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Traveling for the glycosphingolipid path

"Traveler, there is no path.
Paths are made by walking"
Antonio Machado (Spanish Poet, 1875–1939)

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Our studies on glycosphingolipids (GSLs) were initiated through isolation and structural characterization of lacto-series type 1 and 2 GSLs, and globo-series GSLs. Lacto-series structures included histo-blood group ABH and I/i antigens. Our subsequent studies were focused on GSL changes associated with: (i) ontogenic development and differentiation; (ii) oncogenic transformation and tumor progression. Various novel types of GSLs such as extended globo-series, sialyl-Le^x (SLe^x), sialyl-dimeric-Le^x (SLe^x-Le^x), dimeric-Le^x (Le^x-Le^x), Le^y-on-Le^x, dimeric-Le^a (Le^a-Le^a), Le^b-on-Le^a, etc. were identified as tumor-associated antigens. These studies provide an essential basis for up- or down-regulation of key glycosyltransferase genes controlling development, differentiation, and oncogenesis. GSL structures established in our laboratory are summarized in Table 1, and structural changes of GSLs associated with ontogenesis and oncogenesis are summarized in Sections 2 and 3.

Based on these results, we endeavored to find out the cell biological significance of GSL changes, focused on (i) cell adhesion, e.g., the compaction process of preimplantation embryo in which Le^x-to-Le^x, Gb4-to-GalGb4 or -nLc$_4$ play major roles; and (ii) modulation of signal transduction through interaction of growth factor receptor tyrosine kinase with ganglioside, e.g., EGF receptor tyrosine kinase with GM3. Recent trends of studies on i and ii lead to the concept that GSL clusters (microdomains) are organized with various signal transducer molecules to form 'glycosignaling domains' (GSD). GSL-dependent adhesion occurs through clustered GSLs, and is coupled with activation of signal transducers (cSrc, Src family kinase, Rho A, etc.). Clustered GSLs involved in cell adhesion are recognized by GSLs on counterpart cells (carbohydrate-to-carbohydrate interaction), or by lectins (e.g., siglecs, selectins).

Our major effort in utilization of GSLs in medical science has been for: (i) cancer diagnosis and treatment (vaccine development) based on tumor-associated GSLs and glycoepitopes; (ii) genetically defined phenotype for susceptibility to *E. coli* infection; (iii) clear identification of physiological E-selectin epitope (myeloglycan) expressed on neutrophils and myelocytes; (iv) characterization of sialyl poly-LacNAc epitopes recognized as male-specific antigens. Utilization of these GSLs or glycoepitopes in development of anti-adhesion approach to prevent tumor metastasis, infection, inflammation, or fertilization (i.e., contraceptive) is discussed. For each approach, development of mimetics of key GSLs or glycoepitopes is an important subject of future study.

Keywords: **ontogenic development, oncogenic transformation, tumor-associated antigen, cell adhesion, signal transduction, carbohydrate–carbohydrate interaction**

1. Introduction: All begins with structure

Any biochemical study begins with structure. So did the study of glycosphingolipids (GSLs), which was initiated by Johann Ludwig Wilhelm Thudichum (1829–1901). Thudichum discovered two major brain lipids ('cerebroside' and 'sphingomyelin') characterized by a common, novel aliphatic amino alcohol to which the elegant name 'sphingosine' was given [1,2]. The composition and structure claimed by Thudichum have been confirmed and stood for over 100 years despite serious initial criticism[1].

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[1]Thudichum's discovery of sphingosine and sphingolipids was not accepted, and was rejected as 'falsified results' by then-influential biochemist Hoppe-Seyler or his colleagues. This was largely because Thudichum was a surgeon, and had worked on a large variety of topics (e.g., bile pigments, hematoporphyrin, sarcosine, urochrome), had written books on urine, gallstones, wine, cooking, and practiced resection of nasal polyps using a special surgical tool which he had devised. Thudichum was therefore unable to publish his great discovery in any well-established biochemical journal (i.e., Hoppe-Seyler's Zeitschrift). Instead, he published in a barely-known journal (Reports of the Medical Officer of Privy Council and Local Government Board, N. Ser. III:113 (1874) and VIII:117(1876)). For a biography of Thudichum, see Drabkin DL, 'Thudichum: Chemist of the brain,' Univ. of Pennsylvania Press, Philadelphia, 1958.

Table 1. Major GSL structures established in our laboratory. *, site of α1→2 fucosylation (H), addition of A/B determinant to H, or α2→3 and/or α2→6 sialylation. Right column: reference(s) for specific structure and its substituted forms. Ag, antigen

A. Lacto-series type 2 (only core structure)		
1	*Galβ4GlcNAcβ3Galβ4Glcβ1Cer	paragloboside; Siddiqui & Hakomori [174]; α1→2Fuc subst. H_1 Ag, Stellner et al. [175]
2	*Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	i-antigen; Niemann et al. [20]
3	*Galβ4GlcNAcβ6 Galβ4GlcNAcβ3Galβ4Glcβ1Cer *Galβ4GlcNAcβ3	I-antigen; Watanabe et al. [21]; α1→2Fuc subst. H_3 Ag, Watanabe et al. [176]; α2→3SA subst. NUH2 tumor Ag, Nudelman et al. [177]
4	*Galβ4GlcNAcβ6 Galβ4GlcNAcβ3Galβ4Glcβ1Cer *Galβ4GlcNAcβ3Galβ4GlcNAcβ3	α1→2Fuc subst. H4 and A4 antigen; Fukuda MN & Hakomori [178]
5	*Galβ4GlcNAcβ3Galβ4Glcβ1Cer 3 Fucα	accumulated in tumor, later termed Le^x; Yang & Hakomori [179]; α2→3SA subst. SLe^x tumor Ag, Fukushima et al. [180], Fukushi et al. [36]
6	*Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 3 3 Fucα Fucα	dimeric Le^x; Hakomori et al. [33]; α2→3SA subst. tumor Ag (SLe^x-Le^x), Fukushi et al. [36]; α1→2Fuc subst. KH1 tumor Ag, Nudelman et al. [181], Kaizu et al. [37]
7	*Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 3 3 3 Fucα Fucα Fucα	trimeric Le^x; Hakomori et al. [33]
8a	*Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 3 3 SAα2 Fucα	'myeloglycan'; Stroud et al. [101], Handa et al. [102]; enzymatic synthesis [182]
8b	*Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 3 3 3 SAα2 Fucα Fucα	as above
8c	*Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 3 3 3 3 SAα2 Fucα Fucα Fucα	as above

B. Lacto-series type 1

1	Galβ3GlcNAcβ3Galβ3GlcNAcβ3Galβ4Glcβ1Cer 4 4 Fucα Fucα	Le^a-Le^a; Stroud et al. [34]; as tumor Ag, Watanabe et al. [35]
2	Galβ3GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 4 Fucα	Le^a-paragloboside; Kannagi et al. [183]
3	Galβ3GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer 4 3 Fucα Fucα	Le^a-Le^x as tumor Ag; Martensson et al. [184], enz. synthesis, Stroud et al. [39]
4	Galβ3GlcNAcβ3Galβ3GlcNAcβ3Galβ4Glcβ1Cer 2 4 4 Fucα Fucα Fucα	Le^b-Le^a; Stroud et al. [38]; as tumor Ag, Ito et al. [185]
5	SAα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer 6 SAα2	disialyl type 1 (FH9 tumor Ag); Fukushi et al. [186]

C. Globo-series

1	Galα4Galβ4Glcβ1Cer	Gb3 anomeric structure; Hakomori et al. [187]; Burkitt lymphoma Ag, Nudelman et al. [188]
2	GalNAcβ3Galα4Galβ4Glcβ1Cer	globoside (Gb4) anomeric structure; Hakomori et al. [187]
3	GalNAcβ3Galα3Galβ4Glcβ1Cer	isogloboside; Siddiqui et al. [189]
4	GalNAcα3GalNAcβ3Galα3Galβ4Glcβ1Cer	Forssman antigen; Siddiqui et al. [190]
5	Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer	SSEA-3; Kannagi et al. [11]
6	Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer	globo-H; Kannagi et al. [11]; breast cancer Ag, Bremer et al. [191]
7	SAα3Galβ3GalNAcβ3Galα4Galβ4Galβ1Cer	SSEA-4; Kannagi et al. [12]; *E. coli* binding epitope, Stapleton et al. [156]
8	SAα3Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer 6 SAα2	disialyl Gal-globoside; renal cell carcinoma Ag, Saito et al. [192]; metastasis promoter, Satoh et al. [193]

When I began my studies (late 1950s), only four GSLs were well established: galactosylceramide (GalCer), glucosylceramide (GlcCer), sulfatide (sulfated GalCer), and lactosylceramide (LacCer). Leading laboratories such as those of Ernst Klenk, Richard Kuhn, Tamio Yamakawa, Gunnar Blix, and Lars Svennerholm were involved in isolation and characterization of highly complex GSLs containing hexosamines and then-unknown aminosugars with carboxylic group, termed variously as neuraminic acid (Klenk), lactamic acid (Kuhn), hematamic acid (Yamakawa), and sialic acid (Blix), whose structure was later identified as condensation product of pyruvic acid and glucosamine by Alfred Gottschalk; finally, the aminosugar was identified by enzymatic synthesis as mannosamine by Saul Roseman. Methodologies for isolation and characterization of GSLs were crude in those days, and anyone trying to get into this area encountered enormous difficulties in isolation and characterization of GSLs. It was the dream of my young days that I would someday find a new GSL with novel structure; I didn't care about its function.

Studies were accelerated by introduction of a few effective separation techniques: column chromatographies on silica gel, DEAE cellulose, or Sephadex, thin-layer chromatography, and release of oligosaccharide from ceramide. Structural characterization was promoted by development of a few important technologies: improved methylation analysis with GC-MS, sequential hydrolysis by application of exoglycosidases and endoglycosidases, and total MS of permethylated or underivatized GSLs. However, practical usage of ^{1}H-NMR was made possible only by development of 500 MHz equipment in the mid-1980s.

Our own efforts to find new GSL structures were focused on two series of GSLs: lactoseries type 1 and type 2 structures (including histo-blood group antigens), and globo-series structures (particularly extended globo-series) (Table 1). We also focused on GSL changes associated with various stages of ontogenesis and differentiation (Section 2), various types of oncogenesis (Section 3), functional roles of ceramide (Section 4), and functional roles of GSLs (Section 5). These studies were based mainly on structural changes of GSLs, and provided useful information for future studies on up- or down-regulation of glycosyltransferase genes controlling development and oncogenesis. Our own studies on glycosyltransferase genes have been limited to histo-blood group ABO genes, but this topic is not covered here since it was summarized in a recent review [3].

2. Structural concept of differentiation and development

GSL antigens as carriers of species-, organ-, or cell type specificity have a long history, perhaps initiated by unambiguous demonstration of GSL properties for Forssman antigen [4], and 'cytolipin H' and 'cytolipin K' as cell type-specific antigens [5,6]. Species-specific patterns of erythrocyte GSLs were shown by Yamakawa and colleagues [7,8]. These observations indicated that GSLs may play no role in defining erythrocyte function *per se*, although differentiation of erythrocytes and erythroleukemia cells may depend on GSL and other glycosyl epitopes (see Section 2.d).

Several lines of study in our lab, as described below, indicated that dramatic changes in structure of glycoepitopes in GSLs are closely associated with development and differentiation.

a. Switching core structure from globo- to lacto- to ganglio-series: The order of glycoepitopes developed during early embryogenesis

Glycoepitopes defined by mAbs undergo rapid, continuous changes during embryogenesis, as well as during differentiation of cultured embryonal carcinoma cells that resemble early embryo cells [9,10] (for review see [11]). Antigens expressed highly at the earliest (2–4 cell) stage of embryo, defined by 'stage-specific embryonic antigen' (SSEA)-3 and -4 [10], were identified as extended globo-series structures (Gal-globoside, globo-H, globo-A, sialyl Gal-globoside) [12–14]. An antigen expressed highly at 16-to-32-cell (morula) stage and declining later, whose expression is limited to inner cell mass of blastocyst, was termed SSEA-1 [9]. The antigen was identified as Le^x [15] or more exactly Le^x with long-chain poly-LacNAc [16,17]. Ganglio-series antigens are not expressed until neural crest is formed. Switching of core structure from globo- to lacto- and ganglio- was clearly observed by GSL analysis of Tera-2 derived NT2/D1 cells whose differentiation was induced by retinoic acid. SSEA-3, SSEA-4, and globo-A are highly expressed initially, followed by nLc_4 and Le^x, with decline of globo-series. Finally, Le^x-negative cell populations appear showing high expression of ganglio-series antigens defined by mAbs (A2B5 vs. GT3 and GQ1b; R24 vs. GD3; 126 vs. GD2) [14]. Changes in glycoepitopes expressed during early embryogenesis are shown in Figure 1. Dramatic changes of glycoepitopes occurring at morula-stage embryo suggest a functional role of these glycoepitopes in compaction, the first adhesion event in the entire process of ontogenesis (see Section 5a.i).

b. Shifting of glycoepitope expression in transition from unbranched to branched poly-LacNAc

Histo-blood group ABH epitopes carried by unbranched poly-LacNAc are predominant in fetal erythrocytes, whereas those with branched poly-LacNAc become predominant in adult erythrocytes. This phenomenon, initially found for GSLs [18] was later extended to poly-LacNAc carried by band 3 and band 4.5 glycoproteins [19]. Incidentally, around that time, unbranched unsubstituted poly-LacNAc was identified as blood group i antigen expressed in fetal

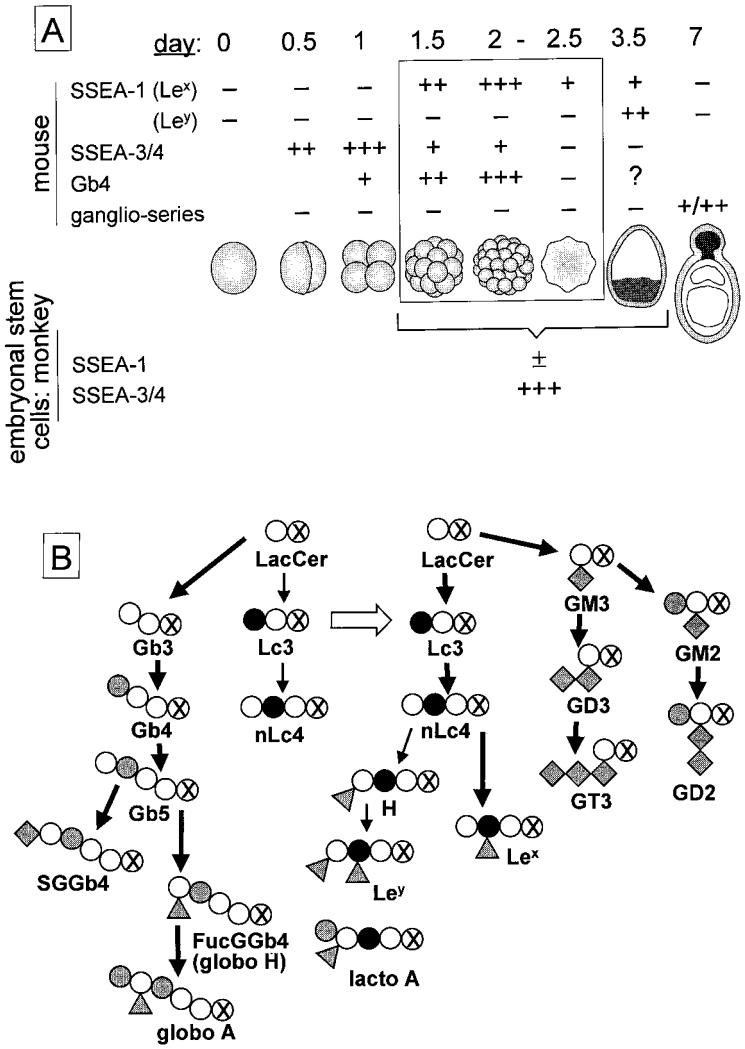

Figure 1. Change of expression patterns of glycoepitopes and GSLs in preimplantation embryo and in human embryonal cell carcinoma Tera-2. *Panel A*: Expression patterns of glycoepitopes in preimplantation embryo. SSEA-1 (Le^x) is not expressed until morula stage, declines after compaction, and is restricted to inner cell mass of blastocyst. SSEA-3 and -4 are expressed highly at four-cell stage and decline later. Globoside (Gb4) is expressed maximally at morula stage. Ganglio-series epitopes are not expressed during preimplantation stage, but appear only upon further differentiation (neural crest formation). There are limited data indicating that SSEA-3 and -4, but not SSEA-1, are highly expressed in morula-stage monkey embryo. Box: morula stage and compacted embryo. Bottom: Reactivity of embryonal stem cells derived from blastocyst but resembling morula stage cells. *Panel B*: Switching of GSL series in Tera-2 derived NT2/D1 cells, from extended globo- to lacto- and finally to ganglio-series during retinoic acid-induced differentiation. Adapted from Fenderson *et al.* [13]. ⊗, Glc. ○, Gal. ●, GalNAc. ●, GlcNAc. ◆, sialic acid (NeuAc). ▲, Fuc. ⟶, major pathway. →, minor pathway.

erythrocytes [20], whereas branched poly-LacNAc was identified as blood group I antigen, a marker of adult erythrocytes [21,22] (Figure 2). Since i-to-I conversion occurs during a period of a few months after birth [23], and I-to-i conversion is associated with hematologic disorders [24], unbranched vs. branched poly-LacNAc may play a significant, but still unknown, role in erythroid cell differentiation and renewal. Transition of i to I or unbranched to branched ABH occurs at almost the same time that fetal hemoglobin (HbF) is converted to adult hemoglobin (HbA). A or B epitopes carried by branched poly-LacNAc are bi- or multivalent, and the binding affinity is much higher than for A or B carried by unbranched poly-LacNAc. Bivalent determinants can bind to two binding sites of IgG antibody; this is termed 'monogamous bivalency' of IgG antibody [25]. Binding of antibody to two or multiple binding sites is favored over binding to a single site by a factor of 10^3 or 10^4 [26,27]. The weak reactivity of fetal erythrocytes with anti-A or anti-B IgG antibodies, due to monovalency, helps prevent hemolytic disorders resulting from ABO-incompatible pregnancy [28]. In fetal erythrocytes, weak A or B reactivity is clearly correlated with deficiency of bivalent determinants, due to lack of 'monogamous bivalency' [29].

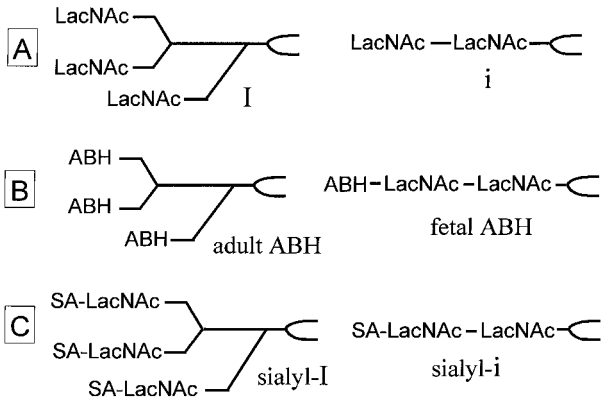

Figure 2. Various developmentally-regulated structures based on poly-LacNAc. Structures are based on common unbranched poly-LacNAc (right) or β1→6 branched poly-LacNAc (left). *Panel A*: unsubstituted, unbranched poly-LacNAc representing i-antigen (Table 1, A.2); unsubstituted, branched poly-LacNAc representing I-antigen (Table 1, A.3,4). *Panel B*: ABH determinant carried by unbranched (left) and branched (right) poly-LacNAc, representing fetal and adult ABH antigen respectively. *Panel C*: unbranched and branched poly-LacNAc having sialyl α2→3 substitution at terminal Gal of poly-LacNAc (with minor degree of α2→6 substitution). Both sialyl-i and sialyl-I are expressed highly in actively-growing cells (eg, placenta, sperm, some tumor cells).

c. Tandemly repeating glycoepitopes

In contrast to mono-, bi-, or multivalent glycoepitopes as described in the preceding section, there are a few distinct structures consisting of tandemly repeating glycoepitopes. Tandemly linked A, i.e., repetitive A, was identified as A_1 antigen [30,31] which is qualitatively distinct from the antigen present in A_2, i.e., H epitope linked to A (Figure 3A) [32]. Another example is tandemly repeating Le^x [33] (Figure 3B) or Le^a [34] (Figure 3C). These repetitive structures were found to be accumulated in human colonic carcinoma, and may represent tumor-associated antigens [33–35]. The nonreducing terminal Gal could be substituted by α1→2Fuc or α2→3NeuAc, leading to formation of sialyl-Le^x-Le^x [36], Le^y-Le^x [37], Le^b-Le^a [38] or Le^a-Le^x [39] (see Section 3.b and Table 1).

d. Switching of GSL series during differentiation of erythroleukemia and myelogenous leukemia

Human erythrocytes are characterized by two major globo-series GSLs, i.e., Gb3 and Gb4 (globoside). Ganglio-series GSLs are absent. In erythroleukemia K562 and HEL cells, ganglio-series GSLs are predominant, but are decreased and replaced by lacto-series GSLs during differentiation. Synthesis of globo-series GSL (particularly Gb3) is induced upon further differentiation [40,41]. Differentiation of mouse myelogenous leukemia cell line M1 into macrophages, induced by various factors, has been used as a model of myeloid cell differentiation [42,43]. Expression of ganglio-series gangliosides in undifferentiated M1 cells declines upon differentiation, and

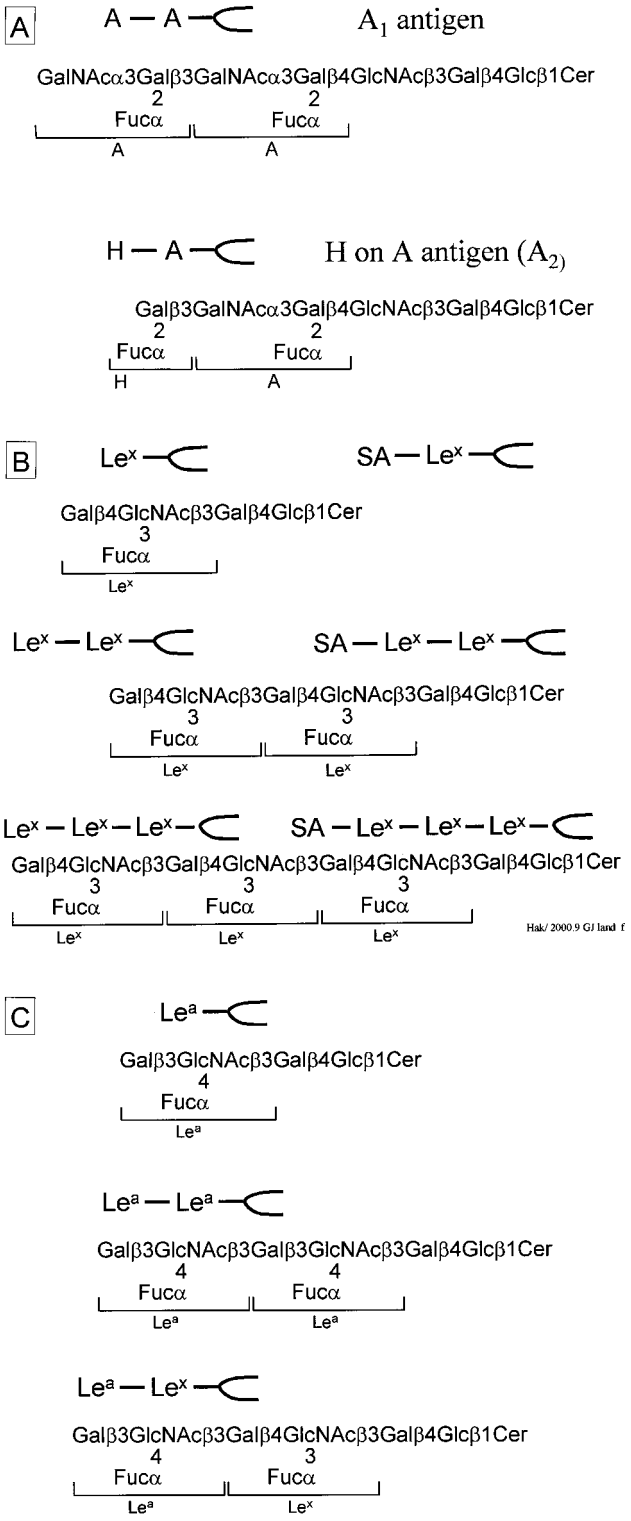

Figure 3. Tandemly repeating glycoepitopes in GSLs. *Panel A*. Repetitive A (tandemly linked A) vs. H on A, representing A_1 and A_2 antigens, respectively. *Panel B*. Singular Le^x vs. tandemly repeating Le^x, and their sialyl derivatives. *Panel C*. Singular Le^a, tandemly repeating Le^a, and Le^a-on-Le^x. Tandemly repeating Le^x, tandemly repeating Le^a, and Le^a-on-Le^x are highly expressed only in certain colonic adenocarcinomas.

they are replaced by lacto-series structures. Finally, globo-series structures represented by Gb3 are detected as major GSL in macrophages. Thus, the order of GSL changes during hematopoietic cell differentiation appears to be the opposite direction from the order in early embryogenesis, i.e., ganglio-→lacto-→globo-series. The significance of this phenomenon is unknown.

3. Structural concept and functional notion of tumor-associated GSL antigens

a. Discovery of tumor-associated GSL antigens

The presence of GSL antigens in polyoma-transformed NIL cells (NIL_{PY}) and their absence in nontransformed NIL cells was revealed by cell surface labeling, and the NIL_{PY} antigen was identified as nLc_4 (paragloboside) [44,45]. Balb/c 3T3 cells transformed by Kirsten strain of murine sarcoma virus (KIMSV) contained Gg3, whereas nontransformed cells did not. KIMSV tumors grown in mice showed accumulation of Gg3 and were stained strongly by anti-Gg3 rabbit antibodies. Gg3 was not expressed in various normal tissues of Balb/c mice. Thus, Gg3 in KIMSV tumors behaves like a tumor-associated antigen [46]. Forssman antigen is detectable in some human gastric, colonic, and lung cancers but is absent in corresponding normal tissues [47,48]. Forssman antigen is absent in tissues of the majority (70–80%) of the human population. However, a minority (20–30%) of humans have this antigen in lung and gastric tissues. Forssman-negative and -positive individuals show presence and absence, respectively, of anti-Forssman antibodies in their sera. Tumors derived from Forssman-negative tissues contain the antigen. Therefore, Forssman can be regarded as a human tumor-associated antigen as well as allogeneic antigen [47,49].

These early studies, all performed prior to establishment of the mAb approach, indicated that GSLs are tumor-associated antigens in both mice and humans. This concept was supported after development of the mAb approach. Surprisingly, a large number of tumor-associated antigens were identified as GSLs or glycoepitopes carried by glycoproteins (for reviews, see [50,51]. Certain anti-GSL IgG mAbs were found to be useful for tumor growth suppression; e.g., T cell lymphoma (L5178) growth in DBA/2 mice was inhibited by anti-Gg3 IgG3 [52], and human melanoma growth was inhibited by infusion of anti-GD3 IgG_3 [53].

b. Mechanism of aberrant glycosylation

Aberrant glycosylation results from either (i) incomplete synthesis of certain GSLs with associated accumulation of precursor, or (ii) enhanced 'neosynthesis' of GSLs which are minimal or absent in normal cells or tissues. Either case i or ii involves appearance of tumor-associated carbohydrate antigens which are defined by mAbs. Typical examples of precursor accumulation are GD2 and GD3 ganglioside in neuroblastoma and melanoma, and Gb3 in Burkitt's lymphoma and ovarian cancer. Appearance of Tn and sialyl-Tn antigens also result from precursor accumulation, although carbohydrate epitopes are restricted to mucin-type, not GSL. Examples of neosynthesis are accumulation of sialyl-Le^x (SLe^x), SLe^x-Le^x, sialyl-Le^a (SLe^a), Le^x, Le^x-Le^x, Le^a, Le^a-Le^a, Le^y, Le^y-Le^x, and Le^a-Le^x (see structures and references in Table 1) in various types of gastrointestinal, colorectal, pancreatic, hepatic, and lung cancer. Globo-H accumulation in breast and prostate cancer is also regarded as neosynthesis.

c. Aberrant glycosylation defining tumor malignancy

Expression of some of these glycosyl epitopes in primary cancer clearly defines outcome of tumor progression and metastasis, as shown in many clinicopathological studies and typically documented in terms of 5- to 7-year patient survival rates following surgical resection of primary tumor (for reviews see [54,55]. Expression of H/Le^y (resulting from deletion of histo-blood group A/B antigens), SLe^x or SLe^a and their analogues, and STn have been most clearly correlated with metastasis and patient survival. Of these epitopes, the mechanism which defines malignancy is known only in the case of SLe^x and SLe^a. I.e., these tumor-associated epitopes mediate binding of tumor cells to microvascular endothelial cells through E-selectin expressed on the endothelial cells, thereby initiating blood-borne metastasis [56–60]. Other epitopes may cause metastasis through yet ill-defined mechanisms, including (i) carbohydrate-carbohydrate interaction [61–63]; (ii) sialic acid-binding protein (siglec) [64,65]; (iii) promotion or inhibition of tumor-associated angiogenesis by ganglioside [66,67]; (iv) activation of platelets by tumor cells and associated release of factor(s) promoting expression of E-selectin at endothelial cells [68]; the factors include lymphokines (IL-1, TNF-α) inducible by tumor cells [69]; (v) enhanced GlcCer synthesis reduces Cer level and confers multi-drug resistance in certain tumor cells [70,71]. This may be ascribable to reduced Cer-dependent apoptosis, consistent with the tumor-inhibitory effect of PDMP based on its inhibition of GlcCer synthase [72].

This research area is still in its early stage of development. After elucidation of these mechanisms, we expect to be able to develop reagents to effectively block metastasis through anti-adhesion or orthosignaling therapy, as proposed some time ago [73]. Functional significance of tumor-associated GSLs is summarized in Figure 4.

4. Ceramides: Not simply a holder of GSLs in membrane, but a definer of glycosylation, antigenicity, and the degree of clustering

a. Cer structure defines glycosylation pattern of GSLs

This novel concept was based on the close relationship between fatty acid profile and terminal glycosylation pattern. Terminal Le^x was clearly associated with Cer having C16:0 fatty acid, whereas terminal H epitope is carried by Cer

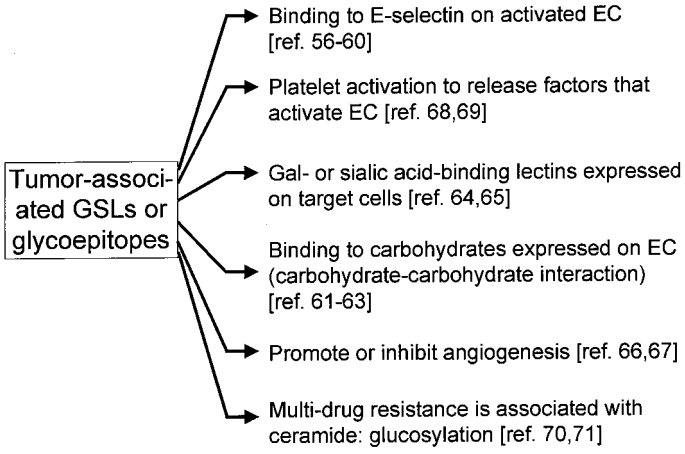

Figure 4. Functional role of tumor-associated GSLs defining tumor malignancy. Expression of tumor-associated GSLs or glycoepitopes in general in primary tumor is strongly correlated with outcome of tumor progression, metastasis, and invasion. Clear evidence is shown for SLe^x or SLe^a-expressing tumors, which bind to E-selectin on activated endothelial cells (EC). Other mechanisms are suggested by the references cited (see text).

containing C22:0, 24:0, or 24:1. Similarly, NeuAcα2→6Gal terminal structure is associated with Cer containing C16:0 and C18:0, whereas those having NeuAcα2→3Gal terminal structure are associated with Cer containing long-chain fatty acid C22:0, 24:0, 24:1 [74]. This novel relationship between terminal glycosylation and fatty acid composition of Cer strongly suggests that specific glycosyltransferases may be preferentially linked to membrane with relatively fluidic (mobile) or rigid (immobile) physical properties. Cer having short-chain fatty acid is more fluidic than those having long-chain fatty acid, which are more rigid.

b. Cer structure defines antigenicity, immunogenicity, and receptor function

A remarkable effect of Cer structure on antigenicity and immunogenicity of GSL carbohydrates was demonstrated by the following studies: (i) fucosyl α1→1Cer isolated from human colonic carcinoma tissue has C20-Sph (eicosasphingosine) and myristic acid (C14) [75]; (ii) fucosyl α1→1Cer having C20-24 fatty acid, its α-hydroxy derivative, and C18-Sph was synthesized [76]. The fucosyl residue in i was not immunogenic, whereas that in ii was strongly immunogenic in rabbit under the same conditions. Antibody raised by ii reacted strongly with ii but only weakly with i [76]. Another study, on reactivity of asialo-GM2 (Gg3) expressed on mouse T cell lymphoma L5178 variant with its mAb 2D4, showed that highly-reactive clones 1C2 and 1B6 contained Cer having α-hydroxy fatty acid with long carbohydrate chain, whereas low-reactive clones 2A1, 2A6 contained Cer having short chain fatty acid [77].

The fatty acid composition of Cer also affects Gb3 receptor function for verotoxin, as demonstrated by C. Lingwood and colleagues. Gb3 with short-chain fatty acid (C12, C14) showed minimal binding, whereas Gb3 with C20:0 or C22:1 had the highest binding ability and cellular susceptibility to verotoxin [78]. Gb3 having synthetic alkyl chain (e.g., monoalkylsulfides, bisalkylsulfides) showed no binding and no cytotoxic susceptibility to verotoxin [79]. Thus, GSL receptor function is influenced greatly by Cer structure, as is GSL antigenicity.

c. Cer may define degree of GSL clustering in membrane

Many lines of evidence have shown that GSLs and sphingomyelin are clustered and organized to form GSL signaling domain (GSD) or GSL-enriched microdomain (GEM) (see Section 5c). The capability of sphingolipids to form clusters may be ascribable to higher *cis* interaction between Cer than between glycerides. Cer can act as hydrogen bond donor as well as acceptor, whereas glyceride can act only as hydrogen bond acceptor [80].

d. Cer as second messenger in signal transduction

Cer, as a catabolite of sphingomyelin, has been regarded as a second messenger in response to cell stimuli, particularly for induction of growth inhibition and apoptosis. Many studies along this line have been performed; for review see [81]. This topic is outside the scope of the present review.

5. Two functional roles of GSLs in membrane

A significant role of GSLs in defining membrane-based cell functions is indicated by quantitative and qualitative changes of GSLs associated with differentiation and development, as described above. Besides the 'classic' function of GSLs as antigens and toxin receptors, we have made major research efforts on two basic GSL functions: involvement in cell adhesion/recognition processes, and in initiation/modulation of signal transduction.

a. GSLs as mediators and modulators of cell adhesion/recognition

Three types of mechanism involved in this process, studied in our laboratory, are summarized below.

i. GSL-dependent cell adhesion/recognition through carbohydrate–carbohydrate interaction. This mechanism was initially revealed by studying Le^x-dependent adhesion of morula-stage mouse embryo (i.e., compaction), or autoaggregation of mouse embryonal carcinoma F9 cells. Since this adhesion process depends highly on Ca^{2+}-mediated Le^x expression [82], we initially looked for an Le^x-binding protein expressed on F9 cells [83]. However, such binding proteins always express Le^x, and we found that the adhesion depends on Ca^{2+}-catalyzed Le^x-to-Le^x interaction [84]. The study was extended to cell adhesion mediated by embryoglycan bearing Le^x [85].

Ca^{2+}-mediated Le^x-Le^x interaction was confirmed by various other procedures, particularly NMR [86–88]. Another striking example of GSL-dependent adhesion is binding of human embryonal carcinoma 2102 cells to Gb4-coated plates, mediated by interaction of Gb4 with GalGb4 or nLc_4 (both expressed on 2102 cell surface). This is a model of autoaggregation of human embryonal carcinoma cells, which may mimic the compaction process of morula-stage embryo of monkey or human [89].

Compaction may proceed via three mediators: (i) E-cadherin [90]; (ii) Le^x-to-Le^x interaction [84]; (iii) Gb4-nLc_4 or Gb4-GalGb4 interaction [89]. Such redundant processes exist because of the importance of compaction. Compaction of E-cadherin knockout mouse embryo proceeds normally [91], as does adhesion of an Le^x-defective variant of F9 embryonal carcinoma [92]. Cadherin and Le^x were found to act cooperatively [88]. Since Le^x is expressed highly in 'embryoglycan' [85,93], it is not surprising that embryogenesis through compaction proceeds in GlcCer synthase knockout mouse [94]. Only globo-series structures (SSEA-3 and -4), but not Le^x, are expressed in primate embryonic stem cells [95]. This indicates a major role of globo-series GSLs in cooperation with cadherin for compaction and embryonic differentiation of primates and perhaps humans.

There may be many cell adhesion/recognition systems in which carbohydrate–carbohydrate interaction plays an essential role. However, only the compaction process as above, and initiation of B16 melanoma metastasis as below, have been elucidated to an appreciable extent.

Adhesion of mouse B16 melanoma cells to LacCer, Gb4, or Gg3 coated plates is mediated by interaction of GM3 (expressed highly on B16 melanoma cells) with the above GSLs [96–98]. The adhesion process is synergistically enhanced when fibronectin or laminin is co-coated with GSL [98]. GM3-dependent adhesion of Bl6 cells to nonactivated mouse endothelial cells (which express LacCer, Gb4, and Gg3) is regarded as the initial step in metastasis of B16 cells [62,63].

GSL adhesion systems based on carbohydrate–carbohydrate interaction have the following characteristics: (i) adhesion process is rapid (within <10 min, compared to >30 min for integrin-dependent adhesion); (ii) specificity is high in some cases, low in others; (iii) most require bivalent cation such as Ca^{2+}, but a few do not; (iv) synergistic with other adhesion systems, e.g., integrins; (v) negative interaction (repulsion) occurs between certain pairs of carbohydrates, e.g., GM3-GM3, Le^y-Le^y. Binding affinity of carbohydrate to carbohydrate depends on degree of clustering of glycosyl epitope presented. Gg3 oligosaccharide (GalNAcβ1→4Galβ1→4Glc) N-linked to polystyrene polymer displayed high binding affinity to GM3 monolayer as determined by surface plasmon resonance (BIAcore). Thus, Gg3-to-GM3 interaction was calculated as $K_a = 2.5 \times 10^6\ M^{-1}$. This value is in the same range as that of many protein-to-protein interactions. In contrast, binding affinity of lactose or cellubiose polystyrene polymer to GM3 was calculated as $K_a = 7.7 \times 10^4$ and $4.4 \times 10^4\ M^{-1}$, respectively [99].

ii. Myeloglycan: A physiological glycoepitope involved in E-selectin mediated myelocyte adhesion. Unambiguous characterization of E-selectin epitope was initially based on inhibitory effect of SLe^x ceramide hexasaccharide or SLe^x-Le^x ceramide octasaccharide on binding of HL60 cells to E-selectin [100]. Later, extensive studies on chemical characterization of large quantities of HL60 cells or human neutrophils indicated that the same GSLs as above were virtually absent; instead, the cells contained a series of long-chain, unbranched poly-LacNAc having α2→3 sialylation at terminal Gal and α1→3 fucosylation at internal GlcNAc but not penultimate GlcNAc [101] (see Table 1A.8.a–c). GSLs with such poly-LacNAc structure, collectively termed 'myeloglycan,' showed strong binding to E-selectin under dynamic flow conditions [102]. A series of myeloglycan therefore represents the physiological E-selectin epitope, although they may also be present bound to O-linked glycoprotein PSGL-1. It remains unclear whether myeloglycan represents a part of physiological P-selectin epitope. An elegant synthetic study by Cummings and associates indicates that a single O-linked SLe^x glycan and three consecutive tyrosine sulfates at N-terminal region of PSGL-1 are essential for strong P-selectin-dependent adhesion [103]. Would higher activity be observable if O-linked SLe^x were replaced by myeloglycan type, particularly under dynamic flow conditions?

iii. Ganglioside-modulated integrin receptor function. During the search for fibronectin (FN) receptors, polysialoganglioside was a candidate for receptor since it inhibits FN-dependent cell adhesion [104]. After FN receptors were identified as $\alpha5\beta1$ and $\alpha4\beta1$ integrin, studies were focused on the possibility that gangliosides modulate integrin function, e.g., GD2 ganglioside enhanced vitronectin receptor activity [105]. GM3 enhanced activity of FN receptor $\alpha5\beta1$ at low doses and inhibited activity at higher doses in reconstituted membrane with PC-cholesterol and varying concentration of GM3, whereas LacCer had no effect [106]. Migration and metastasis of FBJ-LL cells was inhibited by GD1a ganglioside, suggesting that α3 or α6 integrin function is susceptible to GD1a [107].

b. GSLs as initiators/modulators of signal transduction

In view of the remarkable changes of GSL composition and structure associated with differentiation, development, and cancer progression (Section 2), a few preliminary studies were undertaken to test whether GSLs affect cell cycle [108], cell growth [109], and apoptosis [110,111]. Thus, the possibility is opened up that GSLs may modulate function of key receptors for signal transducers controlling various cell physiological processes. GM3 ganglioside in BHK cells inhibited FGF receptor function and blocked FGF internalization [109]. GM1 in 3T3 cells inhibited PDGF-dependent 3T3 cell growth due to

inhibition of tyrosine kinase associated with PDGF receptor [112]. Similarly, EGF-dependent growth of A431 and KB cells was inhibited by GM3, due to inhibition of tyrosine kinase associated with EGF receptor [113]. A number of similar studies since then support the idea that gangliosides inhibit or promote growth factor receptor tyrosine kinase (for review, see [114]. Regarding the physiological relevance of this phenomenon, ldlD cells having EGF receptor are growth-inhibited when grown in Gal-containing medium, whereby GM3 synthesis occurs [115].

c. GEM or GSD as structural units mediating GSL-dependent cell adhesion coupled with signal transduction

Development of the concept of GSL-enriched microdomains (GEM) or glycosignaling domains (GSD) in plasma membranes has a long history. Major inputs for the concept were: (i) clustering of GSLs observed by fluorescent microscopy and electron microscopy; (ii) uneven distribution of GSLs within epithelial cells (apical vs. basolateral); (iii) detergent resistance of GSLs together with cytoskeletal components and other major molecules involved in cell adhesion; (iv) association of Src family kinase with GPI anchor, which is also detergent-resistant; (v) a close similarity of GSL microdomain with caveolar membrane, previously-known invaginations of plasma membrane enriched in cholesterol and caveolin, with which various signal transducer molecules are claimed to be associated [116].

Studies since 1997 show that clusters of GSLs organized with various signal transducer molecules such as cSrc, Src family kinases, small G-proteins (Rho, Ras), and focal adhesion kinase (FAK), are present and separable from caveolar membrane. GSL microdomains are involved in GSL-dependent cell adhesion coupled with signal transduction [117–119] and were termed 'glycosignaling domain' (GSD) (for review see [120]. Caveolar membranes are involved in endocytosis and signal transduction, and disrupted by cholesterol-binding reagents (filipin, nystatin, β-cyclodextrin) [116]. In contrast, function of GSD in B16 melanoma cells is disrupted by lyso-GSLs, particularly sialyl α2→1Sph and lyso-GM3, but not by Galβ1→1Sph (psychosine), lactosyl β1→1Sph, lactosyl β1→1 [lactosyl β1→3]Sph, or lysophosphatidylcholine [119,121,122]. Many investigators have studied GSD in recent years, and a special volume of *Glycoconj J* devoted to the topic is being prepared (Tettamanti G, Sonnino S, eds.). Current concepts of GSL function in terms of GSD structure and function are illustrated in Figure 5.

How is the GSD concept compatible with previous views of ganglioside effect on growth factor receptor function? Receptors for EGF and PDGF were claimed to be associated with caveolar membrane, and are quickly translocated out of caveolar membrane upon stimulation of cells by EGF or PDGF immediately after tyrosine kinase activation [123,124]. However, 'caveolar membrane' in these studies was not clearly distinguished from GSD, and it is possible that the growth factor receptors detected were actually associated with GSD rather than caveolar membrane. The majority of growth factor receptors have been found in high-density rather than low-density membrane fraction. If there is interaction of growth factor receptors with GSLs in GSD, then subtle, unidentified conditions may exist for such interaction.

6. Basic problems pending

a. Molecular genetic approaches for determining functional role of GSLs

Essentially all glycosyltransferase genes for synthesis of all series of GSLs have been cloned. Thus, many glycoepitope changes associated with ontogenesis and oncogenesis (see Sections 2 and 3) can be studied in terms of gene activation/inhibition mechanism. Knockout approach for specific glycosyltransferase genes, or anti-sense approach, may answer questions on function of specific GSLs expressed at defined stages of ontogenesis, or GSLs defining malignancy. An elegant study by RL Proia and colleagues with GlcCer synthase gene knockout mice indicated that GSLs derived from GlcCer play essential roles in differentiation of many types of tissues from primitive germ layers, even though embryogenesis takes place up to primitive germ layers [94] (see Section 5a). A study with GalCer synthase gene knockout mice, defective in synthesis of GalCer and sulfatide in myelin sheath, showed severe defects in mobility of the mice [125,126]. Neuronal tissue developed normally in β1→4GalNAc transferase gene knockout mice which do not synthesize major ganglio-series gangliosides GM2, GM1, GD1a, etc. [127]. However, more recent studies have shown that nervous system of such mice develops axon degeneration and defective myelination, and the animals have impaired motor coordination and behavioral abnormalities [128]. Extension of this approach to each glycosyltransferase involved in synthesis of key GSLs controlling development and oncogenesis is an important pending project.

On the other hand, each glycosyltransferase gene is up- or down-regulated through its promoter region by trans- or cis-acting factors. Only a few studies along this line have been performed so far. Factors involved in promoter regions of β1→4Gal transferase [129], GlcNAc transferase V [130], Fuc transferase IV [131], and blood group A/B transferase [132,133] have been studied. It is important to elucidate factors affecting glycogenes that control development and cancer progression.

b. Does GSL-dependent cell adhesion influence gene expression?

Cell adhesion is not simple physical binding of one cell surface to another. There is much evidence that cell adhesion, particularly mediated by integrins and cadherins, influences gene expression, and consequent changes in various phenotypes, including cell motility. Does a similar mechanism apply for GSL-dependent cell adhesion? This question was partially

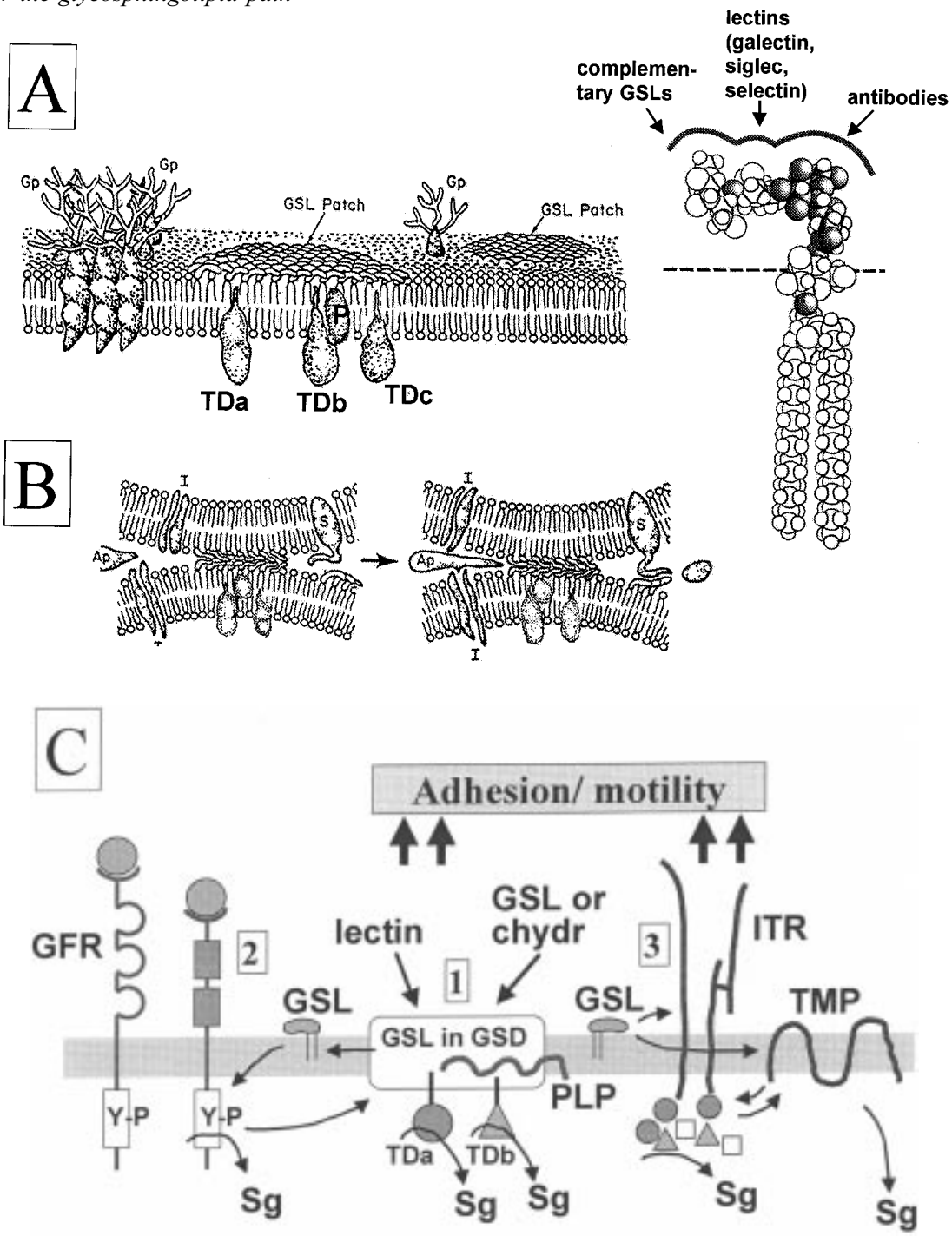

Figure 5. GSL conformation and organization in membrane, as related to GSL function. *Panel A*. **Right**: General conformational structure for globoside. Axis of carbohydrate is perpendicular to that of Cer. Outer surface structure of carbohydrate chain binds to antibodies, lectins, and complementary GSLs. **Left**: Individual GSLs are inserted in outer leaflet of plasma membrane through Cer. Because Cer-to-Cer interaction is stronger than glyceride-to-glyceride interaction in phospholipid bilayer, GSLs cluster to form GSL patches (perhaps with sphingomyelin as well) where various signal transducer molecules (TDa, TDb, TDc) are associated directly or indirectly through hydrophobic proteins (P). GSL patches, forming GSL signaling domain (GSD), are separate from clusters of glycoproteins (Gp) and from caveolar membrane (not shown in this scheme).
Panel B. Scheme for cell-to-cell adhesion through GSL clusters in GSD, through GSL-to-GSL interaction. This takes place early in the adhesion process, prior to involvement of adhesion protein (Ap) or integrin receptor (I). Subsequently, signal transducers associated with GSD are activated, and initiate sending of signals.
Panel C. Functional role of GSD as related to various receptors: (1) clustered GSLs in GSD as major functional unit; (2) GSL interaction with growth factor receptors (GFR); (3) GSL interaction with integrin receptor (ITR), tetraspan membrane protein (TMP), and other proteins. Sg, signaling; PLP, proteolipid protein that binds to GSL and possibly to signal transducers. GSLs in GSD may interact with GFR and thereby modify tyrosine phosphorylation (Y-P), leading to modulation of signaling for cell proliferation and differentiation. Alternatively, GSLs may interact with ITR and TMP to modulate their function, resulting in change of cell adhesion and motility. GSLs in GSD may be directly involved in cell adhesion through carbohydrate-to-carbohydrate interaction or lectin interaction, leading to activation or inhibition of signal transducers (TDa, TDb).

answered by studies on 'GSD' structure and function (Section 5c). GSL-dependent cell adhesion was well studied in the compaction process and in its model, autoaggregation of embryonal carcinoma cells, through Le^x, GalGb4 or Gb4 [89], cadherin [90], or cooperative effect between these glycoepitopes and cadherin [88] (see Sections 2a and 5a). The adhesion may take place at GSD and initiate signal transduction. Gb4-dependent adhesion of 2102 cells induces activation of AP1 and CREB [89]. At each step of differentiation from blastocyst to various 'anlage' (or primordium) through endoderm, ectoderm, and mesoderm, cell-to-cell interaction may proceed through switching on or off of glycosyltransferase genes leading to specific glycosylation pattern at the cell surface. A large variety of developing cells are mobile, and cells having specific surface glycosylation 'code' may selectively interact with each other to form an 'anlage' of a particular organ. Each anlage is assumed to express a specific GSL or glycoepitope pattern which is read by another glycosylation code ('area-code hypothesis') [134].

Code recognition is presumably based on: (i) GSL-to-GSL interaction; (ii) recognition of GSL by its binding protein, e.g., galectin-1 [135], 'siglec' [136]); (iii) modulation by ganglioside of integrin-dependent cell adhesion and motility, which are strongly influenced by tetraspan membrane protein (TMP) [137]. Gangliosides also modulate TMP function (Ono M, Handa K, Hakomori S, unpublished data). Our fragmentary knowledge in this area may be unified into a systematic view in the near future.

c. Physical basis for GSL organization in microdomains: GEM or GSD

GSLs and sphingolipids have unusual 'self-assembling' properties allowing them to form aggregates with various shapes. For example, galactosylceramide having α-hydroxy fatty acid forms tightly-rolled multilayered cylindrical fibers (resembling the myelin sheaths around nerve cells), or long single fibers, under different conditions [138]. Sphingosine forms lipid fibers with different shapes depending on stereoisomeric structure at C1–C3 [139]. Long-chain alkanes or alkenes having terminal asymmetric carbon usually form lipid fibers, some showing clear helical structure [140]. The physical basis for formation of GSL microdomain together with sphingomyelin may require side-by-side (cis) interaction in the plane of the phospholipid/cholesterol layer. Sphingolipids are capable of being hydrogen bond donors as well as acceptors, whereas glycerophospholipids can only be hydrogen bond acceptors [80]. Thus, intrinsic physical properties of sphingolipids appear to provide the basis for formation of sphingolipid microdomains. Extensive further physical studies are necessary for confirmation.

7. GSLs in medical and medicinal problems

Our studies in this area have focused on development of cancer diagnosis and treatment based on our original discoveries of tumor-associated GSL antigens (Section 3). A few collaborations with other groups for study of *E. coli* infection and utilization of male-specific glycosylation will be described here.

a. Diagnostic and prognostic applications and anti-cancer vaccine development based on tumor-associated GSLs and glycoepitopes

i. Diagnosis and prognosis. A number of initial studies on application of mAbs directed to tumor-associated GSL antigens for diagnosis of cancer showed only limited success, since tumor antigen level in serum is only detectable at advanced stages. In contrast, expression of GSL antigens in primary tumor as revealed by immunohistological examination of surgically-resected tumor samples has much more reliable correlation with future survival of patients. Colonic carcinoma expressing SLe^a or SLe^x, non-small cell lung carcinoma expressing H/Le^y, and bladder carcinoma expressing SLe^x in primary tumor show significantly shorter survival than cases not expressing these antigens (for review see [54,55].

ii. Vaccine development. Tumors which express antigens correlated with shorter survival are potential targets for anti-cancer vaccines. GM2 antigen in human melanoma [141], sialyl-Tn in breast cancer [142], and globo-H in prostate cancer [143] have been used for active immunization. Each of these antigens was conjugated to keyhole limpet hemocyanin (KLH), used for immunization of patients, and led to significant increase of survival rate and reduction of clinical symptoms (for review see [55]. However, major immune responses to carbohydrate antigens are IgM and (to a lesser extent) IgG antibody. Cytotoxic T cell response is desirable to suppress tumor cell growth, although such response, restricted to MHC class 1, is observable only when short carbohydrate epitope is linked to a defined site of MHC [144,145]. On the other hand, helper T cell with IL2 response, and associated production of IgG, were observed based on antigen presentation through MHC class 2 [146]. We observed helper T cell response against Tn antigen, i.e., α-GalNAc residues in mucin glycoprotein, and consequent inhibition of TA3-Ha tumor growth in mice when animals were immunized with Tn mucin and pretreated with cyclophosphamide, an inhibitor of suppressor T cells [147]. Similar anti-Tn T cell response with IL2 production was observed with tandem Tn sequence linked to poliovirus peptide [148]. As a carrier to stimulate anti-Tn response, specific lipopeptide was designed [149,150]. Thus, it is important not only to select proper tumor-associated carbohydrate epitope, but also that the epitope be linked to proper polypeptide or other carrier molecule. A polypeptide containing an amino acid sequence common to the major family members of MHC class 2 (Pan DR T helper epitopes; PADRE) has been designed as a common carrier of a specific epitope [151].

In order to overcome the weak immunogenicity of carbohydrate antigens to induce T cell response, peptide 'mimotopes' of Le^x, SLe^x, Le^y, etc. have been used to obtain IgG response in mice [152]. While antibody titer and specificity through this approach have been low so far, they may be improved in future studies.

iii. Rationale for future development of anti-adhesion therapy to block metastasis. The initial step of blood-borne metastasis is binding of tumor cells to endothelial cells or other target cells through interaction of tumor-associated antigen with selectin, galactose or sialic acid-binding lectin (galectin or siglec), or complementary carbohydrate (see Sections 3c and 5a.i). Metastasis of mouse melanoma BL6 cells, mediated by interaction of LacCer, Gb4, or Gg3 expressed on endothelial cells with GM3 expressed on BL6 cells was inhibited by liposomes containing GM3 or Gg3 [63]. Theoretically, carbohydrate epitopes involved in metastasis could also inhibit the metastatic process. However, such carbohydrates are difficult to prepare and unstable *in vivo*. To overcome this difficulty, it is desirable to develop stable mimetics (see Section 8).

b. Susceptibility of *E. coli* infection in urogenital epithelia defined by expression pattern of globo-series antigens and secretor/nonsecretor status

Globo-series structures, expressed highly in urogenital epithelia, are known to be targets of *E. coli* infection causing pyelitis, cystitis, and ureteritis [153–155]. *E. coli* urinary tract infection is much higher in women than men because of anatomical differences, while the incidence of recurrent infection is 4 times higher in nonsecretor compared to secretor individuals [154]. We found, in collaboration with A. Stapleton and W.E. Stamm, that clinical *E. coli* isolates R45 or 1A2 bind to a specific GSL, monosialylgalactosylgloboside (MSGG; the same as SSEA-4; see Table 1c), which is expressed in nonsecretors but virtually absent in secretors [156]. In contrast to salivary and gastrointestinal glands and their secretions, which express lacto-series GSLs and glycoepitopes, globo-series are present exclusively in GSLs and constitute the major epitope in urogenital epithelia that bind to *E. coli*. The mAb directed to sialylgalactosylgloboside is useful for testing susceptibility to recurrent infection [156]. Wild-type clinical isolates of *E. coli* (R45, 1A2) as well as three cloned isolates expressing *pap*-encoded adhesins, all bind preferentially to MSGG over disialylgalactosylgloboside (DSGG) or other globo-series GSLs [157]. MSGG or its mimetics could be targets for anti-adhesive reagents for prevention of recurrent infection. Expression of MSGG and other globo-series antigens in urogenital epithelia, and its relation to secretor/nonsecretor status, are shown schematically in Figure 6.

c. Male-specific GSL epitopes: Can these glycoepitopes help treat infertility and prevent world population explosion?

Sperm-specific antibodies were elicited and infertility was induced in female guinea pigs following immunization with sperm [158]. Anti-human sperm antibodies were detectable in sera of $\sim$17% of human infertility patients, and shown to inhibit motility of sperm [159]. A human mAb H6-3C4 was established by fusion of lymphocytes from a patient having high level of anti-sperm antibody, with mouse myeloma cell line [160]. In collaboration with S. Isojima and Y. Tsuji, we showed that H6-3C4 is directed to sialyl-i regardless of whether sialyl linkage is $\alpha 2 \rightarrow 3$ or $\alpha 2 \rightarrow 6$. The mAb NUH2, established previously against tumor-associated antigen sialyl-I (see Table 1A.3), also bound strongly to sperm and inhibited their motility [161]. Recently, H6-3C4 epitope was found in CD52, a short polypeptide linked to GPI anchor [162,163].

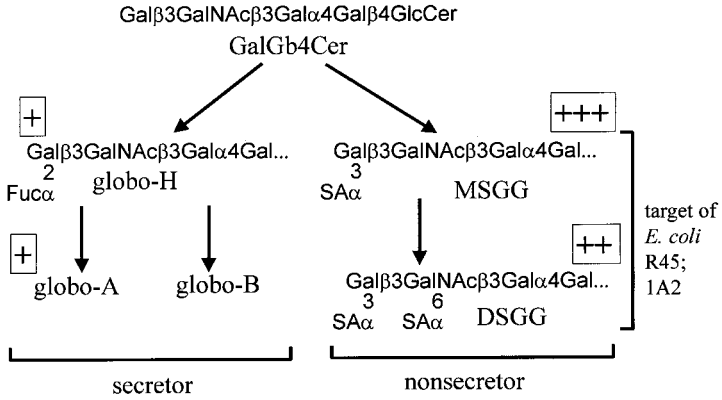

Figure 6. Differences of globo-series GSLs expressed in urogenital epithelia of secretors vs. nonsecretors, leading to different susceptibility to *E. coli* infection. Analysis was made in vaginal epithelial cells, which are readily collectible by tamponing. The major GSLs present in cells from secretor individuals are globo-H, globo-A, or globo-B, depending on histo-blood group status. In contrast, the major GSLs in cells from nonsecretors are mono- and disialosyl galactosylgloboside (MSGG and DSGG). Binding affinity of *E. coli* isolates R45 and IA2 to MSGG is higher than that to DSGG, globo-H, globo-A, or globo-B (see text). +, ++, +++: degree of binding activity of each GSL to *E. coli* isolates.

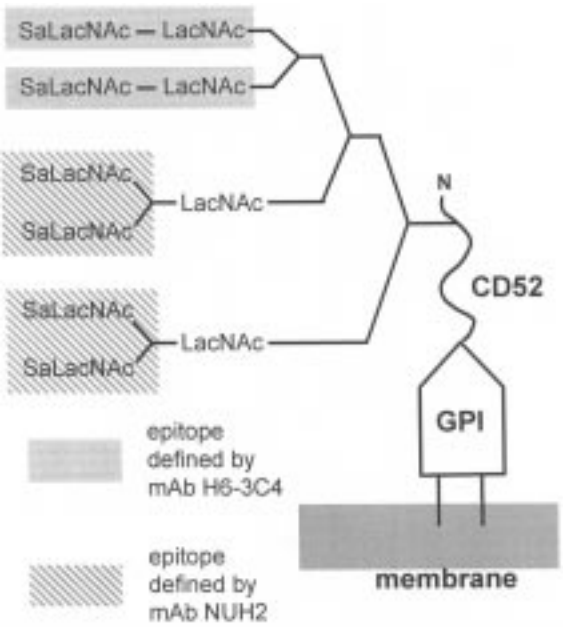

Figure 7. Male-specific glycoepitope of GPI-anchored CD52, expressed in seminal fluid and sperm cells. The scheme is based on the glycoepitope linked to GPI anchor-linked CD52 and dodecapeptide GQNDTSQTSSPS, as well as on the specificity of mAbs H6-3C4 and NUH2 (see text). Sialyl unbranched poly-LacNAc defined by H6-3C4, and sialyl branched poly-LacNAc defined by NUH2, are indicated by two types of shadowing.

The epitopes are not only sialyl-i but also sialyl-I (Figure 7). CD52 is present in lymphocytes but is differently glycosylated and has no epitope which binds to H6-3C4 or NUH2 (see Table 1A.3). It is suggested that CD52 with specific type of glycosylation is present in seminal fluid and absorbed in the sperm cell membrane. The carbohydrate epitope *per se* is by no means specific to sperm, but its organization, density and arrangement within CD52, and presumed its presence in GSD may be unique to human sperm. Epitope organized in such a way could be useful to eliminate anti-sperm antibodies present in infertile women, facilitating pregnancy. On the other hand, mimetics of the above glycoepitope could be useful as contraceptives; nonpeptide mimetics could be administered orally.

8. Glycomimetics

Based on the functional role of GSLs or glycoepitopes in cell adhesion, their oligosaccharides or derivatives have been used to inhibit adhesion involved in initiation of disease processes. Examples are effect of SLe^x oligosaccharide (claimed to be E- and P-selectin epitope; see Section 3c) to prevent acute inflammatory response such as lung injury induced by immune complex [164] or by P-selectin-dependent recruitment of leukocytes [165]. However, SLe^x oligosaccharide is difficult to synthesize in large quantity, and unstable *in vivo*. Therefore, development of practically useful drugs to inhibit selectin-dependent inflammatory response should be based on stable mimetics with binding affinity higher than that of oligosaccharide. A peptide mimetic of SLe^a, DLWDWVVGKPAG, selected from phage display random peptide library, was shown recently to inhibit E-selectin-dependent leukocyte adhesion and to reduce recruitment of neutrophils into the inflammatory region [166].

Metastasis of tumor cells expressing SLe^x, SLe^a, or their analogues, is mediated by binding of these tumor cells to E-selectin expressed on endothelial cells [56–60]. A peptide mimetic of SLe^x, IELLQAR, selected from phage display random peptide library, was recently shown to inhibit metastasis of B16 mouse melanoma cells expressing SLe^x [167]. Mouse lymphosarcoma RAW117-H10, a highly metastatic variant, is characterized by high content of ganglioside GD1α. Its metastasis to liver is mediated by a receptor expressed on sinusoidal endothelial cells [168]. A peptide

A

H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-
Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH

B

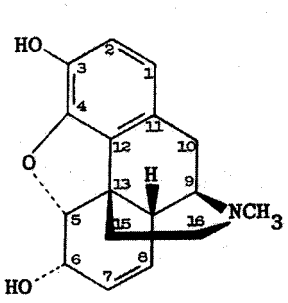

Figure 8. Endorphin (A) and morphine (B). Portions of the surface profiles of these two molecules are quite similar, although it is not obvious by looking at these structures.

mimetic of GD1α, WHWRHRIPLQLAAGR, selected by a similar procedure as above, inhibited liver metastasis of these cells [169]. Le^y is known as a major tumor-associated antigen, and is a target of immunotherapy and drug delivery. A peptide mimetic of Le^y, APWLYGPA, was selected as above and used for immunotoxin delivery and tumor suppression [170]. Peptide mimetics of tumor-associated carbohydrate antigens are expected to induce T cell-dependent immune response as discussed in Section 7a.ii.

Peptide mimetics of carbohydrate antigens are more stable than the original carbohydrate. Morphine is a reagent that strongly mimics endorphin, an endogenous polypeptide, to produce analgesic effect (Figure 8). Similarity in surface structure of morphine and endorphin is difficult to perceive by looking at the figure, but they have a close resemblance. Thus, it is highly desirable to construct and select non-peptide mimetics of carbohydrates, if a defined carbohydrate has a key role in a disease process. This approach is theoretically possible based on anti-carbohydrate antibodies, or on peptide mimetics selected preliminarily from phage display random peptide library. Selection and construction of non-peptide mimetics has been well established where the key structure is either β-turn, strand, or α-helix, e.g., methods for synthesis of mimetics of peptide β-turn [171,172]. If complementarity region of anti-carbohydrate antibody is identified, design and synthesis of mimetics can be performed as described [173]. So far, no nonpeptide mimetics for carbohydrate epitopes have been described. However, this approach is important in view of the increasing applicability of anti-adhesion therapy to prevent tumor cell metastasis, infectious processes, or fertilization, if stable nonpeptide mimetics are available. Because of instability *in vivo*, GSLs, their carbohydrates, or their peptide mimetics, will never be ideal reagents for treatment of disease processes based on GSLs or glycoepitopes. Only nonpeptide mimetics can serve this purpose.

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