Short Communication

Mannosylerythritol lipid increases levels of galactoceramide in and neurite outgrowth from PC12 pheochromocytoma cells

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Abstract

We report here that a microbial extracellular glycolipid, mannosylerythritol lipid (MEL), induces the outgrowth of neurites from and enhances the activity of acetylcholinesterase (AChE) in PC12 pheochromocytoma cells. Furthermore, treatment of PC12 cells with MEL increased levels of galactosylceramide (Gal β 1-1'Cer; GalCer). Exposure of PC12 cells to exogenous GalCer caused the dose-dependent outgrowth of neurites. By contrast, treatment of PC12 cells with nerve growth factor (NGF) did not increase the level of GalCer in the cells. The neurite-related morphological changes induced by GalCer differend from those induced by NGF, indicating differences between the signal transduction pathways triggered by NGF and by GalCer.

Abbreviations: DMEM, Dulbecco modified Eagle's medium; FBS, fetal bovine serum; GSLs, glycosphingolipids; MEL, mannosylerythritol lipid; PBS, phosphate-buffered saline; GalCer, galactosylceramide; NGF, nerve growth factor; AChE, acetylcholinesterase; HPTLC, high performance thin-layer chromatography

Introduction

Glycosphingolipids (GSLs) are components of the outer leaflet of the plasma membrane of eukaryotic cells and play a critical role in cellular interactions and in the control of cell proliferation (Dahms and Schnaar, 1983). Exogenously administered GSLs, including gangliosides, can accelerate the differentiation of primary neurons and neuroblastoma cells *in vitro*, inducing sprouting and extension of neurites (Dahms and Schnaar, 1983). Transfection of neuroblastoma cells with a cDNA that encoded ganglioside GD3 (Neu5Ac α 2-8Neu5Ac α 2-3Gal β 1-1'Cer) synthase induced cholinergic differentiation and sprouting of neurites (Nagai and Tsuji, 1988). These observations suggest that gangliosides might modulate signaling pathways of neuronal differentiation. However, the

molecular mechanisms of induction of differentiation by gangliosides remain unknown.

Mannosylerythritol lipid [MEL; 4-O-(di-O-acetyl-di-O-alkanoyl- β -D-mannopyranosyl)-erythritol], is a microbial extracellular glycolipid produced by *Candida antarctica* T-34 (Kitamoto et al., 1990). We reported recently that MEL induces the differentiation of HL-60 cells (Isoda et al., 1997) and melanoma B16 cells (Zhao et al., 1999). It is noteworthy that MEL, a microbial glycolipid can induce the differentiation of vertebrate cells. In the present study, we found that MEL induced the outgrowth of neurite from and the activation of AChE in PC12 cells. PC12 cells are derived from a rat pheochromocytoma and differentiate into neuron-like cells when treated with nerve growth factor (NGF). Exposure of PC12 cells to MEL increased intracellular levels of galactosylceramide

Figure 1. Effects of NGF and MEL on AChE activity in PC12 cells. PC12 cells were cultured in the absence (control) and in the presence of NGF (40 ng/ml) or/and 5 μ M MEL for 48 h. All values are averages of results of five experiments and the standard deviation for each value is indicated.

(Gal β -1'Cer; GalCer) but NGF did not. Thus, the mechanism of signal transduction triggered by MEL must be different from that by NGF. Furthermore, exogenously added GalCer caused the dose-dependent outgrowth of neurites. This result implies that GalCer can assume a functional role in the differentiation of PC12 cells.

Materials and methods

Culture and treatment of cells

PC12 cells were obtained from the Riken Cell Bank (Tsukuba, Ibaraki, Japan) and grown routinely in DMEM supplemented with 5% fetal bovine serum (FBS), 10% horse serum, 100 μ g/ml streptomycin and 100 units/ml penicillin in collagen-coated tissue culture dishes at 37 °C in 5% CO₂ in air in a humidified incubator. MEL and NGF (Gibco BRL, Rockville, MD, U.S.A.) were dissolved in PBS and GalCer (Wako Pure Chemical Co., Tokyo, Japan) was dissolved in ethanol. Compounds were administered to cells at the indicated concentrations after growth of PC12 cells had been synchronized by incubation overnight in DMEM that contained 0.5% FBS.

Induction of differentiation of PC12 cells

PC12 cells were incubated with 40 ng/ml NGF or 5 μ M MEL for 48 h or with GalCer for 9 h. The induction of cellular differentiation was assessed in terms of neurite formation and AChE activity, which are specific markers of the differentiation of PC12 cells. Cells with one or more neurites with a length greater than 1.5 times of the diameter of the cell body were scored as neurite-positive.

Assay of acetylcholinesterase activity

PC12 cells were washed three times with ice-cold PBS (pH 7.2), suspended into 1 ml of 10 mM phosphate buffer (pH 7.0) and lysed by sonication. AChE activity was measured colorimetrically with acetylthiocholine as substrate (Ellman et al., 1961). AChE activity was expressed as nmol of thiocholine released per min per mg protein.

Analysis of glycosphingolipids

GSLs in cell membranes during the differentiation of PC12 cells were analyzed by HPTLC. GSLs were

Figure 2. Morphological changes in PC12 cells induced by addition of GalCer to the culture medium. PC12 cells were cultured in medium without (A) or with (B) GalCer ($6 \mu\text{M}$) for 48 h. Then each culture was photographed ($\times 100$).

extracted and analyzed by a modified version of previously described method (Tsuruoka et al., 1993). Cells (1×10^7) were washed twice with PBS and GSLs were extracted by sonication with 0.5 ml of a mix-

ture of isopropyl alcohol, hexane and water (55:25:20, v/v/v). The cell pellet was extracted twice and then re-extracted three times with 0.5 ml of a mixture of chloroform and methanol (2:1 v/v). All extracts were

Figure 3. Effects of GalCer on the outgrowth of neurites from PC12 cells. The percentages of neurite-positive cells were calculated as described in the Section Materials and Methods. The total number of cells was taken as 100% at each concentration of GalCer.

combined and evaporated to dryness under a stream of N_2 gas. Each sample was dissolved in 0.5 ml of 0.1 M KCl, passed through a Seppak plus C18 Cartridge (Millipore Corporation, Bedford, MA, U.S.A). This cartridge was washed with 2 ml of 0.1 M KCl and then with 5 ml of distilled water and it was eluted with 2 ml of the mixture of chloroform and methanol (2:1 v/v). The final eluate was evaporated to dryness and the residue was dissolved in 200 μ l of a mixture of chloroform, methanol and water (40:20:1, v/v). The sample was then analyzed on HPTLC plates (HPTLC-Fertigplatten, Kieselgel 60; Merck AG, Darmstadt, Germany) and developed with a mixture of chloroform, methanol and water (50:40:10 v/v) for analysis of gangliosides or chloroform, methanol and water (65:25:4 v/v) for analysis of neutral GSLs. The GSLs were detected by spraying plates with 0.2% orcinol in 2 M sulfuric acid.

Results and discussion

Nerve growth factor inhibits the growth of PC12 cells and promotes the extension of neurites. This study,

treatment of PC12 with 40 ng/ml elicited outgrowth of neurites, as reported previously (Greene and Tischler, 1976). Addition of 5 μ M MEL to the culture medium also resulted in morphological changes and extension of neurites from PC12 cells (data not shown). Treatment of PC12 cells with 40 ng/ml NGF increased the activity of the transmitter-related enzyme AChE (Figure 1). The activity of AChE in PC12 cells after treatment with 5 μ M MEL for 48 h was similar to that after treatment with NGF for the same time. The morphology of the cell extensions and the increase in AChE activity confirmed that incubation of PC12 cells with MEL promoted the outgrowth of neurites. Treatment of PC12 cells with NGF and MEL together had no synergistic effects on the induction of AChE activity (Figure 1).

The structure of MEL, a microbial glycolipid, differs from that of mammalian glycolipids in terms of specific residues but the backbones are similar. Therefore, we postulated that the biosynthetic pathway to membrane glycosphingolipids might be a possible target for MEL. Analysis of the GSLs in PC12 cells by HPTLC revealed that treatment with MEL for 48 h increased the level of a specific GSL, GalCer, but did

not affect the levels of other gangliosides. By contrast, treatment with NGF led to increases in levels of GM1, GD1a, GD1b and GT1b but no change on the level of GalCer.

We next examined the effects of exogenous GalCer on PC12 cells. Addition of GalCer to the culture medium resulted in the obvious extension of neurites from PC12 cells (Figure 2). Furthermore, in PC12 cells treated with GalCer, the outgrowth of neurites was dose-dependent (Figure 3) at concentrations of GalCer below 30 μ M. It is difficult to dissolve GalCer at concentrations much above 30 μ M so the effects of GalCer at higher concentrations could not be analyzed. Our results do, however, provide evidence that GalCer can induce the differentiation of PC12 cells.

We examined whether or not the extension of neurites induced by GalCer was similar to that induced by NGF. We found the two phenomena are distinct from each other. For example, GalCer induced the appearance of thin fibriform neurites, weak adherence of cells to dishes and almost no change in cell shape. By contrast, NGF induced filament-like long neurites and strong adherence of cells to dishes. These observations hint at differences between pathways of signal transduction in PC12 cells triggered by GalCer and by NGF. Since MEL, a microbial extracellular glycolipid, is not known as a precursor of mammalian GalCer, it is possible that the increase in levels of GalCer might result from stimulation of the biosynthesis of GalCer from ceramide or inhibition of the formation of GM4 (Neu5Ac α 2-3Gal β 1-4Glc β 1-1'Cer) from GalCer. Further study of the molecular mechanism of GalCer-mediated differentiation of PC12 cells might lead to an understanding of the signal pathways triggered by glycolipids in animal cells.

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