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Globoside (Gb4) promotes activation of ERK by interaction with the epidermal growth factor receptor

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Abstract

Background—Globoside (Gb4), a globo-series glycosphingolipid (GSL), has been characterized as a stage-specific embryonic antigen (SSEA), and is highly expressed during embryogenesis as well as in cancer tissues. However, the functional role and molecular mechanism of Gb4 is so far unknown.

Methods—GSLs were preferentially inhibited by treatment with D-*threo*-1-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (EtDO-P4), a nanomolar inhibitor of GSL synthesis, in two carcinoma cell lines, HCT116 and MCF7. The effect of EtDO-P4 was examined by MTT assay, FACS analysis, wound assay, western blotting, and RTK array analysis. The functional role of Gb4 was determined by the exogenous addition of various GSLs, and an assay utilizing GSL-coated latex beads.

Results—Both cell lines contained higher levels of neutral GSLs than of sialic acid-containing GSLs. Gb4 was one of the major neutral GSLs. The depletion of total GSLs caused significant reduction of cell proliferation, but had less effect on cell apoptosis or motility. EtDO-P4 treatment also suppressed activation of the epidermal growth factor receptor (EGFR)-induced ERK pathway and various receptor tyrosine kinases (RTKs). The reduced activation of ERK was restored by the exogenous addition of Gb4, but not by the addition of gangliosides (GM1, GM2, GM3, GD1a). The GSL-coated bead assay indicated that Gb4 forms a complex with EGFR, but not with other RTKs.

Conclusions—Gb4 promotes activation of EGFR-induced ERK signaling through direct interaction with EGFR.

General significance—A globo-series GSL, Gb4, promotes EGFR-induced MAPK signaling, resulting in cancer cell proliferation. These findings suggest a possible application of Gb4 in cancer diagnostics and drug targeting.

Keywords

glycosphingolipid; globoside; MAPK; epidermal growth factor receptor

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1. Introduction

Receptor tyrosine kinases (RTKs) play key regulatory roles in critical cellular processes such as proliferation, differentiation, migration, and apoptosis [1]. Upon activation, most RTKs transmit signals through a mitogen-activated protein kinase (MAPK) cascade that consists of Raf, MEK, and ERK [2]. Epidermal growth factor receptor (EGFR), through its dimerization followed by autophosphorylation, transduces signals that regulate cell proliferation, differentiation, and migration [3]. Mutation of EGFR is the basis of many types of cancer, and the expression level of EGFR is often correlated with tumor progression [4]. Activation of RTKs is initiated by various growth factors, hormones, and cytokines. Recent studies indicate that glycosphingolipids (GSLs) promote or inhibit activation of certain RTKs.

GSLs, which are major components of cell surface membranes, are classified on the basis of the presence of a major core structure that includes lacto-series (GlcNAc β 3Gal β 4Glc β Cer), neolacto-series (Gal β 4GlcNAc β 3Gal β 4Glc β Cer), ganglio-series (GlcNAc β 4Gal β 4Glc β Cer), or globo-series (Gal α 4Gal β 4Glc β Cer). Structurally unique GSLs induce distinctive metabolic responses. Their functional roles have been studied increasingly during the past two decades [5, 6]. In particular, gangliosides, which are GSLs containing one or more sialic acid residues, display a variety of biological activities. Gangliosides GM2 and GM3 are capable of binding to membrane components such as RTKs, tetraspanins (TSPs) (including CD9, CD81, CD82), and integrins [7, 8]. The resulting complexes inhibit activation of receptors and consequently reduce cell motility.

Globo-series GSLs, which are neutral GSLs, play important roles in development and other biological processes. Gb3 (Gal α 4Gal β 4Glc β Cer), also known as CD77, was identified as P^k antigen of the P blood group system and serves as a natural receptor for bacterial toxins of the Shiga family (Stx) [9]. Gb3 is highly expressed on immature B-cells and various types of cancer, including Burkitt's lymphoma [10, 11]. Globoside (Gb4; GalNAc β 3Gal α 4Gal β 4Glc β Cer) is highly expressed in human red blood cells (erythrocytes), but its expression in various other types of cells appears to be limited [12]. It is formed by the addition of β 1-3GalNAc residue to Gb3 by β 1,3-N-acetylgalactosaminyltransferase, and expressed predominantly during embryogenesis [13]. Gb4 was reported to bind to nLc₄ (Gal β 4GlcNAc β 3Gal β 4Glc β Cer), inducing signal transduction involved in cell adhesion process [14]. However, the biological roles of Gb4, and details of its mechanisms of action, remain poorly understood.

Here, we present new findings on the functional role of Gb4 expressed in two carcinoma cell lines, HCT116 and MCF7, and the molecular mechanism for the enhancing effect of Gb4 on ERK activation. Gb4 is clearly shown to promote activation of EGFR in 42 types of human RTK. We propose a novel interaction between Gb4 and EGFR activation. Our findings help clarify a molecular mechanism whereby Gb4 is involved in cell development and tumor initiation through RTK-induced cell proliferation.

2. Material and methods

2.1. Antibodies and other materials

The following antibodies were used: mouse anti-EGFR mAb, rabbit polyclonal anti-Met IgG, rabbit polyclonal anti-Tie-2 IgG, rabbit polyclonal anti-FGFR3 IgG, rabbit polyclonal anti-ERK2 IgG, goat anti-mouse IgG-HRP, and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-phospho-ERK1/2 mouse anti-phospho-EGFR (Tyr1068) mAbs (Cell Signaling Technology); mouse anti- β -tubulin mAb (Sigma-Aldrich).

Standard GM1, GM2, GM3, GD1a, Gb3, Gb4, LacCer and rabbit polyclonal anti-Gb4 IgG were purchased from Matreya LLC (Pleasant Gap, PA). EtDO-P4 (D-threo-1-(3',4'-ethylenedioxy)-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol) [15] was provided by Dr. James A. Shayman.

2.2. Cell lines, culture and EtDO-P4 treatment

Colonic carcinoma HCT116 and breast carcinoma MCF7 cell lines were from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% FBS and 1% Gibco™ antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO₂ at 37°C. For EtDO-P4 treatment, cells (5×10^6) were cultured in 100-mm plates in DMEM supplemented with 10% FBS for 24 hr, rinsed twice with serum-free DMEM, added with 1 μ M EtDO-P4/ DMEM + 10% FBS, and incubated for 48 hr.

2.3. GSL extraction, thin-layer chromatography (TLC), and immunostaining

GSL extraction, TLC analysis, and immunostaining were performed as described previously [16, 17]. In brief, HCT116 and MCF7 cells were cultured in DMEM + 10% FBS until confluence and harvested. GSLs were extracted with chloroform/ methanol = (C/M; 2:1, v/v) and isopropanol/ hexane/ water (I/H/W; 55:25:20, v/v/v) under sonication for 30 min. GSL extracts were dissolved in 0.1 M NaOH/ MeOH, incubated at 40°C for 2 hr, neutralized with 1 N HCl, added with hexane, and allowed to stand for 5 min. The lower layer was evaporated under N₂ stream, dissolved in distilled water, and applied to a SepPak C₁₈ cartridge (Varian, Palo Alto, CA). After rinsing with distilled water, total GSLs were eluted with C/M and evaporated. The residues dissolved with C/M were spotted onto TLC plates (Silica Gel 60 F-254, Merck, Whitehouse Station, NJ), developed in C/M/ 0.2% CaCl₂ (55:40:10, v/v/v), and stained by spraying with 2% orcinol in 2 M H₂SO₄.

For further separation based on the amount of sialic acid, the total GSL fraction in chloroform/ methanol/ water (C/M/W; 30:60:8, v/v/v) was applied to DEAE Sephadex A-25 (Sigma-Aldrich). Neutral GSL, monosialosyl-, disialosyl-, and trisialosyl-GSL fractions were sequentially eluted by 0 M, 0.03 M and 0.13 M ammonium acetate in C/M/W, respectively [18]. Each fraction was dialyzed using dialysis membrane (Spectra/Por 3 Dialysis Membrane, MWCO: 3.5 kDa, SpectrumLabs, Shiga, Japan) against double-distilled water. GSLs were lyophilized, dissolved in C/M, and subjected to TLC as described above.

For immunostaining, TLC was fixed with 5% poly(isobutyl-methacrylate) in hexane/ chloroform (H/C; 9:1, v/v) for 90 sec and then blocked with 3% BSA in PBS for 1 hr. After immunoblotting with 1:1,000 diluted rabbit anti-Gb4 antibody overnight at 4°C, TLC was rinsed with T-TBS [TBS (140 mM NaCl, 10 mM Tris-HCl, pH 8.0)/ 0.05% Tween 20] and incubated with 1:3,000 diluted goat anti-rabbit IgG-HRP for 1 hr. TLC was developed by ECL solution (Pierce ECL Western Blotting Substrate, Thermo Science, Rockford, IL). The amounts of gangliosides and neutral GSLs were measured by the 2-thiobarbituric acid method and phenol-sulfuric acid method, respectively [19, 20].

2.4. Determination of cell proliferation, cell apoptosis, and motility

Cell growth was determined by two distinct procedures. (i) After pretreatment with EtDO-P4, cells (5×10^4) were cultured on 96-well plates containing DMEM + 10% FBS supplemented with or without (\pm) 1 μ M EtDO-P4 for 2 days. 10 μ l of MTT reagent (MTT Cell Proliferation Assay kit, Cayman Chemical Co., Ann Arbor, MI) was added to each well and incubated for 3 hr at 37°C in a CO₂ incubator. The culture medium was removed by aspiration and replaced with 100 μ l crystal dissolving solution. Cell proliferation was measured at 570 nm using a microplate reader. (ii) After pretreatment with EtDO-P4, cells

(5×10^5) in a T25 culture plate were incubated in DMEM + 10% FBS \pm 1 μ M EtDO-P4. Viable cells were stained with Trypan Blue and counted in all sections of the hemocytometer under a microscope for each time point.

To assess the cellular apoptosis resulting from the inhibition of GSL synthesis, cells (1×10^6) were rinsed with cold PBS, centrifuged at 1,350 rpm for 5 min at 4°C, rinsed twice with PBS, resuspended with 500 μ l binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4), and incubated with 1.25 μ l FITC Annexin V solution (200 μ g/ mL, BD Biosciences, San Diego, CA) in binding buffer for 10 min at room temp. Following centrifugation, the precipitants were mixed with 10 μ l PI (Propidium iodide, 30 μ g/ mL), incubated for 15 min at room temp, and subjected to flow cytometry analysis (BD FACSCalibur, BD Biosciences, Sparks, MD).

Cell motility was determined by wound assay of semi-confluent cells grown on 24-well plates [21]. After rinsing of the plates with DMEM, cells (5×10^5) pre-incubated with EtDO-P4 were cultured in 24-well plates containing DMEM + 10% FBS \pm 1 μ M EtDO-P4. When cells were at the subconfluent stage, cell layers were wounded by a plastic tip. Each plate was rinsed with serum-free DMEM, kept at 37°C, and photographed.

2.5. SDS-PAGE, western blotting, and receptor tyrosine kinase assay

Cells were lysed with RIPA buffer [1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholic acid, 5 mM tetrasodium pyrophosphate, 50 mM sodium fluoride, 5 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, PhosSTOP Phosphatase Inhibitor (Roche, Mannheim, Germany), and Complete Protease Inhibitor (Roche, Mannheim, Germany)]. The protein amount was measured using a Quant-iT™ Assay Kit (Invitrogen, Carlsbad, CA). A defined amount of cell lysate was subjected to SDS-PAGE and then transferred onto PVDF membrane (Roche, Mannheim, Germany). After blocking with 3% BSA in T-TBS for 1 hr at room temp, PVDF membrane (Millipore Corp., Bedford, MA) was immunoblotted with diluted primary antibodies overnight at 4°C. The membrane was rinsed 3x with T-TBS, incubated with diluted secondary antibodies for 1 hr at room temp, rinsed 3x with T-TBS, and developed using ECL solution. For reblotting, the same membrane was stripped with stripping buffer (Candor Bioscience GmbH, Weissensberg, Germany) for 1 hr at room temp. The membrane was then rinsed 5x with T-TBS, blocked with 3% BSA solution, incubated with diluted primary antibodies, rinsed 3x with T-TBS, incubated with diluted secondary antibodies for 1 hr at room temp, rinsed, and developed using ECL solution.

The activation of various human RTKs was measured by RTK array (R&D System, Minneapolis, MN), according to manufacturer's standard protocol. The phosphorylation level of receptors was measured using Quantity One (Bio-Rad, Hercules, CA) and compared with positive controls at each corner.

2.6. In situ effect of various GSLs on ERK phosphorylation

To determine which GSLs regulate ERK phosphorylation, GSL incorporation was performed as described previously, with slight modification [22, 23]. HCT116 and MCF7 cells (5×10^5), pre-treated or not with 1 μ M EtDO-P4, were cultured in 6-well plates containing serum-free DMEM \pm 1 μ M EtDO-P4 for 12 hr. Separately, a defined amount of various GSLs (GM1, GM2, GM3, GD1a, Gb3, Gb4, and LacCer) was rinsed twice with ethanol, dried under N_2 stream, dissolved in serum-free DMEM \pm 1 μ M EtDO-P4, and sonicated for 10 min. The medium was then changed to serum-free DMEM \pm 1 μ M EtDO-P4 supplemented with GSLs, and the incubation was continued overnight. Incorporated GSLs were determined by fluorescence microscopy (Suppl. Fig. 1). Cells were harvested, lysed, and a defined quantity

of lysate was subjected to western blotting with anti-phospho-ERK antibody and reblotted sequentially with anti-ERK and anti-tubulin antibodies.

2.7. GSL-coated polystyrene latex bead assay

The relationship between Gb4 and EGFR levels was investigated using Gb4-coated polystyrene latex beads by the method described previously, with slight modification [24]. Briefly, 50 μ l polystyrene latex beads (2 wt %, # 461733, Sigma) was rinsed with 1 ml of 10% EtOH in a glass tube. After centrifugation at 3,500 xg for 5 min, the supernatant was carefully aspirated. The precipitated beads were rinsed twice with 1 ml of 30% EtOH. Separately, a defined amount of GSLs in C/M was dried under N₂ stream, rinsed twice with EtOH, and the GSLs were dissolved in 30% EtOH. The GSLs were boiled for 15 sec at 80°C and mixed with polystyrene latex beads for 1 hr at 37°C with agitation. The GSL-coated latex beads were then rinsed 3x with PBS and blocked with 0.1% gelatin in PBS for 1 hr at 37°C. To confirm the GSL coating process, the same amount of beads was resuspended with C/M, sonicated for 15 min, and subjected to TLC (Suppl. Fig. 2). Separately, GSL-coated beads were rinsed 3x with TBS(+) (TBS containing 0.9 mM CaCl₂, 0.5 mM MgSO₄, and 0.1 mM MnCl₂) and incubated with 100 μ g of cell lysate overnight at 4°C. The latex bead components were rinsed 3x with TBS(+), mixed with 2x sample buffer, and subjected to SDS-PAGE followed by western blotting as described above.

3. Results

3.1. GSL composition of two carcinoma cell lines, HCT116 and MCF7

GSLs extracted from HCT116 and MCF7 cells were separated based on their sialic acid content and analyzed by TLC and immunostaining. The overall GSL composition of the two cell lines was similar (Figs. 1A, B). To rule out the possibility that different GSLs had the same motility on TLC plates, we separated GSLs based on their sialic acid content. HCT116 cells displayed a higher content of neutral GSLs and gangliosides than did MCF7 cells (Table 1). In both cell lines, GSLs were found predominantly in the neutral and monosialo-GSL fractions, and the content of neutral GSLs was higher than that of gangliosides. Consistently with a previous report [25], gangliosides GM2 and GM3 were the major acidic GSLs in the monosialo-GSL fraction. Gb4 was identified as a major neutral GSL based on TLC migration and immunostaining with anti-Gb4 antibody (Fig. 1C). Phenol-sulfuric assay showed the presence of 8.85 nmol and 4.35 nmol Gb4 in 1×10^7 HCT116 and MCF7 cells, respectively.

3.2. Effect of EtDO-P4 treatment on cell proliferation, growth, apoptosis, and motility

To explore the roles of GSLs in cellular functions, the synthesis of glucosylceramide-based GSLs was inhibited by treatment with the glucosylceramide synthase inhibitor EtDO-P4 [15]. EtDO-P4 treatment clearly reduced the expression of major GSLs (GM2, GM3, Gb4) (Fig. 2A), as well as cell proliferation (Fig. 2B, C), in both HCT116 and MCF7 cells. In contrast, the depletion of total GSLs by EtDO-P4 treatment marginally affected cell apoptosis and motility (Fig. 2D, E). *I.e.*, for HCT116, the percentages of apoptotic and dead cells were increased from 3.1% to 4.3%, and from 0.2% to 0.7%, respectively. For MCF7, the percentages of apoptotic and dead cells were increased from 2.8% to 4.8% and from 0.3% to 0.9%, respectively. These findings indicate that, in these two carcinoma cell lines, GSLs play an important functional role in proliferation.

3.3. Modification of signaling cascades by inhibition of GSL synthesis

The MAPK and PI3K/Akt signaling pathways are crucial regulators of cell proliferation. We therefore investigated the effects of GSL depletion by EtDO-P4 treatment on ERK and Akt activation. EtDO-P4 treatment significantly reduced ERK phosphorylation (Fig. 3A, B) but

not Akt activation, and reduced phosphorylation of Tyr1068, the binding site of Grb2 adaptor protein, in EGFR. The endogenous protein levels of EGFR, ERK, and Akt were unaffected by EtDO-P4 treatment. An RTK array system was used to examine the effect of GSL depletion by EtDO-P4 on the activation of 42 human RTKs. Phosphorylation of several RTKs (EGFR, HGFR, Tie-2, EphB2, FGFR3) was clearly reduced in treated HCT116 cells as compared to non-treated cells (Fig. 3C, E). The endogenous protein levels of RTKs were unchanged (Fig. 3D).

3.4. Effect of exogenous addition of GSLs on ERK phosphorylation

Several major GSLs were exogenously added to HCT116 and MCF7 cells and the effects on ERK phosphorylation were examined. The phosphorylation level of ERK in both cell lines was significantly decreased by the exogenous addition of several gangliosides (GM1, GM2, GM3, GD1a), but marginally affected by the addition of Gb4 (Fig. 4A, Suppl. Fig. 3). The addition of other neutral GSLs (Gb3, LacCer) had little impact on ERK activation (Fig. 4B). In another experiment, gangliosides GM3 and Gb4 were exogenously added to EtDO-P4-treated MCF7 cells. The reduction in ERK phosphorylation caused by EtDO-P4 treatment was restored by the addition of Gb4, in a dose-dependent manner, but not by the addition of GM3 (Fig. 4C). The addition of 250 μ M Gb4 led to the restoration of ERK phosphorylation to nearly control level (Fig. 4D). These findings clearly indicate that Gb4 promotes ERK phosphorylation and consequently enhances cell proliferation (Suppl. Fig. 4).

3.5. Gb4 interacts with and promotes activation of EGFR

An RTK array was used to investigate the effects of Gb4 on activation of 42 human RTKs. Consistent with the findings shown in Fig. 3C (bottom row), EtDO-P4 treatment suppressed the phosphorylation of most of the RTKs (Fig. 5A, top row). Phosphorylation of three RTKs (EGFR, FGFR3, Tie-2) appears to be increased by exogenous addition of 250 μ M Gb4 to EtDO-P4-treated cells (Fig. 5A, bottom row). In particular, EGFR phosphorylation was increased 3.6-fold by Gb4 addition (Fig. 5B). Gb4-coated polystyrene latex beads displayed an interaction between Gb4 and EGFR (Fig. 6A, top row) but no interactions involving other RTKs, including FGFR3 (Fig. 6A, bottom row). The interaction between Gb4 and EGFR was also observed, to a lesser degree, when beads were coated with 12.5, 25, or 50 μ g Gb4 (Fig. 6B). Beads coated with other neutral GSLs did not bind strongly to EGFR (Fig. 6C). The interaction between EGFR and Gb4 was also demonstrated by co-immunoprecipitation (Fig. 6D). These results, taken together, suggest that Gb4 specifically promotes activation by forming a complex with EGFR.

4. Discussion

The present study demonstrates an important functional role of globotetraosylceramide (Gb4; globoside) in ERK activation. In two carcinoma cell lines, HCT116 and MCF7, the level of neutral GSLs is higher than that of gangliosides, and Gb4 is a major neutral GSL. The depletion of GSLs caused clear suppression of ERK activation and cell proliferation. Reduced activation of ERK was restored by exogenous addition of Gb4, but not of other types of GSLs.

In embryonic development, globo-series structures appear first, followed much later by lacto-series and ganglio-series GSLs [26]. In particular, Gb4, a globo-series GSL, is an inducible molecule activated by TNF- α in vascular endothelial cells and appears to play a functional role in early stages of development [27]. Our findings clearly show that the phosphorylation level of ERK was enhanced by the exogenous addition of Gb4, but not of several gangliosides. Although other types of neutral GSLs (Gb3, LacCer) also appear to be associated with ERK activation, they did not display interaction with EGFR (Figs. 4B, 6C).

The proliferation of both HCT116 and MCF7 cells was increased by the addition of 250 μ M Gb4 (Suppl. Fig. 4). These findings are consistent with previous reports in which gangliosides (including GM2 and GM3) inhibited activation of various RTKs and related signaling pathways in various cell lines [28, 29]. Gb4 was also shown to promote the activities of certain transcription factors (*e.g.*, AP1, CREB) in human embryonal carcinoma 2102 cells [14]. It is not clear why Gb4 did not enhance ERK phosphorylation in the absence of EtDO-P4 pre-treatment (Fig. 4A, C). One possibility is that the level of endogenous Gb4 is sufficient to exert this effect without any exogenous addition.

Whether GSLs located in the outer plasma membrane leaflet affect intracellular signaling molecules is a matter of long-standing debate. GSLs are capable of interacting with various membrane constituents such as Src family tyrosine kinases and small G-proteins [30]. Complexes formed by some gangliosides (GM2, GM3) and various RTKs and TSPs (CD9, CD81, CD82) inhibit the activation of receptors [7, 31]. GD1a induces EGFR activation, with consequent enhancement of cell proliferation [32, 33]. In the present study, the phosphorylation of several RTKs (EGFR, EphB2, FGFR3, HGFR, Tie-2) among 42 human RTKs was reduced by EtDO-P4 treatment. In particular, the reduction in phosphorylation level of EGFR caused by EtDO-P4 treatment was strongly restored in Gb4-treated cell compared with untreated cells.

Although Gb4 has been shown to bind specifically to EGFR, the specific Gb4 binding site(s) on EGFR are unclear. Studies by Hakomori et al. suggest that carbohydrate-to-carbohydrate interaction (CCI) is the underlying mechanism for a variety of glycan-related phenotypic changes (*e.g.*, cell growth, morphology, motility, adhesion) [34, 35]. For example, ganglioside GM3 was shown to interact with GlcNAc termini on N-glycans of EGFR, resulting in inhibition of EGFR dimerization [23]. The function of Trk protein, the high-affinity tyrosine kinase-type receptor for nerve growth factor, was modified by interaction between GM1 and *N*-linked oligosaccharides of Trk [36]. Gb4 was reported to bind to either GalGb4 (Gal β 3GalNAc β 3Gal α 4Gal β 4Glc β Cer) or nLc4Cer (Gal β 4GlcNAc β 3Gal β 4Glc β Cer) [14].

In summary, the present findings in two carcinoma cell lines demonstrate clearly that a globo-series GSL, Gb4, promotes activation of the EGFR-induced MAPK pathway through interaction with EGFR. Detailed studies on the biophysical mechanism of the interaction between Gb4 and EGFR will clarify their roles in initiating tumorigenesis. Investigations on the expression profiles of Gb4 during tumor development and progression in various types of tumor cells and tissues will also be informative. A better understanding of the growth-promoting effect of Gb4 on tumor cells and its relationship with EGFR may lead to the practical development of Gb4-associated cancer diagnostics and drug targeting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EGFR	epidermal growth factor receptor
EtDO-P4	D- <i>threo</i> -1-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidino-1-propanol
ERK	extracellular signal-regulated kinases
Gb4	GalNAc β 3Gal α 4Gal β 4Glc β Cer
GSL	glycosphingolipid
MAPK	mitogen-activated protein kinase
RTK	receptor tyrosine kinase

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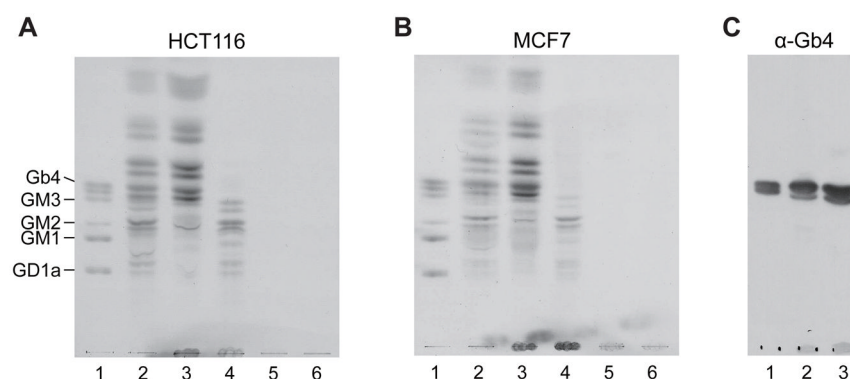


Fig. 1. Thin layer chromatography (TLC) pattern of GSLs extracted from two carcinoma cell lines

HCT116 and MCF7 cells were incubated until confluence, and GSLs were extracted. Total GSLs were separated based on the amount of sialic acid residue. Each fraction was subjected to TLC followed by staining with orcinol/ sulfuric acid or immunostaining with anti-Gb4 antibody. (A-B) orcinol staining of HCT116 and MCF7. Lane 1, mixture of standard GD1a, GM1, GM2, GM3 and Gb4. Lane 2, total GSLs. Lane 3, neutral fraction from total GSLs. Lane 4–6, monosialo-, disialo-, trisialo-ganglioside fraction. (C) immunostaining with anti-Gb4 antibody. Lane 1, mixture of standard GD1a, GM1, GM2, GM3 and Gb4. Lane 2, neutral GSL fraction of HCT116. Lane 3, neutral GSL fraction of MCF7.

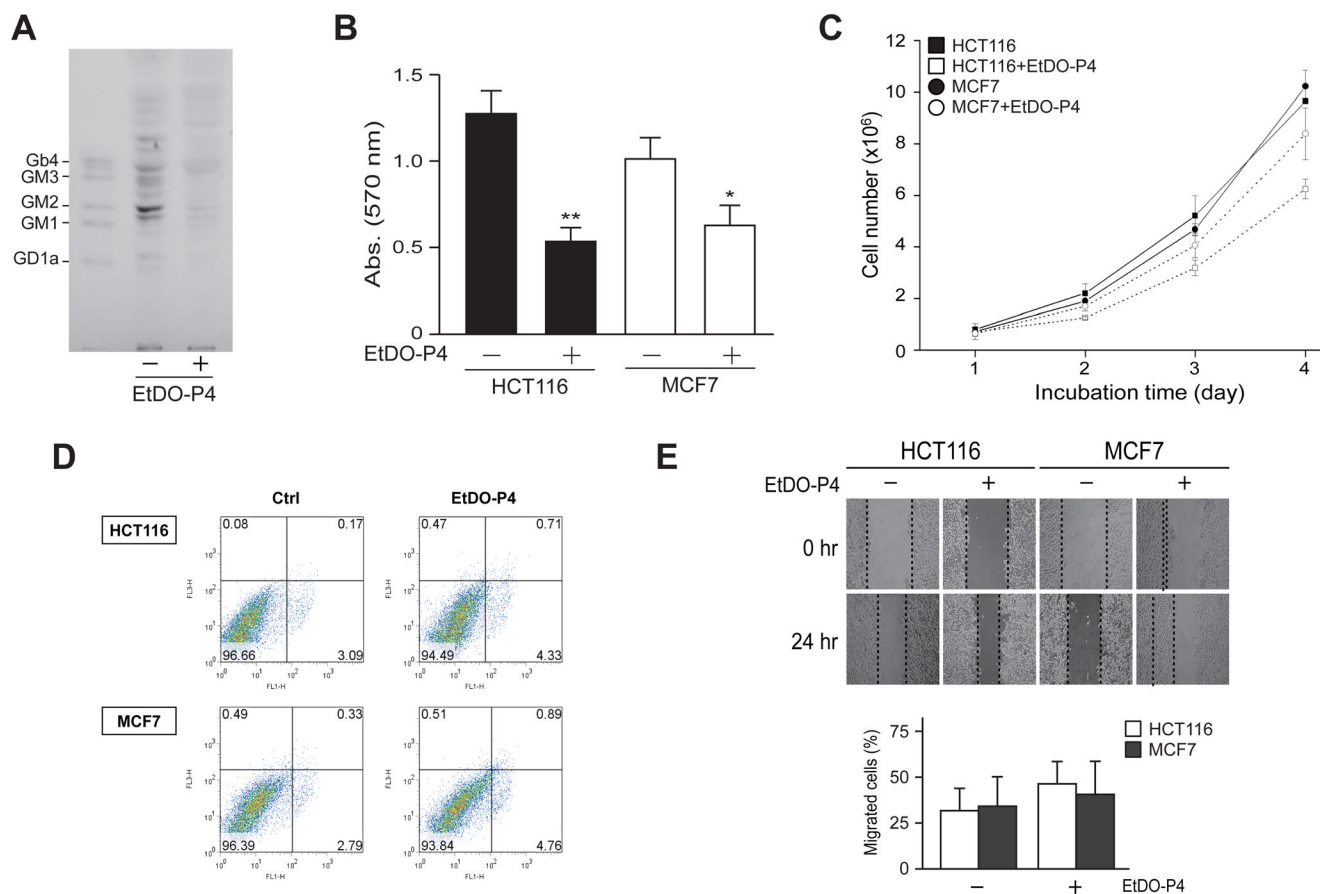


Fig. 2. Effect of EtDO-P4 on proliferation, growth, apoptosis, and motility of HCT116 and MCF7 cells

(A) GSLs were extracted from harvested cells and subjected to TLC followed by orcinol/sulfuric acid staining. Lane 1, standard GSLs. Lane 2, GSLs from HCT116 cells. Lane 3, GSLs from HCT116 cells pre-treated with EtDO-P4. (B) MTT assay for cell proliferation. Black bars, HCT116; white bars, MCF7. (C) Cells (5×10^5) were cultured in DMEM + 10% FBS \pm EtDO-P4. Cell number was estimated by counting with a hemocytometer. Closed squares, HCT116 cells. Open squares, HCT116 cells treated with EtDO-P4. Closed circles, MCF7 cells. Open circles, MCF7 cells treated with EtDO-P4. (D) FACS analysis of cell apoptosis. Cells (1×10^6) treated \pm EtDO-P4 were stained with FITC-Annexin V and PI reagents. Apoptotic cells were measured by flow cytometry. (E) Wound healing assay for cell motility, which was estimated by measurement of wound distance and comparison of percentages of migrated cells vs. control cells at 24 hr. The values shown are mean \pm SD from three or more independent experiments performed in triplicate. Significant differences compared with values from control cells are indicated; *, $P < 0.05$; **, $P < 0.01$.

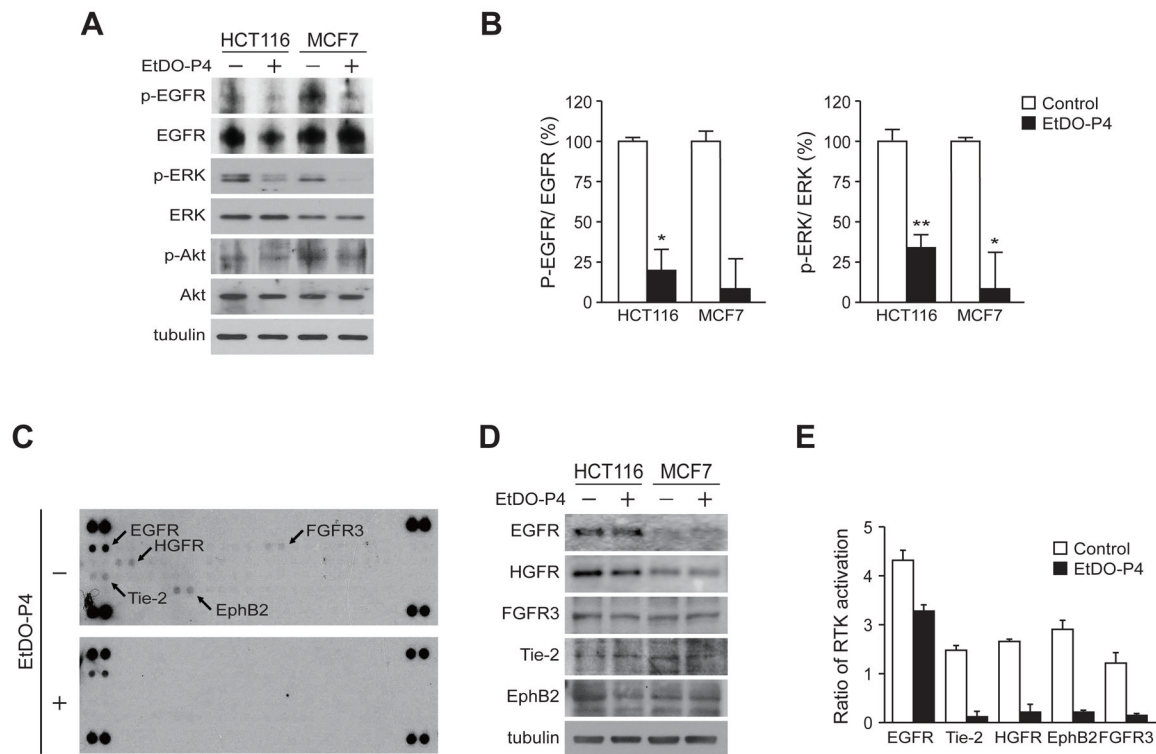


Fig. 3. Inhibitory effect of EtDO-P4 on phosphorylation of RTKs and on related signaling pathways

Cells pre-treated with EtDO-P4 were harvested and lysed in RIPA buffer. (A) Cell lysate (50 μ g) was subjected to western blotting. Lane 1, HCT116 cells. Lane 2, HCT116 cells treated with EtDO-P4. Lane 3, MCF7 cells. Lane 4, MCF7 cells treated with EtDO-P4. (B) Phosphorylation of EGFR and ERK was quantified based on intensity. (C) Effect of EtDO-P4 on activation of RTKs. Cell lysate was analyzed using a RTK array kit (R&D Systems, Minneapolis, MN). Top, HCT116 cells. Bottom, HCT116 cells treated with EtDO-P4. (D) Cell lysate pre-treated \pm EtDO-P4 was subjected to western blotting with anti-EGFR, -HGFR, -FGFR3, -Tie-2, -EphB2 and -tubulin antibodies. (E) The intensity of phosphorylated RTK was normalized to loading control. The values shown are mean \pm SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

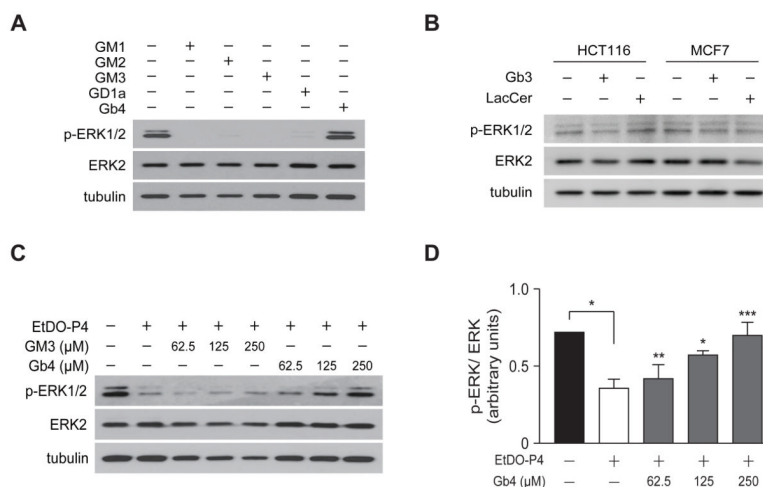


Fig. 4. Effect of exogenous addition of various GSLs on ERK phosphorylation

GSLs (GM1, GM2, GM3, GD1a, Gb3, Gb4, LacCer; each 125 μM) were added separately to culture medium as described in M&M. After incorporation of GSLs, cells were harvested and subjected to western blotting. (A) Effects of GSLs on ERK phosphorylation. Lysate of MCF7 cells was immunoblotted with anti-p-ERK antibody, and the same blot was reblotted with anti-ERK and -tubulin antibodies. Lane 1, control cells. Lane 2, cells treated with GM1. Lane 3, GM2. Lane 4, GM3. Lane 5, GD1a. Lane 6, Gb4. (B) Effects of neutral GSLs on ERK phosphorylation. Lane 1, HCT116 cells. Lanes 2–3, HCT116 cells treated with Gb3 and LacCer. Lane 4, MCF7 cells. Lanes 5–6, MCF7 cells treated with Gb3 and LacCer. (C) MCF7 cells treated with various concentrations of GM3 and Gb4 as indicated. (D) Statistical comparison of data from Panel C. The relative optical density of ERK phosphorylation was divided by that of ERK. The values shown are mean ± SD from three separate experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

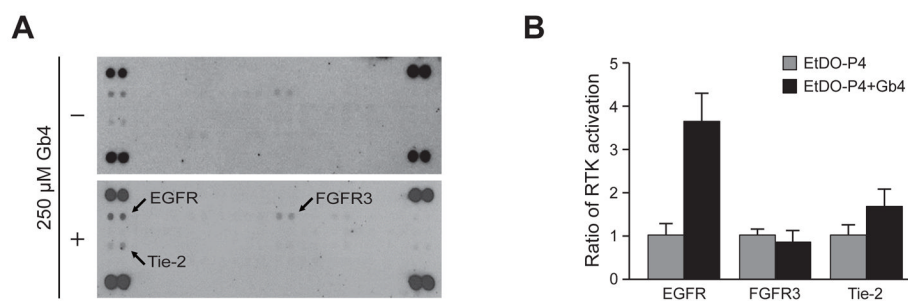


Fig. 5. Exogenous addition of Gb4 promotes phosphorylation of RTKs

(A) HCT116 cells pre-treated with EtDO-P4 were added with 250 μ M Gb4, then harvested and analyzed using RTK array as described in M&M. (B) Based on the results in Panel A, the intensity of phosphorylation of each receptor was measured using Quantity One (Bio-Rad, Hercules, CA), normalized to positive loading control. Gray bars, HCT116 cells pre-treated with EtDO-P4. Black bars, HCT116 cells pre-treated with EtDO-P4, followed by addition of 250 μ M Gb4.

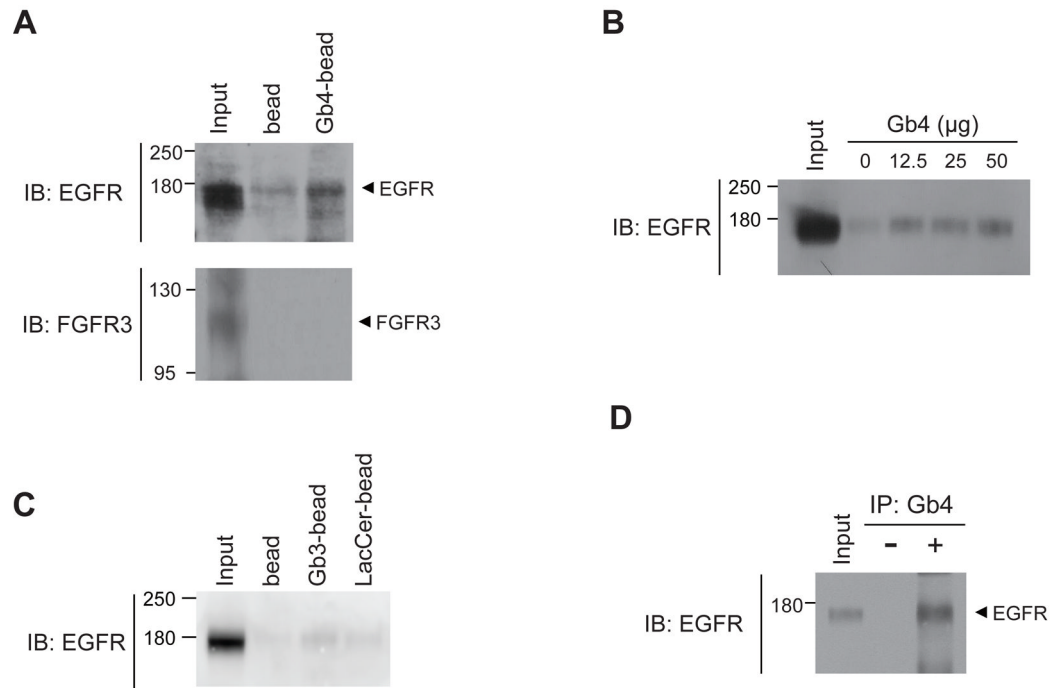


Fig. 6. Gb4 interacts with EGFR

(A) Gb4-coated beads were incubated with cell lysate (100 µg) overnight at 4°C. Top: The precipitated beads were mixed with 2x sample buffer, run on 7% acrylamide gel and immunoblotted (IB) with anti-EGFR antibody. Lane 1, cell lysate as positive control. Lane 2, cell lysate mixed with control beads. Lane 3, cell lysate mixed with Gb4 (100 µg)-coated beads. Bottom: Following the analysis in the top panel, the membrane was stripped with stripping buffer and reblotted with anti-FGFR3 antibody. (B) Latex beads were coated with various amounts of Gb4 (0, 12.5, 25, 50 µg). Lane 1, cell lysate as positive control. Lanes 2–5, cell lysate mixed with beads coated with various amounts of Gb4 as above. (C) Polystyrene bead coated with neutral GSLs (Gb3, LacCer) were used for investigation of possible binding to EGFR. Lane 1, cell lysate as positive control. Lane 2, cell lysate mixed with control beads. Lane 3, cell lysate mixed with beads coated with 100 µg Gb3. Lane 4, cell lysate mixed with beads coated with 100 µg LacCer. (D) Endogenous Gb4 was precipitated by anti-Gb4 antibody and then subjected to western blotting with anti-EGFR antibody.

Table 1
Composition of GSLs in carcinoma cell lines, HCT116 and MCF7

The amount of neutral GSLs and gangliosides was determined by phenol-sulfuric acid and 2-thiobarbituric acid methods, respectively (see M&M).

	Neutral GSL (μg galactose/ 1×10 ⁷ cells)	Gangliosides (μg NeuAc/ 1×10 ⁷ cells)	Gb4 [*]	GM1 [†]	GM2 [‡]	GM3 [‡]
	(nmol GSL/ 1×10 ⁷ cells)					
HCT116	16.27	0.64	8.85	0.04	0.10	0.06
MCF7	8.18	0.45	4.35	0.02	0.08	0.02

^{*}, the amount of Gb4 was calculated based on the intensity relative to neutral GSL fraction.
[†], the amounts of gangliosides GM1, GM2 and GM3 were calculated based on the intensity relative to monosialo-GSL fraction.