主論文の要旨

Ganglioside GD2 Enhances the Malignant Phenotypes of Melanoma Cells by Cooperating with Integrins

（ガングリオシドGD2はインテグリンとの協働作用により
メラノーマの悪性形質を増強する）

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【Abstract】

Cancer-associated gangliosides were reported to enhance the malignant properties of cancer cells. In fact, GD2-positive (GD2+) cells showed increased proliferation, invasion, and adhesion. However, the precise mechanisms by which gangliosides regulate cell signaling in glycolipid-enriched microdomain/rafts GEM/rafts are not well understood. To analyze the roles of GD2 in the malignant properties of melanomas, we searched for GD2-associating molecules on the cell membrane using the enzyme-mediated activation of radical sources combined with mass spectrometry, and integrin β1 was identified as a GD2-associating molecule. Then, we showed the physical association of GD2 and integrin β1 by immunoprecipitation/ immunoblotting. Close localization was also shown by immunocytochemistry and proximity ligation assay. During cell adhesion, GD2+ cells showed multiple phospho-tyrosine bands, epithelial growth factor receptor and focal adhesion kinase.─Knockdown of integrin β1 revealed that the increased malignant phenotypes in GD2+ cells were cancelled. Furthermore, the phosphor-tyrosine bands detected during the adhesion of GD2+ cells disappeared after the knockdown of integrin β1. Finally, immunoblotting of sucrose density gradient-fractionated extracts revealed that large amounts of integrin β1 were localized in GEM/raft in GD2+ cells. All these results suggest that GD2 and integrin β1 cooperate in GEM/rafts, leading to enhanced malignant phenotypes of melanomas.

【Materials and Methods】

Ganglioside GD2-expressing clones were established from a human melanoma cell SK-MEL-28 subline, N1, by transfecting with cDNAs of *ST8SIA1* and *B4GALNT1*.

Expression levels of gangliosides were analyzed by flow cytometry and immunocytochemistry.

Cell proliferation, invasion, and adhesion were analyzed by MTT assay, invasion assay, and RT-CES, respectively.

Suppression of cell growth and adhesion were analyzed using anti-GD2 mAb (220-51).

Identification of GD2-associating molecules was performed by enzyme-mediated activation of radical sources combined with mass spectrometry (EMARS/MS).

Cell surface expression and mRNA expression of integrin β1 were analyzed by flow cytometry and immunoblotting and RT-qPCR, respectively.

Binding of GD2 and integrin β1 was analyzed by immunoprecipitation and subsequent immunoblotting.

Colocalization and association of GD2 and Integrins β1 were examined by immunocytochemistry and proximity ligation assay (PLA), respectively.

Tyrosine-phosphorylated proteins during cell adhesion was analyzed by immunoblotting with mAb PY20.

Knockdown of integrin β1 was performed using siRNA ITG1.

Intracellular distribution of integrin β1 and GD2 was examined by Optiprep gradient ultracentrifugation and immunoblotting.

【Results】

Using SK-MEL-28 (N1), GD2-positive lines were established by transfecting cDNAs of *ST8SIA1* and *B4GALNT1* (Figure 1A). Ganglioside expression was shown (Figure 1B), and by immunocytochemistry (Figure 1C).

MTT assay revealed that GD2+ cells showed a higher proliferation than GD2- cells (*p, < 0.05) (Figure 2A). The invasion assay revealed that GD2+ cells invaded more than GD2- cells (Figure 2B, 2C). RT-CES revealed that the GD2+ cells showed stronger adhesion than GD2- cells (Figure 2D). To clarify the involvement of GD2 in the malignant properties, anti-GD2 mAb 220-51 was added to the culture medium, resulting in the significant growth suppression in GD2+ cells (Figure 3A). When anti-GD2 mAb was added at 0.5 h (Figure 3B, left), cell adhesion in RT-CES was markedly suppressed for ~12 h. When added at 3.0 h (Figure 3C, left), cell adhesion was stably suppressed from 0.5 h to 24 h, while GD2- cells showed no changes (Figure 3A,B, right). Thus, anti-GD2 mAbs strongly suppressed cell adhesion only for GD2+ cells.

EMARS (Figure 4A) was performed with GD2+ S1 cells using mAb220-51. The FITC-labeled molecules were immunoprecipitated with a rabbit anti-FITC antibody, and detected by immunoblotting with a goat anti-FITC antibody (Figure 4B). MS analysis detected more than 30 molecules (Table 1). Among them, integrin β1 was a membrane molecule detected only in mAb-treated cells as demonstrated by MS.

Integrin β1 and its mRNA expression levels were analyzed by flow cytometry and RT-qPCR, showing almost equivalent levels between GD2+ and GD2- cells (Figure 5A,B). Immunoblotting revealed similar integrin β1 levels (Figure 5Ca). Immunoprecipitation/immunoblotting revealed that they were associated on the cell membrane (Figure 5Cb).

Immunocytochemistry revealed that GD2 was colocalized with integrin β1 (Figure 6A, right). The interaction between GD2 and integrin β1 was analyzed by PLA. Amplification products were detected in GD2+ cells (Figure 6B), suggesting that GD2 and integrins cluster on the cell surface.

Cell signals were analyzed by immunoblotting with anti-phosphotyrosine mAb PY20. As shown in Figure 7A, mainly 3 bands at 180, 130, and 100 kDa were detected in GD2+ cells (Figure 7B). These bands were identified to be EGFR and FAK by MS.

After checking effects of 4 siRNAs in the knockdown efficiency (Figure 8A), effects of the knockdown of integrin β1 on cell proliferation and adhesion were examined using ITG1 si-RNA. GD2+ cells show a clear reduction of cell growth, while GD2- cells showed no difference (Figure 8C). As for cell adhesion, the treatment with siRNA resulted in significant decrease in adhesion of GD2+ cells (Figure 8D). For the invasion, the increased invasion of GD2+ cells was strongly suppressed to that of GD2- cells (Figure 8F, 8E).

Immunoblotting of cell lysates treated with si-RNA by mAb PY20 revealed that phosphorylated

bands in GD2+ samples largely disappeared (Figure 9). These results suggested that the increased malignant properties and signals in GD2+ cells are dependent on integrins based on their cooperation on the cell surface.

Cells were detached, and incubated in collagen I-pre-coated plates. Then, cell lysates were fractionated by Optiprep gradient ultracentrifugation, and used for immunoblotting with anti-integrin β1 and -raft markers (Figure 10A). The results indicated that integrin β1 existed in raft fractions and non-raft fractions in GD2+ cells, while it could be found only in non-raft fractions in GD2- cells at 0~5 min (Figure 10B).

【Discussion】

Since characteristic expression of some gangliosides in neuroectoderm-derived cancers was reported, they have been considered to be cancer-associated glycolipids. They have also been reported to be in many other cancers. Recently, GD2 is expected to be a marker of higher malignant cancers, or cancer stem cells. GD2 has been used as a target of antibody therapy and also of CAR-T therapy towards cancers.

EMARS/MS is an efficient approach to investigate the mechanisms by which membrane molecules exert significant effects on the cell membrane. Particularly the identification of physically associating molecules of glycosphingolipids can be crucial to understand their roles in cell signals due to their modes of membrane anchoring. Now, EMARS/MS approach should be powerful in the understanding of the mechanisms for signal regulation by glycosphingolipids.

In this study, we identified integrin β1 as a representative membrane molecule in GD2+ melanoma cells. Previously, we reported that neogenin is a GD3-associated membrane molecule on melanomas. So, it is very interesting that the EMARS-MS revealed different membrane molecules associating with GD3 and GD2, suggesting that individual gangliosides form distinct molecular clusters on the cell membrane, and play individually roles. Thus, molecular clusters around gangliosides can be promising targets of cancer therapy with higher specificity and efficiency.

While close relationships between gangliosides and integrins have been reported, it is surprising that the increased malignant properties based on GD2 expression were cancelled by the knockdown of integrin β1, suggesting that GD2 is essentially involved in cell adhesion by cooperating with integrin β1.

【Conclusion】

Ganglioside GD2 enhances the malignant properties of melanoma cells by cooperating with integrins by forming molecular clusters in the membrane microdomain, lipid rafts.