Inhibition of Endogenous Ganglioside Synthesis Does Not Block Neurite Formation by Retinoic Acid-treated Neuroblastoma Cells*

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Gangliosides are believed to play a critical role in cellular differentiation. To test this concept, we determined the effect of inhibition of endogenous ganglioside synthesis upon neurite formation induced by retinoic acid in LAN-5 human neuroblastoma cells. Ganglioside synthesis and content of LAN-5 cells exposed for 6 days to 10 μM D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) (an inhibitor of glucosylceramide synthase) were reduced by >90%. However, these ganglioside-depleted cells were not blocked from forming neurites when exposed to 10 μM retinoic acid. Even more extensive treatment of LAN-5 cells with 20 μM D-PDMP (6 day pretreatment followed by 6 days together with 10 μM retinoic acid) still did not block the retinoic acid-induced neurite formation. An element of neuroblastoma tumor cell differentiation, neurite formation, is therefore dependent neither on an intact cellular ganglioside complement nor on new ganglioside synthesis.

The high concentrations of gangliosides in the central nervous system have led to the widely held view that these molecules may play an important role in the differentiation of neuronal cells (1). There is evidence supporting this view, in that striking changes in ganglioside metabolism have been observed during both spontaneous and induced cellular differentiation (2–4). The treatment of LAN-5 human neuroblastoma cells with retinoic acid, which causes differentiation of the cells (5, 6), results in a marked increase in cellular ganglioside content (7). An increase in concentration of specific gangliosides (8), a switch in ganglioside biosynthetic pathways and the appearance of new gangliosides (9, 10), and changes in cellular ganglioside patterns (11) are generally considered to be important in the process of cellular differentiation, particularly in neuronal or neuroblastoma cells. Exogenous gangliosides also influence differentiation (12–14). However, the question of whether endogenous gangliosides are essential for cellular differentiation under physiological conditions remains to be answered.

To determine whether endogenous gangliosides are essential for the process of cellular differentiation, we developed the following strategy. In a model system, the LAN-5 human neuroblastoma cell line, which can be induced by retinoic acid to differentiate and form neurites (5, 6), we studied the effect of cellular ganglioside depletion upon neurite formation. D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP),1 a potent inhibitor of glucosylceramide synthase (15, 16), was used to maximally inhibit ganglioside synthesis and deplete cellular ganglioside content. In this situation, despite down-regulation of cellular ganglioside synthesis and content of LAN-5 cells, the formation of neurites induced by exposure of these cells to retinoic acid was not blocked.

**MATERIALS AND METHODS**

**Preparation of Retinoic Acid, D-PDMP, and Nerve Growth Factor Stock Solutions—** Retinoic acid (all-trans form; Sigma) was dissolved in ethanol at a concentration of 10⁻² M and kept as stock solution. For each experiment, retinoic acid was diluted from the stock solution directly into the growth medium. The final concentration of ethanol in the culture medium was ≤0.2% (v/v). D-PDMP was dissolved in distilled water at a concentration of 4 mM (15) and stored at 4 °C. This stock solution was directly added to culture medium (≤0.5%, v/v) in each experiment. Nerve growth factor (human, recombinant NGF-β, Sigma) was dissolved in phosphate-buffered saline with 0.1% bovine serum albumin. This stock solution was filtered and stored at ~70 °C.

**Cell Culture—** LAN-5 human neuroblastoma cells were a generous gift from Dr. Robert Seeger. These cells (passages 94–98) were cultured as adherent monolayers in 75-cm² flasks in Waymouth’s MB 752/1 medium supplemented with 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Cell viability was assessed by trypan blue dye exclusion.

**Induction of LAN-5 Cellular Differentiation—** LAN-5 cells were treated with 10 μM all-trans-retinoic acid to induce cellular differentiation, which was judged by assessing neurite formation, cell proliferation (5, 6), and specific acetylcholinesterase activity (17). Neurite formation was observed and photographed under phase-contrast microscopy. Cells cultured in medium containing 0.2% ethanol were used as the control. Nerve growth factor (250–1000 ng/ml) was also used to induce neurite formation in LAN-5 cells.

**Study of Ganglioside Biosynthesis by Metabolic Radiolabeling—** LAN-5 cells were cultured in the presence or absence of D-PDMP for 72 h. [6-³H]Galactose (specific activity, 5 Ci/mmol) and [6-³H]glucosamine hydrochloride (specific activity, 30.9 Ci/mmol, DuPont NEN, Boston, MA) were added to the culture medium (1 μCi/ml) during the last 24 h. These radiolabeled cells were washed 3 times and harvested by trypsinization. The resulting cell suspension was centrifuged at 300 × g for 10 min, and the cell pellet was washed once with phosphate-buffered saline prior to processing for ganglioside purification (7, 18).

**Ganglioside Purification and Analysis—** The total lipids of the cells were isolated by chloroform/methanol extraction. Gangliosides were purified by diisopropyl ether/1-butanol partition (19) followed by Sephadex G-50 gel filtration. The purified gangliosides were quantified as nanomole of lipid-bound sialic acid (LBSA) by the modified resorcinol method (20), and ganglioside-associated radioactivity was quantified by β-scintillation counting (Betafluor, National Diagnostics, Manville, NJ).

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1 The abbreviations used are: D-PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; LBSA, lipid-bound sialic acid; NGF, nerve growth factor.
**RESULTS**

**Regulation of Cellular Gangliosides by Retinoic Acid and d-PDMP—**LAN-5 human neuroblastoma cells contain GD_{2} (56%), GM_{2} (15%), and GT_{1b} (11%) as the main ganglioside components. The minor ganglioside species also seen are GM_{1}, G_{D3}, and G_{T1b} (18). To provide the basis for studying the influence of endogenous gangliosides on cellular differentiation in this model system, we first established the effects of each agent on ganglioside synthesis and content. Retinoic acid, which induces differentiation, caused an increase in cellular ganglioside content. When LAN-5 cells were treated with 20 μM retinoic acid for 6 days, the cellular ganglioside content increased from 3.95 ± 0.39 (control) to 6.1 ± 0.13 nmol of LBSA/mg of protein (Fig. 1). In contrast, d-PDMP (which blocks ganglioside synthesis) caused depletion of cellular gangliosides.

Exposure of LAN-5 cells to 20 μM d-PDMP for 6 days reduced cellular ganglioside content by 90%, to 0.41 ± 0.29 nmol of LBSA/mg of protein (Fig. 1). Moreover, as shown in Fig. 2, the inhibition of endogenous ganglioside content caused by d-PDMP was rapid and time-dependent; when LAN-5 cells were cultured in medium containing 10 μM d-PDMP, the cellular ganglioside content decreased rapidly, with a 42% reduction in cellular ganglioside content observed after 1 day. By day 6, the total cellular ganglioside content was reduced by 92%, to 0.34 from 4.1 nmol of LBSA/mg of protein. This marked cellular ganglioside depletion was confirmed by a metabolic radiolabeling study, in which we measured the inhibitory effect of d-PDMP on ganglioside synthesis. Following the treatment of LAN-5 cells with 10 μM d-PDMP for 72 h, ganglioside synthesis was already reduced by 90%, to 375 from 3495 dpm/mg of protein by the control cells.

Having found that retinoic acid treatment causes an increase in ganglioside content and that d-PDMP blocks ganglioside synthesis and causes a depletion of cellular gangliosides, it was important to establish the combined effect of these two agents on cellular gangliosides, since the subsequent experiments depended on blocked ganglioside synthesis and cellular ganglioside depletion in order to be able to test the role of endogenous ganglioside synthesis and content in differentiation. Therefore, we treated LAN-5 cells to maximally deplete cellular gangliosides. LAN-5 cells were first cultured in medium containing 20 μM d-PDMP for 6 days and then reseeded and cultured for an additional 6-day period under four different conditions, in medium containing 0.2% ethanol (control), 10 μM retinoic acid, 20 μM d-PDMP, or both 10 μM retinoic acid and 20 μM d-PDMP. As shown in Table I, LAN-5 cells treated with both retinoic acid and d-PDMP during the second 6-day period of culture had essentially the same cellular ganglioside content as the cells treated with d-PDMP alone (0.54 versus 0.40 nmol of LBSA/mg of protein). Thus, the inhibitory effect of d-PDMP on ganglioside synthesis was dominant over the stimulatory effect of retinoic acid on ganglioside synthesis by LAN-5 human neuroblastoma cells. Under this condition, the two agents were not toxic to the cells, as evidenced by cell viability of >95%. Therefore, any effects on differentiation observed in the subsequent experiments would reflect a state of ganglioside depletion.

**Differentiation of Retinoic Acid-treated LAN-5 Cells—**We chose the human neuroblastoma cell line, LAN-5, which can be induced to differentiate by retinoic acid, as a model system to study whether depletion of cellular gangliosides prevents cellular differentiation. As previously shown (5, 21), LAN-5 human neuroblastoma cells propagated in the usual cell culture medium had large cell bodies and few, short processes (Fig. 3). The effect of retinoic acid treatment (10 μM for 6 days) was the induction of striking morphological changes, including rounding up of the cell body, extension of long processes with the appearance of neurites, and cell aggregation into tight clusters (6, 21). The cells still retained their long processes for at least 6 days after retinoic acid was removed (not shown). Biochemically, this neurite formation was accompanied by an increase in cellular ganglioside content, as discussed above.

In contrast, d-PDMP, on the other hand, had no differentiating effect on LAN-5 cells, a finding consistent with a previous report that d-PDMP does not affect differentiation of HL-60 cells (22). Maximal exposure of LAN-5 human neuroblastoma cells to d-PDMP (20 μM for 6 days), which caused 92% reduction in cellular ganglioside content and had a growth inhibitory effect resulting in a lower cell density, did not cause any cell morphological changes in the cells compared to the control culture (Fig. 3). Specifically, the cells bodies remained spread out, the
processes that were present were short, and despite the lower cell density, which resulted from the growth inhibitory effects of D-PDMP, the cells remained evenly distributed on the surface of the flask (i.e. not aggregated).

Study of the combined effects of D-PDMP and retinoic acid tested the necessity of endogenous gangliosides (inhibited by D-PDMP) for cellular differentiation (induced by retinoic acid). Surprisingly, LAN-5 cells cultured in medium containing both 10 μM retinoic acid and 20 μM D-PDMP for 6 days underwent exactly the same morphological changes (neurite formation, rounding up of the cell body, and cell aggregation into tight clusters) as did the cells treated with retinoic acid alone (Fig. 3). It should be emphasized that this neurite formation (Fig. 3) occurred despite the fact that D-PDMP had caused a state of almost completely blocked ganglioside synthesis and depleted cellular gangliosides (Figs. 1 and 2).

The observation of lack of inhibition by D-PDMP of induced cell differentiation was confirmed with a second experimental approach, even more prolonged exposure of LAN-5 cells to D-PDMP. In this experiment, LAN-5 cells were exposed to 20 μM D-PDMP for an initial 6-day period, and then reseeded and cultured for an additional 6-day period under the four different conditions used in the experiment shown in Table I (0.2% ethanol, 10 μM retinoic acid, 20 μM D-PDMP, or 10 μM retinoic acid and 20 μM D-PDMP). By the end of the initial 6-day culture period in D-PDMP alone, i.e. before the addition of retinoic acid, the cells were essentially depleted of gangliosides (Figs. 1 and 2). Exposed for 6 more days to D-PDMP as well as to retinoic acid, these cells once again demonstrated the neurite formation, rounding up, and aggregation associated with retinoic acid-induced cellular differentiation (Fig. 4). Therefore, the neurite formation of LAN-5 cells usually induced by retinoic acid was not blocked by D-PDMP, despite the depletion of cellular gangliosides caused by D-PDMP. L-threo-PDMP, an isomer of D-threo-PDMP, was also studied. It stimulated ganglioside synthesis, increasing cellular ganglioside content from 3.95 ± 0.39 to 5.06 ± 0.64 (n = 3) nmol of LBSA/mg of protein when the cells were exposed to 20 μM L-PDMP for 6 days, which is consistent with a previous report (23). However, it had no effect on neurite formation, nor did it inhibit retinoic acid-induced neurite formation (Fig. 5).

To further assess the influence of D-PDMP on the incidence and length of neurites, LAN-5 cells were seeded at a lower cell density (2 × 10⁴ cells/cm²), and neurite response was quantified as described previously (5). The results (Table II) clearly showed the lack of influence of D-PDMP on the number and length of neurites induced by retinoic acid. In addition, LAN-5 cell differentiation was also assessed by measurement of acetylcholinesterase activity (17). Whereas retinoic acid caused a 2-fold increase in the acetylcholinesterase activity, consistent with previous studies (24), D-PDMP had only a minimal effect. The treatment of the cells with both D-PDMP and retinoic acid did not reduce the retinoic acid-induced increase in acetylcho-

Table I
Effects of retinoic acid and D-PDMP on neuroblastoma cellular gangliosides
LAN-5 cells were treated with 20 μM D-PDMP for 6 days and then were split into four flasks (T-75) in which these cells were further cultured in the medium containing 0.1% ethanol, 10 μM retinoic acid, 20 μM D-PDMP, or 10 μM retinoic acid + 20 μM D-PDMP for 6 days. Then the cells were harvested and the cellular gangliosides purified and quantified as nanomoles of LBSA by the resorcinol assay (20).

| 6-Day D-PDMP treatment followed by 6-day exposure to | Ganglioside content |
|---------------------------------|---------------------|
|                                   | Nmol/flask | Nmol/mg protein |
| 0.1% Ethanol                     | 6.24       | 3.21           |
| 10 μM Retinoic acid              | 3.89       | 3.76           |
| 20 μM D-PDMP                     | 0.44       | 0.40           |
| 10 μM Retinoic acid + 20 μM D-PDMP| 0.38       | 0.54           |

FIG. 3. Effects of retinoic acid and D-PDMP on cellular differentiation of LAN-5 human neuroblastoma cells. LAN-5 cells were cultured for 6 days in medium containing either 0.2% ethanol (control), 10 μM retinoic acid, 20 μM D-PDMP, or 20 μM D-PDMP + 10 μM retinoic acid. Neurite formation indicates that the cells underwent retinoic acid-induced cellular differentiation. Magnification: × 400.
linesterase activity (Table II). Together, these findings lead to the conclusion that induced differentiation of neuroblastoma cells does not require either intact endogenous ganglioside synthesis or content.

**Stimulation of Neurite Formation in Nerve Growth Factor-treated LAN-5 Cells**—We used NGF to induce neurite formation to provide an additional confirmation of these findings, since NGF-dependent trk A tyrosine phosphorylation followed by signal transduction leading to MAP kinase activation (25, 26) has been proposed to be an important mechanism for neuronal cell differentiation. NGF has already been shown to stimulate tyrosine phosphorylation of NGF receptor (p140**trk**A) in LAN-5 cells (25). It seemed likely, therefore, that NGF should stimulate neurite formation in these LAN-5 cells. This was in fact the case; exposure of LAN-5 cells to 250-1000 ng/ml NGF for 8 days resulted in neurite formation. We next determined the effect of inhibition of endogenous ganglioside synthesis by n-PDMP on the NGF-mediated process. Just as in the case of retinoic acid-induced neurite formation, n-PDMP (20 μM, which completely inhibits ganglioside synthesis) did not block neurite formation stimulated by NGF (Fig. 6). These results support the conclusion that induced differentiation of neuroblastoma cells, whether by retinoic acid or by NGF, does not require either intact endogenous ganglioside synthesis or content.

**DISCUSSION**

There is great interest in the elucidation of biological functions of glycosphingolipids in general, and of gangliosides in particular. The involvement of gangliosides in neuronal cell differentiation of the nervous system is suggested by both qualitative and quantitative changes in ganglioside metabolism during brain development (27). Significant changes in human brain gangliosides have been observed in comparing the ganglioside complement of early fetal brain to that of adult brain (3). The ganglioside accretion is largest during the period of dendritic arborization and synaptogenesis (27). Developmen-

**TABLE II**

Quantitative assessment of retinoic acid and n-PDMP effects on neurite response and acetylcholinesterase activity in LAN-5 human neuroblastoma cells

LAN-5 cells were cultured for 7 days in the medium containing 0.1% ethanol, 10 μM retinoic acid, 20 μM n-PDMP, or both 10 μM retinoic acid + 20 μM n-PDMP, and then harvested. Neurite response was assessed as previously described (5): cells were scored as morphologically differentiated if they possessed one or more processes at least twice as long as the soma diameter. Results were recorded as % differentiated cells in each culture: 4+ = > 76%; 3+ = 51-75%; 2+ = 26-50%; 1+ = < 25%. Acetylcholinesterase (AChE) activity was measured spectrophotometrically as described previously (17). The data are the mean ± S.D. of triplicate samples from a typical experiment.

| Treatment                          | Neurite response | AChE activity (pmol/min/mg protein) |
|------------------------------------|------------------|-------------------------------------|
| 0.1% Ethanol                       | 1+               | 14.3 ± 0.4                          |
| 10 μM Retinoic acid                | 4+               | 45.5 ± 1.3                          |
| 20 μM n-PDMP                       | 1+               | 19.6 ± 0.5                          |
| 10 μM Retinoic acid + 20 μM n-PDMP | 4+               | 66.8 ± 1.7                          |

**FIG. 4.** Effect of prolonged n-PDMP treatment on neurite formation induced by retinoic acid in LAN-5 cells. LAN-5 cells were first exposed to 20 μM n-PDMP for 6 days and then reseeded and cultured in medium containing both 10 μM retinoic acid and 20 μM n-PDMP. Even after the extensive exposure of LAN-5 cells to n-PDMP to maximally deplete cellular gangliosides (see Table I) in this experiment, these cells still underwent cellular differentiation. Magnification: × 400.

**FIG. 5.** Effects of l-PDMP on cellular differentiation of LAN-5 cells. LAN-5 cells were cultured for 6 days in medium containing (A) 0.2% ethanol, (B) 10 μM retinoic acid, (C) 20 μM l-PDMP, or (D) both 10 μM retinoic acid and 20 μM l-PDMP. Magnification: × 400.

**FIG. 6.** Effects of nerve growth factor and n-PDMP on neurite formation by LAN-5 cells. LAN-5 cells were cultured for 8 days in control (A) or in medium containing either (B) 1 μg/ml NGF, (C) 20 μM n-PDMP, or (D) both 1 μg/ml NGF and 20 μM n-PDMP. Magnification: × 400.
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no discernable inhibitory effect on the formation of neurites induced by retinoic acid in human neuroblastoma LAN-5 cells. We confirmed this conclusion by showing that NGF-induced neurite formation (47) in LAN-5 cells likewise was not inhibited by blockade of ganglioside synthesis by d-PDMP.

The process of cellular differentiation includes (i) phenotypic changes of tumor cells associated with arrest of their proliferation, and (ii) phenotypic changes of normal cells in vitro, or most significantly in vivo, as part of maturation. Our studies have addressed the necessity of gangliosides in the former case, and have excluded the requirement of intact endogenous ganglioside metabolism and content for tumor cell differentiation. It will be very interesting now to address this same question in the context of normal cell differentiation, and particularly in vivo under physiological conditions, such as in a ganglioside knockout mouse.

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