View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 584 (2010) 1901–1906

FEBS Letters
journal homepage: www.FEBSLetters.org

Review

Glycosynaptic microdomains controlling tumor cell phenotype through alteration of cell growth, adhesion, and motility

Sen-itiroh Hakomori *

Division of Biomembrane Research, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122, USA
Departments of Pathobiology and Global Health, University of Washington, Seattle, WA 98195, USA

ARTICLE INFO

Article history:
Received 21 September 2009
Revised 22 October 2009
Accepted 23 October 2009
Available online 27 October 2009

Edited by Sandro Sonnino

Keywords:
Glycosphingolipid
Tetraspanin
Growth factor receptor
Integrin
Motility
Epithelial–mesenchymal transition
Hypoxia

ABSTRACT

Glycosphingolipids (GSLs) GM3 (NeuAcα3Galβ4Glcβ1Cer) and GM2 (GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer) inhibit (i) cell growth through inhibition of tyrosine kinase associated with growth factor receptor (GFR), (ii) cell adhesion/motility through inhibition of integrin-dependent signaling via Src kinases, or (iii) both cell growth and motility by blocking "cross-talk" between integrins and GFRs. These inhibitory effects are enhanced when GM3 or GM2 are in complex with specific tetraspanins (TSPs) (CD9, CD81, CD82). Processes (i)–(iii) occur through specific organization of GSLs with key molecules (TSPs, caveolins, GFRs, integrins) in the glycosynaptic microdomain. Some of these processes are shared with epithelial–mesenchymal transition induced by TGFβ or under hypoxia, particularly that associated with cancer progression.
© 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Some early studies during the 1970s and 1980s indicated that specific glycosphingolipids (GSLs) are located at specific domains of cellular membranes, e.g., fucose-containing GSLs at apical microvilli membrane, but not at basolateral membrane of intestinal epithelial cells [1]. GM3 (NeuAcα3Galβ4Glcβ1Cer) is present in higher concentration at cell adhesion sites than at other membrane sites of BHK fibroblasts, and GM3 at the adhesion site is insoluble in non-ionic detergent [2]. Sphingolipids and GSLs interact with non-glycosylated proteins in erythrocyte membrane, are insoluble in non-ionic detergent, and display specific morphological profile observed under electron microscopy [3]. At that time, the molecular basis of cell polarity became one of the major topics in cell biology. Specific expression of a glycoprotein, hemagglutinin, was observed after influenza virus infection at apical site, but not basolateral site, of kidney epithelial MDCK cells. A search for possible sorting mechanism indicated that hemagglutinin is delivered directly to the apical surface, rather than "sorting" from basolateral site [4].

These earlier studies suggested that membrane GSLs or glycoproteins must be clustered, providing a basis for interaction with other membrane components. In fact, extensive clustering of GSL Gb4, or of glycophorin, at the surface of erythrocytes, was demonstrated by electron microscopy through freeze/etch procedure [5,6].

Since our long-term research interest is in the structure and function of GSLs, defining tumor growth and motility, our recent studies have focused on organization and interaction of GSLs with functional membrane proteins in microdomain. Results of our major studies during the past decade, together with related studies by other research groups, are described briefly in this review.

2. Microdomain having GSL/caveolin or GSL/tetraspanin, controlling cell adhesion and motility

Following the earlier works as described above, a large number of studies on molecular interaction of various components in microdomains were developed during the late 1980s to early

Abbreviations: Csk, C-terminal Src kinase; EMT, epithelial–mesenchymal transition; FGFR, fibroblast growth factor receptor; FN, fibronectin; GFR, growth factor receptor; Gg3, GalNAcβ4Galβ4Glcβ1Cer (gangliotriaosylceramide); Gg4, Galβ3GalNAcβ4Galβ4Glcβ1Cer (gangliotetraosylceramide); GM2, GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer; GM3, NeuAcα3Galβ4Glcβ1Cer; GSL, glycosphingolipid; LN5, laminin-5; TSP, tetraspanin

* Address: Division of Biomembrane Research, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122, USA. Fax: +1 206 726 1212.
E-mail address: hakomori@u.washington.edu

0014-5793/$36.00 © 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2009.10.065

1990s, indicating the presence of sphingolipid/cholesterol-enriched domains which are resistant to non-ionic detergents. Such microdomains were variously termed as "DRM" (detergent resistant membrane) (for review [7]), caveolae membrane (for review [8]), "raft" (for review [9]), and "glycosignaling domain" ([10,11]; for review [12]). The term "raft" has been used most frequently, to signify an rapidly-moving cell surface signaling platform.

Among various components present in microdomain, caveolin has received great attention as a major adapter molecule affecting cellular phenotype. A few examples are described here. Expression of caveolin-1 may inhibit ovarian cancer progression, and its down-regulation promotes enhanced tumor malignancy through possible interaction with E-cadherin [13]. Caveolin-1 gene is expressed at low level in highly malignant ovarian cancer, and is likely to act as tumor-suppressor gene in human ovarian epithelia [14]. The inhibitory effect of caveolin-1 on tumor progression may be based on its interaction with integrin α subunit, which causes activation of Fyn, in turn activating Shc tyrosine phosphorylation. This sequential process is necessary to open up the Ras/Erk pathway, to control tumor cell motility [15]. These observations indicate that caveolin-1 expression inhibits, and its down-regulation promotes, cancer progression. However, an important question whether gangliosides and GSLs affect caveolin function in microdomain was not considered before.

A recent study [16] addressed this important question. Transfection of cDNA for GM3 synthase (SAT-1) to ovarian cancer A2780 cells caused overexpression of GM3 and (to a lesser extent) GM2 (GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer). Motility of these cells was greatly reduced, in comparison to mock transfectant or wild-type A2780 cells, although growth rate was not affected by enhanced GM3. SAT-1 transfectant expressing high GM3 also displayed high level of caveolin-1 (but not caveolin-2 or -3) which, however, was not accompanied by morphologically distinguishable caveolae. Enhancement of caveolin-1 was closely associated with enhanced expression of GM3 and its synthase gene, since caveolin-1 is the major target of GM3 interaction, as indicated by binding of photoactivatable GM3. Caveolin-1, GM3, and Src are all present in low-density membrane fraction resistant to Triton X-100. The motility-inhibitory effect of caveolin-1 and GM3 is assumed to be mediated by cSrc, since incubation of cells with Src inhibitor, SU6656, inhibited cell motility. Motility inhibition is associated with high GM3 level, while it was lost with low GM3 level, through caveolin-1/GM3 interaction. This study made clear how gangliosides modulate tumor cell motility through interaction with caveolin-1, and activation or inactivation of Src [16].

Interestingly, the motility-inhibitory effect of GM3 together with caveolin was similar to that of GM3 complexed with tetraspanin (TSP) CD9 or CD82 [17–19]. However, caveolin/sphingolipid causes specific membrane architecture and function, as summarized in a recent review [20]. Functional interactions of GSLs with various receptors in microdomains were also extensively reviewed recently [21].

Gangliosides, particularly GM3 and GM2, are known to interact with growth factor receptors (GFRs), and to inhibit the enhanced tyrosine kinase activity of the receptor induced by growth factor. Typical examples are GM3-dependent inhibition of tyrosine kinases associated with epidermal GFR [22], with fibroblast GFR (FGFR) [23,24], and with insulin receptor [25,26]; for reviews see [27,28]. GM3 also interacts with and activates cSrc, Src family kinases, and G-proteins [10]. The relationship of this process with integrin-dependent signaling was subsequently clarified by studies using ldlD cells and their CD9 transfectant [18,19].

During these studies, we noticed that physical and biochemical properties, and functional significance of microdomain containing GSL/TSP complex, are different from those of "raft". Therefore, we applied the term "glycosynapse" or "glycosynaptic microdomain" [29,30], in analogy to "ímmunological synapse" or "ímmunosynapse" [31], the molecular complex surrounding T-cell receptor which is involved in adhesion of antigen-presenting cell to T-cell receptor. Synaptic function, i.e., adhesion process, is involved for both glycosynapse and ímmunosynapse. Contrasting properties of glycosynapse vs. "raft" are summarized in Table 1.

Table 1
Contrasting properties of "raft" vs. "glycosynapse".

Raft	Glycosynapse
Mobile, floating signaling platform	Less-mobile adhesion platform
May not be involved in cell adhesion (not described so far)	Involved in GSL-dependent cell adhesion with concurrent signalling
Involved in control of endocytosis	Unclear (no studies made)
Cholesterol-dependent (disrupted by chol.-binding reagent, e.g., nystatin, filipin, β-cyclodextrin)	Cholesterol-independent (less sensitive to chol.-binding reagent)
Tetraspanin-dependence is not reported	Tetraspanin-dependent
Insoluble in 1% Triton X-100	Soluble in 1% Triton X-100, but insoluble in 1% Brij98

The molecular mechanism of cancer "malignancy", reflecting enhanced cell motility with altered cell adhesion and growth, is assumed to be based on organizational status of GM3 and TSP CD9 in microdomain, as initially suggested by a study using ldlD cells (see subsequent section). This study was extended to interaction of GM2 with TSP CD82 to form GM2/CD82 complex. Such complex inhibits cell motility and growth by blocking functional interaction ("cross-talk") between integrin α3 and cMet [32], or between α3 and FGFR [24]. A basic concept of GSL/TSP complex, and its functional interaction with GFRs and integrins, is shown schematically in Fig. 1.

3. Glycosynapses in various types of cancer cells

Model experiments on the functional role of GM3 and CD9 were initially performed using ldlD cells transfected with CD9 gene (ldlD/CD9) [33]. ldlD is a Chinese Hamster Ovary (CHO) cell mutant with defect of UDP-Gal 4-epimerase, and does not synthesize UDP-Gal from UDP-Glc. Therefore, all glycoconjugates having Gal, including GM3, are not synthesized in this mutant unless Gal is added to chemically-defined ITS culture medium (+Gal) [34]. The ldlD/CD9 grown under +Gal condition expressed both CD9 and GM3. Motility of ldlD/CD9 was inhibited under +Gal condition, but was enhanced under −Gal condition, in which GM3 was not synthesized [33].

Motility of various colorectal cancer cell lines was reduced by exogenous addition of GM3, when the tumor cells expressed high CD9 level. Motility of gastric cancer cell line MKN74 was not susceptible to exogenous GM3 addition, since its CD9 level is low, but motility became inhibitable by GM3 addition when CD9 level was increased through CD9 gene transfection. The motility-inhibitory effect of GM3 in CD9-expressing cells was not replaceable with GM1 or Gb4. These results suggest that co-expression of GM3 and CD9 is essential to inhibit cell motility. CD9, but not integrins β1, α6, α3, or α4, appears to be the primary target of GM3, since ^{3}H-labeled photoactivatable GM3 was found to bind preferentially to CD9 expressed in colonic cancer HRT 18 cells under physiological conditions [17].

In model studies using ldlD/CD9 cells, GM3 and CD9 were found to interact with integrin α3 under +Gal condition, and were located in low-density fraction of glycosynapse, as evidenced by co-immunoprecipitation. Reversible interaction between CD9 and integrin α3, and between CD9 and GM3, was observed by laser beam confocal microscopy in ldlD cells grown under +Gal vs. −Gal condition.

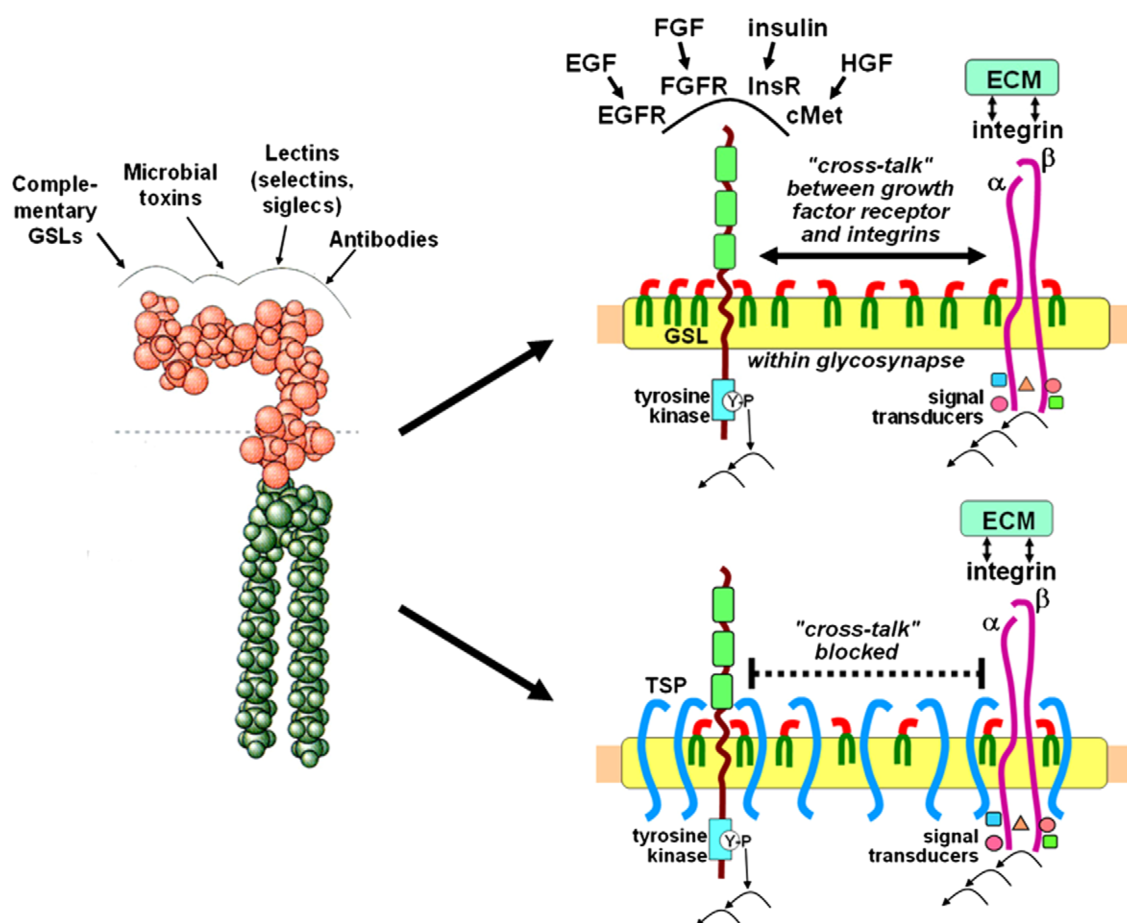

Fig. 1. Interaction of GSLs with tetraspanins, GFRs, and integrins in glycosynapse of tumor cells defines tumor cell growth, adhesion, and motility. *Left panel*: Molecular conformation of GSL. The axis of carbohydrate chain of GSL is perpendicular to the axis of ceramide (*N*-fatty acyl sphingosine), based on X-ray diffraction pattern of crystallized cerebroside [47], and on computerized conformational analysis [48]. The outer surface of oligosaccharide of GSL displays higher hydrophobicity than the inner surface, and consists of a large hydrophobic area adjacent to a small hydrophilic area, providing an ideal binding site for antibodies [49], lectins, and other ligands [50]. A similar mechanism may apply for binding of GSL to complementary carbohydrates [51], which mediates cell–cell adhesion (see Fig. 2). *Right panels*: Upper: GSLs, particularly GM3, interact with: (i) receptors for various growth factors, e.g., EGF, FGF, insulin, cMet, and inhibit the receptor-associated kinase; (ii) integrin receptors α3β1 (for LN5) or α5β1 (for FN) at glycosynapse, and inhibit cell adhesion-mediated activation of signal transduction through Src family kinases or G-proteins; and (iii) besides processes (i) and (ii), a different mechanism based on GSL interaction operates between GFRs and integrins. Such functional interaction (cross-talk) is maintained by GM3 and GM2, i.e., cell adhesion to plates coated with LN5 or FN activates tyrosine kinases associated with FGF or cMet. This process is particularly clear in transformed cells. Lower: Processes (i), (ii), and (iii) are strongly inhibited or blocked when the glycosynapse contains TSP CD9 or CD81 complexed with GM3, i.e., GM3/CD9/CD81, or TSP CD82 complexed with GM2, i.e., GM2/CD82. The inhibitory or blocking effect of such complex is particularly clear for tyrosine kinase of FGFR or that of cMet. The blocking effect of GM3/CD9/CD81 on cross-talk between FGFR and α3β1 is dose-dependent in diploid normal WI38 cells, and is lost completely in transformed VA13 cells due to the loss of CD9/CD81 [23]. GM3 without CD9/CD81, as observed in VA13, or in WI38 with double knockdown of CD9/CD81 by siRNA, causes high cell motility with reduced adhesion [24]. While CD82 was originally found as metastasis suppressor gene product [52], CD9 was originally found as motility-related protein [53], and they are highly expressed in normal cells and depleted in close correlation with degree of malignancy, we found that these TSPs are closely associated with GM3 or GM2, and play essential roles in defining tumor cell motility and invasiveness. The well-known membrane protein caveolin was recently shown to be an important factor defining motility and adhesion when associated with ganglioside, particularly GM3 or GM2 [16] (see text).

Thus, CD9/GM3 complex inhibited laminin-5 dependent cell motility [18].

During this study, CD9 was incidentally found to be soluble in chloroform/methanol, and recovered mainly at lower phase of Folch partition [18]. Such properties are very similar to those of "proteolipid protein" found by Folch over 50 years ago [35].

Composition and function of glycosynapse were further studied in normal diploid human lung fibroblasts WI38, as compared to SV40-transformed VA13 cells. GM3 complexed with two TSPs, CD9 and CD81, was found to be the major component in low-density fraction of glycosynapse prepared in 1% Brij98 detergent. Level of such complex was very high in contact-inhibited, slow-growing WI38, but was greatly reduced in fast-growing VA13 [23].

Comparative Western blotting of low-density fraction of glycosynapse as above, from WI38 vs. VA13 cells, showed that level of FGFR was several times higher in VA13 than in WI38, which was the opposite of results for level of GM3/CD9/CD81 complex. Levels of integrins in the same fraction were similar for the two types of cells. The high level of GM3/CD9/CD81 complex present in WI38 was capable of inhibiting the low level of FGFR tyrosine kinase, whereas the low level of such complex in VA13 was not capable of inhibiting the high level of FGFR tyrosine kinase. Thus, FGF-dependent growth control was well inhibited in WI38, but not in VA13 [23].

In contrast to growth control mechanism as described above, there is a separate mechanism for motility control, based on interaction of GM3 with integrins. Two types of motility control were considered: (i) direct effect of GM3, with or without CD9/CD81, on integrins α3β1 and α5β1, without involvement of FGFR and (ii) indirect effect of GM3, with or without CD9/CD81, on functional interaction of these integrins with FGFR tyrosine kinase. In process (i), motility was strongly inhibited by GM3, through inhibition of integrin-induced activation of Src kinase. This process was assumed to result from translocation of C-terminal Src kinase (Csk) from

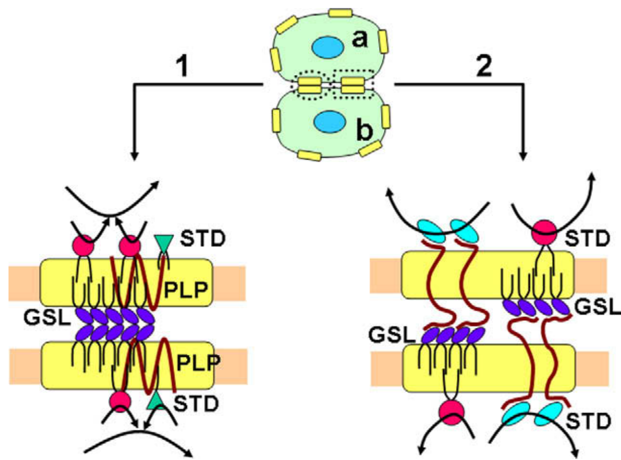

Fig. 2. GSLs in glycosynapse, involved in cell-cell adhesion. When glycosynapse of one cell ("a") contacts glycosynapse of another cell ("b"), cell adhesion occurs by two mechanisms: (1) GSL-to-GSL interaction, which induces activation of signal transducer (STD), leading to change of cellular phenotype. In this process, proteolipid protein (PLP) [18,35] may stabilize conformation of GSL. (2) GSL binds to GSL-binding protein, which induces activation of STD, leading to change of cellular phenotype. Each process is further explained below. *1*. GSL-to-GSL interaction, either homotypic or heterotypic. A typical example of homotypic interaction is cell adhesion based on Le^x-to-Le^x interaction, observed in autoaggregation of embryonic stem cells D3M, or embryonal carcinoma cells F9, in the presence of Ca^{2+} [51,54,55]. Occurrence of such cell adhesion was further confirmed using D3M or F9 cells whose E-cadherin gene was knocked out. These cells still displayed strong Le^x-dependent autoaggregation and adhesion to Le^x GSL-coated plates which were eliminated by siRNA of fucosyltransferase-9, involved in Le^x synthesis [55]. Le^x-to-Le^x interaction has been extensively studied and confirmed by various biophysical procedures, including atomic force microscopy [56], aggregation of gold glyconanoparticles with Le^x [57], and adhesion energy change based on contact angle ($\Delta\theta^c$) of two Le^x vesicles [58]. Heterotypic carbohydrate-to-carbohydrate interactions were found between GM3 and Gg3 [59,60], mediating adhesion of melanoma cells to lymphoma cells; and between GM3 and LacCer [61,62], mediating binding of melanoma cells to microvascular endothelial cells, i.e., cancer metastatic process [61]. Gold lactosyl nanoparticles were found to inhibit melanoma cell metastasis in vivo [63]. Interaction of GalCer with sulfatide (3-O-sulfated GalCer), previously observed on various biophysical bases [64], was found to mediate adhesion of interfacing membranes of oligodendrocytes [65]. Adhesion based on both homotypic and heterotypic carbohydrate interaction induces activation of signal transducers at cytoplasmic site, to alter cellular phenotype. *2*. Structure and function of carbohydrate-binding proteins expressed at cell surface membrane involved in cell–cell adhesion, or interaction of cell with its microenvironment, are well established by many studies. Three major classes of carbohydrate-binding proteins are: (i) Selectins (E-, P-, and L-types) having different structures and functions, and recognizing different glycosyl epitopes containing LacNAc backbone with fucosyl, sialosyl, or sulfate residue. Selectins play a major role in inflammatory processes and cancer progression, particularly metastasis (for review see [66,67]). (ii) Sialic acid-binding lectins, abbreviated as "siglecs", expressed at lymphocytes and myelocytes. Their function is to maintain the internal microenvironment (for review see [68,69]). (iii) Galectins, comprising a huge number of variants, that recognize galactose or galactosamine. Their functions are varied, and many are still unclear (for review see [70]).

outside to inside of glycosynapse. Csk phosphorylates Tyr527 of cSrc, which competitively inhibits Tyr416, and consequently blocks Src kinase/Akt/MAPK signal transduction pathway. In process (ii), GM3 inhibited cross-talk between integrins and FGFR, as indicated by the observation that cell adhesion to plates coated with laminin-5 (LN5) or fibronectin (FN) activated FGFR tyrosine kinase. In both processes (i) and (ii), the inhibitory effect of GM3 was much enhanced when GM3 was complexed with CD9/CD81 [24].

4. Reversion of oncogenic to normal cell phenotype by manipulating GSL/TSP expression in glycosynaptic microdomain

GM3 expression in chick embryonic fibroblast was closely associated with expression of oncogenic phenotype and its reversion. This was initially found in a study with temperature-sensitive mutants of Rous sarcoma virus. GM3 level was high at non-permissive temperature (41 °C) whereby the cells express normal cell phenotype. GM3 expression was down-regulated at permissive temperature (38 °C), whereby transformed phenotype was expressed [36]. This study indicated that transformed phenotype and its reversion are associated with decrease vs. increase of GM3 expression. This concept was further substantiated by studies with transfection of *Jun* oncogene.

Expression of oncogenic phenotype was induced by *Jun* transfection, and was associated with loss of GM3. In contrast, reversion to normal cell phenotype was associated with enhanced expression of GM3 through transfection of GM3 synthase gene. Oncogenic phenotype and its reversion were determined by positive vs. negative growth in soft agar, and reduced vs. enhanced anchorage-dependent cell adhesion. The reversible phenotypic change associated with the presence vs. absence of GM3 was closely related to formation of GM3/CD9/integrin complex. Only absence vs. presence of GM3 appeared to be the major factor defining phenotypic changes of transformation vs. reversion, since CD9 and integrin did not show major changes, and were consistently present [37].

Oncogenic phenotype and its reversion associated with GM3 or GM2 complexed with TSP CD9 or CD82 in glycosynapse were further studied in human tumor cells, as in the following three examples.

1. GM3 is highly expressed in human bladder benign, non-invasive tumor KK47 cells, but GM3 level is very low in highly malignant, invasive bladder cancer YTS1 cells. Both cell lines express TSP CD9, and integrin $\alpha3\beta1$. $\alpha3$/CD9 interaction is much stronger in benign KK47 than in YTS1. This difference is due to the presence of GM3 in KK47, vs. its absence in YTS1. Phenotypic reversion from YTS1 with high motility, to phenotype similar to that of benign KK47 with low motility, was caused by exogenous addition of GM3 to YTS1, whereby $\alpha3$/CD9 interaction was strongly enhanced, and Src kinase activity was strongly reduced. Reduction of Src kinase in glycosynapse is due to translocation of Csk into glycosynaptic domain, whereby Src tyrosine kinase was inhibited. Thus, oncogenic transformation and its reversion can be explained by differences in glycosynaptic organization [19].
2. In contrast to KK47 and YTS1 cells as above, normal bladder epithelial cells HCV29 express ganglioside GM2 and TSP CD82 as major components, which form stable complexes in glycosynapse. Such complex inhibits cMet tyrosine kinase activity induced by hepatocyte growth factor (HGF). Since highly malignant bladder cancer YTS1 has no CD82 but does have low level of GM2, both growth and motility of YTS1 are very high, independent of HGF stimulation. When YTS1 was transfected with CD82 gene, to get high level of CD82, growth and motility were converted to phenotype similar to that of HCV29, i.e., regulated cell growth and low motility, through acquired HGF-dependent cMet tyrosine kinase activity [32].
3. Our studies so far, described above, are focused on the effect of single ganglioside GM3, or GM2, expressed in glycosynapse, and the mechanism of either GM3 or GM2 interacting with TSPs, GFRs, and integrins. However, various normal and tumor cells often express both GM3 and GM2, or other GSLs. Normal bladder epithelial HCV29 cells express both GM2 and GM3, although GM2 is the major component. When GM3 was added to HCV29, HGF-induced cell motility and growth through cMet tyrosine kinase were strongly inhibited. This was found to be based on Ca^{2+}-dependent formation of a heterodimer between GM2 and GM3, as evidenced by electrospray mass spectrometry, and by specific reactivity with newly established mAb 8E11, directed to the heterodimer but not to GM2 or GM3 alone.

Effect of CD82 interaction with GM2/GM3 heterodimer on cMet was further confirmed by model experiments using ldlD cells transfected with CD82 gene and with GM2 synthase (β4GalNAcT4) gene. Growth of ldlD/CD82/β4GalNAcT4 was inhibited under conditions causing appropriate GM2 and GM3 level, when the double transfectant was grown in ITS medium added with 20 μM Gal and 200 μM GalNAc. Such cells were clearly stained by mAb 8E11, indicating the expression of GM2/GM3 heterodimer. Motility and growth of various tumor cells expressing cMet were reduced to a level similar to that of normal cells by treating cells with nanoparticles coated with GM2/GM3 heterodimer [38]. Thus, treatment of tumor cells (in this case bladder cancer) with nanoparticles bearing GM2/GM3 heterodimer is a novel technology to cause reversion of tumor cells to normal cell phenotype.

5. Specific GSLs in glycosynapse mediate epithelial–mesenchymal transition process

Studies initiated by Hay and co-workers [39,40], and her followers, during three decades, showed that epithelial cells change their morphology, growth, and motility when they come in contact with extracellular matrix, or with mesenchymal cells. Consequently, expression of epithelial cell molecules declines, and molecules characteristic of mesenchymal cells increase. This process, termed "epithelial–mesenchymal transition" (EMT), has been widely recognized as a basic process in embryonic development (for review see [41]), and in cancer progression (for review see [42,43]). While changes of glycosylation in developmental processes and cancer progression are well established, no studies have addressed the functional role of GSLs in membrane microdomain, which may mediate EMT.

We recently demonstrated involvement of GSLs in the EMT process, using three epithelial cell lines. Treatment of these cells with transforming growth factor β (TGFβ), the EMT inducer, (i) down-regulated expression of a major epithelial cell marker, E-cadherin; (ii) up-regulated expression of mesenchymal cell markers vimentin, fibronectin, and N-cadherin; (iii) enhanced haptotactic cell motility; and (iv) converted epithelial to fibroblastic morphology. The same changes of these cell lines were also induced by treatment with EtDO-P4, the GlcCer synthase inhibitor, which depletes all GSLs derived from GlcCer.

A close association of specific GSL changes with EMT process, induced by TGFβ or EtDO-P4, is indicated by the following findings: (a) The enhanced cell motility of EtDO-P4-treated cells was abrogated by exogenous addition of GM2 or Gg4 (Galβ3GalNAcβ4Galβ4Glcβ1Cer) (gangliotetraosylceramide), but not GM1 or GM3, in all three cell lines. (b) TGFβ treatment caused depletion or reduction of Gg4 or GM2 in NMuMG, and reduction of GM2 in HCV29. (c) Exogenous addition of Gg4 inhibited TGFβ-induced changes of morphology, motility, and levels of epithelial and mesenchymal markers. These observations indicate that specific GSLs play key roles in defining phenotypes associated with EMT and its reverse process, i.e., mesenchymal-to-epithelial transition [44].

A number of studies during the past decade clearly indicate that molecular changes similar to those in EMT process can also be induced by hypoxia (low O_2) (for reviews see [45,46]). Results of our recent study on Gg4 expression in mouse epithelial NMuMG cells indicate that: (i) gene expression of β1–3 galactosyltransferase-4 (β3GalT4), which adds Gal to Gg3 (GalNAcβ4Galβ4Glcβ1Cer) (gangliotriaosylceramide) to make Gg4, was strongly reduced by TGFβ treatment, as detected by glycogene array analysis and real-time RT-PCR; (ii) similarly to TGFβ treatment, culturing of cells under hypoxia condition, or in the presence of $CoCl_2$, caused activation of hypoxia-inducible factors (HIF), and also induced EMT process: enhancement of cell motility, reduced expression of epithelial marker molecules together with enhancement of mesenchymal molecules, and change of cell morphology; and (iii) associated with these changes, Gg4 level and β3GalT4 gene expression were reduced. These findings further support involvement of GSLs in EMT process and hypoxia (Guan F. et al., *JBC*, under review).

References

[1] Forstner, G.G. and Wherrett, J.R. (1973) Plasma membrane and mucosal glycosphingolipids in the rat intestine. Biochim. Biophys. Acta 306, 446–459.
[2] Okada, Y., Mugnai, G., Bremer, E.G. and Hakomori, S. (1984) Glycosphingolipids in detergent-insoluble substrate attachment matrix (DISAM) prepared from substrate attachment material (SAM): their possible role in regulating cell adhesion. Exp. Cell Res. 155, 448–456.
[3] Yu, J., Fischman, D.A. and Steck, T.L. (1973) Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. J. Supramol. Struct. 3, 233–248.
[4] Matlin, K.S. and Simons, K. (1984) Sorting of an apical plasma membrane glycoprotein occurs before it reaches the cell surface in cultured epithelial cells. J. Cell Biol. 99, 2131–2139.
[5] Tillack, T.W., Allietta, M., Moran, R.E. and Young, W.W.J. (1983) Localization of globoside and Forssman glycolipids on erythrocyte membranes. Biochim. Biophys. Acta 733, 15–24.
[6] Rock, P., Allietta, M., Young, W.W.J., Thompson, T.E. and Tillack, T.W. (1990) Organization of glycosphingolipids in phosphatidylcholine bilayers: use of antibody molecules and Fab fragments as morphologic markers. Biochemistry 29, 8484–8490.
[7] Brown, D.A. and London, E. (1997) Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem. Biophys. Res. Commun. 240, 1–7.
[8] Anderson, R.G.W. (1998) The caveolae membrane system. Annu. Rev. Biochem. 67, 199–225.
[9] Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. Nature 387, 569–572.
[10] Iwabuchi, K., Yamamura, S., Prinetti, A., Handa, K. and Hakomori, S. (1998) GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate–carbohydrate interaction in mouse melanoma B16 cells. J. Biol. Chem. 273, 9130–9138.
[11] Iwabuchi, K., Handa, K. and Hakomori, S. (1998) Separation of "glycosphingolipid signaling domain" from caveolin-containing membrane fraction in mouse melanoma B16 cells and its role in cell adhesion coupled with signaling. J. Biol. Chem. 273, 33766–33773.
[12] Hakomori, S., Handa, K., Iwabuchi, K., Yamamura, S. and Prinetti, A. (1998) New insights in glycosphingolipid function: "glycosignaling domain," a cell surface assembly of glycosphingolipids with signal transducer molecules, involved in cell adhesion coupled with signaling. Glycobiology 8, xi–xviii.
[13] Miotti, S., Tomassetti, A., Facetti, I., Sanna, E., Berno, V. and Canevari, S. (2005) Simultaneous expression of caveolin-1 and E-cadherin in ovarian carcinoma cells stabilizes adherens junctions through inhibition of src-related kinases. Am. J. Pathol. 167, 1411–1427.
[14] Wiechen, K., Diatchenko, L., Agoulnik, A., Scharff, K.M., Schober, H., Arlt, K., Zhumabayeva, B., Siebert, P.D., Dietel, M., Schafer, R. and Sers, C. (2001) Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am. J. Pathol. 159, 1635–1643.
[15] Wary, K.K., Mariotti, A., Zurzolo, C. and Giancotti, F.G. (1998) A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell 94, 625–634.
[16] Prinetti, A., Aureli, M., Illuzzi, G., Prioni, S., Nocco, V., Scandroglio, F., Gagliano, N., Tredici, G., Rodriguez-Menendez, V., Chigorno, V. and Sonnino, S. (2009) GM3 synthase overexpression results in reduced cell motility and in caveolin-1 up-regulation in human ovarian carcinoma cells. Glycobiology. doi:10.1093/glycob/cwp143.
[17] Ono, M., Handa, K., Sonnino, S., Withers, D.A., Nagai, H. and Hakomori, S. (2001) GM3 ganglioside inhibits CD9-facilitated haptotactic cell motility: co-expression of GM3 and CD9 is essential in down-regulation of tumor cell motility and malignancy. Biochemistry 40, 6414–6421.
[18] Kawakami, Y., Kawakami, K., Steelant, W.F.A., Ono, M., Baek, R.C., Handa, K., Withers, D.A. and Hakomori, S. (2002) Tetraspanin CD9 is a "proteolipid", and its interaction with a3 integrin in microdomain is promoted by GM3 ganglioside, leading to inhibition of laminin-5-dependent cell motility. J. Biol. Chem. 277, 34349–34358.
[19] Mitsuzuka, K., Handa, K., Satoh, M., Arai, Y. and Hakomori, S. (2005) A specific microdomain ("glycosynapse 3") controls phenotypic conversion and reversion of bladder cancer cells through GM3-mediated interaction of alpha3beta1 integrin with CD9. J. Biol. Chem. 280, 35545–35553.
[20] Sonnino, S. and Prinetti, A. (2009) Sphingolipids and membrane environments for caveolin. FEBS Lett. 583, 597–606.
[21] Prinetti, A., Loberto, N., Chigorno, V. and Sonnino, S. (2009) Glycosphingolipid behaviour in complex membranes. Biochim. Biophys. Acta 1788, 184–193.
[22] Bremer, E.G., Schlessinger, J. and Hakomori, S. (1986) Ganglioside-mediated modulation of cell growth: specific effects of GM_3 on tyrosine phosphorylation of the epidermal growth factor receptor. J. Biol. Chem. 261, 2434–2440.
[23] Toledo, M.S., Suzuki, E., Handa, K. and Hakomori, S. (2004) Cell growth regulation through GM3-enriched microdomain (glycosynapse) in human

lung embryonal fibroblast WI38 and its oncogenic transformant VA13. J. Biol. Chem. 279, 34655–34664.
[24] Toledo, M.S., Suzuki, E., Handa, K. and Hakomori, S. (2005) Effect of ganglioside and tetraspanins in microdomains on interaction of integrins with fibroblast growth factor receptor. J. Biol. Chem. 280, 16227–16234.
[25] Nojiri, H., Stroud, M.R. and Hakomori, S. (1991) A specific type of ganglioside as a modulator of insulin-dependent cell growth and insulin receptor tyrosine kinase activity: possible association of ganglioside-induced inhibition of insulin receptor function and monocytic differentiation induction in HL60 cells. J. Biol. Chem. 266, 4531–4537.
[26] Yamashita, T., Hashiramoto, A., Haluzik, M., Mizukami, H., Beck, S., Norton, A., Kono, M., Tsuji, S., Daniotti, J.L., Werth, N., Sandhoff, R., Sandhoff, K. and Proia, R.L. (2003) Enhanced insulin sensitivity in mice lacking GM3 ganglioside. Proc. Natl. Acad. Sci. USA 100, 3445–3449.
[27] Yates, A.J. and Rampersaud, A. (1998) in: Sphingolipids as Signaling Modulators in the Nervous System (Ledeen, R.W., Hakomori, S., Yates, A.J., Schneider, J.S. and Yu, R.K., Eds.), Annals of the NY Academy of Sciences, Vol. 845, pp. 57–71, New York Acad. Sci., New York, NY.
[28] Miljan, E.A. and Bremer, E.G. (2002) Regulation of growth factor receptors by gangliosides. Science STKE 2002 (RE15), 1–10.
[29] Hakomori, S. (2002) The glycosynapse. Proc. Natl. Acad. Sci. USA 99, 225–232.
[30] Hakomori, S. (2004) Glycosynapses: microdomains controlling carbohydrate-dependent cell adhesion and signaling. Ann. Braz. Acad. Sci. 76, 553–572.
[31] Bromley, S.K., Burack, W.R., Johnson, K.G., Somersalo, K., Sims, T.N., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M. and Dustin, M.L. (2001) The immunological synapse. Annu. Rev. Immunol. 19, 375–396.
[32] Todeschini, A.R., Dos Santos, J.N., Handa, K. and Hakomori, S. (2007) Ganglioside GM2-tetraspanin CD82 complex inhibits Met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse. J. Biol. Chem. 282, 8123–8133.
[33] Ono, M., Handa, K., Withers, D.A. and Hakomori, S. (1999) Motility inhibition and apoptosis are induced by metastasis-suppressing gene product CD82 and its analogue CD9, with concurrent glycosylation. Cancer Res. 59, 2335–2339.
[34] Kingsley, D.M., Kozarsky, K.F., Hobbie, L. and Krieger, M. (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. Cell 44, 749–759.
[35] Folch, J., Arsove, S. and Meath, J.A. (1951) Isolation of brain strandin, a new type of large molecule tissue component. J. Biol. Chem. 191, 819–831.
[36] Hakomori, S., Wyke, J.A. and Vogt, P.K. (1977) Glycolipids of chick embryo fibroblasts infected with temperature-sensitive mutants of avian sarcoma viruses. Virology 76, 485–493.
[37] Miura, Y., Kainuma, M., Jiang, H., Velasco, H., Vogt, P.K. and Hakomori, S. (2004) Reversion of the Jun-induced oncogenic phenotype by enhanced synthesis of sialosyllactosylceramide (GM3 ganglioside). Proc. Natl. Acad. Sci. USA 101, 16204–16209.
[38] Todeschini, A.R., Dos Santos, J.N., Handa, K. and Hakomori, S. (2008) Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. Proc. Natl. Acad. Sci. USA 105, 1925–1930.
[39] Sugrue, S.P. and Hay, E.D. (1981) Response of basal epithelial cell surface and cytoskeleton to solubilized extracellular matrix molecules. J. Cell Biol. 91, 45–54.
[40] Greenburg, G. and Hay, E.D. (1982) Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J. Cell Biol. 95, 333–339.
[41] Hay, E.D. (2005) The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev. Dyn. 233, 706–720.
[42] Li, L. and Neaves, W.B. (2006) Normal stem cells and cancer stem cells: the niche matters. Cancer Res. 66, 4553–4557.
[43] Polyak, K. and Weinberg, R.A. (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat. Rev. Cancer 9, 265–273.
[44] Guan, F., Handa, K. and Hakomori, S. (2009) Specific glycosphingolipids mediate epithelial-to-mesenchymal transition of human and mouse epithelial cell lines. Proc. Natl. Acad. Sci. USA 106, 7461–7466.
[45] Haase, V.H. (2009) Oxygen regulates epithelial-to-mesenchymal transition: insights into molecular mechanisms and relevance to disease. Kidney Int. 76, 492–499.
[46] Klymkowsky, M.W. and Savagner, P. (2009) Epithelial–mesenchymal transition: a cancer researcher's conceptual friend and foe. Am. J. Pathol. 174, 1588–1593.
[47] Nyholm, P.-G., Pascher, I. and Sundell, S. (1990) The effect of hydrogen bonds on the conformation of glycosphingolipids: methylated and unmethylated cerebroside studied by X-ray single crystal analysis and model calculations. Chem. Phys. Lipids 52, 1–10.
[48] Strömberg, N., Nyholm, P.-G., Pascher, I. and Normark, S. (1991) Saccharide orientation at the cell surface affects glycolipid receptor function. Proc. Natl. Acad. Sci. USA 88, 9340–9344.
[49] Kaizu, T., Levery, S.B., Nudelman, E.D., Stenkamp, R.E. and Hakomori, S. (1986) Novel fucolipids of human adenocarcinoma: monoclonal antibody specific for trifucosyl Le^y ($III^3FucV^3FucVI^2FucnLc_6$), and a possible three-dimensional epitope structure. J. Biol. Chem. 261, 11254–11258.
[50] Spohr, U., Hindsgaul, O. and Lemieux, R.U. (1985) Molecular recognition. II. The binding of the Lewis b and Y human blood group determinants by the lectin IV of *Griffonia simplicifolia*. Can. J. Chem. 63, 2644–2652.
[51] Kojima, N., Fenderson, B.A., Stroud, M.R., Goldberg, R.I., Habermann, R., Toyokuni, T. and Hakomori, S. (1994) Further studies on cell adhesion based on Le^x–Le^x interaction, with new approaches: embryoglycan aggregation of F9 teratocarcinoma cells, and adhesion of various tumour cells based on Le^x expression. Glycoconj. J. 11, 238–248.
[52] Dong, J.T., Suzuki, H., Pin, S.S., Bova, S., Schalken, J.A., Isaacs, W.B., Barrett, J.C. and Isaacs, J.T. (1996) Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res. 56, 4387–4390.
[53] Cajot, J.-F., Sordat, I., Silvestre, T. and Sordat, B. (1997) Differential display cloning identifies motility-related protein (MRP1/CD9) as highly expressed in primary compared to metastatic human colon carcinoma cells. Cancer Res. 57, 2593–2597.
[54] Eggens, I., Fenderson, B.A., Toyokuni, T., Dean, B., Stroud, M.R. and Hakomori, S. (1989) Specific interaction between Le^x and Le^x determinants: a possible basis for cell recognition in preimplantation embryos and in embryonal carcinoma cells. J. Biol. Chem. 264, 9476–9484.
[55] Handa, K., Takatani-Nakase, T., Larue, L., Stemmler, M.P., Kemler, R. and Hakomori, S. (2007) Le^x glycan mediates homotypic adhesion of embryonal cells independently from E cadherin: a preliminary note. Biochem. Biophys. Res. Commun. 358, 247–252.
[56] Tromas, C., Rojo, J., de la Fuente, J.M., Barrientos, A.G., Garcia, R. and Penades, S. (2001) Adhesion forces between $Lewis^x$ determinant antigens as measured by atomic force microscopy. Angew. Chem., Intl. Ed. 40, 3052–3055.
[57] de la Fuente, J.M., Barrientos, A.G., Rojas, T.C., Rojo, J., Canada, J., Fernandez, A. and Penades, S. (2001) Gold glyconanoparticles as water-soluble polyvalent models to study carbohydrate interactions. Angew. Chem., Intl. Ed. 40, 2259–2261.
[58] Gourier, C., Pincet, F., Perez, E., Zhang, Y., Zhu, Z., Mallet, J.M. and Sinay, P. (2005) The natural LewisX-bearing lipids promote membrane adhesion: influence of ceramide on carbohydrate–carbohydrate recognition. Angew. Chem., Intl. Ed. 44, 1683–1687.
[59] Kojima, N. and Hakomori, S. (1989) Specific interaction between gangliotriaosylceramide (Gg3) and sialosyllactosylceramide (GM3) as a basis for specific cellular recognition between lymphoma and melanoma cells. J. Biol. Chem. 264, 20159–20162.
[60] Matsuura, K., Kitakouji, H., Sawada, N., Ishida, H., Kiso, M., Kitajima, K. and Kobayashi, K. (2000) A quantitative estimation of carbohydrate–carbohydrate interaction using clustered oligosaccharides of glycolipid monolayers and of artificial glycoconjugate polymers by surface plasmon resonance. J. Am. Chem. Soc. 122, 7406–7407.
[61] Kojima, N. and Hakomori, S. (1991) Cell adhesion, spreading, and motility of G_{M3}-expressing cells based on glycolipid–glycolipid interaction. J. Biol. Chem. 266, 17552–17558.
[62] Santacroce, P.V. and Basu, A. (2003) Probing specificity in carbohydrate–carbohydrate interactions with micelles and Langmuir monolayers. Angew. Chem., Intl. Ed. 42, 95–98.
[63] Rojo, J., Diaz, V., de la Fuente, J.M., Segura, I., Barrientos, A.G., Riese, H.H., Bernad, A. and Penades, S. (2004) Gold glyconanoparticles as new tools in antiadhesive therapy. Chembiochem 5, 291–297.
[64] Stewart, R.J. and Boggs, J.M. (1993) A carbohydrate–carbohydrate interaction between galactosylceramide-containing liposomes and cerebroside sulfate-containing liposomes: dependence on the glycolipid ceramide composition. Biochemistry 32, 10666–10674.
[65] Boggs, J.M., Gao, W. and Hirahara, Y. (2008) Signal transduction pathways involved in interaction of galactosylceramide/sulfatide-containing liposomes with cultured oligodendrocytes and requirement for myelin basic protein and glycosphingolipids. J. Neurosci. Res. 86, 1448–1458.
[66] Varki, A. (1994) Selectin ligands. Proc. Natl. Acad. Sci. USA 91, 7390–7397.
[67] Kannagi, R. (1997) Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj. J. 14, 577–584.
[68] Crocker, P.R., Paulson, J.C. and Varki, A. (2007) Siglecs and their roles in the immune system. Nat. Rev. Immunol. 7, 255–266.
[69] Varki, A. (2009) Natural ligands for CD33-related Siglecs? Glycobiology 19, 810–812.
[70] Leffler, H. (2004) Special issue on galectins, dedicated to Professor Samuel H. Barondes. Glycoconj. J. 19, 433–629.