EXPERIMENTAL CELL RESEARCH 198, 362–366 (1992)

SHORT NOTE

A Ceramide Analogue (PDMP) Inhibits Glycolipid Synthesis in Fish Embryos

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Glycolipids were depleted from medaka embryos using 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of glucosylceramide synthetase. Embryos cultured in the presence of 20 μM PDMP exhibited a dramatic decline in glycolipid synthesis and cell surface expression. Metabolic labeling of glucosylceramide declined by 87% on Days 3–6 of development and 72% on Days 7–10 (hatching occurred on Day 10). In parallel, PDMP-treated embryos exhibited a striking loss of several tissue-specific glycolipid antigens, including 9-*O*-acetyl GD3 from brain and retina, GT3/GQ1C from brain, neural tube, and retina, and sulfated glycolipid from skin and gut. Despite these changes in glycolipid expression, PDMP-treated embryos were fully viable with no evidence of developmental abnormality. PDMP appears to provide a useful tool for identifying glycolipid antigens in embryos and investigating their role in development. © 1992 Academic Press, Inc.

INTRODUCTION

Glycolipid expression is known to change rapidly during embryonic development [13] and cell differentiation [6, 1]. These changes may reflect the actions of sequential induction signals and serve to facilitate morphogenetic cell interactions. Indeed, there is increasing evidence that glycolipids, and sphingolipid breakdown products regulate a variety of developmental processes, including cell proliferation, recognition, and differentiation (reviewed by Hakomori [9]).

A pharmacologic agent (PDMP) has been developed recently that blocks glycolipid synthesis by competitively inhibiting UDP-glucose:ceramide $\beta 1 \rightarrow$glucosyltransferase [11]. PDMP blocks the formation of glucosylceramide (CMH) and thus prevents the accumulation of complex glycolipids that are derived from CMH by oligosaccharide chain elongation (for PDMP structure, see Fig. 1). PDMP has been shown previously to affect the morphology and glucose transport ability of Balb/c 3T3 cells [14], the attachment of B16 melanoma cells to collagen and laminin [10], and the mitogenetic response of cytotoxic T lymphocyte lines [5].

In the present report, we have investigated the effects of PDMP on glycolipid synthesis and cell surface expression in embryos of a freshwater killifish (medaka). Medaka were used for these experiments because, unlike mammalian embryos, teleost embryos can be cultured easily in small volumes of lipid-free medium and their entire development can be observed readily under a dissecting microscope. Embryos cultured in the presence of 5–40 μM PDMP exhibited a striking loss of many tissue-specific glycolipid antigens without an adverse effect on development.

MATERIALS AND METHODS

Embryo culture. Medaka (*Oryzias latipes*) were obtained from a breeding colony at the School of Fisheries, University of Washington (Seattle, WA). Zygotes were collected within 4 h of ovapositing, rinsed with phosphate-buffered saline (PBS), and placed individually into the wells of 96-well microtiter plates (Corning) in 200 μl of sterile Embryo Rearing Medium (Carolina Biological Supply, Burlington, NC), in the presence or absence of PDMP. D-threo-PDMP (the active enantiomer) was prepared from crystalline powder as a 4 mM stock solution in water, filter-sterilized, and stored at 4°C. The culture medium was changed every 4 days to ensure adequate O_2 tension and minimize risks of contamination.

Metabolic labeling. In order to monitor the effects of PDMP on glycolipid synthesis, embryos were cultured for 3 days in medium (±PDMP) containing D-[6-^{3}H]galactose and D-[6-^{3}H]glucosamine (ICN, Costa Mesa, CA), with specific activities of 25 and 30 Ci/mmol, respectively (used 5 μCi/ml). After labeling, viable embryos were washed thoroughly, dried under N_2, and extracted with 2 ml of chloroform:methanol (2:1). The extract was clarified by centrifugation (100g, 10 min) and the pellet was reextracted twice. Combined extracts were desalted on 0.1 ml C_{18} reverse-phase columns (Analytichem, Harbor City, CA).

Thin-layer chromatography. Glycolipid samples were streaked onto Whatman HP-KF silica-gel plates and subjected to ascending

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chromatography using a solvent system of chloroform:methanol:water (50:40:10) containing 0.05% $CaCl_2$. After developing, thin-layer chromatography (TLC) plates were dried, sprayed with Resolution TLC (EM Corp., Chestnut Hill, MA), and exposed to Kodak X-OMAT AR5 film for 6 days at −80°C. In some experiments the relative amount of specific glycolipid was determined by scanning densitometry using an Ultroscan XL laser densitometer (Pharmacia/LKB, Uppsala, Sweden).

Indirect immunofluorescence. Control and PDMP-treated fry were transferred to O.C.T. embedding compound (Miles, Elkhart, IN), frozen on dry ice, and sectioned at 5 *μM*. Sections on poly-L-lysine-coated slides were postfixed with acetone (1 min), air-dried, and rehydrated with PBS containing 5% (w/v) bovine serum albumin (Fraction V; Sigma, St. Louis, MO). After 30 min, sections were treated with monoclonal antibody (mAb) for 1 h at 4°C, followed by FITC-conjugated rabbit anti-mouse IgG + IgM second antibody (Dakko, Carpinteria, CA) diluted 1/40 for 1 h at 4°C. Sections were then washed with PBS, mounted with 70% (v/v) glycerol in 50 m*M* Tris–HCl (pH 9.6), and examined using a Zeiss fluorescence microscope.

Monoclonal antibodies. The following monoclonal anti-glycolipid antibodies were used: VESP 6.2 specific for sulfated glycolipids [15], A2B5 specific for polysialyl gangliosides GT_3 and GQ1C [4, 6], ME311 specific for 9-*O*-acetyl GD_3 [18], VIN-IS-56 specific for GD_2 [1], and FE-A5 specific for *N*-acetyllactosamine [7].

RESULTS

Effect of PDMP on Glycolipid Synthesis

Embryos were cultured individually in 96-well microtiter plates at 22°C. A beating heart was visible on Day 3 postfertilization and hatching occurred on Day 10. PDMP-induced changes in glycolipid synthesis were investigated by metabolic labeling with [^{3}H]galactose and [^{3}H]glucosamine. Embryos cultured in the presence of 20 *μM* PDMP exhibited a dramatic reduction in the labeling of all glycolipids, including CMH, CDH, CTH, and GM3 (Fig. 2). Scanning densitometry on replicate TLC plates indicated a decline in CMH labeling by 87 ± 3% on Days 3–6 and 72 ± 8% on Days 7–10 of development.

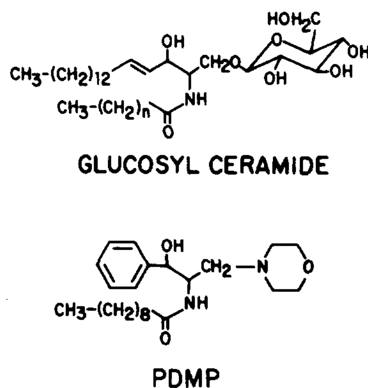

FIG. 1. Schematic representation of glucosylceramide and PDMP. PDMP (molecular weight 427) is a structural analogue of ceramide that blocks the formation of glucosylceramide, leading to a progressive depletion of membrane glycolipids [11].

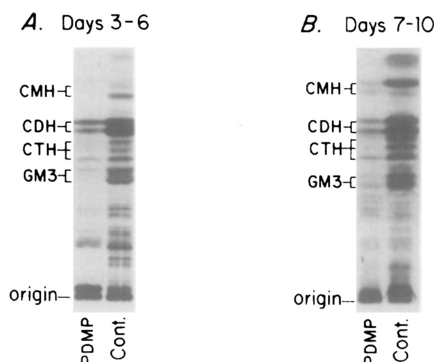

FIG. 2. Effect of PDMP on glycolipid synthesis. Embryos were cultured at 27°C in the absence (Control) or presence (PDMP) of 20 *μM* D-threo-PDMP. Embryos were labeled with [^{3}H]galactose and [^{3}H]glucosamine on Days 3–6 (A) or 7–10 (B) of development. Glycolipids were extracted, partially purified, streaked onto TLC plates, developed, and exposed to X ray film. Each lane represents the total glycolipid content of five embryos. Similar results were obtained in replicate assays. Glycolipids are designated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [12].

Effect of PDMP on Glycolipid Expression

Glycolipids synthesized during development (Fig. 2) may represent only a fraction of glycolipids in the embryo: the majority of glycolipids could be synthesized during oogenesis and stored in egg yolk prior to fertilization. However, analysis of glycolipid content in unfertilized eggs revealed only trace amounts of CDH (<100 ng/egg). Thus, inhibition of glycolipid synthesis should lead to a significant reduction in glycolipid content during development.

Information on glycolipid content in control and PDMP-treated embryos was obtained by indirect immunofluorescence using a panel of anti-glycolipid mAbs. Embryos cultured in the presence of 20 *μM* PDMP exhibited a striking loss of several tissue-specific glycolipid antigens (Fig. 3). Sulfated glycolipid was clearly detected using mAb VESP 6.2 throughout the skin and alimentary canal in control, but not PDMP-treated embryos (Figs. 3a–3d). Ganglioside 9-*O*-acetyl GD3 was detected using mAb ME311 in a small population of peripheral brain cells (possibly astrocytes) in control, but not PDMP-treated embryos (Figs. 3e–3h). Similarly, gangliosides GT3/GQ1C were detected using mAb A2B5 on neurons throughout the central nervous system and neural tube in control, but not PDMP-treated embryos (Figs. 3i–3l). PDMP-treated embryos were labeled with some mAbs, most notably VIN-IS-56 directed to GD2 and FE-A5 directed to *N*-acetyllactosamine; however, this labeling may reflect the presence of shared carbohydrate chains on glycoproteins or (as in

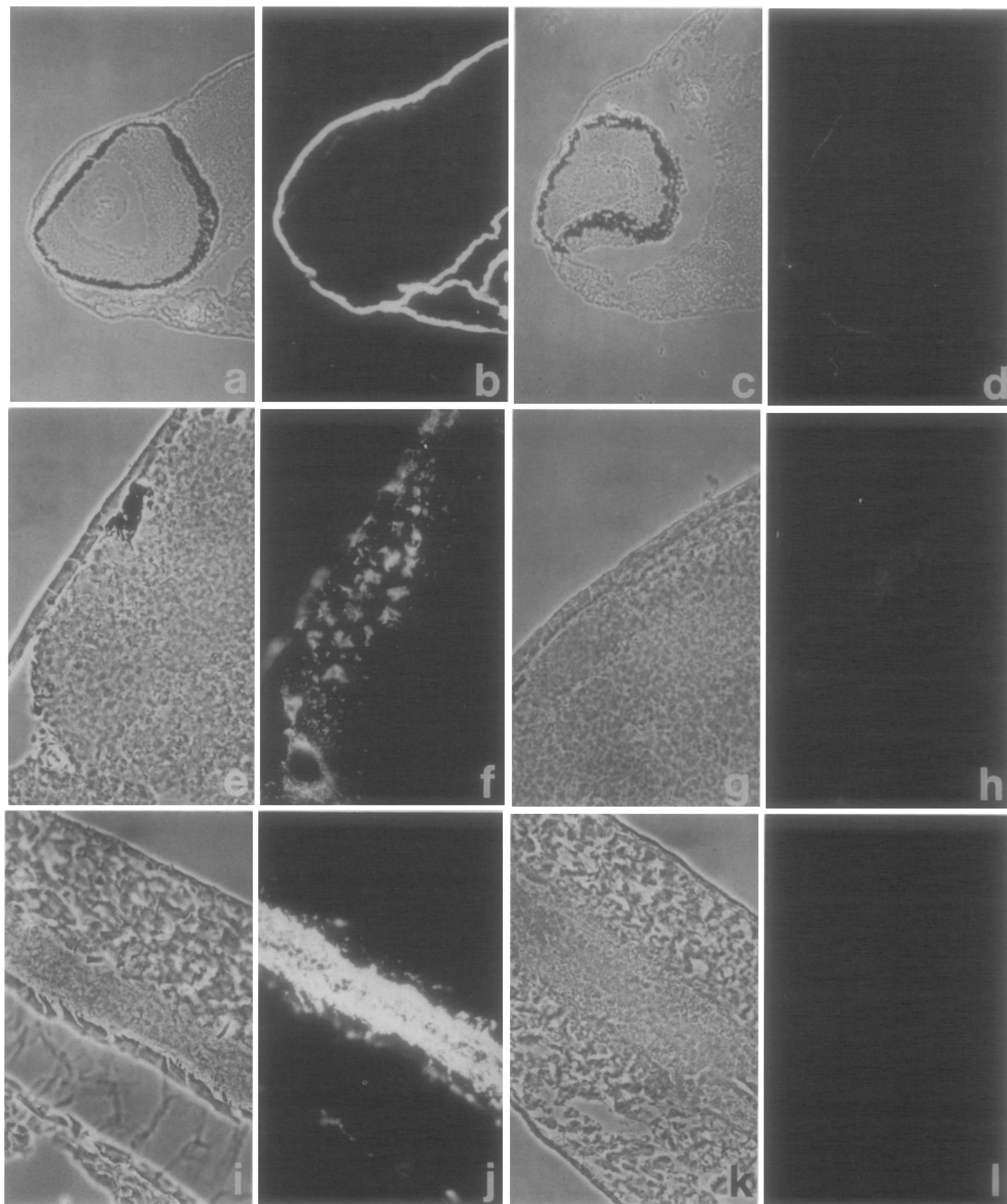

FIG. 3. Effect of PDMP on glycolipid antigen expression. Embryos were cultured in the absence (Control) or presence (PDMP) of 20 μM D-threo-PDMP. On the day of hatching (Day 10), fry were transferred to embedding compound and frozen on dry ice. Sections were labeled with mAb VESP 6.2 specific for sulfated glycolipids (a–d), mAb ME311 specific for 9-*O*-acetyl GD3 (e–h), or mAb A2B5 specific for polylsialylated glycolipids (i–l). Photographs were taken using phase (a, c, e, g, i, k) or fluorescence (b, d, f, h, j, l) microscopy. Magnification ×160 (a–d) or ×400 (e–l).

the case of GD2) may reflect compensatory synthesis of a glycolipid structure on a glycoprotein.

Effects of Glycolipid Depletion on Embryonic Development

Embryos cultured in the absence or presence of 5–40 μM PDMP appeared to be morphologically identical at each stage of development. The time course of hatching in control and PDMP-treated groups at three incubation temperatures was also similar (Fig. 4). After hatching, fry were transferred to 60-mm dishes and maintained in the absence of PDMP for up to 2 weeks. Fry that had undergone embryonic development in the presence of 20 μM PDMP exhibited normal patterns of behavior, including their ability to swim, feed (both flake and live food), respond to bright light, and avoid obstacles. Thus, PDMP-treated embryos appear to have acquired normal neurological and visual functions. PDMP was nonspecifically toxic to embryos at concentrations above 80 μM.

DISCUSSION

The results indicate that medaka embryos can complete normal development despite a significant reduction in cell surface glycolipid. Most notable was the loss of several gangliosides from the nervous system and the loss of sulfated glycolipid from the skin and gut (Fig. 3). These results are surprising because gangliosides such as GD3, GT3, and 9-O-acetyl GD3 are characteristic markers of neuronal tissues [3, 16] and are believed to play an essential role in embryonic development [19].

One hypothesis regarding our data is that the small amounts of glycolipid that remained after PDMP treatment (Figs. 2 and 3) were sufficient to maintain the essential functions of glycolipids in transmembrane signaling and cell interaction. If so, medaka embryos synthesize more glycolipid than needed for development. In this connection, mutant strains of yeast have been isolated that survive without sphingolipids, albeit with a slower growth rate [2]. A second hypothesis is that the adaptive value of membrane glycolipids is not appropriately tested *in vitro*. For example, glycolipids may serve to protect cells in natural environments from pathogenic agents, such as viruses, bacteria, and/or extremes of temperature; however, in our experiments, the time course of hatching in control and PDMP-treated cultures was identical at both high and low temperatures (Fig. 4). A third hypothesis is that embryos employ multiple systems to regulate growth, morphogenesis, and differentiation. The functions of glycolipid-oligosaccharides may be shared in large measure by the oligosaccharide chains of cell surface glycoproteins and proteoglycans. Indeed, protein-bound glycans such as high-molecular weight lactosaminoglycans may play a more important role in mediating cell interactions during early development (reviewed by Fenderson *et al.* [8]).

In summary, PDMP appears to provide a useful tool for studying the glycolipids of cells during development and differentiation. For example, PDMP may allow the identification of carrier molecules for novel carbohydrate differentiation antigens: carbohydrate antigens found exclusively on glycolipids will be lost from cells or embryos following PDMP-treatment, whereas carbohydrate antigens found exclusively on glycoproteins will remain unaffected. Finally, the results presented here identify several glycolipids as neuroectodermal makers that should prove useful for future studies of lineage formation in medaka embryos.

The authors thank Jin-ichi Inokuchi (Fukuoka University, Japan) for kindly providing D-threo-PDMP, William Kresge for expert assistance with egg collection, and Peter Andrews and Brunhilde Felding-Haberman for many helpful suggestions. mAb VESP 6.2 was kindly provided by Klaus Petry (Laboratoire d'Immunologie et de Biologie

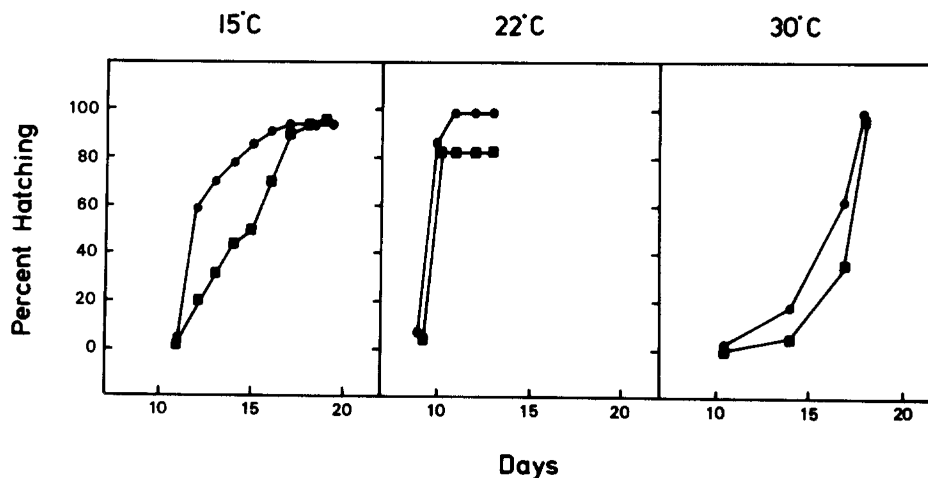

FIG. 4. Effect of PDMP on hatching time. Embryos were cultured in 96-well microtiter plates at 15°C (n = 36 in each group), 22°C (n = 15 in each group), or 30°C (n = 34 in each group). The time course of hatching in control (■) and 20 μM PDMP-treated groups (●) is similar.

Parasitaire, Universite de Bordeaux II, France); mAb ME311 was kindly provided by Jan Thurin (The Wistar Institute, Philadelphia); mAb VIN-IS-56 was kindly provided by Peter Andrews (The Wistar Institute, Philadelphia). This work was supported by NIH Grants CA54590 (G.O.), NS03192 (N.R.), GM23100 (S.H.) and by funds from The Biomembrane Institute (B.F.).

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