Cellular Gangliosides Promote Growth Factor-induced Proliferation of Fibroblasts*

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Cell surface gangliosides have been proposed as modulators of transmembrane signaling. In this study, we used two complementary approaches to investigate the function of cellular gangliosides in the response of mammalian fibroblasts to growth factors. First, inhibition of glucosylceramide synthase by a new specific inhibitor of D-l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (glucosylceramide synthase), which depletes cellular gangliosides at a concentration of 1 μM without causing an increase in ceramide levels, blocked epidermal growth factor-stimulated proliferation of fibroblasts. Similarly, responses to several other growth factors that activate receptor tyrosine kinases, including fibroblast growth factor, insulin-like growth factor-I, and platelet-derived growth factor, were inhibited by 50–100%. Conversely, enrichment of cellular gangliosides by preincubation of the mouse and human fibroblasts with exogenously added gangliosides enhanced growth factor-elicited cell proliferation. Novel findings of this study, distinguishing it from previous similar studies, include differential effects of preincubation versus continuous incubation of cells with gangliosides on growth factor-dependent cell proliferation and the growth factor-like action of NeuNAc α2-3Galβ1-3GalNAcβ1-4(Galα2-3Galβ1-4Glcβ1-1)Cer when cells are pre-treated with the ganglioside.

It has been proposed that gangliosides, which are ubiquitous membrane constituents, act as modulators of transmembrane signaling (1, 2). These molecules exist in glycosphingolipid-enriched microdomains in the plasma membrane, as has been evidenced in human peripheral blood lymphocytes (3). Previous studies have delineated certain effects of exogenous gangliosides upon growth factor signaling. Gangliosides added to the culture medium can insert into the cell plasma membrane (4, 5) and modify the biological responses of cells to various growth factors, including epidermal growth factor (EGF),1 fibroblast growth factor, insulin-like growth factor-I (IGF-I), and platelet-derived growth factor (PDGF) (6). While identifying certain pharmacological effects of gangliosides, studies of the effects of exogenous gangliosides may leave open the question of the physiological role of endogenous gangliosides in growth factor-induced signaling. Thus, we considered it important to assess the influence of gangliosides present in their normal physiological location, i.e., in the cell plasma membrane, upon growth factor-induced signal transduction and proliferation.

Several experimental approaches can be used to study the functions of endogenous gangliosides. One is to assess the effect of eliminating these molecules. Experiments in this direction have included dialidase gene transfection (7), gene transfer (8), the development of a partial ganglioside knockout mouse (9), and the application of enzyme inhibitors (10). We have used the last approach in the present study. Specifically, we used D-l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (PPPP), a newly discovered specific inhibitor of glucosylceramide synthase (11, 12), to inhibit ganglioside synthesis and down-regulate ganglioside expression. Glucosylceramide synthase is the key enzyme for glycosphingolipid synthesis. It catalyzes the synthesis of glucosylceramide from ceramide and glucose, which is the first step in the synthesis of neutral glycosphingolipids and, thus, gangliosides. We used PPPP rather than the widely studied related glucosylceramide synthase inhibitor, D-PDMP, because D-PDMP also causes ceramide accumulation (13), and ceramide (14) and its metabolic products such as sphingosine-1-phosphate (15) may act as second messengers and affect cell growth. In contrast, at a concentration ≤1.0 μM, PPPP has most recently been shown to specifically inhibit glucosylceramide synthesis, leading to the inhibition of ganglioside synthesis, without causing increased ceramide levels (12). By this approach to blocking ganglioside synthesis, it is possible to test the hypothesis that cellular gangliosides are physiological modulators of growth factor-mediated cell proliferation.

As a model system for studying growth control and cell cycle progression, the response of mammalian fibroblasts to growth factors is well established (16). Proliferation of fibroblasts in vitro requires growth factors, which are usually provided by fetal bovine serum. Upon the withdrawal of growth factors, e.g., serum deprivation, fibroblasts enter a nondividing G0 state, characterized by low metabolic activity. The subsequent addition of growth factors induces fibroblast proliferation. Using this model, we examined the role of cellular gangliosides in like growth factor-I; HPTLC, high performance thin-layer chromatography; NHDF, normal human dermal fibroblasts; PDGF, platelet-derived growth factor; PPPP, D-l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (glycosylceramide synthase); D-PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholinol-1-propanol; DMEM, Dulbecco’s modified Eagle’s medium; FGM, fibroblast growth medium; FBEM, fibroblast basal medium.
Gangliosides and Cell Proliferation

Ganglioside-induced proliferation. In fibroblasts that were induced to enter a quiescent state by serum deprivation and then stimulated by the addition of growth factors, the inhibition of cellular ganglioside synthesis effectively blocked the expected proliferative response. Conversely, and consistent with the above findings, enrichment of gangliosides in the plasma membrane by preincubation of the fibroblasts with exogenous gangliosides enhanced the growth factor-induced proliferative response. In addition, G_{1d1a}^2 acted like a growth factor when cells were pretreated with the ganglioside, stimulating both mouse and human fibroblast proliferation. Finally, this study documents differential effects of preincubation versus continuous incubation of cells with gangliosides on growth factor-dependent cell proliferation. Whereas preincubation of cells with gangliosides enhanced growth factor-dependent cell proliferation. Whereas preincubation of cells with gangliosides on growth factors were purchased from Sigma. EGF (6 kDa; mouse submaxillary gland, receptor grade) was reconstituted with Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc., Grand Island, NY). IGF-I (human recombinant) was reconstituted with 0.1 M acetic acid. PDG-F (28 and 31 kd; human platelets) was reconstituted with 0.01 M acetic acid and stored in plastic vials. Basic fibroblast growth factor (bFGF; 16.4 kd; pituitary glands) was reconstituted with DMEM. All stock solutions were stored at −70 °C. Growth factors were directly added to the culture medium by diluting the stock solutions before use. Synthetic G_{4a} (181–18:0), which we previously studied in assays of lymphoproliferation (19, 20), was kindly provided by Dr. Akira Hasegawa of Gifu University (Gifu, Japan). G_{1d1a} (bovine brain), G_{3a} and G_{3b} (bovine buttermilk), and PPPP were purchased from Matreya (Pleasant Gap, PA). Gangliosides were dissolved in serum-free medium and diluted in the culture medium for each assay. PPPP was dissolved in ethanol to a concentration of 1 mg and kept as a stock solution at −20 °C. For each experiment, PPPP was directly added into the culture medium. The final concentration of ethanol in the culture medium was −0.1% (v/v).

Cell Culture—Normal human dermal fibroblasts (NHDF) were purchased from Clonetics (San Diego, CA) and cultured in fibroblast growth medium (FGM) supplemented with 2% fetal bovine serum (FBS; Hyclone, Logan, UT), 0.5 ml of insulin, 0.5 ml of human fibroblast growth factor, and 0.5 ml of GA1000 (Clonetics). For serum-free culture, fibroblast basal medium (FBM) was used. Cells from passages 3–7 were used for this study. The mouse embryonic fibroblast cell lines, Swiss 3T3, Balb/c 3T3 (Balb/3T3 clone A31), and 3T3-L1 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% heat-inactivated FBS (NHDF cells). In selected assays, fibroblasts were also seeded at 3 × 10^3 cells/well in 96-well plates in DMEM with 10% FBS. On the following day, 100 μl of fresh culture medium containing [^3H]-labeled ganglioside (cholesterol-methyl-[^3H]C(sphingomyelin; specific activity, 55 mCi/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO) was added to each well. At different time points, cells were harvested, and the uptake of [^3H]-labeled ganglioside was determined as pmo/10^3 cells.

Measurement of Effects of Cellular Ganglioside Depletion on Cell Proliferation—Mouse embryonic fibroblasts were plated at 1–2 × 10^5 cells/well in 96-well flat bottom culture plates (area = 0.32 cm²; Corning Glass, Corning, NY) in DMEM with 10% FBS and incubated overnight. The culture medium was replaced with fresh culture medium containing either 1 μg PPPP or 0.1% ethanol (solvent vehicle), and the cells were cultured for 3 days. Then, the culture medium was removed, and serum-free medium or low serum medium (0.5 and 1% FBS) containing either 1 μg PPPP + growth factors or 0.1% ethanol + growth factors were added to the plates. After an 18–24 h incubation, the cells were pulsed with 50 μl of [[^3H]thymidine (5 μCi/ml) for 2–3 h and harvested by a cell harvester. [[^3H]Thymidine uptake was quantified by β-scintillation counting (Betafluor; National Diagnostics, Manville, NJ). The data are expressed as the mean ± S.D. of triplicate samples.

Analysis of Sphingolipids—After treatment of Swiss 3T3 cells with 1 μg PPPP or 0.1% ethanol, cellular ceramide and sphingomyelin were analyzed as described previously (10). The cells were washed with phosphate-buffered saline directly in the flask and harvested by trypsinization. After centrifugation, the cell pellet was washed once with phosphate-buffered saline and then extracted twice with 4 ml of chloroform, methanol (1:1). The supernatants were pooled. Chloroform (5 ml) and 0.9% NaCl (4 ml) were added to the pooled supernatants, which were vortexed, sonicated for 2 min, and centrifuged at 1000 × g for 10 min. The lower phase was dried under a stream of nitrogen and resuspended in chloroform, methanol (1:1). For HPTLC analysis of ceramide, the equivalent of 400 μg of cellular protein was spotted on a HPTLC plate and developed in chloroform, acetic acid (9:1, by volume). Ceramide (type III; Sigma) was used as a standard. For HPTLC analysis of sphingomyelin, 400 μg of cellular protein equivalent of sphingomyelin was spotted on an HPTLC plate and developed in chloroform, methanol, water (65:25:4, by volume). Bovine brain sphingomyelin (Megazyme) was used as a standard. After development, the HPTLC plate was submerged in a charring reagent (3% cupric acetate; 10% phosphoric acid) and visualized by charring at 130 °C for 20 min. Densitometric scanning analysis of the bands was performed using a Shimadzu CS-930 dual-wavelength TLC scanner. The protein content of the pellet was determined by the Lowry method (25).

Measurement of Sphingomyelin uptake—Swiss 3T3 cells were plated at 1 × 10^5 cells/well in 96-well plates in DMEM with 10% FBS. On the following day, 100 μl of fresh culture medium containing [[^3H]-labeled sphingomyelin (cholesterol-methyl-[^3H]C(sphingomyelin; specific activity, 55 mCi/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO) was added to each well. At different time points, cells were harvested, and the uptake of [[^3H]-labeled sphingomyelin was determined as pmo/10^3 cells.

Measurement of Effects of Cellular Ganglioside Enrichment and Exogenous Sphingomyelin on Cell Proliferation—Fibroblasts were seeded at 5 × 10^4 cells/well in 96-well plates (area = 0.32 cm²; Corning Glass) in DMEM containing 10% FBS (Swiss 3T3 cells) or FGM containing 2% FBS (NHDF cells). In selected assays, fibroblasts were also seeded at 3 × 10^5 cells/well in the larger 24-well plates (area = 1.9 cm²; Corning Glass). The medium was replaced with fresh medium ± gangliosides or ± sphingomyelin on the following day. After an 18-h incubation, the culture medium was removed, and the cells were starved overnight in serum-free DMEM in the case of mouse embryonic fibroblasts or serum-free FBM in the case of NHDF cells. This medium was then replaced with serum-free medium ± EGF, and the cells were further cultured for 24 h. In some experiments the starvation period was omitted, and the cells were incubated with the gangliosides from the start. The culture medium was replaced directly with serum-free medium ± EGF, and the cells were further cultured for 24 h. During the final 3 h, the cells were pulsed with 50 μl of [[^3H]thymidine (5 μCi/ml) and harvested by a cell harvester. [[^3H]Thymidine uptake was determined by β-scintillation counting. In selected experiments, [[^3H]thymidine incorporation into trichloroacetic acid-insoluble material was also determined as de-
scribed previously (17). Under these same conditions, in parallel experiments cell counts of the 24-well plates were performed after trypsinization of the cells. The data are expressed as the mean ± S.D. of duplicate or triplicate cultures. Scheme 1 shows the two major experimental conditions that were used to evaluate the effect of gangliosides on cell proliferation.

**Statistical Analysis**—The difference between control groups and treatment groups was determined using Student's *t* test (two tailed, unpaired).

**RESULTS**

**Inhibition of Ganglioside Synthesis by PPPP**—To provide the basis for studying the role of cellular gangliosides in growth factor-induced fibroblast proliferation, we modulated ganglioside metabolism by targeting glucosylceramide synthase using the specific enzyme inhibitor, PPPP (11, 12). We studied Swiss 3T3 mouse embryonic fibroblasts that contain GM₃ and GD₁α as the major ganglioside components. By resorcinol assay, the ganglioside content of Swiss 3T3 cells was found to be 7.1 nmol/mg of protein. To determine the kinetics of inhibition of ganglioside synthesis by PPPP, we cultured Swiss 3T3 cells in medium containing either 1 μM PPPP or 0.1% ethanol (solvent control) for up to 4 days. During the final 6 h, radiolabeled sugars were added to the culture medium. As shown in Fig. 1A, exposure of Swiss 3T3 cells to 1 μM PPPP rapidly inhibited ganglioside synthesis. As measured by metabolic radiolabeling, the rate of ganglioside synthesis was reduced from 725 to 164 dpm/mg of protein/h or 22% of the control within one day of treatment (Fig. 1A). By day 4, the rate of ganglioside synthesis was reduced to 6% of the control level. The HPTLC autoradiogram of metabolically radiolabeled gangliosides isolated from PPPP-treated cells confirmed the inhibitory effect of PPPP on ganglioside synthesis (Fig. 1B). When the effect of PPPP on cellular ganglioside content was examined, a rapid and time-dependent depletion of the cellular gangliosides was observed (Fig. 1C). After Swiss 3T3 cells were exposed to 1 μM PPPP for 3 days, the cellular gangliosides were nearly completely depleted. The blocked ganglioside synthesis confirmed that PPPP is a potent inhibitor of ganglioside synthesis in Swiss 3T3 cells.

**Down-regulation of Growth Factor-induced Proliferation by Ganglioside Depletion**—Swiss 3T3 cells are responsive to a number of growth factors, including EGF, bFGF, PDGF, and IGF-1. In the initial experiments, we examined the stimulation of proliferation of Swiss 3T3 cells by EGF. The dose-response curve (Fig. 2A) shows that 2 ng/ml EGF causes optimal stimulation of cell proliferation. This was true over a 10-fold range of cell densities (0.2–2.0 × 10⁴ cells/well, which equals 0.62–6.2 × 10⁴ cells/cm²) (Fig. 2B), demonstrating that this cell line is a good model for studying the role of gangliosides in modulating cell proliferation.

To evaluate the effect of inhibition of ganglioside synthesis and depletion of cellular gangliosides on EGF-induced proliferation, we pretreated Swiss 3T3 cells with 1 μM PPPP or 0.1% ethanol (control) in normal culture medium (DMEM with 10% FBS) for 3 days. The cells were then incubated for 24 h in culture medium containing either 0.5% FBS + 2 ng/ml EGF or 1% FBS + 2 ng/ml EGF (Fig. 2C). Cell proliferation was measured as DNA synthesis by [³²P]thymidine uptake. When cells were incubated with EGF in culture medium containing 0.5% FBS, there was a 6-fold stimulation of [³²P]thymidine uptake as compared with that of cells cultured in the absence of EGF. PPPP treatment of the cells caused 91% inhibition of the EGF-stimulated increase in [³²P]thymidine uptake (Fig. 2C). [³²P]Thymidine uptake was similarly inhibited by PPPP treatment when cells were incubated with EGF in 1% FBS culture medium (Fig. 2C) or in serum-free medium (not shown). The stimulation of proliferation by EGF was blocked whether cells were incubated with EGF in 1% FBS medium for 24 h or for 48 h (Fig. 2D). Thus, in subsequent experiments, we incubated the cells with EGF for 18–24 h in low serum medium. When Swiss 3T3 cells were exposed to 1 μM PPPP for only 3 h during the [³²P]thymidine pulse, [³²P]thymidine uptake was not affected, indicating that PPPP itself did not affect the transport of [³²P]thymidine through the cell plasma membrane. When cells were pretreated with 1 μM PPPP for 3 days and then washed to remove the enzyme inhibitor before incubation with EGF for 24 h, the [³²P]thymidine uptake was 78% of the control (cells treated with EGF in 1% FBS medium + 0.1% ethanol). These results show that the inhibition of cell proliferation by PPPP was reversible and suggest that the inhibition of cell proliferation by PPPP is not toxic to the cells. Together, these results clearly demonstrate that the inhibition of ganglioside synthesis down-regulates EGF-induced proliferation of Swiss 3T3 cells.

We confirmed these findings in a second murine fibroblast cell line, Balb/c 3T3 cells were first exposed to 1 μM PPPP or 0.1% ethanol (control) in normal culture medium for 3 days. The cells were then incubated with or without EGF for 24 h in low serum (1% FBS) medium in the presence or absence of PPPP (1 μM). Under these conditions, the inhibition of cellular ganglioside synthesis completely blocked EGF-induced proliferation (Fig. 3A). In addition, depletion of cellular gangliosides inhibited proliferation induced by optimal concentrations of bFGF, PDGF, and IGF-I by 62, 50, and 69%, respectively (Fig. 3, A and B). Similar inhibitory effects on growth factor-induced
cellular ganglioside synthesis and content. Swiss 3T3 cells were cultured in DMEM with 10% FBS containing either 1 μM [14C]glucosamine hydrochloride during the final 5 h as described under "Materials and Methods." The cells were then harvested, and the gangliosides were analyzed by HPTLC autoradiography (B). 14C-Labeled rat brain gangliosides (RBG; 800 dpm) were used as a standard. The HPTLC plate was exposed to x-ray film for 2 weeks. In Panel C, the total cellular gangliosides were analyzed by HPTLC. Gangliosides were stained with resorcinol hydrochloride reagent. Human brain gangliosides (HBG; 6 nmol) were used as a standard. Lane C in both Panels B and C shows the gangliosides isolated from cells cultured in medium containing vehicle solvent (0.1% ethanol). Approximately 500 μg of protein-equivalent gangliosides were loaded on each lane.

Fig. 1. Time course of inhibitory effect of PPPP on Swiss 3T3 cellular ganglioside synthesis and content. Swiss 3T3 cells were cultured in DMEM with 10% FBS containing either 1 μM PPPP or 0.1% ethanol for up to 4 days and were radiolabeled with [14C]galactose and [14C]glucosamine hydrochloride during the final 5 h as described under "Materials and Methods." The cells were then harvested, and the gangliosides were purified. Ganglioside-associated radioactivity was measured by β-scintillation counting (A) and analyzed by HPTLC autoradiography (B). 14C-Labeled rat brain gangliosides (RBG; 800 dpm) were used as a standard. The HPTLC plate was exposed to x-ray film for 2 weeks. In Panel C, the total cellular gangliosides were analyzed by HPTLC. Gangliosides were stained with resorcinol hydrochloride reagent. Human brain gangliosides (HBG; 6 nmol) were used as a standard. Lane C in both Panels B and C shows the gangliosides isolated from cells cultured in medium containing vehicle solvent (0.1% ethanol). Approximately 500 μg of protein-equivalent gangliosides were loaded on each lane.

Effect of PPPP on Cellular Ceramide and Sphingomyelin Levels—Ceramide is the precursor of glucosylceramide. Inhibition of glucosylceramide synthesis could possibly cause the accumulation of cellular ceramide. Because ceramide has been shown to cause cell growth arrest in a number of cell types (14), we examined whether exposure of the cells to PPPP caused ceramide accumulation. In fact, when Swiss 3T3 cells were incubated with 1 μM PPPP for up to 72 h, the ceramide level remained unchanged from that of control cells (2.22 ± 0.14 versus 2.24 ± 0.12 μg of ceramide/mg of protein, control versus PPPP-treated cells, Fig. 4A). This result is consistent with those of Lee et al. (12). In addition, the multiple bands of ceramide representing different fatty acyl chains also appeared unchanged (Fig. 4A). These findings exclude the possibility that the antiproliferative effects of ganglioside depletion are due to ceramide accumulation.

We further analyzed the effect of PPPP exposure on cellular sphingomyelin. HPTLC analysis (Fig. 4B) shows sphingomyelin to migrate on the HPTLC plate as multiple bands (because of different fatty acyl chains in the ceramide portion). After a 24-h exposure to PPPP, the cellular sphingomyelin content, particularly the fast migrating band that contained the Cys24 fatty acyl chain, increased markedly (Fig. 4B). This increased sphingomyelin level in PPPP-treated cells was persistent over the 72-h experimental period, and at 72 h was twice as high as the control level (11.7 ± 2.6 versus 23.3 ± 2.5 μg of sphingomyelin/mg of protein; control versus P4-treated cells, Fig. 4B). Thus, PPPP does not cause ceramide accumulation but does result in increased sphingomyelin levels.

Effect of Sphingomyelin on Cell Proliferation—To determine whether increased sphingomyelin content in PPPP-treated cells was responsible for the inhibition of growth factor-induced proliferation, we assessed the effect of addition of exogenous sphingomyelin by Swiss 3T3 cells. We first confirmed rapid (<12 h) concentration-related uptake of sphingomyelin by Swiss 3T3 cells (Fig. 5A). To test the effect on proliferation, cells were pretreated with sphingomyelin for 24 h, serum-deprived overnight, and then incubated with 2 ng of EGF/ml for 18 h. Under these conditions, addition of sphingomyelin to the culture medium did not exert any significant effect on EGF-induced proliferation of Swiss 3T3 cells, as measured by [3H]thymidine uptake (Fig. 5B).

Effect of Cellular Ganglioside Enrichment on Cell Proliferation—The consistent inhibition of growth factor-induced cell proliferation that was caused by the depletion of cell gangliosides by PPPP pointed to an enhancing role of endogenous cell gangliosides in growth factor-mediated cell proliferation. This prompted us to examine the effect of enrichment of cellular gangliosides by preincubation of the cells with exogenously added gangliosides (Scheme 1A). Three purified gangliosides, the monosialoganglioside G M3 and the disialogangliosides G D1a and G D1b were used to evaluate the influence on proliferation of enrichment of gangliosides in the cell plasma membrane of Swiss 3T3 murine fibroblasts and NHDF, because they are the main component gangliosides of these cells.

In Swiss 3T3 cells, G D1a but not G M3 had a stimulatory effect on EGF-induced proliferation (Fig. 6). Surprisingly, G D1a also exerted a growth factor-like effect in the absence of EGF and serum. In NHDF cells, all three gangliosides caused concentration-independent stimulation of proliferation. G D1a exerted a stimulatory effect on EGF-stimulated proliferation, demonstrating a strong synergism with EGF in promoting cell proliferation. For example, the [3H]thymidine uptake of the cells that were preincubated with 5 and 10 μM G D1a was 39 and 76% higher, respectively, than that of cells preincubated without the ganglioside. The monosialoganglioside G M3 had a similar effect on NHDF cell proliferation as did G D1 (not shown). Again, G D1a and G M3 also had an independent growth factor
like effect. For example, 10 μM GD1a stimulated NHDF proliferation by nearly 2-fold in the absence of serum and EGF.

In another set of experiments, we further investigated several variables that could influence the observed stimulatory effect of gangliosides upon EGF-induced cell proliferation (Fig. 7). Preincubation of the cells in 96-well plates with ganglioside GD1a (0–100 μM) in FGM containing 2% FBS caused a concentration-dependent stimulation of proliferation either in the presence or absence of EGF (Fig. 7A). Even without serum starvation, there was a strong synergism between ganglioside GD1a and EGF in promoting cell proliferation (Fig. 7B). We also used larger 24-well plates and measured [3H]thymidine incorporation into the trichloroacetic acid-insoluble material (Fig. 7C). A concentration-dependent stimulation of proliferation by GD1a and a strong synergism between added ganglioside and EGF were observed (Fig. 7C), similar to that observed when the cells were cultured in 96-well plates, and [3H]thymidine uptake was determined by β-scintillation counting after the cells were harvested by a cell harvester (Fig. 7A). Finally, we also assessed cell proliferation by direct cell counts (Fig. 7D). Preincubation of the cells with ganglioside GD1a also caused an increase in cell number in this experiment (Fig. 7D), confirming the enhancing effect of enrichment of cellular gangliosides on NHDF proliferation.

To exclude the possibility that any ganglioside effect was caused by contaminating biologically active molecules (e.g., proteins) in the ganglioside preparation, we tested a synthetic (chemically synthesized) ganglioside, GM3 (d18:1–C18:0), which we had previously shown to be equivalent to natural G3M3 in its immunological properties (20). As shown in Fig. 8, this ganglioside also enhanced EGF-induced proliferation and exerted a growth factor-like effect in the absence of EGF and serum. These results confirm the positive stimulatory effect of enrichment of cellular gangliosides on cell proliferation.

To determine whether gangliosides enhance proliferation of fibroblasts in response to other growth factors, we tested the effect of preincubation of NHDF cells with ganglioside GD1a, on bFGF-, PDGF-, and IGF-1-induced proliferation. All were affected. For example, preincubation of the cells with GD1a enhanced bFGF-induced proliferation in a dose-dependent manner (Fig. 9), and there was a strong synergism between ganglioside GD1a and bFGF in promoting cell proliferation. Thus, the enhancing effect of cellular ganglioside enrichment on cell proliferation is not limited to EGF-induced proliferation but appears to be a more general phenomenon.

The results of these experiments seemingly contrast with previous reports that exogenous gangliosides inhibit growth factor-induced proliferation. Because in those studies gangliosides were present during incubation of the cells with growth factors (17), we wondered whether it might be the continuing presence of exogenous gangliosides (which we had washed out) in the culture medium during the incubation with growth factors that underlies these apparently disparate findings. We conducted a set of three experiments to explore this possibility (Fig. 10). Experiment A reproduced our previous design, i.e., cellular ganglioside enrichment followed by removal of unincorporated gangliosides (Fig. 10A). NHDF cells were seeded at 5 × 10^4 cells/well in a 96-well plate in FGM with 2% FBS. The medium was replaced with fresh medium ± ganglioside GD1a on the following day. After 18 h, the medium was removed, and the cells were cultured in serum-free medium (FBM) overnight to starve the cells and were then replaced with serum-free medium ± EGF. The cells were further cultured for 24 h, and cell proliferation was measured by [3H]thymidine uptake (Scheme 1A). Under these conditions, a stimulatory effect of ganglioside GD1a on NHDF proliferation was observed (Fig. 10A), as expected from the experiments described earlier (Figs. 6–9). In experiment B, ganglioside GD1a was present in the culture medium throughout the experiment (Scheme 1B), i.e., during the preincubation, the starvation period, and the incubation of the cells with EGF (Fig. 10B). In experiment C, the cells were preincubated with ganglioside GD1a for 18 h and then starved overnight in the absence of gangliosides. Ganglioside GD1a was added back to the culture medium during the incubation with EGF (Fig. 10C). In both experiments B and C, a clear dose-dependent effect of ganglioside GD1a on cell proliferation was observed. Both in the absence and in the presence of EGF, 10 μM GD1a caused an increase in [3H]thymidine uptake when compared with the ganglioside-untreated cells (Fig. 10, B and C). As the GD1a concentration was increased to 50 μM,
however, the stimulatory effect diminished and was replaced by an inhibitory effect consistent with previous studies (17, 18). The contrast between the effect of a high concentration of the ganglioside when present only during the preincubation versus being present during the assay was even clearer when a composite figure of the data was examined (Fig. 10 D).

To confirm these findings, we studied the effect of ganglioside GD1a on the fibroblast responses to another growth factor, PDGF, using the same experimental design as described in Fig. 10. Similar results were obtained (Fig. 11). Finally, two additional experiments were performed in the larger 24-well plates. In these experiments, the effect of ganglioside GD1a on EGF-induced cell proliferation was evaluated under two conditions, preincubation of cells with the ganglioside (Scheme 1 A) or continuous incubation of cells with the ganglioside (Scheme 1 B). Preincubation of NHDF cells with 50 μM GD1a in FGM containing 2% FBS resulted in a marked (120%) increase in DNA synthesis, measured as [3H]thymidine uptake. In contrast, continuous incubation of cells with the same concentration of GD1a inhibited the [3H]thymidine uptake of the cells by 74% (Fig. 12 A). Likewise, in Swiss 3T3 cells, preincubation with 10 μM GD1a in DMEM containing 10% FBS increased DNA synthesis by 33%, whereas continuous incubation of cells with 10 μM GD1a inhibited the [3H]thymidine uptake of the cells by 59% (Fig. 12 B). These results clearly demonstrate differential effects of ganglioside GD1a preincubation versus continuous incubation on EGF-induced proliferation.

**DISCUSSION**

The present study demonstrates that gangliosides present in cells promote growth factor-induced proliferation and provides strong evidence that the interaction of cell surface gangliosides with growth factor receptors is required for optimal growth factor-induced proliferation of fibroblasts. This was true of all of the growth factors tested, including EGF, bFGF, IGF-I, and PDGF. Our conclusion is supported by data from two complementary experimental approaches. Specific inhibition of glucosylceramide synthase by PPPP without increasing ceramide levels caused inhibition of cellular ganglioside synthesis and blocked growth factor-mediated cell proliferation. Complementing these findings, preincubation of the cells with both natural and a chemically synthesized ganglioside enhanced growth factor-elicited cell proliferation. Novel findings of this study, distinguishing it from previous similar studies, include differential effects of ganglioside GD1a preincubation versus continuous incubation on EGF-induced proliferation.

**Fig. 3.** Inhibition of growth factor-induced proliferation of Balb/c 3T3 cells by PPPP treatment. Cells were seeded at the density of 3.1 × 10⁴ cells/cm² in a 96-well plate in DMEM with 10% FBS and incubated overnight. The medium was replaced with fresh culture medium containing either 1 μM PPPP or 0.1% ethanol, and the cells were cultured for 3 days. The medium was then replaced with 1% FBS-DMEM containing 1 μM PPPP ± growth factors or 0.1% ethanol ± growth factors, and the cells were cultured for 18 h. Cell proliferation was determined by measurement of [3H]thymidine uptake (see “Materials and Methods”). Each bar represents the mean (± S.D.) of triplicate cultures.

with 10 μM GD1a in DMEM containing 10% FBS increased DNA synthesis by 33%, whereas continuous incubation of cells with 10 μM GD1a inhibited the [3H]thymidine uptake of the cells by 59% (Fig. 12 B). These results clearly demonstrate differential effects of ganglioside GD1a preincubation versus continuous incubation on EGF-induced proliferation.
"ing domains" has been proposed to emphasize that clustered glycosphingolipids themselves may also initiate signal transduction through the interaction of aliphatic chains of the transducer molecules with the lipid portion of glycosphingolipids in the microdomains (1). The existence of clustered ganglioside GM3 in the plasma membrane was demonstrated by immunogold electron microscopy in human peripheral blood lymphocytes (3). Depletion of cellular gangliosides conceivably abolishes the formation of glycosphingolipid clusters in the cell plasma membrane, and consequently, this could be one mechanism leading to blocked growth factor-induced proliferation. Conversely, preculture of the cells with gangliosides, which causes enrichment of gangliosides in the plasma membrane and thereby potentially enhances the formation of glycosphingolipid clusters, promoted growth factor-induced proliferation. These studies demonstrate a positive stimulatory role of cellular gangliosides in growth factor-induced transmembrane signaling.

This study was undertaken to investigate the physiological functions of endogenous cellular gangliosides. The use of the enzyme inhibitor, PPPP, is a particularly valuable approach to inhibit ganglioside synthesis. This is because a related glucosylceramide synthase inhibitor, D-PDMP, which has been widely used to inhibit ganglioside synthesis, is also known to cause ceramide accumulation (13, 28). Ceramide (14) and its metabolic products, such as sphingosine-1-phosphate (15), may act as second messengers, thereby affecting cell growth. Sphingosine-1-phosphate may also act as an efficient exogenous effector through its secretion and its binding to Edg receptors (29). Sphingosine and N,N-dimethyl-sphingosine have also

**FIG. 5. Sphingomyelin uptake and its effect on fibroblast proliferation.** In Panel A, Swiss 3T3 cells were seeded at 5 x 10^3 cells/well in 96-well plates in DMEM with 10% FBS. On the following day, 100 μl of fresh culture medium containing 14C-labeled sphingomyelin was added to each well. The uptake of sphingomyelin was measured after incubation of the cells with sphingomyelin for up to 24 h. In Panel B, Swiss 3T3 cells were seeded at 5 x 10^3 cells/well in a 96-well plate in DMEM with 10% FBS. The medium was replaced with the fresh culture medium with or without sphingomyelin on the following day. After an 18-h incubation, the culture medium was removed, and the cells were cultured overnight in serum-free DMEM, which was then replaced with serum-free medium ± EGF. The cells were further cultured for 24 h before the cell proliferation was measured by [3H]thymidine uptake. Each value is the mean (± S.D.) of triplicate measurements.

**FIG. 6. Effect of preincubation with gangliosides on fibroblast [3H]thymidine uptake.** Swiss 3T3 (left panels) and NHDF (right panels) cells were seeded at 5 x 10^3 cells/well in a 96-well plate in DMEM with 10% FBS (Swiss 3T3 cells) or FGM with 2% FBS (NHDF cells). The medium was replaced with fresh culture medium ± ganglioside GD1α, or GM3 on the following day. After an 18-h incubation with gangliosides, the medium was removed, and the cells were cultured in serum-free medium overnight. This medium was then replaced with serum-free medium ± EGF, and the cells were further cultured for 24 h. Cell proliferation was measured by [3H]thymidine uptake. Each bar is the mean (± S.D.) of three separate cultures. *, p < 0.05; **, p < 0.01.
been reported to regulate cell proliferation (30, 31). In contrast to D-PDMP, PPPP at a concentration $\leq 1.0 \, \mu M$ specifically inhibits glucosylceramide synthesis, leading to inhibition of ganglioside synthesis without causing ceramide accumulation (12). Only at $\geq 30 \, \mu M$ does PPPP cause ceramide accumulation by inhibiting 1-O-acylceramide synthase (12). Thus, an effect of ceramide on cell proliferation in PPPP-treated cells can be excluded. The conclusion that it is the inhibition of ganglioside synthesis and depletion of cellular gangliosides that underlie the inhibition of growth factor-stimulated fibroblast proliferation is supported by a recent report of attenuation of the interleukin 2 signal in spleen cells of mice that lack complex gangliosides because of a knockout of the gene for glucosylceramide synthase (9). Transfection of the gene for sialidase, on the other hand, was reported to enhance EGF receptor activity in Al431 cells (7). This may be explained by the fact that transfection of the sialidase gene may not only destroy gangliosides but also alter sialic acid containing glycoproteins (7). Also, in a mutant Chinese hamster ovary cell line that has a reversible defect in

Fig. 7. Effect of preincubation with gangliosides on EGF-induced fibroblast proliferation. NHDF cells were seeded at $5 \times 10^3$ cells/well in 96-well plates (A and B) or at $3 \times 10^4$ cells/well in 24-well plates (C and D) in FGM with 2% FBS. The medium was replaced with fresh culture medium $\leq$ ganglioside GD1a on the following day. After an 18-h incubation with gangliosides, the medium was removed, and the cells were cultured in serum-free medium (FBM) overnight. This medium was then replaced with serum-free medium $\geq$ EGF, and the cells were further cultured for 24 h (A and C). Alternatively, after an 18-h incubation with gangliosides, the medium was directly replaced with serum-free medium $\geq$ EGF, and cells were further cultured for 24 h (B and D). Cell proliferation was measured as $[^3H]$thymidine uptake by scintillation counting after the cells were harvested by a cell harvester (A and B) or by determining $[^3H]$thymidine incorporation into the trichloroacetic acid-insoluble material (C) as described previously (17). The cell number under different culture conditions was also determined (D). The data represent the mean ($\pm$ S.D.) of 2–3 separate measurements.

![Fig. 7](image)

Fig. 8. Effect of preincubation with synthetic ganglioside GM3 on fibroblast proliferation. NHDF cells were cultured exactly as described in the legend to Fig. 6, except cells were treated with synthetic ganglioside GM3 (d18:1–C18:0). Each bar is the mean ($\pm$ S.D.) of triplicate measurements. *, $p < 0.05$; **, $p < 0.01$.

![Fig. 8](image)

Fig. 9. Effect of ganglioside GD1a on bFGF-induced fibroblast proliferation. NHDF cells were seeded at $5 \times 10^3$ cells/well in 96-well plates in FGM containing 2% FBS. The medium was replaced with fresh culture medium $\leq$ ganglioside GD1a on the following day. After an 18-h incubation with gangliosides, the medium was removed, and the cells were further cultured for 24 h. Cell proliferation was measured as $[^3H]$thymidine uptake. Each value is the mean ($\pm$ S.D.) of two separate cultures.

![Fig. 9](image)
Exogenous gangliosides have many effects (33–35). What we have focused on here is the effect on growth factor-induced proliferation of fibroblasts. It has been widely reported that exogenous gangliosides inhibit the proliferative response to several growth factors in a number of cell types (6). For example, ganglioside GM1 inhibited EGF receptor-associated tyrosine kinase activity (18), mitogen-activated protein kinase activity (36), and EGF-dependent growth of KB cells (18) and of the A1S clone of Ala431 human epidermoid carcinoma cells (36). The observed inhibitory effects were considered to result from the direct inhibition of EGF receptor tyrosine phosphorylation (37). Addition of certain gangliosides to the culture medium was also reported to inhibit PDGF-mediated receptor tyrosine phosphorylation and cell growth in Swiss 3T3 cells (17, 38), human glioma U-1232 cells (38, 39), and human neuroblastoma SH-SY5Y cells (40) through the inhibition of receptor dimerization (38–40). GM1 and GM3 (but not GD3) were recently found to inhibit fibroblast growth factor-induced cell proliferation (41) and certain other exogenous gangliosides, e.g. 2–3-sialylparagloboside, were also found to inhibit insulin receptor tyrosine kinase and insulin-dependent growth in HL-60, Lys562, and IM9 cells (42). These studies generally used high concentrations of gangliosides and left them in the cultures during the proliferation assay, and high concentrations of exogenous gangliosides in the form of micelles may bind to growth factors and block the binding of growth factors to their receptors (43).

What is found here is that preincubation with ganglioside GD1a, GM3, and GD3 stimulated fibroblast proliferation when excess exogenous gangliosides were washed out after allowing binding and insertion in the lipid bilayer of the target cells (4, 5) to occur. Purified radiolabeled gangliosides bind to and are stably incorporated in normal human fibroblasts (44) supporting the notion that preincubation of cells with gangliosides is a reasonable approach to enrich cellular gangliosides. This view is further supported by our observation that preincubation of the cells with gangliosides, followed directly by incubation of cells with EGF (without starvation in serum-free medium), also enhanced EGF-induced proliferation (Fig. 7B). To exclude the possibility that the observed enhancing effect was caused by trace amounts of contaminating materials in the ganglioside preparations, we tested a chemically synthesized ganglioside, GM3 (d18:1–C18:0), for its effect on cell proliferation. Because the synthetic ganglioside does not contain any contaminating biological molecules, these results further support the conclusion that preincubation of the cells with gangliosides enhances cell proliferation. Finally, when continuously present in the culture medium during incubation of fibroblasts with growth factors, high concentrations of ganglioside GD3, inhibited cell proliferation, as previously reported (17, 18). Consistent with these results, an enhancement by gangliosides of the effect of bFGF on the growth of cultured bovine capillary endothelial cells has been reported (45).

In conclusion, our findings clearly delineate a positive, stimulating effect of cell membrane gangliosides on growth factor-induced fibroblast proliferation. Several mechanisms of gangli-
Oside action have been proposed (1, 6, 26, 39). The challenge now is to define exactly how gangliosides specifically regulate transmembrane signaling, cell proliferation, and other biological processes. Further studies are also needed to address a possible relationship between the functional role of glycolipid signaling domains and the effect of cellular ganglioside depletion or enrichment on cell proliferation. Analysis of the dynamic interaction of ganglioside molecules with other signaling molecules in the plasma membrane, currently under study, has the goal of fully elucidating the physiological function of ganglioside molecules.

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**FIG. 11. Effect of ganglioside GD1a concentration, and of its presence during incubation with PDGF, on cell proliferation.** The effect of ganglioside GD1a on cell proliferation was evaluated under the following three conditions: (i) preincubation of cells with ganglioside GD1a (Panel A); (ii) continuous incubation of cells with ganglioside GD1a (Panel B); and (iii) ganglioside preincubation, starvation without ganglioside presence, and coinubation of the cells with ganglioside + PDGF (Panel C). NHDF cells were seeded at 5 x 10^5 cells/well in a 96-well plate in FGM with 2% FBS. The medium was replaced with fresh medium ± ganglioside GD1a on the following day. After 18 h, the medium was removed, and the cells were cultured in serum-free medium (FBM) overnight to starve the cells and were then replaced with serum-free medium ± PDGF. The cells were further cultured for 24 h, and cell proliferation was measured by [3H]thymidine uptake (Panel A). In Panel B, ganglioside GD1a was present in the culture medium throughout the experiment, i.e., during the preincubation, the starvation period, and the incubation of the cells with PDGF. In Panel C, the cells were preincubated with ganglioside GD1a for 18 h and then starved overnight in the absence of gangliosides. Ganglioside GD1a was added back to the culture medium during the incubation with PDGF. Panel D is a composite of Panels A–C when PDGF was present. Each value is the mean (± S.D.) of duplicate cultures.

**FIG. 12. Comparison of the effect of ganglioside GD1a preincubation versus continuous incubation on EGF-induced cell proliferation in 24-well plates.** Cells were seeded at 3 x 10^4 cells/well in 24-well plates in FGM with 2% FBS (NHDF cells) or DMEM with 10% FBS (Swiss 3T3 cells). The effect of ganglioside GD1a on EGF-induced cell proliferation was then evaluated under two conditions, preincubation of cells with ganglioside GD1a (Scheme 1A) and continuous incubation of cells with ganglioside GD1a (Scheme 1B). Cell proliferation was measured by determining [3H]thymidine incorporation into the trichloroacetic acid-insoluble material as described previously (17). The [3H]thymidine uptake without EGF stimulation was 1134 ± 135 dpm/well for NHDF cells and 3055 ± 187 dpm/well for Swiss 3T3 cells. Each value is the mean (± S.D.) of two to four cultures.
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