Suppression of Bcl-2 gene expression by sphingosine in the apoptosis of human leukemic HL-60 cells during phorbol ester-induced terminal differentiation

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Abstract Our recent studies have shown that intracellular levels of sphingosine, an endogenous PKC inhibitor, increase during apoptosis resulting from phorbol ester (PMA)-induced terminal differentiation of human myeloid leukemic HL-60 cells, and have suggested that sphingosine may function as an endogenous mediator of apoptosis in these cells [Ohta, et al. (1995) Cancer Res. 55, 691–697]. We report here that apoptosis induced by PMA, sphingosine, and *N,N*-dimethylsphingosine (DMS) was accompanied by a concomitant decrease of bcl-2 expression in both RNA and protein levels in HL-60 cells, while expression of bcl-X_L and bax mRNA did not change, and neither sphingosine nor DMS induced differentiation of HL-60 cells. In contrast, in apoptotic cells induced by pharmaceutical PKC inhibitors H7 or staurosporine, expression of bcl-2 did not change nor did the intracellular sphingosine concentration. These results suggest that sphingosine may function as an endogenous mediator of apoptotic signaling in PMA-induced terminal differentiation of HL-60 cells through bcl-2 down-regulation, probably independent from PKC inhibition.

Key words: Apoptosis; Sphingosine; Differentiation; Phorbol ester (PMA); Bcl-2

1. Introduction

Cellular homeostasis in vertebrates is regulated by cell death as well as by cell proliferation [1–3]. Although the mechanism that induces and executes apoptosis is poorly understood, it is widely accepted that in most cases cell death is controlled by a genetic program which is activated in apoptosis. Recent studies have partially clarified this genetic control [4,5], and individual genes that either promote or inhibit apoptosis have been identified.

In hematopoietic cells, apoptosis can be coupled to terminal differentiation of myeloid progenitors [6,7]. It is generally thought that hematopoietic survival factors, such as GM-CSF or IL-3, can maintain cell survival by inducing the synthesis of cellular proteins capable of suppressing apoptosis [8,9]. One such protein is the bcl-2 proto-oncogene product. Bcl-2 encodes a 26 kDa integral membrane protein that localizes to the outer mitochondria, smooth endoplasmic reticulum, and perinuclear envelop, and is known to be a suppressor gene of apoptosis [10–12]. Within the myeloid lineage, bcl-2 is expressed in early myeloid precursors but is absent in the mature polymorphonuclear leukocytes [13]. Similarly, bcl-2 gene expression is repressed during PMA-induced differentiation in promyelocytic HL-60 cells [14]. The correlation between the expression of bcl-2 and differentiation suggests that down-regulation of the bcl-2 gene may be part of the myeloid maturation pathway, however, the mechanism of the bcl-2 down-regulation itself has not yet been clarified. In our previous studies, we have reported that exogenously added sphingosine induced apoptosis in HL-60 cells, and that in HL-60 cells differentiated by phorbol ester (PMA) sphingosine concentrations were several folds greater than those in the untreated cells [15]. It is of interest to analyze how sphingosine, which is known to be an endogenous PKC inhibitor, is involved in the mechanisms of induction of differentiation and apoptosis, since some pharmaceutical PKC inhibitors cause apoptosis without cell differentiation in HL-60 cells [16]. Therefore, in this study we examined the effect of PMA and exogenously added sphingosine and *N,N*-dimethylsphingosine (DMS) on the regulation of apoptosis-related genes such as bcl-2, bax, and bcl-X_L, and we found a difference in the regulation of bcl-2 expression between sphingosine/DMS and other PKC inhibitors, suggesting that apoptosis induction by sphingosine and DMS is via a mechanism different from PKC inhibition.

2. Materials and methods

2.1. Cell culture

Human myeloid leukemia HL-60 cells were purchased from American Type Culture Collection (ATCC), and maintained in RPMI1640 containing fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamate.

2.2. Analysis of DNA fragmentation

DNA fragmentation was analyzed by agarose gel electrophoresis. HL-60 cells were treated for 6 h at 37°C with one of the following: 5 nM PMA, 5 μM sphingosine, DMS, C2 ceramide, or sphingosine-1-phosphate, 100 nM H7 or 100nM staurosporine. All sphingolipids were dissolved in 50% ethanol. H7 and staurosporine were dissolved in DMSO. DNA was extracted as described previously [15]. Cells were harvested, washed, and incubated in 0.5 ml of 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.25% NP-40, and 0.1% RNase A at 37°C for 30 min. Fifty μl of 10 mg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) were added, and incubation was continued for an additional 30 min. After incubation, 5 μg DNA was applied to each lane in 1.5% agarose gel. DNA in gels was visualized under UV light after staining with ethidium bromide.

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Abbreviations: Sph, sphingosine; DMS, *N,N*-dimethylsphingosine; PMA, 4β-phorbol-12 myristate; PKC, protein kinase C.

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2.3. Northern blot analysis

Total RNA was extracted by using a guanidine isothiocyanate-$CsCl_2$ procedure as described previously [15]. The following DNA probes were used: (a) nt 121 to nt 579 bcl-2 fragment (459 bp), (b) nt 96 to nt 875 bcl-X_L fragment, (780 bp), (c) nt 1 to nt 323 bax fragment, (323 bp).

2.4. Western blot analysis

After treatment with each agent, 1×10^6 cells were washed twice with PBS and lysed in 1% Triton X-100, 0.15 M NaCl and 10 mM Tris-HCl, pH 7.4, with 50 μg/ml PMSF and 2 μg/ml aprotinin at 4°C for 30 min. Lysates were centrifuged at $10,000 \times g$ for 10 min, and boiled in SDS sample buffer for 5 min before running on a 7.5% SDS-polyacrylamide gel. After overnight transfer of SDS-polyacrylamide gel to nitrocellulose membrane (PVDF, MILLIPORE), blots were blocked in 3% BSA for 2 h at room temperature. Rabbit polyclonal antibody to human bcl-2 (DAKO, A/S, Denmark) was used at 1/1000 dilution in PBS with 3% BSA for 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was incubated with the blot at 1/4000 dilution in PBS with 3% BSA for 2 h at room temperature. The blot was visualized with ECL kit (Amersham).

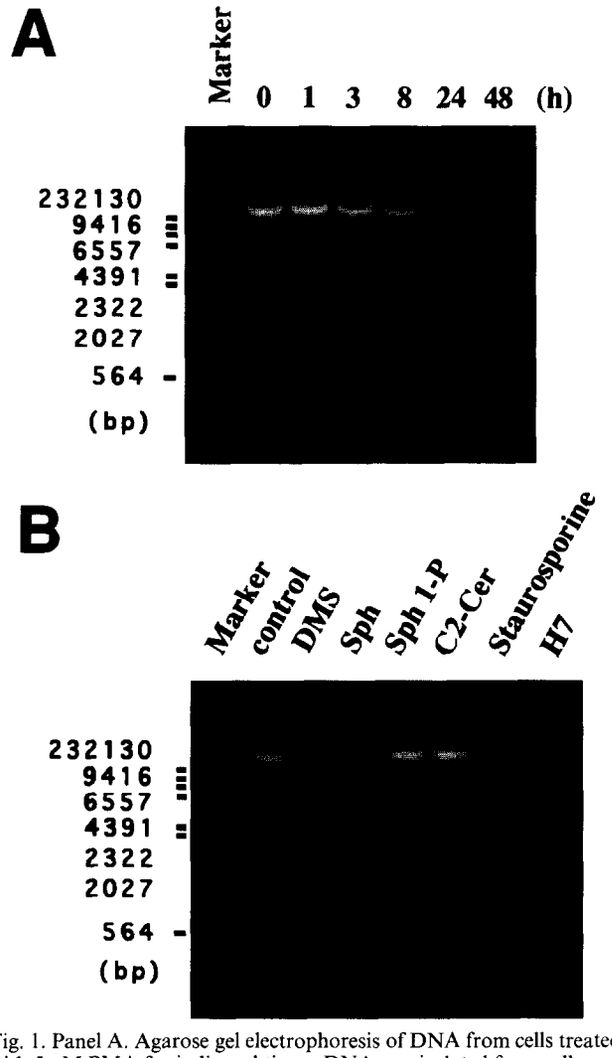

Fig. 1. Panel A. Agarose gel electrophoresis of DNA from cells treated with 5 nM PMA for indicated times. DNA was isolated from cells and analyzed in 1.5% agarose gel electrophoresis. The marker is HindIII digest of λ_DNA. Panel B. Agarose gel electrophoresis of DNA from cells treated with different agents. HL-60 cells were treated with 5 μM sphingosine (Sph), 5 μM DMS, 5 μM C2-ceramide, 5 μM sphingosine-1-phosphate, 100 nM H7, or 100 nM staurosporine for 6 h. DNA was isolated from cells and analyzed in 1.5% agarose gel electrophoresis. The marker is *Hin*dIII digest of λ DNA.

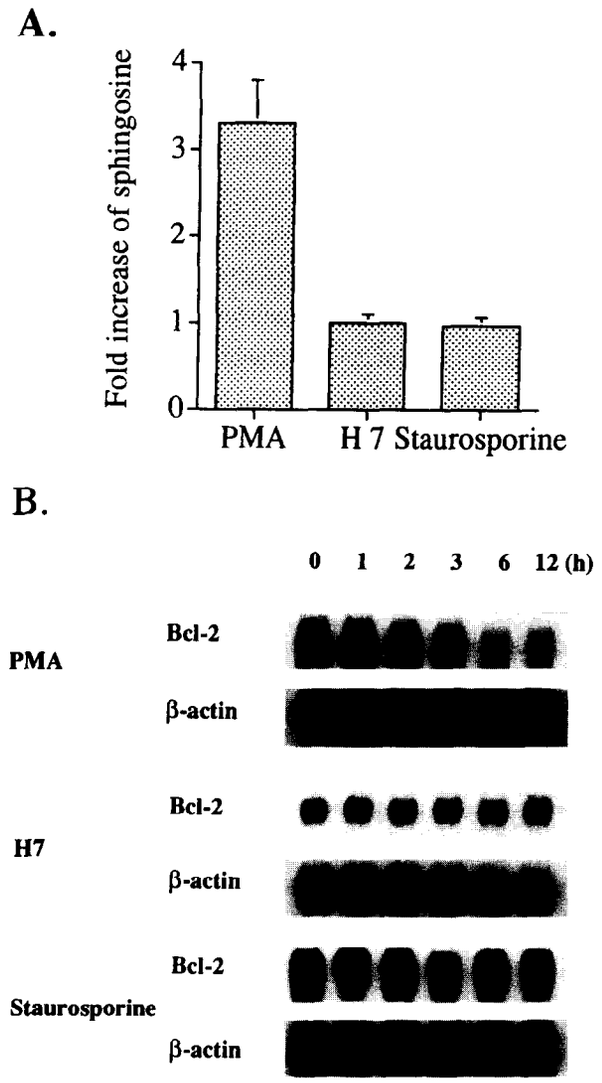

Fig. 2. Panel A. Effect of PMA, H7 and staurosporine on sphingosine concentration in HL-60 cells. HL-60 cells were treated with each agent for 6 h. Cellular lipids were extracted and the concentration of sphingosine was measured as described in section 2. Panel B. Effect of PMA, H7, or staurosporine on expression of bcl-2 mRNA. HL-60 cells were treated with each agent for the indicated time. Northern blots were performed as described in section 2. The β-actin cDNA generated by PCR was used as internal control of the RNA amount in each lane as described previously [15].

2.5. Measurement of sphingosine concentration

Cells were treated with PMA, H7 or staurosporine, and then lipids were extracted from cells. Sphingosine concentrations were measured by conversion to *N*-[^{3}H]acetylated sphingosine with [^{3}H]acetic anhydride as described previously [17]. Radioactive bands were scraped off and counted by Beckman scintillation counter (Beckman).

3. Results and discussion

Endogenous sphingolipid metabolites such as ceramide and sphingosine have been increasingly recognized as lipid mediators of cell growth, differentiation and apoptosis. Sphingosine acts as an endogenous regulator of membrane signaling through protein kinase C or other unknown mechanisms [18–21]. We have shown that sphingosine, and *N,N*-dimethylsphin-

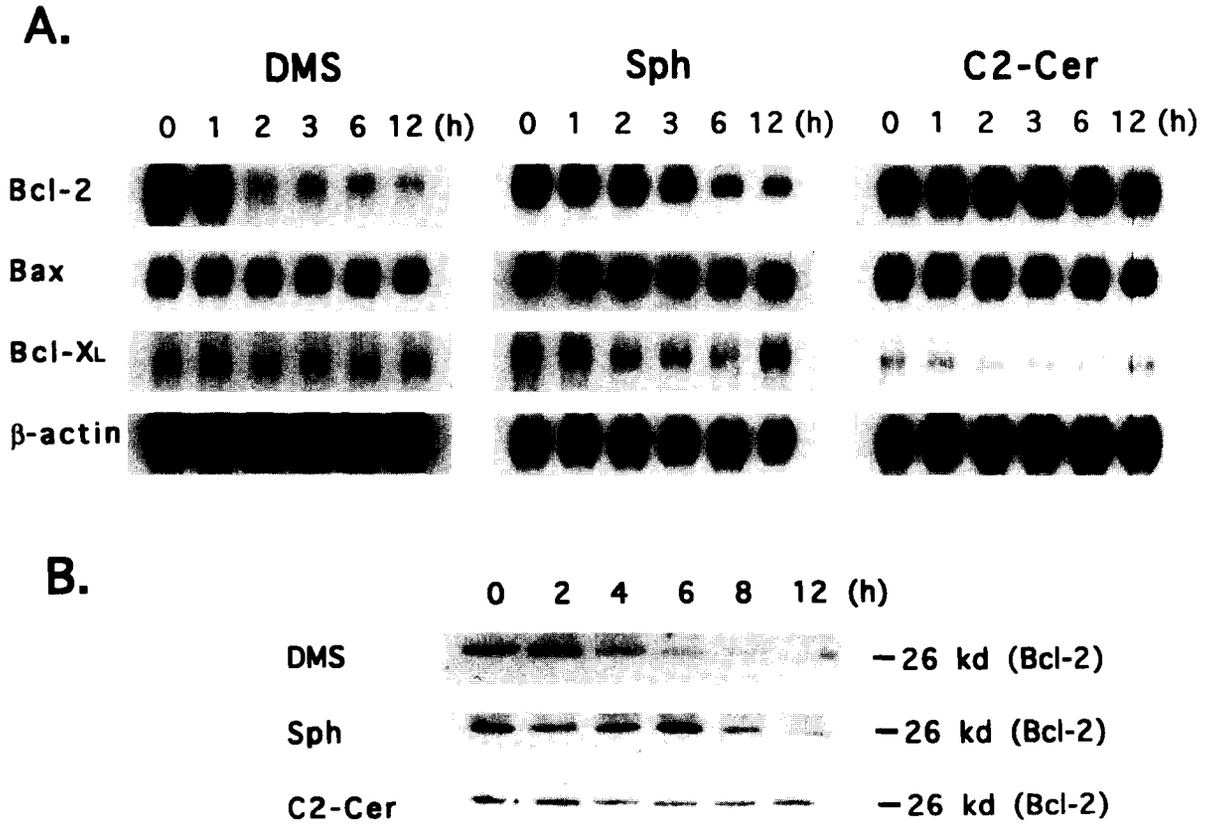

Fig. 3. Panel A. Effect of DMS, sphingosine and C2-ceramide on expression of bcl-2, bax, and bcl-X_L mRNA. HL-60 cells were treated with each agent at a concentration of 5 μM for indicated time. Northern blots were performed as described in section 2. Panel B. Effect of DMS, sphingosine and C2-ceramide on expression of bcl-2 protein. HL-60 cells were treated with each agent at a concentration of 5 μM for the indicated time. Western blots were performed as described in section 2.

gosine, induce apoptosis in HL-60 cells [15] as well as in other solid tumor cells [22,23]. However, involvement of sphingosine in the mechanism to induce apoptosis in terminal differentiation of hematopoietic cells has not yet been clarified.

PMA induces terminal differentiation in HL-60 cells, and as a consequence, induces apoptosis with the characteristic pattern of DNA fragmentation as shown in Fig. 1A. This apoptosis is associated with an increase in intracellular sphingosine concentrations and bcl-2 down-regulation. Both 5 μM sphingosine and DMS induced down-regulation of bcl-2 by 80–90% at 6 h in both mRNA and protein levels (Fig. 3). As shown in Fig. 2 A and B, sphingosine concentrations in HL-60 cells increased 3.3-fold after 1h incubation with PMA, and bcl-2 expression was inhibited with concomitant cell differentiation. H7 and staurosporine are known to be pharmaceutical PKC inhibitors and can induce apoptosis in HL-60 cells as shown in Fig. 1B. However, the apoptotic process is not associated with cell differentiation, increase of intracellular sphingosine concentration (Fig. 2A), or bcl-2 down-regulation (Fig. 2B), suggesting that the mechanism of apoptosis induced by sphingosine may differ from that of other PKC inhibitors. The effect of DMS was stronger than that of sphingosine in the induction of apoptosis and bcl-2 down-regulation. Both compounds are rapidly taken up by cells, but, whereas sphingosine is efficiently converted to Sph-1-phosphate and ceramide, DMS is not metabolically converted into any other compound in most cells [24–26]. The difference in efficiency of sphingosine vs. DMS may be due to their different metabolic fates. Neither ceramide nor sphingosine-1-phosphate, the initial products of sphingosine, caused apoptosis (Fig. 1B) or affected bcl-2 expression (data not shown), although high concentrations of the analogue C2-ceramide does induce apoptosis with down-regulation of bcl-2 in HL-60 cells [27]. One previous report showed that the PKC-inhibitor calphostin C also did not change the expression of bcl-2 [16].

Down-regulation of bcl-2 is associated with differentiation and apoptosis of hematopoietic cells as observed in PMA-treated HL-60 cells (Fig. 2B). Although sphingosine inhibited bcl-2 expression, it did not induce differentiation of HL-60 cells. PMA is PKC activator and sphingosine is PKC inhibitor. Not only bcl-2 down-regulation but also activation of other factors such as PKC are apparently necessary to induce differentiation in HL-60 cells. These observations suggest that sphingosine may function as an endogenous inducer of apoptosis in differentiated hematopoietic cells through down-regulation of bcl-2, but other factors would be involved to differentiate HL-60 cells. That various PKC inhibitors have been shown to inhibit PMA-induced leukemic cell maturation [28,29] and that the induction of apoptosis by H7 or staurosporine was not accompanied by cellular differentiation provides indirect evidence that separate differentiation-dependent and -independent pathways of apoptosis may exist and that differentiation-

dependent apoptosis will be associated with down-regulation of bcl-2.

The mechanism by which bcl-2 and other bcl-2-related proteins inhibit apoptosis is poorly understood. Recent evidence suggests that bcl-2 may prevent apoptosis through the regulation of an antioxidant pathway [30]. Bcl-2 appears to be expressed as part of an intracellular protein complex. For example, bcl-2 has been shown to interact with at least two cellular proteins bax [31] and r-Ras p23 [32], although the significance of these protein interactions is not well understood. Recently, bcl-X, a gene related to bcl-2, has been identified [33]. Alternative splicing of bcl-X produces two mRNA: bcl-X_L and bcl-X_S, the large (L) and small (S) splice forms. Bcl-X_L is a functional analogue of bcl-2, and inhibits apoptosis upon growth factor withdrawal or X-irradiation, whereas overexpression of bcl-X_S counteracts the effect of bcl-2 in preventing apoptosis [31]. As shown in Fig. 3, in contrast to down-regulation of bcl-2 by sphingosine and DMS, neither bax nor bcl-X_L expression was affected during PMA-induced apoptosis or after treatment with sphingosine or DMS.

Together, these results indicate that the regulation of apoptosis within myeloid cells is complex and is modulated by multiple proteins, and sphingolipid metabolites. Intracellular sphingosine may be involved in apoptosis of terminally differentiated HL-60 cells through bcl-2 down-regulation. Thus, the metabolism of sphingolipids, including the generation of sphingosine, is likely to be important in regulating cell growth, differentiation and apoptosis.

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