Mannosylerythritol lipid (MEL), a novel extracellular glycolipid from yeast, was found to induce the proliferation of mouse melanoma B16 cells in a dose-dependent manner and to induce the apoptosis of B16 cells at concentrations higher than 10 μM (Zhao, X., Wakamatsu, Y., Shibahara, M., Nomura, N., Geltinger, C., Nakahara, T., Murata, T., and Yokoyama, K. K. (1999) Cancer Res. 59, 482-486). We show here that exposure of B16 cells to MEL (5 μM) for 2 days resulted in an increase in the levels of differentiation-associated markers of melanoma cells such as melanogenesis and tyrosinase activity, which were accompanied by morphological changes. The MEL-induced differentiation of B16 cells at this concentration was closely associated with arrest of the cell cycle at G1 phase, but no significant population of apoptotic cells was identified. Expression of protein kinase Ca (PKCa) was enhanced after exposure of B16 cells to MEL for 48 h. Antisense oligodeoxynucleotides against the mouse gene for PKCa prevented MEL-induced melanogenesis in B16 cells. Conversely, the effects of the expression of a constitutively active form of PKCa mimicked the effects of MEL on B16 cells. These data suggest that MEL, a yeast-derived glycolipid, triggers the differentiation of B16 melanoma cells through a signaling pathway that involves PKCa.

Abnormal cellular differentiation is a well established characteristic of tumor cells. A promising approach to the treatment of cancer involves the induction of the terminal differentiation and growth arrest of cancer cells. Because the discovery that polar organic compounds can induce the specific differentiation of erythroleukemia cells (1), a variety of reagents, including polar organic compounds, short-chain fatty acids, and retinoids, have been shown to induce features of differentiation in many lines of cancer cells, including melanoma cells (2-6). Our ability to manipulate the phenotypes of neoplastic cells with these reagents provides us with ways to unravel the mechanisms that underlie cellular differentiation, as well as to develop potentially useful therapeutic reagents (7). Melanoma is well known as a chemotherapy-resistant cancer (8), and it has been suggested that melanoma might be a suitable target for therapy with differentiation-inducing agents (9).

Mannosylerythritol lipid (MEL), a novel extracellular glycolipid produced by yeast, induces the granulocytic differentiation of HL-60 promyelocytic leukemia cells and alters the composition of cell surface glycosphingolipids (GSLs) (10). Gangliosides and GSLs, which are ubiquitous constituents of the plasma membrane of mammalian cells, play an important role in the modulation of cellular proliferation, oncogenesis and differentiation (11, 12). Cellular differentiation and oncogenic transformation are accompanied by dramatic changes in both absolute and relative levels of GSLs (13). Yeast-derived glycolipids differ from those derived from mammalian cells in terms of specific substituents, but their backbones are similar. We postulated that MEL might affect other tumor cells, in addition to inducing the cellular differentiation of HL-60 cells.

The molecular mechanisms responsible for the induction of the differentiation of melanoma cells are poorly understood. However, there is evidence that protein kinase C (PKC) might be associated with such differentiation. PKCs form a multigene family of serine- and threonine-specific kinases. They appear to play roles in a wide variety of cellular processes that include the functions of some membrane receptors, and the proliferation and differentiation of cells (14-16). PKC is expressed ubiquitously, while PKCβ is found only in some tissues, and PKCγ appears to be restricted almost exclusively to brain tissue (14). Such distribution suggests possible functional differences among these enzymes. PKC is associated with the differentiation that is induced by retinoic acid (RA), by α-melanocyte-stimulating hormone and by dehydroepiandrosterone in cell lines such as F9 teratocarcinoma cells and melanoma cells (17-19). For example, increased expression of PKCa plays a key role in the RA-triggered differentiation of melanoma cells (20, 21), while PKCβ appears to be critical for the regulation of melanogenesis in both human melanocytes and S91 mouse melanoma cells (17, 22). Thus, the roles of the isoforms of PKC appear to depend on the cell type. Specific activators and inhibitors of PKC have proved useful in attempts to analyze the effects of PKC on various transformation processes. However, such compounds do not discriminate between isozymes. Antisense oligodeoxynucleotides (AS-ODNs) and ribozymes are more suitable for studies of the roles of individual isoforms.

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The abbreviations used are: MEL, mannosylerythritol lipid; AS-ODN, antisense oligodeoxynucleotide; BrdUrd, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; GSL, glycosphingolipid; PKC, protein kinase C; FITC, fluorescein isothiocyanate; RA, retinoic acid; RIPA, radioimmune precipitation assay; S-ODN, sense oligodeoxynucleotide; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; PI, propidium iodide; dimethylsulfoxide, MeSO.
Mannosylerythritol Lipid-induced Differentiation

In this study, we evaluated the physiological effects of MEL on B16 melanoma cells and found that MEL induced cell cycle arrest and the differentiation of the cells in culture. We showed that PKCα was involved in this induced differentiation by using an AS-ODN that was specific to mouse PKCα, as well as by inducing the expression of a constitutively active form of PKCα in B16 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials—**Mouse melanoma B16 4A5 cells (referred to hereafter as B16 cells) were obtained from the Riken Cell Bank (Tsukuba, Ibaraki, Japan) and maintained in DMEM supplemented with 10% fetal bovine serum or in serum-free DMEM-ITES (DMEM plus insulin, transferrin, ethanalamine, and selenite) medium, as described elsewhere (23, 25). Cells were seeded at 5 × 10^4 cells/ml in a humidified atmosphere of 5% CO_2 at 37 °C. B16 cells were then incubated with polyclonal antibodies specific for PKCα, as well as by inducing the expression of a constitutively active form of PKCα in B16 cells.

**Measurements of Melanin Content and Tyrosinase Activity and the Flow Cytometric Analysis of Cultures of B16 Cells—**The methods used for measurements of melanin content and tyrosinase activity and the flow cytometric analysis of cultures of B16 cells have been described elsewhere (23, 25). One unit of tyrosinase activity was defined as the activity that caused an increase in absorbance at 280 nm of 0.001/min. Melanin content was measured at an absorbance of 490 nm, and the concentration of melanin was calibrated with synthetic melanin (Sigma Chemical Co.).

**Immunoblotting of PKC—**Untreated and MEL-treated B16 cells were lysed with ice-cold RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with phenylmethylsulfonyl fluoride (Sigma Chemical Co.; 25 μg/ml), aprotinin (Sigma Chemical Co.; 30 μg/ml) and sodium orthovanadate (Sigma Chemical Co.; 1 μg/ml) at 4 °C for 15 min. Cell lysates were collected and crude samples of protein were purified as described by Wessel et al. (26). Protein concentrations of samples were determined by the Bradford method using a method (Sigma Chemical Co.) as the standard (27). For Western blotting analysis of PKCs, equal amounts of samples were subjected to electrophoresis on a 7.5% polyacrylamide gel in the presence of SDS (SDS-PAGE) and transferred to a nitrocellulose membrane. Immunoblotting was performed with polyclonal antibodies specific to PKCα, PKCβ1, PKCβ2, PKCγ, PKCa, PKCb, and PKCd (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or polyclonal antibodies against PKCα (Promega Co., Madison, WI). The products of immunoreactions were detected with an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech).

**Immunoprecipitation and Assay of PKC Activity in Vitro—**After collection of cell lysates in RIPA buffer, samples were precleared with Protein A/G Plus-agarose beads (Santa Cruz Biotechnology Inc.) at 4 °C for 15 min. The beads were then washed three times with RIPA buffer and suspended in kinase assay buffer for measurement of PKC activity in vitro, using a PKC assay kit (Upstate Biotechnology, Lake Placid, NY). In brief, 10 μl of substrate mixture (500 μl PKC substrate peptide) were mixed with 10 μl of a solution of protein kinase A (PKA) inhibitor, 10 μl of a solution of PKC lipid activator (phosphatidylerine and diglyceride) and 10 μl of the prepared enzyme in a microcentrifuge tube. The reaction was started by the addition of 10 μl of a solution of ATP that contained [γ-32P]ATP (Amersham Pharmacia Biotech, specific activity 3000 Ci/mmol). The mixture was agitated gently and incubated at 30 °C for 10 min. The reaction was stopped by removal of 25 μl of the reaction mixture and placing it in the center of a piece of P81 phosphocellulose paper (Whatman). After 30 s, the paper was immersed in 0.75% phosphoric acid. After washing, the paper was soaked in acetone for 2 min and then transferred to a 5-ml scintillation vial. A scintillation mixture was added and the radioactivity on the paper was measured in a scintillation counter.

**Treatment of B16 Cells with Antisense Oligodeoxynucleotides Directly against PKCa mRNA—**B16 cells were cultured under normal conditions until they reached a 70–80% confluence. Then cells were rinsed with serum-free DMEM and incubated in a mixture of AS-ODN or S-ODN or control oligodeoxynucleotides (Tsukuba Research Laboratory, Toagosei Co., Tsukuba, Japan) and the LipofectAMINE™ reagent (Life Technologies, Inc.) in OPTI-MEMI reduced-serum medium reagent (Life Technologies, Inc.) for 4–5 h. The AS-ODN and the LipofectAMINE™ reagent were then removed, and cells were cultured in normal medium for the indicated times. Cells were used for immunoprecipitation, immunoblotting of PKC, and Northern blotting analysis.

**Northern Blotting Analysis—**Total RNA was isolated from target cells using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Then 25 μg of total RNA from each sample...
was fractionated on a 1% agarose gel that contained 2.2 M formaldehyde and bands of RNA were transferred to a Hybond-N filter (Amersham Pharmacia Biotech). For Northern blotting analysis, filters were incubated in Rapid-hyb buffer (Amersham Pharmacia Biotech) at 65 °C for 30 min before hybridization with a 32P-radiolabeled cDNA probes for mouse PKC isoforms or rat H9252-actin at 65 °C for 18 h. Filters were washed with 0.2X/1000 SSC plus 0.5% SDS for 10 min and then exposed to RX-film (Fuji Film; Tokyo, Japan) with an intensifying screen. Before rehybridization, filters were treated with a solution of boiling 0.5% SDS for 10 min.

Transfection of B16 cells with Plasmids—B16 cells were cultured under normal conditions to 70–80% confluence. Then cells were co-transfected with pcDNA3 (Invitrogen BV, Groningen, The Netherlands), which harbored a gene for neomycin resistance, and the pEF-HisB expression vector (Invitrogen BV) that encoded a His tag-fused constitutively active form of PKCα (28) by calcium precipitation, as described elsewhere (29). Control cells were transfected with pcDNA3 only. After transfection, cells were plated on medium that contained 500 µg/ml of G418 (Life Technologies, Inc.). Clones of resistant cells were selected and stable colonies were examined with monoclonal antibodies against the His tag (Qiagen GmbH, Hilden, Germany) for immunoprecipitation and polyclonal antibodies specific for PKCα for Western blotting. Selected cells were routinely grown in the presence of selective pressure from G418.

RESULTS

The MEL-induced Differentiation of B16 Cells—We investigated the effects of MEL on the differentiation induction of B16 cells by examining the effects of MEL on the production of melanin and tyrosinase activity, which are markers of the differentiation of melanoma cells (30, 31). Fig. 1A shows the

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**FIG. 2.** Flow-cytometric analysis of the cell cycle and apoptosis of B16 cells. Results are shown of the cell cycle analysis of 48-h cultures of untreated B16 cells (A) and 5 µM MEL-treated cells (B). Cells in the S phase were incubated with BrdU and then stained with FITC-conjugated BrdU-specific antibodies. Nuclei were counterstained with PI. **Abcissa**, PI staining for determination of DNA content; ordinate, fluorescence due to FITC. Frequency plots are shown as the number of nuclei in the various cell cycle compartments (G1 phase, bottom left; S phase, top; G2-M phase, bottom right). Panels show representative dot-plots; similar results were obtained in two independent experiments. Panels C and D are representative histograms showing the results of flow cytometric analysis of B16 cells that had been incubated without (C) or with 5 µM MEL (D) for 48 h. Axes represent the fluorescence due to PI (abscissa) and the number of events (ordinate). The sub-G1 peak (indicated as Ap in the histograms) represents the population of apoptotic cells.
The morphology of untreated B16 cells (Fig. 1C) was typical of that of mouse melanoma cells in culture. The cells resembled fibroblasts and were often clumped together. On the second day of cultivation, B16 cells exposed to 5 μM MEL extended dendrites and arranged themselves alongside one another (Fig. 1D), suggesting that cellular differentiation had occurred also at a morphological level.

**MEL Interrupts the Cell Cycle at the G1 Phase**—We next examined whether the MEL-induced differentiation of B16 cells might be accompanied by effects on the cell cycle. As shown in Fig. 2, A and B, flow cytometry revealed that populations of cells exposed to 5 μM MEL for 2 days showed a significant decrease in the relative number of cells in the S phase as compared with untreated cells (from 33.6 to 11.1%) and a marked increase in the relative number of cells in the G1 phase (from 57.0 to 80.8%), while there was little change in the cell population in the G2/M phase (from 6.2 to 5.9%). Thus, the effects of MEL on the differentiation-induction of B16 cells can be attributed, at least in part, to the induction of cell cycle arrest at the G1 phase. Cultures of B16 cells exposed to 5 μM MEL showed no significant increase in the relative number of apoptotic cells, as compared with untreated cells (see Fig. 2, C and D).

Enhanced Expression of PKCa in Response to MEL—As an intracellular calcium-dependent and phospholipid-dependent protein kinase, PKCα has been implicated as a key messenger in the cellular signaling associated with the proliferation and differentiation of cells (14–16). In an attempt to obtain insight into the molecular pathways that lead to cellular differentiation in response to MEL, we examined whether MEL might influence the expression of PKCα. We cultured cells in the presence and in the absence of MEL for the indicated periods of time and harvested them. Then we performed Western blotting analysis to compare relative levels of expression of PKCα. PKCα protein was almost undetectable in MEL-treated and in untreated B16 cells after cultivation for 3 h. After further cultivation, for up to 24 h, there was an increase in the expression of PKCα in both MEL-treated and untreated B16 cells and the increases were time-dependent. There was, moreover, no obvious difference between the levels of PKCα in the two types of culture. However, the expression of PKCα in untreated cells decreased markedly at 48 h, while MEL-treated cells continued to display high-level expression of PKCα (Fig. 3A). The level was ≈2.3-fold higher than that in untreated cells, as determined by densitometric scanning. The enzymatic activities of PKCα during the incubation of cells with MEL were also measured and have a good correlation with the expression levels of PKCα protein (Fig. 3B). Neither PKCβ, PKCγ, nor PKCη were detected in MEL-treated or in untreated cells and the levels of

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**Fig. 3.** Relative level of PKCa protein and phosphorylation activity of PKCa in B16 cells in response to MEL. **A,** Western blotting analysis of PKCα in B16 cells in response to MEL. 20 μg of protein extracts of B16 cells that had been treated without or with 5 μM MEL for the indicated times were fractionated by SDS-PAGE and then immunoblotted with monoclonal antibodies specific for mouse PKCa. **B,** enzymatic activity of PKCa was measured for the indicated times as described in “Experimental Procedures.” Values represent the means of results of three independent experiments. Bars show S.D.

**Fig. 4.** A simplified representation of the primary structure of the gene for PKCα. Each binding domain (BD), the targets of specific AS-ODNs and S-ODNs (arrows) are indicated. The nucleotide sequences of AS-ODNs and S-ODNs are shown in the lower panel.
PKCδ, PKCe, and PKCζ were not significantly altered (see Fig. 5D). These results suggest that PKCα-related pathways might possibly be involved in a MEL-triggered signal pathway in B16 cells.

**Antisense Oligodeoxynucleotides Directed against PKCα mRNA Counteract the MEL-induced Effects on B16 Cells—**To examine the possible relationship between the MEL-triggered differentiation of B16 cells and enhancement of the expression of PKCα, we introduced phosphorothioate AS-ODNs targeted to the mouse gene for PKCα into B16 cells to suppress the expression of PKCα. We used AS-ODNs with sequences that should be specific to the mRNA for PKCα. As shown in Fig. 4, we selected four AS-ODNs directed toward the mRNA for PKCα. To examine the effects of nonspecific suppression by AS-ODNs, we also used a random 23-mer ODN (N23) as a control. B16 cells were exposed to the various AS-ODNs (200 nM) during cultivation. In the case of the four AS-ODNs specific for PKCα, no. 3642 was the most effective in reducing the level of PKCα protein, and it reduced the level to at least 10-fold lower than that in B16 cells treated with N23 (200 nM) and that in untreated cells (Fig. 5A). The AS-ODNs against other target sequences in the mRNA for PKCα did not cause such a significant decrease in the level of PKCα itself. We also examined the effects of sense (S)-ODNs corresponding to nt 960–981 and nt 2636–2659 of PKCα mRNA and found no decrease in the levels of PKCα (Fig. 5B). We next examined the effects of AS-ODN on the level of PKCα mRNA in B16 cells by Northern blotting. Two transcripts of the gene for PKCα were identified with sizes of 10.8 and 3.8 kilobase. The level of PKCα mRNA was dramatically reduced in B16 cells that had been treated with AS-ODN no. 3642 (Fig. 5C). Incubation of B16 cells with S-ODN (no. 3846, 200 nM) had no detectable effect on the level of mRNAs for PKCα (Fig. 5C). The introduction of AS-ODNs or S-ODNs did not change the levels of the expression of other PKCs (Fig. 5D). No expression of PKCβ1, PKCβ2, PKCγ, and PKCζ was detected in B16 cells. The relative levels of other PKC families such as PKCδ, PKCe, and PKCζ were not changed. Thus, an AS-ODN specific for a target site in PKCα transcripts suppressed the expression of PKCα in a sequence-specific manner.

We next performed an assay of PKCα activity in vitro. As shown in Fig. 5E, treatment with 5 μM MEL for 2 days stimulated the phosphorylation activity of PKCα, which reached a level that was 2.5-fold higher than the level in untreated control cells. This result was consistent with the MEL-induced enhancement of the expression of PKCα (Fig. 3). Exposure of B16 cells to AS-ODN no. 3642 resulted in a clear decrease in PKCα activity, while S-ODN no. 3846 had only a minimal effect (Fig. 5E).

We then tested whether B16 cells that lacked PKCα could still respond to MEL. As shown in Fig. 5F, B16 cells treated with 5 μM MEL for 2 days produced more than double the amount of melanin, as compared with the control cells. However, cells exposed to both MEL and AS-ODN no. 3642 exhibited no increase in melanogenesis. B16 cells treated with S-ODN resembled control B16 cells, in terms of the response to MEL (Fig. 5). These results indicated that PKCα plays

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**Fig. 5.** An AS-ODN perturbed the expression of PKCα and prevented MEL-induced melanogenesis. A, Western blotting analysis of PKCα. B16 cells were transfected with the indicated AS-ODN or with ODN N23 (200 nM each). After 48 h, cell lysates (20 μg) were separated on SDS-PAGE, and immunoblotting was performed with polyclonal antibodies against PKCα. Cells exposed only to the LipofectAMINE™ reagent were used as controls. Results from one of three similar experiments are shown. Lane 1, control; lane 2, random ODN N23; lane 3, AS-ODN no. 3642; lane 4, AS-ODN no. 3643; lane 5, AS-ODN no. 3644; and lane 6, AS-ODN no. 3645. B, Western blotting analysis of the effect of S-ODN (200 nM) on the expression of PKCα as described in A. Lane 1, control; lane 2, S-ODN no. 3846; lane 3, AS-ODN no. 3642; and lane 4, S-ODN no. 3849. C, Northern blotting analysis of mRNA for PKCα. B16 cells were cultured with 200 nM AS-ODN no. 3642 or S-ODN no. 3846 and total RNA was isolated on day 1 or day 2. Approximately 25 μg of total RNA was loaded per lane and allowed to hybridize with a labeled cDNA probe for mouse PKCα. Probing for the mRNA for mouse β-actin ensured equal loading of the respective RNAs. The upper film was developed for 26 h and the lower film was developed for 5 h. D, The AS-ODN did not change the levels of expression of other isoforms of PKCα. Western blotting analysis of isoforms of PKCα was indicated. Parent B16 cells were incubated in the presence (lane 2) or absence (lane 1) of MEL as well as 200 nM of AS-ODN for 48 h (lane 3), and the expression level of proteins encoding PKCβ1, PKCβ2, PKCγ, PKCδ, PKCe, PKCζ, and PKCζ were measured. E, assay of PKC activity in vitro. After incubation of cells under the indicated conditions for 2 days, 20 μg of cell lysates were immunoprecipitated with polyclonal antibodies against PKCα. An assay of the kinase activity of immunocomplexes was performed with a specific peptide as the substrate (see text). F, cells treated under the same conditions as in E were analyzed for the production of melanin. Data in E and F are mean results of three independent experiments. Bar shows S.D. G, micrographs of B16 cells exposed to no additive (a); 200 nM AS-ODN no. 3642 (b); 200 nM S-ODN no. 3846 (c); 5 μM MEL (d); 5 μM MEL and 200 nM AS-ODN no. 3642 (e); and 5 μM MEL and 200 nM S-ODN no. 3846 (f). Original magnification: × 250.
a critical role in the MEL-induced differentiation of B16 melanoma cells. We noticed that the morphology of B16 cells incubated with AS-ODN or S-ODN changed slightly. We observed more round-shaped cells in both cases, as compared with the untreated B16 cells (Fig. 5G, panels a–c); however, the number of the melanin-stained cells (see black cells in panels d–f) was significantly reduced after treatment with AS-ODN as compared to S-ODN (panel C). Although we do not know the exact reason for more round-phenotyped cells appearing in the case of AS-ODN or S-ODN as compared with the control cells, this might be caused by the nonspecific effect of oligodeoxynucleotides, because AS-ODN, S-ODN, and N23 resulted in similar changes in the morphology of the cells. However, in terms of the production of melanin, both cases exhibited similar levels to untreated B16 cells (Fig. 5P).

The Effects of Expression of a Constitutively Active Form of PKCa Mimic the Effects of MEL on Melanogenesis in B16 Cells—We introduced the pEFHisB expression vector that included a His-tag fused and constitutively active form of PKCa (28), which had point mutations at amino acid residues 22 and 25, into B16 cells. We selected positive stable clones of B16 cells and evaluated two representative clones, Ao13–1 and Ao13–4, for the expression of the exogenous gene for PKCa (Fig. 6A).

B16 cells that expressed the constitutively active form of PKCa (Ao13–4) had a constitutively high level of melanin (Fig. 6B), which resembled the MEL-triggered melanogenesis in B16 cells. It has also been found that exogenous expression of the constitutively active form of PKCa has no detectable effect on the expression of PKCβ1, PKCβ2, PKCγ, PKCδ, PKCe, and PKCη (no expression) and the expression of PKCβ1 and PKCβ2 decreased after treatment with AS-ODN (Fig. 6D). However, Ao13–4 cells also proliferated at a lower rate than the parent B16 cells (Fig. 6D). This result was consistent with the results in Fig. 6B, which showed that Ao13–4 cells had a greater potential for spontaneous differentiation than the parent cells. However, Ao13–4 cells were less susceptible to MEL in terms of the induction of melanogenesis than the parent B16 cells (data not shown). The reason for this phenomenon remains to be determined. It is possible that, once PKCa has reached a threshold level for commitment to cellular differentiation, further expression of PKCa has no additional effect on the induction of the differentiation of B16 cells.
Mannosylerythritol Lipid-induced Differentiation

DISCUSSION

In the present study, we examined the effects of MEL on B16 melanoma cells. MEL at 5 \( \mu \text{M} \) inhibited the proliferation of B16 cells and stimulated both tyrosinase activity and melanogenesis, with accompanying arrest of the cell cycle at the G1 phase and morphological alterations (Figs. 1 and 2). All of these results suggest that MEL induced arrest of the cell cycle and the differentiation of cells. To our knowledge, there have been no other reports of the induction of the differentiation of melanoma cells by an extracellular microbial glycolipid.

We examined the MEL-triggered signaling pathway in B16 cells, focusing on protein kinase C, which has been reported to play an important role in the regulation of cellular proliferation and differentiation (35–37). Western blotting analysis demonstrated that PKC\( \alpha \), PKC\( \delta \), PKC\( \varepsilon \), and PKC\( \gamma \) were the isoforms of PKC expressed in B16 cells (Fig. 5, A, B, and D). We did not detect the expression of PKC\( \beta_1 \), PKC\( \beta_2 \), PKC\( \gamma \), and PKC\( \eta \) (Fig. 5D). Other groups have reported that only the mRNAs for PKC\( \alpha \) and PKC\( \gamma \) can be detected in B16 cells, with the level of each transcript being extremely low (38). Exposure of B16 cells for 48 h to 5 \( \mu \text{M} \) MEL, which was the optimal concentration for induction of differentiation, resulted in enhanced expression of PKC\( \alpha \), at a level that was ~2.3-fold higher than the level in untreated cells (Fig. 3A). In an attempt to understand the molecular mechanism of the MEL-induced differentiation of B16 cells, we introduced AS-ODNs specific for PKC\( \alpha \) mRNA into B16 cells. Several AS-ODNs inhibited the expression of PKC\( \alpha \) to a varying degree and counteracted MEL-induced melanogenesis (Figs. 4 and 5). Furthermore, the results of the expression of a constitutively active form of PKC\( \alpha \) mimicked the results of the stimulation of B16 cells by MEL (Fig. 6). These results strongly suggest that PKC\( \alpha \) plays a critical role in the MEL-induced differentiation of B16 cells.

Several studies have demonstrated that PKC plays an important role in the growth and progression of certain tumors. For example, in the case of B16-F1 melanoma cells, clones that overexpressed PKC\( \alpha \) have prolonged doubling times and increased melanin production. These phenotypic characteristics are also observed during the RA-induced differentiation of melanoma cells (21). It has been also reported that PKC\( \alpha \) is necessary and sufficient to increase progression through the up-regulation of p21\(^{Cip1}\) in glioma cells (39). By contrast, the exogenously expressed PKC\( \alpha \) in MCF-7 breast cancer cells leads to a more aggressive neoplastic phenotype, and cells exhibit an enhanced rate of proliferation, anchorage-independent growth, and increased tumorigenicity in nude mice (40). Expression after transfection of PKC\( \beta_1 \) and PKC\( \gamma \) in fibroblasts results in a phenotype typical of cellular transformation (41), whereas overexpression of PKC\( \beta_1 \) in colon cells results in growth inhibition (42). Therefore, modified levels of PKC, either elevated or decreased, appear to be closely linked to abnormal proliferation, with the specific effect depending on the type of tumors or isoform in question. Our data imply that increased expression of PKC\( \alpha \) plays a critical role in the MEL-induced differentiation of B16 cells.

It is unclear how MEL triggers the expression of PKC\( \alpha \). MEL was reported previously to perturb the composition of cell-surface glycolipids, such as GM3 and lactosylceramide, in HL-60 promyelocytic leukemia cells (10). It was shown recently that GM3, a kind of GSL, is closely associated with c-Src and Rho in the GM3-enriched microdomain of the surface membrane of B16 melanoma cells. Such organizational units might be directly involved in signal transduction (43). Some glycolipids are known to undergo marked cancer-associated changes (44), and PKC has been reported to be involved in the regulation of glycolipid sulfotransferase activity in renal carcinoma cells (45). The possible link between PKC and cell surface glycolipids remains to be determined and might be the key to an understanding of how signals for differentiation are transmitted to the nucleus to activate target genes.

At a differentiation-inducing concentration (5 \( \mu \text{M} \)), MEL had no significant apoptosis-inducing effect on B16 cells (Fig. 2D). We reported previously that, at concentrations above 10 \( \mu \text{M} \), MEL is a potent inducer of apoptosis in B16 melanoma cells in vitro (23). Therefore, the effects of MEL on B16 cells are concentration-dependent. Because G1 arrest has been observed in MEL-induced differentiation and apoptosis (Fig. 2, A and B; Ref. 23), we speculate that after stimulation of G1 arrest by MEL, B16 cells might respond to different doses of MEL via different, but related signal cascades. The detailed correlations and differences between pathways that lead to MEL-induced differentiation and apoptosis remain to be resolved.

In this study, we selected four AS-ODNs specific for mouse PKC\( \alpha \) mRNA in an attempt to suppress the expression of PKC\( \alpha \) during MEL-induced differentiation of B16 cells. AS-ODN no. 3642 was the most effective in perturbing the expression of PKC\( \alpha \), while S-ODN no. 3846 had no detectable effect on the expression of PKC\( \alpha \). Thus, the AS-ODNs appeared to act in a sequence-specific manner. AS-ODN no. 3642 at 200 nm significantly decreased expression of PKC\( \alpha \) in B16 cells on day 2. However, at above 300 \text{nm} no. 3642 was toxic to the cells. This result supports our previous conclusion that MEL (above 10 \( \mu \text{M} \)) induces apoptosis of B16 cells together with the suppressed expression of PKC activity (46).

MEL induces the differentiation of several different lines of carcinoma cells (10, 46, 47, 48). The details of the molecular mechanisms of MEL-induced differentiation are now being investigated. The data presented here provide evidence that MEL, a yeast-derived glycolipid, triggers the differentiation of malignant B16 melanoma cells in culture and that PKC\( \alpha \) plays a key role in the MEL-induced signaling pathway to cellular differentiation. These results might provide the groundwork for the use of microbial extracellular glycolipids as novel agents for the treatment of melanoma.

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