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Disialyl gangliosides enhance tumor phenotypes with differential modalities

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Abstract Sialic acid-containing glycosphingolipids, gangliosides are highly expressed in human cancer cells and regulate cell signals transduced *via* membrane microdomains. Generally, disialyl gangliosides enhance tumor phenotypes, while monosialyl gangliosides suppress them. In particular, gangliosides GD3 and GD2 are highly expressed in melanomas and small cell lung cancer cells, and their expression cause increased cell growth and invasion. In osteosarcomas, expression of GD3 and GD2 also enhanced cell invasion and motility, and caused increased phosphorylation of focal adhesion kinase and paxillin. In addition to focal adhesion kinase, Lyn kinase was also activated by GD3/GD2 expression, leading to the phosphorylation of paxillin. In contrast with melanoma cells, osteosarcomas showed reduced cell adhesion with increased phosphorylation of paxillin. Thus, increased expression of GD3/GD2 caused enhanced activation of signaling molecules, leading to distinct phenotypes between melanomas and osteosarcomas, *i.e.* increased and decreased adhesion activity. Thus, whole features of glycolipid-enriched microdomain/rafts formed in the individual cancer types seem to determine the main signaling pathway and biological outcome.

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Introduction

It has been reported by many researchers that cancer cells express unique carbohydrate structures in glycoproteins and glycolipids that can not be detected in normal cells and tissues on the cell surface membrane [1]. Since the mechanisms for the synthesis of carbohydrate structures in complex carbohydrates have been well understood mainly due to the progress in the molecular cloning of glycosyltransferase genes, it became relatively easy to understand the changes in the whole features of glycosylation during the transformation of cells to malignant tumors. In particular, the "multistep oncogenesis" theory, *e.g.* accumulated multiple gene alterations in the cells resulting in the evolution of cancer [2], has prompted us to understand the phenotypes of cancers by considering the functions of oncogenes and suppressor genes. Accordingly, the expression and implication of carbohydrate antigens, which are characteristically expressed in particular cancers have been analyzed in the context of cell transformation.

We have analyzed expression and function of cancer-associated glycosphingolipids mainly in neuroectoderm-derived cancers and leukemia cells, and have reported that disialyl gangliosides generally enhance tumor phenotypes such as cell proliferation, invasion and motility [3]. On the other hand, expression of monosialyl gangliosides such as GM2 and GM1 tend to suppress tumor phenotypes not only cell growth and invasion, but also metastatic potential [4, 5]. Hakomori's group also reported that GM3 suppresses tumor phenotypes and regulates EGF receptor-mediated signals [6, 7]. Thus, we have concluded at this moment that disialyl

gangliosides enhance tumor phenotypes, while monosialyl gangliosides generally suppress them [4] whatever the mechanisms are.

Since interactions occurring on the peripheral regions and cell surface between various stimulants and cell receptors are direct and decisive events in the determination of cell responses and fates. Outcome of various interactions taken place here is transmitted to cytoplasmic molecules and/or nuclei, and affects greatly the cell behaviors and responses including epigenetic regulation. In particular, carbohydrates in complex carbohydrates on the cell membrane should function as effecter molecules and/or parts of the effecter molecules in the responses to the environmental changes and extrinsic stimulants to exert fine tuning of signaling [8].

In this review, we would try to introduce recent findings on the regulation of cell signaling by cancer-associated glycosphingolipids with focus on the diversity in the modes of their regulatory actions in several representative cancers.

Disialyl ganglioside GD3 enhances cancer phenotypes of melanoma cells

We have long analyzed functions of sialic acid-containing glycolipids, gangliosides, mainly in malignant melanomas. Actually, gangliosides GD3, GD2 and GM2 have been considered to be cancer-associated antigens, and been expected as target molecules of cancer therapeutics such as antibody therapy [9]. Above all, we have analyzed implication of tandem-type disialyl gangliosides such as GD3 in human melanomas by transfecting GD3 synthase cDNA into a GD3-negative mutant of SK-MEL-28 (N1). Resultant changes in the malignant properties and cell signaling caused by neo-expression of GD3 have been examined. Compared to GD3- control cells, phosphorylation levels of adaptor molecules, p130Cas, paxillin and focal adhesion kinase (FAK) after serum treatment were strongly enhanced in GD3+ cells [10]. Furthermore, we reported that a Src family kinase, Yes was definitely co-precipitated with p130Cas or FAK, and was shown to be in an activated form before serum stimulation in GD3+ cells [11]. Higher amount of Yes was found in glycolipid-enriched microdomain (GEM)/rafts in GD3+ cells than in GD3- cells even without any stimulation. As for integrin-mediated adhesion signals, it was demonstrated that integrin functions were strongly enhanced as analyzed by adhesion to coated fibronectin and collagen type I and IV [12]. As mechanisms, shifts of integrins to GEM/rafts and the cluster formation of integrins in GEM/rafts under GD3 expression [12] were demonstrated. As a most important fact as a GD3 function, it was shown that co-existence of growth factor receptor-mediated signal and adhesion signal are essential for the strong tyrosine phosphorylation of p130Cas and paxillin [13]. These results suggest that two main signaling pathways, *i.e.* growth signal and adhesion signal should merge and converge under GD3 expression, leading to the generation of much stronger signals than those derived from either signaling pathway, forming the basis of cancer phenotypes [14] as shown in Fig. 1.

Enhancement of tumor phenotypes by disialyl gangliosside GD2

On the other hand, it was demonstrated by us that ganglioside GD2 was expressed in small cell lung cancers (SCLCs) (15), while non-small cell lung cancers (NCLCs) generally expressed GM2. Essential difference between SCLC and NSCLC in terms of main active glycosylation pathway was the specific expression of GD3 synthase in SCLCs. GD2 expression in SCLCs resulted in the increased cell growth and invasion activity [15]. A striking difference between GD3 in melanomas and GD2 in SCLCs was that only anti-GD2 antibodies induced apoptosis in SCLC cells [16]. Binding of anti-GD2 monoclonal antibodies triggered dephosphorylation of FAK, leading to the activation of a MAPK, p38 and finally to the induction of anoikis. More over, addition of anti-GD2 monoclonal antibody resulted in the increase of chemosensitivity of lung cancer cells to anti-cancer drugs such as CDDP [17], suggesting that combination therapy of anti-GD2 antibodies and anti-cancer drugs is promising. Delannoy *et al.* also demonstrated effects of GD2 expression in human breast cancer cells on their cancer phenotypes [18]. They showed that GD2 expression induced tyrosine phosphorylation of HGF receptor, c-Met independently from HGF. Only GD2, but not GD3 showed unique

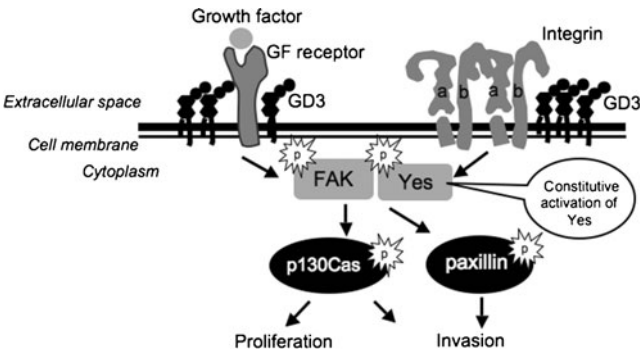

Fig. 1 Enhancement of malignant phenotypes by GD3 at cell membrane of melanoma cells. High expression of GD3 results in the enhancement of growth factor/receptor signals and adhesion signals *via* clustering of integrins. Convergence of both signals by GD3 expression leads cancer phenotypes such as increased proliferation and invasion

function in breast cancers [19]. Although a recent paper by Battula *et al.* reported that GD2 is a stem cell marker in human breast cancers [20], GD2 might not be a mere marker of breast cancer stem cell. Potentially, GD2 should play a crucial role in the survival and/or resistance to therapeutic agents as suggested in the functional analyses as described above.

Disialyl gangliosides GD3/GD2 enhance tumor phenotypes of osteosarcoma cells by unique modalities

Effects of disialyl gangliosides on the tumor phenotypes were further examined in osteosarcoma cells, since they showed high expression levels of GD2 and GD3 [21]. As reported previously, the majority of osteosarcoma cell lines have been considered to express high levels of GD2 [22]. As shown in melanomas, GD2/GD3 expression enhanced tumor invasion and cell motility with increased activation of either FAK or Lyn, resulting in the activation of a common target molecule, paxillin [21] (Fig. 2). Eventually, simultaneous knockdown of FAK and Lyn completely suppressed phosphorylation of paxillin and reduced cell invasion and motility, suggesting the cooperative effects of two parallel signaling pathways in osteosarcomas. A most distinct point from melanoma cells was that cell growth was not affected by the expression of disialyl gangliosides in osteosarcomas [10].

In line with this difference in the effects of ganglioside expression on the tumor phenotypes between melanomas and osteosarcomas, intriguing differences in the cell adhesion were demonstrated. When four subtypes of an osteosarcoma cell line HOS (GD3+, GD2+, GD3+/GD2+, GD2-/GD3-) were compared about their phenotypes and signaling, GD3+/GD2+ cells showed almost no adhesion in real-time cell electronic sensing system [21], while these cells showed the strongest phosphorylation of paxillin during cell "adhesion". So, the intensity in the phosphorylation and that in cell adhesion was completely adverse. It seems slightly hard to explain how the weakest adhesion can induce the strongest activation of paxillin. Whatever the mechanisms are, these results were quite in contrast to those in melanomas, in which strong phosphorylation of p130Cas, paxillin and FAK paralleled with the intensities in cell growth, invasion and adhesion [12].

Recapitulation of the interaction between gangliosides and signaling molecules in the reconstructed membrane-like system

In order to investigate the mechanisms for gangliosides to regulate cell signaling transduced *via* cell membrane, it might be most straightforward to verify direct binding of gangliosides with membrane molecules. For this purpose, many efforts have been performed with limited success [23]. The reason for the failure in the co-precipitation is not known now, but the interaction might not be so strong to be co-precipitated after solubilization, or intervening molecules between them might exist. Even if direct binding between gangliosides and membrane molecules is not verified, there are many cases where functional interactionas are strongly suspected between them. To examine the interaction between gangliosides and membrane molecules in cell membrane, we developed a liposome system in which

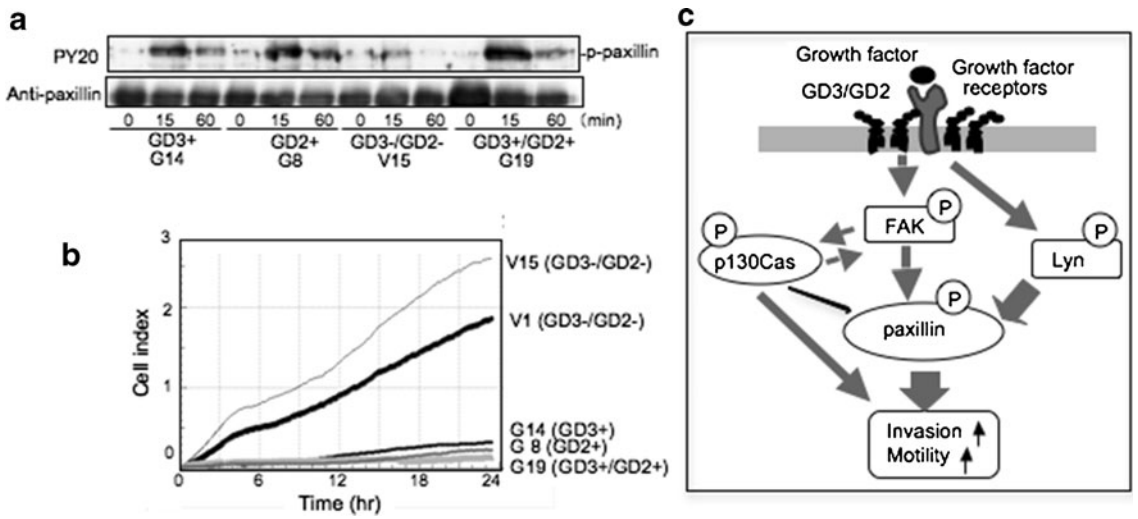

Fig. 2 Cell adhesion and signaling pathway are regulated by GD3/GD2 expression in osteosarcoma cells. **a** Immunoblotting of phosphotyrosine with PY20 using cell lysates from four types of osteosarcoma cell line HOS during cell adhesion. Immunoblotting with anti-paxillin was performed as a control. **b** Cell adhesion patterns of individual cell lines as measured by RT-CES (real time cell electron sensoring system). The resistance of currency by cells was presented as Cell Index. **c** Two major signaling pathways, *i.e.* FAK-paxillin and Lyn-paxillin were defined in the osteosarcoma line based on the results of knockdown experiments (extracted from Ref. 21)

glycolipids and membrane proteins as well as cholesterol and diacylglycerol were embedded [11]. In this system, GD3 added to liposomes could enhance kinase activity of low-active Yes isolated from GD3- cells in a dose dependent manner (1~5 nM) as shown in Fig. 3. The effects of added GM1 to the liposome on relatively active Yes were just opposite, *i.e.* suppression of Yes kinase activity with GM1 along with its amounts was observed. Although the mechanisms for the physical interaction of GD3 and Yes is not clear now, this system seems very promising to investigate direct and/or functional interaction between glycosphingolipids and membrane proteins in the membrane-like environments.

Regulatory mechanisms for cell signaling at GEM/rafts

All these results described as effects of disialyl gangliosides are difficult to understand without the concept of GEM/rafts on the cell membrane. In addition to sphingomyelin, cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins, glycosphingolipids are also major residents in GEM/rafts. Actually, alterations in the carbohydrate moiety of glycolipids crucially affected the architectures and functions of GEM/rafts as demonstrated in a number of studies [19]. Originally, main functions of GEM/rafts were proposed to be platforms for membrane trafficking, cholesterol metabolism and endocytosis *etc.* [24]. In this decade, a number of reports on their roles in the regulation of signaling and as an initiation site for various infections have accumulated [25]. Although there have been arguments on the ambiguity of the concept about GEM/rafts such as defects of visualization of molecular complex on living cell surface [26], the substantial bases of GEM/rafts have been gradually clarified by the progress in chemical analysis of lipid structures and in imaging analysis of membrane molecules with very high magnification and high temporal resolution [27]. From various evidences, it has been suggested that there is compositional and functional heterogeneity in GEM/rafts [28] depending on the carbohydrate structures in glycolipids, or on the GPI-anchored proteins. Individual GEM/rafts seem to contain distinctly assembled membrane proteins. Now, what kind of GEM/rafts exist in one cell, remains to be investigated. Furthermore, dynamic changes in contents and sizes of GEM/rafts are urgent questions to be answered. Recently, Simons *et al.* classified formation processes of lipid rafts into 3 phases [29], *i.e.* phase 1. nanoscale assembly: resting state; phase 2, raft platform: activated, clustered rafts; phase 3, raft phase: large raft cluster visible under current microscope. In phase 2, shift of proteins to GEM/rafts and their interactions with lipids, oligomerization and activation occur. These interactions between glycolipids and their ligand proteins should generate important signals, and GEM/rafts in cancer cells seem to already reach this phase under disialyl gangliosides as described above. The mechanisms for disialyl gangliosides to enhance tumor phenotypes by differential modalities might depend on the features of GEM/rafts formed in

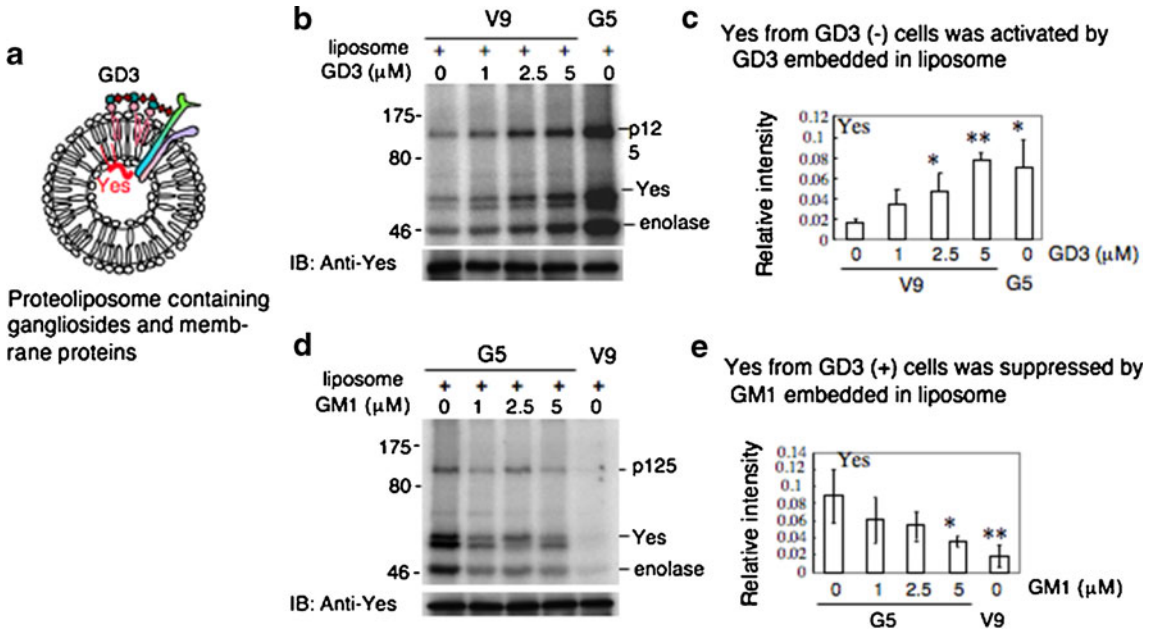

Fig. 3 Regulation of Yes functions by gangliosides. **a** Proteoliposomes as a reconstruction of membrane environment were developed. **b** Yes kinase was enhanced by embedded GD3 in a dose dependent manner. **c** Band intensities of phosphorylated Yes in a were plotted. **d** Yes was, in turn, suppressed by embedded GM1. **e** Band intensities of phosphorylated Yes in d were plotted (modified from Ref. 11)

the individual cancer types. Precise differences in the compositions of GEM/rafts between different types of cancers and their implication remain to be investigated in the near future.

Ending remarks

To construct strategies to overcome cancers, we need to further understand the mechanisms for cancer-associated glycolipids to regulate signals leading tumor phenotypes. In particular, the regulatory mechanisms of GEM/rafts with aberrant architecture and abnormal functions remain to be analyzed in the context of abnormal glycosylation. Simultaneously, various factors determining molecular shapes of glycolipids and features of GEM/rafts should be considered. For example, compositions of fatty acids in food, exposure to UV and natural irradiation and other environmental changes should be important. Since 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 the chemical modification due to the extrinsic factors have been reported to be inherited to daughter cells [30], epigenetic regulation of molecules involved in the glycosylation machineries during receipt of extrinsic stimulants remain to be investigated.

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