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Self-recognition of high-mannose type glycans mediating adhesion of embryonal fibroblasts

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Abstract High-mannose type N-linked glycan with 6 mannosyl residues, termed "M6Gn2", displayed clear binding to the same M6Gn2, conjugated with ceramide mimetic (cer-m) and incorporated in liposome, or coated on polystyrene plates. However, the conjugate of M6Gn2-cer-m did not interact with complex-type N-linked glycan with various structures having multiple GlcNAc termini, conjugated with cer-m. The following observations indicate that hamster embryonic fibroblast NIL-2 K cells display homotypic autoadhesion, mediated through the self-recognition capability of high-mannose type glycans expressed on these cells: (i) NIL-2 K cells display clear binding to lectins capable of binding to high-mannose type glycans (*e.g.*, ConA), but not to other lectins capable of binding to other carbohydrates (*e.g.* GS-II). (ii) NIL-2 K cells adhere strongly to plates coated with M6Gn2-cer-m, but not to plates coated with complex-type N-linked glycans having multiple GlcNAc termini, conjugated with cer-m; (iii) degree of NIL-2 K cell adhesion to plates coated with M6Gn2-cer-m showed a clear dose-dependence on the amount of M6Gn2-cer-m; and (iv) the degree of NIL-2 K adhesion to plates coated with M6Gn2-cer-m was inhibited in a dose-dependent manner by α1,4-L-mannonolactone, the specific inhibitor in high-mannose type glycans addition. These data indicate that adhesion of NIL-2 K is mediated by self-aggregation of high mannose type glycan. Further studies are to be addressed on auto-adhesion of other types of cells based on self interaction of high mannose type glycans.

Keywords High-mannose type N-linked glycans · Hamster embryonal fibroblast NIL-2 K cells · Carbohydrate-carbohydrate interaction (CCI) · Cell adhesion · Ceramide-conjugated oligosaccharides

Abbreviations

CB	cellobiose (Glcβ1-4Glc)
cer-m	ceramide mimetic (tetradecylhexadecane)

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CCI	carbohydrate-to-carbohydrate interaction
C/M/W	a mixture of chloroform methanol and water in a defined volume ratio as indicated case by case
ConA	Concanavalin A
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GS-II	*Griffonia simplicifolia*-II lectin
HPLC	high performance liquid chromatography
Le^x	Galβ1-4[Fucα1-3]GlcNAcβ1-3Gal
M/W	methanol and water mixture
NH_2cer-m	aminoceramide mimetic
ODS column	Shim-pack HRC-octadecyl silica column
Os	oligosaccharide
PC	1,2-dimyristoyl-*sn*-3-phosphocholine
TLC	thin-layer chromatography

Introduction

Adhesion of interfacing cells mediated by carbohydrate-to-carbohydrate interaction (CCI) was originally found in adhesion/ aggregation of mouse embryonic stem cells (ES) cells or embryonal carcinoma (EC) cells, mediated by Le^x to Le^x interaction [1–4]. While species specific adhesion of sponge cells in sea water was known for ~50 years [5], the mechanism of adhesion among the same species has been unclear. This adhesion was found more recently based on the homotypic CCI mediated by oligosaccharides (Os) having a common core structure 3-*0* sulfated GlcNAcβ4Fucα linked to Thr or Ser residue of proteoglycan [6–9]. Since then, various types of cell adhesion mediated by homotypic or heterotypic CCI have been studied by many research groups, using conventional cell biological approaches, or quantitative biochemical and biophysical approaches, including electrospray mass spectrometry [10, 11], NMR spectroscopy [12, 13], atomic force microscopy [14, 15], surface plasmon resonance spectroscopy [7, 16, 17], and aggregation of glycosyl-gold nanoparticles in the presence of Ca^{2+} [9, 18, 19].

These studies were focused on cell to cell adhesion mediated by interfacing glycosphingolipids (GSLs) or glycoproteins, *i.e.*, *trans*-CCI. More recently, N-linked glycans involved in CCI process within the same cell membrane, *i.e. cis*-CCI has been explored, and complex-type N-linked glycans having ≥3 GlcNAc termini were found to interact with ganglioside GM3 of the same cells [20]. This process was shown to cause inhibition of tyrosine kinase associated with epidermal growth factor receptor (EGFR) based on the inhibitory effect of Os having 5-6 GlcNAc termini on interaction of GM3 with EGFR [21].

In contrast to the capability of N-linked glycans with multiple GlcNAc termini, a capability of high-mannose type N-linked glycans involved in CCI process has not been described previously. We report here a novel self-interaction of high-mannose type N-linked glycan, "M6Gn2", mediating the adhesion of hamster fibroblasts NIL-2 K, which express high level of high-mannose type glycans.

Materials and methods

Materials and reagents

All reagents were from Sigma Chemical Co. (St. Louis, MO), except for the following items. *N*-hexanoyl-D-erythro-sphingosine (N-C6:0-D-erythro-ceramide, $C_{24}H_{47}NO_3$, MW 398) was from Matreya (Pleasant Gap, PA). [^{3}H]thymidine (1 mCi/ 1 ml) and [^{3}H]cholesterol (0.25 mCi/0.25 ml) were from PerkinElmer (Waltham, MA). Float-A-Lyzer dialysis membrane (MWCO 500) was from Spectrum Labs (Rancho Dominguez, CA). C_{18}-cartridge was from Varian (Harbor City, CA). ConA-FITC and GS-II-FITC were from EY Laboratories (San Mateo, CA). Solvents (HPLC grade) and Rexyn I-300 resin mixture were from Fisher.

Cells and cell culture

Hamster embryo fibroblasts (NIL-2E cells) were obtained originally from Diamond,L (Wistar Inst., Philadelphia, PA) [22]. NIL-2 K cells were cloned from NIL-2E based on low saturation density (1.0-1.5x10^5/ cm^2, in contrast to 2.0-2.5x10^5/ cm^2 for NIL-2E) [23, 24]. NIL-2 K and BALB/c 3 T3 cells were cultured in Dulbecco's Modified Eagle's Medium (Irvine Scientific, Santa Ana, CA) supplemented with 5 % fetal calf serum (Hyclone, Logan, UT) with addition of 100 units/ml penicillin and 100 μg/ml streptomycin (Mediatech Inc., Herndon, VA) in 5 % CO_2 atmosphere [25].

Separation and purification of oligosaccharides from ovalbumin or fetuin

N-linked glycans from ovalbumin or fetuin were released by hydrazinolysis, *N*-acetylation, separation by cellulose column chromatography, and concanavalin A (ConA) lectin affinity chromatography (for ovalbumin) as described previously [20, 26, 27]. The ConA-non-binding components (including hybrid-type and complex-type Os with multivalent GlcNAc) were separated on ConA-Sepharose 4B

column [20]. The ConA-binding Os fraction (mainly high-mannose type) was eluted with 200 mM α-methylmannoside [26, 27]. The eluate were desalted by a mixture of anionic and cationic resins (Rexyn I-300, H-OH), and α-methylmannoside was separated from ConA-binding Os by limiting dialysis through Float-A-Lyzer dialysis membrane (MWCO 500, Spectrum Labs) for 5 days in distilled water at 4 °C

Cellulose-binding Os from fetuin, and ConA-binding Os from ovalbumin, were subjected to Sephadex G-25 chromatography in distilled water (column 2x150 cm, flow rate 0.8 ml/min, fraction size 10 ml, fraction number 40). Os-containing fractions, monitored by mini-spot test on TLC plate with orcinol spray, were lyophilized, dissolved in distilled water, filtered by Ultrafree-4 (Millipore, Bedford, MA), and subjected to HPLC (see below). TLC was developed in a mixture of 1-propanol/ n-butylacetate/ Solvent B, 8:1:10. Solvent B was a mixture of acetonitrile and 0.2 M acetic acid/ triethylamine, pH 7.2, in a ratio of 3: 7 [20].

Purification of high-mannose type and complex-type oligosaccharides by high-performance liquid chromatography (HPLC)

Os fractions from Sephadex G-25 column chromatography were further separated into various Os components by HPLC through normal-phase Microsorb 100 NH_2 column (Varian, Lake Forest, CA; 0.46 x 25 cm) using a Varian ProStar chromatography system, at room temp.

For purification of high-mannose type Os, mobile phase consisted of Sol A (70 %, v/v, 0.2 M acetic acid/ triethylamine, pH 7.2: acetonitrile=3:7) and Sol B (30 %, v/v, 0.2 M acetic acid/ triethylamine, pH 7.2: acetonitrile=7:3) to start, followed by a linear gradient from 70 % to 40 % over 60 min. During the next 10 min, the percentage of Sol B was maintained at 60 %.

For purification of complex-type Os from fetuin, mobile phase consisted of Sol A and Sol B to start, followed by a linear gradient from 90 % to 78 % over 60 min. During the next 10 min, the percentage of Sol B was maintained at 78 %. The flow rate was 1 ml/min, fraction size was 1 ml, and the effluent was monitored using a Waters 484 tuneable absorbance UV detector at λ=215 nm. The major Os components "M6Gn2" from ConA-binding fraction of ovalbumin and "Os Fet 2" from fetuin were thus separated by HPLC, and subjected to structural analysis (see below).

Characterization of separated Os structures

The Os separated by HPLC were reductively aminated with 2-aminopyridine and sodium cyanoborohydride [27, 28], and their structures were analyzed by the HPLC mapping method, based on elution patterns from two HPLC columns: a Shim-pack HRC-octadecyl silica (ODS) column (Shimazu, Kyoto, Japan) and a TSK-gel Amide-80 column (Tosoh, Tokyo, Japan) as described previously [27, 29, 30]. The PA-Os were identified by comparison with HPLC data of approximately 500 reference PA-Os using a web-based application, GALAXY (http://www.glycoanalysis.info/galaxy2/ENG) [31].

Conjugation of NH_2cer-m to Os, and purification of Os-cer-m conjugates

NH_2cer-m ($C_{30}H_{67}N$, Mw 437) was synthesized, and M6Gn2 and Os Fet 2 were conjugated to NH_2cer-m, as described previously for Os Fr.B [32]. Briefly, 1 mg aliquot of purified Os in vial (Reacti-vial™ Small Reaction Vials, Pierce, Rockford, IL) was completely dried, the residue was dissolved in 60 μl distilled water, 427 μl methanol, and 113 μl (1.3 μmol) NH_2cer-m (5 mg/ml, v/v, in methanol, pH 6.2) was added. The vial was heated at 100 °C for 30 min, cooled, added with 5 μl (12 μmol) sodium cyanoborohydride (150.8 mg/ml, v/v, in distilled water), and incubated at 90 °C for 16 h. The reaction mixture was dried, dissolved in 1.5 ml methanol/ water (M/W) 6:4, and applied to a small C_{18}-cartridge equilibrated with M/W 6:4. The column was washed with M/W 6:4 to eliminate C_{18}-cartridge-non-binding materials. The adsorbed M6Gn2-cer-m conjugate and non-reacted NH_2cer-m were eluted from C_{18} cartridge with isopropanol/ hexane/ water 55:40:5 ("Fraction a") followed by chloroform/ methanol/ water (C/M/W) 2:1:0.1 ("Fraction b"), and dried. Fractions a and b were dissolved in isopropanol/ hexane/ water 55:40:5, and C/M/W 25:25:8, respectively, and centrifuged at 13,000 rpm for 5 min. The pellet from "Fraction a" was dissolved in C/M/W 2:1:0.1, followed by centrifugation. The supernatant was monitored by high performance TLC analysis, developed with C/M/W 50:55:18. Spots were revealed by primulin spray (0.001 % primulin in acetone-water 4:1), and by orcinol/ sulfuric acid reactivity (0.2 % orcinol/ H_2SO_4:H_2O,1:9) [33]. The dried residue was dissolved in a defined volume of C/M/W 25:25:8. The concentration of Os-cer-m conjugate was measured based on primulin and orcinol/ sulfuric acid as above by TLC with Scion image analysis, and aliquots of suitable quantity were used for binding assays [32].

Characterization of structures of N-linked Os-cer-m conjugates with aminoceramide by ESI mass spectrometry

Structures of Os-cer-m conjugates were confirmed by mass spectrometry using an ion trap mass spectrometer Esquire LC (Bruker Daltonics, Billerica, MA) with electrospray ionization source. To confirm the presence of structures of Os after NH_2cer-m-coupling reaction, 24 nmol of Os-cer-m conjugate dissolved in C/M/W 25:25:8 was diluted and

dissolved in 50 % aqueous methanol/ 1 % formic acid/ 5 mM ammonium acetate. This solution was directly infused into the ion source at a flow rate of 1 μl/ min. Spectra were collected in both positive and negative ionization mode. The fragmentation spectra of the analytes were collected with an isolation width of 4 amu, fragmentation amplitude set to 1 volt and SmartFrag On (amplitude automatically varied to 30-200 % of the set fragmentation amplitude of 1 V).

Preparation of PC/ cholesterol liposomes incorporating Os-cer-m, and their binding to Os-cer-m coated on polystyrene plate

Various quantities (0.25-2.0 nmol per well, calculated based on equimolar quantities) of Os-cer-m conjugates in 50 % aqueous ethanol were added to each well of 96-well flat-bottom polystyrene plates (Costar # 9017, Corning Inc., Acton, MA). The coated plates were dried for 5 h at 37 °C, 100 μl 1 % BSA was added to each well and incubated 1 h to block non-specific binding, and wells were washed with TBS solution. ^{3}H-labeled liposomes containing Os-cer-m conjugate were prepared, and binding assay was performed as described previously [20, 32].

Flow cytometric analysis for glycan profile of NIL-2 K cell surface

NIL-2 K and BALB/c 3 T3 cells in monolayer (80 % confluency) were detached with 1 mM EDTA/ PBS, and changes of cell surface glycan profile were analyzed by staining with ConA-FITC, and GS-II-FITC, as described previously [21], followed by flow cytometry (Beckman Coulter, Miami, FL).

Preparation of different adhesion surfaces for NIL-2 K and BALB/c 3 T3 cell binding

Adhesion surfaces were prepared by adsorbing various glycoproteins, lectins, ceramides, or glycolipids on microtiter well surfaces. For cell binding to glycoprotein and lectin, 50 μl of 10 μg/ml ovalbumin, fetuin, and ConA in Salt/Pi buffer (137 mM NaCl/ 2.7 mM KCl/ 0.7 mM $CaCl_2$/ 0.5 mM $MgCl_2$/ 8.1 mM Na_2HPO_4/ 1.5 mM KH_2PO_4, pH 7.4) was added to each well of high-binding 96-well flat-bottom polystyrene plates (Pro-Bind™, Becton Dickinson Labware, Franklin Lakes, NJ) and kept for 3 h at room temp. The wells were washed 3x with 100 μl of Salt/Pi buffer, and used for cell adhesion activity. For cell binding to Os-cer-m conjugates, various quantities (0.25-2.0 nmol/well, based on equimolar quantities) of Os-cer-m conjugates in 50 % aqueous ethanol were added to each well of medium binding 96-well flat-bottom polystyrene plate (Coster #9017, Corning Inc.). The coated plates were dried for 5 h at 37 °C, 200 μl 1 % fetuin in Salt/Pi buffer was added to each well to block nonspecific binding (because fetuin did not show binding to NIL-2 K or BALB/c 3 T3 cells), and incubated 2 h at room temp, and wells were washed 3x with 200 μl Salt/Pi buffer. The plates coated with different adhesion surfaces were used for cell adhesion assays as described below.

Cell adhesion assays

Adhesion assay of NIL-2 K or BALB/c 3 T3 cells was performed as described previously, with slight modification [25, 34]. Briefly, freshly confluent cell monolayers were detached with 1 mM EDTA, mixed with complete culture medium, and transferred to tube. The cells were centrifuged at 1,000 rpm for 5 min and washed 3x with Salt/Pi buffer. Adhesion reactions were started by adding $1.5x10^5$ cells in 50 μl of Salt/Pi buffer to 50 μl of the same buffer in multi-wells coated with various glycoproteins, lectins, ceramides, or glycolipids on multi-well plates as described above. Three wells were prepared for each data point shown. The cell suspensions in Salt/Pi buffer were incubated for 1 h at 37 °C in an atmosphere of 5 % CO_2, and the plate was placed upside-down in PBS containing 1 mM $CaCl_2$/ 0.5 mM $MgSO_4$/ 0.1 mM $MnCl_2$ for 20 min at room temp. After removing all buffer solution in wells, degree of cell attachment for each well was estimated by microscopy.

For determination of cells attached, cells were labeled before assay with 1 μCi/ ml [^{3}H]thymidine in 15 ml of complete culture medium for 20 h. Radioactivity from the attached cells was solubilized for counting by rinsing the multi-well twice with 100 μl of 1 % SDS/ 0.5 N NaOH.

For inhibition studies with sugar in cell adhesion to Os-cer-m conjugates, cells prepared for adhesion reaction as above were suspended in 10 mM α-L-mannose-1,4-lactone/ Salt/Pi buffer, incubated for 15 min at room temp to allow the inhibitors to bind to cells before starting the assay, and added to multi-well containing 50 μl of same concentration of the inhibitor. All procedures after incubation with inhibitor were as described above.

Results

Three types of N-linked oligosaccharide characterized and used for studies of CCI

High-mannose type M6Gn2 from ConA-binding fraction from ovalbumin , complex-type Os with GlcNAc termini Os Fr.B from ConA-non-binding fraction of ovalbumin, and bi-or tri-antennary complex type Os from fetuin were separated as described in M&M. The structure of these Os was

determined by HPLC mapping method, and HPLC elution profiles of Os as 2-amino-pyridylaminated derivatives through ODS column are shown in Fig. 1a. M6Gn2 showed one peak in HPLC (Fig. 1a, peak a), which was identified as high-mannose type with 6 Man residues (Fig. 1b, peak a). Os Fr.B gave two major peaks (Fig. 1a, peaks b, c) as described [20]. Peaks b and c were identified as complex-type N-linked glycans with 6 GlcNAc termini (Fig. 1b, peak b), and one of the 6 GlcNAc termini substituted by Galβ1-4 residue (Fig. 1b, peak c). Os Fet 2 from fetuin showed three major peaks (Fig. 1a, peaks d, e, f) which were identified as complex-type N-linked structures having bi- or tri-antennary with NeuAc termini (Fig. 1b, peaks d, e, f). The elution pattern in Fig. 1a is based on the direct determination of elution time, but not relative time to the elution of glucose (G.U.). TLC patterns of these Os are shown in Fig. 1c.

Conjugation of Os with aminoceramide mimetics (NH_2 cer-m), and structural characterization of Os-cer-m conjugate by mass spectrometry

NH_2cer-m ($C_{30}H_{67}N$, Mw 437) [32] was reacted with M6Gn2, Os Fr.B, and Os Fet 2 for conjugation by reductive amination. Purity of each purified conjugate was confirmed by HPTLC. Spots were detected by orcinol as well as by primulin.

The structure of M6Gn2-cer-m conjugate was determined by ESI-MS with MS-MS. One major signal with *m/z* 1802.1 and minor signal with *m/z* 1639.1 were identified (Fig. 2a). The *m/z* 1802.1 and *m/z* 1639.1 were identified as $[M+H]^+$ of M6Gn2-cer-m (6 Man, 2 GlcNAc, Cer) and one loss of hexose from M6Gn2-cer-m (5 Man, 2 GlcNAc, Cer), respectively. The MS/MS spectrum of M6Gn2-cer-m at *m/z* 1802.1 is shown in Fig. 2b. The MS/MS produced major

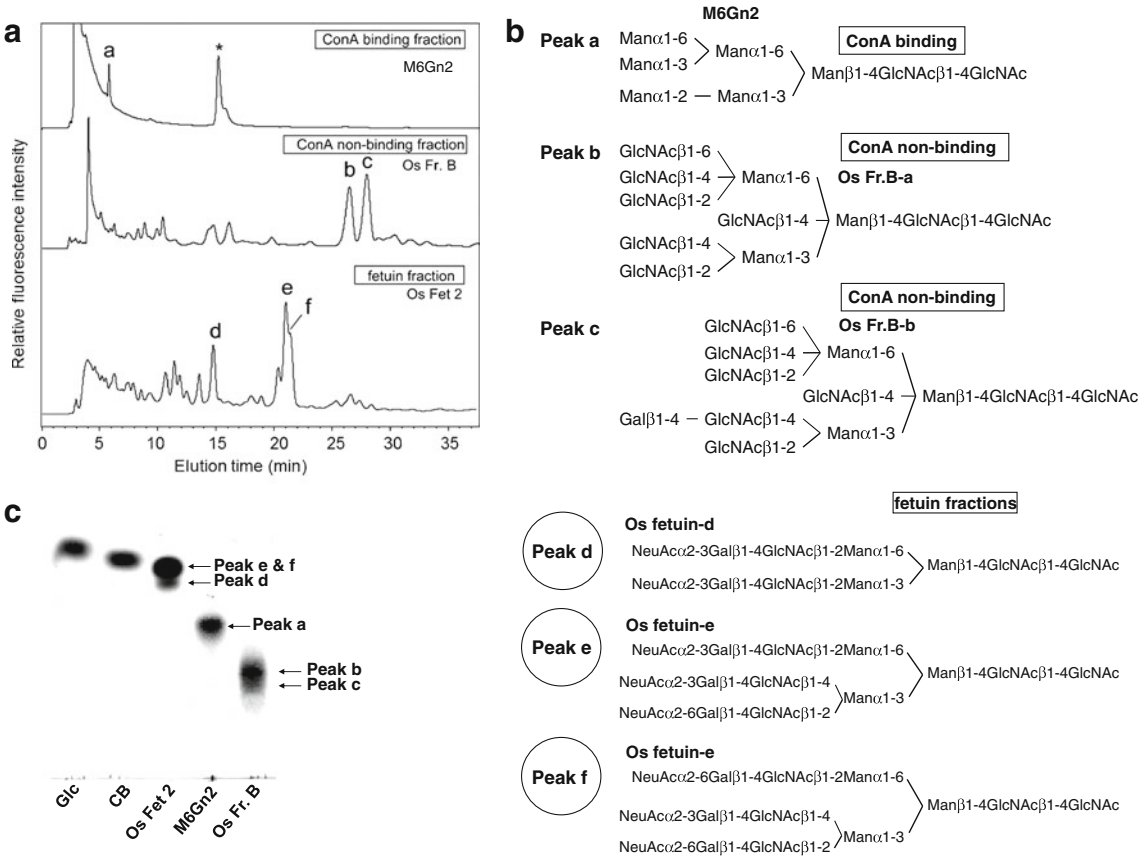

Fig. 1 TLC, HPLC patterns, and structure of N-linked glycans from ovalbumin and fetuin. **a** HPLC patterns of M6Gn2, Os Fr.B, Os Fet 2. Each Os peak was recorded as pyridylaminated (PA) derivative and separated on ODS column. Detailed conditions of HPLC are as described previously [20]. The elution of M6Gn2, Os Fr.B. and Os Fet 2 are expressed in relative elution time in minutes after loading, and not in relative elution time to glucose, *i.e.* "glucose units". The peak marked by asterisk (*), indicates a fraction containing no detectable PA-glycans. The elution pattern of OsFr.B was originally described in "Fr.B"of panel b, of Fig. 1[20], which was expressed as "glucose unit". **b** Structures of peaks corresponding to Os from M6Gn2, Fr.B, and Fet 2, shown in Panel A. Structural assignment was based on HPLC mapping method ("GALAXY"; see M&M), and some were confirmed by ESI-MS analysis, as described previously [20]. **c** TLC patterns of HPLC-separated complex type Os from fetuin, and ConA-binding high-mannose type Os and ConA-non-binding complex type with multivalent GlcNAc termini from ovalbumin. Each Os was separated and revealed by primulin and orcinol H_2SO_4, as described in M&M. R_f: Glc (0.81), CB (0.76), Os Fet 2 (0.74 and 0.68), M6Gn2 (0.52), Os Fr.B (0.38 and 0.33). Possible Os corresponding to each peak in Panel A is indicated for TLC spots

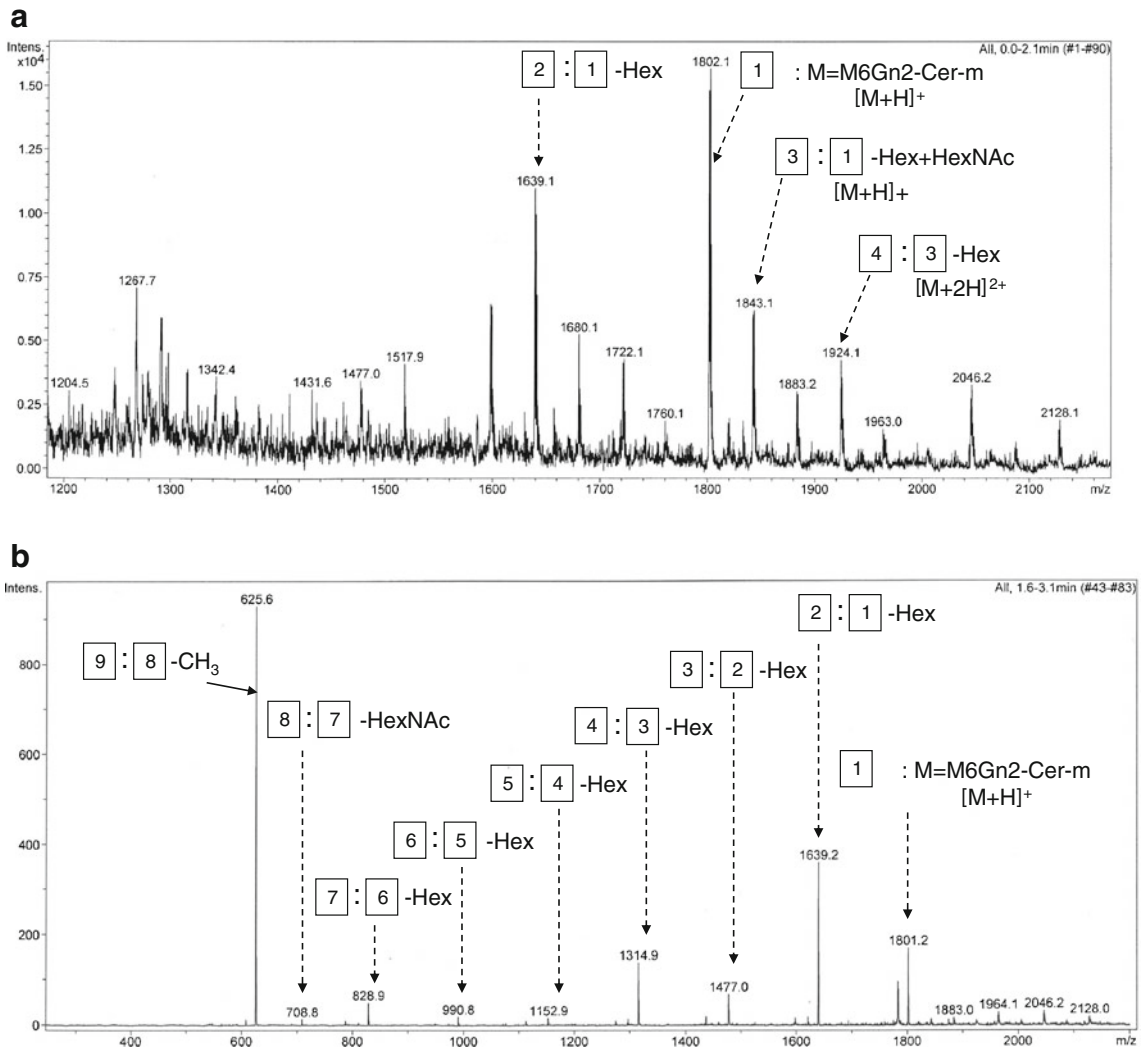

Fig. 2 Positive-ion ESI-MS spectra of aminoceramide conjugates of M6Gn2-cer-m, and MS-MS data for major mass. **a** Aminoceramide conjugates of M6Gn2-cer-m. Note the presence of two major ions, with mass number 1802.1 and 1639.1, corresponding to single charged $[M+H]^+$ major ion with (6 Man, 2 GlcNAc, Cer) and (5 Man, 2 GlcNAc, Cer). **b** MS/MS spectrum of *m/z* 1802.1 corresponding to M6Gn2-cer-m. Product ion of *m/z* 1639.2, *m/z* 1477.0, *m/z* 1314.9, *m/z* 1152.9, *m/z* 990.8, *m/z* 828.9 are based on loss of one Hex with step-by-step from m/z 1802.1, and *m/z* 1314 is based on loss of one HexNAc from *m/z* 828.9

signal with *m/z* 1639.2 assigned to loss of one hexose (5 Man, 2 GlcNAc, Cer), and six minor signals [*m/z* 1477.0 (4 Man, 2 GlcNAc, Cer), *m/z* 1314.9 (3 Man, 2 GlcNAc, Cer), *m/z* 1152.9 (2 Man, 2 GlcNAc, Cer), *m/z* 990.8 (1 Man, 2 GlcNAc, Cer), *m/z* 828.9 (2 GlcNAc, Cer), *and m/z* 708.8 (1 GlcNAc, Cer)] assigned to further loss of hexose. The large peak with *m/z* 625.6 was identified as GlcNAc-Cer minus terminal CH_3.

Self-interaction of M6Gn2 and that of Os Fr.B, as revealed by interaction of cer-m conjugates

Various equimolar quantities (0.25 through 2.0 nmol) of Os-cer-m conjugates (*i.e.*, M6Gn2-cer-m, Os Fr.B-cer-m, and Os Fet 2-cer-m) were placed on each well of polystyrene multi-well plates, to which binding of PC/ [^{3}H]cholesterol liposome incorporating M6Gn2-cer-m was tested as described in M&M. Results are shown in Fig. 3a. Binding of [^{3}H]-liposomes containing M6Gn2-cer-m to plates coated with M6Gn2-cer-m was much higher than to plates coated with Os Fr.B-cer-m or Os Fet 2-cer-m, indicating the occurrence of CCI between M6Gn2, but not CCI of M6Gn2 with Os Fr.B nor with Os Fet 2. M6Gn2-cer-m-liposomes did not show binding to non-coated plates.

Binding of [^{3}H]-labeled liposomes containing M6Gn2-cer-m, or Os Fr. B-cer-m and/or Os Fet 2-cer to the polystyrene plates, coated with respective Os-cer-m were studied. Clear self-interaction was observed between M6Gn2, and between Os Fr.B, but not between Os Fet 2. None of the Os-cer-m liposomes showed binding to non-coated plates (Fig. 3b).

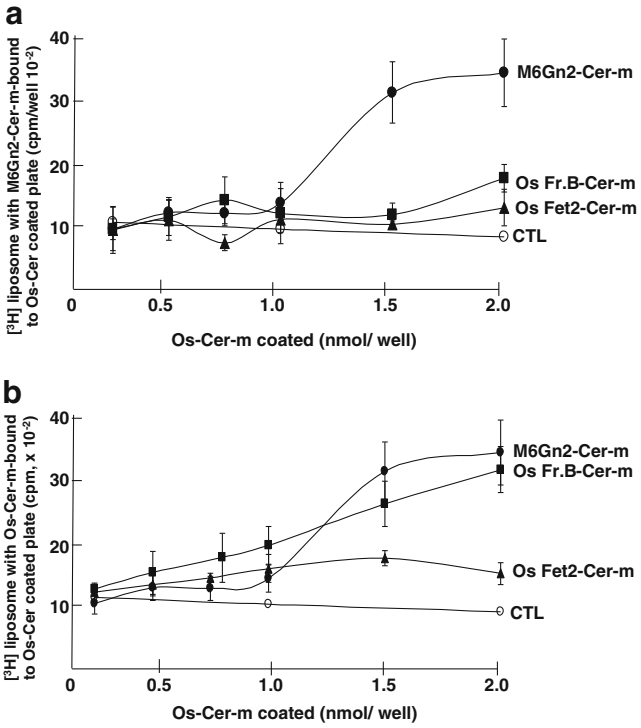

Fig. 3 Binding of [^{3}H]-labeled liposome containing Os-cer-m to various Os-cer-m derivatives coated and affixed on multi-well polystyrene plates. **a** Binding of [^{3}H]-labeled liposome containing M6Gn2-cer-m to various Os-cer-m derivatives coated and affixed on multi-well polystyrene plates. Various quantities (0.25-2.0 nmol/ well) of M6Gn2-cer-m, Os Fet 2-cer-m, and Os Fr.B-cer-m derivatives, as shown on abscissa, were dried at 37 °C, blocked with 1 % BSA for 1 h, and washed. Each well was added with 100 μl of ^{3}H-labeled liposome containing M6Gn2-cer-m, incubated for 16 h, and degree of binding was determined, as described in M&M. Clear M6Gn2-cer-m liposome binding was observed for M6Gn2-cer-m, but not for others. Data shown are typical results from a single triplicate experiment. Similar results were obtained in two other triplicate experiments. Bars indicate standard deviation (S.D.). CTL: 50 % ethanol was added to multi-well polystyrene plates for liposome binding assay. **b** Self-interaction of various Os-cer-m conjugates. [^{3}H]-labeled liposomes containing M6Gn2-cer-m, Os Fet 2-cer-m, or Os Fr.B-cer-m were prepared, added to multi-wells on polystyrene plate coated with the same Os-cer-m derivative at various concentrations, and used for self-binding assay as described in Panel A

Cell adhesion to various adhesion surfaces, and inhibition of high-mannose type Os-mediated cell adhesion

Our previous studies suggest that glycosylhydrolases are present at the cell surface, and may mediate cell adhesion [35]. Hamster embryonic fibroblast NIL-2 K expresses high α-mannosidase activity, which may mediate adhesion of these cells to ovalbumin-coated plates, since such adhesion was inhibited by methyl-α-mannoside or α1,4-L-mannonolactone [35]. These findings may also be interpreted to mean that NIL cells express high level of high-mannose type N-linked structure, which may mediate NIL cell adhesion to ovalbumin-coated plates.

In order to test this possibility, we first determined the expression of high-mannose type glycan *vs.* complex-type glycan with GlcNAc termini, by flow cytometry of NIL-2 K cells with ConA vs lectin GS-II. ConA binding to NIL-2 K was much higher than that of GS-II. BALB/c 3 T3 did not show binding to either ConA or GS-II (Fig. 4a). These results suggest that high-mannose type glycans are expressed strongly in NIL-2 K cells.

Adhesion of NIL-2 K and BALB/c 3 T3 cells to plates coated with various materials considered to affect adhesion was tested. Binding of BALB/c 3 T3 cells to plates coated with ovalbumin, fetuin, M6Gn2-cer-m, or Os Fr. B-cer-m was undetectable, or very low (Fig. 4b). NIL-2 K cells bound strongly to plates coated with 0.5 μg/ well ConA, which specifically binds to high-mannose type glycan, bound weakly to the same amount of ovalbumin, and did not bind to fetuin (Fig. 4c, upper row). NIL-2 K cells adhered strongly to plates coated with 1.5 nmol/ well M6Gn2-cer-m, very weakly to the same amount of Os Fr.B-cer-m, and did not bind to the same amount of cer-m without Os (Fig. 4c, lower row). NIL-2 K cell adhesion to plates coated with M6Gn2-cer-m was dose-dependent, *i.e.*, highest at 2 nmol/ well, and progressively decreased at lower levels (Fig. 4d, upper row). The adhesion was greatly reduced when 10 mM α1,4-L-mannonolactone was added (Fig. 4d, lower row).

Since NIL-2 K cell adhesion to plates coated with M6Gn2-cer-m was dose-dependent, quantitative adhesion of NIL-2 K cells was determined using 0.5 - 2.0 nmol/ well M6Gn2-cer-m, in comparison to adhesion of BALB/c 3 T3 cells (Fig. 5a). The inhibitory effect of α1,4-L-mannonolactone on NIL-2 K cell adhesion to M6Gn2-cer-m-coated plates, and its statistical significance, are shown in Fig. 5b. NIL-2 K cell adhesion was clearly inhibited by treatment of 30 mM concentration of α1,4-L-mannonolactone.

Discussion

Cell to cell contact induces inhibition of cell growth through UDP-Gal dependent changes of Gal metabolism and proposed functional basis of “Cell Society” that is considered to be of primary importance to maintain multi-cellular organisms [36]. Subsequently, cell adhesion and adhesion-dependent signaling with phenotypic changes were studied extensively. Based on the types of molecules involved, cell adhesion mediated by three types of molecular interaction has been considered: protein-to-protein

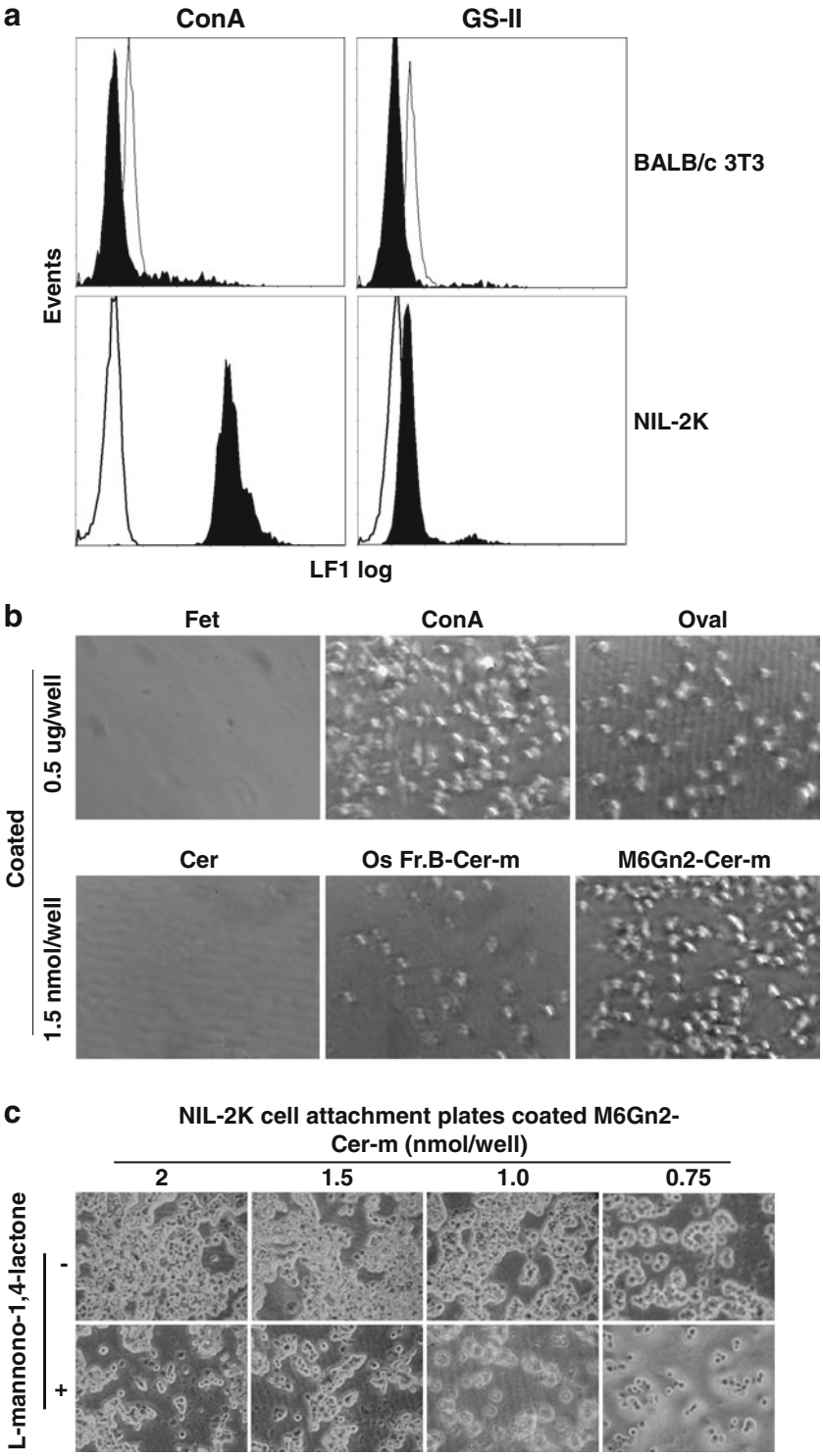

Fig. 4 Photomicrographs showing attachment of BALB/c 3 T3 and NIL-2 K fibroblasts to various adhesion surfaces. **a** Flow cytometric analysis of BALB/c 3 T3 and NIL-2 K cells was performed as described in M&M, using two types of probes: ConA-FITC (left), and GS-II-FITC (right). White pattern: cells without lectin staining. Black pattern: cells with lectin staining. **b** Attachment of BALB/c 3 T3 cells to fetuin, ConA, ovalbumin, aminoceramide, Os Fr.B-cer-m, and M6Gn2-cer-m. NIL-2 K cells were washed with Salt/Pi buffer, added to wells coated with various materials, and incubated as described in M&M. Cells bound to wells were monitored by microscopy. Cer, N-C6:0-D-erythro-ceramide. Fet, fetuin from fetal calf serum. ConA, Concanavalin A. Oval, ovalbumin grade VII. Os Fr.B-cer-m, ceramide conjugate of complex type Os with multivalent GlcNAc termini from ovalbumin. M6Gn2-cer-m, ceramide conjugate of high-mannose type Os from ovalbumin. **c** Inhibitory effect of α1,4-L-mannonolactone on attachment of NIL-2 K cells to M6Gn2-cer-m. NIL-2 K cell binding to M6Gn2-cer-m was tested in the presence of α1,4-L-mannonolactone, as described in M&M. Cells were pre-incubated with α1,4-L-mannonolactone (10 mM) for 15 min at room temp, and then added to wells coated with M6Gn2-cer-m

interaction (PPI), carbohydrate-to-protein interaction (CPI), and carbohydrate-to-carbohydrate interaction (CCI). Cell adhesion mediated by PPI is well studied in the following 2 types of processes:

(1) Cell surface proteins mediating self-adhesion and adhesion mediated differentiation, studies by F. Jacob and his colleagues in the late 1970 [37–39]. However, an essential functional component, Ca^{2+} was overlooked in these early studies. The molecular mechanism through the Ca-dependent adhesion com-ponent "cadherin" was well established by M. Takeichi in early 1980th [40, 41].

(2) Another type of PPI was found associated with cell adhesion based on extracellular matrix (ECM) protein

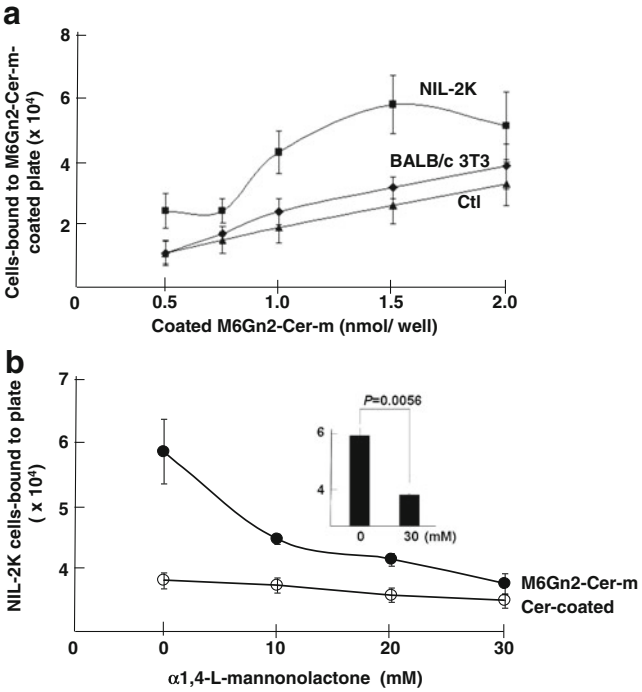

Fig. 5 Adhesion of [^{3}H]thymidine-labelled NIL-2 K cells to M6Gn2-cer-m-coated surfaces. **a** Binding of [^{3}H]thymidine-labeled BALB/c 3 T3 or NIL-2 K cells to multi-well polystyrene plates coated with various quantities (0.50 -2.0 nmol/well) of M6Gn2-cer-m. BALB/c 3 T3 and NIL-2 K cells (1.5x10^5) labeled with 1 μCi/ml [^{3}H]thymidine in 15 ml of complete culture medium for 20 h were suspended in 50 μl of Salt/Pi buffer, added to multi-well polystyrene plates coated with M6Gn2-cer-m or ceramide, and incubated for 1 h at 37 °C. Non-binding cells were removed, and radioactivity from attached cells was counted as described in M&M. NIL-2 K: binding of NIL-2 K cells to M6Gn2-cer-m. BALB/c 3 T3: binding of BALB/c 3 T3 cells to M6Gn2-cer-m. Ctl: binding of NIL-2 K or BALB/c 3 T3 cells to ceramide. Values shown are mean±S.D. from a single triplicate experiment. Similar results were obtained in two other triplicate experiments. **b** Inhibitory effect of α1,4-L-mannonolactone on NIL-2 K cell binding to M6Gn2-cer-m. [^{3}H]thymidine-labeled NIL-2 K cells were added to Cer or M6Gn2-cer-m (1.5 nmol/well) coated multi-well polystylene plate containing 50 μl of various concentrations of α1,4-L-mannonolactone in Salt/Pi buffer. Binding of [^{3}H]thymidine-labeled NIL-2 K cells to Cer or M6Gn2-cer-m was determined as described in Panel A. Cer, ceramide-coated plate. M6Gn2-cer-m: M6Gn2-cer-m-coated plate. Values shown are mean±S.D. from a single triplicate experiment. Similar results were obtained in two other triplicate experiments. The significance of each difference is indicated as *P* value in the inset

and their cell surface protein receptors which are expressed at membrane microdomains. Major ECMs are collagens [42, 43], fibronectins [44–48], and laminin [49, 50], with at least 15 types of integrin receptors [51, 52].

However, many types of proteins involved in cellular adhesion / interactions are known to be glycosylated, which may stabilizes the preferred conformational protein structure, considered to cause effective signaling for changes in cellular phenotype. Glycosyl epitope involved in CPI, or even involved in CCI could be affected by glycosylation at different glycosyl domains, or other glycosyl conjugates associated within the same membrane microdomain [53–57]. For this kind of possibility, many studies on dystrophine and glycoprotein complex, or dystroglycan were addressed on pathogenesis of human diseases [58]. Particularly, involvement of α-dystroglycan bound to laminin is required to inhibit β3GlcNAcT lead to inhibiting tumor growth [59]. Further studies indicate that Fer kinase pathways negatively control α-dystroglycan and promotes tumor growth [60].

Expression of specific gangliosides was clearly reduced by oncogenic transformation of cells [61–64], and oncogenic phenotypes were reverted to normal cells by supplementation of the reduced ganglioside by transfection of specific glycosyltransferase genes [65, 66]. In addition, many types of carbohydrate-binding proteins (lectins), particularly galectins, were observed later in various types of cells [67]. Pathobiological significance in expression of lectins was greatly promoted by discovery of "selectin" that bind to sialyl-Le^x, or sialyl-Le^a [68–71]; or "siglecs", the several types of sialic acid binding lectins, such as sialoadhesin, CD22, CD33, etc [72]. More recent studies on selection of mimetics of short peptides based on phage-display technology focused on specific conformation of anti-carbohydrate antibodies have progressed. This approach provides a useful tool for drug discovery [73, 74].

On the other hand, gangliosides in neuronal cells/tissues were found as differentiation inducers, neuro-protective modulators, and anti-ganglioside antibodies were shown to cause various neuropathies, and neuronal diseases. These processes have been extensively described and discussed [75]. Recent studies have focused on the functional role of gangliosides in the development of Alzheimer's disease [76], and interaction of GM1 ganglioside with amyloid β, the essential component implicated for the development of Alzheimer's disease was analyzed using lyso-GM1 as the model by extensive NMR data [77].

While "SSEA-1" was shown to be expressed at the morula stage of mouse embryos [78], the epitope structure required for cell adhesion was characterized ~6 years later, to be Le^x, and the adhesion process "compaction" was found to be based on Le^x-Le^x interaction [1–3], proposing the concept of CCI,mediating cell adhesion during developmental process. However, Le^x glycans and E-cadherin are co-expressed at embryonal stem (ES) cells and embryonal carcinoma (EC) cells. While E-cadherin is well studied to mediate homotypic adhesion of ES- and EC-cells, functional role of Le^x epitope in Le^x glycan through CCI process has been less clear, and both processes are dependent on Ca^{2+}. To provide stronger evidence for Le^x mediated processes, the Ecad gene in EC and ES cells was knocked out through homologous recombination with proper vectors [79] and establish F9 Ecad (-/-) cells and D3M Ecad (-/-) cells . These

cells do not express Ecad at all, but do express Le^x highly, and displayed strong autoaggregation in the presence of Ca^{2+}, and clearly showed adhesion on polystyrene plates, coated with Le^x-GSL [4]. Thus, clearly established the co-occurrence of CCI mediated by Le^x, and PPI mediated by Ecad.

Another type of cell adhesion mediated by homotypic CCI was found between multivalent GlcNAc termini affixed on cer-m [32], the process is assumed to assist the *cis*-CCI between GM3 to multivalent GlcNAc termini of N-linked glycan of EGFR [21].

Cell adhesion mediated by CCI can be distinguished from CPI or PPI in the following properties: (i) Rapidity of cell adhesion based on CCI or CPI is much faster than that based on PPI . Under dynamic flow conditions, CCI and CPI are clearly observed, but PPI is not. Under static condition, PPI-based adhesion is predominant [80]. (iii) Binding affinity of CCI varies widely ($\sim 10^{-4}$ - 10^{-7}M), and is lower than that of PPI ($\sim 10^{-9}$M) or CPI ($\sim 10^{-8}$M) [7, 16, 81, 82].

Results of this current study indicate a novel type of CCI based on self-recognition of high mannose-type glycans having 6 mannosyl residues, termed "M6Gn2". This is an another example of homotypic CCI, as observed previously between Le^x-to-Le^x glycans. However, the study is limited to NIL-2 K cells expressing high level of high mannose type glycans. Further studies on other types of cells expressing high mannose are to be studied, but suitable cell lines are difficult to find so far.

Finally, we envision that these processes, regardless of PPI, PCI and CCI, may take place at the specific site of the cell surface membrane where the key molecules are organized with specific receptors and signal transducers, to induce adhesion-dependent signal transduction and their inhibitory process, through specific membrane organization, variously termed as raft [53, 54], glycolipid enriched domain [55, 56], glycosynaptic microdomain [57]. The status of such organization is of primary importance when considering the changes in normal *vs.* diseased cells.

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