Phytosphingosine Induces Apoptotic Cell Death via Caspase 8 Activation and Bax Translocation in Human Cancer Cells

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ABSTRACT

Purpose: Sphingolipid metabolites, such as sphingosine and ceramide, are highly bioactive compounds and are involved in diverse cell processes, including cell–cell interaction, cell proliferation, differentiation, and apoptosis. However, the physiological roles of phytosphingosine are poorly understood. In this study, we report that phytosphingosine can potently induce apoptotic cell death in human cancer cells via caspase activation and caspase-independent cytochrome c release.

Experimental Design: Phytosphingosine-induced apoptosis was determined by Hoechst 33258 staining, flow cytometric analysis, and DNA fragmentation assay. Involvement of caspases was determined by immunoblot analysis and cell death detection assays after treatment with synthetic inhibitor z-Val-Ala-Asp-fluoromethyl ketone, z-DEVD-fmk, or z-IETD-fmk. Death receptor (DR) dependency was analyzed by examining expression of DRs (Fas, DR4, DR5, TNFR1, and R2), and interaction of Fas-associated death domain and caspase 8. Involvement of the mitochondria pathway was examined by monitoring of the mitochondrial membrane potential, and the cytochrome c release and Bax translocation.

Results: Phytosphingosine-treated cells displayed several features of apoptosis, including increase of sub-G1 population, DNA fragmentation, and poly(ADP-ribose) polymerase cleavage. We observed that phytosphingosine cause activation of caspase 8 in a DR-independent fashion. Phytosphingosine also induced activation of caspase 9 and 3, loss of mitochondrial membrane potential, and the cytochrome c release from mitochondria. However, we failed to detect Bid cleavage. Moreover, caspase 8 inhibitor z-IETD-fmk did not affect phytosphingosine-induced cytochrome c release and caspase 9 activation, suggesting that phytosphingosine-induced cytochrome c release is caused by caspase 8-independent manner. Phytosphingosine induced mitochondrial translocation of Bax from the cytosol without changes in the protein levels of Bcl-2, Bcl-xL, and Bax. In addition, Bcl-2/Bax interaction was diminished after addition of phytosphingosine.

Conclusion: These findings indicate that phytosphingosine induces apoptotic cell death in human cancer cells by direct activation of caspase 8, and by mitochondrial translocation of Bax and subsequent release of cytochrome c into cytoplasm, providing a potential mechanism for the anticancer activity of phytosphingosine.

INTRODUCTION

Two major cellular apoptotic pathways have been established that mediate apoptosis on exposure to different types of stimuli, such as anticancer drug, ionizing radiation, and viruses (1). One, which is activated by the most anticancer drugs, involves the disruption of mitochondrial membrane potential that leads to the release of cytochrome c, which can trigger caspase 9. The other is activated by the ligation of DRs (1) including TNF receptor gene superfamily. On activation, the DRs recruit the adaptor molecule FADD by the death domain that is also present on FADD, followed by the activation of caspase 8 (2, 3).

Caspase 8 directly activates downstream caspase, caspase 3. Caspase 9, which is activated after the release of cytochrome c from mitochondria, can also activate caspase 3 (2). The mitochondrial activation-mediated pathway has been shown to be required for Fas-induced apoptosis in certain cell types that are classified as type II cells. In these cells, Bid, “BH3 only” protein of the Bcl-2 family, mediates the release of cytochrome c from mitochondria initiated by caspase 8 activation. The release of cytochrome c and other apoptogenic factors from injured mitochondria have been shown to activate caspases (4), and the mitochondrial integrity appears to be regulated, in part, by members of the Bcl-2 family. In response to apoptotic signals, Bax, a proapoptotic member of this family, is redistributed from the cytosol to the mitochondria, where it decreases

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The abbreviations used are: DR, death receptor; FADD, Fas-associated death domain; TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase; DIOC(3), 3,3′-dihexylocarbocyanine iodide; PI, propidium iodide; FBS, fetal bovine serum.
membrane potential leading to cytochrome c release and caspase activation (5).

Recent discoveries have revealed that sphingolipids (ceramide, sphingosine, sphingosine 1-phosphate, phytosphingosine, and so forth) are highly bioactive compounds and are involved in diverse cell processes (6, 7). Ceramide and sphingosine are generated by sphingomyelinase-mediated hydrolysis of sphingomyelin, which resides in the plasma membrane (8). A variety of exogenous stimuli, such as TNF-α, anti-Fas antibody, IL-1β, and γ-radiation, rapidly increase intracellular levels of ceramide and sphingosine through sphingomyelinase, leading to modulation of various cellular events, including apoptosis, proliferation, and differentiation (8–10). Phytosphingosine is also one of the most widely distributed natural sphingoid bases, which is abundant in fungi and plants, and also found in animals including humans. Phytosphingosine is structurally similar to sphingosine; phytosphingosine possesses a hydroxyl group at C-4 of the sphingoid long-chain base, whereas sphingosine has a trans-double bond between C-4 and C-5. However, the physiological roles of phytosphingosine are largely unknown, although recent reports show that phytosphingosine exerts strong cytotoxic effects on Chinese hamster ovary cells and modulates the muscarinic acetylcholine receptor-mediated signal transduction pathway (11).

In this study, we investigated the cellular mechanisms of cell death in human cancer cells induced by phytosphingosine. We report here that phytosphingosine induces apoptotic cell death in human cancer cells both by caspase-dependent and -independent pathways. Our data provide a potential mechanism for the anticancer activity of phytosphingosine and suggest that phytosphingosine may have therapeutic potential for the treatment of human cancer.

**MATERIALS AND METHODS**

**Cell Culture.** Jurkat, human T-cell lymphoma (type II), and NCI-H460, human non-small cell lung cancer cells, were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in PRMI 1640 supplemented with 10% FBS, penicillin, and streptomycin.

**Materials.** Phytosphingosine was purchased from Doo-San Bio-Tech (Seoul, Korea). Polyclonal antibody to caspase 3 and monoclonal antibodies to PARP and cytochrome c were obtained from PharMingen (San Diego, CA), and polyclonal antibodies to caspase 8, 9, Bcl-2, Bax, and Bid were obtained from Santa Cruz (Santa Cruz, CA). The caspase 3 inhibitor, z-DEVD-fmk, the broad-spectrum caspase inhibitor, z-VAD-fmk, and the caspase 8 inhibitor, z-IETD-fmk were obtained from Calbiochem (San Diego, CA).

**Hoechst 33258 Staining.** Hoechst 33258 staining was performed as described previously (12). Briefly, cells were fixed with 4% paraformaldehyde for 30 min in room temperature and then washed once with PBS. Hoechst 33258 (50 ng/ml) was added to the fixed cells, incubated for 30 min at room temperature, and washed with PBS. Cells were mounted and examined by fluorescence microscopy. Apoptotic cells were identified by the condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. At minimum, 500 cells were counted for each treatment.

**Flow Cytometric Analysis of Apoptosis.** Apoptosis was identified and quantified by flow cytometry with PI staining. Both adherent and floating cells were collected after phytosphingosine treatment, washed with ice-cold PBS, and fixed with 70% ice-cold ethanol overnight at 4°C. Fixed cells were washed twice with PBS and treated with 1 mg/ml RNase for 30 min at 37°C. Cellular DNA was stained with 50 μg/ml PI in PBS containing 0.05% NP40. Cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). From the analysis of DNA histograms, the percentages of cells in different cell cycle phases were evaluated. Cells with DNA content less than the G1 phase (sub-G1) were taken as apoptotic cells.

**Extraction of DNA and Agarose Gel Electrophoresis.** Cells were treated with 5 or 10 μg/ml phytosphingosine and were lysed in a lysis buffer [20 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K]. DNA samples were extracted and separated by agarose gel electrophoresis.

**Western Blot Analysis.** Western blot analysis was performed as described (13). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-Cl (pH 8.0), 120 mM NaCl, and 0.1% NP40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (NEC) according to the manufacturer’s recommendation (Boston, MA).

**Measurement of Mitochondrial Membrane Potential.** Mitochondrial membrane potential was determined as the retention of the mitochondria-specific dye DiOC6(3). Cells were loaded with 30 nM DiOC6(3) during the last 30 min of phytosphingosine treatment. After removal of the medium, the cells were washed twice with PBS, and the concentration of retained DiOC6(3) was measured by flow cytometric analysis.

**Preparation of Cytosolic and Mitochondrial Fractions for Cytochrome c and Bax Translocation Measurement.** Cells were collected and washed twice in ice-cold PBS, resuspended in S-100 buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.9 mM MgCl2, 1 mM EGTA, 1 mM EDTA, and a mixture of protease inhibitors and] incubated on ice for 20 min. After a 20-min incubation on ice, the cells were homogenized with a glass Dounce and a loose pestle (Wheaton, Millville, NJ) for 70 strokes. Cell homogenates were spun at 1,000 × g to remove unbroken cells, nuclei, and heavy membranes. The supernatant was resupined at 14,000 × g for 30 min to collect the mitochondria-rich (the pellet) and the cytosolic (the supernatant) fractions. The mitochondria-rich fraction was washed once with the extraction buffer, followed by a final resuspension in lysis buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, and 1 mM EGTA] containing protease inhibitors for Western blot analysis.

**Immunofluorescence Analysis.** For immunofluorescence analysis, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, incubated with
25 nm Mitotracker Red CMXRos (Molecular Probes, Inc., Eugene, OR) for 30 min at room temperature, and then washed three times with PBS. Cells were then incubated with rabbit antihuman Bax diluted 1:200 in 5% FBS/PBS for 1 h at room temperature in a humidified chamber. Excess antibody was removed by washing the coverslips three times with PBS. Cells were then incubated with biotin-labeled goat antirabbit IgG (Zymed Laboratories Inc.) diluted 1:200 in 5% FBS/PBS, and for 1 h protected from light at room temperature. After washing three times with PBS, cells were incubated with FITC-streptavidin diluted 1:200 in 5% FBS/PBS for 4 h. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed by a confocal laser-scanning microscope (Leica Microsystems).

RESULTS
Phytosphingosine Induces Apoptosis in Human T-Cell Lymphoma and Non-Small Cell Lung Cancer Cells. To determine whether phytosphingosine leads to apoptotic cell death in type II cells, Jurkat and NCI-H460 cells were treated with different doses of phytosphingosine, and apoptotic cell death was analyzed by Hoechst 33258 staining, flow-cytometric cell death analysis, and DNA laddering. Fig. 1A shows that there is a dose- and a time-dependent increase of apoptotic cells after phytosphingosine treatment, as measured by Hoechst 33258 staining of nuclei fragmentation and condensation. Apoptosis in Jurkat cells were additionally confirmed by flow-cytometric analysis of cellular DNA contents after staining with PI (Fig. 1B). Phytosphingosine treatment increased cells with sub-G1 contents of DNA, reaching ~75% of the total Jurkat cells after 6 h of the treatment (10 μg/ml). Furthermore, phytosphingosine treatment resulted in internucleosomal DNA fragmentation in Jurkat cells, as evidenced by the formation of a DNA ladder in agarose gel (Fig. 1C). Taken together, these results demonstrate that phytosphingosine induces apoptotic cell death in human T-cell lymphoma and non-small cell lung cancer cells in a dose- and time-dependent manner.

Requirement of Caspase Activities during Phytosphingosine-induced Apoptosis. To determine the requirement of caspase activities during phytosphingosine-induced apoptosis of type II Jurkat and NCI-H460 cells, we used Western blot analysis to analyze the activation of various caspases (Fig. 2A). Treatment of cells with 5 μM phytosphingosine caused activa-

![Fig. 1 Phytosphingosine induces apoptosis in human T-cell lymphoma and lung carcinoma cells. A, Jurkat and NCI-H460 cells were treated with various concentrations of phytosphingosine for the times indicated. Cells were stained with Hoechst 33258, and apoptotic cells were measured by observation under a fluorescence microscope. Apoptotic cells, which contained condensed chromatin fragments, were scored and expressed as a percentage of the total cell number measured. Results from three independent experiments are shown as means; bars, ±SE. B, Jurkat cells were treated with 5 or 10 μg/ml of phytosphingosine for the times indicated. Apoptotic cells were measured by flow cytometric analysis after PI staining. The figure shows a representative result of three independent experiments with similar results. C) Jurkat cells were treated with 5 or 10 μg/ml of phytosphingosine for the time indicated. DNA samples were extracted from cells, subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

tion of caspase 8 in a time-dependent manner, as indicated by reduction in the intensity of the proenzyme. Activation of the caspase 9 by the phytosphingosine was also evidenced by the reduction in the intensity of the proenzyme. Activated caspase-3 was detected after phytosphingosine treatments as a double band representing a p19 proteolytic fragment and the active subunit p17, respectively. In addition, phytosphingosine caused a marked increase in cleavage products of PARP. In NCI-H460 cells, we could not detect cleaved form of caspase 3, and PARP. However, the decrease in proform enzymes was consistently observed. Caspase activation during the course of phytosphingosine-induced apoptosis was additionally confirmed by using a broad-spectrum caspase inhibitor, z-VAD-fmk, and a caspase 3-specific inhibitor, z-DEVD-fmk. As expected, these caspase inhibitors were able to prevent activation of caspases (Fig. 2B) and attenuate apoptosis (Fig. 2C), suggesting that phytosphingosine-induced apoptotic cell death is largely dependent on caspase activation.

Phytosphingosine-induced Caspase 8 Activation Is Independent on DRs. On the basis of the requirement of caspase activities, we first presumed that phytosphingosine induce apoptotic cell death through DR-mediated caspase 8 activation. However, we failed to detect induction of Fas, TNF-related apoptosis inducing ligand receptors (DR4 and DR5), and TNF receptors (TNFR1 and R2) in Jurkat cells treated with phytosphingosine (Fig. 3A). Furthermore, treatment of phytosphingosine did not affect interaction between FADD and caspase 8 (Fig. 3B), strongly suggesting that phytosphingosine-induced caspase 8 activation in these cells occurs in a DR-independent fashion.

Phytosphingosine Induces Caspase-independent Cytochrome c Release from Mitochondria. To evaluate the contribution of the mitochondrial pathway to the induction of apoptosis seen after phytosphingosine treatment, we examined changes in mitochondrial membrane potential and cytochrome c release into the cytosol in the phytosphingosine-treated type II
Jurkat cells. Fig. 4A shows that phytosphingosine treatment significantly disrupted mitochondrial membrane potential. At the same time, the level of cytosolic cytochrome c was markedly increased (Fig. 4B), coinciding with the changes in mitochondrial membrane potential. Caspase 8 has been reported to cleave Bid in type II cells in the presence of apoptotic stimuli. The truncated Bid then triggers the mitochondrial release of cytochrome c into the cytosol and activates caspase-9 (14). To investigate whether caspase 8 activation mediated by phytosphingosine precedes cytochrome c release, we examined Bid cleavage after phytosphingosine treatment. Fig. 4B shows that phytosphingosine did not cause any changes in Bid cleavage in type II Jurkat cells, suggesting that Bid is not involved in cytochrome c release after phytosphingosine treatment. As expected, however, treatment of anti-Fas antibody to the Jurkat cells caused a marked Bid cleavage as well as caspase 8 activation (Fig. 4C). These results suggest that Bid cleavage is not involved in phytosphingosine-induced cytochrome c release.

We next examined the effect of specific caspase inhibitors on phytosphingosine-induced cytochrome c release. Fig. 4, D and E, shows that the broad caspase inhibitor, z-VAD-fmk, and caspase 8 specific inhibitor, z-IETD-fmk, did not prevent the accumulation of cytochrome c in the cytosol of phytosphingosine-treated cells, suggesting that a caspase-independent pathway is involved in cytochrome c release after phytosphingosine treatment.

**Phytosphingosine-induced Cell Death Involves Alterations in the Intracellular Redistribution of Bax Protein.**

Because it has been shown that translocation of Bax from the cytosol to the mitochondria causes caspase activation, a decline of mitochondrial membrane potential and subsequent cytochrome c release (15), we investigated whether phytosphingosine treatment induces mitochondrial translocation of Bax. Fig. 5, A and B, shows that phytosphingosine treatment (5 μM) dramatically redistributes Bax from cytosol to the mitochondria without changing the protein expression levels of Bcl-2, Bcl-xl, and Bax. Indirect immunofluorescence staining of Bax clearly indicated its translocation to the mitochondria (Fig. 5C). In addition, Bax/Bcl-2 interaction was markedly decreased after addition of phytosphingosine (Fig. 5D). These results are consistent with previous data on Bax translocation in several different cell types in response to distinct forms of cell death stimuli (16). To test whether redistribution of Bax protein occurs in a caspase-independent manner, we examined the effect of specific caspase inhibitors on phytosphingosine-induced Bax translocation. The addition of the caspase inhibitors failed to prevent the intracellular redistribution of Bax (data not shown). These results suggest that phytosphingosine induces redistribution of the Bax protein to the mitochondria in a caspase-independent manner during the progression of apoptosis.

**DISCUSSION**

This study was undertaken to characterize the potential mechanism for the anticancer activity of phytosphingosine. The functional mechanism of anticancer activity of sphingosine has been studied extensively. However, despite structural similarity, the physiological roles of phytosphingosine are largely unknown. In this study, we found that phytosphingosine induces apoptotic cell death in human cancer cells through a direct pathway from caspase 8 activation and caspase-independent release of cytochrome c after intracellular redistribution of cell death activator Bax.

Phytosphingosine induces apoptotic cell death in type II Jurkat, a human T-cell lymphoma cell line, and in NCI-H460, a human non-small cell lung cancer cell line, in a concentration- and a time-dependent manner. In the presence of caspase inhibitors, phytosphingosine-induced apoptosis was almost complete...
pletely suppressed, suggesting that phytosphingosine-induced apoptosis is largely dependent on caspase activities. We observed that phytosphingosine causes the activation of caspase 8. However, we failed to detect changes in the expression of DRs (Fas, DR4, DR5, TNFR1, and R2), and the interaction between FADD and caspase 8 after treatment of phytosphingosine. This suggests that phytosphingosine-mediated caspase 8 activation and apoptosis are independent of DRs and/or FADD. Interestingly, we found that phytosphingosine-induced caspase 8 activation is not correlated with Bid cleavage in type II Jurkat cells. However, phytosphingosine did induce a decrease in mitochondrial membrane potential, cytochrome c release into cytosol, and activation of caspase-9. Furthermore, caspase-8 specific inhibitor, N-benzoyloxycarbonyl-IETD-fmk, did not inhibit these events, strongly suggesting that phytosphingosine-induced cytochrome c release from mitochondria. A, mitochondrial membrane potential was determined as the retention of the mitochondria-specific dye DiOC$_6$(3). Jurkat cells were loaded with 30 nM DiOC$_6$(3) during the last 30 min of phytosphingosine treatment. After removal of the medium, the concentration of retained DiOC$_6$(3) was measured by flow cytometric analysis. B, Jurkat cells were treated with 5 μg/ml of phytosphingosine for 3 and 6 h. Whole cell and cytosolic extracts were prepared and assessed by Western blot analysis for cytochrome c and Bid. The data represent a typical experiment conducted at least three times with similar results. C, Jurkat cells were treated with 50 ng/ml of anti-Fas antibody for 3 h. Cell lysates were prepared and assessed by Western blot analysis for caspase-8, -9, -3, and Bid. The data represent a typical experiment conducted three times with similar results. D, Jurkat cells were treated with 5 μg/ml phytosphingosine for 3 h in the presence or absence of z-VAD-fmk. Cytosolic extracts were prepared and assessed by Western blotting for cytochrome c. The data represent a typical experiment conducted three times with similar results. E, Jurkat cells were treated with 5 μg/ml phytosphingosine for 3 h in the presence or absence of z-IETD-fmk. Whole cell and cytosolic extracts were prepared and assessed by Western blotting for caspase 8, Bid, cytochrome c, and caspase 9. The data represent a typical experiment conducted three times with similar results.

Fig. 4 Phytosphingosine induces caspase-independent cytochrome c release from mitochondria.
tochrome c release from the mitochondria and caspase-9 activation are not associated with caspase-8 activation. Thus, it is possible that phytosphingosine-induced cytochrome c release may be initiated by another cytosolic factor(s) other than Bid. On the other hand, caspase-8 specific inhibitor z-IETD-fmk significantly blocked phytosphingosine-induced caspase 3 activation and apoptosis, which correlates well with the previous reports showing that caspase 8 directly activates caspase 3.

Bax translocation to the mitochondria has been shown to reduce mitochondrial membrane potential, enhance cytochrome c release from the mitochondria, and activate caspases (16). Our data show that phytosphingosine induced Bax translocation from the cytosol to the mitochondria without changing the protein levels of the Bax and Bcl-2. We also show that phytosphingosine markedly decreases the level of Bcl-2/Bax complex, suggesting that Bax might be released from Bcl-2/Bax complex by unknown mechanism. Recent data demonstrated that even slight structural modification, such as phosphorylation, can alter function(s) of Bcl-2 as an antiapoptotic molecule (19). In fact, Bcl-2 phosphorylation may cause either disruption of the intermolecular cooperation or increased susceptibility to the protein degradation mediated by intracellular proteolytic enzyme (19). However, we do not know exactly which molecules are involved in the modification of Bcl-2 and/or translocation of Bax protein at this point. Additional studies will be necessary to identify the exact mechanism of regulation of Bcl-2/Bax modulation and Bax translocation induced by phytosphingosine treatment.

Taken together, these results suggest that phytosphingosine induces apoptotic cell death in human cancer cells through caspase 8 activation and caspase-independent cytochrome c release mediated by Bax translocation to the mitochondria. We believe that this is the first report on the potential mechanism for the anticancer activity of phytosphingosine.

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