Induction of Apoptosis by Sphingosine in Human Leukemic HL-60 Cells: A Possible Endogenous Modulator of Apoptotic DNA Fragmentation Occurring during Phorbol Ester-induced Differentiation

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ABSTRACT

The present studies were undertaken to characterize the potential role of sphingosine in the regulation of apoptosis in HL-60 promyelocytic leukemia cells. A 6-h exposure of HL-60 cells to sphingosine or its methy-lated derivative, N,N-dimethylsphingosine, caused internucleosomal DNA fragmentation and stereotypical morphological changes characteristic of apoptosis (i.e., cell shrinkage, nuclear condensation, and the formation of apoptotic bodies), as well as to pharmacological inhibitors of protein kinase C such as 1-(5-isouquinolinesulfonyl)-2-methylpiperezine and staurosporine. Apoptosis by sphingosine and N,N-dimethylsphingosine was measured using a flow cytometric method. The percentages of apoptotic cells in cultures treated with sphingosine (10 μM) and N,N-dimethylsphingosine (10 μM) for 6 h were 55.6 ± 7.8% and 84.2 ± 11.6%, respectively. HL-60 cells were induced to differentiate toward macrophages by treatment with 5 nM 4β-phorbol-12-myristate-13-acetate (PMA). Internucleosomal DNA fragmentation, which was a hallmark of apoptosis, was first detected after 10-h exposure to PMA and increased with longer treatment. Sphingosine concentrations in the cells increased concomitantly with the increasing proportion of apoptotic cells during cell differentiation. Sphingosine level in HL-60 cells differentiated by treatment with PMA for 48 h was about 3.3-fold greater than that in untreated cells. Differentiated HL-60 cells exhibited a markedly increased conversion of exogenously added [3H]ceramide to [3H]sphingosine, indicating elevation of ceramidase activity. Moreover, exposure to sphingosine resulted in down-regulation of c-myc mRNA. These observations suggest the possible role of sphingosine in induction of apoptotic DNA fragmentation during PMA-induced differentiation in myeloid leukemia cells. Sphingosine may function as an endogenous modulator mediating the apoptotic signal.

INTRODUCTION

PKC\(^3\) is involved in intracellular signaling processes including those of cellular proliferation and differentiation in a variety of cells (1). In hematopoietic cell systems, treatment with pharmacological inhibitors of PKC causes the growth inhibition of both normal (2) and leukemic (3) progenitors. Several investigators reported that exposure to PKC inhibitors, such as H\(_7\) and staurosporine, induced apoptosis in HL-60 promyelocytic leukemia cells (4, 5), MOLT-4 lymphoid leukemia cells and normal lymphocytes (6), and a variety of neoplastic cell lines (7). In addition, activation of PKC by exposure to PMA prevented growth factor-deprived hematopoietic cells from undergoing apoptotic cell death (8). These observations suggest the potential role of PKC in the regulation of apoptosis.

Sphingosine, a sphingolipid breakdown product, has been shown to inhibit PKC in vitro and in cells (9–11). We previously reported that sphingosine and its metabolite, DMS, had inhibitory effects on in vitro as well as in vivo tumor cell growth (12–14). DMS also has an inhibitory effect on PKC activity as well as sphingosine (15). Sphingosine and DMS appear to inhibit cell growth and exert cytotoxic activity. It has been postulated that sphingosine functions as an endogenous modulator of PKC and plays important roles in cell growth, differentiation, and oncogenesis (11, 16). However, because of the difficulty of measuring changes in cellular sphingosine levels in response to biological stimuli, whether sphingosine functions physiologically in mediating biological processes including growth suppression has not been determined (17). On the other hand, human myeloid leukemia cell lines, including HL-60 cells, have retained the capacity to respond to inducers of differentiation with the cessation of growth and appearance of a more mature phenotype (18). Recently, evidence of apoptosis has been described in HL-60 cells during both PMA-induced macrophage differentiation (19) and retinoic acid-induced neutrophil differentiation (20). However, it remains unclear what initiates apoptosis during cell differentiation. It is of interest to determine whether sphingosine, a potent endogenous PKC inhibitor, is involved in the mechanism of loss of proliferative capacity and induction of apoptosis during differentiation, since pharmacological PKC inhibitors cause inhibition of cell growth and apoptosis.

The present studies were undertaken to assess the role of sphingosine in the regulation of apoptosis in HL-60 cells. We found that exposure to sphingosine induced DNA fragmentation and morphological changes characteristic of apoptosis in HL-60 cells. Recently, a facile and sensitive method to quantify the mass levels of sphingosine in cells was developed in our laboratory (21). Using this method, we examined changes in sphingosine levels during PMA-induced macrophage differentiation in HL-60 cells. The treatment of HL-60 cells with PMA was associated with the induction of apoptotic DNA fragmentation and increase of the endogenous levels of sphingosine. The results of these experiments suggest the possible role of sphingosine in induction of apoptosis during PMA-induced differentiation in myeloid leukemia cells.

MATERIALS AND METHODS

Chemicals. Sphingosine, DMS, sphingosine-1-phosphate, N-octanoylsphingosine, and [\(^{3}H\)]sphingosine (specific activity, 88 mCi/mmol) were synthesized as described previously (12, 22–24). [\(^{3}H\)]sphingosine was converted to [\(^{3}H\)]C\(_{12}\)-ceramide by acylation with hexanoic anhydride as reported previously (25) and purified by high-performance TLC. Ceramide (type III; from bovine brain), sphingomyelin (from bovine brain), glucosylceramide (CMH; from human Gaucher's spleen), PMA, staurosporine, and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO). H\(_{7}\) was from...
were treated with various sphingolipids or PKC inhibitors for 6 h at 37°C, and containing 1 mM EDTA, 0.25% NP40 (Sigma), and 0.1% RNase A (Sigma) were centrifuged and resuspended in 0.8 ml of 0.9% (w/v) NaCl. Much smaller amounts than sphingosine (27-30).

Dihydrosphingosine (an intermediate in the ile novo pathway of sphingo-}

lipid biosynthesis), dihydrosphingosine would most likely be present in assay for quantification of sphingosine measures both sphingosine and

metabolized on TLC plates using chloroform:methanol:7 N NHjOH:water by centrifugation and resuspended in 0.8 ml of 0.9% (v/v) NaCl. Then, 3 ml of chloroform:methanol (1:2) were added and mixed thoroughly. Phases were separated by adding 1 ml each of chloroform and 1 ml NaCl, and phospholipid content in the lower chloroform phase was estimated according to the method of Ames and Dubin (31).

Metabolism of [3H]Ceramide in HL-60 cells and Their Differentiation Induced Derivatives. The metabolism of exogenously added [3H]Ceramide (a short chain, cell-permeable analogue of ceramide) in HL-60 cells was compared with that in their macrophage differentiation derivatives induced by treatment with PMA for 48 h. Cells (1 X 10^7) were harvested, washed, and resuspended in 1 ml of RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 3 µM [3H]Ceramide. After incubation at 37°C for various periods, cellular lipids were extracted according to the method of Bligh and Dyer (32). Samples from the chloroform phase were dried, dissolved in 40 µl of chloroform:methanol (2:1), and then spotted on TLC plates. Plates were developed with chloroform:methanol:28% NH4OH (80:20:2) or chloroform:methanol:acetic acid:water (100:60:20:5). The bands were visualized and identified under UV light by staining the control lipids with primulin. After spraying with Resolution TLC, autoradiography was performed with Kodak X-Omat film at —80°C. Radioactive spots corresponding to sphingosine, sphingomyelin, or CMH were scraped and counted with a liquid scintillation counter. Northern Blot Analysis. Total cellular RNA was collected according to the method of Chomczynski and Sacchi (33) Poly(A)^+ RNA was isolated by a mRNA purification kit (Pharmacia Biotech, Piscataway, NJ), separated on a 1% agarose formaldehyde gel, and transferred to a nylon-membrane filter (Amersham Corp., Arlington Heights, IL). Blots were hybridized for 16 h at 42°C to the following 32P-labeled DNA probes: (a) the 1.4-kilobase pair ClaI/EcoRI fragment of the c-myc DNA representing exon 3 generated from the plasmid pHSV-1 (34) and (b) the β-actin DNA generated by PCR described previously (35). After the hybridization, the filters were washed twice with 2 X SSC (3 m NaCl, 0.3 m sodium citrate, pH 7.0) and 0.1% SDS at room temperature for 10 min followed by one washing with 0.2X SSC and 0.1% SDS at 42°C for 30 min.

RESULTS

DNA agarose gel electrophoresis revealed that a 6-h exposure of HL-60 cells to sphingosine caused DNA fragmentation with a pattern characteristic of internucleosomal fragmentation as well as pharmacological inhibitors of PKC such as H7 or staurosporine (Fig. 1). DNA

![Fig. 1. Agarose gel electrophoresis of DNA from HL-60 cells treated with different agents.](https://example.com/agarose-gel-electrophoresis.png)
INDUCTION OF APOPTOSIS BY SPHINGOSINE

Fig. 2. Agarose gel electrophoresis of DNA from HL-60 cells. A, effect of sphingosine on DNA fragmentation. The cells were treated for 6 h with an ethanol vehicle or with the indicated concentrations of sphingosine. DNA was isolated from cells and analyzed by 2.0% agarose gel electrophoresis as described in “Materials and Methods.” Lane 1, HindIII digest of φX174DNA. B, specificity of action of sphingosine. The cells were treated for 6 h with an ethanol vehicle (Con), 10 μM sphingosine (Sph), 10 μM N-octanoylsphingosine (C₈-cer), 10 μM sphingosine-1-phosphate (S-1-P), 10 μM CMH, or 10 μM DMS.

Fig. 3. Morphological appearance of HL-60 cells treated for 6 h with an ethanol vehicle (A), 10 μM sphingosine (B), 50 μM H7 (C), or 10 μM DMS (D). The cells were stained by Wright-Giemsa as described in “Materials and Methods.” Exposure to sphingosine, H7, and DMS induced apoptotic changes such as cell shrinkage, condensed chromatin, and fragmented nuclei (Fig. 3B). Exposure to H7 and DMS

However, exposure to DMS (10 μM) induced internucleosomal DNA fragmentation.

The appearance of morphological features characteristic of apoptotic cell death was monitored in HL-60 cells treated with sphingosine (10 μM), H7 (50 μM), and DMS (10 μM) for 6 h (Fig. 3). The ethanol vehicle had no effect on HL-60 cell morphology as shown in Fig. 3A. Cells treated with sphingosine revealed the abundance of apoptotic cells in culture, which were characterized by stereotypical morphological changes of apoptosis such as cell shrinkage, condensed chromatin, and fragmented nuclei (Fig. 3B). Exposure to H7 and DMS

from cells treated with an ethanol vehicle for 6 h was unfragmented. Increases in sphingosine concentrations resulted in increased DNA fragmentation (Fig. 2A). The effects of sphingosine (10 μM) were first detected after 2 h and increased with longer treatment (data not shown). Exogenously added sphingosine is converted to other sphingolipids such as ceramide and sphingosine-1-phosphate in cells (36).

To evaluate the specificity of action of sphingosine, HL-60 cells were treated with various sphingolipids (Fig. 2B). Treatment for 6 h with cell-permeable N-octanoylsphingosine (10 μM), sphingosine-1-phosphate (10 μM), or CMH (10 μM) failed to cause DNA fragmentation.
induced more prominent apoptotic changes than did sphingosine (Fig. 3, C and D). On the other hand, exposure to sphingosine, H7, or DMS did not induce cellular differentiation toward macrophages or neutrophils (data not shown).

After treatment of HL-60 cells with different agents, the percentages of apoptotic cells were measured using a flow cytometric method (Fig. 4). The reduced DNA content of apoptotic cells resulted in an unequivocal hypodiploid DNA peak which was apparently discernible from the diploid DNA peak. Although treatment of HL-60 cells with an ethanol vehicle did not induce apoptosis (Fig. 4A), an apparent hypodiploid DNA peak of apoptotic cells was detected in cultures treated with sphingosine (10 μM), H7 (50 μM), and DMS (10 μM) (Fig. 4, B-D). The percentages of apoptotic cells in cultures treated with an ethanol vehicle, sphingosine (10 μM), H7 (50 μM), and DMS (10 μM) were 1.1 ± 0.2, 55.6 ± 7.8, 72.8 ± 10.5, and 84.2 ± 11.6%, respectively. Exposure to DMS (10 μM) caused larger percentages of apoptotic cells than did that of sphingosine (10 μM).

As previously described (19), internucleosomal DNA fragmentation occurred during PMA-induced differentiation into macrophages (Fig. 5). When HL-60 cells were cultured with 5 nM PMA, the cells were induced to differentiate into macrophage-like adherent cells showing prominent pseudopods. Internucleosomal DNA fragmentation was first detected after treatment for 10 h with PMA and increased with longer treatment. There was no detectable DNA fragmentation in untreated HL-60 cells. Cells with characteristic features of apoptosis were observed during PMA-induced differentiation by light microscopy (data not shown). In addition, differentiated HL-60 cells into macrophages were seen to engulf apoptotic cells. Moreover, the flow cytometric analysis revealed that the percentages of apoptotic cells were 1.9, 5.0, 8.6, and 9.4% after treatment with PMA for 3, 10, 24, and 48 h, respectively.

Cellular concentrations of sphingosine, which functioned as an inducer of apoptosis, were measured in HL-60 cells induced to differentiate by treatment with PMA for the indicated times (Fig. 6). To correct for possible losses during extraction, mass levels of sphingosine, which partitions in membranes, are expressed as molar percentages of phospholipids. The phospholipid levels were unchanged during PMA-induced cell differentiation (data not shown). The sphingosine level in untreated HL-60 cells was 0.0394 ± 0.0078 mol %/phospholipid (6.5 ± 0.4 pmol/10⁶ cells). Sphingosine levels in the cells increased concomitantly with an increasing proportion of apoptotic cells during cell differentiation. The sphingosine level in differentiated HL-60 cells after 48-h exposure to PMA was about 3.3-fold greater than that in untreated cells. On the other hand, the level of sphingosine in H7-treated apoptotic cells (percentage of apoptotic cells was 73% at 50 μM, after 6-h incubation) did not show any increase. Namely, sphingosine content of control and H7-treated cells was 6.0 and 5.5 pmol/10⁶ cells, respectively.

Sphingosine and complex sphingolipids such as sphingomyelin and glycosphingolipids are formed from ceramide (27–29) (Fig. 7). To
evaluate the alteration between sphingolipid metabolism in HL-60 cells and that in their macrophage-like differentiation-induced derivatives, cell-permeable [\(^{3}H\)C\(_{6}\)-ceramide was added to the cell suspension and its metabolism was examined (Fig. 8). \([^{3}H\)C\(_{6}\)-ceramide was rapidly metabolized to \([^{3}H]\)sphingosine, \([^{3}H]\)CMH, and \([^{3}H]\)sphingomyelin. Formation of sphingosine and CMH in HL-60 cells differentiated by treatment for 48 h with PMA markedly increased as compared to that in untreated cells. In contrast, at each time point, sphingomyelin formation in differentiated HL-60 cells was less than 50% of that in untreated cells.

HL-60 cells have been reported to overexpress the c-myc proto-oncogene (37). Overexpression of c-myc may play an essential role in maintaining the high proliferative rate of HL-60 cells. The down-regulation of mRNA for c-myc has been shown to be related to induction of apoptosis (38). We investigated the effects of sphingosine on c-myc expression. The c-myc gene was constitutively expressed in HL-60 cells and exposure to sphingosine resulted in down-regulation of c-myc mRNA that was detected as early as 1 h (Fig. 9). Sphingosine (10 \(\mu\)M) was capable of c-myc down-regulation by 60–70% at 4 h.

**DISCUSSION**

The present study showed that sphingosine and its methylated derivative, DMS, induced apoptosis in HL-60 human promyelocytic leukemia cells. An exposure (2–6 h) to sphingosine or DMS is sufficient to cause DNA fragmentation and morphological changes characteristic of apoptosis. Both sphingosine and DMS have an inhibitory effect on PKC activity (9–15); pharmacological PKC inhibitors, such as H7 and staurosporine, also induced apoptosis. These observations suggest that induction of apoptosis by sphingosine and DMS may be related to inhibition of PKC activity. Induction of apoptosis by sphingosine and DMS, even in the presence of normal serum, was found in many tumor cell lines including CMK-7 megakaryocytic leukemia cells, Colo 205 colon tumor cells, and A431 epidermoid carcinoma cells. The apoptotic capacity of PKC inhibitors including sphingosine and DMS may involve cell cycle-related factors (4). For example, exposure of HL-60 cells to sphingosine resulted in down-regulation of c-myc gene expression (as shown in Fig. 9), which played an important role in regulation of both cell proliferation and apoptosis (39). HL-60 cells differentiation toward macrophages has also been shown to be associated with the down-regulation of c-myc mRNA (40). A further possibility is that PKC inhibitors may affect the cell cycle by inhibiting topoisomerase II, since topoisomerase II is phosphorylated and activated by PKC (41). In fact, topoisomerase II inhibitors, as well as PKC inhibitors, strongly induce apoptosis in HL-60 cells (4). On the other hand, sphingosine and DMS have also been known to regulate a number of biological processes including growth suppression in PKC-independent pathways (17). Chao et al. (42) reported that sphingosine induced dephosphorylation of the Rb gene product, and the effect of sphingosine on Rb was independent of inhibition of PKC. The potent and specific activation of Rb by sphingosine correlated with inhibition of cell growth and with arrest at the \(G_0/G_1\) of the cell cycle. In addition, sphingosine has been shown to activate protein kinases, which were distinct from PKC, cyclic nucleotide-activated kinases, and calcium-dependent kinases, with high specificity for \(d\)-erythro-sphingosine (43). Existence of DMS-dependent protein kinase was...
concomitantly with the proportion of apoptotic cells. In contrast, in apoptotic cells induced by H7, there was no increase observed in sphingosine content after 6 h. Taken together the fact that exogenously added sphingosine could induce the apoptosis of the cells, these observations suggest that sphingosine may function as an endogenous inducer of apoptosis occurring in differentiated cells, and that the sphingosine increase might not be the simple result of apoptosis or cell death, although further study of intracellular function of sphingosine is necessary in order to characterize its role in the regulation of apoptosis.

Sphingosine is produced from ceramide by ceramidase in cells (Fig. 7). Cellular ceramide is thought to be formed by the acylation of de novo biosynthesized dihydrosphingosine, followed by desaturation (27–29), or by the hydrolysis of sphingomyelin in response to stimulation with agents such as TNF-α and IFN-γ (52). The treatment with PMA caused no hydrolysis of sphingomyelin. Increase of sphingosine levels in HL-60 cells differentiated by the treatment with PMA may be due to changes in activity of ceramidase or biosynthesis of sphingolipids during cell differentiation. We examined the metabolism of [3H]Cer-ceramide added exogenously in HL-60 cells and their macrophage differentiation derivatives (Fig. 8). Ceramide was rapidly metabolized to sphingosine, CMH, and sphingomyelin. Differentiated HL-60 cells exhibited a markedly increased conversion of [3H]ceramide to sphingosine, suggesting elevated ceramidase activity in the cells. By contrast, the conversion of [3H]ceramide to sphingomyelin in differentiated HL-60 cells was less than 50% of that in untreated cells. Decrease of sphingomyelin synthesis from ceramide in differentiated HL-60 cells may result in a decrease of formation of diacylglycerol, an endogenous PKC activator (53), since sphingomyelin is synthesized by phosphatidylcholine:ceramide phosphocholine transferase (sphingomyelin synthase), which transfers the phosphocholine head group from the phospholipid phosphatidylcholine to ceramide, yielding sphingomyelin and diacylglycerol as shown in Fig. 7. Some of the reactions involved in sphingolipid metabolism may regulate PKC activity through the generation or removal of PKC modulators such as sphingosine and diacylglycerol as previously proposed (11). On the other hand, [3H]ceramide conversion to CMH in differentiated HL-60 cells markedly increased as compared with that in untreated cells. CMH and GM₃ have been shown to increase remarkably in the process of cell differentiation into macrophages with PMA (54). PDMP, a competitive inhibitor of UDP-glucose:ceramide glucosyltransferase, which markedly reduced basal levels of CMH and GM₃, selectively blocked adherence, but not growth inhibition during macrophage differentiation (55). A pathway to glycosphingolipids with a shorter sugar chain from ceramide might be related to cell differentiation into macrophages with PMA. Thus, the metabolism of sphingolipids including generation of sphingosine might be important to regulate cell growth, differentiation, and apoptosis.

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INDUCTION OF APOPTOSIS BY SPHINGOSINE


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