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JB Reflections and Perspectives

Glycosphingolipids as mediators of phenotypic changes associated with development and cancer progression

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Sen-itiroh Hakomori has been involved for the past ~40 years in studies on structure and function of glycosphingolipids (GSLs). The following is a brief summary of highlights of his research, performed in association with his colleagues.

Keywords: oncogenic progression/ontogenic development/blood group/carbohydrate-carbohydrate interaction/epithelial-mesenchymal transition/glycosphingolipid.

Abbreviations: CCI, carbohydrate–carbohydrate interaction; EC, embryonal carcinoma; Ecad, E-cadherin; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ES, embryonic stem; FGFR, fibroblast growth factor receptor; Gb4, GalNAcβ3Galα4Galβ4Glcβ1Cer; Gb5, Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer; GFR, growth factor receptor; Gg3, GalNAcβ4Galβ4Glcβ1Cer; Gg4, Galβ3GalNAcβ4Galβ4Glcβ1Cer; GM3, NeuAcα3Galβ4Glcβ1Cer; GM2, GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer; GM1, Galβ3GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer; GSL, glycosphingolipid; LacCer, Galβ4Glcβ1Cer; Le^a, Galβ3[Fucα4]GlcNAcβ3Galβ4Glcβ1Cer; Le^x, Galβ4[Fucα3]GlcNAcβ3Galβ-R; mAb, monoclonal antibody; SSEA, stage-specific embryonic antigen; TSP, tetraspanin.

GSLs defining oncogenic progression and its reversion

A clear reduction of ganglioside GM3, associated with oncogenic transformation, was observed initially in BHK cells transformed by polyoma virus (*1*), and subsequently in chicken embryonic fibroblasts transformed by Rous sarcoma virus (*2*), particularly those with temperature sensitive mutants (*3*). These studies indicated that transformed phenotype and its reversion are associated, respectively, with decrease *versus* increase of GM3 expression. This concept was further substantiated by studies with mouse or chicken fibroblasts transformed by viral *Jun* oncoprotein (v-*Jun*). Expression of v-*Jun* induced oncogenic phenotype in fibroblasts, with the loss of GM3 synthesis, whereas transfection of GM3 synthase gene caused reversion of oncogenic to normal cell phenotype, with appearance of GM3 (*4*). Oncogenic *versus* normal cell phenotype was determined based on presence *versus* absence of colony formation in soft agar, and of anchorage-dependent cell growth. In this process, GM3/tetraspanin (TSP) CD9/integrin complex plays a key role, although CD9 and integrin are not changed; only GM3 disappears or appears (*4*). These studies (*2–4*) were performed in collaboration with P.K. Vogt's lab at Scripps Research Institute, La Jolla, CA, USA.

Further studies on inhibitory effect of GM3 and/or GM2 on degree of malignancy of human cancers indicated that: (i) GM3 complexed with TSP CD9 inhibited motility, growth, and invasiveness of colorectal cancer (*5*), and of bladder cancer cells (*6*). GM3 complexed with CD9/CD81 inhibited the same phenotypes of normal lung fibroblasts (*7*, *8*). (ii) GM2 complexed with TSP CD82, in normal bladder epithelial HCV29 cells, inhibited cell motility and growth through inhibition of hepatocyte growth factor (HGF)-dependent cMet kinase. In contrast, bladder cancer YTS1 had high cMet activity, to induce high

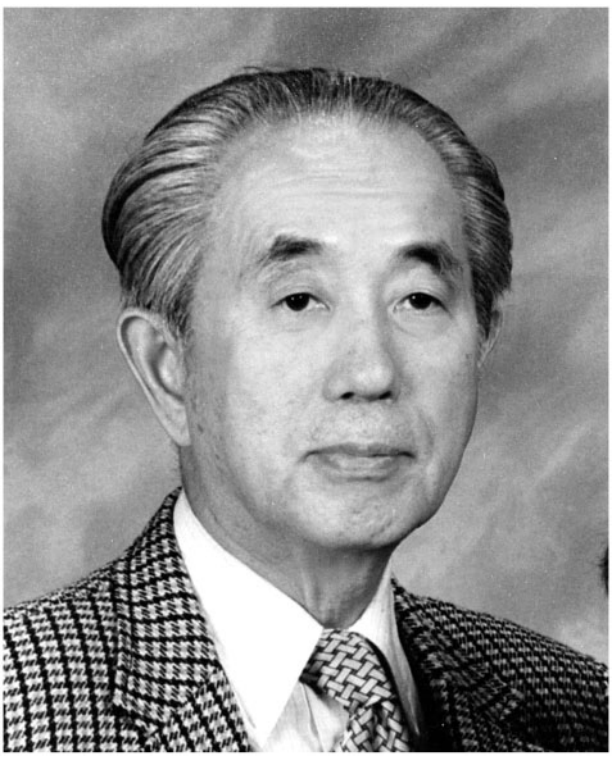

Fig. 1 Prof. Sen-itiroh Hakomori.

motility and growth, since GM2/CD82 complex was absent. Transfection of CD82 gene to YTS1 caused complex formation, which reverted oncogenic to normal phenotype, similar to HCV29 cells (*9*). (iii) The above complex also inhibited functional interaction (cross-talk) between integrin α3 and cMet, *i.e*. cMet activation through tumour cell adhesion to laminin 5 (Ln5)-coated plate was inhibited by this complex (*9*).

GSLs defining ontogenic development

Compaction of morula-stage embryo, the first cell-cell adhesion event during mouse embryogenesis, is assumed to be mediated by both glycosyl epitope Le^x (*10*) and E-cadherin (Ecad) (*11*, *12*). In order to further assess separate occurrence of these two processes, Ecad gene of EC F9 and ES D3M cells was knocked out. These F9 $Ecad^{(-/-)}$ and D3M $Ecad^{(-/-)}$ cells displayed clear autoaggregation, and dose-dependent adhesion to plates coated with various quantities of Le^x GSL (*13*).

In order to identify the molecules which may define embryonic phenotypes, D. Solter & B.B. Knowles established SSEA-1 (*14*), -3 and -4 (*15*) by mAb approach. They used mouse EC cells or mouse embryo at 4–8 cell stage as immunogen in rats or mice, to obtain mouse or rat mAbs directed to these SSEAs, which were identified as Le^x (fucosyl LacNAc) (*16*, *17*), Gb5/or fucosyl-Gb5 (*18*) and sialosyl-Gb5 (*19*), respectively. Expression of SSEA-3 and -4 was observed at 2–4 cell stage, before morula. SSEA-1 appeared at 8–16 cell stage, and declined after compaction (*15*). Thus, globo-series structures appear at the 2–4 cell stage, and decline, followed by appearance of lacto-series at morula stage, which then decline after compaction. Ganglio-series structures GM3, GM2, GM1, GD1a, *etc*. appear only at the stage of neural crest formation. The biological significance of these embryonic antigens is largely unknown, except that SSEA-1 may be involved in Ca^{2+}-dependent embryonic cell adhesion, *i.e*., compaction.

Blood group A and H antigens, and genetic basis of their expression

Structures

Blood group A and B activities were initially found by T. Yamakawa & T. Iida (*20*) to be associated with neutral GSLs from erythrocyte 'ghosts', before blood group ABH epitopes of mucin-type glycoproteins were well established biochemically (*21*). Subsequently, A and H epitopes were found to be carried by lacto-series type 2 with varying chain length and degree of branching, which were named as A^a, A^b, A^c, A^d and H_1, H_2, H_3, H_4, based on thin-layer chromatography (TLC) pattern (*22*). Structures of blood group A subtypes A1 *versus* A2 were identified later as repetitive A (type 3 chain A) (*23*) and A-associated H (type 3 chain H) (*24*), *i.e*. GalNAcα3[Fucα2]Galβ3GalNAcα3[Fucα2]Galβ3GlcNAcβ3Galβ4Glcβ1Cer as A1, and Fucα2Galβ3GalNAcα3[Fucα2]Galβ3GlcNAcβ3Galβ4Glcβ1Cer as A2. These structures are expressed exclusively in GSLs but not in glycoproteins (*23*, *24*).

Genes (ABO alleles)

A few cDNAs encoding A enzyme (UDP-GalNAc: H-α-GalNAc transferase) were cloned based on amino acid sequence of purified A enzyme, and their structures were compared with those of homologous cDNA from blood cells of B and O individuals (genotype *BB*, *OO*) (*25*). Four nucleotide substitutions and four corresponding amino acid sequences essential for expression of A^1 allele and *B* allele, and differences between A and B enzymes, were identified. Amino acids 266 and 268, *i.e*. Leu and Gly for A enzyme *versus* Met and Ala for B enzyme, were dominant in determining A *versus* B activity (presumably recognizing UDP-GalNAc *versus* UDP-Gal) (*26*). Typical *O* allele (O^1) was characterized by deletion of nucleotide 261G, causing frame shift and encoding of an entirely different, short polypeptide, due to appearance of early termination codon at nucleotide 354 (*26*).

The genomic structure of ABO genes consists of seven exons which span ~19 kb of genomic DNA on chromosome 9, band q34. Most of the coding sequence is located in exon 7 (*27*). Deletion or reduction of A antigen in cancer cells is often associated with enhanced malignancy of cancer (*28*). Analysis of the 5′ upstream region indicated reduced promoter activity of CBF/NF-Y binding region, and possibly enhanced DNA methylation of A transferase promoter (*29*).

Cell adhesion and signal transduction mediated by carbohydrate–carbohydrate interaction

Le^x-to-Le^x interaction

A few lines of evidence indicated that Le^x epitope may mediate adhesion of ES cells or auto-aggregation of EC cells. Since carbohydrate-binding proteins are often considered to mediate carbohydrate-dependent cell adhesion, a few experiments were performed to determine whether Le^x-binding proteins are involved in EC F9 cell adhesion. However, no such protein was found. Instead, a component having a large quantity of Le^x was found to inhibit the autoaggregation of F9 cells (*30*). This suggested a novel possibility that Le^x-to-Le^x interaction mediates F9 cell autoaggregation. Subsequently, studies along this line clearly indicated the occurrence of Le^x self-interaction as a basis of F9 cell autoaggregation in the presence of Ca^{2+}, which was inhibited by EDTA (*31*). Further studies indicated that multi-merization of poly-Le^x glycan, termed 'embryoglycan', occurred based on Le^x-to-Le^x interaction (*32*). Le^x-to-Le^x, but not Le^x-to-lactosyl, interaction was further confirmed by atomic force microscopy (*33*), and by aggregation of Le^x- *versus* lactosyl-gold nanoparticles (*34*). Le^x-to-Le^x, but not Le^x-to-Le^a, or Le^a-to-Le^a interaction was determined by contact of two vesicles containing various combination of GSLs based on change of vesicular angle θ_c, which reflects adhesion energy involved (*35*).

GM3-to-Gg3 or GM3-to-LacCer interaction

Adhesion of mouse B16 melanoma cells to mouse L5178 lymphoma cells was found to be based on interaction between GM3 (expressed on B16) and Gg3 (expressed on L5178), in the presence of Ca^{2+} (*36*). GM3-to-Gg3 interaction was quantitatively determined by surface plasmon resonance (SPR) spectroscopy (*37*).

GM3-to-LacCer interaction was suggested to mediate B16 melanoma cell metastasis (*38*). This possibility was supported by observed inhibition of melanoma metastasis by administration of lactosyl nanoparticles, but not glucosyl nanoparticles (*39*). GM3-to-LacCer interaction was further supported by quantitative interaction of micellar solution of LacCer or lactosylsphingosine with GM3-coated plate, by dose-dependent change of surface pressure, $\Delta\pi$ (*40*).

GalCer-to-sulphatide interaction

This interaction was initially suggested in a brief note (*41*). A series of studies on this specific CCI were performed by J.M. Boggs and colleagues, using various methods (*42*,*43*). They observed significant interaction between oligodendrocytes and myelin sheath membranes, based on this CCI (*44*). Addition of liposomes containing GalCer and/or sulphatide to cultured oligodendrocytes, caused depolymerization of actin and microtubule filaments. This is assumed to be induced by signal transduction through cell surface glycosynaptic domain (*45*) by this specific CCI.

GM3 interaction with N-linked glycans of growth factor receptors

Ganglioside GM3, but not GM1 or Gb4, was found to interact with epidermal growth factor receptor (EGFR), and to inhibit EGF-induced tyrosine kinase activity associated with EGFR (*46*), although the mechanism of GM3 interaction with EGFR and tyrosine kinase has been unclear. Recently, GM3 was found to interact with N-linked glycans having $\geqslant 3$ GlcNAc termini, through a novel Ca^{2+}-dependent CCI (*47*). The interaction has provided a possible basis for GM3 binding capability with EGFR, as well as inhibitory effect of GM3 on tyrosine kinase associated with EGFR, as below.

(i) EGFR purified from A431 cells binds to the lectin GS-II or to the monoclonal antibody J1. Both GS-II and J1 bind to GlcNAc, particularly multivalent GlcNAc termini of N-linked glycans. (ii) Polystyrene beads coated with GM3, when incubated with A431 cell lysate, could pull down EGFR. This process did not occur with beads coated with GM1 or Gb4. (iii) This process was inhibited by pre-incubation of GM3-coated beads with a specific oligosaccharide, termed "Os Fr.B", which has 5–6 GlcNAc termini, but not another oligosaccharide having 2 GlcNAc termini (*48*).

Perspectives

Following the discovery of sphingosine,sphingomyelin, and galactosylceramide (cerebroside), the simplest form of glycosphingolipids (GSLs), by J.L.W. Thudichum (1829–1901), over 100 years ago, structural variation of GSLs was studied by a few pioneers: Gunnar Blix, Ernst Klenk, Richard Kuhn, Herbert Carter, Tamio Yamakawa and their colleagues. Further studies on GSL structure were carried on by the 'next generation'—which includes Hakomori. Thus, three series of GSL backbone structures, ganglio- (GalNAcβ4Galβ4GlcCer core), lacto- (GlcNAcβ3/4Galβ4GlcCer core) and globo-series (Galα4Galβ4GlcCer core), were established. So far, ~50 ganglio-, ~80 lacto- and ~10 globo-series structures are known.

Current trends of GSL studies are focused on their functional roles in defining cellular phenotype associated with developmental process and disease progression. A few of them are described below.

(i) GSLs, organized at "glycosynaptic microdomains", control expression of various cellular phenotypes through their interactions with growth factor receptors (GFRs), integrins, caveolins, tetraspanins (TSPs), Src family kinases, and various other signal transducers. In previous studies along this line, as described in the first section, types of microdomain components and their combinations were limited. GSLs used were GM3 and GM2, or their hybrid; GFRs were EGFR, FGFR, and cMet; TSPs were CD9 and CD82; integrins were α3β1 and α5β1. In future studies, we expect that combinations of basic components in such microdomains will be increased, and found to affect a large variety of phenotypes, depending on microenvironment of cells, and will be correlated with developmental stage, and with type of disease status. Inhibitory effect of sialyl-nLc4 on insulin receptor and its kinase is much higher than that of GM3 (49). Plasmalopsychosine, a novel conjugate of plasmal (fatty aldehyde) to galactosylsphingosine, displays activation of nerve growth factor receptor kinase. In contrast, psychosine inhibited this receptor kinase (50). Extension of these studies may open new vistas for control of diabetes or neuronal diseases. There are many possibilities for application of other GSLs with different receptors and signal transducers.

(ii) Epithelial cells change their morphology, motility, and growth when they come in contact with a new microenvironment, whereby epithelial molecules (*e.g.*, Ecad, desmoplakin) decline, and mesenchymal molecules (*e.g.*, fibronectin, vimentin, Ncad) increase. This process, termed "epithelial-mesenchymal transition" (EMT), is considered to provide basic molecular changes associated with developmental processes and cancer progression (*51*, *52*). GSL function in "defining oncogenesis and its reversion", as described in the first section, is closely associated with EMT/hypoxia process, and better understood in that context.

We found recently that reduction or deletion of specific GSLs (*e.g.*, Gg4 or GM2) is closely associated with EMT process of mouse and human epithelial cells induced by treatment of cells with TGFβ, the well-known EMT inducer. A possibility is opened that Gg4 or GM2 is required for expression of epithelial cell phenotype, and reduction or deletion of such GSL causes EMT process associated with decline of epithelial molecules, with enhanced expression of

mesenchymal molecules. In fact, exogenous addition of Gg4 or GM2 inhibited EMT process, in terms of changes of morphology, motility, expression of epithelial and mesenchymal molecules (*53*). More recent studies showed that transfection of Gg4 synthase gene caused enhancement of endogenous Gg4, causing similar phenotypic reversion of EMT process as exogenous addition.

(iii) Some approaches, previously developed for determining expression pattern of glycogenes and glycosyl epitopes in cells and tissues, have been and will be increasingly utilized: (a) RNAs, extracted from given cells and tissues, are probed by glycogene microarray, typically provided by Consortium for Functional Glycomics, Scripps Research Institute, La Jolla, CA, USA. A total of ~1246 glycogenes are known, covering glycosyltransferases, hydrolases, carbohydrate-binding proteins, sugar nucleotide synthases, and their transporters. (b) Tissue microarrays from normal and diseased regions of tissues, including benign and malignant cancers, and their metastatic deposits, are commercially available. *In situ* expression of glycans is examined by immunohistochemical procedure using specific anti-glycan antibodies, or *in situ* expression of specific mRNAs is examined by *in situ* hybridization with specific DNA probes using these tissue microarrays. Results of these studies will be used to evaluate the significance of glycan changes in cancer and various other disease processes, and finally for therapeutic application and to prevent disease progression.

Reduction or deletion of specific GSLs (*e.g.* Gg4 or GM2) expressed in epithelial cells affects the EMT process induced by TGFβ, and exogenous addition of such GSLs inhibits the EMT process (*53*). Recent studies indicate that expression of specific GSLs, synthases and their genes involved in EMT are the same as those involved in hypoxia (Guan, F., Schaffer, L., Handa, K., and Hakomori, S., unpublished data). GSL function in 'defining oncogenesis and its reversion', as described in the first section, is closely associated with EMT/hypoxia process, and better understood in that context.

A specific glycosyl epitope in tumour cells whose expression is affected during EMT process or in hypoxia may be associated with the capability of inducing cancer stem cells (CSC), which is known to provide self-renewing, invasive property of tumour cells (*54*, *55*). To explore this possibility, a pair of cell lines, termed 'a' (expressing antigen A) and 'b' (not expressing antigen A) can be cultured in soft agar under hypoxia, in order to determine whether those glycosyl epitopes expressed in tumours are capable of inducing CSC, which may be characterized by expression of SSEA stem cell markers, particularly SSEA-3 and -4.

Conflict of interest
None declared.

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