

Sphingosine induces apoptosis in androgen-independent human prostatic carcinoma DU-145 cells by suppression of bcl-X_L gene expression

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Abstract Our recent studies have suggested that sphingosine, an endogenous protein kinase C (PKC) inhibitor, may mediate apoptosis induced by a phorbol ester (PMA) in human promyelocytic leukemia HL-60 cells [Ohta et al. *Cancer Res.* 1995;55:691–697], and that the apoptotic induction by both PMA and sphingosine is accompanied by down-regulation of bcl-2, a gene which acts to prevent apoptotic cell death [Sakakura et al. *FEBS Lett.* 1996;397:177–180]. In this study, we examined the sphingosine-induced apoptosis of the androgen-independent human prostatic carcinoma cell line DU-145, which expresses bcl-X_L and Bax but not bcl-2, and found that treatment of DU-145 cells with sphingosine suppressed bcl-X_L in both mRNA and protein levels but did not change bax expression at all. In contrast, in apoptotic cells treated with a PKC inhibitor, staurosporine, no effect on bcl-X_L or bax expression was observed. The initial metabolites of sphingosine in the cells, ceramide and sphingosine 1-phosphate, failed to induce apoptosis. These results indicate that, in DU-145 cells, sphingosine, but not its metabolites, induces apoptosis through down-regulation of bcl-X_L, independently of PKC inhibition. Our present results, together with previous observations, strongly suggest that apoptosis regulatory genes differ according to cell type and apoptosis induction through sphingosine is accompanied by inhibition of either bcl-2 or bcl-X_L activity in these cells.

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Key words: Apoptosis; Sphingosine; Protein kinase C; Prostatic carcinoma; Bcl-X_L

1. Introduction

Sphingomyelin hydrolysis products, ceramide, sphingosine and their metabolites, play important roles in the regulation of cell growth, differentiation and apoptosis [1–4]. Intracellular levels of sphingosine, an endogenous PKC inhibitor [5,6], increased in HL-60 cells during the PMA-induced apoptotic process [7,8]. Similarly, it was observed that treatments of human neutrophils [9] and cardiac myocytes [10] with tumor necrosis factor- α (TNF- α) induced apoptosis and enhanced concomitantly the endogenous sphingosine concentration. In these studies, exogenously added sphingosine also induced apoptosis in these cells and it was proposed that sphingosine,

produced in response to apoptotic stimuli, functions as an endogenous inducer of apoptosis. Recently, we reported that sphingosine and its *N*-methylated derivative, *N,N*-dimethyl-sphingosine, is able to induce apoptosis in cancer cells of both hematopoietic and solid tumor origin ([11,12]; Sakakura, Sweeney, Shirahama, Solca, Kohno, Hakomori, Fisher, Igarashi, submitted). Sphingosine did not, however, induce apoptosis in normal epithelial cells such as HUVECs or rat mesangial cells, but did induce apoptosis in their transformed counterparts ([11]; Sakakura, Sweeney, Shirahama, Solca, Kohno, Hakomori, Fisher, Igarashi, submitted).

The apoptotic process is encoded by an endogenous program, and deregulation is considered to be involved in human disease states including acquired immunodeficiency syndrome, neurodegenerative disorders and cancer [13,14]. Cell death by apoptosis occurs when a cell activates an internally encoded suicide program as a result of either extrinsic or intrinsic signals. The bcl-2 proto-oncogene has been identified as a molecule which counters a variety of apoptotic stimuli [15–17], suggesting that it might be a central death repressor in the apoptotic pathway. However, bcl-2 has failed to protect some hematopoietic cells from apoptosis induced by cytokine deprivation or receptor-mediated signaling [18]. Recently, a new bcl-2-related gene, bcl-X, has been identified [19,20]. Alternative splicing produces two species of mRNA, named bcl-X_L and bcl-X_S. Bcl-X_L acts as a cell death repressor, while bcl-X_S acts as a cell death promoter, and both genes appear to be involved in some bcl-2-independent apoptosis [18,19]. Another gene implicated in the apoptotic pathway is bax [21–23]. Bax is able to homodimerize as well as heterodimerize with bcl-2 or bcl-X_L. When in excess, bax counters the ability of bcl-2 or bcl-X_L to prevent apoptotic cell death. We previously reported that sphingosine down-regulates bcl-2 expression in mRNA as well as protein levels, resulting in apoptosis induction in HL-60 cells, but that bcl-X_L expression level is not affected by sphingosine treatment [8]. Since apoptosis is known to be regulated through both bcl-2-dependent and -independent (including those involving bcl-X_L) pathways, in different cell types [18,19], the effects of sphingosine on bcl-X_L in these cells were unclear.

To address this issue, we chose androgen-independent human prostatic carcinoma DU-145 cells which express bcl-X_L but not bcl-2 at the protein level. We show here that sphingosine, but not its metabolites, induced apoptosis in these cells by down-regulation of bcl-X_L, most probably independently of PKC inhibition.

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2. Materials and methods

2.1. Cell culture

Human prostatic carcinoma DU-145 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD), and maintained in DMEM medium containing 10% heat-inactivated fetal bovine serum supplemented with 2 mM L-glutamine.

2.2. Sphingolipids and other chemicals

Sph was purchased from Sigma (St. Louis, MO). Sphingosine-1-phosphate [24] and C2-ceramide [25] were synthesized as previously described. All sphingolipids were dissolved in 50% ethanol and were utilized by proper dilution. Staurosporine (Sigma, St. Louis, MO) was dissolved in DMSO.

2.3. Measurement of apoptosis by flow cytometry

Cultured cells were treated with various agents including Sph as described above. Apoptotic cells were quantitatively evaluated by flow cytometry as described previously [12]. After centrifugation, the resultant cell pellet was resuspended in 0.3 ml hypotonic fluorochrome solution containing 50 µg/ml propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100. The fluorescence of individual nuclei was analyzed using an EPICS flow cytometer (Coulter Electronics, Hialeah, FL).

2.4. Analysis of DNA fragmentation

DNA fragmentation was analyzed by agarose gel electrophoresis as described in our previous paper [8], using DU-145 cells treated for 15 h at 37°C with one of the following: 15 µM Sph, C2-ceramide, or sphingosine-1-phosphate, or 100 nM staurosporine.

2.5. Northern blot analysis

Total RNA was prepared by the acid guanidine thiocyanate-phenol-chloroform method [7]. For Northern blot analysis, 20 µg of total RNA was denatured with glyoxal, subjected to 1.2% agarose gel electrophoresis, and transferred to a Hybond nylon membrane (Amersham, St. Louis, MO). The membrane was probed with a 875 bp bcl-X_L cDNA probe (nucleotides 96–875), which was labeled with [α -³²P]dCTP by the random oligonucleotide priming method. Human β -actin cDNA (Clontech, Palo Alto, CA) probe was used as a control.

2.6. Western blot analysis

After treatment with each reagent, the cells were washed 3 times with PBS(–) and lysed in Laemmli's sample buffer [26]. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel and were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% skimmed milk at room temperature for 2 h, blots were allowed to react with rabbit polyclonal antibody to human bcl-X_L (L-19) or mouse bax (P-19) (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/100 dilution in PBS(–) with 5% skimmed milk for 2 h at room temperature. The blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) at 1/100 dilution in PBS(–) with 5% skimmed milk at room temperature for 2 h, and visualized with ECL kit (Amersham, St. Louis, MO).

3. Results and discussion

The aim of the present study was to examine the effects of sphingosine on apoptotic induction in androgen-independent prostatic carcinoma DU-145 cells and its regulatory effect on bcl-2-related genes. Flow cytometric analysis showed that sphingosine induces apoptosis in these cells in both a dose- and a time-dependent manner (Fig. 1). Exogenously added sphingosine is metabolically converted to sphingosine-1-P or ceramide [12] which is thought to be an endogenous mediator for apoptotic signaling induced by radiation or TNF- α in leukemic cells such as HL-60 and U-937 cells [27–29]. Apoptotic induction was usually observed with exogenously added short-chain cell permeable ceramides, such as C2- or C6-ceramide, but not with long-chain natural ceramides [4,11].

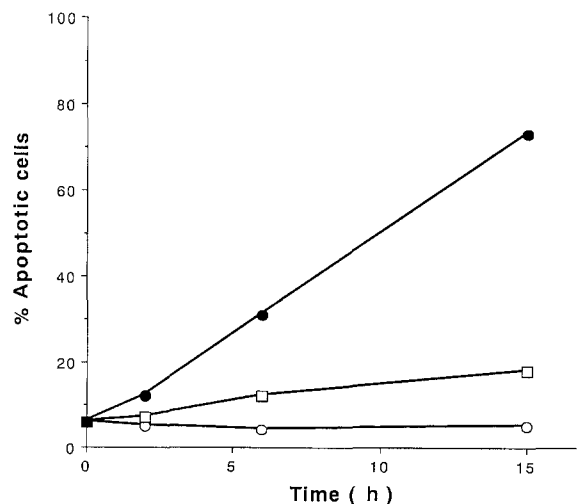


Fig. 1. Percentage of apoptotic cells after treatment for 2, 6 or 15 h with ethanol vehicle (0.1%, control) (○), 5 µM (□) or 15 µM sphingosine (●), as determined by flow cytometry.

However, neither sphingosine-1-phosphate nor cell permeable ceramide, C2-ceramide, caused apoptosis in DU-145 cells under the present experimental conditions (Fig. 2). The apoptotic induction by sphingosine was also confirmed by internucleosomal DNA fragmentation which is a hallmark of apoptosis (data not shown) [30]. The protein kinase C inhibitor, staurosporine, also induced apoptosis, although the number of apoptotic cells seen was much less in the cells treated with staurosporine than in those with sphingosine (Fig. 2).

Sphingosine has been proposed as an endogenous mediator for apoptosis [7,9], but the mechanism involved has not been established. We have previously shown that apoptosis resulting from PMA-induced terminal differentiation of human leukemic HL-60 cells was accompanied by an increase in intracellular sphingosine levels and down-regulation of bcl-2 in both mRNA and protein levels [8]. Exogenously added sphingosine did not induce cellular differentiation in these cells, but did induce apoptosis with down-regulation of bcl-2 expression. However, neither treatment with PMA nor sphingosine affected the expression levels of the death promoter, bax and the death repressor, bcl-X_L. We suggest that down-regulation of bcl-2 by sphingosine might be responsible for apoptosis resulting from PMA-induced terminal differentiation of HL-60 cells. Also, these results suggest that only bcl-2 was responsible for apoptotic induction in HL-60 cells and that down-regulation of bcl-2 was enough to induce apoptosis, although HL-60 cells express another apoptosis repressor gene, bcl-X_L. This possibility may be supported by the observation that TNF- α as well as ceramide, an endogenous mediator for apoptosis caused by TNF- α , also induces apoptosis in HL-60 cells with down-regulation of bcl-2 but without any changes in bcl-X_L expression [29].

In the present experiment using DU-145 cells, sphingosine induced apoptosis with a concomitant decrease of bcl-X_L expression in both mRNA and protein levels, but without any changes in bax expression (Fig. 3). These changes in the apoptosis-related genes produce a decreased ratio of bcl-X_L/bax heterodimers to bax/bax homodimers, a situation suggested to be sufficient to induce apoptosis [21,23]. From the present results and those of our previous studies [8], we conclude that

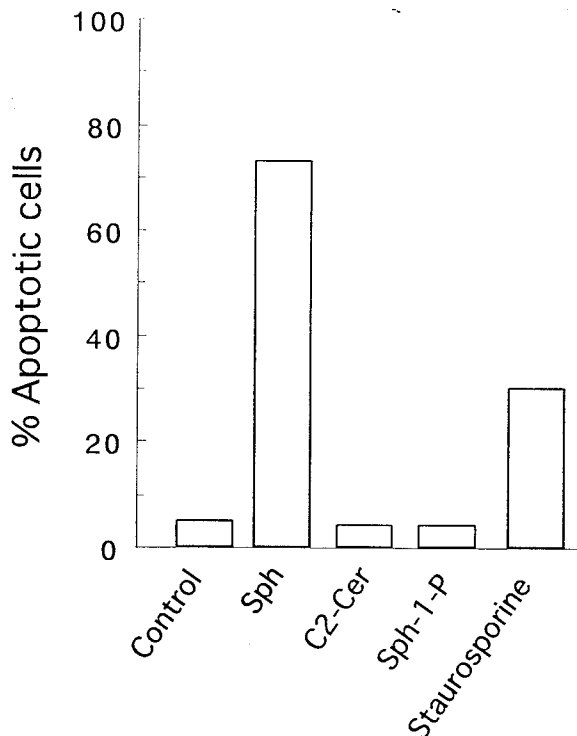


Fig. 2. Percentage of apoptotic cells after treatment for 15 h with ethanol (control), 15 μ M sphingosine, 15 μ M C2-ceramide, 15 μ M sphingosine-1-phosphate (Sph-1-P) and 100 nM staurosporine, as determined by flow cytometry. Data are the means of two independent experiments with similar values.

apoptosis regulatory genes differ according to cell type and that sphingosine induces apoptosis by inhibiting either bcl-2 or bcl-X_L activity. In apoptosis induced by the PKC inhibitor

staurosporine, neither bcl-X_L nor bax expression was changed, indicating that the apoptotic mechanism induced by PKC inhibition differs from that induced by sphingosine.

A key signal transducer, p21Ras, is known to regulate the differentiation and proliferation of eukaryotic cells [31,32]. It has also been demonstrated that p21Ras interacts with the serine/threonine kinase Raf-1, which in turn regulates the activity of a kinase cascade that includes MEK and mitogen-activated protein kinases (MAPK) [33,34]. Recently, it was reported that Ras-generating signals lead not only toward cell growth, but can also initiate apoptosis, depending upon the state of other cellular signaling mediators such as bcl-2 and PKC [35,36]. Ras-induced apoptosis was triggered after suppression of cellular PKC activity and was blocked by bcl-2. We previously showed that sphingosine suppressed MAPK in solid tumor cells, resulting in the induction of apoptosis and suggesting that sphingosine-induced apoptotic signaling may be related to Ras/Raf-1/MAPK cascade (Sakakura, Sweeney, Shirahama, Solca, Kohno, Hakomori, Fisher, Igashi, submitted).

Normal prostatic epithelium and prostatic carcinoma cells proliferate in response to androgen stimulation [37]. This androgen dependence has been the basis of androgen ablation therapy for prostatic carcinomas. However, most prostatic carcinomas become resistant to androgen ablation therapy within 2 years through androgen receptor mutation or some other unknown mechanism [38,39]. Androgen-independent prostatic carcinomas are also resistant to anti-cancer agents, which is thought to be a major cause of the failure of prostatic carcinoma treatment. Because many anti-cancer agents have been found to suppress tumor growth by inducing apoptosis [40], it is important to understand the relationship between androgen independence in prostatic carcinoma cells and the expression of apoptosis-related genes.

Recently, it was shown by RT-PCR that apoptosis repres-

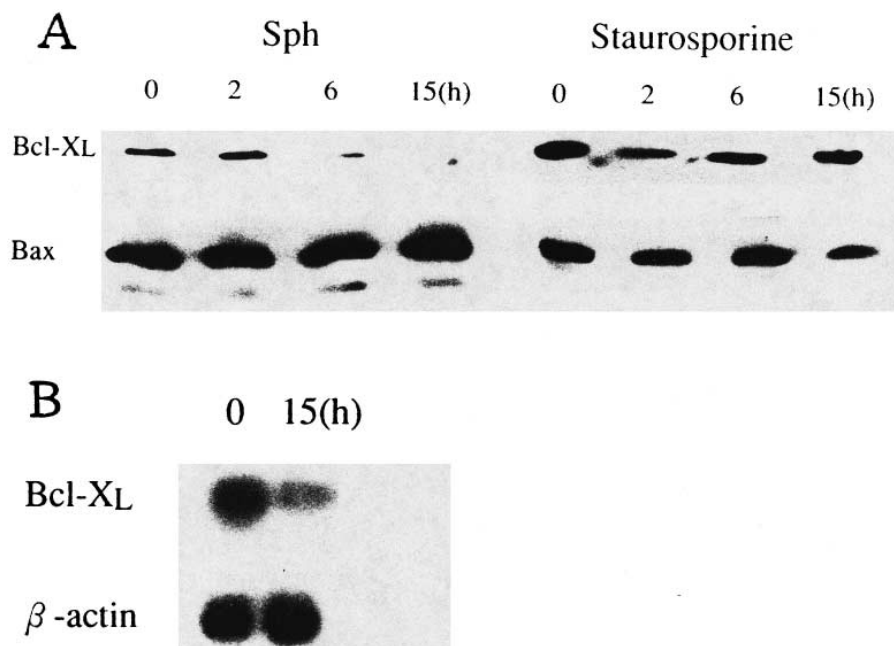


Fig. 3. Immunoblotting analysis. A: Western blot analysis. Lysates from control cells (0.1% ethanol) or cells treated with 15 μ M sphingosine, or 100 nM staurosporine for 2, 6 or 15 h were immunoblotted with anti-human-bcl-2 antibody or anti-human-bcl-X_L antibody. B: Northern blot analysis of bcl-X_L mRNA. Total RNA (20 μ g) from cells treated for 15 h with or without 15 μ M sphingosine was subjected to 1.2% agarose gel electrophoresis and probed with bcl-X_L cDNA probe. Human β -actin cDNA probe was used as a control.

genes including bcl-2 and bcl-X_L were expressed in DU-145 cells, but anti-sense bcl-2 oligonucleotides failed to enhance the apoptosis caused by staurosporine [41]. However, we detected bcl-X_L protein but not bcl-2 protein in DU-145 cells, suggesting that anti-sense bcl-2 oligonucleotides would not be effective in these cells. A similar expression in both bcl-2 and bcl-X_L proteins was also observed in another androgen-independent prostatic carcinoma cell line, PC-3, in which sphingosine also induced apoptosis with down-regulation of bcl-X_L (data not shown). These results suggest that bcl-X_L may be an apoptosis repressor gene in androgen-independent prostatic carcinoma cells, and that sphingosine or its derivatives such as *N,N*-dimethylsphingosine might be effective in the therapeutic treatment of this disease.

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