Treatment of mouse melanoma cells with phorbol 12-myristate 13-acetate counteracts mannosylerythritol lipid-induced growth arrest and apoptosis

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Abstract

Mannosylerythritol lipid (MEL), an extracellular glycolipid from yeast, induces the differentiation of HL-60 promyelocytic leukemia cells towards granulocytes. We show here that MEL is also a potent inhibitor of the proliferation of mouse melanoma B16 cells. Flow-cytometric analysis of the cell cycle of MEL-treated B16 cells revealed the accumulation of cells in the sub-G₀/G₁ phase, which is a hallmark of cells undergoing apoptosis. Treatment of B16 cells for 24 h with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), did not interfere with the growth and survival of the cells, but it effectively counteracted the MEL-induced growth arrest and apoptosis. The activity of PKC was reduced in B16 cells treated with MEL at a concentration at which MEL induced apoptosis. However, incubation with PMA in addition to MEL reversed this reduction in the activity of PKC. These results suggest that converging signaling pathways are triggered independently by MEL and PMA and that the signals might both be mediated by PKC.

Abbreviations: DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GSL, glycosphingolipid; ITES, insulin, iron-free transferrin, ethanolamine and selenite; MEL, mannosylerythritol lipid; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; PI, propidium iodide.

Introduction

Mannosylerythritol lipid (MEL) is an extracellular glycolipid that is produced in large amounts by *Candida antarctica* T-34 when this yeast is grown with myristic acid as a source of carbon. The hydrophilic moiety of MEL has been identified as 4-O-(di-O-acetyl-di-O-alkanoyl- β -D-mannopyranosyl)-erythritol (Kitamoto et al., 1990). Microbial glycolipids differ from mammalian glycolipids in terms of specific side chains, but their backbones are similar. Ubiquitous components of the plasma membranes of mammalian cells, such as gangliosides and glycosphingolipids (GSLs), have been shown to modulate cell growth, cell

adhesion, and transmembrane signaling. Moreover, changes in the composition and metabolism of GSLs have been observed during oncogenesis, differentiation and oncogenic transformation (Hannun and Bell, 1989; Hakomori and Igarashi, 1992). The induction of the differentiation of HL-60 promyelocytic leukemia cells towards granulocytes by exposure of MEL has also been reported (Isoda et al., 1997).

Apoptosis is considered to be the major process responsible for the death of cells that is induced by physiological events such as the end of the life span of differentiated cells (Applady and Modak, 1977). Apoptosis is also thought to play a key role in oncogenesis since disrupted regulation of the apoptotic

process can result in the development of malignant tumors (Carson and Ribeiro, 1993). Among such tumors, melanoma is known to be particularly resistant to chemotherapeutic agents (Koh, 1991). In numerous systems, members of the protein kinase C (PKC) family, which consists of close to a dozen serine/threonine kinases, are key factors in cell differentiation, the control of growth, the promotion of tumors and cell death. (Livneh and Fishman, 1997). For example, PKC α plays a crucial role in the retinoic acid-induced differentiation of B16 melanoma cells (Gruber et al., 1992). However, the role of PKC in apoptosis in melanoma cells remains to be elucidated.

In the present study, we found that exposure of cultured melanoma B16 cells to MEL resulted in growth arrest and apoptosis. Simultaneous exposure to phorbol 12-myristate 13-acetate (PMA) counteracted both the MEL-induced growth arrest and apoptosis. These findings suggest that it might be pertinent to examine the possible use of MEL, a yeast extracellular glycolipid, as a novel therapeutic agent for the treatment of melanoma. They also suggest a role for PKC in the signaling pathway that is triggered in mouse melanoma cells by MEL.

Materials and methods

Preparation of MEL

MEL was prepared and purified as described elsewhere (Kitamoto et al., 1990).

Culture and treatment of cells

Mouse melanoma B16 4A5 cells (referred to hereinafter as B16 cells), obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan), were maintained in culture in DMEM supplemented with 10% FBS at 37 °C. For treatment with MEL and PMA (Sigma Chemical Co., St. Louis, MO, U.S.A.), cells were cultured in serum-free DMEM-ITES medium, in which ITES consisted of insulin (5 μ g/ml), iron-free human transferrin (10 μ g/ml), ethanolamine (25 μ M) and selenite (25 nM; RD-1; Kyokuto Pharmaceutical Kogyo Co., Tokyo, Japan). MEL and PMA were dissolved in distilled water and in ethanol, respectively, and were administered to cells at the indicated concentrations, in the presence or absence of the inhibitor of PKC, GF109203, at 0.2 μ M. A cell-counting kit (WST-1; Dojin Laboratories, Kumamoto, Japan) was used to monitor the number of viable cells.

Flow-cytometric analysis of apoptotic cells

B16 cells were cultured in 100-mm tissue culture dishes (2.4×10^4 cells/cm²) in DMEM supplemented with 10% FBS for about 8 h. Then they were washed once in PBS and cultured for one further day in DMEM-ITES that contained MEL or PMA at the indicated concentrations. Aliquots of 1×10^6 cells were then washed with PBS, fixed in 70% ethanol, resuspended in 1 ml of PBS and stained with PI (50 μ g/ml) for 30 min at room temperature. Flow-cytometric analysis of a minimum of 10 000 cells was performed with a FACSort flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Data were analyzed with LYSIS II software (Becton Dickinson).

Detection of apoptotic cells by fluorescence microscopy

Untreated B16 cells or cells treated with 10 μ M MEL with or without 10 nM PMA and 0.2 μ M GF109203X (an inhibitor of PKC; CalBiochem. Novabiochem Intl., La Jolla, CA, U.S.A.) for 24 h were collected and washed with PBS. Cells were fixed in 37% formaldehyde for 4 h. Then they were pelleted by centrifugation and resuspended in PBS. Cells in 10 μ l of each suspension of cells were double-stained with 2 μ l of 100 μ M Hoechst 33342 (Sigma Chemical Co.) and 1 μ l of a solution of PI (1 mg/ml). Finally cells were examined with a fluorescence microscope (Olympus Inc., Tokyo, Japan).

Assay of protein kinase C in vitro

Preparation of cell lysates and immunoprecipitation with PKC-specific antibodies were performed as described elsewhere (Isoda et al., 1997). The activity of PKC was measured *in vitro* with a PKC kit (Upstate Biotechnology, Lake Placid, NY, U.S.A.). In brief, the immunoprecipitate obtained with PKC-specific antibodies was mixed, in a microcentrifuge tube, with 10 μ l of substrate cocktail (500 μ M substrate peptide for PKC), 10 μ l of a solution of an inhibitor of protein kinase A, 10 μ l of lipid activators (phosphatidyl serine and diglyceride) of PKC. The reaction was started by addition of 10 μ l of a solution of ATP that contained [γ -³²P]ATP at 30 °C. After incubation for 10 min, 25 μ l of the reaction mixture were removed and spotted on a disc of phosphocellulose paper (no. 81; Nihon Millipore Ltd., Tokyo, Japan). After the paper had been washed several times with

Figure 1. MEL inhibits the growth of B16 cells. Cells were seeded in 96-well microplates at an initial concentration of 1.2×10^5 cells/ml and grown in DMEM-ITES medium without or with $10 \mu\text{M}$ MEL. Numbers of viable cells were counted at 24-h intervals. The means and standard deviations (where appropriate) of five measurements are shown.

0.75% phosphoric acid and acetone, radioactivity was measured in a liquid scintillation counter.

Results

MEL induces apoptosis of B16 cells

We studied the effects of MEL on the proliferation of B16 cells by adding MEL directly to cells in culture. Treatment of B16 cells with $10 \mu\text{M}$ MEL resulted in a significant reduction in the number of viable cells within 48 h (Figure 1). At concentrations below $10 \mu\text{M}$, MEL had dose- and time-dependent inhibitory effects on the proliferation of B16 cells (data not shown). More than 30% of cells were stained with trypan blue after exposure to $10 \mu\text{M}$ MEL for one day. The toxicity of MEL became more apparent after a two-day incubation, with the number of viable cells decreasing approximately 10- to 20-fold.

We next examined whether the MEL-induced inhibition of proliferation and the reduction in cell viability were associated with apoptotic cell death. B16 cells that had been exposed to $10 \mu\text{M}$ MEL for 24 h were analyzed by flow cytometry since cells in the sub- G_0/G_1 peak are recognized as cells undergoing

apoptosis (Dive et al., 1992; Darzynkiewicz et al., 1992). The DNA histogram in Figure 2A (upper right panel) shows a prominent sub- G_0/G_1 peak in the case of cells treated with MEL. No such peak was observed in the case of untreated cells (upper left panel). Quantitative analysis revealed a significant increase in the relative number of apoptotic cells from less than 10% to almost 30% after exposure to MEL (Figure 2B). Treatment with MEL also caused fragmentation of DNA and the condensation of chromatin (Zhao et al., 1999), which confirmed the apoptosis-inducing potential of MEL in B16 melanoma cells. Treatment of B16 cells with $10 \mu\text{M}$ MEL for 48 h increased the percentage of apoptotic cells to more than 50% (data not shown). Flow-cytometric analysis revealed that treatment with PMA alone did not alter the relative number of apoptotic cells as compared with that in the population of untreated cells (Figure 2). Exposure to PMA (10 nM) and MEL ($10 \mu\text{M}$) together markedly decreased the relative number of apoptotic cells as compared with the effect of MEL alone.

Phorbol 12-myristate 13-acetate counteracts the antiproliferative and apoptotic effects of MEL

The retinoic acid-induced differentiation of B16 cells can be blocked by prolonged treatment of the cells with PMA, a result that suggests that PKC might play a key role in the differentiation of melanoma cells (Gruber et al., 1992). This finding, together with our recent observation that MEL induces the differentiation of HL-60 promyelocytic leukemia cells (Isoda et al., 1997), prompted us to examine the effects of treatment of B16 cells with PMA, either alone or in combination with MEL. Exposure to PMA at various concentrations (1 to 100 nM) over a two-day period had no obvious effects on the proliferation of B16 cells (Figure 3A). However, treatment with PMA and MEL together increased the viability of B16 cells as compared with that of cells treated with MEL alone (Figure 3B). Although the protective effect of PMA was most apparent at concentrations of MEL below $10 \mu\text{M}$, the viability of cells treated with $10 \mu\text{M}$ MEL was significantly enhanced by simultaneous exposure to 10 nM PMA (Figure 3B).

The activity of PKC reflects the antiproliferative and apoptotic effects of MEL and the extent of the modification of these effects by PMA

The protective effects of PMA against the MEL-triggered cessation of growth and apoptosis suggest

Figure 2. Treatment of B16 cells with MEL enhances apoptosis and the effect of MEL is antagonized by PMA. (A) Representative histograms showing results of flow-cytometric analysis of B16 cells that had been treated for 24 h without or with 10 μ M MEL, 10 nM PMA, or both. Axes represent fluorescence from propidium iodide (abscissa) and the number of events (ordinate). The sub- G_0/G_1 peak (indicated as 'Ap' in the histograms) represents the population of apoptotic cells. (B) Quantification of the results shown in (A). The means and standard deviations of the results of three experiments are shown.

Figure 3. Effects of PMA on the growth of B16 cells or MEL-treated B16 cells. (A) Effects of PMA on the growth of B16 cells. Cells were seeded in 96-well microplates at an initial concentration of 1×10^5 cells/ml and grown in DMEM-ITES medium without or with PMA at the indicated concentrations. Data points represent the means of results of three experiments. Standard deviations were less than 10%. (B) Effects of simultaneous treatment of B16 cells with MEL and PMA on cell growth. Numbers of viable cells were determined after 24 h. The numbers of viable cells are shown relative to the number of viable untreated cells. Data points represent the means of results of three experiments. Standard deviations were less than 10%.

Table 1. The activity of PKC and the MEL-induced apoptosis of B16 cells

Sample	PKC activity (pmol phosphate incorporated into substrate min/mg protein in cell lysate)	Percentage of apoptotic cells (%)	
		a*	b*
B16	138	7	6.2
B16/MEL	116	30	36.8
B16/PMA	157	7.5	5.9
B16/MEL/PMA	140	17	22.7
B16/MEL/PMA/GF109203X	112	33	34.3

B16 cells were treated with 10 μ M MEL and/or 10 nM PMA in the presence or absence of 0.2 μ M GF109203X for 24 h, as indicated. Cells were collected and the activity of PKC was determined as described in Materials and Methods. Data are means of results of three separate analyses. a*: Data are means of results of three independent flow-cytometric analyses. b*: Data obtained by counting cells stained with both Hoechst 33342 and PI. All the data are average means of three separate experiments.

a role for PKC in the signal transduction pathway in B16 cells that follows exposure to MEL. Therefore, we examined the activity of PKC in B16 cells. Several isoforms of PKC have been implicated in the apoptotic responses of cells to a variety of stimuli that induce programmed cell death (Newton et al., 1995; Emoto et al., 1996; Shao et al., 1997). As shown in Table 1, exposure of B16 cells to 10 μ M MEL for 24 h resulted in a reduction in the activity of PKC. However, treatment of B16 cells with PMA and MEL together reversed the negative effects of MEL on the activity of PKC. This result was consistent with the protection by PMA of B16 cells from apoptosis. Moreover, treatment with PMA and MEL together in the presence of GF109203X, an inhibitor of PKC, abolish the protection by PMA against MEL-induced apoptosis. These results suggest that regulation of the activity of PKC might be closely related to the regulation of apoptosis in this system.

Discussion

Mannosylerythritol lipid (MEL) from yeast inhibited the proliferation of mouse melanoma B16 cells and triggered apoptosis (Figures 1 and 2). Furthermore, B16 cells were protected from MEL-induced growth arrest and apoptosis by PMA. These results suggest that treatment with MEL stimulates pathways that lead to, or are at least related to, the regulation of PKC activity in B16 cells.

Activation of PKC is one of the earliest events in the signal transduction cascades that lead to a variety of cellular responses. However, there are contradict-

ory reports about the role of PKC in apoptosis. For example, treatment with a phorbol ester protects cells from apoptosis induced by TNF α (Obeid et al., 1993; Jarvis et al., 1994), by chemotherapeutic agents (Grant et al., 1992), by withdrawal of growth factors (Lotem et al., 1991) and by irradiation (Haimovitz-Friedman et al., 1994). By contrast, phorbol esters have been shown to induce apoptosis in several types of cell via activation of PKC (Young et al., 1994; Day et al., 1994; Kizaki et al., 1989; De Vente et al., 1995; Garzotto et al., 1998). We measured the enzymatic activity of PKC in B16 cells after treatment with MEL and after treatment with both MEL and PMA together (Table 1). We found that the activity of PKC in B16 cells that had been reduced by treatment with MEL was enhanced when PMA was applied simultaneously with MEL to B16 cells (Table 1). In the presence of GF109203, an inhibitor of PKC, the protective effects of PMA on the activity of PKC and against apoptosis induced by treatment with MEL were lost. These data suggest that regulation of the activity of PKC might be associated with apoptosis that is induced by MEL in B16 cells. The molecular mechanisms of the effects of MEL remain to be determined. Since MEL is a glycolipid biosurfactant, the mode of action of MEL might involve qualitative and/or quantitative changes in membrane-associated moleculars. Changes in the composition of glycosphingolipids during the MEL-induced differentiation of HL-60 cells have been described (Isoda et al., 1997). Activation of PKC and its subsequent translocation to the nucleus can be triggered by growth factors via the generation of diacylglycerol (DAG), a membrane-bound second messenger (Nishizuka, 1992). Furthermore, PKC reg-

ulates the level of nuclear DAG by inhibiting entry into the nucleus of DAG kinase- ζ , the overexpression of which has a profound growth-inhibitory effect (Topham et al., 1998). It remains to be determined whether the MEL-induced inhibition of growth and MEL-induced apoptosis are linked directly to the DAG/PKC pathway and whether PMA affects the signaling cascade of this pathway directly.

In conclusion, we have shown that MEL, a microbial extracellular glycolipids, is a novel mediator of the growth arrest and apoptosis of melanoma cells. Since melanomas are known as being resistant to chemotherapeutic intervention, our findings suggest that it might be useful to pursue the development of novel therapeutic reagents based on MEL. Further studies of the molecular mechanisms involved in the responses to MEL should be the first steps in this direction.

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References

- Appleby DW and Modak SP (1997) DNA degradation in terminally differentiating lens fiber cells from chick embryos. *Proc Natl Acad Sci USA* 74: 5579–5583.
- Carson DA and Ribeiro JM (1993) Apoptosis and disease. *Lancet* 341: 1251–1254.
- Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P and Traganos F (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* 13: 795–808.
- Day ML, Zhao X, Wu S, Swanson PE and Humphrey PA (1994) Phorbol ester-induced apoptosis is accompanied by NGFI-A and *c-fos* activation in androgen-sensitive prostate cancer cells. *Cell Growth Differ* 5: 735–741.
- De Vente JE, Kukoly CA, Bryant WO, Posekany KJ, Chen J, Fletcher DJ, Parker PJ, Pettit GJ, Lozano G, Cook PP and Ways DK (1995) Phorbol esters induce death in MCF-7 breast cancer cells with altered expression of protein kinase C isoforms: Role of p53-independent induction of gadd-45 in initiating death. *J Clin Invest* 96: 1874–1886.
- Dive C, Gregory CD, Phipps DJ, Evans DL, Melner AE and Wyllie AH (1992) Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochem Biophys Acta* 1133: 275–285.
- Emoto Y, Kisaki H, Manome Y, Kharbanda S and Kufe D (1996) Activation of protein kinase C delta in human myeloid leukemia cells treated with 1-beta-D-arabinofuranosylcytosine. *Blood* 87: 1990–1996.
- Garzotto M, White-Jones M, Jiang Y, Ehleiter D, Liao WC, Haimovitz-Friedman A, Fuks Z and Kolesnick R (1998) 12-O-Tetradecanoylphorbol-13-acetate-induced apoptosis in LNCaP cells is mediated through ceramide synthase. *Cancer Res* 58: 2260–2264.
- Grant S, Jarvis WD, Swerdlow P, Turner A, Traylor R, Wallace H, Lin P, Pettit G and Gewirtz DA (1992) Potentiation of the activity of 1-beta-D-arabinofuranosylcytosine by the protein kinase C activator bryostatin 1 in HL-60 cells: association with enhanced fragmentation of mature DNA. *Cancer Res* 52: 6270–6278.
- Gruber J R, Ohno S and Niles RM (1992) Increased expression of protein kinase C α plays a key role in retinoic acid-induced melanoma differentiation. *J Biol Chem* 267: 13356–13360.
- Haimovitz-Friedman FA, Balaban N, McLoughlin M, Ehleiter D, Micheali J, Vlodavsky I and Fuks Z (1994) Protein kinase C mediates basic fibroblast growth factor protection of endothelial cells against radiation-induced apoptosis. *Cancer Res* 54: 2591–2597.
- Hakomori S (1981) Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *Annu Rev Biochem* 50: 733–764.
- Hakomori S and Igarashi Y (1992) Gangliosides and glycosphingolipids as modulators of cell growth, adhesion and transmembrane signaling. *Adv Lipid Res* 25: 147–162.
- Hannun YA and Bell RM (1989) Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* 243: 500–507.
- Isoda H, Shinmoto H, Kitamoto D, Matsumura M and Nakahara T (1997) Differentiation of human promyelocytic leukemia cell line HL-60 by microbial extracellular glycolipids. *Lipids* 32: 263–271.
- Jarvis WD, Kolesnick PN, Fornari FA, Traylor RS, Gewirtz DA and Grant S (1994) Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. *Proc Natl Acad Sci USA* 91: 73–77.
- Kitamoto D, Akiba S, Hioki T and Tabuchi T (1990) Extracellular accumulation of mannosylerythritol lipid by a strain of *Candida antarctica*. *Agric Biol Chem* 54: 31–36.
- Kizaki H, Tadakura T, Odaka C, Muramatsu J and Ishimura Y (1989) Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. *J Immunol* 143: 1790–1794.
- Koh HK (1991) Cutaneous melanoma. *N Engl J Med* 325: 171–182.
- Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258: 607–614.
- Livneh E and Fishman DD (1997) Linking protein kinase C to cell-cycle control. *Eur J Biochem* 248: 1–9.
- Lotem J, Cragoe EJ and Sachs L (1991) Rescue from programmed cell death in leukemic and normal myeloid cells. *Blood* 78: 953–960.
- Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* 270: 28495–28498.
- Obeid LM, Linardic CM, Karolak LK and Hannun YA (1993) Programmed cell death induced by ceramide. *Science* 259: 1769–1771.
- Topham MK, Bunting M, Zimmerman GA, McIntyre TM, Blackshear PJ and Prescott SM (1998) Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. *Nature* 394: 697–700.
- Young CY, Murtha PE and Zhang J (1994) Tumor-promoting phor-

bol ester-induced cell death and gene expression in a human prostate adenocarcinoma cell line. *Oncol Res* 6: 203–210.
Zhao XX, Wakamatsu Y, Shibahara M, Nomura N, Geltinger C, Na-

kahara T, Murata T and Yokoyama KK (1999) Mannosylerythritol lipid is a potent inducer of apoptosis and differentiation of mouse melanoma cells in culture. *Cancer Res* 59: 482–486.