Glycosphingolipid GM3, a known suppressor of epidermal growth factor receptor (EGFR) phosphorylation, inhibits cell proliferation. Valproic acid, conversely, is known as an up-regulator of GM3 synthase gene (ST3GAL5). To test the possibility that valproic acid could inhibit EGFR phosphorylation by increasing the level of GM3 in cells, we treated A431 epidermoid carcinoma cells with valproic acid and found that valproic acid treatment caused an about 6-fold increase in the GM3 level but only a marginal increase in the GM2 level in these cells and that the observed increase in GM3 level was valproic acid dose-dependent. Consistent with this observation, valproic acid treatment induced GM3 synthase gene expression by about 8-fold. Furthermore, phosphorylation of EGFR was reduced, and cell proliferation was inhibited following valproic acid treatment. Consistent with these results, transient expression of GM3 synthase gene in A431 cells also increased cellular level of GM3, reduced phosphorylation of EGFR, and inhibited cell proliferation. Treatment with l-phenyl-2-decanoylamino-3-morpholino-l-propanol, an inhibitor of glucosylceramide synthesis, decreased the cellular level of GM3 and reduced the inhibitory effects of valproic acid on EGFR phosphorylation and cell proliferation. These results suggested that induction of GM3 synthesis was enough to inhibit proliferation of cancer cells by suppressing EGFR activity. Valproic acid treatment similarly increased the GM3 level and reduced phosphorylation of EGFR in U87MG glioma cells and inhibited their proliferation. These results suggested that up-regulators of GM3 synthase gene, such as valproic acid, are potential suppressors of cancer cell proliferation.

Glycosphingolipid GM3 is one of the well studied gangliosides. GM3 is synthesized by transferring sialic acid to lactosylceramide, a step catalyzed by a sialyltransferase known as GM3 synthase, which is encoded by the ST3GAL5 gene (1). GM3 is a common precursor for the synthesis of all gangliosides. GM3 is known as a modulator of growth factor receptors and insulin receptor (2–4). Particularly, modulation of epidermal growth factor receptor (EGFR) by GM3 has been well studied. For modulation of receptor activity, it is essential that GM3 binds to its receptor. There are two possible ways GM3 can bind to a receptor: 1) binding via sialic acid of GM3 and basic amino acid residues of the receptor as has been found in the case of insulin receptor and GM3 (5) and 2) binding via carbohydrate-to-carbohydrate interaction as has been observed between GM3 and N-glycans of EGFR (6, 7). The binding of GM3 to these receptors prevented receptor dimerization and inhibited phosphorylation of receptors (8).

Several previous studies have shown that GM3 level in cells changes on transformation (9, 10). For example, transformation of fibroblast cells by v-Jun greatly reduced GM3 levels in transformed cells, and increased synthesis of GM3 reversed the oncogenic properties of the cell (11). In addition, Noguchi et al. (12) reported that the GM3 synthase gene was highly expressed in adenocarcinoma tissues obtained from non-small cell lung cancer patients. Furthermore, adenocarcinoma cells, which expressed high levels of GM3 synthase mRNA, exhibited more sensitivity to EGFR tyrosine kinase inhibitors and a decreased level of EGFR phosphorylation (12). These results led to the suggestion that GM3 might suppress cancer cell proliferation.

In contrast to GM3, valproic acid, which is known as an anti-convulsant and mood-stabilizing drug and a histone deacetylase inhibitor (13–15), has been shown to up-regulate ST3GAL5 gene expression (16). In a recent clinical trial, the potential application of valproic acid in treating cancers, especially high grade gliomas, was examined, and it was observed that valproic acid had some therapeutic effect on glioma (17).

Based on the above discussed results, we hypothesized that valproic acid might induce the expression of GM3 synthase as a result of which more GM3 would be produced, and this increase in GM3 production might suppress EGFR phosphorylation and prevent growth of cancer cells. To test this hypothesis, we examined the effects of valproic acid on cancer cell lines. In this study, we report that treatment of cells with valproic acid led to an increase in the GM3 level on the cell surface, which in turn inhibited cell proliferation by reducing the EGFR phosphorylation.
Results

Increase in the Expression Level of GM3 Synthase Gene (ST3GAL5) by Valproic Acid Treatment—To determine whether valproic acid would affect the transcriptional expression of ganglioside synthesis enzyme genes, reverse transcription-polymerase chain reaction (RT-PCR) and real time quantitative polymerase chain reaction (RT-qPCR) assays were performed. For these analyses, in addition to the GM3 synthase gene (ST3GAL5), we also chose GM2 synthase gene (B4GALNT1) and GD3 synthase gene (ST8SIA1), which are key enzymes for the synthesis of gangliosides and are reported as histone acetylation-regulated genes (19, 20).

RT-PCR results shown in Fig. 1A revealed that the expression level of ST3GAL5 gene increased as the concentration of valproic acid was increased from 0 to 10 mM. In contrast, the expression level of B4GALNT1 gene hardly changed with the increase in valproic acid concentration. In addition, no amplified band was observed for the ST8SIA1 gene, and no change in the expression level of the internal control GAPDH gene was observed. The expression levels of ST3GAL5 and B4GALNT1 genes were then quantified by RT-qPCR. As shown in Fig. 1B, the expression of ST3GAL5 gene increased ~4-fold when the valproic acid concentration was 1 mM and over 8-fold when the valproic acid concentration was either 5 or 10 mM. The expression level of B4GALNT1 gene, conversely, increased only by about 2-fold under the same experimental conditions. These results suggested that valproic acid strongly induced the expression of GM3 synthase gene ST3GAL5, whereas the expression levels of GM2 synthase gene B4GALNT1 and GD3 synthase gene ST8SIA1 were marginally induced and not induced at all, respectively.

Increase in Glycosphingolipid GM3 Level by Valproic Acid Treatment—A431 cells were cultured in 10-cm-diameter culture dishes until they became 80% confluent, and then the cells were treated with various concentrations (0–10 mM) of valproic acid for 24 h. Cells were then collected, glycosphingolipids (GSLs) were extracted and subsequently analyzed by thin layer chromatography (TLC) and immuno-TLC. As shown in Fig. 2, although the relative levels of GSLs remained unchanged (Fig. 2, upper panel), the GM3 level increased in a dose-dependent manner as the concentration of valproic acid also increased (Fig. 2, middle panel). This increase in the GM3 level might be due to the induction of ST3GAL5 gene expression by valproic acid (Fig. 1). In fact, the observed GM3 level in cells treated with 10 mM valproic acid was 6-fold greater than that of the control cells (Fig. 2, middle panel); this increase in GM3 level was similar to the valproic acid-induced increase in the ST3GAL5 gene expression level (Fig. 1B). GM2 level also increased in cells treated with 5 and 10 mM valproic acid, but the increase was marginal (Fig. 2, bottom panel); this result paralleled the valoric acid.
Valproic acid-induced increase in the B4GALNT1 gene expression level (Fig. 1B). The increase in the GM2 level may be due to the increase in the GM3 level. No GD3 was, however, detected by immuno-TLC in these samples (data not shown). This result is consistent with the observation that no ST8SIA1 expression was detected by RT-PCR (Fig. 1A).

**Inhibition of EGFR Phosphorylation by Valproic Acid**—Phosphorylation of EGFR in A431 cells treated with various concentrations of valproic acid was assessed by Western blotting assay using the anti-phospho-EGFR antibody. The amount of EGFR in each sample was also analyzed by Western blotting assay using an anti-EGFR antibody, and the relative phosphorylation level of EGFR in each sample was normalized with respect to the amount of EGFR. As shown in Fig. 3, phosphorylation of EGFR in valproic acid-treated cells was reduced in a dose-dependent manner. Thus, the relative phosphorylation levels of EGFR in A431 cells treated with 1, 5, and 10 mM valproic acid were 72, 52, and 34%, respectively, of that of the control cells (Fig. 3, right panel). These results, taken together with the results described in the previous paragraph, suggested that the valproic acid-induced increase in GM3 production inhibited EGFR phosphorylation.

**Inhibition of Proliferation of A431 Cells by Valproic Acid Treatment**—As shown in Fig. 4 (left panel), incorporation of 5-ethyl-2'-deoxyuridine (EdU), a nucleoside analog of thymidine, into A431 cells was reduced in cells treated for 48 h with valproic acid compared with that in the untreated control cells, and this observed decrease in EdU incorporation increased with the increase in the concentration of valproic acid. As EdU incorporation indicates DNA synthesis activity of the cells, reduced incorporation of EdU indicates a decrease in cell proliferation. Similar results were observed in the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Fig. 4, right panel). As shown, control A431 cells reached the stationary phase within 6 days; however, the growth of A431 cells treated with 1 mM valproic acid was slow compared with that of the control cells. Moreover, growth was completely inhibited in cells treated with 5 and 10 mM valproic acid, respectively. These results suggested that valproic acid treatment prevented cell proliferation.

**Effects of Transient Transfection of A431 Cells with pcDNA/ST3GAL5 Plasmid**—To further confirm that only induction of GM3 synthase, which would be expected to cause an increase in the GM3 level, would inhibit cell proliferation because of reduced EGFR phosphorylation, A431 cells were transiently transfected with an ST3GAL5 expression plasmid in which the expression of the ST3GAL5 gene was under the control of CMV promoter. As expected, the amount of GM3 increased (Fig. 5A), and the relative phosphorylation level of EGFR in these ST3GAL5 expression plasmid-transfected cells was reduced compared with that in the mock plasmid-transfected (control) cells (Fig. 5B). In addition, these transiently transfected cells grew more slowly than the control cells (Fig. 5C, left and right panels, respectively). It is noteworthy that cell proliferation, GM3 level, and EGFR phosphorylation level in ST3GAL5 expression plasmid-transfected cells were similar to those observed in cells treated with 1 mM valproic acid. These results suggested that up-regulation of GM3 synthase prevented growth of A431 cells as a result of an increase in the GM3 level.

**1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) Treatment**—To further confirm that the increase in GM3 inhibited EGFR phosphorylation and cell proliferation, A431 cells were treated with UDP-glucose:N-acylsphingosine glucosyltransferase inhibitor PDMP, which reduces glycosphingolipid content in cells (21). Indeed, treatment with 20 μM PDMP reduced GSLs and GM3 levels in A431 cells (Fig. 6A, 20 μM PDMP- and 0 mM valproic acid-treated sample lane in orcinol and anti-GM3 panels). Remarkably, when A431 cells were simultaneously treated with 20 μM PDMP and 5 or 10 mM valproic acid, the GM3 level in these cells increased even though the total GSLs levels were slightly reduced (Fig. 6A). In contrast, both GM3 and total GSLs levels were reduced in cells treated with 20 μM PDMP and 1 mM valproic acid (Fig. 6A). Consistent with these results, EGFR phosphorylation (Fig. 6B) and cell proliferation (Fig. 6C, left and right panels) were not inhibited in cells treated only with 20 μM PDMP and in cells treated with 20...
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**FIGURE 3. In situ inhibition of EGFR phosphorylation by valproic acid.** A431 cells were treated with various concentrations (0–10 mM) of valproic acid for 24 h, and the treated cells were cultured for 48 h at 37 °C. Cell proliferation was monitored by following the incorporation of EdU into cells using the Click-iT EdU Alexa Fluor 555 HCS (EdU) assay kit as described under “Experimental Procedures.” The phosphorylation of EGFR was analyzed by Western blotting with anti-phospho-EGFR (Tyr-1068) and anti-EGFR (left panel) antibodies. Band densities were quantified, and the amount of phosphorylated EGFR (pEGFR) was calculated as the density of the anti-Tyr(P)-1068-bound EGFR band divided by the density of the anti-EGFR-bound EGFR band. In the right panel, this ratio was plotted with respect to that of the control (no valproic acid treatment) cells. Cells were treated as follows: lane 1, no EGF, no valproic acid; lane 2, 100 ng of EGF, no valproic acid; lane 3, 100 ng of EGF, 1 mM valproic acid; lane 4, 100 ng of EGF, 5 mM valproic acid; lane 5, 100 ng of EGF, 10 mM valproic acid. Results are presented as means ± S.E. of two independent experiments (n = 2). *, p < 0.05; **, p < 0.01. Error bars represent S.E.

**FIGURE 4. Effect of valproic acid on the proliferation of A431 cells.** Left panel, cells in DMEM, 10% FBS were treated with 0–10 mM valproic acid in 96-well plates, and treated cells were cultured for 48 h at 37 °C. Cell proliferation was monitored by following the incorporation of EdU into cells using the Click-iT EdU Alexa Fluor 555 HCS (EdU) assay kit as described under “Experimental Procedures.” The phosphorylation of EGFR was analyzed by Western blotting with anti-phospho-EGFR (Tyr-1068) and anti-EGFR (left panel) antibodies. Band densities were quantified, and the amount of phosphorylated EGFR (pEGFR) was calculated as the density of the anti-Tyr(P)-1068-bound EGFR band divided by the density of the anti-EGFR-bound EGFR band. In the right panel, this ratio was plotted with respect to that of the control (no valproic acid treatment) cells. Cells were treated as follows: lane 1, no EGF, no valproic acid; lane 2, 100 ng of EGF, no valproic acid; lane 3, 100 ng of EGF, 1 mM valproic acid; lane 4, 100 ng of EGF, 5 mM valproic acid; lane 5, 100 ng of EGF, 10 mM valproic acid. Results are presented as means ± S.E. of two independent experiments (n = 3). *, p < 0.05; **, p < 0.01. Error bars represent S.E.

µM PDMP and 1 mM valproic acid. Furthermore, proliferation of cells treated with 20 µM PDMP and 5 mM valproic acid was less inhibited compared with that of the cells not treated with PDMP (compare results shown in Fig. 6C with results shown in Fig. 4). Together, these results suggested that the increase in GM3 level was mainly responsible for the valproic acid-induced inhibition of cell proliferation.

**Effect of Valproic Acid on the Proliferation of U87MG Cells—** U87MG cells are glioblastoma cells, and their proliferation was dependent on the EGFR activity. Therefore, the effect of valproic acid on U87MG cells was examined. However, because U87MG cells do not express enough EGFR for detection by immunoblotting, for this study we used U87MG.WtEGFR cells, which harbored an EGFR expression plasmid (22).

As seen above with the A431 cells, the GM3 levels in valproic acid-treated U87MG.WtEGFR cells also increased with the concomitant increase in the concentration of valproic acid (Fig. 7A). The relative phosphorylation level of EGFR in these cells was also reduced inversely with the GM3 level (Fig. 7B). Furthermore, valproic acid treatment inhibited proliferation of U87MG.WtEGFR cells (Fig. 7C). However, valproic acid was less effective in inhibiting the proliferation of U87MG.WtEGFR cells than that of the A431 cells (Fig. 4 versus 7C). The reduced level of inhibition in proliferation of U87MG.WtEGFR cells might be due to the fact that the increase in the GM3 level (Fig. 2 versus 7A) and decrease in the EGFR phosphorylation level (Fig. 3 versus 7B) were lesser in valproic acid–treated U87MG.WtEGFR cells than in the A431 cells.

**Discussion**

In this work, we successfully demonstrated that the induction of GM3 synthase gene by valproic acid suppressed the proliferation of ovarian epidermoid carcinoma A431 cells by inhibiting EGFR phosphorylation (Figs. 1–4). Valproic acid is a known up-regulator of GM3 synthase gene (16), and it is also known as an inhibitor of histone deacetylase (14, 15). Therefore, it is possible that valproic acid may control the expression of other genes. To test the idea that only induction of GM3 synthase gene could inhibit proliferation of A431 cells, we transfected these cells with the ST3GAL5 expression plasmid and showed that these transfected cells exhibited a phenotype similar to that of the cells treated with 1 mM valproic acid (Fig. 5). Furthermore, we observed that addition of PDMP during the valproic acid treatment caused less inhibition of cell proliferation due to reduced GM3 level (Fig. 6). These results suggest
FIGURE 5. Effect of increasing the expression level of GM3 synthase gene. A431 cells were transiently transfected with a GM3 synthase expression plasmid, and transfected cells were used for analyzing GM3 and GM2 content, EGFR phosphorylation level, and cell proliferation. A, analysis of GM3 and GM2 levels. A431 cells were transfected with mock plasmid vector or GM3 synthase expression plasmid (pcDNA/ST3GAL5). Total GSLs were extracted from these cells, extracted GSLs were analyzed by HPTLC and detected by orcinol staining (left panel), and GM3 and GM2 were detected using the anti-GM3 and anti-GM2 antibodies, respectively (middle and right panels). Band densities were quantified as before to determine the total amount of GSLs, GM3, and GM2. Lane 1, mock-transfected cells; lane 2, pcDNA/ST3GAL5-transfected cells. Results shown are means ± S.E. of two independent experiments. *, p < 0.05; **, p < 0.01. B, in situ EGFR phosphorylation assay. A431 cells were transfected with the control or pcDNA/ST3GAL5 plasmid and transfected cells were used for the EGFR phosphorylation assay as described in Experimental Procedures. Phosphorylation was analyzed by Western blotting with anti-phosphoEGFR (Tyr1068) and anti-EGFR (left panel). Band densities were quantified, amount of phosphorylated EGFR was calculated, and data were plotted as before (right panel). Lane 1: mock-transfected cells, no EGF added; lane 2: mock-transfected cells, 100 ng EGF added; and lane 3: pcDNA/ST3GAL5 transfected cells, 100 ng EGF added. Results are presented as means ± S.E. of two independent experiments (n = 2). *, p < 0.05; **, p < 0.01. Error bars represent S.E. C, cell proliferation assay. A431 cells were transfected with the control plasmid (mock) or pcDNA/ST3GAL5 plasmid, and transfected cells were seeded on 96-well plates and cultured for 48 h (for EdU incorporation assay) or 3–7 days (for MTT assay) at 37 °C. Cell proliferation was analyzed using the EdU assay kit (left panel) or MTT assay kit (right panel). Circles, control (mock-transfected) cells; crosses, pcDNA/ST3GAL5-transfected cells. Results shown are means ± S.E. of three independent experiments (n = 3). *, p < 0.05. Error bars represent S.E.
that by increasing the cellular GM3 level it is possible to effectively inhibit cell proliferation.

We also examined the expression levels of GM2 synthase and GD3 synthase genes, which are other key enzymes for ganglioside synthesis, because histone acetylation is known as a regulatory factor of glycosyltransferase genes (19, 20). Addition of valproic acid led to the induction of GM2 synthase gene (B4GALNT1) expression, but its expression level was significantly less than that of the GM3 synthase gene (Fig. 1), and consequently less GM2 ganglioside was synthesized (Fig. 2).
FIGURE 7. Effects of valproic acid on U87MG.WtEGFR glioma cells. Cells were treated with the indicated concentration of valproic acid, and treated cells were analyzed for GM3 level, EGFR phosphorylation level, and cell proliferation. A, increase in GM3 level by valproic acid treatment. U87MG.WtEGFR cells were treated with valproic acid for 24 h, and GSLs were extracted from the treated cells. Total GSLs were analyzed by HPTLC and detected by orcinol staining (upper panel), and GM3 was detected using the anti-GM3 antibody (lower panel). In the right panel, results were plotted as described in the legend of Fig. 2. Cells were treated as follows: lane 1, none; lane 2, 1 mM valproic acid; lane 3, 5 mM valproic acid; lane 4, 10 mM valproic acid. Results shown are means ± S.E. of two independent experiments (n = 2). *, p < 0.05; **, p < 0.01. B, in situ inhibition of EGFR phosphorylation by valproic acid treatment. U87MG.WtEGFR cells were treated with 0–10 mM valproic acid for 24 h. Phosphorylation of EGFR (pEGFR) was assayed by Western blotting (left panel), quantified, and plotted (right panel) as described under “Experimental Procedures”. Cells were treated as follows: lane 1, none; lane 2, 100 ng of EGF; lane 3, 100 ng of EGF and 1 mM valproic acid; lane 4, 100 ng of EGF and 5 mM valproic acid; lane 5, 100 ng of EGF and 10 mM valproic acid. Results shown are means ± S.E. of two independent experiments (n = 2). *, p < 0.05; **, p < 0.01. C, proliferation of U87MG.WtEGFR cells treated with or without valproic acid. Cells in DMEM containing 10% FBS were treated with 0–10 mM valproic acid and then cultured for 3–7 days at 37 °C. Cell proliferation was analyzed using the MTT assay kit. Circles, control (untreated) cells; squares, 1 mM valproic acid-treated cells; triangles, 5 mM valproic acid-treated cells; diamonds, 10 mM valproic acid-treated cells. Results shown are means ± S.E. of three independent experiments (n = 3). *, p < 0.05. Error bars represent S.E.
The expression of GD3 synthase gene (ST8SIA1), in contrast, was not detected in untreated control cells, and its expression was not induced following the addition of valproic acid (Fig. 1). These results indicate that valproic acid treatment induces only the synthesis of GM3 as a ganglioside in A431 cells. This observation also supports our idea that an increase in the GM3 level could inhibit cell proliferation effectively.

No difference was observed between the expression levels of ST3GAL5 in cells treated with 5 and 10 mM valproic acid (Fig. 1). This result may indicate that the induced expression of ST3GAL5 gene became saturated following treatment with 5 mM valproic acid for 24 h. However, the amount of GM3 ganglioside synthesized was higher in cells treated with 10 mM valproic acid than in cells treated with 5 mM (Fig. 2). This could be due to a difference between the early stage induction of ST3GAL5 expression following the addition of 5 and 10 mM valproic acid. As the glycolipid is usually synthesized after the synthesis of GM3 as a ganglioside in A431 cells. This observation also supports our idea that an increase in the GM3 level could inhibit cell proliferation effectively.

Furthermore, Osuka et al. (24) have also observed that valproic acid inhibited angiogenesis and exhibited an antitumor effect against glioma. They observed that proliferation of gliomas and endothelial cells was inhibited as a direct effect of valproic acid. They also observed that valproic acid inhibited secretion of VEGF from glioma cells that might have resulted from an indirect effect of valproic acid on angiogenesis (24). Valproic acid is a known histone deacetylase inhibitor (14, 15), and expression of several genes (including ST3GAL5) has been shown to be altered by valproic acid treatment (14, 16). Many mechanisms have been proposed to explain the antitumor effect of valproic acid (25, 26). We believe that the results of our study provide further evidence that valproic acid has antitumor activity. Our findings, along with the results from other studies described above, clearly suggest that induction of GM3 synthase gene expression by agents such as valproic acid might be a potential method for treating tumors. Based on our observations, we have proposed a novel mechanism by which valproic acid might inhibit proliferation of cancer cells (Fig. 8). Support for this proposed model comes mainly from the results of two experiments. 1) Transient transfection of cells with ST3GAL5 expression plasmid increased the cellular GM3 level as a result of which cell proliferation was inhibited (Fig. 5), and 2) the PDMP treatment experiment showed that the main effect of valproic acid was to increase the cellular GM3 level, which in turn inhibited cell proliferation (Fig. 6). Therefore, the observed increase in the GM3 level seems to be one of the main reasons why valproic acid prevented cell proliferation (Fig. 8). Taken together, results obtained from our studies suggest that screening for targeted up-regulators of GM3 synthesis may open up new avenues for the development of novel antitumor drugs.

Experimental Procedures

Cell Culture—Human ovarian epidermoid carcinoma A431 cells and human glioblastoma U87MG.WtEGFR cells (generously provided by Dr. Cavenee) were used in this study. These cells were cultured in DMEM (Sigma, D5796) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biowest, S05417S1820), 100 μg/ml penicillin, 50 μg/ml streptomycin, and 110 μg/ml sodium pyruvate at 37 °C in a humidified 5% CO₂ incubator.

RT-PCR and RT-qPCR—A431 cells (6 × 10⁶ cells), cultured in 10-cm-diameter culture dishes, were incubated in DMEM containing 0–10 mM valproic acid for 24 h. Cells were washed twice with 4 ml of PBS, and total RNAs from treated and untreated control A431 cells were isolated using the RNeasy Mini kit (Qiagen, 74104). Reverse transcription was carried out using 2 μg of each total RNA and a High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, 4387406) following the manufacturer’s instructions. The following gene-specific primer sets were designed and used for PCR: 5’-CTTCTTCTCTACGAGCTCTCTAG-3’ and 5’-CTAAGA-
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CAACGGCAATGACACC-3′ (forward and reverse primers, respectively, for GM3 synthase), 5′-TCTACCCCTTGTCAGCACC-3′ and 5′-AAGACCGCTCATGTCCTCCTC-3′ (forward and reverse primers, respectively, for GM1 synthase), 5′-GGAAGACCTGTCGACCCCTG-3′ and 5′-GAGCAGCCA-CAGCACCCTTCCTC-3′ (forward and reverse primers, respectively, for GD3 synthase), and 5′-TCTCCTGAGCTGAACGGGAA-3′ and 5′-GAGGAGTGGGTGTCGCC-3′ (forward and reverse primers, respectively, for GAPDH, an endogenous control gene). RT-qPCR was performed using the following reaction condition: 95 °C for 10 min (denaturation and polymerase activation) followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The changes in the expression levels of the target genes were determined by quantifying relative gene expression, which is defined as \(2^{-\Delta\Delta C_t}\). The threshold cycle (Ct) of a target genes was normalized by Ct of GAPDH gene at each valproic acid concentration (\(\Delta C_t\) target gene = Ct target gene – Ct GAPDH). \(\Delta C_t\) of a target gene in cells treated with valproic acid was then calibrated by \(\Delta C_t\) of the target gene in the untreated cells (\(\Delta\Delta C_t\) target gene = \(\Delta C_t\) target gene – \(\Delta C_t\) untreated target gene).

**Cell Proliferation Assay**—Cell proliferation assays were performed using the Click-iT® EdU Alexa Fluor® 555 HCS assay kit (Thermo Fisher Scientific, C10352) and the MTT assay kit (Nakarai Co., Japan, 23506-80). For the Click-iT® EdU Alexa Fluor 555 HCS (EdU) assay, A431 cells were seeded at a density (Nakarai Co., Japan, 23506-80). For the Click-iT EdU Alexa Fluor 555 HCS assay, A431 cells were seeded at a density of 10,000 cells/well (in 100 μl of DMEM containing 10% FBS) of a 96-well culture plate. Cells were treated with various concentrations (0–10 mM) of valproic acid (sodium salt) for 48 h, then the cells were incubated with 10 μM EdU for 3 h. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, and EdU incorporated into newly synthesized DNA was detected after staining with the fluorescent Alexa Fluor 555 azide supplied in the assay kit. Quantitative analysis of the fluorescence signal was performed using the Typhoon FLA 9000 and ImageQuant TL (GE Healthcare). For the MTT assay, A431 and U87MG.WtEGFR cells were seeded at a density of 4 × 10^5 cells/well (in 100 μl of DMEM containing 10% FBS) of a 96-well culture plate. Cells were treated with various concentrations (0–10 mM) of valproic acid (sodium salt) for 48 h, and then the cells were incubated with 10 μM EdU for 3 h. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, and EdU incorporated into newly synthesized DNA was detected after staining with the fluorescent Alexa Fluor 555 azide supplied in the assay kit. Quantitative analysis of the fluorescence signal was performed using the Typhoon FLA 9000 and ImageQuant TL (GE Healthcare). For the MTT assay, A431 and U87MG.WtEGFR cells were seeded at a density of 4 × 10^5 cells/well (in 100 μl of DMEM containing 10% FBS) of a 96-well culture plate. Various concentrations (0–10 mM) of valproic acid (sodium salt) were added to these cells, and cells were cultured for an additional 3–7 days. 10 μl of MTT solution (5 mg/ml in PBS) was added to each well, and cells were incubated for 2 h in a humidified 5% CO2 incubator. The amount of formazan formed in the assay mixture was solubilized with 100 μl of 0.04 M HCl in 2-propanol, and its concentration in each well was measured by measuring the absorbance at 570 nm using a SpectraMax Paradigm (Molecular Devices) microtiter reader.

**Extraction of Gangliosides and Immuno-TLC**—Gangliosides were extracted using a mixture of isopropyl alcohol/hexane/water. Briefly, ~6 × 10^6 cells (grown in 10-cm-diameter culture dishes) were washed with ice-cold PBS and resuspended in 400 μl of water, and then 4 volumes of isopropyl alcohol/hexane (2:2:1, v/v) was added to the cells. After sonication for 1 min followed by incubation on ice for another minute, cells were centrifuged at 3,500 rpm for 5 min. The supernatant was collected as the total lipid fraction. Cell pellets were re-extracted with 2.0 ml of a mixture of isopropyl alcohol/hexane/water (2:2:1, v/v). Two extracted lipid fractions, containing gangliosides, were combined, and the liquid was evaporated off. To the dried lipid fraction, 1.5 ml of 0.1 M NaOH, methanol was added, and the mixture was incubated at 40 °C for 2 h. After neutralizing the mixture with 180 μl of 1 M acetic acid in methanol, the hydrolyzed fatty acid of diacylglycerol was extracted twice with 1.5 ml of hexane. Methanol was removed by evaporation, the resulting solid residue was dissolved in water, and the solution was applied to a Sep-Pak C18 1-ml Vac Cartridge (Waters, WAT023590). Gangliosides were eluted using methanol and chloroform/methanol (2:1, v/v). Gangliosides were dried under vacuum, and the dried residue was dissolved in 50 μl of chloroform/methanol (2:1, v/v). 10 μl of the ganglioside solution was analyzed using high performance TLC plates (Merck). A mixture of chloroform, methanol, 0.2% aqueous CaCl2 solution (60:35:8, v/v) was used as the mobile phase. TLC plates were developed by staining with orcinol/sulfuric acid. Immuno-TLC analysis was performed using anti-GM3 (GMR6, Seikagaku Biobusiness Co., 370695) (1:500 dilution), anti-GM2 (MKL-16, Seikagaku Biobusiness Co., 370640) (1:500 dilution), and anti-GD3 (GMR19, Seikagaku Biobusiness Co., 370635) (1:500 dilution) monoclonal antibodies. HRP-conjugated (goat) anti-mouse IgM(μ) antibody (Chemicon International, AP128P) (1:3000 dilution) was used as the secondary antibody. The bound antibody was detected by enhanced chemiluminescence using SuperSignal West Dura (Pierce, 34076) and quantified by TLC-densitometry using LAS3000mini (FujiFilm).

**In Situ EGFR Phosphorylation Assay**—The in situ EGFR phosphorylation assay was performed as described earlier (6, 8). A431 and U87MG.WtEGFR cells, which were cultured in 24-well plates in DMEM containing 10% FBS, were incubated in serum-free DMEM containing 0–10 mM valproic acid for 24 h after which cells were washed twice with 0.5 ml of PBS. Phosphorylation was induced by incubation with 200 μl of EGF (0.1 μg/ml), and cells were incubated for 1 h at 4 °C. After incubation, cells were washed twice with 0.5 ml of PBS, and then cells were resuspended in 30 μl of lysis buffer (1% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM Na2VO4) and incubated for 30 min at 4 °C. The cell lysate was subjected to SDS-PAGE analysis and then analyzed by Western blotting using anti-EGFR and anti-phospho-EGFR (Tyr-1068) antibodies. HRP-conjugated (goat) anti-rabbit IgG antibody (1:1000 dilution) was used as the secondary antibody. The bound antibody was detected by enhanced chemiluminescence using SuperSignal West Dura (Pierce, 34076) and quantified by densitometry using LAS3000mini (FujiFilm).

**Transfection with ST3GAL5 Expression Plasmid**—A431 cells were cultured in 3.5-cm-diameter culture dishes in DMEM containing 10% FBS until they became 80% confluent. 2 μg of ST3GAL5 expression plasmid (pcDNA/ST3GAL5) or mock plasmid vector (pcDNA3.1) was mixed with 100 μl of Opti-
MEM and 8 μl of FuGENE, and the mixture was incubated for 15 min at room temperature. This FuGENE/plasmid mixture was added to A431 cells and gently mixed well. After 24-h incubation in a CO₂ incubator at 37 °C, cells were used for conducting various (cell proliferation, GM3 level, and EGFR phosphorylation) experiments.

**PDM Treatment**—PDM treatment was performed as described earlier by Makino et al. (18). Briefly, A431 cells were cultured in 10-cm-diameter culture dishes in DMEM containing 10% FBS until they became 50% confluent. PDM was added to the culture medium at a final concentration of 20 μM, and cells were incubated for 48 h in a CO₂ incubator at 37 °C. Different amounts (0–10 mM) of valproic acid were added to each dish, and cells were incubated for another 24 h in the CO₂ incubator at 37 °C. These cells were used for GSL analysis and the EGFR phosphorylation assay.

**Author Contributions**—N. K. conducted most of the experiments, analyzed the results, and wrote most of the paper. Y. N. conducted RT-PCR and RT-qPCR and experiments on GM3 levels in A431 and U87MG.WtEGFR cells. S. T. performed transfection of A431 cells with pcDNA/ST3GAL5 plasmid. K. N. conceived the idea for the project, conducted experiments on cell proliferation, and wrote the paper with N. K.

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