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Neuroblastoma GOTO cells are hypersensitive to disruption of lipid rafts

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ABSTRACT

GOTO cells, a neuroblastoma cell line retaining the ability to differentiate into neuronal or Schwann cells, were found to be rich in membrane rafts containing ganglioside GM2 and hypersensitive to lipid raft-disrupting methyl-β-cyclodextrin (MβCD); the GM2-rich rafts and sensitivity to MβCD were markedly diminished upon their differentiation into Schwann cells. We first raised a monoclonal antibody that specifically binds to GOTO cells but not to differentiated Schwann cells and determined its target antigen as ganglioside GM2, which was shown to be highly concentrated in lipid rafts by its colocalization with flotillin, a marker protein of rafts. Disturbance of normal structure of the lipid raft by depleting its major constituent, cholesterol, with MβCD resulted in acute apoptotic cell death of GOTO cells, but little effects were seen on differentiated Schwann cells. Until this study, GM2-rich rafts are poorly characterized and MβCD hypersensitivity, which may have clinical implications, has not been reported.

Introduction

GOTO cells are a cultured cell line established from the neuroblastoma of a child [1] and retain the ability to differentiate into Schwann cells and neuronal cells, a property common to neuroblastomas that derive from neural crest stem cells [2,3]. Hypercoagulability has been observed in many cancer patients and accumulating evidence suggests that there is a strong link between metastatic potential of cancer cells and activation of the coagulation cascade. For studying the mechanism of cancer-associated thrombosis, the GOTO cell seems to be useful since we previously demonstrated that it has a Factor X (FX)-dependent thrombin-generating activity, which disappears upon its differentiation into the Schwann cell [4]. As an approach to identify the molecules involved in the cancer-associated thrombosis, we raised monoclonal antibodies (mAbs) that bind to the surface of GOTO cells but not to that of differentiated Schwann cells, and characterized one of such antibodies, mAb I-13. Contrary to our expectations, the antibody turned out to be anti-GM2 IgM. Of the gangliosides (sialic acid-containing glycosphingolipids), GM2 is a minor ganglioside in most normal tissues, but is highly expressed in certain cancer cells, and is a strong immunogenic ganglioside as reflected by the fact that anti-GM2 IgM antibodies have been detected in some patients with dysimmune neuropathy or lower motor neuron syndrome [5–8].

Gangliosides are concentrated within small subdomains of the plasma membrane, which are distributed in the fluid mosaic membrane as lipid microdomains, like rafts floating in the sea, and are therefore called lipid rafts. We therefore thought that the neuroblastoma GOTO cell and our anti-GM2 antibody (mAb I-13) are useful for studying lipid rafts. In the present study, we characterized GOTO cells in terms of lipid rafts and their components. Fluorescence-activated cell sorting (FACS) indicated that the anti-GM2 antibody binds avidly to GOTO cells but very weakly to differentiated Schwann cells. While determining the effects of depletion of cholesterol, another major component of lipid rafts, on mAb I-13 staining of the surface of GOTO cells, we found that GOTO cells are extremely sensitive to depletion of cholesterol and undergo acute apoptotic cell death upon treatment with cyclodextrin, a cyclic oligosaccharide that removes specifically cholesterol from the cell surface plasma membrane [9]. Such a high sensitivity to cholesterol depletion, namely acute cell death induced by cyclodextrin, has not been reported in any cells yet and suggests that

Abbreviations: BrdU, bromo-deoxyuridine; mAb, monoclonal antibody; MβCD, methyl-β-cyclodextrin; PI, propidium iodide; TLC-IB, thin-layer chromatography immunoblotting; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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GOTO cells have a unique type of rafts rich in GM2 and cholesterol. Moreover, the concomitant reduction of the hypersensitivity and the amount of cell surface GM2 upon Schwannian differentiation suggests that this unique character of rafts depends on GM2. The cyclodextrin-sensitive nature was found to be shared among GM2-rich tumor cell lines including IMR-32 (a neuroblastoma), K562 (a chronic myelogenous leukemia), and Lu-135 (a small cell lung carcinoma). Our results may also have clinical implications.

Materials and methods

Cell culture and differentiation. The following cells were obtained from the Health Science Research Resources Bank (Osaka, Japan): GOTO, a human neuroblastoma cell; IMR-32, a human neuroblastoma cell; THP-1, a human acute monocytic leukemia cell; HL60, a human acute promyelocytic leukemia cell; MOLT-3, a human acute lymphoblastic leukemia cell; MEG-01, a human megakaryoblastic leukemia cell; K562, a human chronic myelogenous leukemia cell; U937, a human histiocytic lymphoma cell; TIG-1, a normal fibroblast obtained from a human fetal lung; and G-361, a human melanoma cell. A human small cell lung carcinoma cell line, Lu-135, was obtained from RIKEN cell bank (Tsukuba, Japan). Cells were maintained in 1:1 mixture of Eagle's minimum essential medium (MEM) and RPMI Media 1640 supplemented with penicillin (50 U/ml), streptomycin (50 mg/ml), and 10% fetal calf serum. For inducing differentiation of GOTO cells into Schwann cells, GOTO cells were cultured for 20 days by growing cells in normal growth medium contain-

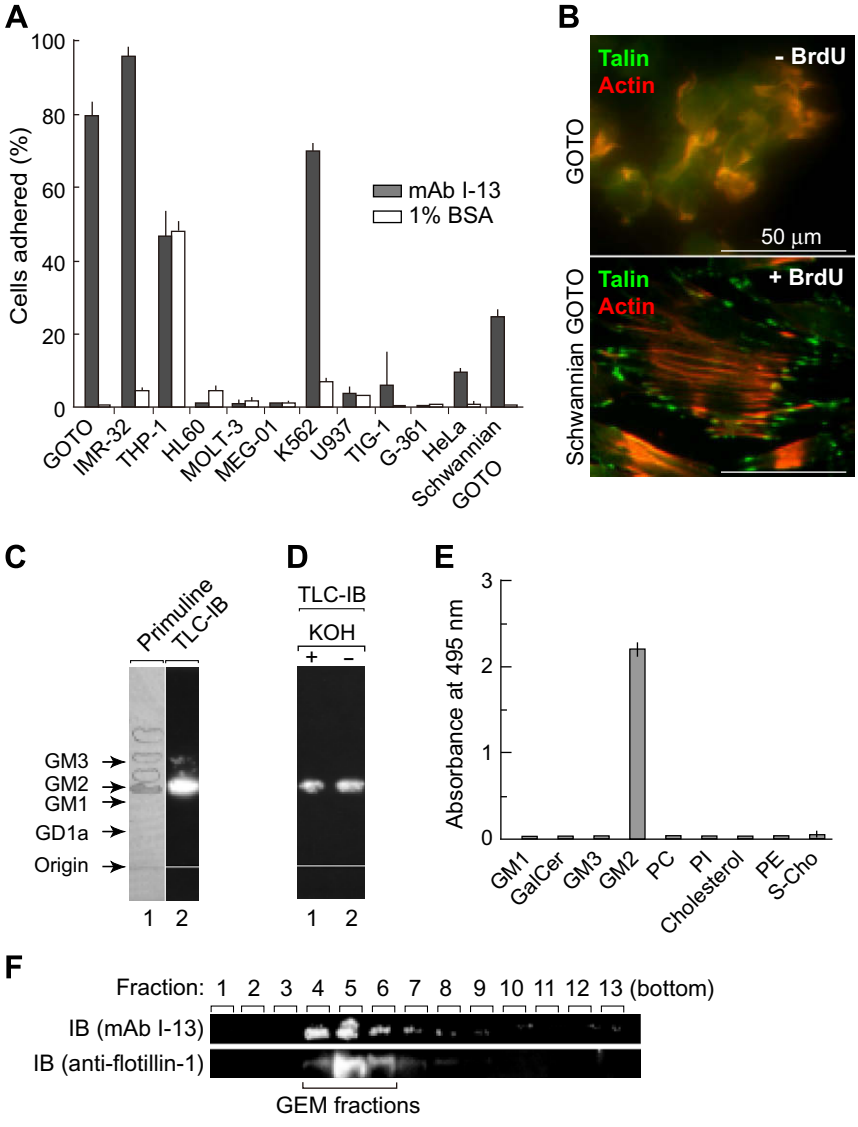

Fig. 1. Reactivity of mAb I-13 with GOTO cells and identification of its antigen molecule. (A) Cell adhesion assay to measure the abilities to bind to I-13 (closed columns) or BSA (hollow columns). Bound cells were quantified by phosphatase assay. Bars represent means of three experiments ± SEM. (B) BrdU-induced differentiation of GOTO cells into Schwann cells. Cells were stained with anti-talin (green) and fluorescence-labeled phalloidin (red). (C–E) Identification of the antigen for mAb I-13. (C) Detection of mAb I-13 antigen lipid by TLC immunoblotting (TLC-IB). Lane 1, whole lipids extracted from GOTO cells; and lane 2, TLC-IB of the lipids of GOTO cells stained with mAb I-13. Arrows indicate the positions of reference standards of known gangliosides, of which only GM2 reacted with mAb I-13. (D) Effect of alkali treatment on the mobility of the antigen lipid on TLC plate. The lipids of GOTO cells were treated with (+) or without (–) 0.2 M KOH and analyzed by TLC-IB with mAb I-13. (E) Relative binding abilities of mAb I-13 to various lipids determined by ELISA. GM1, Galβ1 → 3GalNAcβ1 → 4(Neu5Acα2 → 3)Galβ1 → 4Glcβ1 → 1Cer; GM2, GalNAcβ1 → 4(Neu5Acα2 → 3)Galβ1 → 4Glcβ1 → 1-Cer; GM3, Neu5Acα2 → 3Galβ1 → 4Glcβ1 → 1Cer; GalCer, α-galactosylceramide; PC, phosphatidyl choline; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; S-Cho, cholesterol-3-sulfate. (F) Detection of mAb I-13 antigen lipid in glycolipid-enriched membrane (GEM) fractions. Homogenates of GOTO cells were subjected to subcellular fractionation, and the fractions were analyzed by TLC-IB with mAb I-13 (upper panel) and Western blotting with anti-flotillin-1 (lower panel). IB, immunoblotting.

ing 5 mg/ml 5-bromo-2′-deoxyuridine (BrdU). The Schwannian differentiation was confirmed by staining of focal adhesion components talin and actin as described in Supplementary Materials and Methods.

Generation of monoclonal antibody (mAb) I-13 and antigen identification. For details refer to Supplementary Materials and Methods. In brief, a mAb I-13 was generated by immunizing BALB/c mice with GOTO cells as described previously [10,11], evaluated by cell adhesion assay, and prepared as ascitic fluid. Lipids of various cells were extracted by a modification of the Bligh–Dyer method [12] and the antigenicity was confirmed by thin-layer chromatography (TLC) immunoblotting (TLC-IB) [13]. Alkali resistance of antigen lipid was confirmed by TLC-IB after alkali treatment of extracted lipids. To define antigen lipid, an enzyme-linked immunosorbent assay (ELISA) was performed using several commercial lipids. Glycolipid-enriched membrane fractions (GEM fractions) of GOTO cells were purified by detergent-free sucrose density gradient ultracentrifugation [14]. Existences of antigen lipid and flotillin-1 in GEM fractions were confirmed by TLC-IB or Western blotting to demonstrate predominant presence of antigen lipid in lipid rafts in GOTO cells.

Assessment of cell death. For details refer to Supplementary Materials and Methods. In brief, methyl-β-cyclodextrin (MβCD)-induced morphological changes in GOTO cells were observed by differential interference contrast (DIC) optics. Propidium iodide (PI) and CytoTox-Glo Cytotoxicity Assay kit (Promega, Madison, WI, USA) were used for assessment of MβCD-induced cell death in several cell lines. Cell images were obtained using a fluorescence microscope. Fluorescence of PI and luminescence of CytoTox-Glo reagent were quantified using a 96-well microplate reader. Terminal dUTP nick end-labeling (TUNEL) assay was performed to detect the apoptotic chromosomal DNA fragmentation using TUNEL Enzyme (Roche Diagnostics, Mannheim, Germany).

Fluorescence-activated cell sorting (FACS). Antigen lipid on cell surface was quantified by FACS analysis. Harvested cells were washed extensively with PBS and incubated with mAb I-13 (ascites fluid diluted 1:5000) for 30 min at 4 °C. After washing with PBS, the cells were further incubated with 1:2000 diluted Alexa-488-conjugated goat anti-mouse IgM (Invitrogen, Carlsbad, CA, USA) for 30 min at 4 °C. Analysis was performed using FACSCaliber (Becton Dickinson, San Jose, CA, USA) and the data were analyzed with WinMDI software.

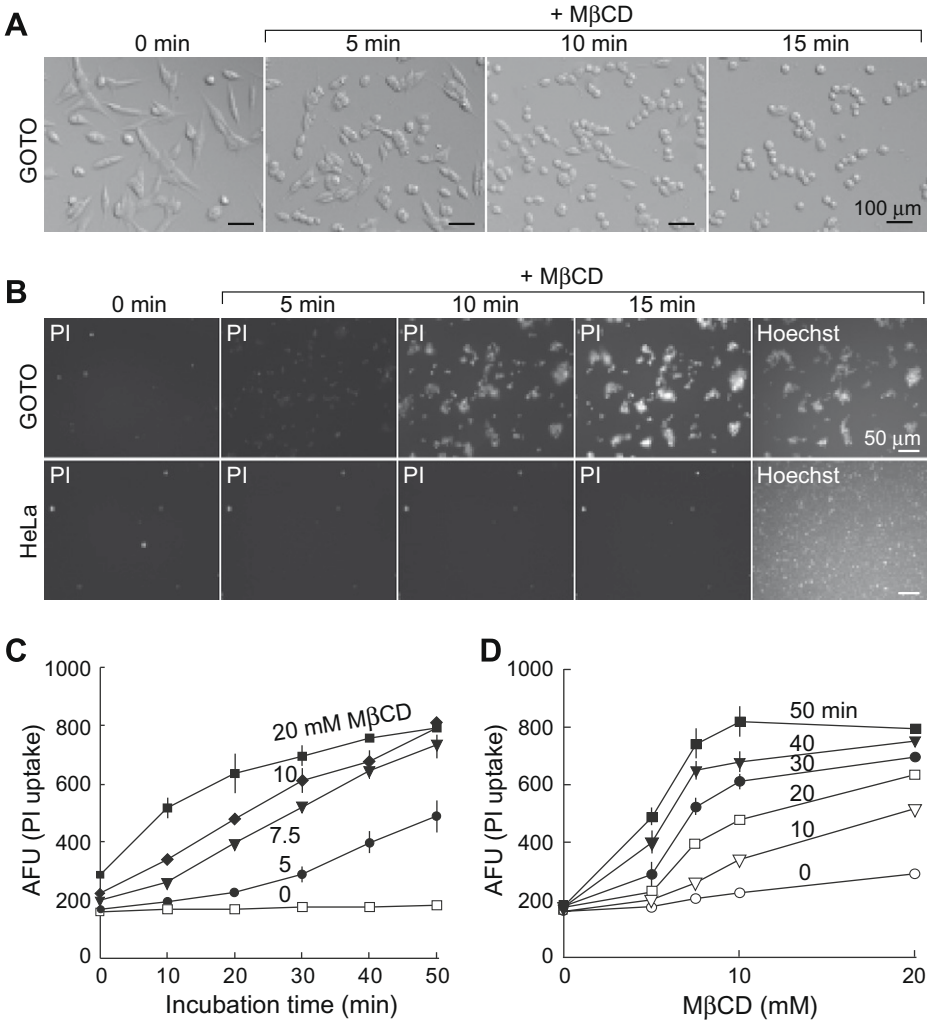

Fig. 2. Hypersensitivity of GOTO cells to cholesterol depletion with MβCD. (A) Effects of MβCD treatment on morphology of GOTO cells. Cells were treated with 10 mM MβCD for 0–15 min before fixation and observed using DIC optics. (B) Propidium iodide (PI) staining for determining viable versus dead cells. GOTO and HeLa cells were treated with 10 mM MβCD, 1 μg/ml PI, and 10 mg/ml Hoechst 33342 (Hoechst). Hoechst was used for visualizing both viable and dead cells. (C,D) Time- and dose-dependency of MβCD-induced cell death measured by PI uptake assay. GOTO cells were treated with 0–20 mM MβCD and 1 μg/ml PI for 0–50 min. Bars represent means ± SEM of four experiments. AFU, arbitrary fluorescence units.

Results

Ganglioside GM2 as a target antigen of anti-GOTO cell antibody (mAb I-13)

Monoclonal antibodies were generated by immunizing BALB/c mice with cultured GOTO cells as previously described [10,11]. One clone, termed I-13, was selected by cell adhesion assay and FACS screening as a promising one that recognizes a molecule highly expressed on the surface of GOTO cells but in low levels on differentiated Schwann cells (Figs. 1A and 4B). The binding potency of GOTO cells, IMR-32 cells, and K562 cells was exceptionally strong compared to that of other cells including THP-1, HL60, MOLT-3, MEG-01, U937, TIG-1, G-361, HeLa, and differentiated Schwann cells (Fig. 1A). FACS analysis showed that the mAb I-13 strongly bound to the surface of GOTO cells but weakly to differentiated Schwann cells (Fig. 4B). Subsequent characterization of the monoclonal antibody revealed that I-13 is IgM. To determine its antigen, we first extensively performed Western blot analysis using whole cell extracts or membrane preparations of GOTO cells but failed to obtain positive results, and it became conceivable that the antigen might not be a protein. We next performed TLC immunoblotting using lipid extracts of GOTO cells. A positive spot of low R_f value was observed, suggesting that the antigen is a very polar lipid (Fig. 1C). The antigen lipid was resistant to alkali treatment (Fig. 1D). These results suggest that the antigen is a sphingolipid. We finally carried out ELISA and established that the antigen molecule of mAb I-13 is a ganglioside, GM2 (Fig. 1E).

Predominant presence of GM2 in lipid rafts in GOTO cells

The lipid raft is a membrane microdomain enriched in cholesterol and glycosphingolipids such as gangliosides [15]. The presence of cholesterol confers resistance to solubilization with nonionic detergents or sodium carbonate at low temperatures and these detergent/carbonate-insoluble microdomains float to low-density membrane fractions on ultracentrifugation. By exploiting these properties, a widely used method has been established for isolation of glycolipid-enriched membrane fractions (GEM fractions) or lipid rafts [16,17]. Using this standard method, we isolated GEM fractions from GOTO cells and determined whether GM2 is indeed enriched in the GEM fractions of GOTO cells. TLC immunoblotting of fractions separated by ultracentrifugation indicated that most of GM2 is concentrated in a low-density fraction whose identity as the raft-enriched fraction was established by the colocalization of flotillin-1 (Fig. 1F), a well-established marker protein of lipid rafts.

Acute apoptosis of GOTO cells induced by disruption of rafts

Cyclodextrin is known to disrupt lipid rafts by specifically depleting cholesterol from the rafts [9,18]. Treatment with 2,6-di-*O*-methyl-β-cyclodextrin (MβCD) caused a marked change in the shape of GOTO cells (cell rounding; Fig. 2A) followed by cell death, in a time- and concentration-dependent manner, as assessed by propidium iodide uptake (Fig. 2B–D); ultimately most cells detached from the culture plate. The shape change became apparent as early as at 5 min of treatment and most cells underwent morphological changes from a spindle-like to a round shape at ~10 min, and cell death was induced at ~10 min after addition of 10 mM MβCD (Fig. 2B). Such acute damages were not seen in HeLa cells (Fig. 2B) and only weakly in differentiated Schwann cells (Fig. 4A). The Schwann cell differentiation was induced by treating GOTO cells with 5-bromo-2′-deoxyuridine (BrdU) and confirmed by the presence of focal adhesions visualized by immunostaining for its components, talin and actin microfilaments

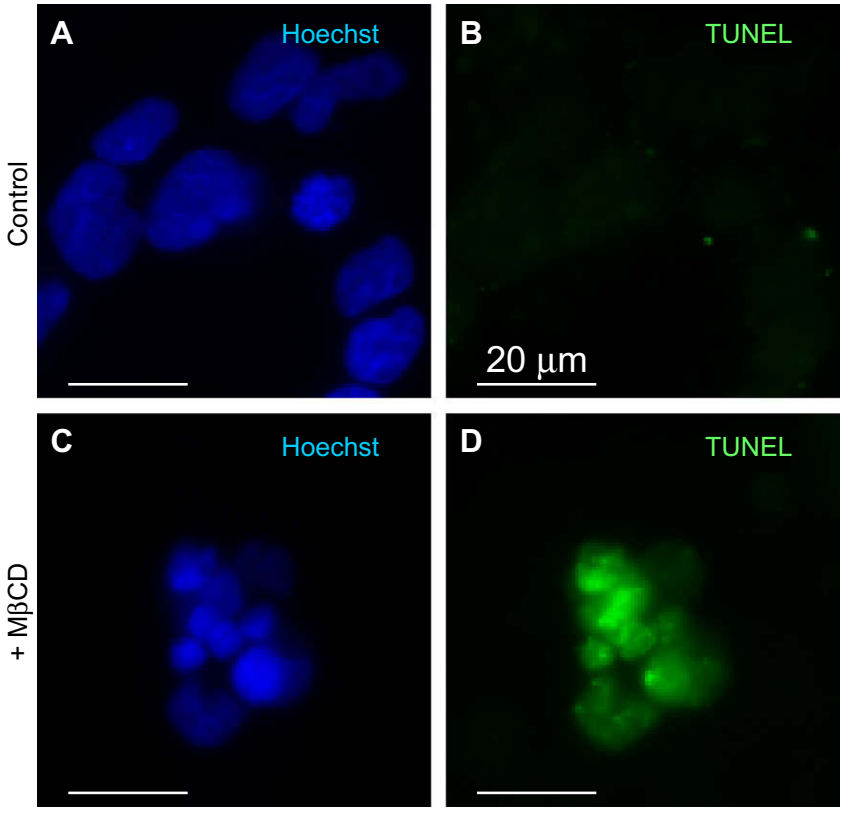

Fig. 3. TUNEL assay showing apoptotic cell death of GOTO cells induced by MβCD. GOTO cells were incubated in the presence (C,D) and absence (A,B) of 10 mM MβCD for 10 min and subjected to TUNEL apoptosis assay.

(Fig. 1B). TUNEL assay for detecting DNA fragmentation revealed that the MβCD-induced cell death was mediated via apoptosis (Fig. 3).

Acute apoptotic cell death induced by MβCD in GM2-rich tumor cells

At the initial screening stage of the monoclonal antibody, we noticed that, in addition to GOTO cells, certain tumor cells bind to 96-well plastic plates coated with mAb I-13 (Fig. 1A). Those cells include the human neuroblastoma cell line IMR-32 and chronic myelogenous leukemia cell line K562. High level expression of GM2 in these cells was confirmed by FACS analysis (Fig. 4B). A literature search revealed another cell line, small cell lung carcinoma cell line Lu-135, as a tumor cell line rich in GM2 [19]. We confirmed the GM2-rich nature of Lu-135 by FACS analysis (Fig. 4B). These results suggested that IMR-32, K562, and Lu-135 cells may have GM2-rich lipid rafts and sensitive to MβCD. Indeed, all these cells, like GOTO cells, underwent acute cell death on MβCD treatment (Fig. 4A).

Discussion

By using a newly generated monoclonal antibody against ganglioside GM2, a major component of lipid rafts, we characterized the neuroblastoma GOTO cell and found that GM2 is exceptionally highly enriched on the surface of GOTO cells compared to BrdU-differentiated Schwann cells (Fig. 1) and other cells including THP-1, HL60, MOLT-3, MEG-01, U937, TIG-1, G-361, and HeLa cells (Fig. 1A). Raft localization of GM2 was confirmed by its colocalization with flotillin-1 in the GEM (raft) fractions separated by sucrose density gradient fractionation of GOTO cells homogenized in sodium carbonate (Fig. 1F). The flotillin family consists of two homologous members: flotillin-1 and flotillin-2. Both are ubiquitously expressed and associated with membrane microdomains (lipid rafts) by means of fatty acid modifications and hydrophobic amino acid stretches [20–22]. The results reported here strongly suggest that the neuroblastoma GOTO cell contains lipid rafts rich in GM2. This property of the GOTO cell would make it useful for studying lipid rafts since (i) lipid rafts are attracting much attention as platforms that are crucial for certain types of cell signaling [23,24] and endocytosis [25–27] and (ii) GM2-rich rafts have not been characterized in detail yet while GM1-rich rafts are relatively well characterized using cholera toxin subunit B (CTB) as a probe for ganglioside GM1 [28].

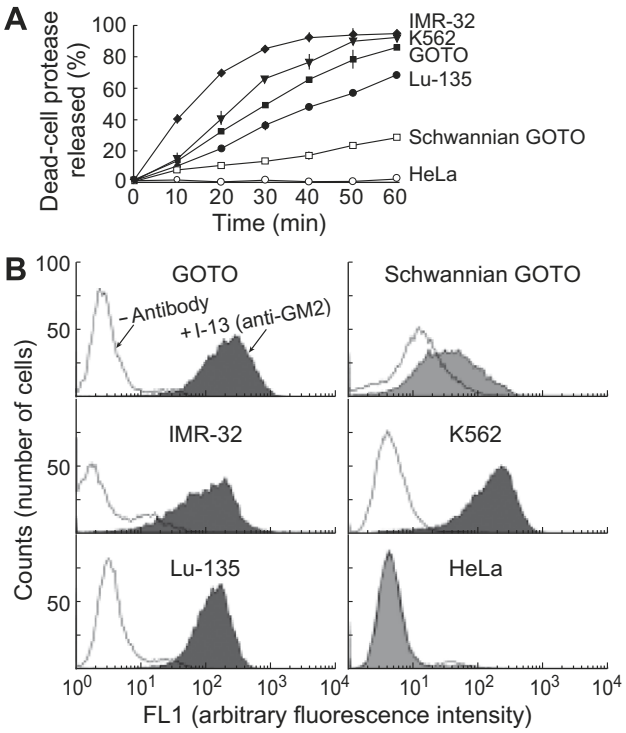

Fig. 4. Correlation of acute MβCD-induced apoptosis and high level cell surface expression of GM2. (A) MβCD-induced cell death monitored with a CytoTox-Glo cytotoxicity assay kit using IMR-32, K562, GOTO, Lu-135, Schwannian GOTO, and HeLa cells. Cells were treated with 10 mM MβCD and subjected to the viability assay at the indicated time points. Lethality of the cells was calculated as described in Supplementary Materials and Methods. Bars represent means ± SEM of four experiments. (B) FACS analysis to measure the cell surface GM2 of the indicated cells. Cells were processed in the presence and absence of mAb I-13 and subjected to FACS analysis. GOTO cells exhibited a striking reduction in the reactivity toward I-13 upon differentiation into Schwann cells, suggesting differentiation-dependent alteration in GM2 contents.

The marked alterations in the abundance of GM2-rich rafts, observed here on differentiation of GOTO cells into Schwann cells, is not surprising but rather expected, but our demonstration is the first experimental evidence for such an alteration (i.e. differentiation-dependent marked difference in the physicochemical properties of lipid rafts). Another interesting point is that the Schwannian differentiation was accompanied by a formation of a large number of focal adhesions (Fig. 1B) and by a striking resistance to MβCD (Fig. 4A), an observation that is somehow consistent with previous reports that (i) overexpression of focal adhesion kinase (FAK) renders the host cell resistant to MβCD-induced apoptosis and (ii) cholesterol depletion by MβCD treatment induces apoptosis of A431 cells through downregulation of FAK and internalization of lipid rafts/caveolae [29].

Methylated β-cyclodextrin (MβCD) is a water soluble derivative of β-cyclodextrin (β-CD), a cyclic oligosaccharide formed by seven units of α-D-(+)-glucopyranose, and possesses the ability to encapsulate a variety of compounds in its hydrophobic cavity [30]. In the field of membrane lipid research, this property of MβCD has been used to deplete cholesterol from the plasma membrane. For example, it has been successfully used to perturb the function of lipid rafts by decreasing their cholesterol content, which led to retardation of cellular functions and to the implication that lipid rafts act as an organization center of proteins for signal transduction [23,24], endocytosis [25–27], apoptosis [31,32] and also a docking site for the entry of viruses, bacteria, and toxins [33,34]. In the present study, we demonstrated that GOTO cells rapidly undergo apoptotic cell death when treated with MβCD (Figs. 2–4). It has been reported that MβCD has the potential to induce relatively slow apoptosis of several human cell lines including human keratinocyte, epidermoid carcinoma, prostate cancer, and breast cancer cells [35,36]. Additionally, our results indicated that human neuroblastoma, chronic myelogenous leukemia, and small cell lung carcinoma cell lines are extremely hypersensitive to MβCD-mediate cholesterol depletion (Fig. 4). One possible explanation for this hypersensitivity is that GM2/cholesterol-rich rafts are associated with the apoptosis signaling cascade and destabilization of the rafts by cholesterol depletion leads to activation of apoptosis. Since the GM2/cholesterol-rich nature is a property common to most neuroblastoma cells, the MβCD-induced cell death may have clinical implications. Neuroblastoma is one of the most malignant tumors of childhood, which frequently shows spontaneous regression. The high rate of spontaneous regression in infants is currently explained in relation to delayed activation of normal apoptotic pathways resulting from the absence of nerve growth factor in their microenvironments [37]. The unique property of the lipid rafts on GOTO cells may also contribute to the regression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.105.

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