer in Burkitt's lymphoma (92) and Le^y in various types of human cancers (93) were identified as inducers of apoptosis.

(*d*) Ganglioside G_{M3} or G_{D3}, at optimal concentrations, greatly enhance integrin-dependent adhesion, which may promote tumor cell invasion (60, 61).

Transmembrane Signal Control by GSLs and Its Catabolites. We and others observed that gangliosides affect cellular signaling through major signal transducers (*e.g.*, tyrosine-kinase linked receptors or protein kinase C; reviewed in Ref. 94). Subsequent studies showed that lyso-G_{M3} strongly inhibits EGF receptor kinase, whereas de-*N*-acetyl-G_{M3} enhances activity of this kinase (95, 96) and of serine kinase and induces cell proliferation (97). These primary degradation products of gangliosides are detectable as physiological components of cells (95, 96). De-*N*-acetyl-G_{D3} was found in human melanoma in addition to de-N-acetyl-G_{M3}. These catabolites may play an important role in modulation of tumor cell growth. Conversion of G_{M3} to de-*N*-acetyl-G_{M3} or G_{D3} to de-*N*-acetyl-G_{D3}, catalyzed by *N*-acetylase, is susceptible to the tyrosine kinase inhibitor genistein (98).

Shedding of gangliosides from tumor cells is greater than from normal cells, and sera of patients with cancer (compared to sera of normal subjects) have much higher levels of gangliosides (99). Shedded gangliosides may inhibit immune response *in vitro* as well as *in vivo*. Tumor progression is associated with increased ganglioside levels in blood, which may inhibit host immune response and thereby promote tumor growth through "escape" of tumor cells from this immune response (100).

Levels of Sph, Cer, and their derivatives in specific types of tumor cells with different metastatic/invasive properties have not been systematically studied. Cer accumulation was reported in Rous sarcoma-transformed fibroblasts (101). However, these sphingolipids are now recognized as important second messengers and as modulators of transmembrane signaling (reviewed in Refs. 102–104). Understanding of tumor cell growth control by gangliosides, their catabolites, Sph, and Cer is important for development of ortho-signaling therapy (see below).

Anti-Adhesion and Ortho-Signaling Therapies Based on Aberrant Glycosylation and Signaling in Tumor Cells

Anti-Adhesion Therapy. Some, if not all, TACAs have been functionally identified as adhesion molecules that bind lectins or selectins expressed on ECs or on target cells of specific organs.

[5] N. Kojima and S. Hakomori, unpublished data.

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TACAs can also be recognized by complementary carbohydrates expressed on target cells, as described in a preceding section. Whatever the mechanism, if carbohydrate-dependent tumor cell adhesion triggers or promotes invasion and/or metastasis, the use of oligosaccharide derivatives or GSL antigens incorporated in liposomes should theoretically block metastasis. One successful example is inhibition of mouse melanoma metastasis by administration of G_{M3} or Gg3Cer liposomes. Spontaneous metastasis from s.c. grown B16/BL6 tumors to lung was strongly inhibited by G_{M3} or Gg3Cer liposomes but not by other GSL liposomes (105). The success of this approach was based on the fact that melanoma cells adhere to mouse ECs through interaction between G_{M3} expressed on the former and LacCer (or Gg3Cer) on the latter. LacCer does not inhibit B16/BL6 metastasis, presumably because LacCer liposomes are cleared rapidly from blood (*i.e.*, taken up by liver). Methyl-β-lactoside, however, when preincubated with B16/F10 cells and i.v. injected, significantly suppressed lung colonization (106). In a similar study, the addition to drinking water of citrus pectin containing many galactosyl residues resulted in inhibition of metastasis in a rat prostate cancer model (107). These results were interpreted as blocking of galectin-3 by the galactosyl residues of citrus pectin. However, inhibition of melanoma metastasis by liposomes containing G_{M3} or Gg3Cer can be interpreted only as blocking of G_{M3}-Gg3Cer interaction.

Thus, development of anti-adhesion therapy for blocking of metastasis is realistically possible if a sufficient quantity of an appropriate TACA that promotes adhesion of human tumors (see above) can be mass produced and clinically applied. TACA mimetics, designed to be resistant to glycosidases and to maintain high affinity with binding proteins and carbohydrates, are ideal anti-adhesion reagents.

Aggressive cell social behavior of tumor cells depends greatly on enhanced β1→6GlcNAc antenna to form multi-antennary *N*-linked glycosylation expressed on cadherin, integrin, CD44, LAMP-1, and other receptors. The aggressive cell behavior can be reduced or eliminated by modification of *N*-linked glycosylation, *i.e.*, application of *N*-glycosylation processing inhibitors such as castanospermine, *N*-methylnojirimycin, swainsonine, and others. Because of its lower toxicity, swainsonine and its analogues have often been used for this purpose. Swainsonine restores contact inhibition and thereby suppresses tumor growth in soft agar and induces normal function of LAMP and integrin receptors (108). Swainsonine reduces α-mannosidase activity and *N*-glycosylation processing in MDAY-D2 mouse tumor and thereby strongly inhibits metastatic potential (133). A few lipophilic analogues of swainsonine showed higher inhibitory activity of glycosylation processing and enhanced antimetastatic effects (110). A Phase I clinical study on toxicity of swainsonine and effect of this drug on cancer-associated symptoms and on tumor size in patients with advanced cancer was reported recently (109). Of 19 cases, a few showed remarkable effects in terms of tumor shrinkage and improvement of symptoms (*e.g.*, cough and shortness of breath). Toxicity was minimal.

Another promising compound that inhibits glycosylation processing is 1,6-*epi*-cyclophellitol. The compound inhibits α-glucosidase activity similarly to castanospermine but had much less toxicity. It inhibited B16/F10 cell adhesion/migration in the Boyden chamber assay and inhibited B16/F10 lung metastasis *in vivo* (111).

Ortho-Signaling Therapy. Administration of D-PDMP to tumor-bearing mice inhibited tumor growth *in situ* and metastasis, in association with inhibition of GSL synthesis. PDMP inhibits synthesis of essentially all GSLs except galactosylceramide, di-galactosylceramide, and sulfatide, and displays associated tumor growth inhibition (112). Metastasis of mouse Lewis lung carcinoma was strongly inhibited by D-PDMP (113). Radin and Inokuchi (114) have emphasized the importance of appropriate design of anticancer drugs to inhibit glucosylation of Cer, the initial step in all GSL synthesis. This may be an effective approach, since malignancy of many experimental and human cancers is defined by aberrant accumulation of specific GSLs, which leads to aberrant cell adhesion and cell social behavior (see "Structural and Metabolic Features of GSLs Accumulated in Tumor Cells . . . "). *N*-Butyldeoxygalactonojirimycin specifically inhibits synthesis of glucosylceramide from Cer but has no effect on glycosylation processing of *N*-linked oligosaccharides (115). This reagent is expected to display an effect similar to that of PDMP for reduction of tumor growth and malignancy. A recent study (116) indicates that D-PDMP inhibits GSL shedding from tumor cells and can, therefore, be used for suppression of tumor growth *in vivo*.

The effect of D-PDMP is due in part to induced accumulation of Cer, Sph, and DMS (117), which may in turn inhibit PKC-mediated transmembrane signaling. DMS was used successfully to inhibit growth of various human tumor cell lines in nude mice (118). TMS was subsequently used in place of DMS, because TMS has a stronger inhibitory effect on PKC and platelet activation by tumor cells and gives a stable aqueous solution. TMS strongly inhibited spontaneous metastasis of mouse melanoma B16/BL6 (119). Sph-1-P is a strong inhibitor of cell motility in transformed cells (69). A combination of TMS and Sph-1-P in liposomes was a better inhibitor of BL6 metastasis than TMS alone because Sph-1-P inhibits tumor cell motility (120).

The mechanism for TMS-dependent inhibition of metastasis remains to be clarified. P-selectin expression in platelets is strongly inhibited by DMS and TMS (121). i.v. infusion of TMS inhibits P-selectin expression at systemic vascular endothelia (122). TMS also inhibits E-selectin expression at endothelial cells through blocking of NF-κB activation (123). Cer may also modulate interleukin 1-dependent NF-κB activation, and Cer and interleukin 1 cooperatively induce E-selectin expression (124). These findings suggest that the metastasis-inhibitory effect of TMS is due to inhibition of tumor cell-dependent activation of platelets and to inhibition of E-selectin expression by ECs.

The exogenous addition of Cer analogue C2-Cer to hematopoietic tumor cells (HL60 and U937) induces apoptosis (125). Phorbol myristate acetate-induced apoptosis in HL60 cells is associated with enhanced release of Sph, rather than accumulation of Cer (126). Apoptosis of five human adherent cell lines was induced strongly by Sph and DMS but to a much lower degree by C2-, C6-, or C8-Cer (127). Gb3 (CD77) expression on germinal center B lymphocytes (128) and on Burkitt lymphoma (92) is correlated with the degree of apoptosis. Treatment of Gb3-expressing cells with Gb3-binding subunit B of verotoxin (not the toxic subunit) induces internalization of Gb3 and rapid apoptosis. CD19 has a potential Gb3-binding site with sequence similarity to verotoxin subunit B. CD19-Gb3 interaction produces signal transduction specific for B-cell development, differentiation, and apoptosis (129). The component of bacterial colicin having anticancer effect was recently identified as verotoxin-1, which binds to Gb3 and induces apoptosis (130).

Taken together, these recent observations suggest that effective reagents for ortho-signaling therapy of cancer will be found through a search for GSL or sphingolipid analogues that modify GSL synthesis or Sph or Cer metabolism or that block enhanced transmembrane signaling in tumor cells.

Acknowledgments

I thank Dr. Stephen Anderson for scientific editing and preparation of the manuscript and Prof. Tatsuro Irimura and Dr. Tsutomu Tsuji (Faculty of Pharmaceutical Science, University of Tokyo, Tokyo, Japan) for useful discussions and information.

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