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Lyso-GM3, its dimer, and multimer: their synthesis, and their effect on epidermal growth factor-induced receptor tyrosine kinase

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Abstract Glycosphingolipids, particularly gangliosides, are known to modulate growth factor receptor tyrosine kinase. A well-documented example is the inhibitory effect of GM3 on kinase associated with epidermal growth factor receptor (EGFR) in human epidermoid carcinoma A431 cells. Lyso-GM3 was detected as a minor component in A431 cells, and may function as an auxiliary factor in GM3-dependent inhibition of EGFR. We studied the inhibitory effect of chemically synthesized GM3, lyso-GM3, and its derivatives, on EGFR function, based on their interaction in membrane microdomain, with the following major findings: (1) GM3, EGFR, and caveolin coexist, but tetraspanins CD9 and CD82 are essentially absent, within the same low-density membrane fraction, separated by sucrose density gradient ultracentrifugation. (2) Strong interaction between EGFR and GM3 was indicated by increasing binding of EGFR to GM3-coated polystyrene beads, in a GM3 dose-dependent manner. Confocal microscopy results suggested that three components in the microdomain (GM3, EGFR, and caveolin) are closely associated. (3) Lyso-GM3 or lyso-GM3 dimer strongly inhibited EGFR kinase activity, in a dose-dependent manner, while lyso-GM3 trimer and tetramer did not. >50 μM lyso-GM3 was cytolytic, while >50 μM lyso-GM3 dimer was not cytolytic, yet inhibited EGFR kinase strongly. Thus, lyso-GM3 and its dimer exert an auxiliary effect on GM3-induced inhibition of EGFR kinase and cell growth, and lyso-GM3 dimer may be a good candidate for pharmacological inhibitor of epidermal tumor growth.

Keywords Lyso-GM3 dimer · Receptor tyrosine kinase · Epidermal growth factor receptor · GM3 · Cytotoxicity

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
GFR	growth factor receptor
GM3	NeuAcα3Galβ4Glcβ1Cer
HPTLC	high-performance thin-layer chromatography
HRP	horseradish peroxidase
mAb	monoclonal antibody
MALDI-TOF	matrix assisted laser desorption/ionization
MS	time-of-flight mass spectrometry
PBS	phosphate-buffered saline
PNF	post-nuclear fraction
r.t.	room temperature
TBS	Tris-buffered saline

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Introduction

Cell growth is regulated primarily by growth factors and their receptors, organized at the cell surface membrane. Function of each type of growth factor receptor is inhibited or enhanced by a certain type of glycosphingolipid or ganglioside, surrounding the receptor in the membrane microdomain [1–3]. As a well-studied model, epidermal growth factor (EGF) activates tyrosine kinase at cytoplasmic domain of the EGF receptor (EGFR), when EGF binds to extracellular domain 2 of the receptor [4–7]. The inhibitory effect of ganglioside GM3 on EGF-dependent activation of EGFR tyrosine kinase [2, 8, 9] is assumed to result from interaction between GM3 and EGFR, as indicated by binding of GM3-coated fluorescent microspheres to EGFR [10]. A possible mechanism based on interaction of GM3 with a specific N-linked glycan of EGFR was recently proposed [11].

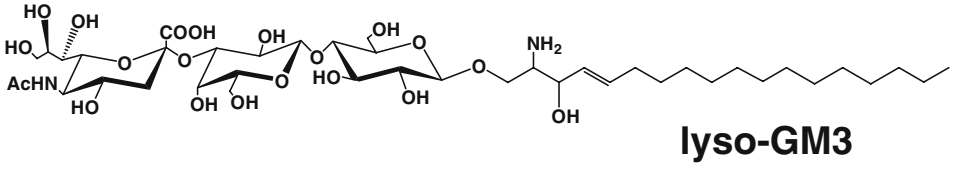
Sialyllactosyl sphingosine (lyso-GM3) was detected as a very minor component in A431 cells, and showed a stronger inhibitory effect than GM3 on EGFR kinase under physiological concentration of ATP and low detergent concentration (<0.05% Triton X-100) [9, 12]. The present study is focused on the following points: (1) Organization of GM3, EGFR, and other components in membrane microdomain of A431 cells, in terms of functional interaction of GM3 with EGFR, GM3 with caveolin, and caveolin with EGFR. (2) Quantitative interaction of GM3 with EGFR, using GM3-coated polystyrene beads. (3) Comparative inhibitory effect of lyso-GM3, lyso-GM3 dimer, trimer, and tetramer on EGF-induced tyrosine kinase associated with EGFR. Structures of lyso-GM3 dimer, trimer, and tetramer are illustrated in Fig. 3.

Results of these studies confirm that lyso-GM3 is a much stronger inhibitor of EGFR kinase than GM3, but displays strong cytotoxicity. Lyso-GM3 dimer showed lower cytotoxicity, but had similar inhibitory effect on EGFR as lyso-GM3. Lyso-GM3 trimer and tetramer showed essentially no inhibitory effect. Thus,lyso-GM3 dimer is a potential pharmacological inhibitor of EGFR tyrosine kinase activity, which is enhanced in various types of tumor cells.

Materials and methods

Chemicals, antibodies, and other reagents

Chemicals Methyl(methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-glycero-D-galacto-2-nonulopyranosid)onate was from Peptide Institute, Inc. (Osaka, Japan). (*2S*, *3R*, *4E*)-2-azido-3-*O*-benzoyl-4-octadecene-1,3-diol was kindly donated by Yutaka Ohhira (Daikin Industries, Ltd., Tsukuba, Japan).



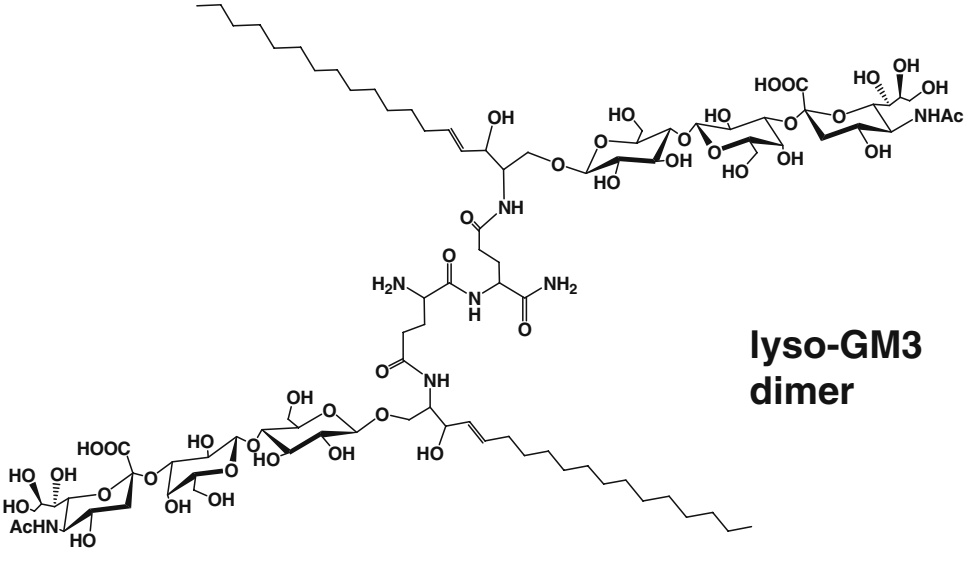


Fig. 1 Structures of lyso-GM3 and lyso-GM3 dimer

Antibodies Mouse mAb (clone F4) to human EGFR (cytoplasmic region) was from Sigma (St. Louis, MO). Mouse mAb PY20 to tyrosine phosphate from BD Transduction Labs (San Diego, CA). Mouse mAb to human γ-tubulin from Sigma. Mouse IgG3 mAb DH2 to GM3 (immunogen, GM3 lactone) established in this lab [13]. Rabbit antibodies to human caveolin from BD Transduction Labs. Normal mouse and rabbit IgG from Santa Cruz Biotech (Santa Cruz, CA).

Secondary antibodies (1) *for Western blotting*: goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) from Southern Biotech (Birmingham, AL). Micro-BCA kit and SuperSignal West Pico chemiluminescent substrate from Pierce (Rockford, IL). (2) *for confocal microscopy*: goat anti-mouse IgG labeled with Alexa Fluor 488 (IgG H+L), 488 (IgG3 γ3), or 594 (IgG1 γ1), and chicken anti-rabbit IgG labeled with Alexa Fluor 594 (IgG H+L), from Molecular Probes (Eugene, OR).

Ganglioside GM3 used as reference compound from Matreya (Pleasant Gap, PA). HPTLC plates from Merck. EGF and other reagents from Sigma, unless indicated otherwise.

Cells and cell culture

Human epidermoid carcinoma cell line A431 was purchased from American Type Culture Collection (ATCC, Rockville, MD) and grown in Dulbecco's modified Eagle's

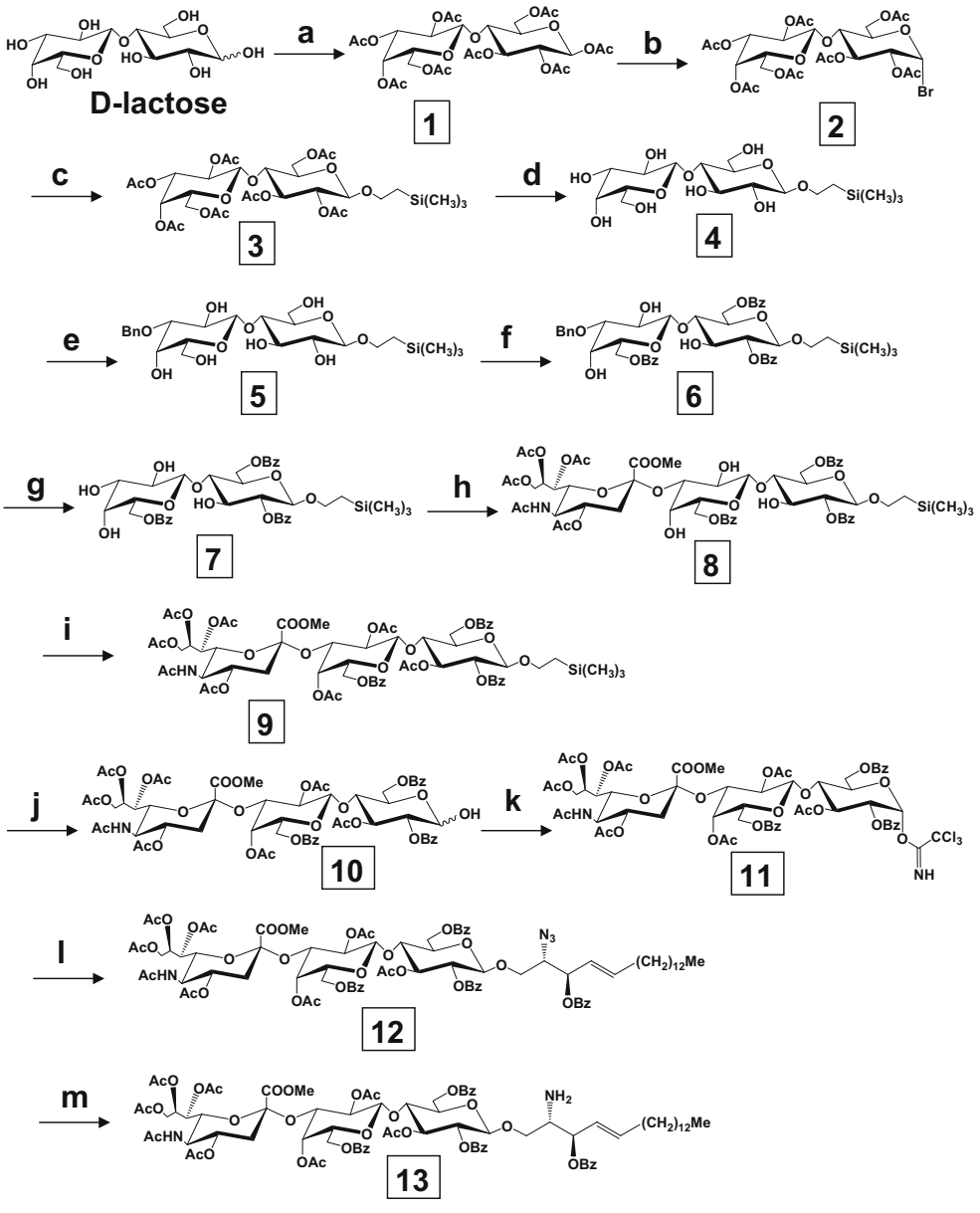


Fig. 2 Synthesis of lyso-GM3 derivative. Reagents and conditions: **a** Ac_2O, pyridine, 65°C, 4 h; **b** HBr/AcOH, CH_2Cl_2, r.t., 1.5 h; **c** TMEt, Ag_2ClO_4, Ag_2ClO_3, molecular sieves, CH_2Cl_2, r.t., 12 h, **d** NaOMe, MeOH, r.t., 2.5 h; **e** *1* dibutyltin oxide, methanol, reflux, 3 h; *2* Bu_4NBr, $PhCH_2Br$, benzene, reflux, 3 h; **f** BzCl, pyridine, CH_2Cl_2, −50°C, 30 min; **g** Pd-C, formic acid, MeOH, reflux, 2 h; **h** methyl α-thioglycoside of Neu5Ac, NIS-TfOH, molecular sieves, CH_3CN, r.t., 3 h, **i** Ac_2O, pyridine, r.t., 4 h; **j** $BF_3{\cdot}(C_2H_5)_2O$, CH_2Cl_2, 0–20°C, 7 h; **k** Cl_3CCN, DBU, CH_2Cl_2, 0°C, 2 h; **l** (*2S*, *3R*, *4E*)-2-azido-3-*O*-benzoyl-4-octadecene-1,3-diol, $BF_3{\cdot}(C_2H_5)_2O$, molecular sieves, CH_2Cl_2, 0°C, 4 h; (m) PPh_3, H_2O, benzene, 45°C, 10 h

medium (DMEM) with 10% fetal bovine serum (FBS). These cells were used for studying the effects of GM3 or lyso-GM3 derivatives on EGF-induced tyrosine kinase activity associated with EGFR, and on level of cell growth determined by [^{3}H]thymidine incorporation, as described previously [8, 9]. Methodologies applied for analysis of phenotypic change are described in specific subsections below.

Synthesis of lyso-GM3 and its derivatives

Lyso-GM3, lyso-GM3 dimer (for structures, Fig. 1), and various other derivatives were synthesized as outlined in Fig. 2. These products were characterized by ^{1}H-NMR spectra, determined by Varian Gemini-100 and UNITY plus 400 spectrometers for solutions in chloroform-*d* and methanol-d_4 with tetramethylsilane as internal standard. Optical rotations were measured by JASCO LIP 1000 digital polarimeter. Merck Silica gel was used for column chromatography. The molecular mass was measured by Bruker Autoflex MALDI-TOF mass spectrometer.

Conjugation of lyso-GM3 to glutaminylglutamic acid (Glu–Glu) or other glutamic acid oligomers

Lyso-GM3 having 2-azido-Sph, and all hydroxyl and carboxyl groups protected (compound **12**), was synthesized, and reductively converted to compound having 2-amino group (compound **13**) without further purification, as described above. The compound **13** was used for conjugation to glutamic acid dimer (Glu–Glu), trimer (Glu–Glu–Glu), or tetramer (Glu–Glu–Glu–Glu), as shown in Fig. 3 and explained in its legend.

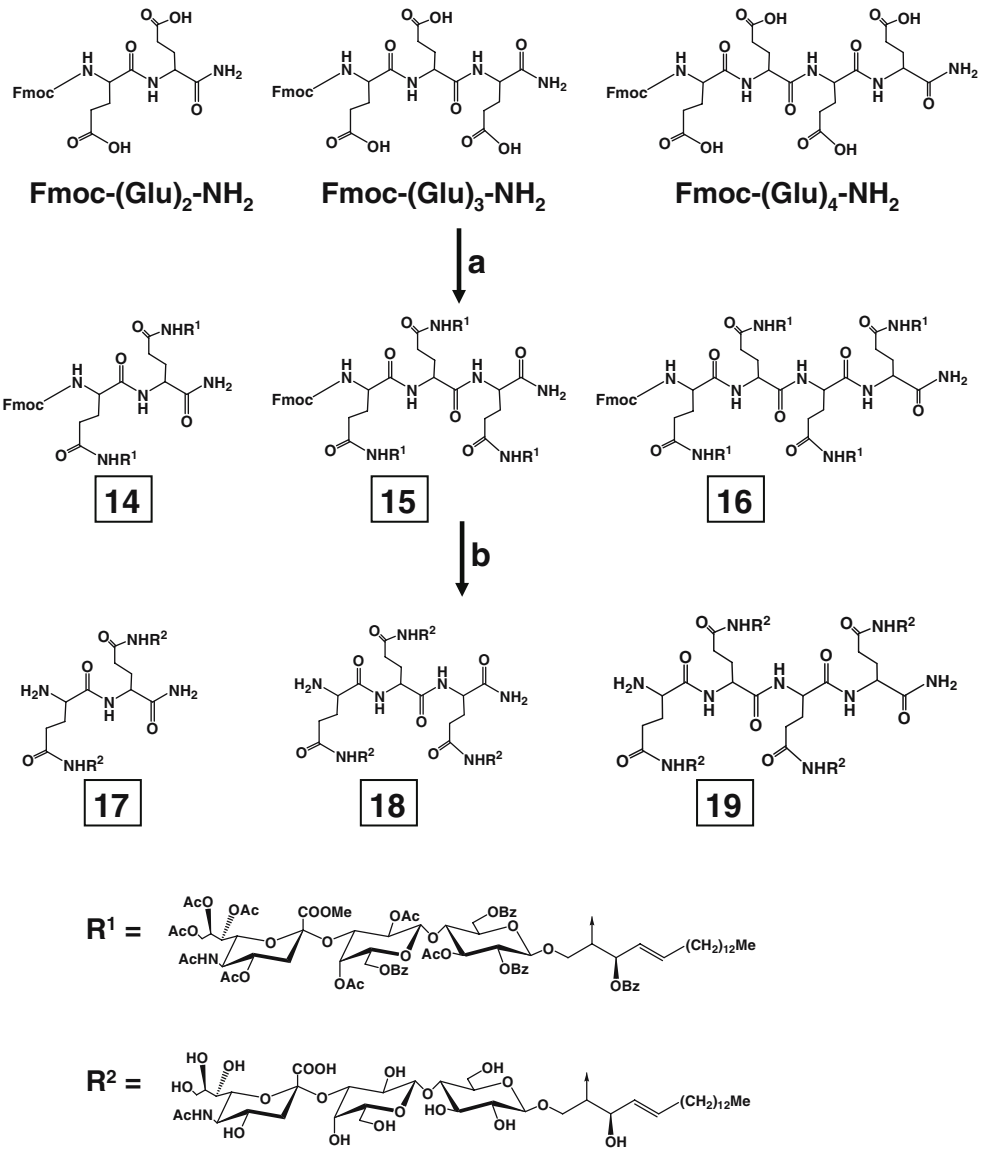


Fig. 3 Condensation reaction of lyso-GM3 derivative to glutamic acid dimer (Glu–Glu), trimer (Glu–Glu–Glu), and tetramer (Glu–Glu–Glu–Glu), and their Fmoc derivatives. Reagents and conditions: **a** **13**, WSC, CH_2Cl_2, DMF, r.t., 15 h; **d** NaOMe, MeOH, r.t., 18 h

Two residues of compound **13** were conjugated to Fmoc–Glu–Glu (Fmoc–$(Glu)_2$–NH_2) by carbodiimide method to yield two lyso-GM3 residues with all hydroxyl groups protected linked to this carrier (compound **14**), which was characterized by molecular ion in MALDI-TOF MS (positive) at 3779.96 (calculated: $[M+K]^+=3780.09$). The yield was 32%. Similarly, three residues of compound **13** were conjugated to Fmoc–Glu–Glu–Glu (Fmoc–$(Glu)_3$–NH_2) to yield three lyso-GM3 residues with all hydroxyl groups protected, linked to this carrier (compound **15**). This compound was characterized by molecular ion in MALDI-TOF MS (positive) at 5516.95, 5531.51 (calculated : $[M+Na]^+ = 5514.83, [M+K]^+ = 5530.94$). The yield was 18%. Four residues of compound **13** were conjugated to Fmoc–Glu–Glu–Glu–Glu (Fmoc–$(Glu)_4$–NH_2) to yield four lyso-GM3 residues with all hydroxyl groups protected linked to this carrier (compound **16**). This compound was characterized by molecular ion in MALDI-TOF MS (positive) at 7265.56 $\left(\text{calculated} : [M+Na]^+ = 7265.69\right)$. The yield was 13%. MALDI-TOF MS data for compounds **14**, **15**, and **16** were found to be essentially identical or close to theoretical values, and the yield was very reasonable.

Deprotection of all hydroxyl groups of compounds **14**, **15**, and **16** was performed by dissolving the protected compound in methanol, and adding sodium methoxide (final concentration 1%). The reaction mixture was stirred overnight at r.t.., then neutralized with a small amount of Dowex HCR-W2 (H^+) resin. Thus, protected groups of all hydroxyl groups of compounds **14**, **15**, and **16** were de-protected, resulting respectively in lyso-GM3 dimer (compound **17**), lyso-GM3 trimer (compound **18**), and lyso-GM3 tetramer (compound **19**). Yields were 27% for dimer, 16% for trimer, and 10% for tetramer.

HPTLC pattern of lyso-GM3 (lane 2) and its dimer (lane 3), in comparison to GM3 (lane 1), is shown in Fig. 4. The fractions containing lyso-GM3 trimer and tetramer (lanes 4, 5) are also shown. Each fraction contained a major, slow-migrating band (marked by asterisk), in addition to minor, faster-migrating bands (see Fig. 4 legend).

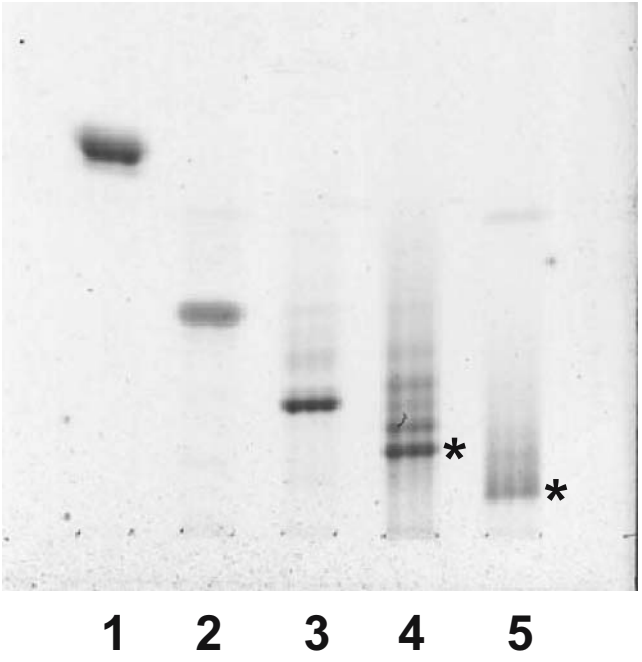


Fig. 4 HPTLC pattern of GM3, lyso-GM3, and its derivatives. Developed with chloroform/methanol/aqueous 0.2% $CaCl_2$, 5: 4: 1 (*v*/*v*/*v*), and stained with 0.5% orcinol in aqueous 10% H_2SO_4. *lane 1*: GM3 (Rf 0.42). *lane 2*: lyso-GM3 monomer (Rf 0.35). *lane 3*: lyso-GM3 dimer (Rf 0.14). *lane 4*: lyso-GM3 trimer (Rf 0.092). *lane 5*: lyso-GM3 tetramer (Rf 0.046). Properties of each compound are described under the Materials and methods section. The fractions for trimer (*lane 4*) and for tetramer (*lane 5*) contained a major band (*marked by asterisk*) in addition to minor bands. MALDI-TOF MS data for the compounds 14, 15, and 16 in Fig. 3, the immediate precursors of dimer, trimer, and tetramer, indicate that parental mass number of each compound corresponded to theoretical value (see Materials and methods section, "Conjugation of lyso-GM3 to glutaminylglutamine ..."). This HPTLC pattern was determined ~5 years after sample preparation, although the sample was stored in freezer. It is possible that minor components above the major band were created during storage

Preparation of post-nuclear fraction (PNF) and glycolipid-enriched microdomain (GEM) fractions for determination of their components (methods for results shown in Fig. 5)

PNF and GEM were prepared as described previously [14, 15], except for use of 1% Brij 98 instead of Triton X-100. Briefly, A431 cells (5×10^6/15-cm dish; Nunc) were cultured in DMEM with 10% FBS for 48 h, washed 2× with sf DMEM, incubated in 15 ml sf DMEM for 24 h at 37°C. Cells (~1×10^8) were washed with PBS, collected by scraping, centrifuged, and the pellet was suspended in 5 ml Brij 98 lysis buffer (1% Brij 98, 25 mM HEPES buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA) containing 75 U aprotinin, 2 mM phenylmethanesulfonyl fluoride, and 100 μg/ml leupeptin. The suspension was kept on ice for 30 min and Dounce homogenized (ten strokes). The lysate was centrifuged at 2,500 rpm for 7 min at 4°C to remove nuclei and debris from the supernatant fraction (PNF). PNF was further separated into 12 fractions, from top (Fr. 1; lowest density) to bottom (Fr. 12; highest density) by sucrose density gradient ultracentrifugation [14, 15]. Aliquots of each fraction, containing equal protein content (~0.2 μg), were analyzed by SDS-PAGE and Western blot with antibodies directed to specific proteins (e.g., EGFR). For analysis of GSLs, each fraction (or combinations of a few fractions) were applied to BondElut C18 column (Varian, Harbor City, CA), subjected to HPTLC, and immunostained by DH2 as described in the legend for Fig. 5b and c.

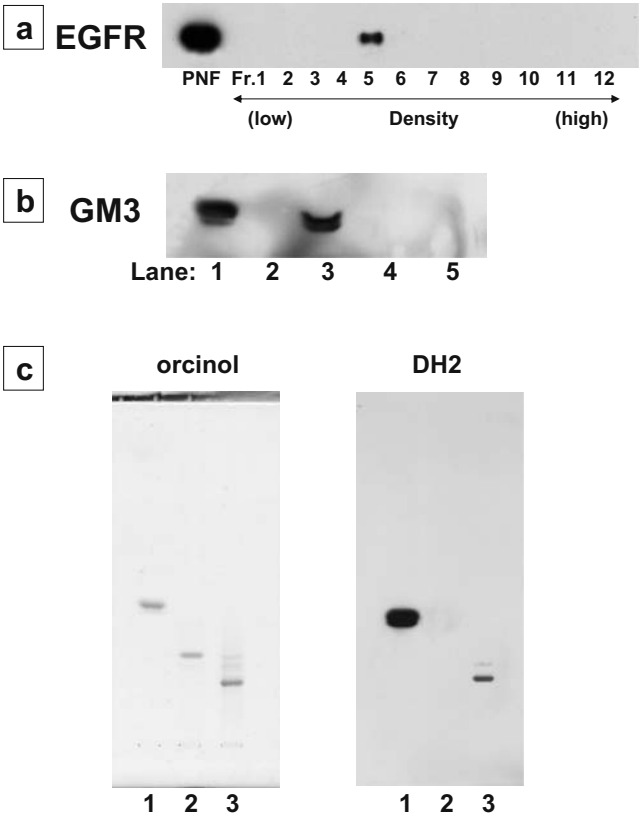


Fig. 5 Distribution patterns of EGFR (**a**) and GSLs (**b**) in membrane fractions with low to high density and TLC reactivity of lyso-GM3 and its dimer with anti-GM3 mAb DH2 (**c**). **a** Western blot pattern of EGFR in each fraction (Fr. 1–12) separated by sucrose density gradient ultracentrifugation of PNF of A431 cells prepared in 1% Brij 98 as described in the Materials and methods section. **b** HPTLC pattern of respectively combined Fr. 1–3, 4–6, 7–9, and 10–12. Each fraction 1–12 prepared as in panel (**a**). GSL fraction from each Fr. 1–12, respectively, was separated using C18 column as described in the Materials and methods section, and analyzed by HPTLC. *lane 1*: GM3 (reference). *lane 2*: Fr. 1–3. *lane 3*: Fr. 4–6. *lane 4*: Fr. 7–9. *lane 5*: Fr. 10–12. For TLC immunostaining, TLC plate was fixed by 1 min immersion in 0.5% polyisobutyl–methacrylate solution in a mixture of chloroform/hexane 1:9, dried, blocked with 3% BSA in PBS for 1 h at r.t., and reacted overnight with mAb DH2 (hybridoma supernatant) at 4°C. The plate was washed with PBS, then incubated with HRP-conjugated secondary antibody for 1 h, followed by detection with SuperSignal substrate. **c** HPTLC pattern of GM3 (*lane 1*), lyso-GM3 (*lane 2*), and lyso-GM3 dimer (*lane 3*). Developed with solvent chloroform/methanol/0.2% $CaCl_2$ in water, 5:4:1 (*v*/*v*/*v*) for both left and right panel. The pattern in left panel stained by spraying with 0.5% orcinol in aqueous 10% H_2SO_4, then heating at 120°C for 10 min. The pattern in right panel immunostained with DH2 as in (**b**)

Determination of distribution of lyso-GM3 dimer and EGFR by laser scanning confocal microscopy (methods for results shown in Fig. 6)

A431 cells (2×10^4 cells) were grown on cover glass (12 mm diameter) placed in 24-well plates (Corning) in DMEM containing 5% FBS for 24 h. Cells were gently washed 2× with sf DMEM, and incubated in 250 µl sf DMEM with or without 50 µM GM3 or lyso-GM3 dimer

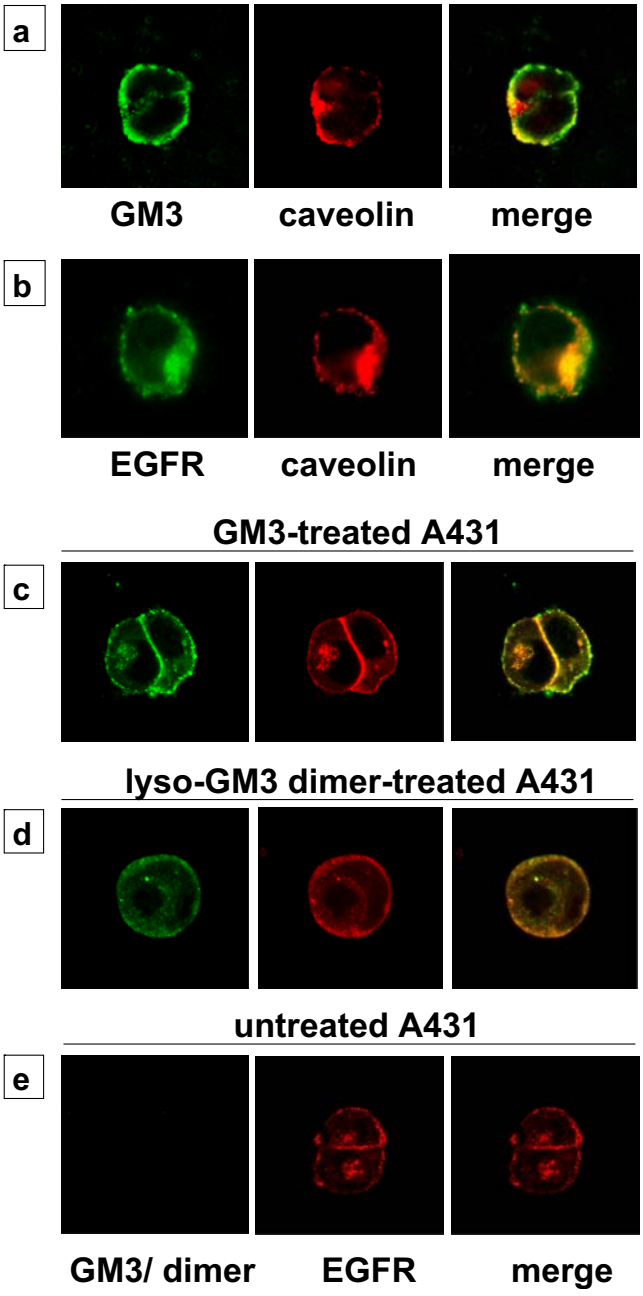


Fig. 6 Co-localization of membrane components in A431 cells. **a**, **b**: Co-localization of GM3 and caveolin, and that of EGFR and caveolin, as revealed by confocal microscopy. A431 cells were cultured on cover glass, treated with GM3, double-stained with anti-GM3 mAb DH2 and anti-caveolin, or anti-EGFR and anti-caveolin, and differentially labeled with Alexa Fluor 488 goat anti-mouse IgG (H+L) and Alexa Fluor 594 chicken anti-rabbit IgG (H+L) as described in the Materials and methods section. **c**–**e**: Co-localization of GM3 and EGFR using GM3-treated A431 cells (**c**) and lyso-GM3 dimer-treated cells (**d**). Co-localization was not observed for untreated A431 cells (**e**). These cells were double-stained with anti-GM3 mAb DH2 (mouse IgG3) and anti-EGFR (mouse IgG1), and differentially labeled with Alexa Fluor 488 goat anti-mouse IgG3 and Alexa Fluor 594 goat anti-mouse IgG1

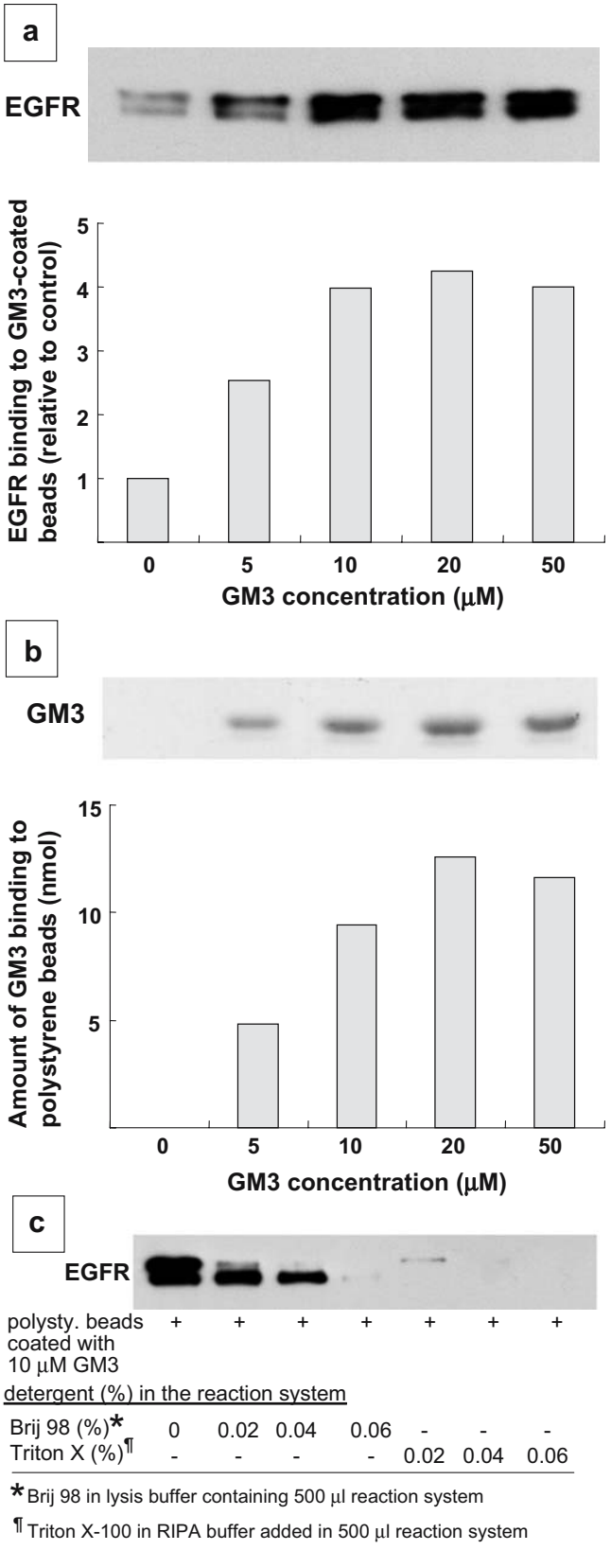


Fig. 7 Adsorption of GM3 and EGFR on polystyrene beads. **a** GM3 dose-dependent adsorption of EGFR on GM3-coated polystyrene beads. Method: see Materials and methods section, "Determination of GM3 binding to EGFR". **b** Quantity of GM3 adsorbed on a defined quantity of polystyrene beads. GM3 adsorbed on polystyrene beads under conditions as described for **(a)** were extracted by methanol. The extract was separated by centrifugation, concentrated, and stained by orcinol reagent. **c** Effect of detergents, in Brij 98 lysis buffer or RIPA buffer, on binding of EGFR to GM3-coated beads. GM3-coated beads (incubated with 10 μM GM3) were resuspended in 500 μl TBS(+) containing A431 cell membrane (20 μg protein, prepared as described previously) and various amounts (in μl) of Brij 98 lysis buffer or RIPA buffer. Compositions of these buffers: see Materials and methods section. The concentration of both detergents (Brij 98 in "Brij 98 lysis buffer", and Triton X-100 in RIPA buffer) was 1% in total volume 500 μl of reaction system containing A431 cell extract. Therefore, concentrations of detergent in both Brij 98 lysis buffer and RIPA buffer were 0.02, 0.04, and 0.06% respectively. Preparation of reaction system, and conditions of reaction: see Materials and methods section

for 2 h at 37°C. Then, cells were washed with PBS and fixed with 2% paraformaldehyde/ PBS for 15 min, washed 2× with PBS, permeabilized with 0.1% Triton X-100 for 5 min, washed 2× with PBS, and incubated with 1% BSA/ PBS with 0.05% NaN_3 for 30 min. For double staining, cells were incubated with 250 μl of antibody mixture for 1 h at r.t.; antibodies used were anti-GM3 DH2 (mouse IgG3), anti-EGFR (mouse IgG1), and anti-caveolin (rabbit pAb). Cells were incubated with a mixture of Alexa Fluor 488 goat anti-mouse IgG3 and Alexa Fluor 594 goat anti-mouse IgG1, or Alexa Fluor 488 goat anti-mouse IgG (H+L) and Alexa Fluor 594 chicken anti-rabbit IgG (H+L) for 1 h at r.t., washed, mounted with a drop of Glycergel (Dako Corp., Carpinteria, CA) containing 1% 1,4-diazabicyclo [2,2,2]octane, and observed by laser scanning confocal microscopy (FluoView, Oympus, Tokyo, Japan) using an appropriate filter set.

Control staining was performed with normal mouse IgG or normal rabbit IgG to measure possible cross-reaction or nonspecific binding. All controls gave negative results.

Determination of GM3 binding to EGFR (methods for results shown in Fig. 7)

GM3 binding to EGFR was previously determined based on adsorption of EGFR to GM3-coated polystyrene beads with 1 μm diameter [11, 16]. This method was modified as below, using 4.2 μm diameter beads, which we found to be more appropriate. We also studied the effect of detergent in lysis buffer on adsorption of EGFR on the GM3-coated beads.

Polystyrene beads (4.2 μm diameter; Interfacial Dynamics Corp., Portland, OR) were washed 3× with TBS(−) and once with ethanol (each washing by 3,500 rpm (~1,800×*g*) centrifugation). Beads were resuspended in ethanol, and aliquoted into microfuge tubes (~5×10^6 beads per tube) with various concentration of GM3 in 1 ml of ethanol/water 9:1

(*v*/*v*). Beads were incubated with tumbling for 1 h at r.t., dried under N_2 stream, washed 3× with TBS(−), and incubated with 300 μl of 0.1% gelatin/TBS with tumbling for 1 h at r.t., to block nonspecific binding sites. Then, beads were centrifuged, washed with TBS(+) [10 mM Tris–HCl (pH 8.0), 130 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 0.1 mM $MnCl_2$], and resuspended in 500 μl of TBS(+) containing PNF (30 μg protein). The mixture was kept overnight at 4°C with tumbling, and then washed 3× with TBS(+), resuspended in SDS-PAGE sample buffer, boiled, and analyzed by Western blotting with anti-EGFR antibody.

For determining amount of GM3 bound to polystyrene beads, GM3-coated beads were prepared as above, and extracted 3× with 500 μl methanol (each time sonicated for 20 min, and centrifuged). The extracts were dried under N_2 stream, dissolved in chloroform/methanol (2:1), placed on HPTLC plate (Merck), developed with chloroform/methanol/0.2% $CaCl_2$ in H_2O (50:40:10), and stained with orcinol reagent.

For determining effects of detergent on EGFR binding to GM3-coated beads, beads coated by incubation with 10 μM GM3 were resuspended in 500 μl TBS(+) containing A431 cell membrane (20 μg protein, prepared as described previously [11, 16]) and various amounts of Brij 98 lysis buffer or RIPA buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris, pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM pyrophosphate, 50 mM NaF). Conditions for determining effect of detergent: see Fig. 7 legend.

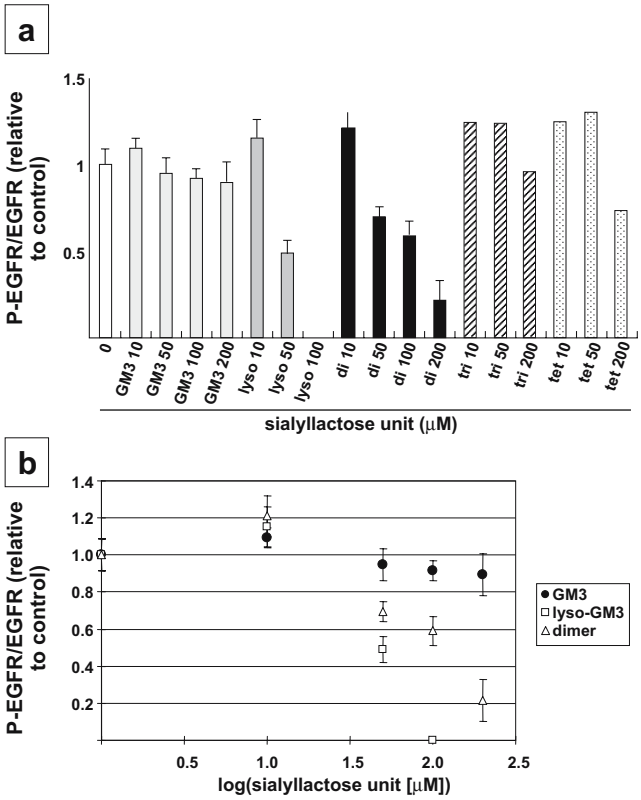


Fig. 8 Effect of lyso-GM3 dimer, trimer, and tetramer, in comparison with GM3, on activity of EGF-associated tyrosine kinase in cultured A431 cells. **a** Relative effects of GM3, lyso-GM3 ("lyso"), dimeric lyso-GM3 ("di"), trimeric lyso-GM3 ("tri"), and tetrameric lyso-GM3 ("tet"). *Ordinate*: ratio of band density of tyrosine phosphorylated EGFR, divided by band density of EGFR. *Numbers on abscissa* indicate concentration of sialyllactose units in micromoles (μM). *0* no compound. **b** Relative effect as in **a**, expressed as sialyllactose units. *Abscissa*, log [sialyllactose unit (μM)]. *Ordinate* ratio of band density of tyrosine phosphorylated EGFR, divided by band density of EGFR

Effects of GM3, lyso-GM3, lyso-GM3 dimer, trimer, and tetramer on EGF-induced EGFR tyrosine kinase in cultured A431 cells (methods for results shown in Fig. 8)

A431 cells (1.5×10^5) were seeded and cultured in 24-well plates in DMEM containing 5% FBS for 24 h, then in serum-free (sf) DMEM for another 24 h prior to confluence. Then, cells were washed twice with sf DMEM, incubated in 200 μl sf DMEM containing various concentrations of GM3, lyso-GM3, lyso-GM3 dimer (compound **17** in Fig. 3), lyso-GM3 trimer (compound **18**), or lyso-GM3 tetramer (compound **19**), for 2 h at 37°C. After incubation, the medium was removed and 200 μl of 100 ng/ml human EGF in PBS was added to the well and incubated for 1 h at 4°C. Cells were then washed and lysed in 100 μl RIPA buffer containing 1 mM Na_3VO_4, 75 U aprotinin, and 2 mM phenylmethanesulfonyl fluoride, at 4°C. The lysate was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was subjected to 7% SDS-PAGE and Western blot after determination of protein concentration. The intensity of Western blot was determined by densitometry using Scion image program.

Effects of lyso-GM3 dimer on cell proliferation, determined by ^{3}H-thymidine incorporation (methods for results shown in Fig. 9)

A431 cells (5×10^4 cells/well) were seeded and cultured in 48-well plates (Corning) as above. Cells were then washed 2× with sf DMEM, incubated in 200 μl sf DMEM with or without 50 μM lyso-GM3 dimer (sialyllactose unit concentration; 100 μM) for 2 h at 37°C. After incubation, the medium was removed and 180 μl of 10 pg/ml (1.7 pM) human EGF in sf DMEM was added to the well and incubated for 23 h. 20 μl of ^{3}H-thymidine (100 μCi/ml, 2 μCi/well) was then added and incubated for 1 h at 37°C. Triplicate samples of each cell culture were washed with PBS and harvested by trypsinization, and ^{3}H-thymidine incorporated in cells was measured by scintillation counter, as described previously [17].

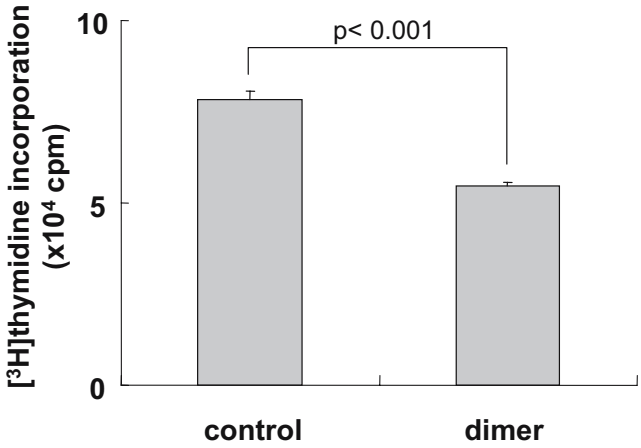


Fig. 9 Inhibitory effect of lyso-GM3 dimer on A431 cell proliferation, determined by [^{3}H] thymidine incorporation A431 cells were seeded, cultured, pre-incubated with 50 μM lyso-GM3 dimer, and processed for [^{3}H]thymidine incorporation as described in the Materials and methods section, "Effects of lyso-GM3 dimer on cell proliferation ...". [^{3}H]thymidine incorporation expressed in cpm (ordinate) in nontreated ("control") cells, and in lyso-GM3 dimer ("dimer") treated cells. The difference between the means is statistically significant ($p<0.001$)

Cell viability (methods for results shown in Fig. 10)

A431 cells (1×10^5) were seeded and cultured in 48-well plate in DMEM containing 5% FBS for 24 h, and in sf DMEM for 24 h, until confluence. Then, cells were washed twice with sf DMEM, incubated in 100 μl sf DMEM containing various concentration of GM3, lyso-GM3, or lyso-GM3 dimer, for 2 h at 37°C. After incubation, cells were washed with PBS and harvested with trypsin–EDTA, centrifuged, and suspended in 100 μl PBS. Number of living cells, excluding those stained by Trypan Blue, was counted. Viability was expressed as described in the Fig. 10 legend.

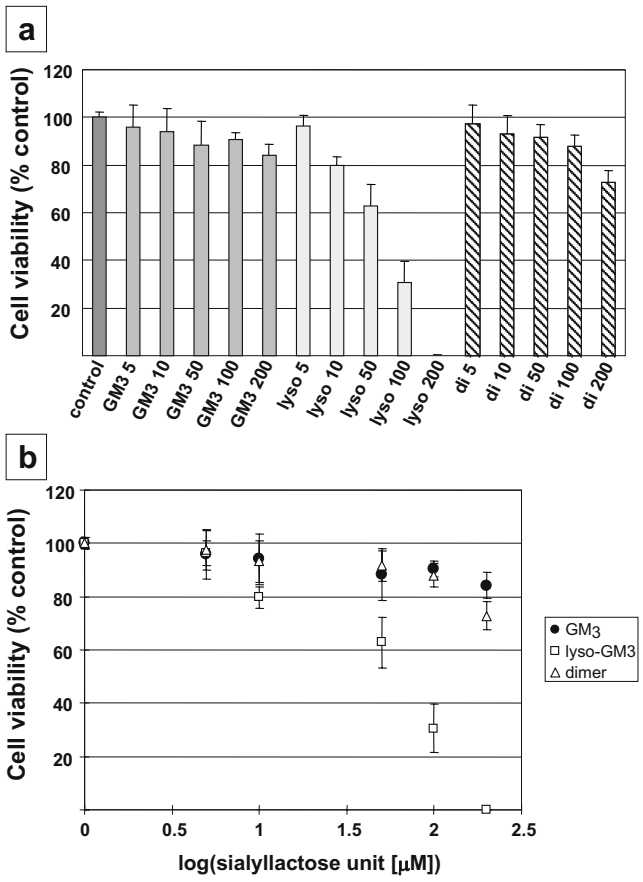


Fig. 10 Cytotoxic effect of GM3, lyso-GM3, and lyso-GM3 dimer on A431 cells. **a** A431 cells were grown to confluency on 24-well plates, washed, plated in sf DMEM containing various concentrations (5, 10, 50, 100, 200 μM) of each compound as above (*abscissa*), incubated 2 h, and subjected to Trypan Blue exclusion assay (see Materials and methods section). Ratio of number of treated cells to control (nontreated) cells (defined as 100%) was calculated (ordinate). "lyso", lyso-GM3; "di", lyso-GM3 dimer. **b** Relative effect as in **a**, expressed as sialyllactose units. "dimer", lyso-GM3 dimer

Results

Co-presence of EGFR, GM3, and caveolin in low-density membrane fraction from A431 cells, and possible interaction of EGFR with exogenously added GM3 or lyso-GM3 dimer

Postnuclear fractions prepared from A431 cells were separated into 12 fractions with different densities as described in M&M. EGFR and GM3 were found exclusively in low-density Fr. 5 (Fig. 5a and b). Caveolin was found in low-density Fr. 4, 5, 6, but also present in high-density Fr. 7–10 (data not shown). In these studies, expression of GM3 was determined by mAb DH2 which reacts with GM3 and lyso-GM3 dimer, but not with lyso-GM3 (Fig. 5c). The highly novel reactivity observed with GM3 and lyso-GM3 dimer but not with lyso-GM3 may indicate that anti-GSL antibody recognizes not only the glycosyl residue but also its orientation (see Discussion section).

The apparent association of these components was further investigated using confocal microscopy. Co-localization of GM3 and caveolin, EGFR and caveolin, and GM3 and EGFR, was clearly indicated by clear "merge image" when A431 cells were treated with exogenous GM3 (Fig. 6a–c).

When cells were treated with exogenous lyso-GM3 dimer, which also reacts with anti-GM3 mAb DH2 (as above; Fig. 5c), merge image was observed, but was relatively weak (Fig. 6d). Untreated A431 cells reacted very weakly with DH2, and therefore no merge image was observed (Fig. 6e).

Since tetraspanins CD9 and CD82 are often present in various types of cells, controlling cell motility and cell

growth, their possible presence was studied by flow cytometry and by Western blot analysis using antibodies directed to these tetraspanins. CD9 and CD82 were essentially absent in A431 cells (data not shown).

Direct interaction of GM3 with EGFR in A431 cells

In view of the possible interaction of GM3 with EGFR indicated by their presence in the same low-density membrane microdomain, and by "merge image" in confocal microscopy as above, further evidence of such interaction was demonstrated based on adsorption of EGFR on GM3-coated polystyrene beads, as described under M&M. GM3 dose-dependent increase of EGFR adsorption was clearly observed. EGFR adsorption reached a plateau when GM3 concentration reached 10 nmol/ml (*i.e.*, ~10 μM) (Fig. 7a). The amount of GM3 adsorbed on a given quantity of polystyrene beads, determined by TLC immunostaining, was similar for 10, 20, or 50 μM concentration (Fig. 7b). The validity of this method for determining interaction of GM3 with EGFR was assessed based on the effect of Brij 98 lysis buffer or RIPA buffer, as shown in Fig. 7c. Brij 98 at low concentration (0.02–0.04%) slightly reduced EGFR binding to GM3-coated polystyrene beads, while Triton X-100 in RIPA buffer strongly blocked such binding. In many of our previous studies, Brij 98 (polyoxyethylene 20 oleyl ether) had much less effect than Triton X-100 (octyl phenoxy polyoxyethanol), in terms of protecting interaction of GSL with signal transducer molecules in microdomain [18–20].

Comparative inhibitory effect of GM3, lyso-GM3, lyso-GM3 dimer, lyso-GM3 trimer, and lyso-GM3 tetramer, on EGF-induced EGFR tyrosine kinase activity in cultured A431 cells

The inhibitory effects of lyso-GM3 and its derivatives on EGF-induced EGFR tyrosine kinase activity are shown in Fig. 8a. Under conditions in which GM3 did not clearly inhibit the kinase activity (50–100 μM concentration), lyso-GM3 dimer showed clear inhibition at 50 μM (P-EGFR/EGFR ratio 0.5), and stronger inhibition at 200 μM (P-EGFR/EGFR ratio 0.2, or in some cases <0.01). Unexpectedly, lyso-GM3 trimer and tetramer showed no inhibitory effect at 10 or 50 μM, and only very weak effect at 200 μM. Inhibitory effects of GM3, lyso-GM3, and lyso-GM3 dimer were compared in terms of sialyllactose units (μM), as shown in Fig. 8b. The inhibitory effect of lyso-GM3 was stronger than that of lyso-GM3 dimer, which was much stronger than that of GM3.

Since lyso-GM3 is clearly cytotoxic at 50–100 μM (see following section), the inhibitory effect on cell proliferation of lyso-GM3 dimer as compared to nontreated control A431 cells was determined by [^{3}H]thymidine incorporation. Lyso-GM3 dimer clearly inhibited cell proliferation (Fig. 9)].

Cytotoxic effects of GM3, lyso-GM3 monomer, and lyso-GM3 dimer on A431 cells

Since lyso-GM3 and its dimer clearly inhibit EGF-induced EGFR tyrosine kinase activity in A431 cells, we compared cytotoxicity of these two compounds with that of GM3 at various concentrations. Viability of cells under each experimental condition is shown in Fig. 10a as percent of control cell number, and in Fig. 10b in terms of log sialyllactose units. Only lyso-GM3 displayed a clear dose-dependent cytotoxicity, whereas GM3 and lyso-GM3 dimer displayed strikingly lower cytotoxicity.

Discussion

The inhibitory effect of GM3 on EGFR tyrosine kinase activity in epidermoid carcinoma A431 and KB cells was demonstrated initially for the receptor kinase activity present in solubilized membrane [8, 9], and subsequently with *in situ* effect of GM3 on A431 cells in culture [21]. The modulatory effect of gangliosides has been extended to various other growth factor receptors, such as platelet-derived growth factor receptor [22, 23], insulin receptor [24–26], and nerve growth factor receptor [27, 28]. These studies indicate that the basic modulatory effect of sphingolipids and gangliosides on receptor function is common [1–3].

Present studies suggest that EGFR is present exclusively in low-density, GM3-enriched microdomain of A431 cells. A similar association of GM3, Src family kinase, and G-proteins with low-density membrane fraction of B16 melanoma cells [14], and of T-cell lymphoma [29], was observed previously (for review see [30, 31]. Interaction of GM3 with EGFR, and with other GEM components, was suggested by "merge image" of these components in confocal microscopy, and was further confirmed by quantitative, direct interaction of EGFR with GM3-coated polystyrene beads.

Noteworthy observations were as follows: (1) lyso-GM3 and lyso-GM3 dimer have strong inhibitory effect on EGF-induced EGFR tyrosine kinase in A431 cells in culture; (2) the degree of inhibition by lyso-GM3 and its dimer are much higher than that of GM3; (3) lyso-GM3 displayed high cytotoxic effect, whereas lyso-GM3 dimer had minimal cytotoxic effect; (4) lyso-GM3 trimer and tetramer have much less inhibitory effect on EGFR kinase.

Effects of lyso-GM3 trimer and tetramer on EGFR tyrosine kinase were much weaker than that of lyso-GM3 dimer, as shown in Fig. 8a and b. We are confident that

lyso-GM3 trimer (compound 18) and lyso-GM3 tetramer (compound 19) showed much weaker inhibitory effect than lyso-GM3 dimer (compound 14), based on the following evidence: (1) The chemical yields from compound 13 to 14, or 13 to 15, or 13 to 16, were close to theoretical values, and the molecular mass, determined by MALDI-TOF MS, was close to theoretical. (2) Conversion of compound 14 to 17, 15 to 18, and 16 to 19, was simple de-acetylation with sodium methoxide in methanol. In many cases, such reaction is quantitative. Yield and molecular mass by MALDI-TOF MS of compounds 17, 18, and 19 was very reasonable. All these preparations and determinations by mass spectrometry were made when the compounds were freshly synthesized. (3) Effect of lyso-GM3 dimer (compound 14) on EGFR tyrosine kinase was determined by T. Hikita and K. Handa, when the synthesized compound had not spent many years in storage in freezer. We are therefore confident that low activity of lyso-GM3 trimer and tetramer is not due to degradation of the compounds during long period of storage.

The exact mechanism of the stronger inhibitory effect of lyso-GM3 or its dimer, compared to GM3, on EGFR kinase is unknown and remains to be elucidated. While both lyso-GM3 and lyso-GM3 dimer strongly inhibit EGFR kinase, the mechanisms of their effects may be different. Lyso-GM3, similar to other lyso-GSLs or lysophospholipids which are cytotoxic, may disrupt membrane organization, making it impossible to show interaction with EGFR. In contrast, lyso-GM3 dimer may directly inhibit EGFR kinase. In fact, exogenously added lyso-GM3 dimer displayed “merge image” with EGFR.

A provision that inhibitory effect of lyso-GM3 and lyso-GM3 dimer on EGFR tyrosine kinase is basically different, *i.e.*, (1) Lyso-GM3 disrupts membrane organization of microdomain and thereby interferes with GM3 to EGFR interaction, as is observed in trace amount of RIPA buffer. Glycosyl residue of lyso-GM3 is oriented entirely differently from GM3, and therefore interaction with EGFR is difficult. (2) Lyso-GM3 dimer and to a lesser extent GM3 are present in microdomain in orderly manner, in which glycosyl residue of both GM3 and lyso-GM3 dimer are in different orientation than lyso-GM3. Therefore, interaction of glycosyl residue of GM3 is greatly affected by carrier molecule, *i.e.*, carrier molecule of GSL [32].

In previous studies, a clear functional difference of reconstituted membrane vesicles (1) containing GM3, phosphatidylcholine, sphingomyelin, cholesterol, and cSrc, vs. (2) as above, but also containing lyso-GM3, was demonstrated. Adhesion of vesicles (2) to Gg3-coated plate caused strong activation of cSrc, whereas adhesion of vesicles (2) to Gg3-coated plate blocked Src activation, *i.e.*, presence of lyso-GM3 in vesicles (2) blocked adhesion-induced signaling. This blocking effect may not be due simply to membrane disruption, since psychosine (Gal-sphingosine) or lactosylsphingosine did not block Src activation [33]. This finding suggests that lyso-GM3 may interact with GM3, and thereby block interaction of GM3 with EGFR. *I.e.*, the mechanism for the effect of lyso-GM3 is not simply due to its cytotoxicity, but rather to modulatory effect of lyso-GM3 on GM3-to-EGFR interaction. Further extensive studies along this line, using lyso-GM3 dimer, may clarify the mechanism.

Lyso-GM3 trimer and tetramer were expected to show stronger inhibitory effect than lyso-GM3 dimer, since previous studies have shown that ligand-dependent interaction with receptor is enhanced by multivalency of the ligand [34]. However, lyso-GM3 trimer and tetramer had much lower inhibitory effect as compared to lyso-GM3 dimer. Orientation and position of three or four sialosyl–lactosyl–Sph groups in trimer or tetramer may not be the same as that in dimer. Orientation and position of sialosyl–lactosyl–Sph groups in dimer may be highly effective to interact with EGFR. Conformational analysis of these molecules may provide an important basis for future synthesis of more effective inhibitors of receptor kinase.

Many invasive cancer cells express high level of EGFR and activated tyrosine kinase. Therefore, tyrosine kinase associated with EGFR has been a target of cancer chemotherapy. For example, ZD1839 (gefitinib; Iressa®) [35–38] is a selected inhibitor of EGFR-associated tyrosine kinase, and has been used in clinical trials. So far, toxicity of this drug is high in some cases, and evaluation for cancer treatment is still continuing. Results of the present study, focused on GM3 analogues, suggest that lyso-GM3 dimer, which displays minimal cytotoxicity but stronger inhibitory effect than GM3 on EGFR kinase, may be a good candidate for pharmacological inhibitor of epidermal tumor growth, since it is presumably less cytotoxic for normal cells than ZD1839 or similar drugs.

Recently, glycosyl mimetics of sialyl–Le^a and sialyl–Le^x were shown to successfully block neutrophil interaction with E-selectin, and thereby suppress various types of inflammatory responses [39]. Similarly, selection of glycosyl mimetics of GM3 or lyso-GM3 dimer may lead to more effective inhibitors of EGFR function. This is an important approach for the future.

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