Glycoconjugate Journal 21, 125–137, 2004

Carbohydrate-to-carbohydrate interaction, through glycosynapse, as a basis of cell recognition and membrane organization

Senitiroh Hakomori

Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122-4302; and Departments of Pathobiology and Microbiology, University of Washington, Seattle, WA 98195

Cell adhesion mediated by carbohydrate-to-carbohydrate interaction (CCI), or cell adhesion with concurrent signal transduction, are discussed in three contexts.

1. Types of cell adhesion based on interaction of several combinations of glycosphingolipids (GSLs) at the surface of interfacing cells ("*trans* interaction") are reviewed critically, to exclude the possible involvement of GSL-binding proteins. Special emphasis is on: (i) autoaggregation of mouse teratocarcinoma F9 cells mediated by Le^x-to-Le^x interaction, in which presence of Le^x-binding protein is ruled out; (ii) adhesion of GM3-expressing cells to Gg3-expressing cells, in which involvement of GM3- or Gg3-binding protein is ruled out.

2. Characteristic features and requirements of CCI, as compared with carbohydrate-to-protein interaction (CPI) and protein-to-protein interaction (PPI), are summarized, including: (i) specificity and requirement of bivalent cation; (ii) reaction velocity of CCI as compared to PPI; (iii) negative (repulsive) interaction; (iv) synergistic or cooperative effect of CCI and PPI, particularly GM3-to-Gg3 or GM3-to-LacCer interaction in synergy with integrin-dependent adhesion, or Le^x-to-Le^x interaction in cooperation with E-cadherin-dependent adhesion.

3. Microdomains at the cell surface are formed based on clustering of GSLs or glycoproteins organized with signal transducers. Among such microdomains, those involved in adhesion coupled with signal transduction to alter cellular phenotype are termed "glycosynapse". In some glycosynapses, growth factor receptors or integrin receptors are also involved, and their function is modulated by GSLs only when the receptor is *N*-glycosylated. This modulation may occur in part via interaction of GSLs with *N*-linked glycans of the receptor, termed "*cis* interaction".
Published in 2004.

Keywords: **glycosphingolipid, Le^x, GM3, Gb4, Gg3, *trans* interaction, *cis* interaction, glycosynapse, binding protein, cell adhesion, autoaggregation, bivalent cation**

Abbreviations: **AFM, atomic force microscopy; CAM, cell adhesion molecule; chydr, carbohydrate; CCI, carbohydrate-to-carbohydrate interaction; CPI, carbohydrate-to-protein interaction; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FN, fibronectin; Gb4, GalNAcβ3Galα4Galβ4Glcβ1Cer; Gb5, Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer; Gg3, GalNAcβ4Galβ4Glcβ1Cer; GSL, glycosphingolipid; H, Fucα2Gal; Le^a, Galβ3[Fucα4]GlcNAcβ3Galβ4Glcβ1Cer; Le^x, Galβ4[Fucα3]GlcNAcβ3Galβ4Glcβ1Cer; Le^y, [Fucα2]Galβ4[Fucα3]GlcNAcβ3Galβ4Glcβ1Cer; PPI, protein-to-protein interaction; SPR, surface plasmon resonance; SSEA, stage-specific embryonic antigen; TSP, tetraspanin.**

1. Introduction

Prior to development of the current concept of cell recognition based on carbohydrate-to-carbohydrate interaction (CCI), temperature-dependent gelification of agarose was well known. Self-aggregation of various plant polysaccharides based on CCI was studied over 40 years ago, mainly by D.A. Rees and colleagues, using changes in optical rotation or ^{13}C-NMR associated with gelification of various polysaccharides (*e.g.* [1,2]. Interaction of chondroitin sulfate and hyaluronic acid was claimed subsequently [3]. In these early studies of polysaccharide interaction, the biological significance was unclear.

To whom correspondence should be addressed: Senitiroh Hakomori, PNRI, 720 Broadway, Seattle, WA 98122-4302; E-mail: hakomori@u.washington.edu

The majority of cellular glycoconjugates (∼70–80%) are located at the cell surface to form a "glycocalyx". They were therefore imagined to be involved in cell recognition, for many years. Galectins, discovered in the mid-1970s, were suspected to mediate cell recognition, although the majority of galectins were found intracellularly. The mechanism for involvement of galectins in cell recognition was more complex than initially considered [4–7]; for extensive review, see [8]. During the past decade, selectins (for review see [9–11] and siglecs [12,13] have been added to the list of carbohydrate (chydr)-binding proteins that mediate cell recognition.

Cell recognition based on CCI is unique in that it displays (i) high variability depending on degree of clustering; (ii) reactivity more rapid than that of protein-to-protein interaction (PPI) that typically occurs through integrins, cadherins, or various types of cell adhesion molecules (CAMs); and (iii) synergistic effect with PPI. Therefore, CCI is not "alternative" or "supplemental" to PPI or chydr-to-protein interaction (CPI). Rather, CCI is the initial step leading to multiple, redundant mechanisms, including PPI (see Section 2.2 below).

Despite the increasing evidence for cell adhesion based on CCI, it is difficult to completely rule out possible co-occurrence of CPI with CCI.

Here, I will briefly review (i) our previous studies on cell-to-cell interaction mediated by *trans*-CCI; (ii) our current studies suggesting occurrence of CCI between integrins or growth factor receptors and surrounding gangliosides, *i.e.*, *cis*-CCI; (iii) assembly of glycoconjugates involved in *trans*-CCI and in *cis*-CCI within the same microdomain or between interfacing microdomains, termed "glycosynapse" [14,15]. This term implies chydr-dependent cell adhesion with concurrent signal transduction to alter cellular phenotype, in analogy to "immunological synapse" [16,17].

2. Examples of cell adhesion mediated by *trans*-CCI

2.1. Two well-studied types of homotypic cell adhesion through *trans*-CCI, based on self-recognition of specific chydr epitopes

Studies on molecular mechanisms of well-known homotypic animal cell adhesion systems revealed the involvement of self-recognition of chydr epitopes in two adhesion systems, *i.e.*, (i) species-specific aggregation of marine sponge cells, in the presence of Ca^{2+}, in which the glycan part of proteoglycan plays a major role in self-recognition, and possible involvement of the protein part in cell adhesion was ruled out in recent studies [18]; (ii) autoaggregation of mouse teratocarcinoma F9 cells, also in the presence of Ca^{2+}, which mimics the "compaction" process of preimplantation mouse embryo. Based on "common sense" considerations, the possible involvement of chydr-binding proteins through CPI was investigated, but was ruled out by extensive studies. Steps in these studies, which led to the concept of *trans*-CCI, are summarized in Tables 1 and 2.

Table 1. Species-specific autoaggregation of sponge cells is due to *trans*-CCI based on self-recognition of specific oligosaccharides. Studies conducted primarily in Max M. Burger's lab

a. Species-specific aggregation of marine sponge cells is based on Ca^{2+}-dependent proteoglycan-to-proteoglycan interaction [19–21], in which the glycan part is responsible for aggregation through CCI [22]. The protein part could be a glycan carrier but is not functionally involved in aggregation [23,24].
b. Glycan structures involved in CCI were studied in *Microciona prolifera*. Multiples of Galβ1,4GlcNAcβ1,3Fuc having 6,4-pyruvation at terminal Gal [25], and GlcNAcβ1,3Fuc having 3-O-sulfation at terminal GlcNAc [26], defined respectively by "Block 1" and "Block 2" mAbs, were identified as aggregation-mediating epitopes.
c. The epitope defined by Block 2 mAb was shown to bind to itself (self-recognition) by quantitative SPR spectroscopy using multiple 3-O-sulfated GlcNAcβ1,3Fuc linked to BSA [27].
d. Self-recognition of glycans derived from four genera of sponge (*Microciona*, *Halichondria*, *Suberites*, *Cliona*), as a basis of species-specific cell aggregation, was studied by molecular force microscopy. Strong (190–310 pN) adhesive force was observed for glycan self-recognition in each case [18].

Table 2. Autoaggregation of mouse teratocarcinoma F9 cells, which mimics compaction of morula-stage mouse embryo, is due to *trans*-CCI based on self-recognition of Le^x by Le^x. Studies conducted in our lab

a. Both F9 cells and morula-stage mouse embryo highly express α1-3 fucosyl *N*-acetyllactosamine (Le^x) [28–31]. Trivalent Le^x inhibited F9 autoaggregation or embryo compaction, and also caused "de-compaction" of once-compacted embryo [32].
b. (i) Major proteins separated from F9 cell extract did not show Le^x-binding ability [33]. (ii) Two fractions (Mr ∼30 K, ∼18 K) in F9 cell extract did show Le^x-binding ability, which was maintained even after exhaustive "pronase" digestion [33–35]. (iii) The Le^x-binding molecules extracted from [^{3}H]GlcNH$_2$-labeled F9 cells were identified as Le^x-GSL as well as poly-LacNAc bearing high level of Le^x, previously described as "embryoglycan" [36,37].
c. Liposomes containing Le^x-GSL interact specifically with Le^x-GSL-coated plate in the presence of Ca^{2+} [34].
d. Thus, the Le^x binding partner is Le^x *per se*, and the autoaggregation is based on Ca^{2+}-dependent Le^x-to-Le^x self-recognition. The phenomenon was shown by liposome binding, equilibrium dialysis, and aggregation of Le^x-coated microspheres [34]. It was further confirmed by other investigators, based on atomic force microscopy [38], SPR spectroscopy [39], and crystallography of Le^x trisaccharide [40].

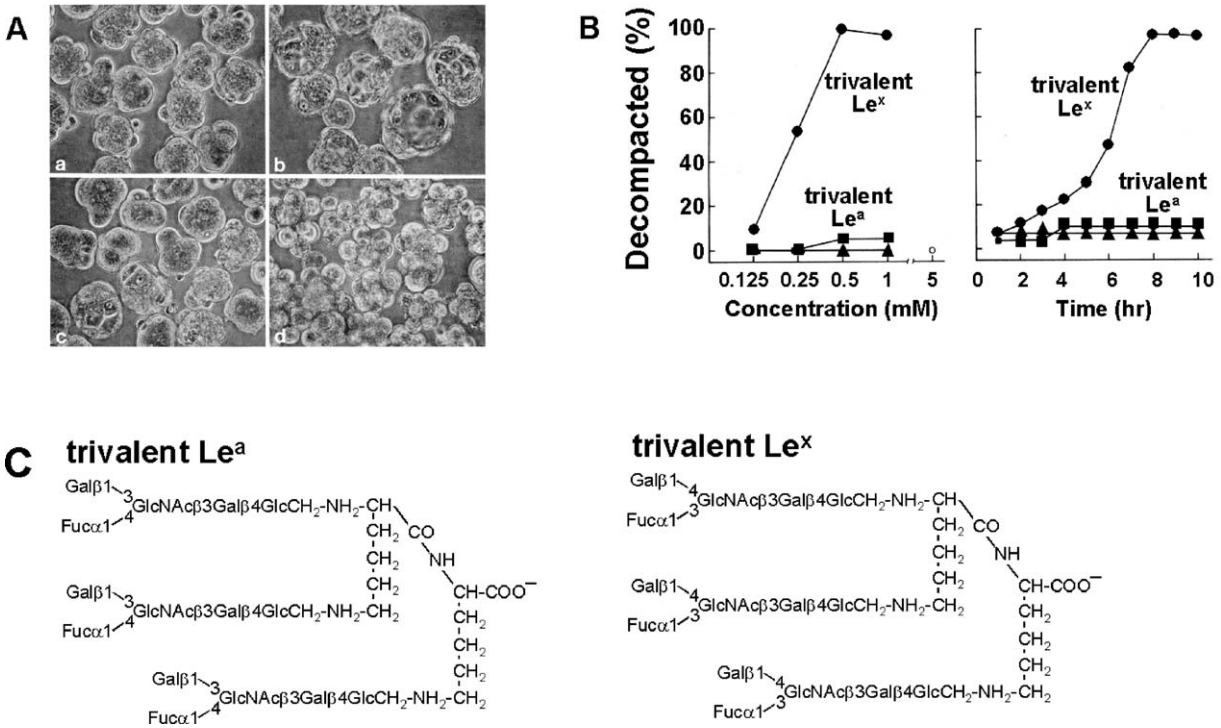

Figure 1. Inhibition of compaction, or "de-compaction" of mouse embryo, by trivalent Le^x but not by trivalent Le^a. Panel A: Compacted embryos (a) were cultured in Whitten's medium alone (b) or in Whitten's medium containing either 1 mM trivalent Le^a (c), or 1 mM trivalent Le^x (d). Embryos are shown after 8–12 h (c, d) or 24 h (b) of culture. Note that only trivalent Le^a "de-compacted" the once-compacted mouse embryo. Panel B: Dose response (left) and time course response (right) for decompaction by trivalent Le^x conjugate, and by other positional isomer conjugates. Compacted embryos were cultured in the presence of trivalent Le^x (●), trivalent Le^a (■), or chitotriose-lysyllysine (▲). No effect was seen for monovalent Le^x (5 mM) plus lysyllysine (1 mM). Percentage of decompacted embryos was scored after 10–12 h of culture (left). From Fenderson *et al.* [32]. Panel C: Structures of trivalent Le^a (left) and trivalent Le^x (right).

2.2. Compaction of mouse embryo, and autoaggregation of mouse teratocarcinoma F9 cells: Cooperative effect of Le^x-dependent and E-cadherin-dependent adhesion

F9 cell autoaggregation, in the presence of Ca^{2+}, mimics compaction of morula-stage mouse embryo, the first of the series of adhesion events during embryogenesis. Both F9 cell autoaggregation and the compaction process were inhibited by trivalent Le^x but not trivalent Le^a, in a dose-dependent and time-dependent manner (Figure 1). In addition, this adhesion system is controlled by E-cadherin [41], although compaction proceeded well in an E-cadherin knockout mouse [42]. Autoaggregation of teratocarcinoma proceeded normally in an Le^x-defective variant [43]. These findings suggest that embryonal compaction and teratocarcinoma autoaggregation depend on a cooperative effect of Le^x- and E-cadherin-dependent adhesion [44]. In general, cell adhesion essential for important functions consists of multiple steps, initiated by CCI and followed by PPI; this is termed "redundancy of adhesion systems".

We studied the molecular mechanism of the adhesion process using autoaggregation of Le^x-positive F9 cells, in comparison with control Le^x-negative PYS-2, a differentiated teratocarcinoma cell line derived from the same parental teratocarcinoma cell line OTT 6030 (from mouse strain 129) as F9. Autoaggregation of F9 cells in the presence of bivalent cation (Figure 2A-a) was inhibited by addition of EDTA (Figure 2A-b) or trivalent Le^x (Figure 2A-c), but not by trivalent Le^a (Figure 2A-d). Such autoprecipitation of F9 cell extract was due to autoaggregation of glycoprotein containing poly-LacNAc with high level of Le^x (Figure 2B), previously described as "embryoglycan" [36,37]. The autoaggregation property of the glycan was abolished by treatment with pig liver fucosidase (Figure 2C).

These results (autoaggregation of Le^x-positive F9 cells, its absence in Le^x-negative PYS-2 cells, and inhibition of F9 cell autoaggregation by Le^x but not by Le^a oligosaccharide) suggested two possibilities: (i) an Le^x-binding protein present in F9 cells mediates Le^x-dependent aggregation (*i.e.*, based on standard concept of CPI); (ii) Le^x interacts with Le^x in the presence of bivalent cation, based on a novel mechanism. Possibility (i) was explored by extensive studies searching for protein(s) showing Le^x-binding ability. However, major proteins separated from F9 cell extract showed no Le^x-binding ability, and two Le^x-binding fractions with Mr 30 K and 18 K

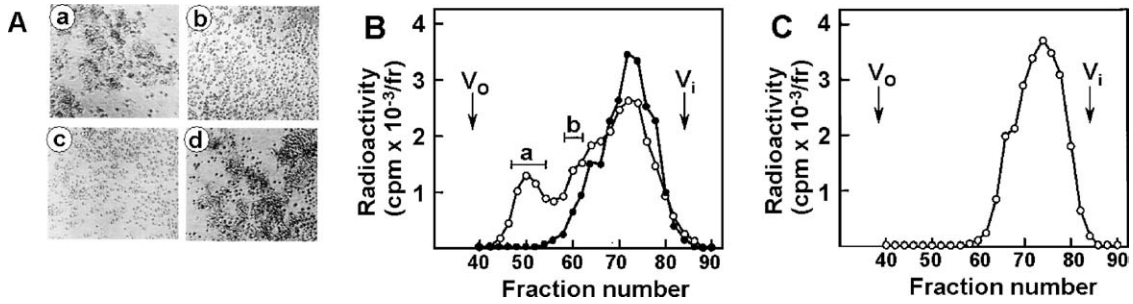

Figure 2. Autoaggregation of F9 cells (A) and F9 cell glycan in cell extract (B). Such aggregated peak (a, and part of b) is absent in the presence of EDTA in B, and in defucosylated F9 cell glycan (C). Panel A: Inhibition of F9 cell autoaggregation by trivalent Le^x but not by trivalent Le^a. (a) F9 cells cultured in bivalent cation; (b) culture containing 50 mM EDTA; (c) culture of (a) in 2 mM trivalent Le^x; (d) culture of (a) in 2 mM trivalent Le^a. From Eggens *et al.* [34]. Panel B: Autoaggregation of labeled glycoprotein in F9 cell extract, as demonstrated by change of gel filtration pattern. F9 cells were metabolically [^{3}H]$GlcNH_2$-labeled, extracted with buffer A containing 1% octylglucoside and 5 mM EDTA as in Panel B, dialyzed against TBS containing (i) 5 mM $CaCl_2$, or (ii) 10 mM EDTA, and subjected to gel filtration on Sephacryl CL-6B (1.5 × 120 cm) with continuous elution in medium (i) (○) or (ii) (●) respectively. 2 ml fractions were collected, and 0.1 ml aliquots of each fraction were determined for radioactivity. Note that the elution curve in the presence of $CaCl_2$ shows autoaggregation peaks a and b (○), which are absent in the elution curve in the presence of EDTA (●). Panel C: Elution of the same material with medium (i) as above, but treated with 0.1 U bovine liver α-fucosidase (37°C, 24 h), which destroys Le^x structure. Panels B and C from Kojima et al. [35].

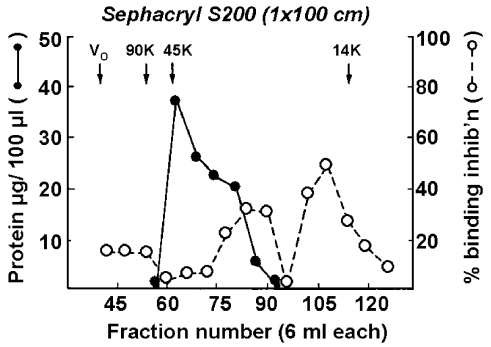

Figure 3. Proteins or glycoproteins in F9 cell extract, having Le^x-binding activity. F9 cell membranes were extracted with 10 mM CHAPSO, clarified by ultracentrifugation followed by dialysis to remove detergent, and subjected to gel filtration on Sephacryl S200 (1 × 100 cm). Fractions (6 ml) were separated, and aliquots were assayed for protein content (left ordinate; solid circle). Other aliquots were tested for Le^x binding activity in terms of inhibition of antibody binding to Le^x-coated plate (right ordinate; open circle). Note that major protein peaks separated had no Le^x-binding activity. Two bands, with Mr 30 K and 18 K, showed binding to Le^x; these had very low or undetectable protein content. From Rosenman *et al.* [33].

had no measurable protein level (Figure 3), suggesting that the Le^x-binding molecule may not be protein. In order to confirm this point, F9 cell glycoproteins and GSLs, including the 30 K and 18 K components, were metabolically labeled with [^{3}H]$GlcNH_2$, extracted by CHAPSO, dialyzed, subjected to gel filtration (see Figure 3 legend), lyophilized and extracted with isopropanol/hexane for GSLs, extensively digested with pronase, and separated by gel filtration. Le^x-binding activity and protein content of each fraction were compared. Le^x-binding activity was highly resistant to extensive pronase digestion, and the Le^x-binding substance had properties similar to those of embryoglycan. The ratio of embryoglycan to Le^x-GSL in F9 cells was ~4:1. These results supported possibility (ii) as above. Le^x-to-Le^x interaction *in vitro* was confirmed initially by binding of Le^x-GSL liposomes to Le^x-coated plates [34], and later by a number of other studies [38–40,45].

2.3. GSL-dependent adhesion

2.3.1. Cell adhesion possibly mediated by GSLs: Early studies

We [46] and others [47,48] discovered a cell surface protein (later termed "fibronectin"; FN) that may mediate cell adhesion. However, its cell surface receptor was not identified at that time. Since gangliosides inhibit FN-dependent cell adhesion [49], they were once considered as FN receptors. The real receptor was later identified as protein (integrin) [50,51]. Complexity arose from the fact that integrin interacts with gangliosides, and such interaction is required for integrin function as FN receptor [52,53].

An alternative possibility was suggested that GSL-binding protein (rather than integrin) may be widely present, and mediate cell adhesion. We performed experiments to test this hypothesis and found that certain cells adhere to plates coated with a defined GSL. Our attempts to demonstrate specific GSL-binding protein in such cases were unsuccessful. However, we found that a cell expressing a specific GSL ("GSL 1") adhered well to plates coated with a second GSL ("GSL 2"), but not with other GSLs ("GSL 3"). Interaction of GSL 1 with GSL 2, but not with GSL 3, was confirmed based on GSL-liposome binding to GSL-coated plate. Melanoma B16 cells adhered to mouse lymphoma L5178V, based on GM3-to-Gg3 interaction.

Table 3. Adhesion of GM3-expressing cells to Gg3-expressing cells or to Gg3-coated plate

a. Mouse melanoma B16 cells (expressing high level of GM3) adhere strongly to mouse lymphoma L5178V (expressing Gg3; particularly clone AA12) but not to L5178 AV27 (variant not expressing Gg3) [54] (Figure 4A).
b. Adhesion of B16 to L5178V cells was blocked by pretreatment of B16 with anti-GM3 mAb DH2 or pretreatment of L5178V with anti-Gg3 mAb 2D4 [54]. Also, B16 did not bind to L5178 AV27. Thus, B16 adhesion to L5178V is based solely on GM3-to-Gg3 interaction (Figure 4B).
c. Adhesion of not only B16 but also other GM3-expressing cells to Gg3SAM was observed, and the degree of adhesion was in the same order as degree of GM3 expression, *i.e.*, B16 $\gg$ HEL299 $>$ BHK $>$ WA4 $>$ 3T3. The binding was abolished by sialidase, by treatment of cells with mAb DH2, or by treatment of plates with mAb 2D4 [55] (Figure 4C).
d. GM3-to-Gg3 interaction was determined quantitatively by SPR spectroscopy using Langmuir monolayer of GM3 and multiple Gg3 trisaccharide bound to polystyrene [56].

Experimental data are summarized in Table 3, and examples are shown in Figure 4A and B.

2.3.2 *Search for GSL-binding proteins that mediate GSL-dependent adhesion*

We studied the possible existence of GSL-binding proteins by the use of specific GSLs (LacCer, Gb4, Gb3, nLc_4, GM3, Gg3) covalently linked to amino glass bead columns through oxidized double bond of Sph [57]. [^{35}S]Met metabolically-labeled cellular proteins were extracted with ethydium bromide or dialyzable detergents (CHAPS, octylglucoside), and their possible interaction with each type of GSL was examined by their binding to respective GSL-glass bead columns. Non-adsorbed or adsorbed (eluted with potassium iodide) ^{35}S-labeled proteins were analyzed by SDS-PAGE followed by autoradiography. Although this approach to search for specific GSL-binding proteins was ultimately unsuccessful, we observed that: (i) there were several proteins that bound non-specifically to multiple GSLs [58]; (ii) no proteins bound specifically to any particular GSL, including Gg3 [58]; Lingwood CA, Hakomori S, unpubl. data). GSL-glass bead columns were used for purification of specific anti-GSL antibodies [57]. However, GSLs covalently bound to amino glass beads are probably not ideal for detection of GSL-binding proteins, since the covalent linking procedure oxidizes double bond of Sph and the original GSL structure is thereby greatly modified.

2.3.3 *Gb4-dependent autoaggregation of human teratocarcinoma 2102 or TERA-2 cells*

A model of human or primate embryonic compaction process is autoaggregation of human teratocarcinoma 2102 or TERA-2 cells [59–61]. Le^x is expressed minimally on these cells. Instead, they express extended globo-series structures such as SSEA-3 (identified as a mixture of Gb5Cer and $\alpha 1 \rightarrow 2$ fucosyl-Gb5 (globo-H)) [62] and SSEA-4 (identified as sialyl-Gb5) [63]. SSEA-3 and SSEA-4 are maximally expressed at morula stage of primate embryo [61]. These structures may mediate the compaction process of primate (presumably including human) embryo. Our studies indicate that autoaggregation of 2102 cells is mediated by Gb4-to-Gb5 as well as

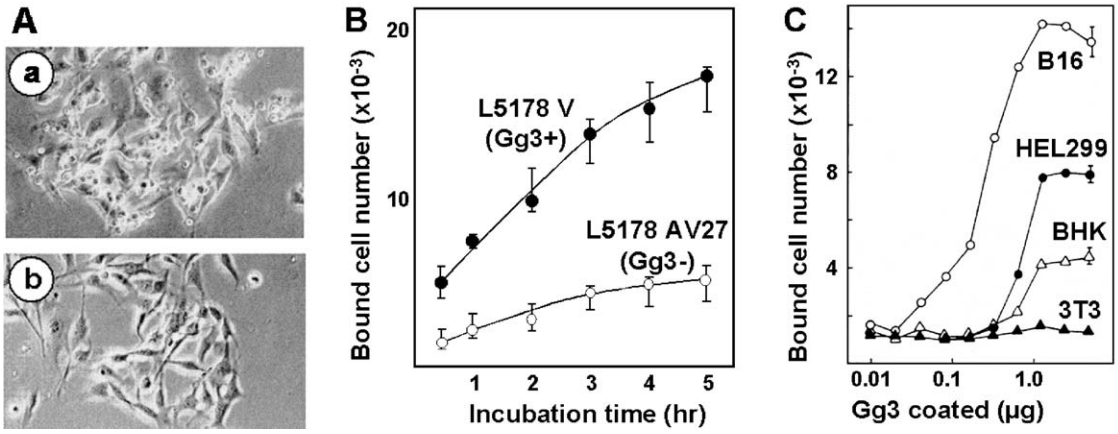

Figure 4. Cell adhesion based on GM3-to-Gg3 interaction. Panel A: Aggregation of mouse melanoma B16 cells with mouse lymphoma L5178V (high level of Gg3 expression) (A-a), and absence of such aggregation with variant L5178 AV27 (not expressing Gg3) (A-b), when co-cultured with melanoma B16 cells (spindle shape) for 24 h. Panel B: Time course change in adhesion of [^{3}H]$GlcNH_2$-labeled L5178V and L5178 AV27 to B16 cells grown as monolayer. Number of cells was calculated based on metabolic labeling activity. From Kojima and Hakomori [54]. Panel C: Adhesion of [^{3}H]thymidine-labeled cell lines with different degrees of GM3 expression to Gg3-coated plate. Number of cells was calculated based on metabolic labeling activity. Different amounts of Gg3 (μg per plate, abscissa) were added on 24-well plates and dried. Cells were incubated 15 min at 37°C. From Kojima and Hakomori [55].

Gb4-to-nLc$_4$ interaction. CCI based on interaction of these two pairs of structures (both including common core Gb4), assumed to be characteristic of the human/ primate compaction process, is termed "Gb4-dependent adhesion". Such adhesion induces signaling leading to activation of transcription factors CREB and AP1 [64]. In this adhesion system, the possibility of CPI through Gb4 or Gb5 binding protein remains to be studied (see Section 2.2).

2.3.4 Adhesion of Le^y-expressing cells to H-expressing cells

Mouse blastocyst expresses high level of Le^y at the surface layer, whereas inner cell mass expresses Le^x. The process of implantation of blastocyst to endometrium of mouse uterus was inhibited by anti-Le^y mAb AH6, indicating that Le^y at the blastocyst surface is recognized by a counterpart structure at endometrium. Since H (Fucα1 $\rightarrow$ 2Gal) is expressed in endometrium, and Le^y-to-H interaction was shown in liposome-binding assay, a possibility was suggested that the implantation process is initiated by CCI between Le^y and H epitopes [65]. It is important to note that both H type 1 and H type 2 interact strongly with Le^y, but Le^y-to-Le^y interaction is repulsive (see Section 5.3).

3. Binding affinity and specificity of CCI, comparable to CPI or PPI

In general, CCI is regarded as weak *in vitro*, when tested in comparison to CPI or PPI. However, the strength of CCI varies extensively depending on the degree of clustering. As shown in recent quantitative analytical studies of CCI using SPR spectroscopy, atomic force microscopy (AFM), and micellar interaction in Langmuir monolayers, CCI binding affinity in some cases is as high as that observed for CPI or PPI, depending on degree of clustering. Under SPR spectroscopy, GM3 monolayer prepared from Langmuir interface interacted strongly with Gg3 oligosaccharide conjugate linked to polystyrene (Ka 1.1×10^8 M^{-1}), but to a much lesser degree with lactose conjugate linked to the same carrier (Ka 7.7×10^4 M^{-1}) [56]. In another SPR study, Le^x trisaccharides with arms linked to SAM of alkanethiolate on gold film strongly bound to Le^x trisaccharides with arms linked to gold nanosphere (Kd 5.4×10^7 M^{-1}) [39]. SPR spectroscopy was also applied to study self-recognition of sulfated disaccharide units of proteoglycan causing species-specific aggregation of a type of sponge cell [26]. The armed disaccharide, 3-O-sulfated GlcNAcβ1 $\rightarrow$ 3Fucα, linked to BSA, showed strong self-interaction on SPR (Ka estimated as $\sim 10^6$ M^{-1}) [27]. The range of binding affinity of CCI as determined by SPR in these experiments was similar to that of CCI or PPI.

AFM was developed to determine the exact force of molecular interaction [66], and has been used for measurement of ligand-receptor interaction [67,68]. This approach was recently applied to determine adhesion force between Le^x trisaccharide carried by mercaptoundecanol linked to gold film surfaces. The force of a single Le^x-to-Le^x interaction was recorded as 20 ± 4 piconewton (pN), whereas no interaction force was found for Le^x-to-lactose or lactose-to-lactose [38]. Since 200-300 pN is considered sufficient to ensure cell-cell adhesion, 10-15 pairs of Le^x clusters are enough to cause clear cell adhesion. Finally, interaction of GSL Langmuir monolayer with GSL oligosaccharide conjugate or with GSL micellar solution was determined in terms of quantitative changes in $\Delta\pi$ value (pressure-area (π-A) isotherm) [69,70].

4. *Cis*-CCI: Implications for interaction of membrane receptors with other membrane glycoconjugate components

A few lines of recent study suggest that CCI may be involved in interaction of membrane receptors with other membrane components. Receptors so far studied are those involved in cell adhesion (integrins) and in cell growth (EGF receptor; EGFR).

4.1 Fibronectin (FN)-dependent adhesion may be controlled by interaction of *N*-linked glycan of integrin $\alpha 5\beta 1$ with ganglioside

$\alpha 5\beta 1$ of keratinocytes is inhibited by gangliosides GD3 and GT1b. Since these gangliosides bind to *N*-glycosylated $\alpha 5\beta 1$, but not to de-*N*-glycosylated $\alpha 5\beta 1$, the inhibition is presumably due to CCI between $\alpha 5\beta 1$ and ganglioside at the same cell surface membrane (*cis*-CCI) [71]. However, direct evidence for interaction between *N*-linked glycans of integrin and gangliosides has not been presented.

4.2 Laminin-5 (LN5)-dependent motility of ldlD/CD9 cells is controlled by $\alpha 3$/CD9/GM3 ganglioside complex

ldlD, a mutant of CHO cells deficient in UDP:Gal 4-epimerase is incapable of synthesizing complete *N*-linked glycan or GM3 ganglioside unless cells are grown in medium with added Gal (+Gal condition). Using ldlD cells transfected with TSP CD9 (ldlD/CD9), we found that LN5-dependent cell motility is greatly enhanced under—Gal condition whereby endogenous GM3 synthesis and *N*-glycosylation are blocked, and that the motility is inhibited when GM3 synthesis occurs and *N*-glycosylation of $\alpha 3$ is completed. Thus, GM3 may mediate *cis*-CCI between N-glycan of $\alpha 3$ and CD9, to form stable GM3/CD9/$\alpha 3$ complex [72].

4.3 EGF receptor requires *N*-glycosylation for its function, and its activation is inhibited by GM3

N-glycosylation is required for EGFR function [73,74], and a specific *N*-linked glycan in EGFR suppresses ligand-dependent oligomerization [75]. Down-regulation of EGFR function by GM3 [76] may require interaction of GM3 with *N*-linked glycan structure at domain IV of EGFR. Pretreatment of A431 cells with 1-deoxymannojirimycin, which blocks synthesis

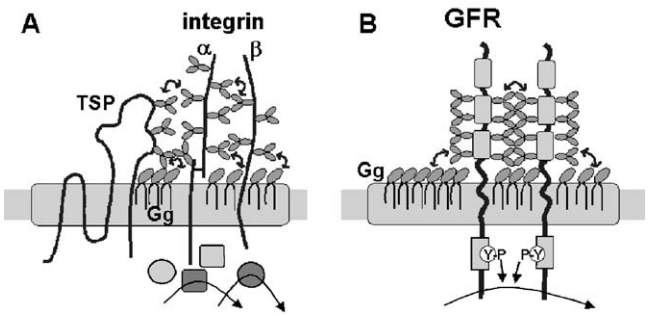

Figure 5. Hypothetical model of *cis*-CCI controlling interaction of integrin receptor with TSP, and interaction of growth factor receptors (GFR) with surrounding gangliosides. Panel A: Integrin subunits α and β are associated with TSP. Such interaction is mediated by *N*-linked glycans and surrounding gangliosides (Gg). Panel B: GFR and their interaction with surrounding gangliosides through *N*-linked glycans. From Hakomori and Handa [15].

of complex-type and causes accumulation of high-mannose type glycan, reduces binding of EGFR to GM3 and degree of GM3-dependent inhibition of EGFR tyrosine kinase activity (Hikita T, Handa K, Hakomori S, unpubl. data).

The findings described in a–d above suggest that N-glycans of integrins or EGFR mediate interaction of the receptor with ganglioside or associated TSP, and that such interaction modulates receptor function. A model of *cis*-CCI in maintenance of integrin and EGFR function is illustrated in Figure 5.

5. Common, characteristic features and requirements of CCI

5.1 Specificity, and requirement of bivalent cation

5.1.1 Specificity

CCI depends highly on structural specificity of the pair of chydrs. Typical examples based on our preliminary studies of GSL-to-GSL interaction are shown in Figure 6. Some pairs are highly specific (*e.g.*, Le^x-to-Le^x) [34], whereas others are less specific (*e.g.*, Gb4-to-Gb5 or -nLc_4 [64]; GM3-to-Gg3 or -LacCer [55]).

5.1.2 Clustering proper orientation, and effect of carrier

In all cases, CCI requires clustering of the chydr epitopes involved. The clustering is maintained by binding of chydr epitope to ceramide, which may also promote proper assembly and orientation of chydr epitope. GM3 liposome bound strongly to Gg3Cer coated on plastic surface, whereas sialyllactose (the same as GM3 chydr) carried by polyacrylamide bound only weakly to Gg3Cer-coated surface (Ito A, Hakomori S, unpubl. data). Le^xCer bound strongly to Le^xCer liposome, whereas Le^x carried by diacylglycerol bound only weakly to Le^xCer [77].

Oligosaccharides without carrier do not show CCI by themselves, rather, they inhibit CCI. For example, Le^x oligosaccharide (LFP-III) does not exhibit self-interaction, determined by change of ^{1}H-NMR pattern, in the presence of Ca^{2+} [78], although it does inhibit Le^x-to-Le^x interaction or Le^x-dependent cell adhesion [34].

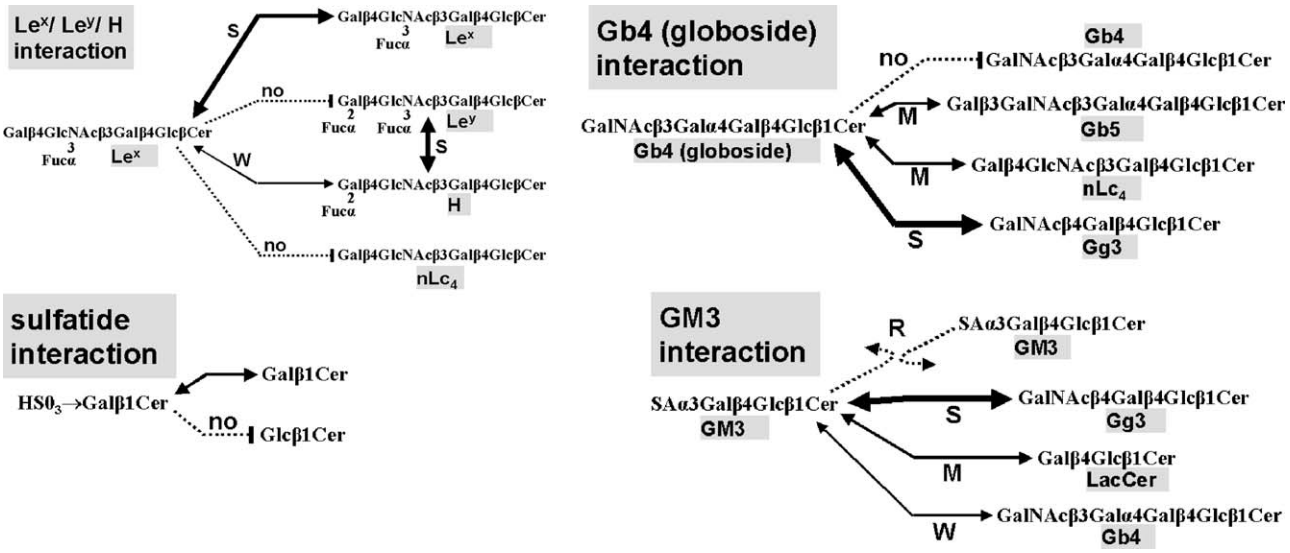

Figure 6. Structures of various carbohydrate epitopes showing strong (S), medium (M), weak (W), no, or repulsive (R) interactions with one another.

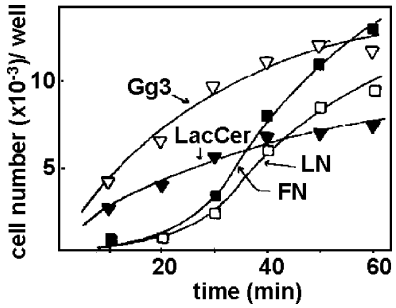

Figure 7. CCI-based adhesion (GM3 interaction with Gg3 or LacCer) of BL6 cells is faster than PPI-based adhesion (α4, α3 integrin interaction with FN or LN). BL6 cells show rapid adhesion to plates coated with Gg3 (▽) or LacCer (▼). This adhesion process (CCI based on GM3-to-Gg3 or GM3-to-LacCer interaction) starts at 10–20 min and is completed at 30 min. In contrast, adhesion to FN (■) and LN (□) (PPI) of the same cells which have α4 and α3 integrins starts at 30 min and is completed at 60 min. From Kojima *et al.* [79].

5.1.3 Bivalent cation requirement

Bivalent cation, particularly Ca^{2+}, displays promoting (catalytic) effect in most cases of CCI [34,54,55,79,80], but a few cases do not require bivalent cation [64,81].

5.2 Reaction velocity of CCI as compared to PPI: Basis of redundancy of adhesion systems

This phenomenon reflects time course change of cell adhesion based on CCI as compared to PPI, as typically observed for adhesion of GM3-expressing B16/BL6 melanoma cells to Gg3- or LacCer-coated vs. FN- or LN-coated plate. The former adhesion (CCI between GM3 and Gg3 or LacCer) became obvious at 10–20 min and was completed at 30 min, whereas the latter (PPI between integrin α3 or α5 and FN or LN) started at 30–40 min and finished at 60 min (Figure 7) [79]. Reaction velocity reflects degree of cell adhesion under dynamic flow conditions. *I.e.*, slower adhesion process is not manifested under dynamic flow, even though binding affinity is high under static condition (Figure 8).

Another example of redundant systems is co-existence of Le^x-dependent and E-cadherin-dependent adhesion in F9 teratocarcinoma cells or mouse preimplantation embryo (see Section 2.2 above).

5.3 Negative (repulsive) interaction

An interesting characteristic of CCI is repulsive interaction, as clearly shown in the case of GM3-to-GM3 and Le^y-to-Le^y. A typical example of GM3-to-GM3 repulsive interaction, in comparison with positive GM3-to-Gg3 interaction and non-interaction of GM3-to-nLc_4 etc., is shown in Figure 9. Since repulsive interaction is assumed to be equally important as positive interaction in biological systems, particularly during developmental processes, extensive further studies along this line

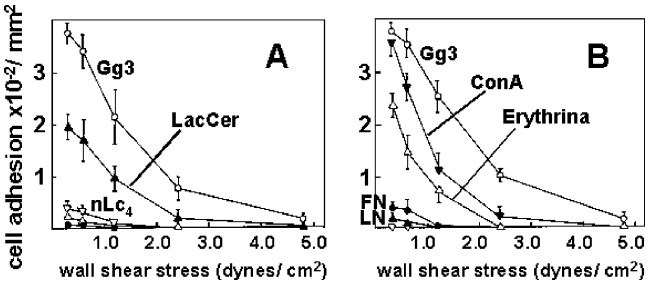

Figure 8. BL6 cell adhesion based on CCI, vs. CPI or PPI, under dynamic flow condition. A defined area of glass wall in laminar-flow chamber was coated with various quantities of GSLs (Gg3, LacCer, nLc_4, GM3), lectins (ConA, *Erythrina* lectin), or adhesive proteins (FN, LN). Number of BL6 cells adhered per mm^2 (ordinate) was determined and shown as a function of increasing wall shear stress (1.0–5.0 dynes/cm^2) (abscissa) during 3 min. Panel A. Adhesion was stronger to Gg3- and LacCer-coated plate than to nLc_4- or GM3-coated plate. Panel B. Adhesion to Gg3-coated plate (CCI) was stronger than that to ConA- or *Erythrina* lectin-coated plate (CPI), particularly at higher shear stress (1.5–5.0 dynes/cm^2). Adhesion to FN- or LN-coated plate (PPI) was minimal even at low shear stress (<1.0 dynes/ cm^2). From Kojima *et al.* [79].

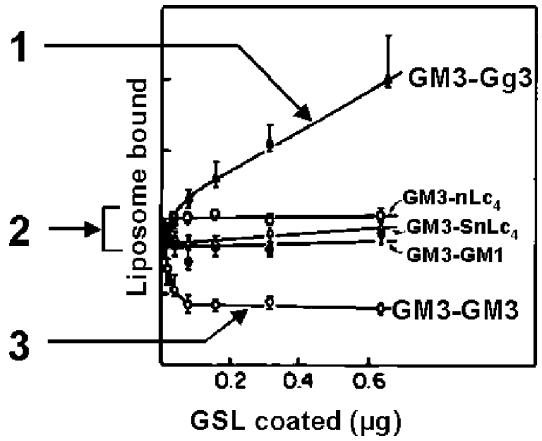

Figure 9. Negative interaction (repulsion), positive interaction, and absence of interaction, shown by GM3-liposomes added on plates coated with various GSLs. Line 3: Negative (repulsive) interaction between GM3 and GM3, indicated by significantly lower binding of labeled GM3-liposome to GM3-coated plate as compared to binding of GM3-liposome to non-coated plate. Line 1: Positive interaction of GM3 with Gg3. Line 2: Absence of interaction of GM3 with non-coated plate, or with plate coated with nLc_4, sialyl-nLc_4, or GM1. From Kojima and Hakomori [54].

are important. We are not aware of any documented cases of repulsive interaction in CPI or PPI.

5.4 Synergistic effects of CCI with PPI

Another important characteristic of CCI is its cooperative or synergistic effect with other, co-existing adhesion systems. A cooperative effect was shown between Le^x-based CCI and

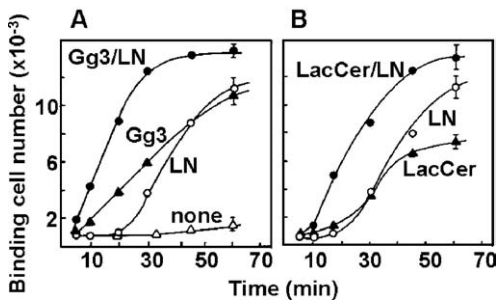

Figure 10. Example of synergistic effect of GSL and adhesive protein LN: Strong enhancing effect of GSL and LN co-coating on BL6 cell adhesion. Panel A: Time course change of BL6 adhesion to 96-well plates coated in four ways: with Gg3 alone (▲), with LN alone (○), with Gg3 plus LN (●), or with no coating (△). Panel B: Same adhesion conditions as in A, but using LacCer instead of Gg3. Note that adhesion was more rapid, and number of adhered cells was greater, for wells in which LN was co-coated with Gg3 or with LacCer (●), compared to wells coated with LN alone (○) or LacCer alone (▲). Similar results were obtained using FN instead of LN (not shown). From Kojima and Hakomori [83].

E-cadherin-based PPI [82]. GM3-dependent adhesion of BL6 cells to Gg3- or LacCer-coated plates was synergistic with integrin-dependent adhesion of the same cells to FN- or LN-coated plates [83]. Some of these data are shown in Figure 10.

6. Organization of glycoconjugates involved in *trans*- and *cis*-CCI at cell surface microdomains: Glycosynapses controlling chydr-dependent or chydr-modulated cell adhesion with concurrent signal transduction

Three types of cell adhesion processes are known: (i) Based on homotypic interaction between protein receptors (*e.g.*, Ig-like CAM, cadherins) or heterotypic interaction between adhesion receptors (*e.g.*, integrin to ECM; integrin to ICAM). Both these processes are essentially PPI [41,50,51,84]. (ii) Mediated by chydr-binding receptors (lectins): galectins [5], selectins [9], and siglecs [12,85]. (iii) Based on *trans*- or *cis*-CCI as a new mechanism of cell recognition/ adhesion, discussed above.

Cell adhesion based on *trans*-CCI is not a process additional or supplemental to processes mediated by CPI or PPI, but rather provides unique, characteristic features such as high variability

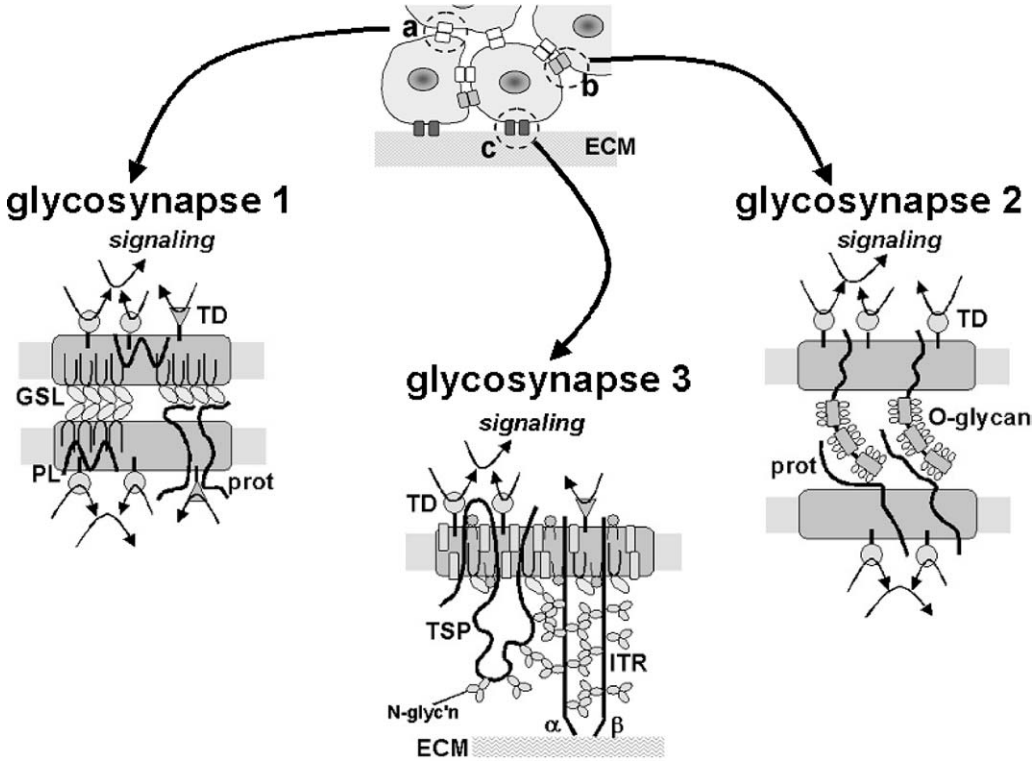

Figure 11. Three types of glycosynapse, microdomains controlling chydr-dependent or chydr-modulated cell adhesion and concurrent signal transduction. Cell-to-cell adhesion sites (a and b at top left) consist of interfacing microdomains, shown as two contacting oblong shapes. Cell-to-ECM adhesion site (c) involves a different microdomain structure shown as a pair of dark oblongs. Examples of adhesion site a, termed "glycosynapse 1", are based on GSL-to-GSL interaction or GSL-to-binding protein ("prot") interaction, in which GSLs are associated with signal transducer (TD) and stabilized by proteolipid protein (PL). "Glycosynapse 2", another cell-to-cell adhesion site, is based on O-linked mucin-type glycoproteins which are recognized by chydr-binding proteins. Both O-linked glycans and their recognizing proteins are associated with TD. Adhesion of cell to ECM (FN, collagen, LN) is mediated by *N*-glycosylated adhesion receptor (*e.g.*, integrin receptor, ITR) complexed with TSP and ganglioside ("glycosynapse 3"). Cell adhesion and motility are greatly inhibited when glycosynapse 3 complex is stabilized by glycosylation.

in binding affinity, high specificity, high reaction velocity, and occurrence of negative (repulsive) interaction, as summarized in Section 5. Importantly, a cooperative effect of CCI with PPI is clearly observable.

Processes mediated by *cis*-CCI are at an early stage of study, and our knowledge is therefore highly limited. Interaction of *N*-linked glycans of integrin receptors with surrounding gangliosides, and similar interaction of growth factor receptors with gangliosides in the same microdomain, are of particular interest. Microdomains containing GSL as major component, displaying interaction of gangliosides with growth factor receptors, and modulating and regulating cell growth through inhibition or activation of signal transduction, are termed "glycosynapse 1" [14,15]. Our recent findings indicate that glycosynapse 1 regulates cell growth and contact inhibition. Such regulatory function is lost in transformed cells, through change of components, their organization, and interaction in glycosynapse 1 [86].

Microdomains containing mucin-type O-glycans and Src family kinases were found in T-cell lines [87], and are involved in activation of T-cell function through IL2 production (Handa K, Andersen S, Hakomori S, unpubl. data). Similar microdomains containing mucin-type glycoproteins, termed "glycosynapse 2" [14,15], must be widely distributed in tumors, but their functions have not been well characterized.

Complexes consisting of *N*-glycosylated integrins, TSPs CD9/CD82, and GM3, termed "glycosynapse 3" [14,15], were found originally in an ldlD cell model system. They were subsequently found in human colorectal and bladder cancers, and may be widely present in various types of cells. Glycosynapse 3 inhibits tumor cell motility and invasiveness, and may play an important role in metastasis. Both CD9 and CD82 were discovered originally as motility-inhibitory factors of tumor cells, whose function was not manifested unless integrins were fully *N*-glycosylated and surrounding GM3 was present. Glycosynapse 3 is probably formed through *cis*-CCI.

Types of glycosynapses and their functional roles are shown schematically in Figure 11. Function of glycosynapse 1 is mediated mainly by *trans*-CCI, whereas that of glycosynapse 3 is mediated by *cis*-CCI.

Acknowledgment

Studies described were supported by NIH/NCI grants OIG CA42505 and R01 CA80054.

References

1 Arnott S, Fulmer A, Scott WE, Dea ICM, Moorhouse R, Rees DA, The agarose double helix and its function in agarose gel structure, *J Mol Biol* **90**, 269–84 (1974).

2 Bryce TA, McKinnon AA, Morris ER, Rees DA, Thom D, Chain conformations in the sol-gel transitions for polysaccharide systems, and their characterisation by spectroscopic methods, *Faraday Disc Chem Soc* **57**, 221–9 (1974).

3 Turley EA, Roth S, Interactions between the carbohydrate chains of hyaluronate and chondroitin sulphate, *Nature* **283**, 268–71 (1980).

4 Kopitz J, von Reitzenstein C, Burchert M, Cantz M, Gabius HJ, Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture, *J Biol Chem* **273**, 11205–11 (1998).

5 Perillo NL, Marcus ME, Baum LG, Galectins: Versatile modulators of cell adhesion, cell proliferation, and cell death, *J Mol Med* **76**(6), 402–12 (1998).

6 Kopitz J, von Reitzenstein C, Andre S, Kaltner H, Uhl J, Ehemann V, Cantz M, Gabius H-J, Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3, *J Biol Chem* **276**(38), 35917–23 (2001).

7 Amano M, Galvan M, He J, Baum LG, The ST6Gal I sialyltransferase selectively modifies N-glycans on CD45 to negatively regulate galectin-1-induced CD45 clustering, phosphatase modulation, and T cell death, *J Biol Chem* **278**(9), 7469–75 (2003).

8 Leffler H, Glycoconjugate Journal, Vol. 19, No. 7/8/9, "Special issue on galectins", 2004.

9 Varki A, Selectin ligands, *Proc Natl Acad Sci USA* **91**, 7390–97 (1994).

10 McEver RP, Cummings RD, Cell adhesion in vascular biology: Role of PSGL-1 binding to selectins in leukocyte recruitment, *J Clin Invest* **100**(3), 485–91 (1997).

11 Kannagi R, Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer, *Glycoconj J* **14**, 577–84 (1997).

12 Crocker PR, Floyd H, Ferguson DJP, Nitschke L, Sialic acid-dependent cell-cell interactions mediated by siglecs: Roles of sialoadhesin and CD22 in macrophage adhesion and B cell homing to the bone marrow, In, *Sialobiology and Other Novel Forms of Glycosylation*, edited by Inoue Y, Lee YC, Troy FA. Osaka (Gakushin Publ. Co., Japan, 1999), pp. 111–20.

13 Crocker PR, Varki A, Siglecs in the immune system, *Immunology* **103**(2), 137–45 (2001).

14 Hakomori S, The glycosynapse, *Proc Natl Acad Sci USA* **99**(1), 225–32 (2002).

15 Hakomori S, Handa K, Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cells: Essential basis to define tumor malignancy, *FEBS Lett* **531**(1), 88–92 (2002).

16 Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML, The immunological synapse, *Annu Rev Immunol* **19**, 375–96 (2001).

17 Krummel MF, Davis MM, Dynamics of the immunological synapse: Finding, establishing and solidifying a connection, *Curr Opin Immunol* **14**, 66–74 (2002).

18 Bucior I, Scheuring S, Engel A, Burger MM, Carbohydrate-carbohydrate interaction provides adhesion force and specificity for cellular recognition, *J Cell Biol* **165**(4), 529–37 (2004).

19 Humphreys T, Chemical dissolution and in vitro reconstruction of sponge cell adhesions: I. Isolation and functional demonstration of the components involved, *Dev Biol* **8**, 27–47 (1963).

20 Henkart P, Humphreys S, Humphreys T, Characterization of sponge aggregation factor: A unique proteoglycan complex, *Biochemistry* **12**(16), 3045–50 (1973).

21 Jumblatt JE, Schlup V, Burger MM, Cell-cell recognition: Specific binding of Microciona sponge aggregation factor to homotypic cells and the role of calcium ions, *Biochemistry* **19**(5), 1038–42 (1980).

22 Misevic GN, Burger MM, The species-specific cell-binding site of the aggregation factor from the sponge *Microciona prolifera* is a highly repetitive novel glycan containing glucuronic acid, fucose, and mannose, *J Biol Chem* **265**(33), 20577–84 (1990).

23 Misevic GN, Burger MM, Carbohydrate-carbohydrate interactions of a novel acidic glycan can mediate sponge cell adhesion, *J Biol Chem* **268**, 4922–29 (1993).

24 Fernandez-Busquets X, Kammerer RA, Burger MM, A 35-kDa protein is the basic unit of the core from the 2×10^4-kDa aggregation factor responsible for species-specific cell adhesion in the marine sponge *Microciona prolifera*, *J Biol Chem* **271**(38), 23558–65 (1996).

25 Spillmann D, Hard K, Thomas-Oates J, Vliegenthart JFG, Misevic G, Burger MM, Finne J, Characterization of a novel pyruvylated carbohydrate unit implicated in the cell aggregation of the marine sponge *Microciona prolifera*, *J Biol Chem* **268**(18), 13378–87 (1993).

26 Spillmann D, Thomas-Oates JE, van Kuik JA, Vliegenthart JFG, Misevic G, Burger MM, Finne J, Characterization of a novel sulfated carbohydrate unit implicated in the carbohydrate-carbohydrate-mediated cell aggregation of the marine sponge *Microciona prolifera*, *J Biol Chem* **270**(10), 5089–97 (1995).

27 Haseley SR, Vermeer HJ, Kamerling JP, Vliegenthart JFG, Carbohydrate self-recognition mediates marine sponge cellular adhesion, *Proc Natl Acad Sci USA* **98**(16), 9419–24 (2001).

28 Solter D, Knowles BB, Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1), *Proc Natl Acad Sci USA* **75**, 5565–69 (1978).

29 Hakomori S, Nudelman ED, Levery SB, Solter D, Knowles BB, The hapten structure of a developmentally regulated glycolipid antigen (SSEA-1) isolated from human erythrocytes and adenocarcinoma: A preliminary note, *Biochem Biophys Res Commun* **100**, 1578–86 (1981).

30 Kannagi R, Nudelman ED, Levery SB, Hakomori S, A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen, SSEA-1, *J Biol Chem* **257**, 14865–74 (1982).

31 Gooi HC, Feizi T, Kapadia A, Knowles BB, Solter D, Evans MJ, Stage-specific embryonic antigen involves a1-3 fucosylated type 2 blood group chains, *Nature* **292**, 156–8 (1981).

32 Fenderson BA, Zehavi U, Hakomori S, A multivalent lacto-N-fucopentaose III-lysyllysine conjugate decompacts preimplantation mouse embryos, while the free oligosaccharide is ineffective, *J Exp Med* **160**, 1591–6 (1984).

33 Rosenman SJ, Fenderson BA, Hakomori S, The role of glycoconjugates in embryogenesis, In *Glycoconjugates in Medicine*, edited by Ohyama M, Muramatsu T (Professional Postgraduate Services, Japan, 1988, pp. 43–50).

34 Eggens I, Fenderson BA, Toyokuni T, Dean B, Stroud MR, Hakomori S, 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**, 9476–84 (1989).

35 Kojima N, Fenderson BA, Stroud MR, Goldberg RI, Habermann R, Toyokuni T, Hakomori S, 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**, 238–48 (1994).

36 Muramatsu T, Gachelin G, Damonneville M, Delarbre C, Jacob F, Cell surface carbohydrates of embryonal carcinoma cells: Polysaccharidic side chains of F9 antigens and of receptors to two lectins, FBP and PNA, *Cell* **18**, 183–91 (1979).

37 Muramatsu T, Muramatsu H, Gachelin G, Jacob F, High molecular weight carbohydrates on embryonal carcinoma cells, In *Teratocarcinoma and embryonic cell interactions*, edited by Muramatsu T, Gachelin G, Moscona AA, Ikawa Y, Japan Scientific Societies Press (Tokyo, p. 143–56), 1982.

38 Tromas C, Rojo J, de la Fuente JM, Barrientos AG, Garcia R, Penades S, Adhesion forces between $Lewis^x$ determinant antigens as measured by atomic force microscopy, *Angew Chem Intl Ed* **40**(16), 3052–5 (2001).

39 Hernaiz MJ, de la Fuente JM, Barrientos AG, Penades S, A model system mimicking glycosphingolipid clusters to quantify carbohydrate self-interactions by surface plasmon resonance, *Angew Chem Intl Ed* **41**(9), 1554–7 (2002).

40 Yvelin F, Zhang Y, Mallet J, Robert F, Jeannin Y, Sinay P, Crystal structure of the $Lewis^x$ trisaccharide, *Carbohydr Lett* **1**, 475–82 (1996).

41 Takeichi M, Cadherin cell adhesion receptors as a morphogenetic regulator, *Science* **251**, 1451–5 (1991).

42 Larue L, Ohsugi M, Hirchenhain J, Kemler R, E-cadherin null mutant embryos fail to form a trophectoderm epithelium, *Proc Natl Acad Sci USA* **91**, 8263–7 (1994).

43 Draber P, Maly P, Mutants of embryonal carcinoma cells defective in the expression of embryoglycan, *Proc Natl Acad Sci USA* **84**, 5798–802 (1987).

44 Boubelik M, Floryk D, Bohata J, Dráberová L, Macák J, Smíd F, Draber P, Le^x glycosphingolipids-mediated cell aggregation, *Glycobiology* **8**, 139–46 (1998).

45 Siuzdak G, Ichikawa Y, Caulfield TJ, Munoz B, Wong C-H, Nicolaou KC, Evidence of Ca^{2+}-dependent carbohydrate association through ion spray mass spectrometry, *J Am Chem Soc* **115**, 2877–81 (1993).

46 Gahmberg CG, Hakomori S, Altered growth behavior of malignant cells associated with changes in externally labeled glycoprotein and glycolipids, *Proc Natl Acad Sci USA* **70**, 3329–33 (1973).

47 Hynes RO, Alteration of cell surface proteins by viral transformation and proteolysis, *Proc Natl Acad Sci USA* **70**, 3170–4 (1973).

48 Ruoslahti E, Vaheri A, Kuusela P, Linder E, Fibroblast surface antigen: A new serum protein, *Biochim Biophys Acta* **322**, 352–8 (1973).

49 Kleinman HK, Martin GR, Fishman PH, Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen, *Proc Natl Acad Sci USA* **76**, 3367–71 (1979).

50 Ruoslahti E, Integrins, *J Clin Invest* **87**, 1–5 (1991).

51 Hynes RO, Integrins: Versatility, modulation, and signaling in cell adhesion, *Cell* **69**, 11–25 (1992).

52 Cheresh DA, Pytela R, Pierschbacher MD, Klier FG, Ruoslahti E, Reisfeld RA, An Arg-Gly-Asp-directed receptor on the surface of human melanoma cells exists in a divalent cation-dependent functional complex with the disialoganglioside GD2, *J Cell Biol* **105**, 1163–73 (1987).

53 Zheng M, Fang H, Tsuruoka T, Tsuji T, Sasaki T, Hakomori S, Regulatory role of G_{M3} ganglioside in a5b1 integrin receptor for

fibronectin-mediated adhesion of FUA169 cells, *J Biol Chem* **268**, 2217–22 (1993).
54 Kojima N, Hakomori S, Specific interaction between gangliotriaosylceramide (Gg3) and sialosyllactosylceramide (GM3) as a basis for specific cellular recognition between lymphoma and melanoma cells, *J Biol Chem* **264**, 20159–62 (1989).
55 Kojima N, Hakomori S, Cell adhesion, spreading, and motility of G_{M3}-expressing cells based on glycolipid-glycolipid interaction, *J Biol Chem* **266**, 17552–58 (1991).
56 Matsuura K, Kitakouji H, Sawada N, Ishida H, Kiso M, Kitajima K, Kobayashi K, 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**(30), 7406–7 (2000).
57 Laine RA, Yogeeswaran G, Hakomori S, Glycosphingolipids covalently linked to agarose gel or glass beads, *J Biol Chem* **249**, 4460–6 (1974).
58 Carter WG, Fukuda M, Lingwood C, Hakomori S, Chemical composition, gross structure, and organization of transformation-sensitive glycoproteins, *Ann NY Acad Sci* **312**, 160–77 (1978).
59 Andrews PW, Goodfellow PN, Shevinsky LH, Bronson DL, Knowles BB, Cell surface antigens of a clonal human embryonal carcinoma cell line: Morphological and antigenic differentiation in culture, *Int J Cancer* **29**, 523–31 (1982).
60 Andrews PW, Damjanov I, Simon D, Banting G, Carlin C, Dracopoli NC, Fogh J, Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line TERA-2: Differentiation *in vivo* and *in vitro*, *Lab Invest* **50**, 147–62 (1984).
61 Andrews PW, Fenderson BA, Hakomori S, Human embryonal carcinoma cells and their differentiation in culture, *Int J Androl* **10**, 95–104 (1987).
62 Kannagi R, Levery SB, Ishigami F, Hakomori S, Shevinsky LH, Knowles BB, Solter D, New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3, *J Biol Chem* **258**, 8934–42 (1983).
63 Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, Solter D, Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells, *EMBO J* **2**, 2355–61 (1983).
64 Yu S, Withers DA, Hakomori S, Globoside-dependent adhesion of human embryonal carcinoma cells, based on carbohydrate-carbohydrate interaction, initiates signal transduction and induces enhanced activity of transcription factors AP1 and CREB, *J Biol Chem* **273**, 2517–25 (1998).
65 Zhu Z, Kojima N, Stroud MR, Hakomori S, Fenderson BA, Monoclonal antibody directed to Le^y oligosaccharide inhibits implantation in the mouse, *Biol Repro* **52**, 903–12 (1995).
66 Israelachvili JN, Adams GE, Measurement of forces between two mica surfaces in aqueous electrolyte solutions in the range 0-100 nm, *J Chem Soc Faraday Trans* **74**, 975–1001 (1978).
67 Helm CA, Knoll W, Israelachvili JN, Measurement of ligand-receptor interactions, *Proc Natl Acad Sci USA* **88**, 8169–73 (1991).
68 Dammer U, Popescu O, Wagner P, Anselmetti D, Guntherodt H-J, Misevic GN, Binding strength between cell adhesion proteoglycans measured by atomic force microscopy, *Science* **267**, 1173–75 (1995).
69 Matsuura K, Kitakouji H, Tsuchida A, Sawada N, Ishida H, Kiso M, Kobayashi K, Carbohydrate-carbohydrate interaction between glycolipids and glycoconjugate polystyrenes at the air-water interface, *Chem Letters* **1998**, 1293–4 (1998).
70 Santacroce PV, Basu A, Probing specificity in carbohydrate-carbohydrate interactions with micelles and Langmuir monolayers, *Angew Chem Intl Ed* **42**(1), 95–8 (2003).
71 Wang X, Sun P, Al-Qamari A, Tai T, Kawashima I, Paller AS, Carbohydrate-carbohydrate binding of ganglioside to integrin a5 modulates a5b1 function, *J Biol Chem* **276**(11), 8436–44 (2001).
72 Kawakami Y, Kawakami K, Steelant WFA, Ono M, Baek RC, Handa K, Withers DA, Hakomori S, 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**(37), 34349–58 (2002).
73 Wang X-Q, Sun P, O'Gorman M, Tai T, Paller AS, Epidermal growth factor receptor glycosylation is required for ganglioside GM3 binding and GM3-mediated suppression of activation, *Glycobiology* **11**(7), 515–22 (2001).
74 Fernandes H, Cohen S, Bishayee S, Glycosylation-induced conformational modification positively regulates receptor-receptor association: A study with an aberrant epidermal growth factor receptor (EGFRvIII/DEGFR) expressed in cancer cells, *J Biol Chem* **276**(7), 5375–83 (2001).
75 Tsuda T, Ikeda Y, Taniguchi N, The Asn-420-linked sugar chain in human epidermal growth factor receptor suppresses ligand-independent spontaneous oligomerization, *J Biol Chem* **275**, 21988–94 (2000).
76 Bremer EG, Schlessinger J, Hakomori S, Ganglioside-mediated modulation of cell growth: Specific effects of GM_3 on tyrosine phosphorylation of the epidermal growth factor receptor, *J Biol Chem* **261**, 2434–40 (1986).
77 Geyer A, Gege C, Schmidt RR, Carbohydrate-carbohydrate recognition between $Lewis^x$ glycoconjugates, *Angew Chem Intl Ed* **38**(10), 1466–8 (1999).
78 Wormald MR, Edge CJ, Dwek RA, The solution conformation of the Le^x group, *Biochem Biophys Res Commun* **180**, 1214–21 (1991).
79 Kojima N, Shiota M, Sadahira Y, Handa K, Hakomori S, Cell adhesion in a dynamic flow system as compared to static system: Glycosphingolipid-glycosphingolipid interaction in the dynamic system predominates over lectin- or integrin-based mechanisms in adhesion of B16 melanoma cells to non-activated endothelial cells, *J Biol Chem* **267**, 17264–70 (1992).
80 Menikh A, Nyholm P-G, Boggs JM, Characterization of the interaction of Ca^{2+} with hydroxy and non-hydroxy fatty acid species of cerebroside sulfate by Fourier transform infrared spectroscopy and molecular modeling, *Biochemistry* **36**, 3438–47 (1997).
81 Yu S, Kojima N, Hakomori S, Kudo S, Inoue S, Inoue Y, Binding of rainbow trout sperm to egg is mediated by strong carbohydrate-to-carbohydrate interaction between (KDN)GM3 (deaminated neuraminyl ganglioside) and Gg3-like epitope, *Proc Natl Acad Sci USA* **99**(5), 2854–59 (2002).
82 Boubelik M, Dráberová L, Draber P, Carbohydrate-mediated sorting in aggregating embryonal carcinoma cells, *Biochem Biophys Res Commun* **224**, 283–88 (1996).
83 Kojima N, Hakomori S, Synergistic effect of two cell recognition systems: Glycosphingolipid-glycosphingolipid interaction

and integrin receptor interaction with pericellular matrix protein, *Glycobiology* **1**, 623–30 (1991).

84 Edelman GM, Crossin KL, Cell adhesion molecules: Implications for a molecular histology, *Annu Rev Biochem* **60**, 155–90 (1991).

85 Crocker PR, Clark EA, Filbin M, Gordon S, Jones Y, Varki A, Siglecs: A family of sialic acid-binding lectins, *Glycobiology* **8**(2), v-vi (1998).

86 Toledo MS, Suzuki E, Handa K, Hakomori S, Cell growth regulation through GM3-enriched microdomain in human lung embryonal fibroblast WI38 and its oncogenic transformant VA13, *J Biol Chem* (in press) (2004).

87 Handa K, Jacobs F, Longenecker BM, Hakomori S, Association of MUC-1 and PSGL-1 with low-density microdomain in T-lymphocytes: A preliminary note, *Biochem Biophys Res Commun* **285**, 788–94 (2001).