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Le^x glycan mediates homotypic adhesion of embryonal cells independently from E-cadherin: A preliminary note ☆

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Abstract

Le^x glycan and E-cadherin (Ecad) are co-expressed at embryonal stem (ES) cells and embryonal carcinoma (EC) cells. While the structure and function of Ecad mediating homotypic adhesion of these cells have been well established, evidence that Le^x glycan also mediates such adhesion is weak, despite the fact that Le^x oligosaccharide inhibits the compaction process. To provide stronger evidence, we knocked out Ecad gene in EC and ES cells to establish F9 Ecad (−/−) and D3M Ecad (−/−) cells, which highly express Le^x glycan but do not express Ecad at all. Both F9 Ecad (−/−) and D3M Ecad (−/−) cells displayed strong autoaggregation in the presence of Ca^{2+}, while PYS-2 cells, which express trace amount of Ecad and undetectable level of Le^x glycan, did not display autoaggregation. In addition, F9 Ecad (−/−) and D3M Ecad (−/−) cells displayed strong adhesion to plates coated with Le^x glycosphingolipid ($III^3FucnLc4Cer$), in dose-dependent manner, in the presence of Ca^{2+}. Thus, ES or EC cells display autoaggregation and strong adhesion to Le^x-coated plates in the absence of Ecad, further supporting the notion of Le^x self-recognition (*i.e.*, Le^x-to-Le^x interaction) in cell adhesion.
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Keywords: E-cadherin; Le^x glycan; SSEA-1; Autoaggregation; Embryonal stem cells; Embryonal carcinoma cells; Le^x glycosphingolipid; Knockout; Carbohydrate-to-carbohydrate interaction; Cell adhesion

A cell surface protein, originally termed ''uvomorulin'', highly expressed at morula stage of mouse embryo, mediates adhesion of embryonic cells to induce compaction [1–3]. The protein was later termed ''cadherin'' or ''E-cadherin (Ecad)'', since its function depends on presence of Ca^{2+}, and is abolished by calcium-chelating reagents [4,5]. Structure, function, and molecular variants of cadherin as homotypic adhesion receptor, and its cytoplasmic organization with catenin and plakoglobin, have been extensively studied during the past two decades (for review see [6–8]. Further functional notion of Ecad during embryogenesis was addressed by approaches using Ecad null mice [9] or Ecad gene knockout embryonal stem (ES) cells [10].

On the other hand, Le^x glycan [11,12] carried by stage-specific embryonic antigen-1 (SSEA-1) [13], was also found

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; Ecad, E-cadherin; EC, embryonal carcinoma; ES cells, embryonal stem cells; FBS, fetal bovine serum; glycan, a short term for a polysaccharide; GSL, glycosphingolipid; Le^x, Galβ4[Fucα3]GlcNAcβ3Galβ-R; Le^x GSL, Galβ4[Fucα3]GlcNAcβ3Galβ4Glcβ1Cer ($III^3FucnLc4Cer$); Le^x glycan, an uncharacterized polysaccharide having multiple Le^x epitopes; anti-SSEA-1 mAb reacts with such structure; mAb, monoclonal antibody; Os, oligosaccharide; SSEA-1, stage-specific embryonic antigen-1.

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to be highly expressed at morula stage of mouse embryo. It is assumed to be involved in the compaction process, since Le^x oligosaccharide (Os), but not its positional isomer H or Le^a Os, inhibited compaction, or induced de-compaction of compacted embryo [14,15].

Questions arose why two entirely different adhesion systems—one a single transmembrane protein, the other a glycan with multiple Le^x—(i) are co-expressed at morula stage embryo and in embryonal carcinoma cells; (ii) are both involved in the compaction process and in autoaggregation of F9 cells; (iii) both display homotypic self-recognition in the presence of bivalent cation, particularly Ca^{2+}.

Another concern is that Le^x glycan is intrinsically heterogeneous, and apparent Le^x glycan function to cause self-recognition could be due to contamination of Ecad (see Discussion). In order to address these points, we established variant lines of F9 EC cells or D3M ES cells whose Ecad gene is knocked out. Ecad expression was completely

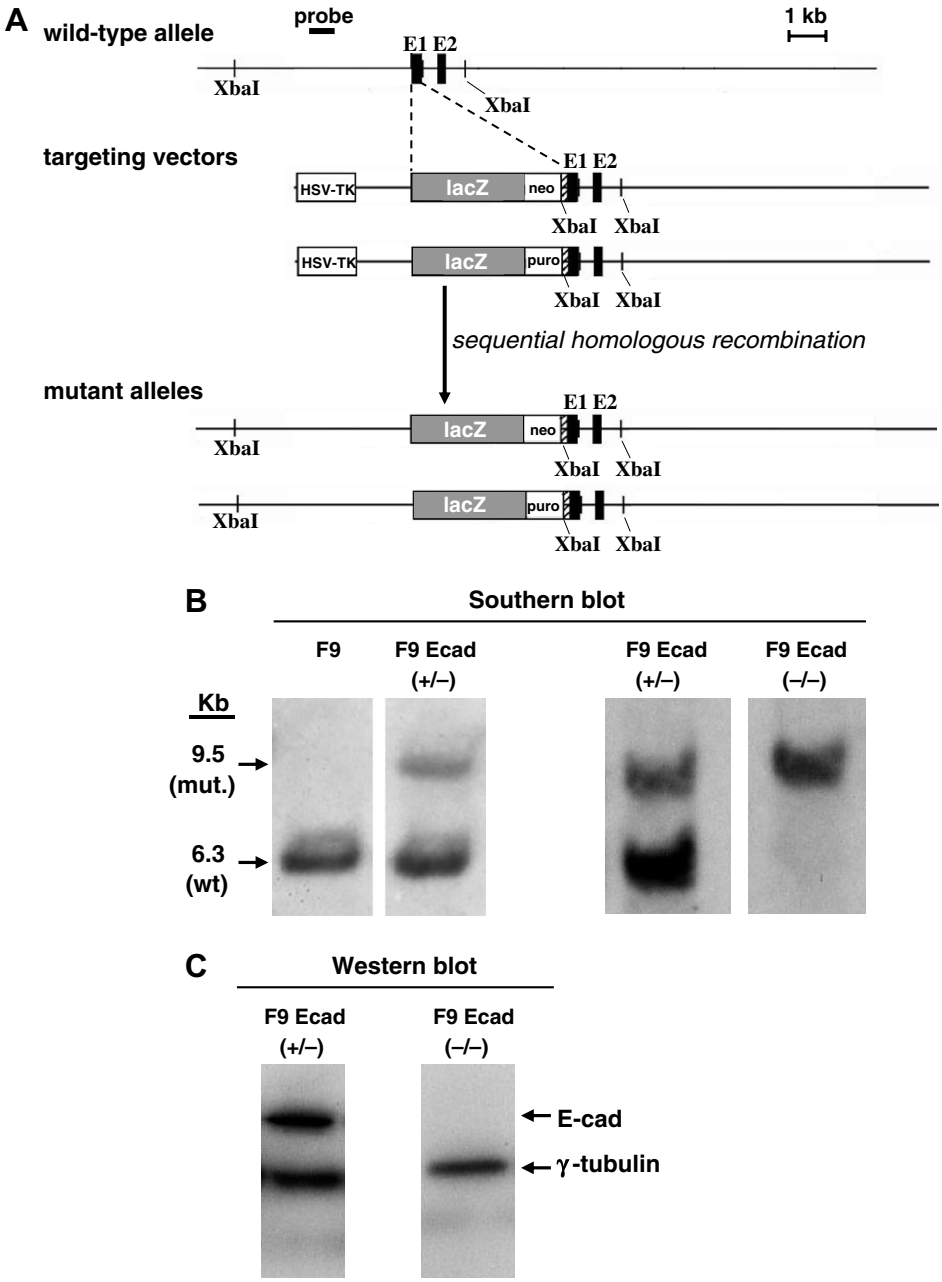

Fig. 1. (A) Strategy for targeted disruption of Ecad in F9 cells. Schematic presentation of wild-type Ecad allele, with location of DNA probe used for Southern blot analysis (top line), two targeting vectors (middle lines) [16], and mutated alleles after sequential homologous recombination. E-cad null cells were produced by insertion of a lacZ gene and neomycin or puromycin resistance cassette into exon 1 just upstream of the initiation codon of the gene. E, exon. (B) Southern blot hybridization analysis of genomic DNA. Genomic DNA isolated from indicated cells was digested with XbaI (see A) and processed for Southern blot analysis, as described in M&M. (C) Western blot analysis of Ecad (−/−), showing no Ecad expression. γ-Tubulin was stained as loading control. Western blot was performed as described previously [17]. Briefly, total cell lysates from equal number of cells were analyzed by Western blot with a mixture of rat anti-Ecad and mouse anti-γ-tubulin, followed by a mixture of secondary antibodies.

absent in these cells, but Le^x glycan carried by SSEA-1 is highly expressed, and these cells display clear autoaggregation and adhesion to Le^x GSL-coated plates.

Materials and methods

Cell culture. F9 cells and F9 Ecad (−/−) EC cells were grown in DMEM (Irvine, Santa Ana, CA) containing 10% FBS (Hyclone, Logan, UT) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycinin in 0.1% gelatin-coated flasks. The following cell lines were purchased, established in our lab, or transferred from other labs.

PYS-2, a differentiated cell line derived from mouse 129 embryonal carcinoma OTT6050 (from which F9 cells were derived), which do not express Le^x glycan (SSEA-1), and express minimal level of Ecad, were grown in the culture medium described above.

Mouse ES clone D3M, and its Ecad null variant D3M Ecad (−/−), were established as described previously [10], their cell biological properties were elaborated in L. Larue's lab (Institut Curie, UMR146 CNRS, Orsay, France), and cells were transferred to the lab in Seattle, WA. These cells were grown in 60% conditioned medium from buffalo rat liver (BRL) cells and 40% ES medium (22% FBS, leukemia inhibitory factor (LIF, 1000 U/ml), 2-ME (1 μl/100 ml)-DMEM) as described previously [10].

Preparation of F9 Ecad (−/−) cells. Ecad null F9 cells were produced by sequential targeted gene disruption using two targeting vectors, Ecad-neo and E-cad-puro, which contain neomycin-resistance gene and puromycin-resistance gene, respectively. These plasmids were established in R. Kemler's lab, Max-Planck Institut für Immunbiology, Freiburg, Germany, as described by Stemmler et al. [16]. First, 25 μg of linearized Ecad-neo was transfected into 1×10^7 F9 Ecad (+/+) cells by electroporation to obtain heterozygous cells. After culture in selection medium containing G418 (400 μg/ml) and Ganciclovir (2 μM) for ~2 weeks, growing colonies were screened for homologous recombination by Southern blot analysis using AlkPos Labelling Kit and CDP-Star reagent (GE HealthCare, Piscataway, NJ), and the DNA probe described in Fig. 1A, following the manufacturer's instruction. Second, linearized Ecad-puro was transfected into F9 Ecad (+/−) cells by similar procedures as above. After selection with above medium containing puromycin (0.8 μg/ml), F9 Ecad (−/−) cells were sorted by FACS at Fred Hutchinson Cancer Research Center (Seattle, WA), after staining with rat anti-Ecad mAb (Invitrogen, Carlsbad, CA) and with Alexa 488 labeled anti-rat IgG (Invitrogen). F9 Ecad (−/−) cells were further cloned in the selection media, while established cells used for autoaggregation and adhesion assays were cultured in regular culture media.

Ecad and Le^x expression on the cell surface. After detaching with 0.01% trypsin in DMEM, cells were stained with rat anti-Ecad mAb as above or with anti-SSEA-1 IgM mAb [13]. After staining with appropriate fluorescence-labeled secondary antibodies, cells were analyzed by flow cytometry (Beckman Coulter, Fullerton, CA) as described previously [17].

Autoaggregation and adhesion of cells to $III^3FucnLc4Cer$-coated plates. Among various conditions tested, we found that autoaggregation of F9 Ecad (−/−) cells or D3M Ecad (−/−) cells was clearly observed by "hanging drop" method [18] as described in the Fig. 3A legend. Adhesion of these cells to plates coated with various quantities of Le^x GSL ($III^3FucnLc4Cer$) was determined under the same conditions as described previously for adhesion of F9 or PYS-2 cells to plates coated with $III^3FucnLc4Cer$ [19], as detailed in the Fig. 3B legend. $III^3FucnLc4Cer$ was prepared from a large quantity of liver metastatic deposit from colorectal cancer, and its structure was well characterized by methylation analysis and mass spectrometry [20].

Results

Establishment of F9 cells whose Ecad expression is knocked out

Knockout of Ecad gene in F9 cells by sequential targeting gene disruption, as described by Stemmler et al. [16] and in Fig. 1A, resulted in successful establishment of cells whose Ecad expression was eliminated (termed "F9 Ecad (−/−)"), as evidenced by Southern blot and Western blot analyses (Fig. 1B and C). Ecad level of F9 Ecad (+/−) was similar to that of wild-type F9 by Western blot analysis (data not shown). Mouse ES D3M cells with Ecad gene knockout (termed "D3M Ecad (−/−)") were established as described previously [10]. Flow cytometric patterns of wild-type F9, F9 Ecad (−/−), and D3M Ecad (−/−) in comparison to PYS-2 cells clearly indicate that both Le^x glycan and Ecad are highly expressed in wild-type F9. Le^x glycan expressed in F9 Ecad (−/−) and D3M Ecad (−/−) was as high as in wild-type F9, but Ecad expression in both these cell lines was completely negative. Le^x glycan expression in PYS-2 cells was also completely negative, while Ecad was expressed at very low level (Fig. 2).

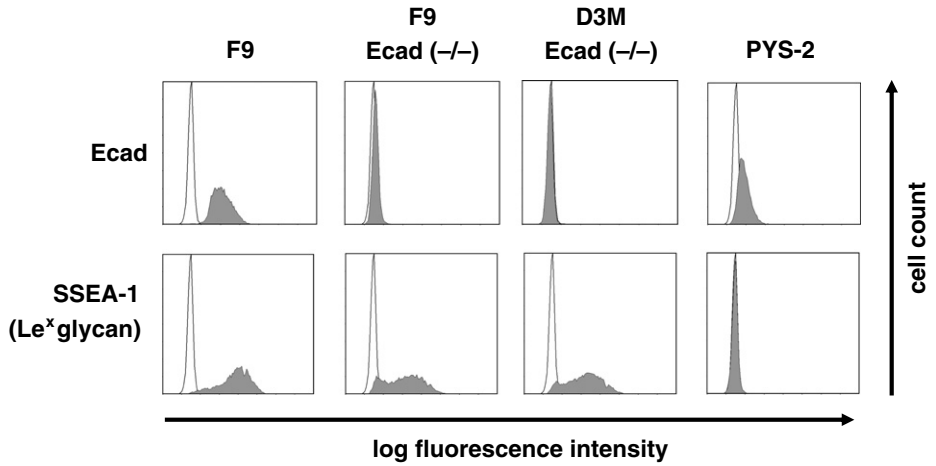

Fig. 2. Cell surface expression of Le^x glycan and Ecad in F9, F9 Ecad (−/−), ES D3M Ecad (−/−), and PYS-2 cells, by flow cytometric analysis. Cells were stained with rat anti-Ecad mAb and mouse anti-SSEA-1 mAb, followed by appropriate fluorescence-labeled secondary Ab. Open curve: secondary Ab only, as control.

Autoaggregation of F9 Ecad (−/−) cells or ES D3M Ecad (−/−) cells, and adhesion of these cells to plates coated with Le^x GSL ($III^3FucnLc4Cer$)

Both F9 Ecad (−/−) and D3M Ecad (−/−) cells, which express Le^x glycan but not Ecad, showed strong autoaggregation by "hanging drop" method during 5 h in the presence of Ca^{2+}, under the conditions described in M&M. Autoaggregation increased, particularly for F9 Ecad (−/−) cells, during 18 h incubation. In contrast, PYS-2, which do not express Le^x glycan but express minimal level of Ecad, did not display autoaggregation under the same conditions (Fig. 3A).

Both F9 Ecad (−/−) and D3M Ecad (−/−) cells adhered, in dose-dependent manner, to plates coated with various quantities of Le^x GSL in the presence of Ca^{2+}. Adhesion of both cells reached a plateau at 1.0–2.0 μg/ well Le^x GSL (Fig. 3B-1 and -2). PYS-2 cells did not show adhesion to Le^x GSL-coated plates even at >3.0 μg/ well Le^x GSL (Fig. 3B-3). Similar adhesion of wild-type F9, but not PYS-2 cells, to Le^x GSL-coated plates was observed previously [19]. Thus, adhesion of F9 Ecad (−/−), D3M

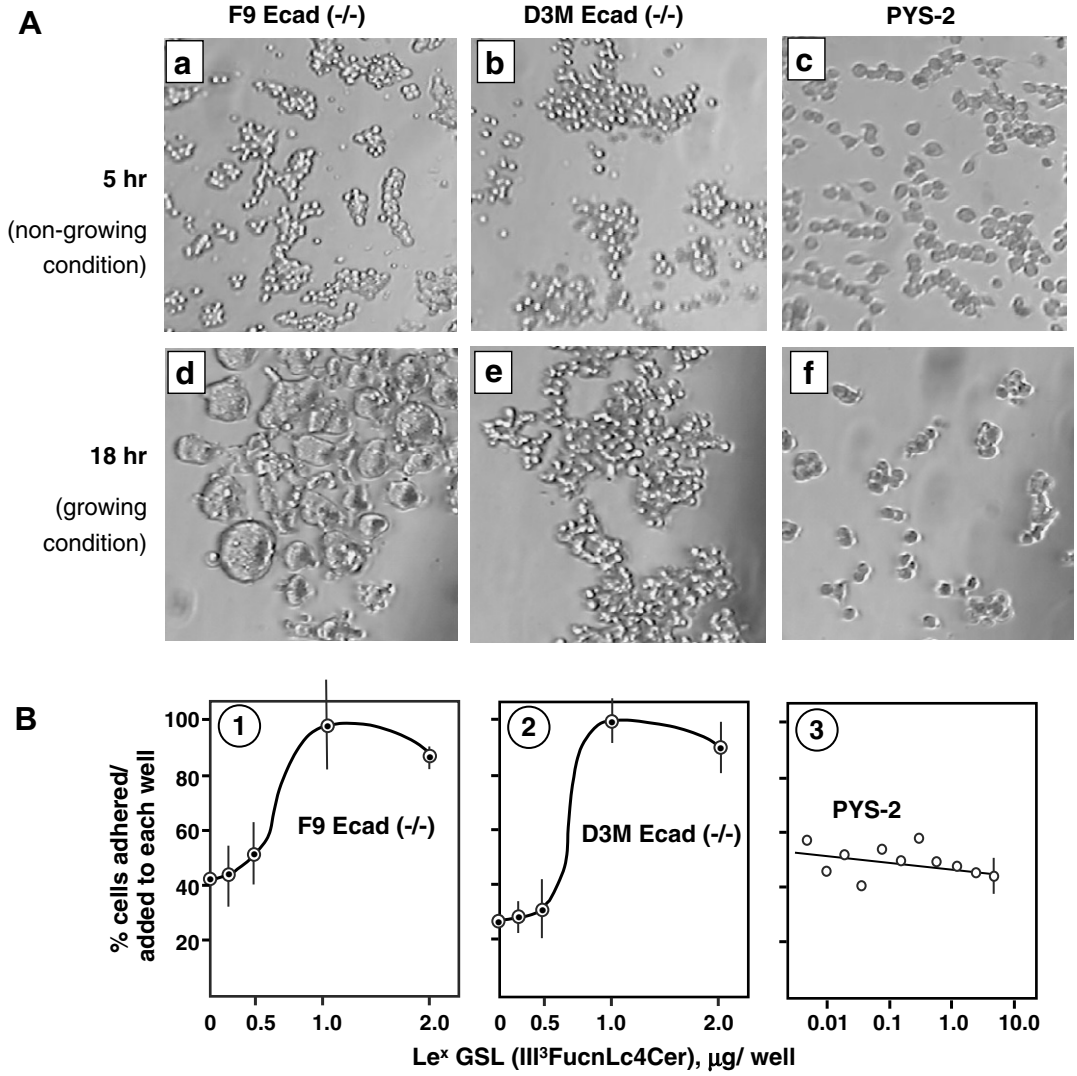

Fig. 3. Autoaggregation of F9 Ecad (−/−) and D3M Ecad (−/−), or adhesion of these cells to plates coated with $III^3FucnLc4Cer$. (A) Autoaggregation of F9 Ecad (−/−) cells or D3M Ecad (−/−) cells, compared to PYS-2 cells as control. Each type of cell in culture, at subconfluency, was detached and suspended in FBS-DMEM as described in M&M. Autoaggregation was observed under two different conditions. (i) After washing with DMEM, cells were resuspended in 1% BSA-DMEM. Cell suspension (3×10^4 in 30 μl) was placed on non-adherent plastic surface and subjected to "hanging drop" procedure [18], *i.e.*, placed "upside-down" in moist chamber. After 5 h incubation at 37 °C, cells in droplet were turned "right side up", incubated ~5 min at room temp, and observed under microscope. (a) F9 Ecad (−/−); (b) D3M Ecad (−/−); (c) PYS-2. (ii) After washing with DMEM, cells were resuspended in their respective culture media (see M&M). Cell suspension (1×10^3 in 30 μl) was placed as above, incubated for 18 h at 37 °C under growing condition, and observed as above. (d) F9 Ecad (−/−); (e) D3M Ecad (−/−); (f) PYS-2. (B) Quantitative Le^x-mediated adhesion of F9 Ecad (−/−) and D3M Ecad (−/−). (1) F9 Ecad (−/−) adhesion. (2) D3M Ecad (−/−) adhesion. Plates were coated with 0.25, 0.5, 1.0, or 2.0 μg/ well of Le^x GSL ($III^3FucnLc4Cer$), and adhesion was measured as detailed in M&M. Note that both cells, in the absence of Ecad expression, adhered strongly to Le^x GSL coated >1.0 μg/well, but minimally to Le^x GSL coated <0.5 μg/well.

Ecad (−/−), and wild-type F9 cells to Le^x GSL-coated plates is considered to be mediated by Le^x-to-Le^x interaction.

Discussion

While the current concept that cadherin mediates homotypic adhesion of ES and EC cells has been well established (for review see [6,7], our knowledge on the functional role of Le^x glycan is still fragmentary. Even though Le^x Os inhibits adhesion of ES cells at morula stage mouse embryo [14,15], it has been difficult to provide definitive evidence that Le^x glycan really causes homotypic adhesion in ES or EC cells, since (i) these cells highly express both Le^x glycan and Ecad, and function of both these molecules requires Ca^{2+}; (ii) there was a possibility that Le^x glycan could be contaminated by Ecad, since Le^x glycan used in some studies was often highly heterogeneous, in spite of the fact that self-recognition ability of extensively purified or synthetic Le^x Os or Le^x glycan has been well documented, based on Le^x-to-Le^x interaction in the presence of bivalent cation (see below).

In order to clarify the functional role of Le^x glycan co-expressed with Ecad in EC F9 or ES D3M cells, we knocked out Ecad gene in order to eliminate Ecad function. The results clearly indicate that: (i) Ecad expression was completely eliminated in both F9 Ecad (−/−) and D3M Ecad (−/−) cells. Le^x glycan was highly expressed in both these cells, at a level similar to that of wild-type F9 or D3M cells. (ii) Autoaggregation of F9 Ecad (−/−) cells was clearly observed even at 5 h under non-growing conditions (1% BSA in DMEM), and was greatly enhanced at 18 h under growing conditions. Autoaggregation of D3M Ecad (−/−) cells was clearly observed at both 5 h and 18 h. Control PYS-2 cells, which do not express Le^x glycan, did not display autoaggregation under 5 h nor 18 h incubation. (iii) Adhesion of both F9 Ecad (−/−) and D3M Ecad (−/−) cells to plates coated with III^3-FucnLc4Cer was clearly observed, particularly at 1.0–2.0 μg/ well concentration.

Le^x-mediated F9 cell adhesion was initially considered to be based on presence of Le^x-binding protein. This possibility was ruled out by demonstration of Le^x-binding components in F9 cell extract after exhaustive pronase digestion [21]. The Le^x-binding component in F9 cells was finally identified as Le^x *per se* [22]. Cell adhesion based on Le^x-to-Le^x interaction has been further extended to WA4 variant of B16 melanoma in the presence of Ca^{2+} [19].

The concept of specific Le^x-to-Le^x interaction has been recently supported by many elegant studies to elucidate the molecular basis of cellular interactions. For example, atomic force between a single pair of Le^x epitopes was found as 22 piconewton (pN), while the force between a pair of lactosyl epitopes was <1 pN. Fifteen to 20 pairs of Le^x is sufficient to cause cell adhesion [23]. Autoaggregation of Le^x-nanoparticles was observed in the presence of Ca^{2+}, but no such aggregation was found for lactosyl-nanoparticles [24]. Binding affinity (K_d value) of Le^x-nanoparticles to Le^x affixed on gold film was 5.4×10^{-7}, while that of Le^x to lactose, or lactose to lactose, was in the range $8–15 \times 10^{-2}$ [25]. Adhesion energy (W_{adh}) of two interfacing membrane vesicles containing Le^x GSL or various other GSLs was determined by change of contact angle (θ_c). Only vesicles containing Le^x, but not those containing Le^a or other structures, showed high W_{adh}, indicating strong adhesion [26].

The concept of carbohydrate-to-carbohydrate interaction has been further extended to other homotypic or heterotypic cell adhesion, widely observed in cells ranging from primitive sponges [27,28] to higher vertebrates [29–32] (for review see [33,34]. In all these studies, multiple glycosyl epitopes were organized in order to observe clear binding. In the case of Le^x-mediated adhesion, multiple Le^x epitope assembled in SSEA-1 is essential, although exact structure of SSEA-1 remains to be elucidated.

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