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JB Review

Fine tuning of cell signals by glycosylation

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Carbohydrates on the glycoproteins and glycosphingolipids expressed on the cell surface membrane play crucial roles in the determination of cell fates by being involved in the fine tuning of cell signalling as reaction molecules in the front line to various extrinsic stimulants. In glycoproteins, modification of proteins is performed by substitution of sugar chains to one or multiple sites of individual proteins, leading to quantitative and qualitative changes of receptor functions in the cell membrane. As for glycosphingolipids, majority of them consist of two moieties, i.e. carbohydrates and ceramides, and are localized in the microdomains such as lipid rafts or detergent-resistant microdomains. They generate and/or modulate cell signals to determine the cell fates by interacting with various carbohydrate-recognizing proteins. Modes of glycosylation and mechanisms by which glycosylation is involved in the regulation of cell signals are now hot subjects in glycobiology.

Keywords: glycosylation/carbohydrate/lipid raft/microdomain/glycolipid.

It is long time since the facts were demonstrated that main factors involved in the evolution of cancers are composed of gene mutation on chromosome, gene deletion, or gene amplification. In particular, the 'multistep oncogenesis' theory, e.g. accumulated multiple gene alterations in the cells result in the evolution of cancer, has been widely accepted (*1*) with colon cancers as representative examples. During the progress of these studies, involvement of many oncogenes and suppressor genes has been demonstrated. Simultaneously, implication of genetic background and extrinsic factors, such as mutagenic chemicals, UV and irradiation, and infectious media like viruses, and biological factors, such as chromosomal translocation in the cancer evolution, has been well understood (*2*). Furthermore, in addition to the changes in the gene expression and function based on the altered base sequences, chemical modification of DNA such as DNA methylation and of histone proteins such as methylation, acetylation and phosphorylation have been demonstrated to be involved in the regulation of gene expression, and these chemical modification due to the extrinsic factors have been reported to be inherited to daughter cells (*3*).

These facts indicate that DNA and its regulatory factors in nuclei play crucial roles in the expression of cellular functions not only in cancers but also in many other cells as a 'playmaker'.

Nevertheless, interactions occurring on the peripheral regions and cell surface with extrinsic factors are direct and decisive events in the determination of cell responses and fates. Outcome of various phenomena taken place here is transmitted to nuclei as signals and affects greatly the contents and features of genetic information in nuclei. In particular, carbohydrates in complex carbohydrates such as glycoproteins and glycolipids on the cell membrane should function as effector molecules and/or parts of the effector molecules in the responses to the environmental changes and extrinsic stimulants to exert fine tuning of signalling (*4*). Recently, a number of these examples have been reported, and mechanisms for these regulations with glycosylation are quite diverse.

In this review, we would try to introduce recent notable reports in this field and to draw common scientific principles shared among independent studies with focus on the regulation of cell signalling and its implication in the individual cell functions.

Types of Glycosylation and Their Implication

Among complex carbohydrates, there are glycoproteins and some proteoglycans, which penetrate lipid bilayer membrane, and glycosphingolipids and glycosylphosphoinositide (GPI)-anchored proteins, both of which anchored in the outer layer of the membrane. In almost all cases, carbohydrates are attached to outside portion of the membrane molecules. When *N*-glycans cannot be attached to membrane proteins for some reasons, those proteins are often unable to be expressed on the cell surface.

Generally speaking, importance of carbohydrates in the functions of glycoproteins is relatively low in *N*-glycans since functions of carrier proteins are predominant, and glycosylation often plays as a modulator of the protein functions. In the case of *O*-glycans, roles of carbohydrates are usually dominant. As for

proteoglycans, chemical structures, length of sugar chains, and sulfation patterns of the carbohydrates are more important in their biological functions than the core peptides. Moreover, it is not rare that no core proteins are associated with glycosaminoglycans.

Carbohydrates-Mediated Signalling via Extrinsic and Intrinsic Factors

When some complex carbohydrates exert functions, it becomes possible only by the presence of ligand molecules that recognize specific structures of carbohydrates and bind with them. To date, a number of endogenous lectin families (*5*) such as selectins, galectins, siglecs and C-type lectins have been identified and are being investigated for their functions. However, majority of them except some lectins such as selectins are not clear in their binding specificities. In turn, we have to say that there have been no endogenous ligand proteins defined that specifically recognize individual carbohydrate structures. On the other hand, it is well known that some bacteria-derived toxins recognize particular sugar chains, particularly those on glycolipids, and utilize them as their receptors. High specificity of the interaction between toxins and glycolipids is well-known, and the functional processes of the toxic effects via those receptors have been well studied (*6*). For instances, GM1 for cholera toxin, b-series gangliosides for tetanus toxin, Gb3/CD77 for Shiga-like toxin (verotoxin) are well-known, and some of them are being utilized in the experimental and clinical fields (*7*,*8*). Although it is not clear why the binding specificity of carbohydrate structures to the intrinsic ligands are not definite compared to that to the extrinsic factors, weak binding between carbohydrates and endogenous ligand molecules with gradual intensities might have advantageous aspects for our bodies. It seems also true that we have not necessarily made systematic efforts to search ligand molecules for individual carbohydrate structures so far. Therefore, it is likely that intrinsic genuine carbohydrate-recognizing molecules will be found in the future.

Regulation of Protein Functions by Attached Carbohydrates

There have been a number of studies on the roles of carbohydrates in membrane glycoproteins as regulatory mechanisms for the protein functions. Here, we would introduce prominent studies performed recently. Ohtsubo *et al.* demonstrated that modification of *N*-glycans on Glut-2, that is important effector of insulin, by GnT-IVa regulates the localization and function of the molecule (*9*). For the mechanisms, they showed that transcription factors such as Foxa3 and Hnf1a regulate expression levels of GnT-IVa by sensing levels of blood sugar and fatty acids. On the other hand, Taniguchi's group has demonstrated that two major modification of *N*-glycans, i.e. bisecting structure with GnT-III and tetra-anntenary structures with GnT-V are playing a role of switch to determine the malignancy of cancer cells (*10*). Furthermore, they found that the presence or absence of core fucose at the initial site of *N*-glycans regulates function of TGFβ receptors, and a defect in the fucosylation causes emphysema (*11*). Gu *et al.* reported that *N*-glycan structures on integrins play roles in the regulation of quantity and quality of the adhesion signals (*12*).

Regulation of Cell Signalling by Glycosphingolipids

It has been known that GM1 enhances differentiation signals mediated via NGF/TrkA in neuronal cells and protects apoptosis signals induced by serum deprivation (*13*). These results were obtained by the experiments in which exogenous GM1 was added to a cultured rat pheochromocytoma cell line, PC12 (*13*). On the other hand, PC12 cell lines transfected by GM1/GD1b/GA1synthase cDNA showed that GM1 rather suppresses the differentiation signals by NGF (*14*). In this case, phosphorylation and dimerization of TrkA after NGF stimulation were strongly suppressed, and the phosphorylation reaction of Erk1/2 was also markedly suppressed. More interestingly, over-expression of GM1 resulted in the shift of TrkA from lipid rafts to non-lipid rafts, suggesting that this changes in the intracellular localization of TrkA might be a main cause for the lowered transmission of NGF signalling.

As for growth signals, GM1 expression resulted in the suppression of cell growth and growth signals caused by exogenous stimulations in Swiss 3T3 (*15*), Lewis lung cancer (LLC) (*16*) and SK-MEL-37 (*17*). In LLC, GM1 also suppressed metastatic potential (*16*), indicating that GM1 and GM1 synthase generally suppresses malignant properties in cancer cells. Furthermore, gene silencing of GM1 synthase in the parent cell of LLC resulted in the enhancement of cell growth, invasion and metastatic potential (*16*), providing an evidence for the suppressive function of GM1 to the malignant properties. Taken together with GM1 effects in PC12 cells, it was concluded that expression of GM1 synthase disturbs assembly of signalling molecules in lipid rafts, and suppresses growth/differentiation signals. In addition, not only GM1, but GM2 also suppressed metastatic potential of LLC by reducing phosphorylation levels of FAK (*18*). This result supported that monosialyl gangliosides generally suppress cancer phenotypes as summarized in Fig. 1.

Hakomori *et al.* reported that ganglioside GM3 suppresses functions of EGF receptor and its phosphorylation signals upon EGF stimulation (*19*). Similarly, Inokuchi *et al.* reported that GM3 expression in adipose tissues suppresses functions of insulin receptors (*20*). Taken all these findings together, it is suggested that monosialyl compounds generally suppress cell signals with minor differences in their mechanisms.

All these results are in good contrast with functions of disialyl glycolipids, which will be described below (Fig. 1).

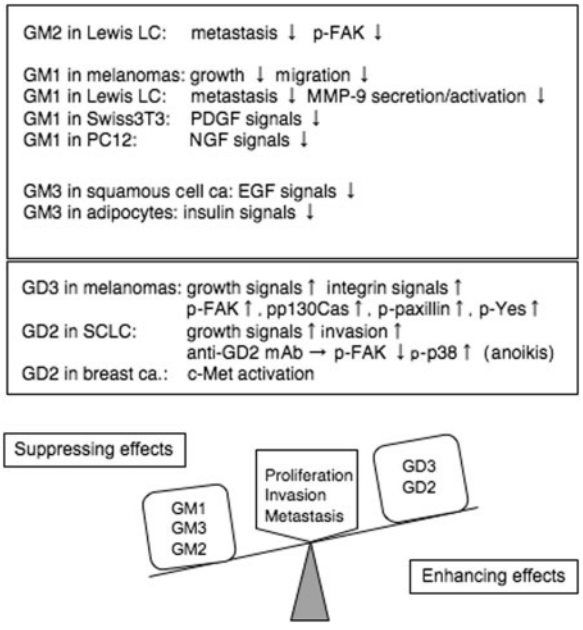

Fig. 1 Contrastive effects of the expression of glycosphingolipids on the cell phenotypes were found between monosialyl and tandem-type disialyl compounds. Mouse Swiss 3T3, Lewis lung cancer and human melanoma SK-MEL-37 showed suppressed phenotypes by the introduction of GM1 synthase cDNA. Metastatic potential was also suppressed in Lewis lung cancer, suggesting that GM1 synthase expression leads cancer cells to rather suppressed malignant properties. On the other hand, disialyl compounds generally enhanced cell growth and invasion and activated related signal molecules. This figure was reproduced with modification of Fig. 1 in Experimental Medicine (Extra Issue), Vol. 30, No. 5, pp113, 2012 with permission of Yodosha Co., Ltd, Tokyo, Japan.

Enhancement of Growth Signals and Adhesion Signals by Disialyl Gangliosides

Our group has studied on the functions of tandem-type disialyl glycolipids mainly in malignant melanomas. Above all, gangliosides GD3, GD2 and GM2 have been considered to be cancer-associated carbohydrate antigens and been expected as target molecules of cancer therapeutics. We have analysed implication of GD3 in human melanomas by establishing transfectant cells of GD3 synthase cDNA into GD3-negative mutant of SK-MEL-28 (N1). Resultant changes in the malignant properties and cell signalling caused under neo-expression of GD3 have been examined. Consequently, it was demonstrated that phosphorylation levels of adaptor molecules, p130Cas, paxillin or FAK (focal adhesion kinase) were strongly enhanced in GD3+ cells (*21*). Furthermore, a Src family kinase, Yes, is constitutively activated and tightly bound to p130Cas and FAK in GD3+ cells (*22*). Higher amount of Yes was found in lipid rafts in GD3+ cells than in GD3- cells even before any stimulation. As for adhesion signals, it was demonstrated that adhesion signals via integrins were strongly enhanced based on shifts of integrins to lipid rafts and on the cluster formation of integrins in lipid rafts under GD3 expression (*23*). Interestingly, it was shown that co-existence of growth stimulation and adhesion signal is essential for the tyrosine phosphorylation of p130Cas and paxillin. These results suggest that signals from growth factor receptors and those from adhesion receptors merge and converge under GD3 expression, leading to the generation of much stronger signals than those derived from either signalling pathway (Fig. 2).

On the other hand, it was demonstrated by our group that unique ganglioside GD2 was expressed in small cell lung cancers (SCLCs) (*24*). On the other hand, non-small cell lung cancers (NCLC) expressed GM2. Essential difference between SCLC and NSCLC in terms of main glycosylation was the specific expression of GD3 synthase in SCLCs. As shown in melanoma study, GD2 expression in SCLCs resulted in the increased cell growth and invasion activity. Striking difference between melanomas and SCLCs was that anti-GD2 antibodies induced apoptosis in SCLC cells (*25*). Binding of anti-GD2 monoclonal antibodies triggered dephosphorylation of FAK, leading to the activation of p38 and finally to the induction of anoikis. Delannoy *et al*. also examined effects of GD2 expression in human breast cancer cells on their cancer phenotypes (*26*). They showed that GD2 expression induced phosphorylation of c-Met independently from HGF, and it was unique function of GD2, not of GD3.

Regulatory Mechanisms for Cell Signalling at Lipid Rafts

All these results described above are difficult to understand without considering lipid rafts on the cell membrane. In particular, glycosphingolipids are one of major resident components in lipid rafts, and the facts that alterations in the carbohydrate moiety of glycolipids crucially affect the architectures and functions of lipid rafts have been shown in a number of studies (*27*). Originally, main functions of lipid rafts were proposed to be sites of membrane trafficking, cholesterol metabolism and endocytosis etc (*28*). Recently, a number of reports on its role in the regulation of signal transduction and in a clue of various infections have been markedly accumulated (*29*). Although there have been arguments on the ambiguity of the concept about lipid rafts, particularly about defects of visualization of molecular complex on living cell surface (*30*), analyses on the substantial basis of lipid rafts have been enormously developed by progress in chemical analysis of lipid structures and in visualization of membrane molecules with one-molecule imaging (*31*). Consequently, understanding of polymorphic natures of lipid rafts and of their hierarchical architecture has been markedly advanced. Simons *et al*. classified formation processes of lipid rafts into three phases (*32*), namely, Phase 1: nanoscale assembly, resting state; Phase 2: raft platform, activated, clustered rafts; Phase 3, raft phase, large raft cluster (Fig. 3). In this Phase 2, shift of proteins to lipid rafts and their interactions with lipids, oligomerization and activation occur, and these interactions between carbohydrates on glycolipids and their ligand proteins generate important signals. Furthermore,

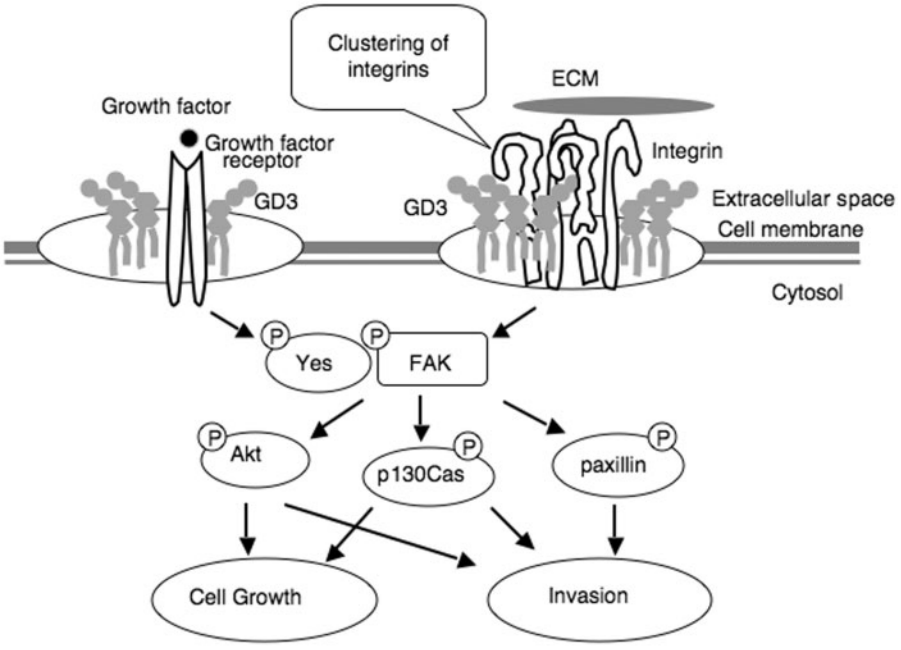

Fig. 2 Two major signaling pathways are merged and converged under expression of GD3 in melanoma cells. GD3 is localized in lipid rafts, playing a role for convergence of growth signals and adhesion signals to generate high magnitude of malignant signals in melanomas. This figure was reproduced with modification of Figure 2 in Experimental Medicine (Extra Issue), Vol. 30, No. 5, pp114, 2012 with permission of Yodosha Co., Ltd, Tokyo, Japan.

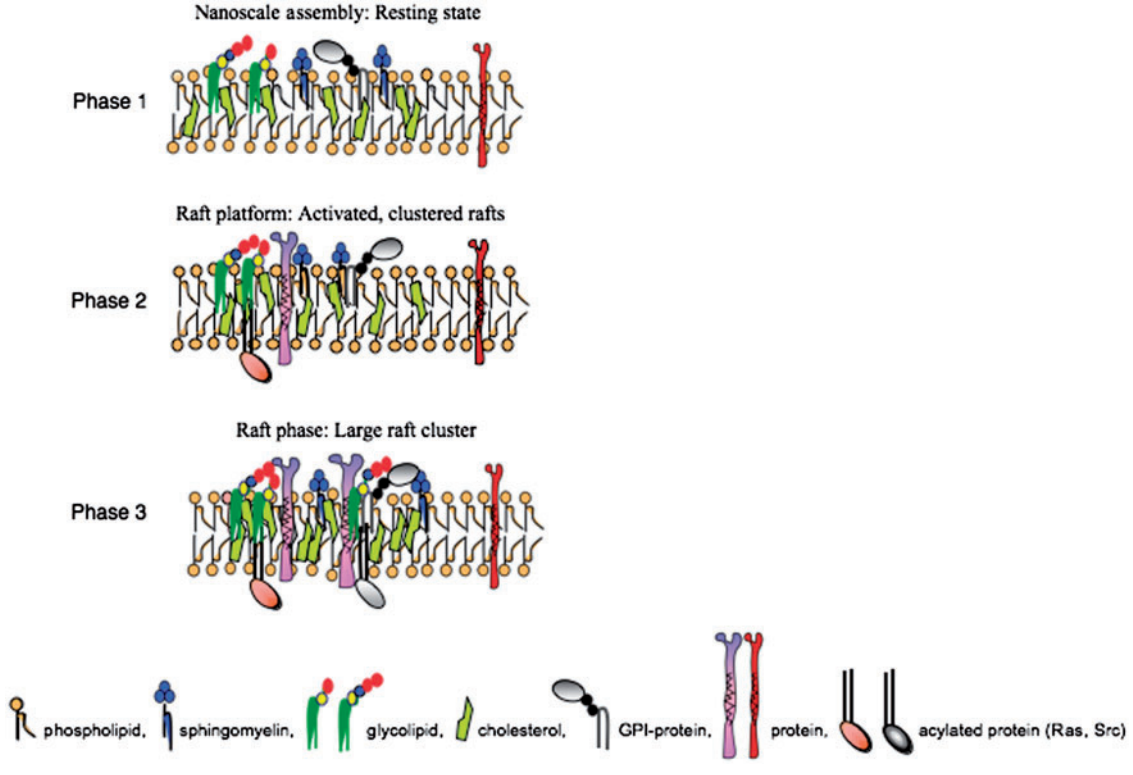

Fig. 3 Three phases of lipid rafts formation. Processes of lipid rafts formation are classified into three phases depending on the assembly of protein molecules and sizes of the microdomains (*31*). Phase 1: nanoscale assembly, resting state; Phase 2: raft platform, activated, clustered rafts; Phase 3: raft phase, large raft cluster. Modified from Ref. (*31*). This figure was reproduced with modification of Figure 3 in Experimental Medicine (Extra Issue), Vol. 30, No. 5, pp115, 2012 with permission of Yodosha Co., Ltd, Tokyo, Japan.

lipid rafts in cancer cells seem to be already at this Phase 2. As described above, melanoma cells expressing GD3 are considered to be just in this situation.

Regulation of Differentiation and Growth Signals by Proteoglycan Glycosaminoglycans

It has been long known that proteoglycans regulate cell differentiation and growth signals by forming molecular complexes with growth factors and their receptors on the cell surface membrane (*33*). However, it is not necessarily clear at this moment whether these molecular complexes are a machinery to promote the binding of various growth factors to their receptors or to facilitate the accessibility of factors to the receptors and assembly of them. They might be sorts of device for the storage of the factors. Probably, all these explanations might express true aspects of the facts, but unknown factors and unknown mechanisms should exist and regulate reasonable complex formation and its degradation. In particular, elucidation of the physiological degradation systems for glycosaminoglycans is strongly expected.

Conclusion

All results described above suggest that 'glycosylation' plays as a fine tuner of cell signalling. However, 'fine' does not necessarily mean that the range of the tuning is minute. 'Fine' alterations in chemical structures in carbohydrates may frequently result in dramatic changes, we think. Recently, Contreras *et al*. reported that membrane-anchoring proteins contain a common motif for the binding with particular forms of shingolipids in transmenbrane domains (*34*). These results strongly support our findings that membrane proteins accumulate in lipid rafts and efficiently transduce cell signals during the signal introduction, and glycosphingolipids modulate these processes as described above. Their findings concretely suggest the mechanisms for the regulation of signals by glycosphingolipids. To date, substantial basis for the concept of lipid rafts has been weak. But it seems to become increasingly realistic with apparent experimental evidences. In these processes, importance of the heterogenous lipid structures in ceramide portions is now increasingly recognized as well as that of carbohydrate moiety. Thus, the meaning of whole structures of individual glycolipids in their unique functions is now being clarified.

Conflict of interest

None declared.

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