Suppression of Extracellular Signal-related Kinase and Activation of p38 MAPK Are Two Critical Events Leading to Caspase-8- and Mitochondria-mediated Cell Death in Phytosphingosine-treated Human Cancer Cells*

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We previously demonstrated that the phytosphingosine-induced apoptosis was accompanied by the concomitant induction of both the caspase-8-mediated and mitochondrial activation-mediated apoptosis pathways. In the present study, we investigated the role of mitogen-activated protein kinases (MAPKs) in the activation of these two distinct cell death pathways induced by phytosphingosine in human cancer cells. Phytosphingosine caused strong induction of caspase-8 activity and caspase-independent Bax translocation to the mitochondria. A rapid decrease of phosphorylated ERK1/2 and a marked increase of p38 MAPK phosphorylation were observed within 10 min after phytosphingosine treatment. Activation of ERK1/2 by pretreatment with phorbol 12-myristate 13-acetate or forced expression of ERK1/2 attenuated phytosphingosine-induced caspase-8 activation. However, Bax translocation and caspase-9 activation was unaffected, indicating that down-regulation of the ERK activity is specifically required for the phytosphingosine-induced caspase-8-dependent cell death pathway. On the other hand, treatment with SB203580, a p38 MAPK-specific inhibitor, or expression of a dominant negative form of p38 MAPK suppressed phytosphingosine-induced translocation of the pro-apoptotic protein, Bax, from the cytosol to mitochondria, cytochrome c release, and subsequent caspase-9 activation but did not affect caspase-8 activation, indicating that activation of p38 MAPK is involved in the mitochondrial activation-mediated cell death pathway. Our results suggest that phytosphingosine can utilize two different MAPK signaling pathways for amplifying the apoptosis cascade, enhancing the understanding of the molecular mechanisms utilized by naturally occurring metabolites to regulate cell death. Molecular dissection of the signaling pathways that activate the apoptotic cell death machinery is critical for both our understanding of cell death events and development of cancer therapeutic agents.

From the Laboratory of Radiation Effect and Laboratory of Experimental Pathology, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea, the Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea, and the Department of Biochemistry, College of Medicine, Hanyang University, Seoul 133-791, Korea

Apoptosis can be induced via two distinct intracellular signaling pathways, the death receptor-mediated or the mitochondrial activation-mediated pathway (1–3). The former pathway is triggered by ligation of death receptors such as Fas and TNFR. In this case, caspase-8 is recruited to the ligated receptors via FADD, an adaptor molecule, leading to its proteolytic activation. The activated caspase-8 in turn cleaves and activates caspase-3, which then initiates a set of events that culminate in apoptosis (1, 2). In contrast to the death receptor pathway, the mitochondrial pathway responds to anticancer drugs and multiple classes of environmental stresses (3). These stimuli cause redistribution of the proapoptotic Bcl-2 members, such as Bax, from the cytosol to mitochondria. This event is followed by reduction of the mitochondrial transmembrane potential and the release of mitochondrial cytochrome c to the cytosol. The released cytochrome c participates in the process leading to caspase-9 activation, followed by activation of caspase-3 (4). 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, a “BH3 only” protein of the Bcl-2 family, mediates the release of cytochrome c from mitochondria initiated by caspase-8 activation (5). Taken together, it appears that the significance of each of the apoptotic pathways varies depending on both stimulus and cell types.

The mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases, are mediators of intracellular signals in response to various stimuli. MAPKs include extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK. The ERK pathway is activated by mitogenic stimuli, such as growth factors, cytokines, and phorbol esters and plays a major role in regulating cell growth and differentiation (6–9). The activation of the ERK pathway is generally considered to be a survival signal induced by growth factors against apoptotic signals (10, 11). It has recently been reported that the ERK activation plays antiapoptotic roles in Fas-, TRAIL-, chemotherapeutic drug- and serum deprivation-induced apoptosis (12). Moreover, the MEK/ERK pathway

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** To whom correspondence should be addressed: Laboratory of Radiation Effect, Korea Institute of Radiological and Medical Sciences, Gongneung-Dong, Nowon-Ku, Seoul 139-706, Korea. Tel.: 82-2-970-1324; Fax: 82-2-977-0381; E-mail: sjlee@kcri.re.kr

† The abbreviations used are: TNFR, tumor necrosis factor receptor; FADD, Fas-associated death domain protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADPribos) polymerase; DIOC6(3), 3,3′-dihexyloxacarbocyanine iodide; Z-, benzyloxycarbonyl-; fmk, fluoromethylketone; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.
blocks apoptosis by inhibiting the activation of caspase-8, a key component of the death receptor pathway, induced by extrinsic stimulation of Fas, TNFR, and TRAIL receptor (13, 14). In contrast to ERKs, JNK and p38 MAPK are weakly activated by growth factors but respond strongly to a variety of stress signals including tumor necrosis factor, interleukin-1, ionizing and UV irradiation, hyperosmotic stress, and chemotherapeutic drugs (15–19). Activation of these kinases has shown to correlate well with the apoptosis induced by these stress stimuli. Additionally, studies using the inhibitors of JNK and p38 MAPK have suggested that JNK and/or p38 MAPK activation is necessary for UV-, cytokine-, ceramide-, and chemotherapeutic drug-induced apoptosis (20). However, the basis for cross-talk between MAPKs and apoptotic cell death machinery remains largely unclear.

Sphingolipid metabolites such as ceramides, sphingosines, and sphingosine 1-phosphates have emerged as key regulators of apoptosis (21). Ceramide is generated as a product of the sphingomyelin hydrolysis, which is triggered by a variety of apoptotic stimuli, including tumor necrosis factor-α, anti-Fas, and γ-radiation (22–27). Importantly, the apoptotic actions of these stimuli are attenuated when the ceramide generation is blocked. Moreover, direct exposure of cells to the cell-permeable ceramide analogs was shown to induce apoptosis, suggesting that ceramide can act as a mediator of apoptosis. It has been reported that ceramide induces apoptosis by the activation of the JNK signaling pathway (28). Ceramide can be further metabolized by ceramidase to sphingosine, which can then be phosphorylated by sphingosine kinase to form sphingosine 1-phosphate (29). Whereas sphingosine induces apoptosis in various cell types (30), sphingosine 1-phosphate antagonizes the apoptotic actions of ceramide (30).

Given the well established role of these three sphingolipid metabolites in apoptosis, other sphingolipid derivatives may also contribute to controlling apoptosis. Among these derivatives, phytosphingosine is one of the most widely distributed natural sphingoid bases that is abundant in fungi and plants and also found in animals including humans. The structure of phytosphingosine is very 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 role of phytosphingosine is largely unknown, although it has recently been reported that phytosphingosine exerts strong cytotoxic effects on Chinese hamster ovary cells and modulates the muscarinic acetylcholine receptor-mediated signal transduction pathway (31).

Previously, we have shown that phytosphingosine employs two distinct apoptotic pathways, the caspase-8-dependent and mitochondrial activation-dependent pathway, to induce apoptosis in two different cell types, human T cell lymphoma, Jurkat, and non-small cell lung cancer cell line, NCI-H460 (32). This bifurcation of the signaling pathway induced by phytosphingosine differs from those seen with other agents. Given this unique feature of phytosphingosine, further analysis of its action is expected to significantly advance understandings not only of physiological functions of sphingolipid but also of the apoptotic cell death control.

In this study, we investigate the mechanisms underlying the simultaneous induction of both the caspase-8-mediated and mitochondrial activation-mediated cell death by phytosphingosine. The data suggest that down-regulation of the ERK pathway is critical in the death receptor-independent activation of caspase-8, and activation of p38 MAPK is essential for the induction of mitochondrial translocation of Bax and cytochrome c release during phytosphingosine-induced apoptosis. Since phytosphingosine appears to utilize two distinctive MAPK signaling pathways for killing cells, understanding the signaling mechanisms of phytosphingosine-induced cell death may provide a better knowledge of the interrelationship between MAPKs and cell death machinery in mammalian cell systems.

**EXPERIMENTAL PROCEDURES**

**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 (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin, and streptomycin at 37 °C in a humidified incubator with 5% CO₂.

**Plasmids—**pCMV5-DN-p38 MAPK, an expression vector encoding the dominant-negative mutant of p38 MAPK, and pLCEP4-DN-ERK, the dominant-negative mutants of ERK, were gifts of Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA).

**Materials—**Phytosphingosine was purchased from Avanti (Alabaster, AL). 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 and -9, Bcl-2, Bax, Bid, and HSP60 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to phospho-p38 MAPK, caspase-3, and PARP were obtained from Cell Signaling Technology (Beverly, MA). The p38 MAPK-specific inhibitor, SB203580, a broad spectrum caspase inhibitor, Z-VAD-fmk, and the caspase-8 inhibitor, Z-IETD-fmk, were obtained from Calbiochem.

**Hoechst 33258 Staining—**Hoechst 33258 staining was performed as described previously (32). 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 propidium iodide 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 ng/ml propidium iodide in PBS containing 0.05% Nonidet P-40. Cells were then analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). From the analysis of DNA histograms, the percentages of cells in different phases of cell cycle 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/HCl, pH 8.0, 0.1% 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 (33). Briefly, cell lysates were prepared by extracting proteins with lysis buffer (40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet P-40) 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 ECL procedures (Amersham Biosciences) according to the manufacturer’s recommendation.

**Measurement of Mitochondrial Membrane Potential—**Mitochondrial membrane potential was determined as the retention of the mitochondrial-specific dye DiOC₆(3) (Molecular Probes, Inc., Eugene, OR). 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 relative amount of retained DiOC₆(3) was measured by flow cytometric analysis.

**Preparation of Cytosolic and Mitochondrial Protein Fractions for the Measurement of Cytochrome c and Bax Translocation—**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, mixture of protease inhibitors), and incubated on ice for 20 min. After a 20-min incubation on ice, the cells were homogenized with a...
Dounce glass homogenizer 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 spun again at 14,000 g for 30 min to collect the mitochondria-rich (pellet) and cytosolic (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% Nonidet P-40, 0.25% sodium deoxycholate, 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, and incubated with 25 nM Mitotracker Red CMXRos (Molecular Probes) for 30 min at room temperature and then washed three times with PBS. Cells were then incubated with rabbit anti-human 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 anti-rabbit IgG (Zymed Laboratories Inc.) diluted 1:200 in PBS/PBS, for 1 h protected from light at room temperature. After washing three times with PBS, coverslips were mounted onto microscopic slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed by a confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

Immunocomplex Kinase Assay—ERK activity in phytosphingosine-treated cell lysates was measured by the ability of these lysates to phosphorylate PHAS-1 (Stratagene, La Jolla, CA), a substrate for ERK (34). Briefly, cell lysates were prepared by extracting proteins with lysis buffer (40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet P-40) supplemented with protease inhibitors. Immunoprecipitation was performed by incubating 200 μg of lysate with anti-ERK-1 or ERK-2 antibody for 90 min and then adding 50 μl of protein A-agarose (Sigma) after a 90-min incubation at 4 °C. The immune complex was recovered by centrifugation and washed three times with lysis buffer and then two times with kinase reaction buffer (250 mM HEPES (pH 7.5), 10 mM MgCl₂, 200 μM Na₃VO₄). The immune complex was resuspended in kinase reaction buffer containing 120 μg of PHAS-1 substrate along with 2 μCi of [γ-³²P]ATP (ICN). The reaction was allowed to proceed for 30 min at room temperature and was terminated by the addition of SDS-loading buffer and boiling for 3 min. Phosphorylated PHAS was resolved on 12.5% PAGE gels, dried, and autoradiographed.

RESULTS

Phytosphingosine Induces Death Receptor-independent Caspase-8 Activation—We have previously shown that phytosphingosine induces apoptosis in the human cancer cell line, Jurkat. To confirm, we treated these cells with different doses of phytosphingosine, and induction of apoptotic cell death was analyzed. Fig. 1A shows that there is a dose- and time-dependent increase of apoptotic cells after the phytosphingosine treat-
ment, as measured by Hoechst 33258 staining for nuclei fragmentation and condensation. Several studies have suggested that activation of caspase-8 depends upon its oligomerization, which is mediated by the association of the DED domains of the adaptor molecule, FADD, and caspase-8. This association requires the interaction of FADD, either directly or indirectly with surface receptors such as Fas or TNFR, which possess death domains (1, 2). To determine whether death receptors are involved in phytosphingosine-induced apoptosis, we performed co-immunoprecipitation assays to analyze the association of FADD and caspase-8 in Jurkat cells after treatment with phytosphingosine. As shown in Fig. 1B, caspase-8 activation and interaction between FADD and caspase-8 were detected in cells treated with Ch11, a soluble Fas ligand. In contrast, caspase-8 was not found to be associated with FADD upon phytosphingosine-mediated activation of caspase-8. This suggests that caspase-8 activation triggered by phytosphingosine occurs in a death receptor-independent fashion.

**Phytosphingosine Induces Caspase-independent Bax Translocation, Cytochrome c Release, and Caspase-9 Activation in Human Cancer Cells**—We previously reported that treatment of cells with phytosphingosine promoted cytochrome c release and caspase-9 activation through acceleration of Bax translocation to the mitochondria (32). To determine whether phytosphingosine-induced Bax translocation is affected by the caspase-8 activation, we examined the effect of Z-IETD-fmk, a caspase-8-specific inhibitor, on the Bax translocation induced by phytosphingosine. As shown in Fig. 1C, phytosphingosine treatment dramatically redistributes Bax from the cytosol to mitochondria, and the addition of Z-IETD-fmk failed to prevent the intracellular redistribution of Bax. These results suggest that Bax translocation, cytochrome c release, and caspase-9 activation occur in a caspase-8-independent fashion during the apoptotic process triggered by phytosphingosine.

**Phytosphingosine Suppresses the ERK Signaling Pathway**—MAPKs have been implicated in regulation of apoptosis in response to various stimuli. To investigate a potential involvement of ERKs in phytosphingosine-induced cell death, we first examined their activities following phytosphingosine treatment. ERK1/2 activities were determined by immunoblot analysis using anti-phospho-ERK1/2 antibody to detect the activated phosphorylated forms of ERK1/2 in Jurkat and NCI-H460 cells. As shown in Fig. 2A, phytosphingosine treatment led to a dramatic down-regulation of phosphorylated ERK1/2. The phosphorylated ERK started to diminish within 2 min of the phytosphingosine treatment (5 or 10 μM, respectively, in Jurkat and NCI-H460 cells). Suppression of ERK activity was sustained for 3 or 6 h, respectively, in Jurkat and NCI-H460 cells, whereas the total expression level of ERK did not change significantly upon treatment with 5 or 10 μM phytosphingosine (Fig. 2B). Furthermore, PD98059 treatment markedly suppressed the phytosphingosine-induced caspase-8 and caspase-3 activation, PARP cleavage, and subsequent apoptosis (Fig. 3, A and B). However, cytochrome c release, caspase-9 activation, and Bax translocation triggered by phytosphingosine were not affected by PMA treatment (Fig. 3A). To further clarify the relationship between ERK activation and phytosphingosine-induced cell death, we employed a specific inhibitor of MEK, PD98059. A simultaneous treatment of cells with PD98059, PMA, and phytosphingosine effectively reversed the inhibitory effect of PMA and phytosphingosine on caspase-8 and caspase-3 activation, PARP cleavage, and subsequent apoptosis (Fig. 3A). Furthermore, PD98059 treatment reversed the inhibitory effect of PMA on caspase-3 and PARP cleavage (Fig. 3B). In fact, treating cells with PD98059 alone suppressed caspase-8, caspase-3 activation, and PARP cleavage caused by phytosphingosine (Fig. 3C).
To further investigate the relationship between ERK activity and caspase-8 activation, we examined effects of the ectopically expressed ERK1 and ERK2 on biochemical features of the phytosphingosine-induced apoptosis. As shown in Fig. 5A, caspase-8 activation induced by phytosphingosine treatment was significantly inhibited by overexpression of ERK1 or ERK2, whereas it did not affect the phytosphingosine-induced caspase-9 activation. Furthermore, ERK1 or ERK2 overexpression partially prevented the phytosphingosine-induced apoptotic cell death (Fig. 5B). In addition, ectopic expression of dominant negative forms of ERK1 or ERK2 significantly enhanced the phytosphingosine-induced cell death and caspase-8 activation and reversed the PMA-induced suppression of phytosphingosine-mediated cell death and caspase-8 activation (Fig. 5C). Taken together, the caspase-8-dependent apoptotic process induced by phytosphingosine requires suppression of the MEK/ERK signaling pathway. However, this signaling pathway does not seem to be critical in the mitochondria-mediated apoptosis pathway.

Selective Activation of p38 MAPK during Phytosphingosine-induced Apoptosis—To determine the role of p38 MAPK and JNK in phytosphingosine-induced apoptosis, we first analyzed the activation status of p38 MAPK and JNK by Western blot analysis with antibodies specific to the phosphorylated form of these two kinases. As shown in Fig. 6A, treatment with phytosphingosine resulted in a dramatic increase of the phosphoryl-

FIG. 3. PMA blocks the activation of caspase-8 by phytosphingosine but did not affect phytosphingosine-induced caspase-9 activation. A, Jurkat cells were treated with 5 μg/ml of phytosphingosine in the presence or absence of 100 nM PMA for 3 h. Cells were harvested, lysed, and subjected to Western blot analysis using anti-phospho-ERK1/2, caspase-8, caspase-9, caspase-3, or PARP antibodies. Cytosolic fraction was used for analysis of the cytochrome c expression, whereas Bax and HSP60 proteins were detected by Western blot analysis of the mitochondrial fraction. The data represent a typical experiment conducted three times with similar results. B, Jurkat cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number counted. Results from three independent experiments are shown as means ± S.E. The data represent a typical experiment conducted three times with similar results.

FIG. 4. PD98059 attenuates the inhibitory effect of PMA in phytosphingosine-induced cell death. Jurkat cells were pretreated with 100 nM PMA and/or 30 μM PD98059 for 30 min and then with 5 μg/ml phytosphingosine for 3 h. A, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy as described above. Results from three independent experiments are shown as means ± S.E. B, cell lysates were prepared, and Western blot analysis was performed using anti-caspase-8, caspase-3, or PARP antibodies. The data represent a typical experiment conducted three times with similar results.
ated form of p38 MAPK, indicating its activation in Jurkat as well as NCI-H460 cells. p38 MAPK activation was apparent at 5 min, peaked at 1 h after phytosphingosine treatment, and was sustained for 3 or 6 h, respectively in Jurkat and NCI-H460 cells. In contrast, the level of the phosphorylated form of JNK did not alter over the time course examined in both cell lines. The total cellular level of p38 MAPK and JNK also remained constant. These results suggest that phytosphingosine can selectively induce activation of p38 MAPK during the apoptotic process of human cancer cells.

Activation of p38 MAPK Is Independent of Caspase Activity—

It has been reported that p38 MAPK acts at a step prior to caspase activation during the apoptotic process induced by certain stimuli (35, 36). In disagreement with these reports, activation of p38 MAPK has been observed to be dependent on caspase activity under different experimental conditions (37). To determine whether caspase activities are required for the p38 MAPK activation in phytosphingosine-mediated apoptosis, we examined the effect of a broad spectrum caspase inhibitor, Z-VAD-fmk, on the p38 MAPK activation by phytosphingosine. As shown in Fig. 6B, phytosphingosine-induced p38 MAPK activation was not inhibited by Z-VAD-fmk. Additionally, Z-DEVD-fmk, a caspase-3-specific inhibitor, also did not affect the activation of p38 MAPK induced by phytosphingosine (data

Fig. 5. Overexpression of ERK1 or ERK2 inhibits phytosphingosine-induced caspase-8 activation and apoptosis but not caspase-9 activation. A, NCI-H460 cells were transfected with either empty vector (PCEP4) or the expression vector for ERK1 or ERK2. These cells were treated with 5 μg/ml phytosphingosine for 3 h. Cell lysates were prepared, and Western blot analysis was performed using anti-phospho-ERK1/2, caspase-8, or caspase-9 antibodies. The data represent a typical experiment conducted three times with similar results. B, control and ERK1 or ERK2-overexpressing cells were treated with 5 μg/ml phytosphingosine for 3 h. These cells were stained with Hoechst 33258 dye and analyzed as described above (Fig. 3B). Results represent averages of three independent experiments ± S.E. C, NCI-H460 cells overexpressing the dominant negative form of ERK1 or ERK2 were treated with or without 100 nM PMA for 30 min followed by 5 μg/ml phytosphingosine for 3 h. After staining with Hoechst 33258, apoptotic cells were quantitated as described above. Results represent averages of three independent experiments ± S.E. (upper panel). Cell lysates were subjected to Western blot analysis using anti-caspase-8 antibody (bottom panel). The data represent a typical experiment conducted three times with similar results.
not shown). However, both inhibitors completely blocked the phytosphingosine-induced caspase activation and apoptotic cell death (data not shown). These data strongly suggest that induction of the p38 MAPK activity by phytosphingosine is independent of caspase activation, and it is likely to be an upstream event of caspase activation in phytosphingosine-induced apoptotic cascade.

Interference with the p38 MAPK Pathway Prevents Induction of Mitochondria-mediated Apoptosis by Phytosphingosine—Using a p38 MAPK inhibitor, SB203580, we investigated effects of the p38 MAPK inhibition on apoptotic cell death induced by phytosphingosine. As shown in Fig. 7A, SB203580, at a concentration sufficient to completely inhibit p38 MAPK activity, inhibited two representative aspects of apoptosis (38), namely loss of mitochondrial transmembrane potential and increase in the plasma membrane permeabilization. Moreover, preincubation with SB203580 markedly inhibited DNA fragmentation and apoptotic cell death induced by phytosphingosine (Fig. 7, B and C). These data indicate that p38 MAPK is involved in the mitochondria-mediated apoptotic process induced by phytosphingosine.

Activation of p38 MAPK Is Required for Phytosphingosine-mediated Release of Cytochrome c and Intracellular Redistribution of the Cell Death Activator, Bax—We next investigated effects of inhibition of the p38 MAPK pathway on the caspase activation induced by phytosphingosine. As shown in Fig. 8, A and B, treatment with SB203580 or forced expression of a dominant negative form of p38 MAPK did not alter the caspase-8 cleavage induced by phytosphingosine. However, the phytosphingosine-induced caspase-9 activation was completely blocked by SB203580 treatment or by overexpression of a dominant negative p38 MAPK. These results suggest that activation of p38 MAPK is required for the caspase-9 but not for the caspase-8 activation during phytosphingosine-induced apoptosis. Furthermore, p38 MAPK may regulate some aspects of mitochondrial function, such as cytochrome c release in response to phytosphingosine, as proposed by other studies using different experimental systems (39, 40). Therefore, we investigated the role of p38 MAPK in cytochrome c release from mitochondria induced by phytosphingosine treatment. As predicted, SB203580 treatment or overexpression of dominant negative p38 MAPK effectively blocked the phytosphingosine-induced cytochrome c release (Fig. 8, A and B). These results indicate that p38 MAPK acts as an important mediator of the phytosphingosine-induced cytochrome c release from mitochondria, independent of caspase-8 activation.

We next studied whether p38 activation is required for the mitochondrial translocation of Bax proteins after phytosphingosine treatment. The intracellular redistribution of Bax proteins was evaluated by immunoblot analysis of mitochondrial fractions and by confocal laser-scanning microscopy. Phytosphingosine treatment (5 μg/ml) induced significant changes in distribution of Bax proteins (Fig. 8). In contrast, inhibition of the p38 MAPK pathway by SB203580 or by ectopic expression of a dominant negative p38 MAPK dramatically suppressed the phytosphingosine-induced mitochondrial translocation of Bax proteins in Jurkat cells (Fig. 8, A and B). In agreement with these data, SB203580 effectively blocked the phytosphingosine-induced cytochrome c release from mitochondria, independent of caspase-8 activation.

Fig. 6. Phytosphingosine induces caspase-independent activation of p38 MAPK in Jurkat or NCI-H460 cells. A. Jurkat and NCI-H460 cells were treated with 5 or 10 μg/ml of phytosphingosine for the time periods indicated. Cell lysates were prepared, and Western blot analysis was performed using anti-p38 MAPK, phospho-p38 MAPK, JNK, or phospho-JNK antibodies. The data represent a typical experiment conducted at least three times with similar results. B. Jurkat cells were treated with 5 μg/ml phytosphingosine for the time indicated in the presence or absence of 30 μM z-VAD-fmk. Expression of p38 MAPK and phosphorylated p38 MAPK was analyzed by Western blot analysis. The data represent a typical experiment conducted three times with similar results.
gosine-induced Bax translocation to mitochondria observed by confocal laser-scanning microscopy (Fig. 8C). Interestingly, PD98059, a MEK inhibitor, which enhanced phytosphingosine-induced apoptotic cell death, did not affect the intracellular redistribution of Bax proteins (data not shown). Taken together, these results suggest that activation of the p38 MAPK signaling pathway is critical in regulating translocation of Bax proteins from the cytosol to mitochondria during phytosphingosine-induced apoptosis.

**DISCUSSION**

The functional mechanism of anticancer activity of ceramide or sphingosine has been studied extensively. Despite the structural similarity to ceramide and sphingosine, however, physiological roles of phytosphingosine are still largely unknown. Moreover, the functional mechanism by which phytosphingosine induces apoptotic cell death is unclear. In this study, we found that phytosphingosine treatment resulted in a marked attenuation of ERK activity and a rapid activation of p38 MAPK. We also demonstrated that suppression of the ERK pathway is critical in regulating translocation of Bax proteins from the cytosol to mitochondria during phytosphingosine-induced apoptosis.

It has been known that the ERKs are activated in response to various growth factor stimulations, whereas the JNK and p38 MAP kinase are activated by various forms of stress signals (41). These studies support the general view that activation of the ERK pathway delivers a survival signal that counteracts proapoptotic effects associated with JNK and p38 MAPK activation (42–47). The prosurvival function of ERK was demonstrated again in a recent report that showed that inhibition of ERK signaling leads to an increased sensitivity to cisplatin in ovarian cancer cells (48). In addition, activation of the MEK/ERK signaling pathway in activated Jurkat cells has been shown to suppress TRAIL-mediated apoptosis in a similar fashion as it suppresses Fas-mediated apoptosis (13).

It is now well established that an exogenous treatment of sphingosine causes apoptosis in a variety of leukemic or solid tumor cell lines, and a large body of knowledge has been accumulated regarding the role of MAPKs in sphingosine-induced apoptosis. For instance, sphingosine treatment results in complete inhibition of ERK activity in leukemic and solid cancer cells (49–51), indicating that inhibition of the ERK pathway may be required for sphingosine-induced apoptosis. In contrast to sphingosine or ceramide, sphingosine 1-phosphate promotes cell survival in response to various apoptotic stimuli.
such as tumor necrosis factor-α, Fas ligation, serum deprivation, ionizing radiation, anticancer drugs, or ceramide (30, 52–61). The protective effect of sphingosine 1-phosphate from cell death stimuli is accomplished through the activation of the ERK pathway (23, 62). In the present study, we provide evidence that inhibition of the MEK/ERK signaling pathway is important for induction of apoptotic cell death by phytosphingosine in Jurkat and NCI-H460 cells. We demonstrate that inhibition of the MEK/ERK signaling pathway is required for the phytosphingosine-induced caspase-8 activation. A rapid decrease of phosphorylated-ERK1/2 is observed within 10 min after treatment with phytosphingosine. Pretreatment with PMA or overexpression of ERK1/2 attenuates phytosphingosine-induced caspase-8 activation and apoptotic cell death but does not affect Bax translocation or caspase-9 activation, indicating that suppression of ERK activity during phytosphingosine-induced apoptosis is required only for the caspase-8 activation. We have previously reported that the phytosphingosine-mediated caspase-8 activation and apoptosis were independent of the death receptors and/or FADD (32). In this study, we show that the adaptor molecule, FADD, and caspase-8 do not interact during phytosphingosine-induced apoptosis. However, both, the precursor and activated forms of caspase-8 co-precipitate with FADD after treatment with soluble Fas ligand. These results support the emerging hypothesis that caspase-8 activation is not strictly dependent on death receptor engagement. In death receptor-mediated apoptosis, caspase-8 activity is often responsible for the cleavage of a cytosolic substrate (e.g. Bid), which then triggers the cytochrome c release from mitochondria. In contrast, phytosphingosine induced cy-
Role of MAPKs in the Phytosphingosine-induced Apoptosis

FIG. 9. Schematic model of the roles of MAPKs in phytosphingosine-induced cell death. Phytosphingosine suppresses ERK1/2 and activates p38 MAPK. Suppression of ERK1/2 induces the activation of caspase-8, which leads to caspase-3 activation resulting in apoptotic cell death. Activation of p38 MAPK induces translocation of Bax from the cytosol to mitochondria, reducing mitochondrial membrane potential. This leads to cytochrome c release, which results in caspase-9 and -3 activation and, eventually, apoptotic cell death. Since phytosphingosine appears to utilize two different signaling pathways for killing cells, understanding the signaling mechanisms of phytosphingosine-induced cell death may provide the basis for cross-talk between MAPKs and the apoptotic machinery.
Role of MAPKs in the Phytosphingosine-induced Apoptosis

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