Carbohydrate-carbohydrate interaction as an initial step in cell recognition

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Abstract: Specific interaction between specific carbohydrate moieties of glycosphingolipids (GSLs) has been demonstrated based on interaction of GSL-containing liposomes with GSL-coated solid phase, and affinity adsorption of multivalent GSL oligosaccharide on solid-phase GSL column. Evidence is presented that such GSL-GSL interaction is an initial step in the cell recognition process. Examples are LeX-LeX interaction at the morula stage of mouse embryogenesis, Gg3-GM3 interaction in melanoma/lymphoma cell interaction, and galactosylceramide-sulfatide interaction in myelin sheath membrane formation. The molecular basis of such interactions is discussed.

INTRODUCTION

An orderly interaction of cell-cell or cell-substratum is the basic and essential process in formation of tissues and organs in multicellular systems, and defects in this process could provide a common basis for malformation or oncogenic transformation. A possible role of cell surface glycosphingolipids (GSLs) as cell-cell or cell-substrate recognition sites in determining the specificity of interactions has been suggested. This assumption, however, was based on the occurrence of dramatic changes in quantity and quality of GSLs during ontogenesis and oncogenesis (refs. 1,2) on one hand, and inhibition of cell recognition in morphogenesis by specific GSLs or their oligosaccharide sequences on the other (refs. 3-7). During the past decade, a number of specific adhesion proteins (fibronectin, laminin, thrombospondin, von Wildebrand factor, cadherin, N-CAM, etc.) have been discovered, and the cell surface receptors which recognize these proteins have been characterized (ref. 8). A large variety of specific cell recognition events can be achieved via various adhesive proteins and their receptors, as exemplified by the integrin superfamily receptor (refs. 9-11). In contrast to the rapid and dramatic progress in studies of protein cell recognition through protein-protein interaction as above, studies on the role of carbohydrates (CHOs) in cell recognition have been hampered because the nature of CHO recognition molecules at the cell surface has remained ill-defined (see Discussion). My purpose in this lecture is to summarize some preliminary findings indicating the existence of a novel cell recognition system based on CHO-CHO interactions.

RECOGNITION OF LeX BY LeX AT EARLY STAGES OF EMBRYOGENESIS AND F9 TERATOCARCINOMA CELL AGGREGATION

A remarkable functional role of CHO sugars during embryogenesis has been clearly indicated by dramatic changes of carbohydrate antigens, as defined by specific monoclonal antibodies (MAbs), at various stages of ontogenesis in pre- and post-implantation mouse embryos (see ref. 2 for review). Changes of expression of various CHO antigens during early stages of embryogenesis are illustrated in Fig. 1. Galectosyl globoside (SSEA-3; Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer) and sialosyl galactosyl globoside (SSEA-4; NeuAcα2→3Galβ1→4GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer) were maximally expressed at the 2- to 4-cell stage, and declined rapidly at the morula stage (ref. 12). In contrast, LeX (SSEA-1; Galβ1→4(Fucα1→2)[GalNAcβ1→R]) was not expressed until the 8-cell stage, showed maximal expression at the 16- to 32-cell stage, and declined rapidly after compaction (refs. 13,14). As LeX declined, LeX (Fucα1→2Galβ1→4(Fucα1→3)[GalNAcβ1→3Gal]) became expressed after compaction, and showed high expression on the surface of the blastocyst (ref. 15). This pattern may indicate a role of LeX expression at the 16- to 32-cell stage in preparation for compaction, the first Ca2+-dependent cell adhesion event.
Fig. 1. Reciprocal change in cell surface expression of three major carbohydrate antigens during development of preimplantation embryo. Panel A: Glycosylation changes during development of preimplantation mouse embryo. SSEA-3 and -4 (dashed line) (which are essentially glycolipid antigens with globo-core structure) disappear at the 8- to 16-cell stage, while SSEA-1 (thick solid line) and Le\textsuperscript{y} antigen (thin solid line) (which are essentially lactosaminoglycan) appear at this stage. Le\textsuperscript{y} disappears from the outer cell compartment following compaction, as the cells differentiate into trophectoderm.

Panel B: Le\textsuperscript{x} and Le\textsuperscript{y} expression in mouse morula and blastocyst as determined by immunofluorescence. 8- to 16-cell stage morula (a, b, e, f) was strongly stained by anti-Le\textsuperscript{x} (anti-SSEA-1) MAbs, but not stained by anti-Le\textsuperscript{y} MAbs. In contrast, post-compaction and blastocyst stage (c, d, g, h) were strongly stained by anti-Le\textsuperscript{y} MAbs but not stained by anti-Le\textsuperscript{x} MAbs.

Without this adhesion, subsequent development of the embryo may not occur. Thus, cell adhesion is seen as a prerequisite for further development of the morula stage embryo, and perhaps at every stage of development as well, although the molecular mechanisms involving cell adhesion and subsequent program switching of cellular differentiation are totally unexplored. The role of Le\textsuperscript{x} in the compaction process was further elucidated by the observation that compaction was inhibited by Le\textsuperscript{x} oligosaccharide (lacto-N-fucopentaose III; LNF III), particularly trivalent Le\textsuperscript{x}, i.e., LNF III bound to lysyllysine (LL). Other oligosaccharide conjugates did not inhibit compaction (ref. 6). On the basis of these findings, we searched for cell surface molecules recognizing Le\textsuperscript{x}, using undifferentiated mouse teratocarcinoma F9 cells which mimic the morula-stage preimplantation embryo and show Ca\textsuperscript{2+}-dependent cell aggregation (ref. 16). In our preliminary studies, the recognition molecule was identified as an Le\textsuperscript{x}-bearing glycoprotein (ref. 17). We therefore suspected that Le\textsuperscript{x}-dependent cell recognition was mediated by a glycoprotein having lectin-like affinity to Le\textsuperscript{x}, or Le\textsuperscript{x} by itself. The latter possibility was supported by various experimental findings based on glycolipids. [Note: Since CHO chains in glycoproteins are extremely heterogeneous, determination of CHO-CHO interaction in a particular structure is difficult unless glycoproteins and oligosaccharides derived therefrom are available in large quantity. Therefore, all experiments demonstrating CHO-CHO interaction should be designed on a glycolipid basis.] (i) Le\textsuperscript{x} liposome, but not paragloboside (PG; Gal\textsubscript{β}1→4GlcNAc\textsubscript{β}1→3Gal\textsubscript{β}1→4Glc\textsubscript{β}1→Cer) or sialosylparagloboside (SPG; NeuAca2→3Gal\textsubscript{β}1→4GlcNAc\textsubscript{β}1→3Gal\textsubscript{β}1→4Glc\textsubscript{β}1→Cer) liposome, adhered on Le\textsuperscript{x}-coated plastic.
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(i) Conversely, LeX liposome adhered only on LeX-coated surface, not on PG- or SPG-coated surface (Fig. 2). (ii) Only LeX showed self-aggregation, i.e., clearly measurable changes by aggregometer or microscopic examination. (iii) Radiolabeled lactofucopentaose III-LL conjugate was adsorbed on LeX-octylsepharose column and specifically eluted with EDTA (Fig. 3). (iv) Only lactofucopentaose III could be more retained with LeX liposome-containing suspension on equilibrium dialysis. These four findings, indicating LeX-LeX interaction, all required the presence of bivalent cation (Ca$^{2+}$ or Mg$^{2+}$), and the interactions were inhibited by EDTA.

Fig. 2. Interaction of liposomes containing LeX glycolipid with glycolipid-coated solid phase (A), and interaction of liposomes containing various glycolipids with LeX-coated solid phase (B). Panel A: LeX glycolipid liposomes were incubated with solid phase coated with: △, LeX; ▲, PG; ●, SPG; ○, no glycolipid. Panel B: LeX-coated solid phase was incubated with liposomes containing: △, LeX; ▲, PG; ●, SPG; ○, no glycolipid. Note that adhesion of PG and SPG liposome on LeX-coated surface was less than that on non-coated surface.

Fig. 3. Affinity chromatography of LNF III-LL conjugate on LeX glycolipid or C18 silica gel column. Synthetic LeX pentasaccharide glycolipid (5 mg) was adsorbed on C18 column (0.5x0.7 cm) equilibrated in 20 mM Tris-HCl, pH 7.4, 1 mM CaCl$_2$, 150 mM NaCl. LNF III-LL conjugate (radiolabeled at the carboxy-terminal of LL; 150 cpm/nmol) was dissolved in the same buffer and applied on the column. Elution with the same buffer was followed by elution with buffer containing 5 mM EDTA (arrow). A, LNF III-LL conjugate applied on LeX glycolipid column. B, lactosyl-LL conjugate applied on LeX glycolipid column. C, LNF III-LL conjugate applied on C18 column. D, lactosyl-LL conjugate applied on C18 column.

The role of Ca$^{2+}$-sensitive cell adhesion proteins cadherin or uvomodulin during the compaction process has been previously demonstrated (refs. 8,18,19). LeX-dependent cell adhesion may take place earlier than the event in which adhesion proteins are involved, since only LeX (not cadherin) inhibited the compaction process. This hypothesis is illustrated in Fig. 4. [Note: LeX-LeX interaction may precede protein interaction as shown in Fig. 4, but this is still hypothetical. However, it is logical to imagine that multiple weak interactions, which are essentially reversible, take place prior to irreversible, stable interactions. Experiments designed to inhibit the compaction process using adhesive proteins such as cadherin and uvomodulin may be difficult to perform unless binding fragment can be obtained in sufficient quantity.]
Fig. 4. Idealized model indicating various steps in the cell recognition process. Specific interaction between two homotypic cells could be initially mediated by multiple carbohydrate-carbohydrate interactions, followed by interaction of pericellular adhesive proteins and their receptors, and finally a cell-cell communication channel could be established through a gap junction protein. Panel A: LeX-LeX interaction between two LeX glycolipids, or the LeX side chain in glycoprotein (Gp) on one cell interacting with LeX on the counterpart glycoprotein or glycolipid. ○, galactose; ●, N-acetylglucosamine; △, fucose. This stage of interaction could be defined by LeX-LeX complementarity, but the strength of interaction could be a function of the number of such weak interactions, and could be enhanced greatly in the presence of bivalent cation. According to this model, density of carbohydrate chains organized at the cell surface is essential to define such interaction. Panel B: subsequent non-specific but stronger interactions mediated by various adhesive proteins (ADP) (e.g., fibronectins, laminin, thrombospondin, cadherin) through integrin receptor protein (ITG). Cell surface carbohydrate binding protein (SBP) (e.g., endogenous lectin), glycosyltransferase, or hydrolase may also recognize certain types of carbohydrate chain at this stage. This stage of interaction is also mediated by Ca2+ and other bivalent cations. Panel C: possible establishment of a cell-cell communication channel within gap junction. JP, gap junction protein.

APPLICATION OF LeX-LeX INTERACTION FOR TARGETING OF LeX LIPOSOME TO LeX-BEARING TUMORS

LeX-dependent cell aggregation, as described in the previous section, may indicate a possible application of LeX-LeX interaction in targeting of LeX liposome to LeX-bearing tumor cells. This possibility has been supported by experiments performed in vitro as well as in vivo, using LeX-expressing human colonic cancer cell line HRT-18 as a target. Tumor cells interacted specifically with LeX liposomes, but not with control liposomes or those containing PG or SPG (Fig. 5A). HRT-18 tumors grown in nu/nu mice and injected intravenously with 125I-labeled liposomes showed accumulation of LeX liposome only; this accumulation was maximal after 24 hr (Fig. 5B).

Fig. 5. Panel A: time-dependent accumulation of liposomes on HRT-18 cells in vitro.

**Targeting effect of 125I-labeled LeX liposomes on LeX-bearing tumors in vivo**

Fig. 5. Panel B. HRT-18 cells were inoculated in nu/nu mice and tumors were formed. Mice were injected with liposomes containing LeX (solid column), PG (shaded column), or no glycolipid (open column). Accumulation of radioactive liposome is expressed by tissue/blood activity ratio. Time course is indicated on abscissa. Significant differences (p<0.005) are indicated by dashed brackets.
Prompted by the observed Le^x-Le^x interaction, we searched systematically for other glycolipid interactions. A highly specific interaction was found between GM3 (sialosylactosylceramide; NeuAcα2-3Galβ1-4Glcβ1-1Cer) and Gg3 (gangliotriaosylceramide; GalNAcβ1-4Galβ1-4Glcβ1-1Cer). This interaction, similar to that of Le^x-Le^x, was obvious only in the presence of bivalent cation, and was abolished by the presence of EDTA. The GM3-Gg3 interaction was verified by the following observations: (i) adhesion of GM3-containing liposome on Gg3-coated solid phase and vice versa, and the observation that GM3 concentration in liposome as well as Gg3 density coated were highly density-dependent (Fig. 6) (ref. 20); (ii) NeuAcα2-3Galβ1-4Glc linked to LL was adsorbed specifically on Gg3-C18 column and was eluted by EDTA (Fig. 7) (Kojima N, Stroud M, Hakomori S, unpublished data); (iii) specific interaction of Gg3 liposome with GM3 liposome (data not shown).

**Fig. 6.** Interaction of glycolipid-containing liposome with glycolipids (or glycolipid-liposome) coated on plastic surface. **Panel A:** Liposomes labeled with [14C]-cholesterol and containing GM3 (○), SPG (△), GM1 (●), Gg3 (▲), or PG (□) were incubated in 96-well flat-bottom polystyrene plastic plates coated with various quantities of Gg3 as indicated on the abscissa. For quantification of effect of 2D4 antibody, Gg3-coated wells were treated with MAb 2D4 (5 μg/ml) at room temperature for 1 hr, washed with TBS, and incubated with GM3 liposome (X). **Panel B:** GM3 liposome containing 14C-labeled cholesterol was incubated with various quantities (abscissa) of GM3, SPG, GM1, Gg3, or PG coated on solid phase. Symbols as in Panel A.

**Fig. 6, Panel C.** Liposomes containing different densities of GM3 were prepared from 50 nmol 14C-labeled cholesterol, 50 nmol phosphatidylcholine, and various molarities of GM3. Symbols indicate the molar ratios of cholesterol, phosphatidylcholine, and GM3 in liposomes as follows: 10:10:0.5 (●), 10:10:1.0 (○), 10:10:1.5 (▲), 10:10:2.0 (△), and 10:10:2.5 (■).
Fig. 7. Interaction of $[^3H]$-labeled oligosaccharide-LL conjugate with glycolipid-C18 column. Lactosyl-LL or 2-3sialosyllactose-LL conjugate labeled at the carboxyl group of LL by reductive tritiation were prepared. Glycolipids (Gg3, GM3) or phospholipids (PC) were adsorbed onto a C18-silica gel column, extensively washed with TBS containing 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$, and equilibrated well. 100 nmole of oligosaccharide-LL conjugate (10,000 cpm) was applied on glycolipid-C18 column and eluted with 7.5 ml of TBS containing 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$, followed by 7.5 ml of TBS containing 10 mM EDTA and 1% propanol. Fractions (0.5 ml) were collected and aliquots of 0.1 ml were counted by scintillation counter. Arrows indicate the fraction number in which the buffer was changed from TBS containing Mg$^{2+}$ and Ca$^{2+}$ to TBS containing EDTA. Panels A-C: elution pattern of lactosyl-LL, 2-3sialosyllactose-LL, and 2-3 sialosyllactitol respectively. Panels D-F: peak A in panel B was loaded onto Gg3-C18, PC-C18, and GM3-C18 column, respectively.

In experiments based on these preliminary findings, mouse melanoma B16 (expressing high level of GM3) was found to interact with mouse lymphoma L5178 clone AA12 (expressing high level of Gg3) but not with L5178 clone AV27 (not expressing Gg3) (Fig. 8). The B16-AA12 interaction was inhibited by pretreatment of B16 cells with anti-GM3 MAb DH2, or pretreatment of AA12 cells with anti-Gg3 MAb 2D4. The interaction was inhibited by presence of NeuAca$_{2,3}$Galp$_{1,4}$Glc, but not NeuAca$_{2,6}$Galp$_{1,4}$Glc (data not shown).
These findings clearly indicate that the specific cell adhesion between AA12 and B16 cells is mediated by specific interaction between Gg3 and GM3. This was further confirmed by specific cell adhesion as well as the subsequent spreading of B16 cells on Gg3-coated surface. Comparison with two other melanoma cell lines, F10 and F1, showed that BL6 (which has the highest metastatic potential) had greatest cell adhesion on Gg3-coated surface. F1 cells (lowest metastatic potential), which show low GM3 expression at the cell surface, also showed poor adhesion on Gg3-coated surface (data not shown). Adhesion of BL6 cells on Gg3-coated surface was not affected by presence of RGDS peptide sequence, which is an important recognition site for the integrin receptor. In contrast, BL6 adhesion on non-coated plastic surface was clearly inhibited by RGDS. Furthermore, Gg3-dependent BL6 cell adhesion was greatly enhanced on fibronectin/Gg3 co-coated surface (data not shown).

**SPECIFIC INTERACTION OF GALACTOSYLCERAMIDE AND SULFATIDE**

In tests with various other glycolipid systems, a clear interaction was also observed between liposomes containing galactosylceramide and sulfatide-coated surface (Fig. 9A). In contrast, there was no interaction of liposomes containing glucosylceramide, lactosylceramide (CDH), or globotriaosylceramide (CTH). In view of the fact that both galactosylceramide and sulfatide are important major components of myelin sheath membrane, which displays characteristic lamellar structure, it is conceivable that the galactosylceramide-sulfatide interaction plays some role in morphogenesis of myelin sheath (Fig. 9B).

**Panel A:** 14C-cholesterol-labeled liposomes containing galactosylceramide (●), globotriaosylceramide (△), lactosylceramide (▲), and glucosylceramide (□) were incubated with plastic surface coated with various amounts of sulfatide. After incubation, wells were washed with PBS and liposome binding was counted. Only Gal-Cer showed specific reactivity with sulfatide.

**Panel B:** Since sulfatide and Gal-Cer are particularly abundant in myelin sheath membrane (MSM), we hypothesize that morphogenesis of MSM involves interaction of these two glycolipids between membrane layers. Density of the two glycolipids (represented by black and white symbols in the bottom drawing) may define lamellar structure of MSM. [Adapted from A. Peters, The structure and function of nervous tissue, Academic Press, NY, 1968.]
All living organisms (i.e., displaying self-replication), including viruses, rickettsiae, chlamydiae, mycoplasma, bacteria, and higher plant and animal cells, are characterized by highly glycosylated cell surface membranes; the pattern of glycosylation is species- or strain-specific. Although molecular species of cell surface carbohydrates and their chemical structures have been well identified as polysaccharides, peptidoglycans, proteoglycans, glycoproteins, or glycolipids, the function of these glycoconjugates has been a long-standing enigma. Their extensive variation may reflect corresponding differences in cellular interaction processes, although direct evidence for this hypothesis has been lacking. The process of cell recognition via cell surfaces plays an essential role in multicellular system formation, organogenesis, and morphogenesis; its failure typically leads to malformation and/or cancer. Studies on the role of cell surface CHOs in cell recognition have been focused on possible involvement of cell surface proteins that interact with specific CHOs, i.e., lectins, or glycosyltransferases and hydrolases (refs. 21-23). Only a few animal cell lectins have been found to be expressed at the cell surface, e.g., Ashwell receptor on hepatocytes (ref. 24), tumor-associated lectins (ref. 25), and more recently "selectin family" receptor expressed on endothelial cells as well as granulocytes/monocytes and activated platelets (refs. 26,27). It is probable, however, that the majority of lectins found in muscles and parenchymatous cells in major organs are not expressed at the cell surface, but have other roles in defining presently-unknown cellular functions unrelated to cell-cell recognition, e.g., protein mobilization within cells (refs. 28,29). In terms of glycosyltransferases, there is no clear evidence for their expression at the cell surface, except for the recent observation that B cell antigen CD75 is a cell surface sialyltransferase (ref. 30). Application of molecular-biological techniques in sequencing various cell surface molecules may reveal an increasing number of sequence homologies with lectins, glycosyltransferases, and hydrolases. This trend of study may eventually elucidate the real functional roles of cell surface lectins and transferases.

Cell surface CHOs vary extensively and show continuous changes in association with differentiation and oncogenesis (refs. 1,2). Thus, if lectins and glycosyltransferases were involved in every step of (and type of) cell recognition, their specificities would presumably also show extensive variation and continuous changes corresponding to those of cell surface CHOs. This possibility cannot be definitively ruled out at this time, but appears extremely unlikely. The hypothesis presented here (i.e., that various CHOs expressed at the cell surface are recognized by complementary CHOs) provides a more plausible mechanism for recognition of highly variable glycosylation patterns.

Obviously, mechanisms for recognition of intercellular information are crucial to understanding the development of multicellular organisms, and the pathobiological genesis of degenerative, inflammatory, and neoplastic diseases. As mentioned in the Introduction, great advances have been made recently in understanding the mechanism of cell recognition mediated by adhesive proteins and their cell surface receptors (refs. 8-11). Following the initial proposal by Roseman (ref. 31), there has been considerable discussion of the role of cell surface CHOs in defining specificity of cell recognition, based on interaction of CHOs with complementary proteins, as briefly described earlier. In contrast, specific cell recognition based on CHO-CHO interaction as currently envisioned is uniquely characterized by: (i) high specificity; (ii) weak affinity; (iii) high flexibility; and (iv) the possibility of repellant as well as attractive interaction. Although a single CHO-CHO interaction is weak, the combination of multiple sites could produce reasonably strong binding affinity. Changes in conformation and orientation of CHO chains could allow for variable intensity of interaction during morphogenesis and development. Repellent activity between cells is equally important as binding activity. Repellent forces at the molecular level (e.g., GM3-GM3, Le<sup>x</sup>-Le<sup>y</sup>) are in striking contrast to attractive forces (e.g., GM3-Gg3, Le<sup>x</sup>-Le<sup>y</sup>), and are an important consideration in cell-cell interactions (Fig. 10).
The molecular mechanism for CHO-CHO interaction is not clearly understood at this time. However, in the cases studied (e.g., GM3-Gg3 and Leα-Leα), the two interacting CHOs on GSLs are characterized by two contrasting surface profiles based on molecular models constructed according to hard sphere exo-anomeric calculations: (i) one surface showing a large hydrophobic domain surrounded by a hydrophilic area; (ii) another surface which is primarily hydrophilic. Complementarity of two interacting CHOs could be based on: (i) hydrophobic interaction between the respective hydrophobic surfaces; (ii) hydrogen bonding between hydrophilic groups; (iii) "matching" conformation of the interacting surfaces. The GM3-Gg3 interaction is shown as an example in Fig. 11. It is important to note that bivalent cation is always required for observed interactions; this may be related to stabilization of hydrogen bonds. A clear demonstration of CHO-CHO interaction can only be obtained based on solid-phase presentation of GSLs (i.e., GSLs in liposomes, adsorbed on column or plastic surface) or on equilibrium dialysis of GSL liposomes (or micelles) and oligosaccharides, particularly multivalent oligosaccharides. Interaction of monovalent oligosaccharides by themselves in solution is extremely difficult to demonstrate. Obviously, the same approach used for GSL in liposomes or adsorbed on solid phase could be extended to other classes of glycoconjugates (i.e., a large variety of CHO chains held on proteins or polysaccharides), although this is technically difficult at present. Studies along this line are nonetheless of crucial importance, since an essential part of cell recognition mediated by CHO-CHO interaction must involve glycoprotein CHOs.

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