Phytosphingosine in combination with TRAIL sensitizes cancer cells to TRAIL through synergistic up-regulation of DR4 and DR5

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Received June 16, 2006; Accepted October 3, 2006

Abstract. Sensitization of cancer cells to TRAIL could improve the effectiveness of TRAIL as an anticancer agent. We explored whether TRAIL in combination with phytosphingosine could sensitize cancer cells to TRAIL. The combined treatment enhanced synergistic apoptotic cell death of Jurkat T cells, compared to TRAIL or phytosphingosine alone. Enhanced apoptosis in response to the combination treatment was associated with caspase-8 activation-mediated Bax and Bak activation and mitochondrial dysfunction. The combination treatment also resulted in synergistic up-regulation of TRAIL receptor R1 (DR4) and R2 (DR5). siRNA targeting of DR5 significantly attenuated the combination treatment-induced caspase-8 activation, mitochondrial dysfunction, and apoptotic cell death. Upon stimulation of cells with the combination treatment, NF-κB was activated. Moreover, siRNA targeting of NF-κB significantly attenuated the combination treatment-induced DR4 and DR5 expression and receptor-mediated caspase-8 activation. These results indicate that phytosphingosine sensitizes cancer cells to TRAIL through the synergistic up-regulation of DR4 and DR5 in an NF-κB-dependent fashion resulting in caspase-8 activation and subsequent mitochondrial dysfunction. These findings support the potential application of combination treatment with TRAIL and phytosphingosine in the treatment of cancers that are less sensitive to TRAIL.

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Abbreviations: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; DR, death receptor; DcR, decoy receptor; FADD, Fas-associated death domain protein; PARP, poly(ADP-ribose) polymerase; $DiOC_6(3)$, 3,3'dihexylocarbocyanine iodide; siRNA, small interfering RNA

Key words: Phytosphingosine, TRAIL, DR4, DR5

Introduction

Cytotoxic molecules have been considered as potential new therapeutics for drug-resistant cancer cells. Tumor necrosis factor (TNF)[1]-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, which includes TNF, FasL, CD27L, OX40, CD30L and CD40L. All members in this superfamily are type II membrane proteins, and many of them play an important role in regulating important biological processes such as apoptosis, cytokine production, and other cellular activities (1,2). Whereas other anti-cancer molecules such as TNF-α and Fas3L cause life-threatening toxicities, TRAIL induces apoptosis in various tumor cells but not in normal cells (3-5). Due to its apparent highly selective tumoricidal activity, TRAIL may be useful as a new therapeutic agent against cancer (6-8).

TRAIL can interact with at least 4 cell membrane-anchored receptors, including the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which contain an intracytoplasmic death domain and are therefore capable of triggering the apoptotic death signal, and the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which do not contain an intact cytoplasmic death domain and are therefore incapable of transmitting a death signal. Triggering of proapoptotic receptors by TRAIL induces recruitment of a DISC consisting of FADD and procaspase-8, resulting in cleavage and activation of caspase-8 followed by proteolytic initiation of effector caspases, such as caspase-3 (9).

Although TRAIL is a potent inducer of apoptosis in certain tumor cell types, some cancer cells are not sensitive to TRAIL-mediated apoptosis (10-12). We previously showed that the expression levels of DR5, the activation levels of caspase-8 and -3 and RIP were critical factors in determining TRAIL-sensitivities in Jurkat T cells (12). Interestingly, *in vitro* application of chemotherapeutic drugs augments the apoptotic activity of TRAIL in a variety of tumors, such as Kaposi's

sarcoma, breast carcinoma, leukaemia and glioma (10,13-16). Conflicting results for the acting mechanism of combined treatment of TRAIL and apoptosis-inducing chemotherapeutic agents have been reported. It was suggested that activation of TRAIL receptor was directly involved in etoposide- and radiation-mediated cell death in certain cancer cells (17,18). On the other hand, the combined treatment of TRAIL and topoisomerase I inhibitor CPT-11 induced synergistic cytotoxicity in prostate cancer cells through regulation of Bcl-2 family proteins and potent activation of caspases (19).

Sphingolipid metabolites such as ceramides, sphingosines, sphingosine 1-phosphates, and phytosphingosine have emerged as key regulators of apoptosis (20). Previously we observed that phytosphingosine, a long-chain sphingoid base, induces apoptosis in human T cell leukemia and non-small cell lung carcinoma cells (21,22). Moreover, phytosphingosine in combination with ionizing radiation synergistically enhanced radiation response in radiation-resistant cancer cells (23). Although many reports emphasized the contributions of sphingolipid metabolites in radiation-induced cell death, the role in the modulation of anticancer drug sensitivity and their precise action mechanisms were largely unknown.

Here we explored whether phytosphingosine sensitizes TRAIL-induced apoptotic cell death in human cancer cells. We showed that phytosphingosine in combination with TRAIL synergistically enhanced TRAIL-induced apoptotic cell death in human T cell leukemia through the enhancement of DR expression in an NF-κB-dependent fashion. The mechanism that we elucidated in this study may provide insight into the design of future combination therapies for cancers that are less sensitive to TRAIL treatment.

Materials and methods

Cells and reagents. Jurkat, human T cell lymphoma (Type II), FADD-deficient Jurkat T cells, and NCI-H460, human non-small cell lung cancer cell lines, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and routinely maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2 mM glutamine, 100 mg/ml penicillin/streptomycin (Gibco BRL). Cells were maintained at 37˚C in a humidified atmosphere with 5% CO_2. Recombinant TRAIL was a gift from Dr Byung-Ha Oh at POSTECH, Korea. Antibodies to caspase-8, caspase-9, caspase-3 and PARP were purchased from Upstate Biotechnology. Polyclonal antibody to caspase-3 and monoclonal antibodies to PARP and cytochrome *c* were obtained from Pharmingen (San Diego, CA, USA). Polyclonal antibody to Bid was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ß-actin and α-tubulin was purchased from Sigma. z-IETD-fmk was obtained from Calbiochem (San Diego, CA, USA).

Hoechst 33258 staining. Hoechst 33258 staining was performed as described previously (23). Briefly, cells were fixed with 4% paraformaldehyde for 30 min at 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.

*Measurement of mitochondrial membrane potentia*l. For the measurement of mitochondrial membrane potential, cells were incubated in 30 nmol/l 3,3'-dihexyloxacarboxyanine iodide [$DiOC_6(3)$] and 10 μmol/l at 37˚C for 30 min and harvested by trypsinization and washed with cold PBS solution three times. Mitochondrial membrane potential was then analyzed with a FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ).

Surface TRAIL receptor expression. Cells ($0.5x10^6$) were resuspended in 100 μl of DR4 and DR5 antibodies from Santa Cruz Biotechnology at dilutions of 1:500 and incubated for 30 min on ice. After washing with PBS/1% BSA, the cells were resuspended in 200 μl of an FITC conjugated anti-goat and anti-mouse antibody from Molecular Probe (Dianova, Hamburg, Germany) at dilutions of 1:200 and incubated for a further 30 min on ice. After washing twice, the cells were resuspended in 200 μl PBS and quantified by FACS with FL-1 (560 SP) employing a FACScan flow cytometer (Beckton Dickinson).

Isolation of cytosolic and mitochondrial fractions. Cells were collected and washed twice in ice-cold PBS, and were resuspended in S-100 buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and a mixture of protease inhibitors] and incubated on ice for 20 min. After 20-min incubation on ice, the cells were homogenized with a Dounce glass homogenizer with a loose pestle (Wheaton, Millville, NJ, USA) for 70 strokes. Cell homogenates were spun at 1000 x g to remove unbroken cells, nuclei and heavy membranes. The supernatant was respun at 14,000 x g for 30 min to collect the mitochondria-rich (the pellet) and cytosolic (the supernatant) fractions.

Western blot analysis. Western blot analysis was performed as described (17). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-Cl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] 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 (ECL) procedures (NEN) according to the manufacturer's recommendations.

Flow cytometric analysis of Bax and Bak activation. Bak- or Bax-associated conformational changes were assessed as previously described (24). Briefly, after fixation (0.25% PFA, 5 min) and washing, cells were incubated for 30 min in the presence of digitonin (100 μg/ml) with antibodies recognizing N-terminal epitopes of Bak (AM03. TC100; Oncogene Research Products) or Bax (clone 6A7; Pharmingen). After incubation with an FITC-conjugated anti-mouse antibody for

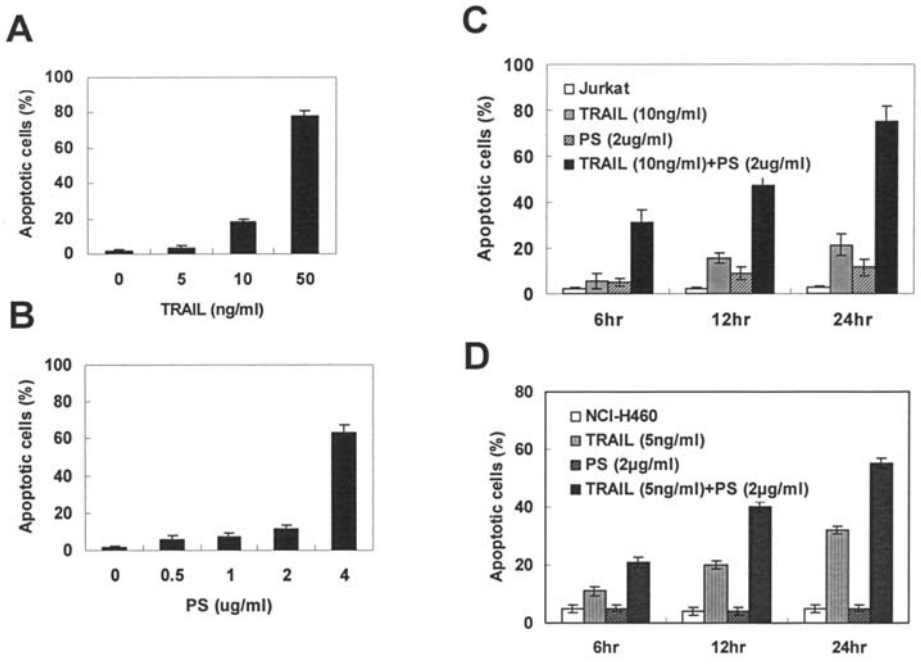

Figure 1. Combination treatment with TRAIL and phytosphingosine induced synergistic apoptotic cell death in Jurkat T cells. (A) TRAIL-induced apoptosis. Jurkat T cells were treated with increasing doses of TRAIL (5, 10, and 50 ng/ml), and cultured for 24 h. Cells were stained with Hoechst 33258, and apoptotic cells were analyzed by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell numbers measured. Results from three independent experiments are shown as means ± SEM. (B) Phytosphingosine-induced apoptosis. Jurkat T cells were treated with increasing doses of phytosphingosine (0.5, 1, 2, and 4 μg/ml). After 24 h, cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± SEM. (C) Phytosphingosine sensitizes Jurkat T cells to TRAIL-induced apoptotic cell death. Jurkat T cells were treated with 10 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 6, 12, and 24 h, cells were stained with Hoechst 33258 and apoptotic cells were measured by fluorescence microscopy. Results from three independent experiments are shown as means ± SEM. (D) Phytosphingosine sensitizes NCI-H460 cells to TRAIL. Cells were treated with 5 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or a combination of TRAIL (5 ng/ml) and phytosphingosine (2 μg/ml). After 6, 12, and 24 h, cells were stained with Hoechst 33258 and apoptotic cells were measured by fluorescence microscopy. Results from three independent experiments are shown as means ± SEM.

30 min, cells (10,000 per sample) were analyzed on a FACScan flow cytometer, using Cell Quest software.

Small interfering RNA (siRNA) transfection. RNA interferences of DR5, p53 and NF-κB were performed using 21-base pair (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Ambion (Austin, TX). The nucleotide sequence for DR5 siRNA was AAGACCCTTGTGCTCGTTGTCdTdT and p53 siRNA was GGCCUUGGAACUCAAGGAUtt. The nucleotide sequence for NF-κB siRNA was CCAUCAACUA UGAUGAGUUtt. A control siRNA specific to the green fluorescent protein DNA sequence CCACTACCTGAGCAC CCAGdTdT was used as a negative control. For transfection, Jurkat T cells were seeded in 10-cm dishes at 30% confluency, and siRNA duplexes (200 nM) were introduced into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Preparation of nuclear extracts. Cells were washed with PBS, harvested, pelleted, and resuspended in lysis buffer [10 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 20 mg/ml aprotinin, 20 mM leupeptin, and 0.5% NP-40]. After 5 min on ice, the lysates were centrifuged at 2500 rpm at 4˚C for 5 min. The supernatant was removed and the pellet was briefly washed in lysis buffer free of NP-40, and centrifuged at 1200 rpm. The pellet was resuspended in an equal volume of nuclear extract buffer [20 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol). After a 10-min incubation at 4˚C, the suspension was briefly vortexed and centrifuged at 14000 rpm for 5 min. The supernatant was removed, and used as a nuclear extract. Protein concentrations were determined by the same method as described for whole-cell extracts.

Electrophoretic mobility shift assays (EMSA). The sequence of probes was as follows: NF-κB binding site 5'-CAGAGGG ACTTTCCGAGA-3. The oligonucleotides were labeled with γ-[^{32}P]ATP and T4 polynucleotide kinase. The labeled probes were then incubated with nuclear extract at RT for 30 min. The reaction mixture consisted of γ-[^{32}P]ATP-labeled deoxy-oligonucleotides (45000 cpm), 2 mg of poly(dl-dc) and 10 mg of nuclear protein extract with 10 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 12.5% glycerol. After incubation for 30 min at RT, the reaction mixtures were electrophoresed on a 5% polyacrylamide gel with 0.5 TBE (100 V for 1 h). The gel was dried and auto-radiographed. Nuclear extracts were also incubated with a 10- or 100-fold molar excess of cold competitor

Results

Combination treatment of TRAIL with phytosphingosine enhanced synergistic apoptotic cell death in Jurkat T cells. We first examined TRAIL or phytosphingosine-induced

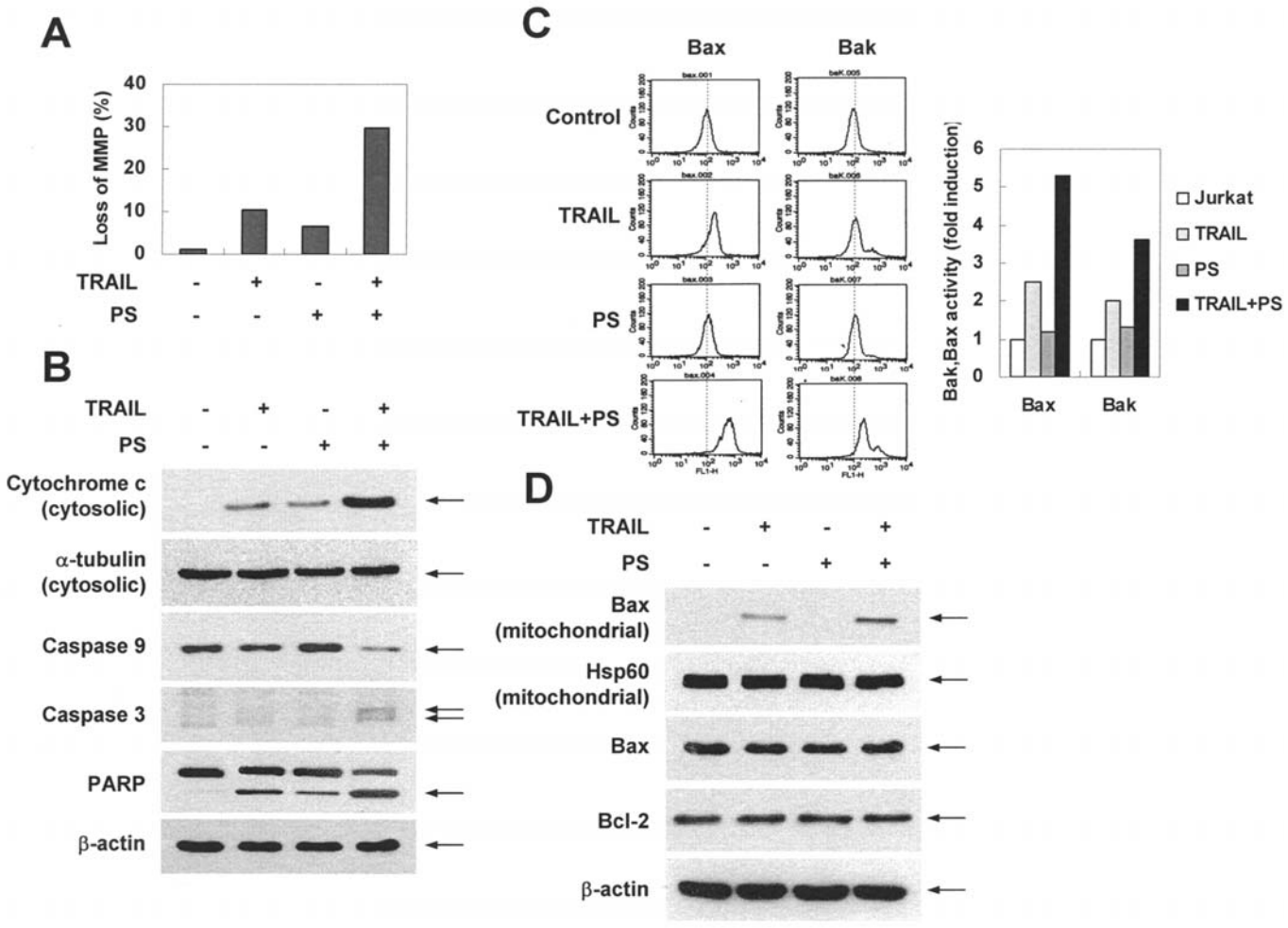

Figure 2. The combination treatment induced apoptotic conformational changes of Bax and Bak, and mitochondrial membrane potential loss. Jurkat T cells were treated with 10 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). (A) Mitochondrial transmembrane potential loss by the combination treatment. After 24 h, the mitochondrial transmembrane potential of the cells treated with TRAIL and/or phytosphingosine was determined by retention of $DioC_6(3)$ added during the last 30 min of treatment. After removal of the medium, the amount of retained $DioC_6(3)$ was measured by flow cytometry. (B) After 24 h, cytochrome *c* release from mitochondria into the cytosol, caspase-9, and -3 activation, and PARP cleavage were determined by Western blot analysis with anti-cytochrome *c*, -caspase-9, -caspase-3, and -PARP antibodies. α-tubulin was used as a cytosolic marker protein. (C) The combination treatment-induced apoptotic conformation of Bax and Bak. Conformational changes of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitope of Bak or Bax as described in Materials and methods. (D) Analysis of Bax translocation by subcellular fractionation. After 3 h, mitochondrial fraction and total cell lysates were subjected to Western blot analysis with anti-Bax and -Bcl-2 antibodies. HSP 60 and ß-actin were used as a mitochondrial marker protein and loading control, respectively.

apoptotic cell death. Jurkat T cells were treated with TRAIL or phytosphingosine in a range of 10 to 100 ng/ml and 0.5 to 4 μg/ml, respectively, for 24 h, and cell death was measured by Hoechst 33258 staining. As shown in Fig. 1A, at the concentration of 50 ng/ml of TRAIL, approximately 80% of Jurkat cells were killed, but 10 ng/ml of TRAIL showed less than 20% of cytotoxicity. Phytosphingosine treatment at the concentration of 2 μg/ml did not induce apoptotic cell death (Fig. 1B). We next examined whether TRAIL treatment in combination with phytosphingosine had a sensitizing effect on cell death in Jurkat T cells. As shown in Fig. 1C, the combination treatment with 10 ng/ml TRAIL and 2 μg/ml phytosphingosine indeed synergistically enhanced the apoptotic cell death of Jurkat T cells. The combination treatment-induced synergistic apoptotic cell death was also observed in human non-small cell lung cancer cells (Fig. 1D). The synergistic cytotoxic effect was constantly observed in 12 h as well as 24 h after the combination treatment. These results indicated that phytosphingosine treatment could sensitize human cancer cells to TRAIL.

The combination treatment induced Bax and Bak activation-mediated mitochondrial dysfunction. To determine the contribution of the mitochondrial pathway to the induction of apoptotic cell death observed by the combination treatment of TRAIL with phytosphingosine, we examined changes in mitochondrial membrane potential, cytochrome *c* release from mitochondria into the cytosol, and subsequent activation of caspase-9 and -3. Fig. 2A shows a marked loss of mitochondrial membrane potential in Jurkat T cells treated with TRAIL and phytosphingosine. At the same time, the level of the cytosolic cytochrome *c* was markedly increased (Fig. 2B), coinciding with changes in mitochondrial membrane potential. Combination treatment also caused activation of caspase-9 and -3 and cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 2B). However, treatment of cells with phytosphingosine (2 μg/ml)

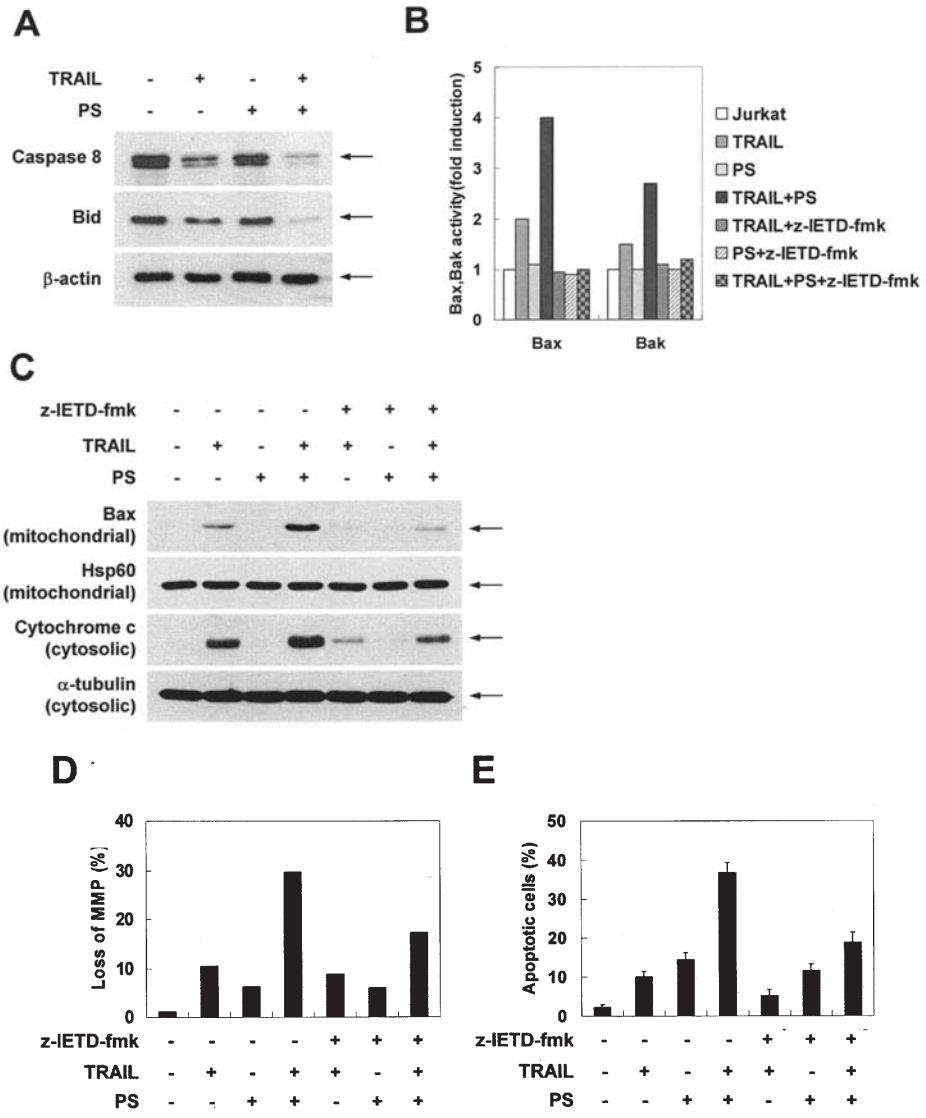

Figure 3. Enhanced activation of caspase-8 and Bid contributed to Bax and Bak activation and subsequent mitochondrial dysfunction after the combination treatment. (A) Activation of caspase-8 and Bid after the combination treatment. Jurkat T cells were treated with 10 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, caspase-8 activation and Bid cleavage were determined by Western blot analysis with anti-caspase-8 and -Bid antibodies. ß-actin was used as a loading control. (B) Caspase-8 dependent activation of Bax and Bak. Jurkat T cells were treated with 10 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml) in the presence or absence of 30 μM of z-IETD-fmk. Conformational changes of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitope of Bak or Bax as described in Materials and methods. (C) Analysis of Bax translocation and cytochrome *c* release by subcellular fractionation in the presence or absence of 30 μM of z-IETD-fmk. After 3 h, mitochondrial and cytosolic fractions were subjected to Western blot analysis with anti-Bax and -cytochrome *c* antibodies. HSP60 and α-tubulin were used as mitochondrial and cytosolic marker proteins, respectively. (D) Mitochondrial transmembrane potential loss by the combination treatment in the presence or absence of 30 μM of z-IETD-fmk. After 24 h, mitochondrial transmembrane potentials of the cells were determined by retention of $DioC_6(3)$ added during the last 30 min of treatment. After removal of the medium, the amount of retained $DioC_6(3)$ was measured by flow cytometry. (E) The combination treatment-induced cell death in the presence or absence of 30 μM of z-IETD-fmk. After 24 h, cells were stained with Hoechst 33258 and apoptotic cells were measured by fluorescence microscopy. Results from three independent experiments are shown as means ± SEM.

or TRAIL treatment alone (10 ng/ml) showed a subtle effect on cell death. These results suggest that mitochondrial dysfunction-mediated release of cytochrome *c* and subsequent caspase activation might be involved in the sensitization effect of the combination treatment in Jurkat T cells.

Since it has been shown that the proapoptotic Bcl-2 family members, Bax and Bak are crucial to the mitochondrial dysfunction (25,26), we investigated whether combination treatment with TRAIL and phytosphingosine induces activation of Bax or Bak. To determine activation of Bax and Bak after the combination treatment, flow cytometric analysis was performed with antibodies recognizing N-terminal epitope of Bax or Bak. As shown in Fig. 2C, the combination treatment resulted in conformational changes of both Bax and Bak, seen as shifts to the right in the resulting histograms. Moreover, combination treatment with TRAIL and phytosphingosine also resulted in a marked redistribution of Bax from cytosol to the mitochondria without changing the total protein expression levels of Bax (Fig. 2D). In addition, we failed to detect any changes in the level of anti-apoptotic protein Bcl-2. These results indicate that combination treatment with TRAIL and phytosphingosine synergistically enhances mitochondrial

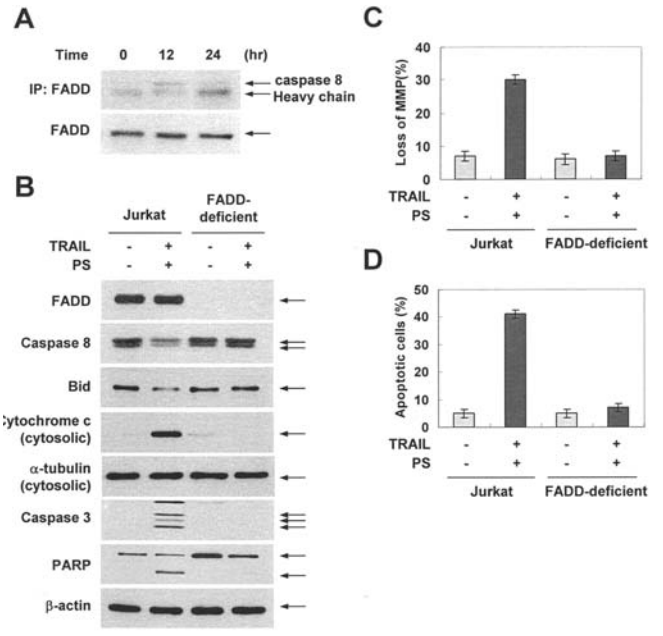

Figure 4. The combination treatment-induced mitochondrial apoptotic cell death was death receptor-dependent. (A) Jurkat T cells were treated with a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 12 and 24 h, whole cell extracts were immunoprecipitated with anti-FADD antibody and immune complexes were separated by SDS-PAGE and probed with anti-caspase-8 antibody. (B) Wild-type or FADD-deficient Jurkat cells were treated with a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, whole cell and cytosolic extracts were prepared and subjected to Western blot analysis with anti-caspase-8, -Bid, -cytochrome *c*, -α-tubulin, -capsase 3, -PARP, and -ß-actin antibodies. α-tubulin and ß-actin were used as a cytosolic marker protein and a loading control, respectively. The data represent a typical experiment conducted three times with similar results. (C) After 24 h, wild-type or FADD-deficient Jurkat 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 ± SEM. (D) Wild-type or FADD-deficient Jurkat cells were treated with a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, mitochondrial transmembrane potentials of the cells were determined by retention of $DioC_6(3)$ added during the last 30 min of treatment. After removal of the medium, the amount of retained $DioC_6(3)$ was measured by flow cytometry.

dysfunction through the activation of pro-apoptotic proteins Bax and Bak.

Enhanced activation of caspase-8 and Bid contributed to Bax and Bak activation and mitochondrial dysfunction in Jurkat T cells treated with TRAIL in combination with phytosphingosine. In the Type II cancer cells, Bid, a 'BH3 only' protein of the Bcl-2 family, mediates the conformational changes of Bax and Bak, and this process is initiated by caspase-8 activation. To examine whether caspase-8-dependent Bid activation is involved in the Bax and Bak activation induced by the combination treatment, we examined caspase-8 activation and Bid cleavage after the combination treatment. As shown in Fig. 3A, phytosphingosine in combination with TRAIL markedly induced caspase-8 activation and Bid cleavage. Whereas, TRAIL alone showed only slight induction of caspase-8 activation and Bid cleavage, and phytosphingosine alone did not show any effect. Moreover, pretreatment of z-IETD-fmk, a caspase-8-specific inhibitor, significantly attenuated the combination treatment-induced activation of Bax and Bak (Fig. 3B), Bax translocation to the mitochondria (Fig. 3C), dissipation of mitochondrial membrane potential (Fig. 3D), cytochrome *c* release (Fig. 3C), and apoptotic cell death (Fig. 3E). These observations suggest that enhanced activation of caspase-8 and Bid by the combination treatment contributes to Bax and Bak activation and subsequent mitochondrial dysfunction-mediated apoptotic cell death.

The combination treatment-induced caspase-8 activation was death receptor-dependent. Many studies have suggested that caspase-8 activation is dependent on the oligomerization of caspase-8 by its association with the adaptor molecule, FADD, via the DEDs of the two molecules. This requires the interaction of FADD either directly or indirectly with surface receptors such as Fas, TNF-R, or TRAIL-R which possess death domains (3,4). In order to examine the involvement of death receptor (DR) in the combination treatment-induced apoptosis, we immunoprecitated FADD followed by immunoblotting for caspase-8 from the immunoprecipitates. As shown in Fig. 4A, the interaction between FADD and caspase-8 was detected in response to the combination treatment. To further confirm death receptor-dependent caspase-8 activation, we examined caspase-8 activation and cell death in FADD-deficient Jurkat T cells in response to the combination treatment. As shown in Fig. 4B, the combination treatment failed to induce caspase-8 activation and Bid cleavage in FADD-deficient Jurkat cells. In addition, in FADD-deficient Jurkat cells, the combination treatment did not induce mitochondrial membrane potential loss (Fig. 4C), cytochrome *c* release, caspase-3 activation (Fig. 4B), and apoptotic cell death (Fig. 4D). These results suggest that the combination treatment-induced caspase-8 activation occurs in a death receptor-dependent fashion.

Combination treatment with phytosphingosine and TRAIL induced synergistic up-regulation of TRAIL receptors R1 (DR4) and R2 (DR5). TRAIL-induced apoptosis is known to depend on expression of TRAIL receptors, DR4 and DR5. To determine whether enhanced activation of caspase-8 by combination treatment of TRAIL and phytosphingosine is affected by TRAIL receptor expression in Jurkat T cells, we examined the surface expression of DR4 and DR5 by flow cytometry. As shown in Fig. 5A, the combination of TRAIL and phytosphingosine resulted in a marked up-regulation of DR5 protein level, and slight alteration of DR4 level. No significant increase was observed in cells treated with TRAIL or phytosphingosine alone. Moreover, siRNA targeting of DR5 significantly attenuated the combination treatment-induced caspse-8 activation, Bid cleavage (Fig. 5B), mitochondrial membrane potential loss (Fig, 5C), cytochrome *c* release, caspase-3 activation (Fig. 5B), and apoptotic cell death (Fig. 5D). These results suggest that synergistic up-regulation of TRAIL receptors, DR4 and DR5, might be involved in the combination treatment-induced caspase-8 activation and subsequent mitochondrial apoptotic cell death in Jurkat T cells.

NF-κB is involved in the combination treatment-induced up-regulation of DR4 and DR5. It has been reported that NF-κB

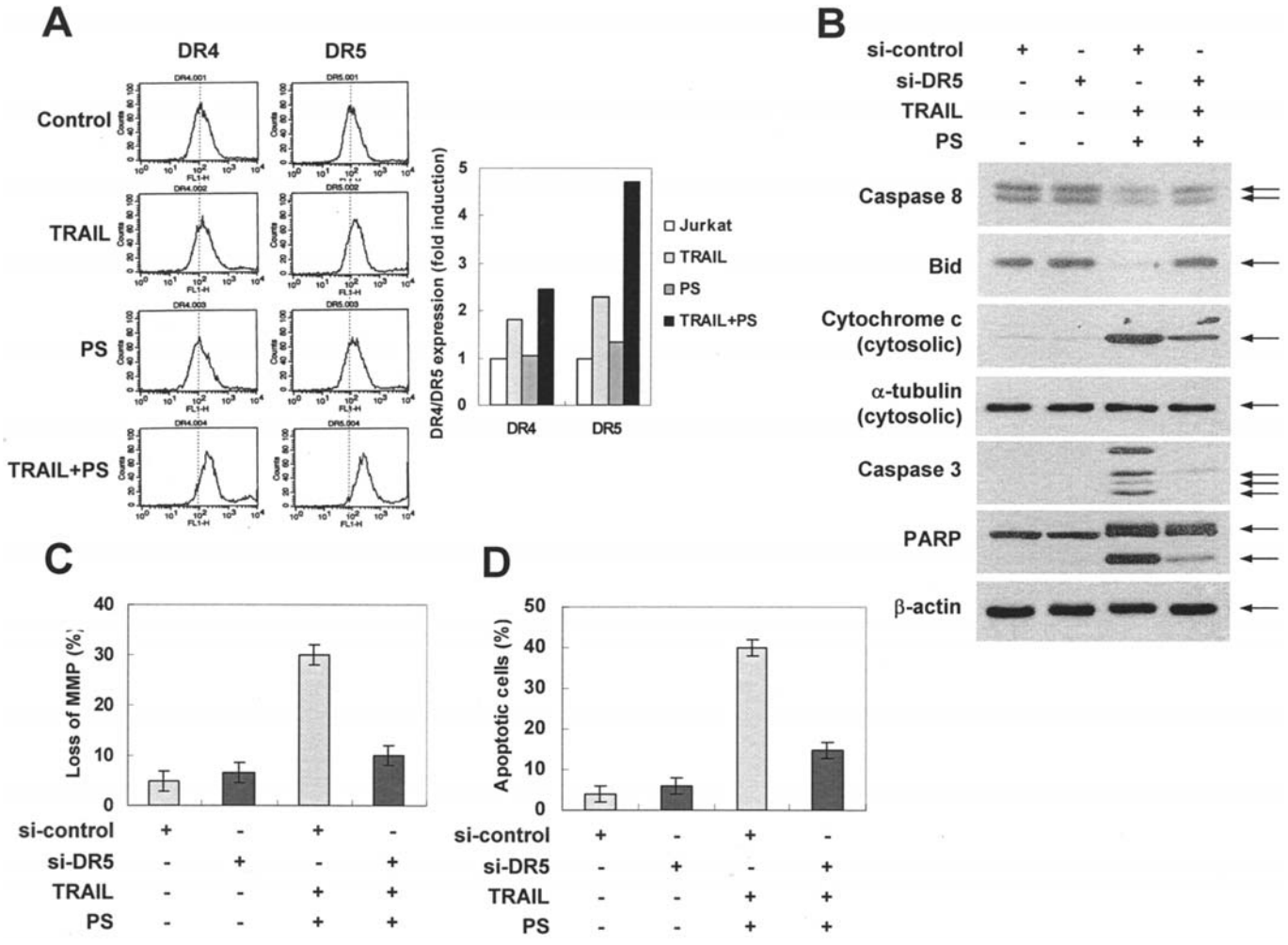

Figure 5. Combination treatment with phytosphingosine and TRAIL induced synergistic up-regulation of DR4 and DR5. (A) Changes of protein level of DR4 and DR5 after the combination treatment. Jurkat T cells were treated with 10 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, cell surface levels of DR4 and DR5 were measured by flow cytometry after staining the cells with anti-DR4 or -DR5 antibody. (B) Effect of DR5 siRNA on the combination treatment-induced caspase-8 activation. Jurkat T cells transfected with DR5 siRNA were treated with a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, mitochondrial and cytosolic fractions were subjected to Western blot analysis with anti-caspase-8, -Bid, -cytochrome *c*, -α-tubulin, -capsase 3, PARP, and -ß-actin antibodies. α-tubulin and ß-actin were used as a cytosolic marker protein and a loading control, respectively. (C) Effect of DR5 siRNA on the combination treatment-induced mitochondrial membrane potential loss. Jurkat T cells transfected with DR5 siRNA were treated with combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, the mitochondrial transmembrane potential of the cells was determined by retention of $DioC_6(3)$ added during the last 30 min of treatment. After removal of the medium, the amount of retained $DioC_6(3)$ was measured by flow cytometry. (D) Effect of DR5 siRNA on the combination treatment-induced apoptotic cell death. Jurkat T cells transfected with DR5 siRNA were treated with a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, cells were stained with Hoechst 33258 and apoptotic cells were measured by fluorescence microscopy. Results from three independent experiments are shown as means ± SEM.

and p53 binding sites are present in the promoter of the human TRAIL receptor gene, and NF-κB or p53 transcription factors participate directly in transcriptional regulation of DR4 and/or DR5 (18,27). To determine whether these transcription factors are involved in the combination treatment-induced DR4 and DR5 expression and subsequent cell death pathways, electrophoretic gel mobility shift assay with NF-κB or p53 binding sites was performed. As shown in Fig. 6A, in response to the combination treatment, the NF-κB binding site-specific DNA binding complexes had shifted. But, any shifted bands with p53 binding sites were not observed (data not shown). To further confirm whether NF-κB transcription factor is essential for the combination treatment-induced DR4 and DR5 expression, NF-κB siRNA was transfected, and DR4 and DR5 expression after the combination treatment was measured by flow cytometric analysis. siRNA targeting of NF-κB completely inhibited DR4 and DR5 expression (Fig. 6C) as well as the NF-κB binding complex (Fig. 6B) induced by the combination treatment. These results suggest that up-regulation of DR4 and DR5 in response to the combination treatment is NF-κB-dependent.

Discussion

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor ligand family that exerts potent cytotoxic activity in a variety of human cancer cells but not in normal cells. Because of this unique merit, TRAIL may have a potential use in cancer treatment. Therefore, sensitization of cancer cells to TRAIL could improve the effectiveness of

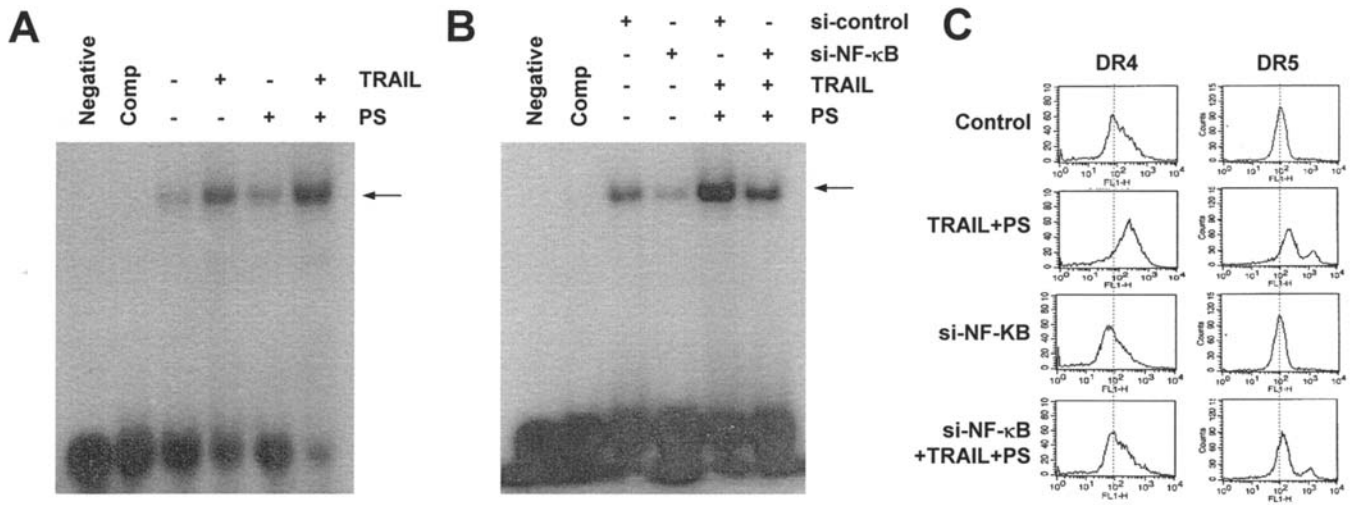

Figure 6. The combination treatment-induced up-regulation of DR4 and DR5 was NF-κB dependent. (A) Activation of NF-κB in response to the combination treatment. Jurkat T cells were treated with 10 ng/ml TRAIL alone, 2 μg/ml of phytosphingosine alone, or combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, nuclear fraction was prepared and subjected to electrophoretic mobility shift assay (EMSA) with DNA oligomers specific for NF-κB binding sites as described in Materials and methods. Comp, the competition lane (100-fold excess of cold oligonucleotide in the reaction mixture. (B) Effect of NF-κB siRNA on the combination treatment-induced NF-κB activation. Jurkat T cells transfected with NF-κB siRNA were treated with combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, nuclear fractions were prepared and subjected to EMSA with DNA oligomers specific for NF-κB binding sites. (C) Effect of NF-κB siRNA on the combination treatment-induced DR4 and DR5 expression. Jurkat T cells transfected with NF-κB siRNA were treated with a combination of TRAIL (10 ng/ml) and phytosphingosine (2 μg/ml). After 24 h, protein levels of DR4 and DR5 were determined by flow cytometric analysis using anti-DR4 and -DR5 antibodies as described in Materials and methods.

TRAIL as an anticancer agent. In this study, we demonstrated that phytosphingosine can sensitize cancer cells to TRAIL through the up-regulation of DR4 and DR5 resulting in caspase-8 activation and subsequent mitochondrial dysfunction, and that up-regulation of DR4 and DR5 was NF-κB dependent.

Previously we have reported that phytosphingosine can potently induce apoptotic cell death in Jurkat T cells and non-small cell lung cancer cells (21,22). We showed that 5-20 μg/ml of phytosphingosine treatment induced a strong apoptotic cell death in Jurkat T cells within 3 h. However, 2 μg/ml of phytosphingosine did not effectively induce cell death even after 24 h. Treatment of cells with 10 ng/ml of TRAIL induced only slight induction of apoptosis at 24 h. However, combination treatment with phytosphingosine (2 μg/ml) and TRAIL (10 ng/ml) induced synergistic apoptotic cell death, suggesting that phytosphingosine markedly sensitizes Jurkat T cells to TRAIL. Several reports demonstrated that DNA damaging chemotherapeutic agents, such as doxorubicin, cisplatin, camptothecin or etoposide, and radiation enhanced TRAIL sensitivity in certain cancer types (13-16,28,29). Moreover, the topoisomerase I inhibitor CPT-11 also was shown to augment TRAIL-induced apoptosis in several different cancer cell lines (10,19,30). However, there have been conflicting reports on the acting mechanism of the combination of TRAIL and other apoptosis-inducing chemotherapeutic agents. For example, it was suggested that aggregation (activation) of TRAIL receptor was directly involved in cell death induced by the combination treatment of etoposide or camptothecin with TRAIL in certain cancer cells (31). However, the combined treatment of TRAIL and CPT-11 was shown to induce synergistic cytotoxicity towards prostate cancer cells through regulation of Bcl-2 family proteins and potent activation of caspases (19). Consistent with this finding, we found that the combination treatment with TRAIL and phytosphingosine synergistically induced conformational changes of multidomain proapototic members of the Bcl-2 family, Bax and Bak, mitochondrial relocalization of Bax, and subsequent mitochondrial dysfunction. The mitochondrial apoptotic cell death pathway has been shown to be required for Fas- or TRAIL-induced apoptosis in certain cell types that are classified as type II cells (25,32). In these cells, Bid, a 'BH3 only' protein of the Bcl-2 family, mediates activation of Bax and/or Bak and subsequent dissipation of the mitochondrial membrane potential initiated by caspase-8 activation (33). We also observed marked activation of caspase-8 and Bid in response to the combination treatment with TRAIL and phytosphingosine. Moreover, inhibition of caspase-8 completely blocked the combination treatment-induced Bax and Bak activations and apoptotic cell death. These observations suggest that the combination treatment of TRAIL and phytosphingosine induces synergistic apoptosis in Jurkat T cells through mitochondrial dysfunction mediated by caspase-8-mediated Bax and Bak activation.

Recent reports have demonstrated that several anticancer treatments, such as camptothecin, etoposide, or ionizing radiation-mediated cell death resulted in aggregation of TRAIL receptor in certain cancer cells (17,18,31). The same studies showed that the expression level of TRAIL receptor did not change as a result of anti-cancer treatment. We also could not detect any changes in TRAIL receptor level in response to TRAIL or phytosphingosine alone. Interestingly, however, we found that TRAIL treatment in combination with phytosphingosine markedly up-regulated the DR5 protein level, and also slightly increased the DR4 level. Moreover, siRNA targeting of DR5 significantly attenuated the combination treatment-induced caspase-8 activation and mitochondrial apoptotic cell death. These results suggest that the up-regulation

of DR4 and DR5 might be involved in the combination treatment-induced synergistic apoptotic cell death of Jurkat T cells.

It has been suggested that p53 is involved in transcriptional regulation of DR5 (27), and others suggested that differential activation of NF-κB could induce both DR4 and DR5 expression (18). We also observed a marked activation of NF-κB in response to the combination treatment with TRAIL and phytosphingosine. Moreover, inhibition of NF-κB by siRNA targeting of NF-κB completely blocked the combination treatment-induced up-regulation of DR4 and DR5 and receptor-mediated caspase-8 activation. However, any changes in the activity of p53 in response to the combination treatment of Jurkat T cells were not detected (data not shown). These observations suggest that NF-κB might be involved in the up-regulation of DR4 and DR5 in response to the combination treatment with TRAIL and phytosphingosine. We are currently exploring the precise signaling pathway for the combination treatment-induced NF-κB activation.

In conclusion, in the present study we explored whether TRAIL treatment in combination with phytosphingosine could sensitize cancer cells to TRAIL. We showed that the combination treatment enhanced synergistic mitochondrial apoptotic cell death through NF-κB-mediated up-regulation of DR4 and DR5 and subsequent activation of caspase-8. The mechanism that we elucidated in this study may provide insight into the design of future combination therapies for cancers that are less sensitive to TRAIL treatment.

Acknowledgements

We thank Dr Byung-Ha Oh at POSTECH, Korea, for kindly providing recombinant TRAIL. This study was supported by a Korea Research Foundation grant (KRF-2003-005-C00025, and by Korea Science and Engineering Foundation (KOSEF) and Ministry of Science and Technology (MOST), Korean government, through its National Nuclear Technology Program.

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