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Carbohydrate to carbohydrate interaction in development process and cancer progression

Kazuko Handa · Sen-itiroh Hakomori

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Abstract Two types of carbohydrate to carbohydrate interaction (CCI) have been known to be involved in biological processes. One is the CCI between molecules expressed on interfacing cell membranes of different cells to mediate cell to cell adhesion, and subsequently induce cell signaling, and is termed *trans*-CCI. It has been indicated that the Le^x to Le^x interaction at the morula stage in mouse embryos plays an important role in the compaction process in embryonic development. GM3 to Gg3 or GM3 to LacCer interaction has been suggested to be involved in adhesion of tumor cells to endothelial cells, which is considered a crucial step in tumor metastasis. The other is the CCI between molecules expressed within the same microdomain of the cell surface membrane, and is termed *cis*-CCI. The interaction between ganglioside GM3, and multi (>3) GlcNAc termini of N-linked glycans of epidermal growth factor receptor (EGFR), has been indicated as the molecular mechanism for the inhibitory effect of GM3 on EGFR activation. Also, the complex with GM3 and GM2 has been shown to inhibit the activation of hepatocyte growth factor (HGF) receptor, cMet, through its association with tetraspanin CD82, and results in the inhibition of cell motility. Since CCI research is still limited, more examples of CCI in biological processes in development, and cancer progression will be revealed in the future.

K. Handa · S.-i. Hakomori (✉)
Pacific Northwest Research Institude,
720 Broadway,
Seattle, WA 98122, USA
e-mail: hakomori@u.washington.edu

S.-i. Hakomori
Departments of Pathobiology and Global Health,
University of Washington,
Seattle, WA 98195, USA

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Abbreviations

CCI	carbohydrate-to-carbohydrate interaction
Cer	ceramide
CD82	one of tetraspanin superfamily (TM4), interacting membrane components to control cellular phenotype
CHO	Chinese hamster ovary
CPI	carbohydrate-to-protein interaction
Ecad	E-cadherin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ES	embryonal stem
Fuc	fucose
GFR	growth factor receptor
GalCer	β-galactosylceramide
GalNAc	*N*-acetylgalactosamine
GSL	glycosphingolipid
HGF	human growth factor
HGFR	hepatocyte growth factor receptor
HPLC	high-performance liquid chromatography
IgG	immunoglobulin G
LacCer	lactosylceramide
LacNAc	Galβ4GlcNAc
M	mannose
mAb	monoclonal antibodies
NeuAc	*N*-acetylneuraminic acid
NMR	nuclear magnetic resonance
Os	oligosaccharide
PPI	protein-to-protein interaction

RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSEA	stage-specific embryogenic antigen
TBS	tris-buffered saline (tris, tris(hydroxymethyl) aminomethane)

Nomenclature of glycosphingolipids and glycan structures

GSLs are abbreviated as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem J* 171: 21–35, 1978, Table I); however, the suffix -Ose or -OseCer is omitted. Ganglio-series gangliosides are abbreviated according to the extended version of Svennerholm's list (*e.g.*, Holmgren *et al.*, *Proc Natl Acad Sci USA* 77: 1947–1950, 1980).

Glycosphingolipid abbreviations used

Gb3	Galα4Galβ4Glcβ1Cer
Gb4	GalNAcβ3Galα4Galβ4Glcβ1Cer
Gb5	Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Gg3	GalNAcβ4Galβ4Glcβ1Cer
Gg4	Galβ3GalNAcβ4Galβ4Glcβ1Cer
GD1a	[NeuAcα3]Galβ3GalNAcβ4[NeuAcα3] Galβ4Glcβ1Cer
GD1b	Galβ3GalNAcβ4[NeuAcα8NeuAcα3] Galβ4Glcβ1Cer
GD2	NeuAcα2-8NeuAcα2-3[GalNAcβ1-4] Galβ1-4Glc1Cer
GD3	NeuAcα2-8NeuAcα2-3Galβ4Glc1Cer
GT1a	NeuAcα8NeuAcα3Galβ3GalNAcβ4 [NeuAcα3]Galβ4Glcβ1Cer
GT1b	NeuAcα3Galβ3GalNAcβ4[NeuAcα8 NeuAcα3]Galβ4Glcβ1Cer
GM1	Galβ3GalNAcβ4[NeuAcα3]Gal β4Glcβ1Cer
GM2	GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer
GM3	NeuAcα3Galβ4Glcβ1Cer
LacCer	Galβ4Glcβ1Cer
nLc4	Galβ4GlcNAcβ3Galβ4GlcβCer
Lea	Galβ3[Fucα4]GlcNAcβ3Galβ→R
Lex	Galβ4[Fucα3]GlcNAcβ3Galβ→R
Ley	[Fucα2]Galβ3[Fucα4]GlcNAcβ3Galβ→R
NGcGM3	N-glycolylneuraminyl-lactosylCer
NAcGM3	N-acetylneuraminyl-lactosylCer
PG	paragloboside: Galβ4GlcNAcβ3Gal β1-4Glcβ1-Cer
SSEA-3	Fucα2Galβ3NAcβ3Galα4Gal β4Glcβ1Cer
SSEA-4	NeuAcα3Galβ3GalNAcβ3Galα4Gal β4Glcβ1Cer
H antigen	Fucα1-2Galβ-R

Introduction

Interactions between molecules expressed on cell membranes are known to control various types of cellular functions, cell growth, adhesion, differentiation, motility, and so on. Various types of glycosylations, glycosyltransferases involved for the modification, and their functional roles, including interactions between carbohydrates and proteins, have been extensively studied and well documented [1, 2]. However, little attention was paid on carbohydrate to carbohydrate interaction (CCI), and its roles in cell adhesion and cell signaling. While CCI is a novel concept, and has been demonstrated in a relatively limited combination of carbohydrate structures in limited types of cells, ~10 independent groups have been involved in this area of research. In this review, we will describe studies from our own group, and others, on CCI involved in cell-cell adhesions and in ganglioside-mediated inhibition of growth factor receptor activation.

Carbohydrate to carbohydrate interactions involved in cell adhesion

Sponges, the simplest and earliest multicellular organisms are considered to have developed from mono-cellular ancesters in the sea, ~1 billion years ago. Various species of sponges with different colors are known to develop through species-specific auto-aggregation. These bio-chemical mechanisms initially were studied by Humphreys about 45 years ago [3]. Subsequent and extensive biochemical studies on the molecular mechanism of the auto-aggregation by Burger, Spillman, Misevic, Vliegenthart and their associates, demonstrated that red sponge cells (*Microciona prolifera*) aggregate through CCI between oligosaccharide (Os), particularly with the structure 3-O-sulfated GlcNAcβ1-3Fucα1-O-Ser/Thr, assembled on "proteoglycan", but not with the same Os structure with β-anomeric linkages to Ser/Thr of proteoglycan [4]. A clear piece of evidence along this line of studies, was shown by self-aggregation of the Os with α- *vs.* β-anomeric linkages, when linked to gold nanospheres [5]. The same approach with glycosyl-nanospheres was previously developed by Rojo *et al.* [6]. The cell adhesion force between proteoglycan oligosaccharide in sponge cells was determined by atomic force microscopy (AFM) and the adhesion force between two proteoglycans was found to be upto 400 pN. This indicates a single pair of molecules could hold the weight of 1600 cells [7].

In mammalian cells, we demonstrated that mouse embryonic stem cells at the morula stage induce compaction. Our studies started with the characterization of an epitope defined by the monoclonal antibody (mAb), stage-specific embryogenic antigen (SSEA)-1, which was established by immunizing Balb C mice with embryonic carcinoma F9 cells [8]. The

epitope was identified as Galβ4[Fucα3]GlcNAcβ3Gal, *i.e.* internally fucosylated lacto-series type 2 structure, the positional isomer of blood group Le^a, and therefore termed as Le^x [9], and better reactivity was shown with the long, non-branched structure with Le^x at terminal structure [10]. Also, the trisaccharide inhibited the binding of the mAb [11]. Later, other stage specific antigens, SSEA-3, and −4, defined by respective mAb were characterized as extended globo-series structures [12, 13], indicating a high impact on functional significance of glycosylation defining the developmental process [14, 15].

SSEA-1 antigen is not expressed in zygotes up to the 2- to 4-cell stage (0.5-1 day), but is expressed at 8–32 cells, *i.e.*,"morula" stage (1.5-2 days), and declines after "compaction" [8, 15] (Fig. 1a). Such a clear change of expression pattern suggested that Le^x may mediate the compaction. Indeed, the compaction process was inhibited by trivalent Le^x, but not by trivalant Le^a (Fig. 1b-b *vs.* b-c; see c for structures) in mouse embryogenesis [16]. Subsequent studies showed that homotypic interaction of mouse teratocarcinoma F9 cells is inhibited with Le^x oligosaccharide (lactofucopentaose III) and that liposomes containing Le^x GSL selectively bind to not only F9 cells but also to Le^x GSL-coated plates. These results strongly suggested CCI in cell-cell interaction [17]. The possible interaction between Le^x was further tested with Le^x-conjugated gold sol nanoparticles; auto-aggregation of the nanoparticles was observed in the presence of $CaCl_2$, and such auto-aggregation was abolished by EDTA [18], as shown in Fig. 1d. While the data in Fig. 1 suggested the occurrence of Le^x to Le^x interaction, it did not exclude the possibility for the presence of Le^x-binding proteins that may mediate the cell aggregation. Using mouse embryonal carcinoma F9 cells, which highly express SSEA-1, we searched for a possible

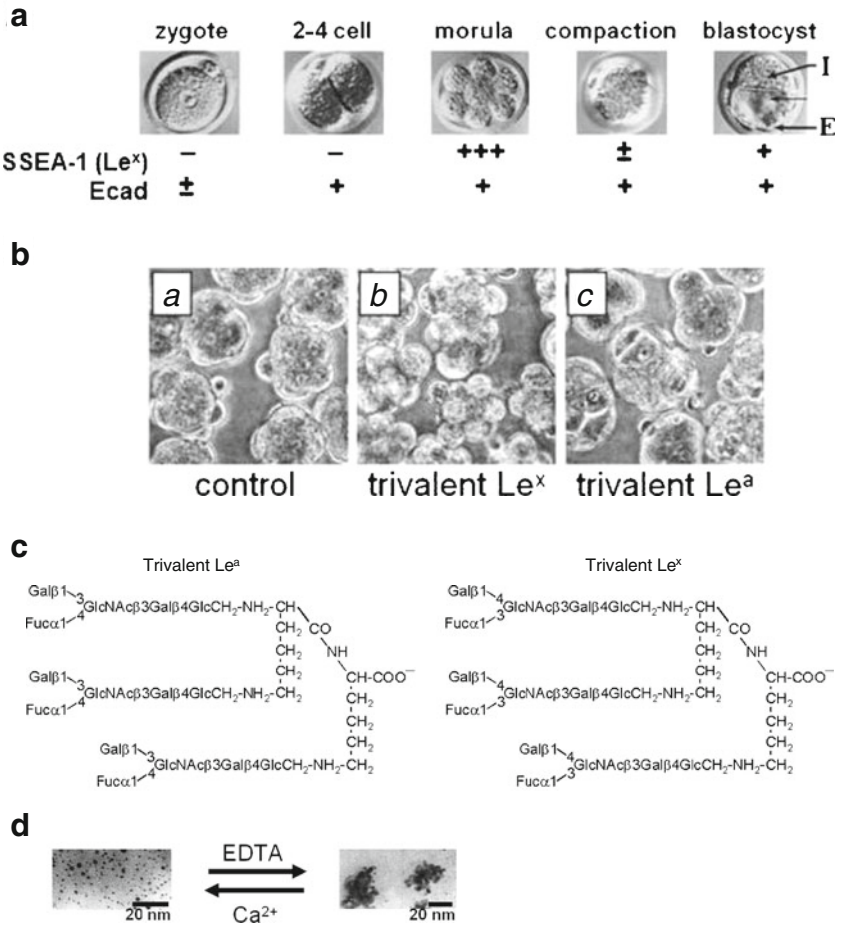

Fig. 1 Le^x -Le^x interaction. **a**: Le^x expression during mouse embryogenesis. Le^x, which is not expressed in zygote, or 2–4 cell, is expressed maximally at morula, and declines at "compaction" stage. At blastocyst stage, Le^x expression is limited to inner cell mass *(I)* and endodermal surface *(E)*, whereas E-cadherin (Ecad) is expressed fairly consistently. Composed from data by Solter & Knowles [8], and Handyside [15]. **b**: De-compaction effect of trivalent Le^x but not trivalent Le^a. Compacted mouse embryonic stem cells in Whitten medium (*a*) were decompacted in the presence of trivalent Le^x (*b*), but not trivalent Le^a (*c*). Data from Fenderson *et al.* [16]. **c**: Trivalent Le^a and Le^x . Left: Trivalent Le^a, three Le^a oligosaccharides are conjugated to three amino groups of lysyllysine. Right: Trivalent Le^x, three Le^x oligosaccharides are conjugated to three amino groups of lysyllysine. **d**: Ca^{2+} dependent auto-aggregation of Le^x-gold nanoparticles. Auto-aggregation of gold (Au) nanoparticles conjugated with Le^x epitope in the presence of 10 mM $CaCl_2$, observed by transmission electron microscopy. The aggregation was reversed by the addition of 10 mM EDTA. Data from de la Fuente *et al.* [18]

Le^x-binding protein that may block binding mAb SSEA-1 to F9 cells. We found that components with molecular mass 15–25 kD display the inhibitory activity. Proteinase treatment had no effect on their inhibitory activity [9, 19], and no protein was detectable in the components, suggesting that these could be poly-LacNAc, though further analysis needs to be done.

Subsequent studies on molecules carrying the Le^x-glycan in F9 cells, were performed [20] following previous studies on "embryoglycan" [21, 22], which was shown to have a high molecular mass, while structural information about the glycan was limited at that time. Our observations were: (i) Comparative adhesion studies of Le^x-expressing tumor cells *vs.* non-expressing variants showed that only Le^x-expressing cells adhere to Le^x-coated plates, and display cell aggregation in analogy to F9 cell aggregation. (ii) The major carrier of Le^x-determinants in F9 cells was not GSLs, but rather polylactosaminoglycan with high mass range in "embryoglycan", and they demonstrated auto-aggregation in the presence of Ca^{2+}, and reversible dissociation by addition of EDTA. Furthermore, defucosylation caused the loss of auto-aggregation activity (Fig. 2).

In addition, the importance of Le^x–carrying molecules and of E-cadherin (Ecad) expressed in embyonal stem (ES) cells and F9 cells were compared, since both Le^x and Ecad are expressed during embryogenesis and cell adhesion through either process is known to require the presence of Ca^{2+}. For this purpose, Ecad gene was knocked out by homologous recombination in both F9 and ES cell line, D3M, to establish F9 Ecad (−/−), and D3M Ecad (−/−) cells. The Ecad-deficient cells still retained the ability of cell aggregation, suggesting the importance of Le^x dependent interaction in the cell aggregation with these cells [23].

Our further search for CCI between other glycans in addition to the Le^x-Le^x interaction, showed a strong interaction between GM3 to Gg3, and to lactosyl-ceramide (LacCer), but not other GSLs tested. In liposome binding assay, as shown in Fig. 3, and in cell adhesion assay using B16 mouse melanoma cells, as shown in Fig. 4 [24]. In both assays, Gg3 showed higher binding than LacCer. Also, in the lung colonization assay in mice, pre-incubation of B16 cells with liposomes containing GM3- or Gg3 resulted in clear inhibition, while liposomes containing LacCer was found to be much less effective [25]. In contrast, the binding assay based on Langmuir monolayers, demonstrated the most clear binding between LacCer and GM3 [26]. Indeed, a following study showed that lacosyl-bound to gold-nanoparticles (Fig. 5a) inhibit the colonization of B16 mouse melanoma cells in lung and liver most effectively (Fig. 5b)[6]. It is reasonable that lactosyl residues linked to gold nanoparticles through the long alkyl chain are more efficient in blocking tumor cells adhering to vascular endothelial cell surfaces than lactosylceramide liposomes directly and randomly linked to PC-cholesterol surfaces. Thus the approaches with glycosyl-gold nanosphere, originally found by Penades and her group are well justified for inhibition of cancer progression.

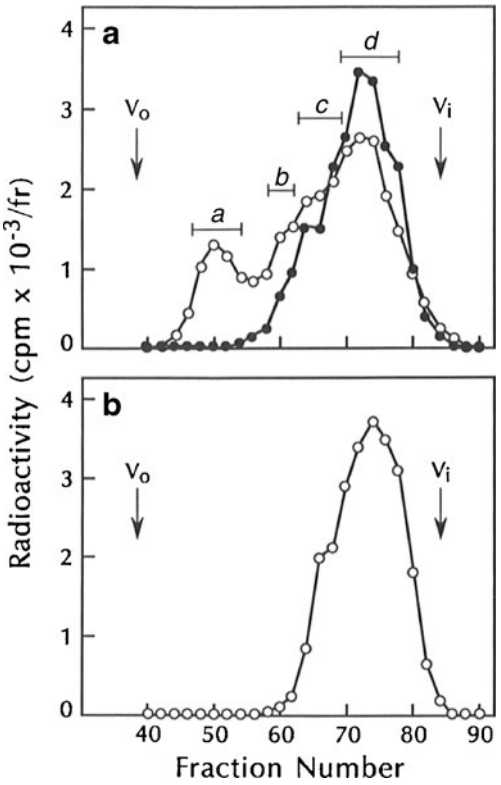

Fig. 2 Aggregation of Lactosaminoglycan-Glycopeptide (LAG-GP) as demonstrated by gel filtration. **a**: LAG-GP fraction prepared from F9 cells metabolically labeled with [^{3}H]GlcN, was dialysed in the presence of $CaCl_2$ or EDTA, then subjected to gel filtration on Sephacryl CL-6B colum, and eluted with buffer solution containing $CaCl_2$ or EDTA. Fractions were collected and radioactivity in each fraction was determined. (○), gel filtration pattern in the presence of $CaCl_2$. (●) in the presence of EDTA. v_0, void volume. v_i, column volume. In the presence of Ca^{2+}, LAG-GP fraction showed three peaks (termed *a*, *b* and *c*) corresponding to high-M_r aggregates, in addition to the major peak *d*. In the presence of EDTA, peaks *a* and *b* completely disappeared, and *c* was greatly reduced. **b**: LAG-GAP fraction was pre-treated with bovine liver α-fucosidase, which cleaves off the fucosyl residue of Le^x, and then analyzed by gel filtration in the presence in the presence of $CaCl_2$. Peaks *a*, *b*, and *c* disappeared. Data from Kojima *et al.* [20]

In addition, a different *trans*-CCI was found based on a series of studies using GalCer and 3-*O*-sulfated GalCer (sulfatide), which are expressed in the myelin sheath membrane, by Boggs and her colleagues [27, 28]. Nerve axons are surrounded by multilayers of myelin sheath. They showed that a strong interaction between cerebroside and sulfatide expressed at interfacing membranes, may cause mutual membrane binding, and demonstrateg the CCI between the two GSLs induces signal transduction with various signaling pathways, involving Src family kinases, P13K, MAPK, *etc.*, using selective inhibitors for these signaling molecules [28]. GSL expression at uterine endometrium was found to change on

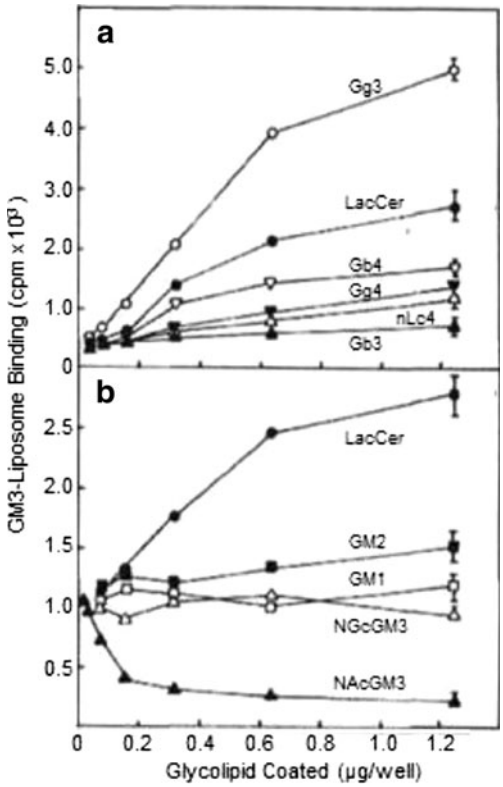

Fig. 3 Interaction of GM3 liposomes to various GSLs coated on solid phase wells. **a**: GM3-liposomes labeled with [^{14}C]-cholesterol are added to 96-well polystyrene plastic plates coated with various quantities of Gg3 (○), LacCer (●), Gb4 (∇), Gg4 (▼), nLc4 (Δ), and Gb3 (▲). Each well is added with 100 μl (25,000 cpm) of GM3 liposomes in TBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and incubated at room temperature for 16 h. After washing, bound ^{14}C activity was measured [24]. **b**: A similar analysis as in A, including *N*-acetyl GM3 (regular GM3) and *N*-glycolyl GM3. It is noticeable that negative interaction (repulsion) was found between *N*-acetyl GM3, but no repulsion between *N*-acetyl GM3 and *N*-glycolyl GM3. Data from Kojima *et al.* [24]

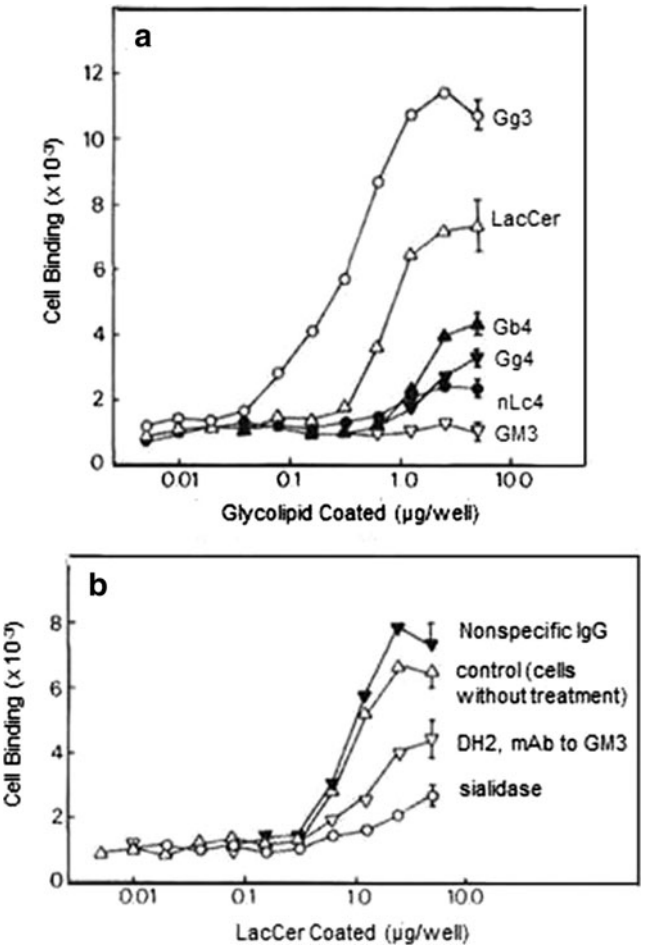

Fig. 4 Adhesion of B16 melanoma cells on LacCer-coated (in comparison with other GSL-coated) solid phase and effects of anti-GM3 mAb and sialidase treatment. **a**: Adhesion of B16/BL6 cells on GSL-coated 96-well plates: Gg3 (○), LacCer (CDH) (Δ), Gb4 (▲), Gg4 (▼), PG (●) GM3 (∇). [^{3}H]Thymidine-labeled cells (2×10^4) were added to the wells. After incubation for 15 min in serum-free medium at 37°C, the plates were washed. The bound cells were harvested and counted. **b**: Adhesion of BL6 melanoma cells on LacCer-coated 96-well plates and effects of treatment of cells with various reagents. [^{3}H] Thymidine-labeled cells (1×10^5/ml) were treated with sialidase (0.1 unit/ml in PBS) (○), mAb DH2 (anti-GM3, 20 μg/ml) (∇), or nonspecific mouse IgG (20 μg/ml) (▼), for 30 min at room temperature. After washing, the treated cells (2×10^4) were added to the coated-wells. After 15 min of incubation, plates were washed. The bound cells were harvested and counted

pregnancy, as shown in rabbit experiments; GM3, GD3 decreased and Gb3, Gb4 increased, which may provide a better chance for the embryo to get embedded in the endometrium [29]. Further studies in mic showed that blastocysts express Le^y, while endometrium express blood group O-associated H epitope, and that intra-uterine injection of anti-Le^y mAb AH6 blocks blastocyst cell adhesion and their continuous development [30]; suggesting a possible CCI between Le^y and H in adhesion of blastocysts to endometrium.

Further physicochemical studies by various labs support the specific Le^x-Le^x interaction: An example 2-dimensional ^{1}H-NMR [31]; atomic force microscopy between a pair of single Le^x was 20±4 piconewtons (pN), whereas no force (0 pN) was found for lactose-to-Le^x, or lactose-to-lactose [32]; vesicular adhesion energy, determined by the change of contact angle θ_c between vesicles [33], was much higher for two vesicles both containing Le^x-Cer, than for two vesicles both containing Le^a-Cer, or one vesicle containing Le^x-Cer and the other Le^a-Cer [34].

Human embryonic carcinoma cells like 2102Ep are characterized by high expression of nLc4, Gb4 and the extended Gb4 (Gb5, fucosyl or sialosyl Gb5), termed SSEA-3, and −4. Our previous study indicated 2102Ep cells bind to Gb4-coated plates through interaction between Gb4 and nLc4 or Gb5, and that the binding activates transcription factors AP1 and CREB [35].

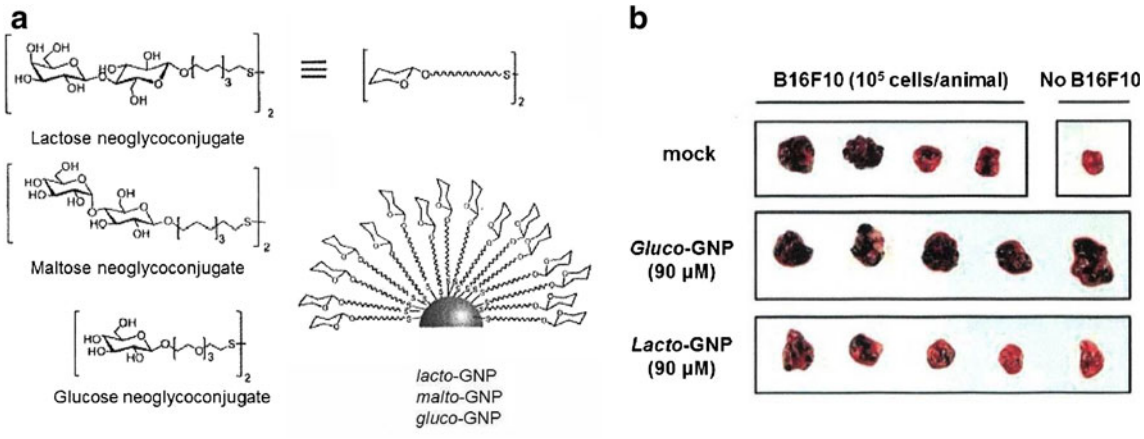

Fig. 5 Inhibition of the colonization of B16F10 melanoma cells in *in vivo*. **a**: Neoglycoconjugates and glyconanoparticles used in this study. **b**: Specific inhibitory effect of lacto-GNPs on the lung colonization of B16F10 cells. Representative pictures of lungs from animals in each group in comparison with the lung obtained from a control animal not injected with B16F10 cells. Data from Rojo *et al.* [6]

Summary of CCIs currently known is shown in Fig. 6. Some of them were observed based on strong cell adhesion assay, although some others were based on biochemical assays *in vitro*. Futher extensive studies are needed to demonstrate that these CCIs are really involved in cell adhesion, and have some functional roles in development and/or cancer.

Carbohydrate to carbohydrate interaction in regulation of growth factor receptor

Previous works by our lab and others, showed that GM3 strongly interacts with epidermal growth factor receptor (EGFR), and inhibits EGF-induced activation of EGFR [1, 36, 37]. Subsequent studies to compare the activity of various gangliosides indicated that GM3 displays much higher inhibitory effect than other gangliosides; and the order of the inhibitory effect was: GM3>>>GM2, GD3, GM4>GM1> GD1a, GD1b in A431 cells or KB cells [38]. However, the molecular mechanism underlying the specific inhibition of EGFR with GM3 was not known.

Based on our previous finding of CCI, we examined the possibility that GM3 interacts with N-linked glycans of EGFR as the first step for the inhibition. Human EGFR has 11 potential N-glycosylation sites, and at least 8 sites are known to carry N-glycan in A431 cells [39]. The preparation

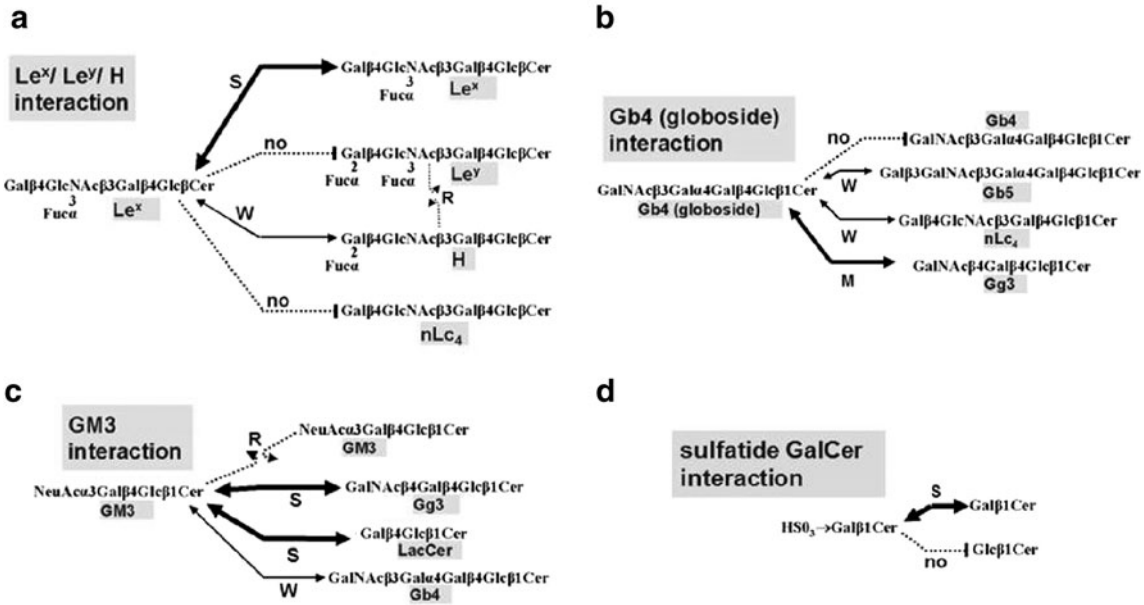

Fig. 6 Structures of various carbohydrate epitopes showing strong (S), medium (M), weak (W), no, or repulsive (R) interactions with one another. **a**: $Le^x/Le^y/H$ interaction [16, 17, 25, 29, 30]. **b**: Gb4 (globoside) interaction [35]. **c**: GM3 interaction [25, 67] **d**: sulfatide GalCer interaction [27, 28]

of enough N-glycans to analyze their binding to GM3 requires a large amount of A431 cells; therefore, we prepared and purified N-glycans from readily available sources, ovalbumin and fetuin. Since a series of methodologies for isolation and characterization of N-glycans were well established, we first separated a sufficient amount of purified N-glycans from ovalbumin, and analyzed which types of N-glycans display GM3 binding. This was to apply such information in the future for analysis of N-glycans expressed and involved in GM3-mediated inhibition of EGFR activation in A431 cells. Total N-glycans from ovalbumin were isolated following the fractionation method, as outlined by Shimizu *et al.* [40], including lectin-affinity chromatography [41, 42], and then further separated into N-glycan components by high performance liquid chromatography (HPLC) using "Varian Prostar" column. These purified N-glycans were covalently bound to amino-group of phosphatidyl-ethanolamine (PE) as previously reported [43, 44]. The PE-bound glycans were coated/affixed on to polystyrene plates, and their binding with GM3 and other GSLs were determined using liposomes containing each GSL. With this method, we found that N-glycan containing 5 or 6 GlcNAc termini, termed as "OsFr.B" (Fig. 7a), binds to GM3 at highest degree among N-glycans tested. These penta-hexa antennary N-linked structures and their incomplete form with multiple GlcNAc termini were previously isolated from oviduct epithelial cells and their structures were fully characterized [45]. The biantennary N-glycan carrying 2 GlcNAc termini "Os 1" did not show any detectable GM3 binding. Also, OsFr. B did not show any significant binding to other GSLs tested so far [46].

Next, we examined if N-linked glycan with multi-GlcNAc termini is expressed in A431 cells and whether it is really involved in GM3-mediated inhibition of EGFR activation, using OsFr.B, described above. As shown in Fig. 7: (i) Polystyrene beads coated with GM3, but not with GM1 or Gb4, could bind EGFR in A431 cell lysate. The specific binding of EGFR to GM3 was blocked by preincubation of GM3-coated beads with Os Fr.B, but not with

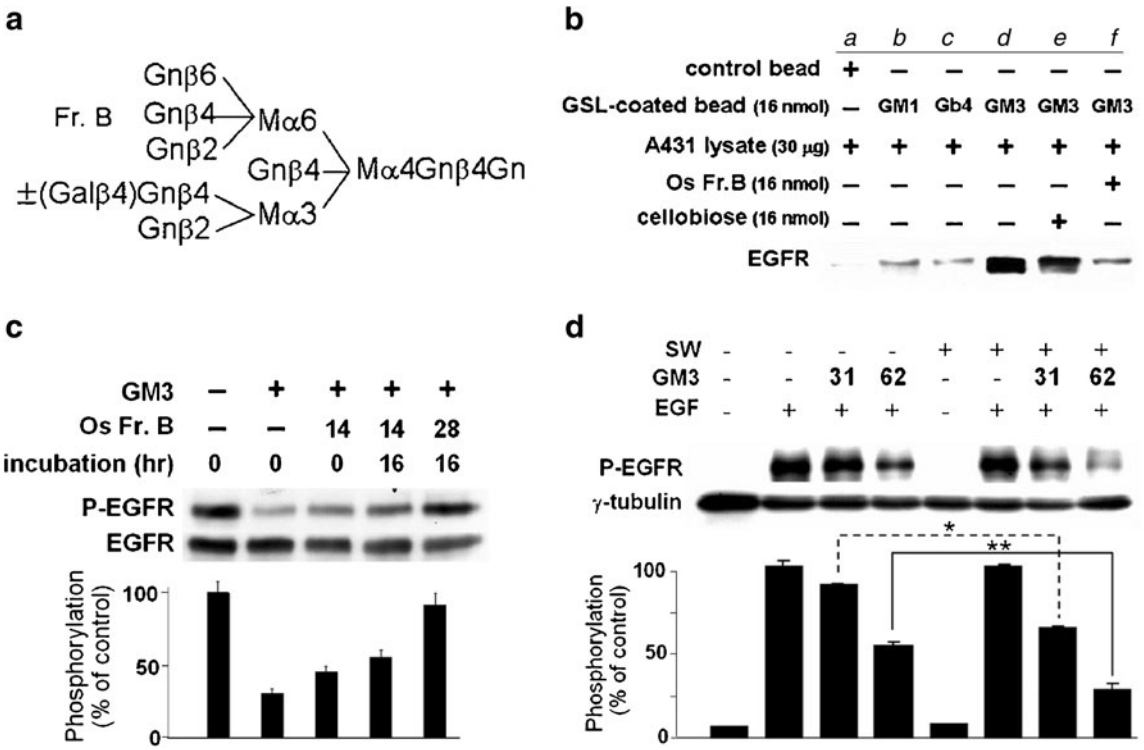

Fig. 7 Interaction between GM3 and EGFR, and effects of "Os Fr.B" glycan with multiple GlcNAc termini. **a**: Structure of Os Fr.B, having 5 or 6 GlcNAc(Gn) termini. The oligosaccharide (Os) was separated, purified from obalbumin, and its structure was determined as in Yoon *et al.* [46] and Yamashita *et al.* [45]. Gn, *N*-acetylglucosamine (GlcNAc). M, mannose. **b**: Interaction of GSL-coated polystyrene beads with EGFR, and inhibition of this interaction by Os Fr.B. Polystyrene beads (1 μm in diameter), which were coated with GM3, GM1, or Gb4 were mixed with cell lysate prepared from A431 cells. After washing, EGFR bound to the beads were detected by Western blot with anti-EGFR mAb. Os Fr.B, or cellobiose were added in the cell lysate to analyze the specific effect of Os Fr.B on the binding of EGFR to GM3-coated beads. **c**: Os Fr.B was mixed with GM3 micelles and then incubated with membrane fraction prepared from A431 cells. EGF was added and the mixture was incubated for 10 min at 22°C. Phosphorylation of EGFR was analyzed by Western blot with mAb PY20, and re-blotted with anti-EGFR mAb. EGF-induced EGFR activation was calculated as P-EGFR/EGFR. **d**: Effect of swainsonine (SW) treatment on GM3-mediated inhibition of EGFR activation. Control and SW-treated A431 cells were preincubated with GM3 and then stimulated with EGF. EGFR activation was detected with mAb PY20, using γ-tubulin as loading control. Significance of differences: *, $p=0.025$; **, $p=0.0025$. Data from Yoon *et al.* [49]

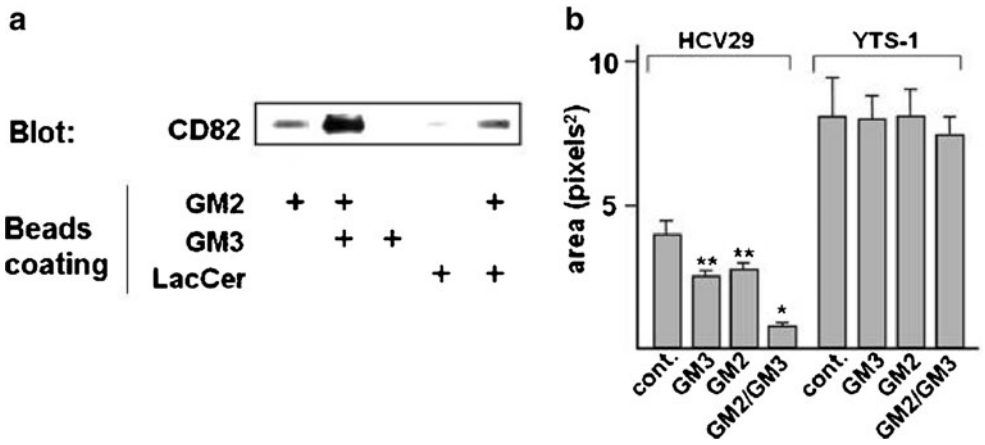

Fig. 8 Inhibitory role of GM2/GM3 complex in cell motility through its association with CD82. **a**: Interaction of GM2/GM3 complex with CD82. Polystyrene beads (1 μm diameter) coated with GM2, GM2/GM3, GM3, LacCer or GM2/LacCer, were mixed with cell lysates prepared from YTS-1 cells overexpressing CD82. After washing, the amount of CD82 bound to the coated beads was determined by Western blot with anti-CD82. **b**: Inhibitory effect of GM2/GM3 complex on cell motility. Silica nanospheres (50 nm diameter) were coated with GM3, GM2, or GM2/GM3. HCV29 cells (CD82 positive) and YTS-1 cells (CD82 negative) were mixed with the coated nanospheres and their phagokinetic cell motility activities were analyzed on gold-sol monolayers and expressed as moved area. * $p<0.001$. ** $p<0.01$. Data from Adriane Todechini *et al.* [52]

cellobiose, which has no GlcNac termini (B). (ii) Preincubation of GM3 micelles with Os Fr.B reduced the inhibitory effect of GM3 on EGFR activation (C). (iii) Immunoprecipitated EGFR from the cell lysate reacted with lectin GS-II [47] and mAb J1 [48], both of which are known to react with GlcNAc termini of N-linked glycan. Treatment of the cells with swainsonine, an inhibitor for mannosidase II, enhanced the inhibitory effect of GM3 on EGFR activation (D), along with increased expression of glycan with GlcNAc termini on the cell surface. The data shown in Fig. 7 which was constructed from various results shown in Yoon *et al.* [49].

These findings indicate the existence of a novel type of CCI, which takes place between GM3 and GlcNAc termini of N-linked glycan of EGFR within the same cells, and its functional role in EGFR activation. We termed this type of CCI as "*cis*-CCI".

Subsequent works in this area support the CCI between GM3 and N-glycans with GlcNAc on EGFR as the molecular mechanism for GM3-mediated inhibition of EGFR activation, essentially supporting our results as above. In studies by Nakayama and his colleagues [50], the sequential treatments of A431 cells with sialidase and β-galactosidase resulted in enhanced inhibition with GM3 of EGFR activation, and enhanced binding of lectin GS-II to EGFR. In addition, GM3 did not bind to EGFR from α-mannosidase IB-knocked down A431 cells, and at the same time, GM3-mediated EGFR activation was not detectable in the knockdown cells. The concept was also supported by our recent study with ldlD14 cells, UDP-Gal/UDP-GalNAc 4′-epimerase-deficient mutant of Chinese hamster ovary (CHO) cells. The cells were initially used by Davis and his colleagues to demonstrate that induced GM3 expression causes a reduction of EGFR activation [37], indicating that EGFR activation is physiologically regulated

Table 1 Cell adhesion mediated by

	PPI (protein-to protein interaction)	CPI (carbohydrate-to-protein interaction)	CCI (carbohydrate-to-carbohydrate interaction)
Examples	Integrin α3β1 to LN5 α5β1 to FN αLβ2 to ICAM-1 or −2	SLe^x-to-E-selectin SLe^a-to-E-selectin Sialoglycan to siglec	Le^x-to-Le^x GM3-to-Gg3
Rapidity	Slow [55]	Fast	Very Fast [55]
Affinity (K_a in M)	~10^{-8} to 10^{-9}	10^{-8}	~10^{-5} to 10^{-8} [18, 63–65]
Variability	Low	Low	Very High; depends on degree of clustering and orientation of epitopes [20, 24, 34]
Repulsion	Yes [66]	Not known	Yes [55, 67]
Synergism between two adhesion systems	Yes, with CCI	Not known	Yes, with PPI

by endogenous GM3, and supporting the findings of the inhibitory activity of GM3 [36]. In our study, EGFR-transfected ldlD14 cells expressed higher level of GlcNAc termini on the cell surface and exogenously-added GM3 showed inhibitory activity on EGFR activation, when the cells were cultured in the absence of Gal. The addition of excessive amounts of Gal in the culture media could block the expression of GlcNAc termini, and the inhibitory effect of GM3 on EGFR activation was much weaker in these cells [51]. Identification of the structure of the N-glycan that exists on EGFR and binds to GM3 remains to be studied, although N-glycan carrying 6 GalNAc termini was identified as a minor component expressed in EGFR from A431 cells after treatment with sialidase and β-galactosidase [50].

In addition to *cis*-CCI between ganglioside and N-linked glycans in EGFR activation, we recently found regulator activity of the complex of gangliosides on hepatocyte growth factor receptor (HGFR), cMet; using human bladder cell lines with different degree of malignancy. Hetero-dimeric complex of GM2 and GM3 was closely associated with cMet-CD82 complex, and the ganglioside complex negatively regulated HGF-induced cMet activation, cell motility and growth (Fig. 8) [52]. Other examples of ganglioside complexes have been reported; antibodies to ganglioside complexes, GM1-GD1a, GD1a-GD1b, and GT1a-GT1b, were detected in sera of patients with Guillain-Barre syndrome, suggesting connection of the complexes with acute motor nerve paralysis in this autoimmune disease [53, 54].

Discussion/perspective

Processes mediated by CCI show many key differences from those mediated by protein to protein interaction (PPI) or carbohydrate to protein interaction (CPI). Some of the major distinctions, for *trans*-CCI, are summarized in Table 1. PPI is the slowest process, since it requires conformational adaptation between two interacting proteins (*e.g.*, integrin and fibronectin), whereas in CPI carbohydrate conformation is pre-defined and ready to bind to a single protein conformation. In CCI, the reaction is most rapid since conformations of two carbohydrates are pre-defined. Under physiological dynamic flow condition, cell adhesion based on CCI or CPI is clearly observed, but adhesion based on PPI is very weak or undetectable [55].

Functional roles of PPI and CPI have been extensively investigated, and their importance in a wide range of cellular functions (cell growth, motility, adhesion, apoptosis, *etc.*), in both physiological and pathological conditions including cancer, has been well documented. In contrast, comparable studies on CCI so far are quite limited. Binding affinity of CCI varied extensively, possibly based on degree of cluster, and display high affinity when presented on gold nanoparticles. Binding affinities of PPI and CPI are consistently higher than CCI. Further extensive research in this direction will reveal more examples of CCI and its role in regulation of cell functions.

It is well documented that expression and activity of EGFR, and other GFRs in the ErbB family, are enhanced in many human cancers and that they play important roles in mediating tumor cell growth and progression. These tyrosine kinase associated growth factor receptors, particularly EGFR, are therefore considered prime targets for cancer treatment, and tyrosine kinase inhibitors, such as gefitinib and erlotinib have been developed for the treatment of cancer. However, it has been reported that the treatment with such drugs induces mutations in the kinase domain of EGFR, leading to resistance to the drugs [56–61]. Since the mechanism of the inhibition with GM3 through CCI is completely different from that of the tyrosine kinase inhibitors, it might be possible to consider GM3 or its synthetic mimetics as therapeutic agents in future. In fact, *N*-trifluoroacetyl-GM3 synthesized by Yoshino and Halfpap strongly inhibited sialidase activity and oncogenic phenotype in morphology. Cell growth and "contact inhibition" [62]; further studies along this line are under way.

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