Glucosylceramide Synthase Inhibitor Inhibits the Action of Nerve Growth Factor in PC12 Cells*

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Previous studies have shown that the ceramide analogue, D-threo-1-phenyl-2-decanoylamin-3-morpholino-propanol (D-PDMP), inhibits glucosylceramide synthase and thus leads to extensive depletion of glycosphingolipids derived from glucosyl ceramide. Our previous studies have shown that choler toxin B subunit, which specifically binds to the cell surface ganglioside GM1, and GM1 itself can enhance the action of nerve growth factor (NGF) in responsive cells by enhancing the NGF-induced autophosphorylation of the high affinity NGF receptor, Trk. Using D-PDMP, we examined the effects of the inhibition of the biosynthesis of glycosphingolipids on intracellular NGF signaling pathway. D-PDMP was found to inhibit NGF-induced neurite outgrowth of PC12 cells. Moreover, D-PDMP clearly inhibited NGF-induced autophosphorylation of Trk and prevented the activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase, downstream targets of Trk-initiated intracellular protein kinase cascades. These effects of D-PDMP were abolished by the addition of GM1 but not by the addition of other ganglioside subspecies to the culture medium. Furthermore, the effect of D-PDMP seemed to be specific for the Trk receptor, because intracellular signaling pathway of epidermal growth factor was not affected by D-PDMP. Dimethylsphingosine and the cell-permeable analogue, C2-ceramide, did not show such a strong inhibitory effect on neurite outgrowth or on the autophosphorylation of Trk. The present results and our previous observations clearly demonstrate that Trk requires endogenous gangliosides, especially GM1, for its normal function in mediating the neurotrophic activity of NGF at least in PC12 cells.

Sialic acid-containing acid glycosphingolipids, gangliosides, are found largely in central nervous system. Also neuronal cell differentiation and development are known to be closely correlated with the biosynthesis and expression of gangliosides (1, 2). Heretofore, it has been demonstrated that these gangliosides are not only structural constituents of plasma membrane lipids but are also involved in several molecular events. For example, the monosialoganglioside GM1 is known to enhance the activity of nerve growth factor (NGF)† on NGF-responsive cells and stimulate neural sprouting both in vitro and in vivo (3–9). The biological effects of NGF are mediated by high affinity binding to cell surface glycoprotein receptors called Trk encoded by the trk protooncogene (10, 11). The initial step of the intracellular signal transduction of NGF is believed to the activation of Trk-associated tyrosine kinase activity by the autophosphorylation on tyrosine residue (10–13). Our previous studies have shown that GM1 enhances the action of NGF by enhancing NGF-induced autophosphorylation of the high affinity NGF receptor, Trk (9, 14). These enhancing effect of GM1 is at least in part due to tight association of GM1 with Trk in a way that is SDS-resistant (14). Subsequent study has demonstrated that GM1 enhances NGF-induced receptor dimerization and thereby enhances the inter-receptor tyrosine kinase reaction (15), although it cannot explain the positive effect of GM1 observed in the in vitro tyrosine kinase assay (14). At present, however, it remains to be elucidated whether or not GM1 is essential for Trk-initiated intracellular signal transduction mechanism upon NGF binding.

D-Threo-1-phenyl-2-decanoylamin-3-morpholino-propanol (D-PDMP) is ceramide analogue and inhibits glucosylceramide synthase activity (16). D-PDMP leads to extensive depletion of endogenous glycosphingolipids, including gangliosides biosynthesized from glucosyl ceramide, and causes accumulation of ceramide and sphingosine, and it has proved to be useful as a tool for studying various functional roles of endogenous glycosphingolipids (17). Inhibition of cell proliferation has been reported in concert with these changes in sphingolipids level by this compound (18). In vivo studies also demonstrated that D-PDMP treatment inhibits tumor growth and metastasis as well as renal hypertrophy associated with diabetes mellitus (19). In NIH3T3 fibroblasts, which have overexpressing receptors for insulin-like growth factor-1, D-PDMP has been reported to prevent the proliferative response to either insulin-like growth factor-1 or serum by blocking cell cycle progression in association with an inhibition of cell cycle-dependent kinases (20, 21), although it did not affect early signal transduction events in response to these stimuli. In neuronal cell system, D-PDMP has been reported to affect growth of murine neuroblastoma (22). D-PDMP also inhibits the axonal growth of hippocampal neurons (23). More recently, Mizutani et al. (24) reported that functional synapse formation in cultured rat cerebral cortical neurons is inhibited by the depletion of the endogenous gangliosides with D-PDMP, and the exogenous supplement of ganglioside GQ1b in culture medium reverses the effect of D-PDMP on the synapse formation. These previous

† The abbreviations used are: NGF, nerve growth factor; D-PDMP, D-threo-1-phenyl-2-decanoylamin-3-morpholino-propanol; PI, phosphatidylinositol; PVDF, polyvinylidene difluoride; MAP, mitogen-activated protein; EGF, epidermal growth factor; DMS, dimethylsphingosine.
studies have suggested that glycosphingolipids, especially gangliosides, play an important role in the maintenance of normal neuronal functions and differentiation. At present, however, the detailed molecular mechanism for these previous observations remains to be elucidated.

In this study, we examined the effect of D-PDMP-treatment on the signal transduction pathway of NGF, which has been known to be the essential trophic factor for neurons in dorsal root ganglion and in some cortical neurons. The data clearly demonstrated that D-PDMP inhibits NGF-induced neurite outgrowth in PC12 cells. D-PDMP also inhibited the responses in both autophosphorylation of Trk and the activation of PI 3-kinase and MAP kinase in response to NGF. Exogenous ganglioside GM1 but not other gangliosides reverses the effect of D-PDMP on morphological differentiation and the autophosphorylation of Trk. These results showed that GM1 is an essential endogenous factor for the normal function of the Trk receptor.

MATERIALS AND METHODS

Cell Culture—Rat pheochromocytoma cell line PC12 cells were cultured in Dulbecco’s modified Eagle’s medium as described (9, 14). Cells were preincubated in serum-free medium for 1 h at 37 °C and treated with NGF (50 ng/ml) for 5 min for immunoprecipitation and immunocomplex kinase assay of Trk. For morphological studies, the cells were cultured on collagen/poly-l-lysine (25:1)-coated 24-well plates with serum containing regular medium and pretreated, when indicated, with 5–30 μM D-PDMP for 1 week. After exposure to D-PDMP for 1 week, the cells were then treated with NGF at 50 ng/ml for 36 h at 37 °C in serum-free medium. In some cases, cells pretreated with D-PDMP in regular medium for 1 week were serum-starved for 4 h in the presence of various kinds of gangliosides such as GM1 (25 μM), GM3 (25 μM), GD1b (25 μM), GT1b (25 μM), and GQ1b (25 μM) before addition of NGF with a continuous presence of D-PDMP in culture medium.

Immunoprecipitation and Immunocomplex Kinase Assay—Immunoprecipitation and immunocomplex kinase assay were performed essentially as described previously (9, 14). In brief, control and NGF-treated PC12 cells precultured in the presence or absence of 20 μM D-PDMP for 1 week were lysed in lysis buffer (20 mM Hepes, pH 7.2, 1% Nonidet P-40, 10% (v/v) glycerol, 50 mM NaF, 1 mM phenylmethysulfonyl fluoride, 1 mM Na3VO4, 10 μg/ml leupeptin). Cell-free lysates were normalized for proteins and immunoprecipitated with an anti-Trk antibody (14). The immunocomplex kinase assay was performed as described previously (14). PI 3-kinase activity was measured with Trk immunoprecipitate phosphatidylinositol as a substrate (25).

Immunoblot Analyses—Trk was immunoprecipitated with an anti-
Trk antibody from cell-free lysates as described above. The resulting immunoprecipitates were electrophoretically transferred to polycrylilde difluoride (PVDF) membranes after SDS/polyacrylamide gel electrophoresis with 10% gels and were probed with an anti-Trk antibody or anti-phosphotyrosine antibody (4G10, Upstate Biotechnology Inc.). Detection of positive bands was performed using the ECL detection system (Amersham Pharmacl Biotech). For the detection of activated MAP kinase, equal amounts of cell-free lysates were subjected to SDS/polyacrylamide gel electrophoresis followed by Western blotting. The PVDF membranes were probed with either an anti-phosphospecific MAP kinase or ERK1/2 antibody (New England Biolabs).

Ganglioside Purification and Analysis—The total lipids of the cells were isolated by chloroform/methanol extraction. Gangliosides were purified by diisopropyl ether/butanol partition (26) followed by DEAE-Sephadex A-25 column chromatography (bed volume, 3 ml). Acidic gangliosides fraction was desalted with Sephadex LH-20 column and further analyzed with ascending thin layer chromatography on high performance TLC plates. The plates were stained with either resorcinol reagents or horseradish peroxidase-conjugated B subunit of cholera toxin. For the study of ganglioside biosynthesis, cellular lipids were isolated by chloroform/methanol extraction. Gangliosides were purified by diisopropyl ether/1-butanol partition (26) followed by DEAE-Sephadex A-25 column chromatography (bed volume, 3 ml). Acidic gangliosides fraction was desalted with Sephadex LH-20 column and further analyzed with ascending thin layer chromatography on high performance TLC plates.

RESULTS
Morphology—We examined the effect of D-PDMP on NGF-induced neurite outgrowth in PC12 cells. After preincubation of PC12 cells with D-PDMP (10–30 µM) for 1 week in serum-containing regular medium, the cells were stimulated with 50 ng/ml NGF for 36 h in the continuous presence (Fig. 1, D–F) or absence of D-PDMP (Fig. 1C). D-PDMP clearly inhibited NGF-induced neurite outgrowth (Fig. 1, D–F), although D-PDMP alone did not cause any morphological alteration (Fig. 1, compare A and B). On the other hand, the supplement of 25 µM GM1 along with 50 ng/ml NGF in culture medium of the cells pretreated with 20 µM D-PDMP for 1 week caused neurite outgrowth (Fig. 1G). We also assessed the effects of dimethylsphingosine (DMS) and C₄-ceramide on the morphological differentiation of the cells by NGF. We did not observe any effect on NGF-induced neurite outgrowth in C₄-ceramide-loaded cells (Fig. 2C, panel 4), but we found a slight inhibitory effect in DMS-loaded cells (Fig. 2C, panel 3). Under these conditions, we did not observe any significant difference in alive cell numbers versus control cells as judged by trypan blue dye exclusion assay. More than 50 µM D-PDMP was toxic to PC12 cells under the present conditions as assessed by the trypan blue dye exclusion method.

Effects of DMS and C₄-ceramide on Trk-associated Tyrosine

FIG. 2. Effect of dimethylsphingosine and C₄-ceramide on Trk autophosphorylation (A) and morphological differentiation (C). A. PC12 cells were cultured on 175-cm² flasks in the regular medium and pretreated with either 5 µM DMS (lane 3) or 10 µM C₄-ceramide (lane 4) for 4 h at 37 °C in serum-free medium. Then cells were stimulated with 50 ng/ml NGF for 5 min at 37 °C (lanes 2–4) or unstimulated (lane 1) with a continuous presence of either DMS or C₄-ceramide in culture medium. Trk was immunoprecipitated with an anti-Trk antibody, and Western blot analyses were performed on these Trk immunoprecipitates with an anti-phosphotyrosine (A) as described under “Materials and Methods.” B. PVDF membranes used in A were reprobed with an anti-Trk antibody after stripping the first antibody (anti-phosphotyrosine antibody) as specified in a manual from the manufacturer. C. PC12 cells were cultured on 25:1 collagen/poly-L-lysine-coated 24-well plates in the regular medium and pretreated with 5 µM DMS (panel 3) or 10 µM C₄-ceramide (panel 4) for 4 h at 37 °C in serum-free medium. Then the cells were stimulated with 50 ng/ml NGF (panels 2–4) for an additional 36 h at 37 °C with the continuous presence of either DMS or C₄-ceramide in culture medium. The pictures are shown as phase contrast micrographs made from typical areas of these cultures.
Kinase Activity—DMS and ceramide as well as sphingosine are reported to be accumulated in d-PDMP-treated cells (17, 28). Therefore, we examined the effects of such lipids on both morphology and Trk-associated tyrosine kinase activity. The cell-permeable analogue of ceramide, C2-ceramide (10 μM), did not affect NGF-induced neurite outgrowth nor NGF-induced Trk autophosphorylation (Fig. 2A, lane 4). On the contrary, DMS (5 μM) had weak inhibitory effects on both parameters (Fig. 2A, lane 3).

Effect of d-PDMP on Trk-associated Tyrosine Kinase Activity—The effect of d-PDMP on receptor tyrosine kinase activity was examined. d-PDMP pretreatment for 1 week inhibits NGF-induced Trk autophosphorylation in both immunocomplex kinase assay and immunoblot analysis (Fig. 3), although the addition of d-PDMP into the in vitro kinase assay mixture did not cause any change in kinase activity (data not shown). To further examine this finding, we checked the effect of d-PDMP on the downstream of Trk-associated tyrosine kinase-initiated intracellular kinase cascade. d-PDMP also inhibited both PI 3-kinase and MAP kinase activation in response to NGF (Fig. 4).

Effects of d-PDMP on Glycosphingolipids and Neutral Lipids Biosynthesis and Metabolism—PC12 cells were cultured in the presence or absence of 20 μM d-PDMP for 1 week and then labeled with 10 μCi of [3H]palmitic acid for 24 h at 37°C. The total lipids were further fractionated into acidic and neutral lipids as described previously (26). The analysis of neutral lipids with thin layer chromatography revealed that glucosylceramide is actually depleted in d-PDMP-treated cells as compared with that of control cells (Fig. 5C, lane 2). Acidic lipids fraction was also analyzed on TLC plates, and we verified substantial decrement of disialo and monosialo ganglioside in d-PDMP-treated cells (Fig. 5, A and B).

The Distribution of GM1 in the Cells—We further assessed the distribution of GM1 by use of fluorescein isothiocyanate-conjugated B subunit of cholera toxin as a specific probe in cells pre-treated with 20 μM d-PDMP for 1 week by confocal laser microscope. The data showed a similar staining pattern of GM1 in d-PDMP-pretreated cells but severe reduction of the intensity when compared with those of control cells with a dose-dependent manner (Fig. 6, panels 2 and 3). These findings strongly suggest that, in fact, GM1 is strongly down-regulated in d-PDMP-treated cells.

Detection of GM1 in Trk Immunoprecipitates—Our earlier observation has revealed that GM1 binds to the Trk protein and regulates the receptor function. Therefore, we checked the effect of d-PDMP on the GM1 binding to Trk receptor as reported previously (14). In d-PDMP-treated cells, we could not detect any GM1 in Trk immunoprecipitates even from NGF-treated cells (Fig. 7, lane 4). This effect seems to be due to the depletion of GM1 in d-PDMP-treated cells, because the supplement of GM1 in the culture medium resulted in the detection of GM1 in Trk immunoprecipitates (Fig. 7, lanes 3 and 5).

S6 Kinase Assay of d-PDMP-pretreated Cells—We examined the specificity of the effect of d-PDMP on the growth factor receptor-initiated intracellular signaling pathway. NGF stimulates S6 kinase activity (Fig. 8, lane 4), whereas d-PDMP treatment inhibits NGF-induced S6 kinase activation (Fig. 8, lane 5). On the other hand, d-PDMP does not affect EGF-induced S6 kinase activation (Fig. 8, compare lanes 2 and 3).

DISCUSSION

Striking changes in synthesis and expression patterns of gangliosides have been observed during cellular differentiation and development as well as pharmacological agents (1, 2, 28). Exposure of neuroblastoma cells to retinoic acid induces differentiation of the cells as well as a marked increase in cellular gangliosides content (29–31). A recent report has revealed that both induced and spontaneous neurotogenesis are associated with enhanced expression of ganglioside GM1 in the nuclear
membrane (32). It has been shown that GM1 functions as a neurotrophic agent and that it potentiated the action of NGF in responsive cells both in vitro and in vivo (5–10). There is increasing attention and interest in the elucidation of biological functions and their mechanisms. In 1995, several groups including ours found that GM1 enhances the action of NGF by augmenting NGF-induced Trk autophosphorylation and rescues neuronal cells from apoptotic death elicited by withdrawal.
of trophic support by NGF (14, 33, 34). Furthermore, we also demonstrated that GM1 binds to Trk, and its strong binding to Trk is thought to be the molecular mechanism for the observed enhancing effects of GM1 on the responsibility of Trk to NGF (14). These studies highlighted the significance of GM1 on the regulation of Trk receptor function as well as on neuronal survival. Hereofore, the data on the reactivity of the Trk protein to NGF in gangliosides-depleted or even down-regulated cell system have not been available. It is important to characterize the changes in the behaviors of cells that are lacking in gangliosides, especially GM1. For this purpose, we utilized n-PDMP to deplete gangliosides in the cells. n-PDMP has been reported to lead to extensive depletion of endogenous glycosphingolipids, including gangliosides biosynthesized from glucosyl ceramide, and causes accumulation of ceramide (17, 34). In neuronal cell system, n-PDMP treatment inhibits neurite outgrowth from several neuroblastoma cell lines (22) and inhibits the functional synapse formation in cultured rat cortical neurons (24), although the molecular sequences of these observed effects remain to be elucidated.

In this study, we demonstrated the following. 1) Pretreatment of the cells with n-PDMP prevents NGF-induced neurite outgrowth from PC12 cells. 2) This inhibitory effect of n-PDMP on the neurite outgrowth is abolished by the supplement of GM1 but not by other gangliosides tested (GM3, GD1b, GT1b, and GQ1b) in the culture medium. 3) In such n-PDMP-treated cells, we can hardly observe Trk autophosphorylation in response to NGF, but again supplement of GM1 in the culture medium makes cells to respond to NGF stimulation. 4) The activation of PI 3-kinase and MAP kinase, which lies in the down stream of Trk-initiated kinase cascade, in response to NGF is also missing in n-PDMP-pretreated cells. 5) DMS and C2-ceramide, a cell-permeable analogue of ceramide, which are proposed to be accumulated in the cells pretreated with n-PDMP (17, 28), do not affect the reactivity of the cells to NGF in the latter; the former case showed a slight inhibitory effect. At this point, we should point out that a relatively high concentration of ceramide can induce apoptotic cell death of PC12 cells (35). In addition, exogenous addition of more than 20 μM C2-ceramide into culture medium has been reported to show weak inhibitory effect on NGF-induced Trk autophosphorylation (36). In this study, however, prolonged exposure of n-PDMP for 1 week did not seem to affect cell viability, because trypan blue dye exclusion assay revealed that such long term exposure of the cells to n-PDMP did not induce any more accelerating cell death nor internucleosomal DNA fragmentation than naive cells (data not shown). Furthermore, GM1 replacement into culture medium reverses the strong inhibitory effects of n-PDMP on neurite outgrowth and Trk autophosphorylation (Figs. 1 and 3). These results suggest that these lipids theoretically proposed to accumulate might contribute little if any to the inhibitory effects of n-PDMP shown in this study. 6) n-PDMP treatment causes down-regulation of endogenous gangliosides as well as binding of GM1 to the Trk protein. These data strongly suggest that endogenous GM1 regulates the reactivity of Trk receptor to NGF in at least PC12 cells. Recent report by Li and Ladisch (37) on retinoic acid-induced neurite outgrowth appears to be inconsistent with the previous reports (22, 24) and our present findings. Although we do not know the exact molecular mechanism for such inconsistency, it might be due to the difference in the culture system employed in each studies and the difference of neurites (axon versus dendrite) elicited by these different stimuli. Growth of axon and dendrite is not always accompanied. Some stimuli induce axonal growth but not dendritic growth. These different types of neurites might have different sensitivity to gangliosides. Alternatively, basal requirement of some ganglioside, especially GM1, for the normal function of Trk receptor might be different among cell types or special spacial distribution of GM1 and Trk receptor just like as those found in GM3 and c-Src and Rho proteins in other cell systems (38) might be different. Further studies to answer these questions are now in progress in this laboratory.

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