Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells

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The cytotoxic effect of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is limited in some cancer cells, including A549 lung adenocarcinoma cells. However, treatment with TRAIL in combination with subtoxic concentrations of sulforaphane (SFN) sensitizes TRAIL-resistant A549 cells to TRAIL-mediated apoptosis. Combined treatment with SFN and TRAIL induced chromatin condensation, DNA fragmentation, annexin V staining and sub-G1 phase DNA content. These indicators of apoptosis correlate with the induction of caspase-3 activity that results in the cleavage of poly(ADP-ribose) polymerase and the release of lactate dehydrogenase. Both the cytotoxic effect and apoptotic characteristics induced by combined treatment were significantly inhibited by z-DEVD-fmk, a caspase-3 inhibitor, demonstrating the important role of caspase-3 in the observed cytotoxic effect. Combined treatment also triggered the activation of p38 MAPK and JNK, and downregulation of ERK and Akt. Inhibitors of ERK (PD98059) or Akt (LY294002), but not p38 MAPK, resulted in significantly decreased cell viability. Although the activation of JNK was increased in response to combined treatment, inhibition of the JNK pathway significantly attenuated cell viability. These results indicate that caspase-3 is a key regulator of apoptosis in response to combined SFN and TRAIL, in human lung adenocarcinoma A549 cells through downregulation of ERK and Akt.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is known to induce apoptosis in several cell lines. Cellular sensitivity to TRAIL is dependent on expression of cell membrane TRAIL receptors and caspase-8 (1,2). Caspase-8 is activated in response to TRAIL.

Abbreviations: DAPI, 4, 6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide; NSCLC, non-small cell lung cancer; PARP, poly(ADP-ribose) polymerase; PI, pleiopodium isodide; PI3K, phosphoinositide 3-kinase; SD–SAGE, sodium dodecyl sulfate-polyacylamide gel electrophoresis; SFN, sulforaphane; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

These authors contributed equally to this work.

The cytotoxicity resulting from combined treatment with SFN and TRAIL was partially prevented by z-DEVD-fmk, a specific inhibitor of caspase-3. These results indicate that caspase-3 is a key regulator of apoptosis in response to combined SFN and TRAIL, in human lung adenocarcinoma A549 cells through downregulation of ERK and Akt.

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In the current report, we use the SFN- and TRAIL-resistant lung adenocarcinoma A549 cell line as model system to investigate the sensitizing effects of SFN on TRAIL-induced apoptosis. Our observations demonstrate that treatment with a combination of TRAIL and SFN may be a safe and effective strategy to treat apoptosis-resistant carcinoma.

Materials and methods

Materials

Propidium iodide (PI), 4, 6-diamidino-2-phenylindole (DAPI), and 3-(4, 5-Dimethyl-2-thiazoly)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma (St Louis, MO). Caspase activity assay kit was obtained from R&D Systems (Minneapolis, MN). An enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL). Caspase-3 inhibitor I (z-DEVD-fmk), PD98059, SB203580, SP600125 and LY294002 were obtained from Calbiochem (San Diego, CA). RPMI 1640 medium was purchased from Invitrogen Corp. (Carlsbad, CA) and fetal bovine serum (FBS) was purchased from Gibco BRL (Gaithersburg, MD). All other chemicals not specifically cited here were purchased from Sigma.

Antibodies

Antibodies against cIAP-1, cIAP-2, XIAP, Bcl-2, Bax, Bid, Bcl-XL, poly (ADP-ribose) polymerase (PARP), PLCγ, caspase-3, caspase-8 and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ERK, phospho (p)-ERK, p38, p-p38, JNK, p-JNK, Akt and p-Akt were purchased from PharMingen (San Diego, CA). Antibody against β-actin was from Sigma. Peroxidase-labeled donkey antirabbit and sheep antimouse immunoglobulin were purchased from Amersham.

Cell culture

Human lung adenocarcinoma A549 cells and human leukemic U937 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in a 5% CO2 humidified incubator, and maintained in RPMI 1640 culture medium containing 10% heat-inactivated fetal bovine serum.

Cell viability and growth

The cells were grown to 70% confluence and treated with the indicated concentrations of SFN, TRAIL or combined treatment (SFN + TRAIL). Control cells were supplemented with complete media containing 0.1% dimethyl sulfoxide (DMSO) (vehicle control) for 24 h. Following treatment, cell number and viability were determined by trypan blue exclusion and MTT assays, respectively.

Nuclear staining

After treatment with SFN + TRAIL for 24 h, the cells were harvested, washed in ice-cold PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were permeabilized with saponin and stained with DAPI solution for 10 min at room temperature. Nuclear morphology of the cells was examined by fluorescence microscopy.

Cell cycle analysis

The cells were serum starved for 18 h to synchronize them in the G0 phase of cell cycle. Synchronous populations of cells were subsequently treated in the absence or presence of SFN + TRAIL for 24 h. The cells were washed twice with cold PBS and then centrifuged. The pellet was fixed in 75% (vol/vol) ethanol for 1 h at 4°C. The cells were washed once with PBS and resuspended in cold PI solution (50 μg/ml) containing RNase A (0.1 mg/ml) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry analyses were performed using FACS-Calibur (Becton Dickinson, San Jose, CA). Forward light scatter characteristics were used to exclude the cell debris from the analysis. The sub-G1 population was calculated to estimate the apoptotic cell population.

DNA fragmentation assay

The cells were treated with SFN and/or TRAIL for 24 h and were lysed on ice in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min. Lysates were vortexed and cleared by centrifugation at 10,000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl-alcohol (25:24:1, vol/vol/vol) and analyzed electrophoretically on 1.5% agarose gel containing ethidium bromide.

Annexin V analysis

For the analysis of apoptosis, DC were stimulated with LPS or left without any stimuli and apoptosis was analyzed over time by staining of phosphatidylserine translocation with FITC-annexin V (PharMingen) according to the manufacturer’s instructions.

Protein extraction and western blot analysis

Cells were harvested, washed once with ice-cold PBS and gently lysed for 2 min in 80 μl ice-cold lysis buffer (20 mM Tris Cl, pH 7.2, 1 mM dithiothreitol, 10 mM KCl, 1.5 mM MgCl2, 5 μg/ml pepstatin A, 10 μg/ml leupeptin and 2 μg/ml aprotinin). Supernatants were collected and protein concentrations determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at −80°C or immediately used for immunoblotting. Aliquots containing 30 μg of total protein were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) for immunoblot analysis using the indicated primary antibodies. HRP-conjugated secondary antibodies were detected using an enhanced chemiluminescence detection system (Amersham).

Determination of caspase activity

Caspase activities were determined by colorimetric assays using caspase-3, caspase-8 and caspase-9 activation kits according to the manufacturer’s protocol. The kits utilize synthetic tetrapeptides labeled with p-nitroanilide. Briefly, the cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37°C. The caspase activity was determined by measuring changes in absorbance at 405 nm using the microplate reader.

Statistical analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student’s t-test. A value of P < 0.05 was accepted as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments.

Results

Non-toxic dose of SFN significantly triggers TRAIL-induced apoptosis in TRAIL-resistant A549 cells

To investigate the effects of SFN, TRAIL or their combined treatment (SFN + TRAIL) on cell viability, A549 cells were treated with the indicated agents and subjected to MTT assays, chromatin condensation, DNA fragmentation, annexin V staining and cell cycle analyses. As shown in Figure 1A, treatment with 10 μM SFN alone for 24 h was not able to induce death in A549 cells and treatment with 100 ng/ml TRAIL alone resulted in only a slight decrease in cell viability (84 ± 5%). Notably, A549 cells treated with a combination of TRAIL and varying concentrations of SFN reduced cell viability significantly more than treatment with TRAIL alone. Treating cells with 100 ng/ml TRAIL and 2.5, 5 or 10 μM of SFN reduced cell viability to 71 ± 5%, 51 ± 6% and 31 ± 8% of control levels at 24 h, respectively. We also observed the cytotoxic effects of combined treatment by varying the concentration of TRAIL (25, 50 or 100 ng/ml) and holding the concentration of SFN (10 μM) constant. This also resulted in a significant reduction in cell viability in a TRAIL dose-dependent manner, with cell viability reduced to 83 ± 6%, 72 ± 5% and 50 ± 8% of control viability, respectively (Figure 1B). As shown in Figure 1C, treatment with up to 10 μM SFN alone did not induce any morphologic features indicative of cell death and treatment with up to 100 ng/ml TRAIL alone induced only a slight increase in such morphological changes. However, combined treatment with 10 μM SFN and 100 ng/ml of TRAIL induced significant morphological changes and cell shrinkage in A549 cells. In order to obtain a quantitative measure of apoptosis induction, we next investigated the amount of cells with sub-G1 DNA content using flow cytometric analysis. Combined treatment with 10 μM SFN and 100 ng/ml TRAIL resulted in significant accumulation of cells with sub-G1 DNA content (34 ± 5%), whereas treatment with SFN or TRAIL alone did not (Figure 1D). For a further assessment of apoptosis, we examined the exposure of phosphatidylserine on the cell surface by using annexin V staining. Flow cytometric analysis revealed that the percentage of annexin V-staining cells increased with combined treatment (Figure 1E). Additionally, Figure 3F demonstrated that treatment with 5 μM SFN and 100 ng/ml TRAIL significantly increased fragmentation. In this experiment, the number of cells with sub-G1 DNA content was identical to that in vehicle.
These results suggest that SFN significantly stimulates TRAIL-induced apoptosis in SFN- and TRAIL-resistant A549 cells. Combined treatment with SFN and TRAIL upregulates proapoptotic Bax, truncated Bid, caspase activity and subsequent cleavage of PARP. The proapoptotic and antiapoptotic members of the Bcl-2 and IAP protein families play pivotal roles in regulating cell viability. These proteins interpret a wide array of diverse upstream survival and distress signals in order to decide the fate of the cells (22). Therefore, we investigated whether combined treatment induces apoptosis by modulating the expression of Bcl-2 and IAP protein family members. As shown in Figure 2A, combined treatment of 10 μM SFN and 100 ng/ml TRAIL did not affect the expression levels of antiapoptotic XIAP, cIAP-1, cIAP-2, Bcl-2 or Bcl-XL. In contrast, expression of apoptotic Bax was upregulated significantly, whereas the apoptotic protein, Bid, was truncated. Caspases are also known to act as important mediators of apoptosis and contribute to the overall apoptotic morphology by cleavage of various cellular substrates. Therefore, we investigated the cleavage of caspase-3, caspase-8 and caspase-9, and the subsequent proteolytic cleavage of PARP in A549 cells treated with SFN and TRAIL for 24 h. As shown in Figure 2B, western blot analysis reveals that treatment with TRAIL alone slightly affects the cleavage of caspases and PARP. However, combined treatment with 100 ng/ml TRAIL and concentration of SFN >5 μM significantly induced the cleavage of caspases and PARP in a SFN concentration-dependent manner. Next, cell lysates containing equal amounts of total protein from cells treated with SFN and TRAIL were assayed for in vitro caspase activity. As shown in Figure 2C, combined treatment with 100 ng/ml TRAIL and concentrations of SFN >5 μM increased caspase-3, caspase-8 and caspase-9 activity significantly. These results indicate that combined treatment induces apoptotic death in A549 cells, at least in part through a caspase-dependent pathway.

Inhibition of caspase-3 activity restores cell viability following combined treatment with SFN and TRAIL.

Caspase-3 represents one of the key proteases responsible for cleavage of PARP and subsequent apoptosis. To further evaluate the significance of caspase activation in combined treatment, we used a general and potent inhibitor of caspase-3, z-DEVD-fmk. As shown in Figure 3A and B, SFN-mediated enhancement of TRAIL-induced cell death was significantly suppressed by z-DEVD-fmk, indicating that the TRAIL-induced apoptosis by SFN was mediated through caspase-3 activation. We also assessed the effect of combined treatment on cell cycle distribution in the presence of z-DEVD-fmk by flow cytometry. As shown in Figure 3C, 24 h of combined treatment resulted in significant accumulation of cells with sub-G1 DNA content.
(31 ± 3%). However, cells treated with SFN and TRAIL in the presence of z-DEVD-fmk had a normal cell cycle profile and did not exhibit an increase in cells with sub-G1 DNA content. Consistent with the flow cytometry results, an analysis by light microscopy showed that z-DEVD-fmk treatment decreased membrane shrinkage, cell rounding (upper panel) and the appearance of apoptotic bodies (lower panel) following combined treatment (Figure 3D). Furthermore, combined treatment with SNF and TRAIL significantly induced cleavage of PARP and caspase-3 (Figure 3E), and caspase-3 activity (Figure 3F), whereas z-DEVD-fmk pretreatment significantly inhibited cleavage of PARP and caspase-3, and caspase-3 activity. These results clearly indicate that SFN partially sensitizes A549 cells to TRAIL-induced apoptosis through caspase-3 activation.

The ERK pathway is important for the induction of apoptosis in response to combined treatment with SFN and TRAIL.

We next investigated the effect of combined SFN and TRAIL treatment on the expression and activity of MAPKs in order to determine whether this signaling pathway is involved in mediating the observed apoptotic response. As shown in Figure 4A, the phosphorylation of p38 MAPK and JNK increased significantly after 0.5 h of combined treatment. In contrast, however, ERK activation gradually decreased after 8 h of treatment. Resulting also suggest that SFN exerts a dose-dependent effect on MAPKs treated with a fixed concentration of TRAIL (Figure 4B). We next evaluated the possible roles of MAPKs in combined treatment-induced apoptosis. As shown in Figure 4C, pretreatment with SP600125 (a potent inhibitor of JNK) or PD98059 (a potent inhibitor of ERK) significantly increased the number of cells with sub-G1 DNA content from 28 ± 5% to 73 ± 3% or 66 ± 5%, respectively. However, pretreatment with SB203589, a specific inhibitor of p38 MAPK, did not exert a statistically significant effect on the combined treatment. In addition, A549 cells cultured in the presence of SP600125 or PD98059 showed a marked change in morphology and condensed chromatin in the nuclei following 24 h of combined treatment (Figure 4D). Consistent with the increase in sub-G1 DNA content, pretreatment with SP600125 or PD98059 could significantly increased combined treatment-induced cell death and antiproliferation, however, SB203580 was not able to upregulate the appearance of apoptotic features (Figure 4E and F). These results led us to believe that combined treatment is probably to inhibit ERK activation, suggesting that this pathway is probably to be involved in apoptosis. However, low-dose SP600125 (<10 μM) could not block apoptosis and high-dose SP600125 (<15 μM) increased apoptosis in combined treatment. These results also indicate a different role for JNK activation in response to combined treatment of A549 cells, because high-dose SP600125 alone has significant cytotoxicity.

Fig. 2. The expression levels of apoptosis-related proteins by combined treatment in A549 cells. A549 cells were treated with indicated concentrations of agents for 24 h. (A and B) Equal amounts of cell lysates (30 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with specific antibodies (anti-Bcl-2, anti-Bax, anti-Bcl-XL, anti-Bid, anti-XIAP, anti-cIAP-1, anti-cIAP-2, caspase-3, caspase-8 and caspase-9). A representative study is shown, and two additional experiments yielded similar results. The experiment was quantitated by densitometry and represented as the indicated protein:actin ratio. (C) Caspases (-3, -8 and -9) activities were determined using a caspase assay kit obtained from R&D following the manufacturer’s protocol. Data are expressed as mean ± SD of three independent experiments. The significance was determined by Student’s t-test (*P < 0.05 versus untreated control); CF, cleavages form.
Blockage of the Akt pathway increases apoptosis induced by combined treatment with SFN and TRAIL

In order to investigate significance of the Akt pathway in response to combined treatment, we performed time- and SFN dose-dependent experiments in order to determine the expression and phosphorylation levels of Akt. As shown in Figure 5A, the levels of phosphorylated Akt significantly decreased in response to SFN and TRAIL at 4 h. Consistent with this result, levels of phosphorylated Akt also decreased at 8 h in a SFN concentration-dependent manner at concentrations >5 μM (Figure 5B). Total Akt protein levels remained constant throughout the course of the experiment. We next investigated whether activation of the Akt pathways is necessary for apoptosis induced by combined treatment. The PI3K inhibitor LY294002 (Akt-upstream inhibitor) was used to determine whether the inhibition of Akt phosphorylation was responsible for the induction of apoptosis. As shown in Figure 5C and D, pretreatment with LY294002 alone resulted in increased G1 arrest without apoptosis. Treatment with the inhibitor in combination with SFN and resulted in a marked increase in apoptosis, as determined cells with sub-G1 DNA content (63 ± 6%) and chromatin condensation. We next analyzed cell viability and cell number, in order to further elucidate the relationship between the Akt pathway and combined treatment-induced apoptosis in A549 cells. Treatment with 25 μM LY294002 significantly decreased cell viability (from 39 ± 4% to 5 ± 2%) in the presence SFN and TRAIL (Figure 5E). We also found that cotreatment of LY294002 with SFN and TRAIL resulted in a significant decrease in cell number (Figure 5F). These results indicate that combined treatment-induced apoptosis may be associated with downregulation of the Akt-signaling pathway.

SFN triggers TRAIL-sensitive U937 cells at subtoxic concentrations of TRAIL

As the final step of our investigation, we assessed whether SFN-induced apoptosis in TRAIL-sensitive human leukemic U937 cells can be more effectively induced at subtoxic doses of combined treatment. The cells were treated with the various concentrations of SFN or TRAIL for 24 h and the analysis of cell viability was measured with MTT assay. As shown in Figure 6A and B, SFN or TRAIL significantly decreased cell viability at a dose-dependent manner.
viability was decreased at >4 μM SFN (78 ± 5 μM) or 15 μM TRAIL (69 ± 6 μM), respectively. We next investigated the cell death of combined treatment of holding-subtoxic concentration of TRAIL (10 ng/ml) and several of subtoxic concentrations of SFN (0.5, 1 and 2 μM). This also resulted in a significant reduction in cell viability in a SFN dose-dependent manner, with cell viability reduced to 65 ± 6%, 44 ± 5% and 29 ± 7% of control viability, respectively (Figure 6C). Taken together, these results indicate that treatment with subtoxic concentration of SFN sensitizes TRAIL-sensitive human leukemic U937 cells to low dose of TRAIL-mediated apoptosis.

Discussion

Lung cancer is the most frequent cause of cancer-related death worldwide. Non-small cell lung cancers (NSCLCs) arise via multiple mechanisms and are currently being treated with a wide variety of novel pharmaceutical agents (23,24). However, the efficacy and mechanisms of action for these potential therapeutic agents are still under study. Recently, several groups identified TRAIL as a family and that induces apoptosis in many cancer and transformed cells (25–27). Activation of caspase-8 by TRAIL leads to two different apoptotic pathways, depending on the cell type (3). TRAIL induces apoptosis in a mitochondrial-independent manner, activating downstream effector caspases such as caspase-3 (28), whereas a mitochondrial-dependent pathway proceeds via activation of caspase-9, which then induces the execution phase of apoptosis (29). Our results indicate that combined treatment of SFN and TRAIL simultaneously induces mitochondrial-dependent (caspase-9) and -independent (caspase-8) apoptosis. TRAIL may be a safe and effective biological agent for cancer therapy in humans, however, some NSCLC-derived cells, such as A549, are known to be resistant to TRAIL-induced apoptosis through activation of the PI3K/Akt pathway (30). Several reports have also shown that chemotherapeutic agents sensitized TRAIL-induced synergistic cytotoxicity in TRAIL-resistant cells (31,32). Consequently, it is very important to seek an agent that can sensitize TRAIL-induced apoptosis in TRAIL-resistant NSCLC.
In the present study, we demonstrated that combined treatment with SFN and TRAIL triggers apoptosis in A549 cells that are normally resistant to either agent alone. Furthermore, this sensitizing effect occurred in a SFN dose-dependent manner. Combined treatment induced cell death followed by cell shrinkage and chromat condensation that are hallmark features of apoptosis. Recently, some researchers reported that SFN sensitizes TRAIL-mediated apoptosis through the induction of DR5 expression in human osteosarcoma cells (33) and TRAIL-resistant hepatoma cells (12). Caspases belong to a family of cysteine proteases that are integral parts of the apoptotic

Fig. 5. Combined treatment significantly sensitizes LY294002-induced apoptosis in A549 cells. The cells were treated with SFN + TRAIL in time- or dose-dependent manner. LY294002 was treated 1 h before combined treatment. (A and B) Equal amounts of cell lysate (30 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with specific antibodies (anti-p-Akt and Akt). β-Actin was used as an internal loading control. The experiment was quantitated by densitometry and represented as the indicated protein:actin ratio. (C) The cells were stained with PI (50 μg/ml) and analyzed by flow cytometry. The results are from one representative experiment of three performed that showed similar patterns. (D) Cells were harvested after 24 h of treatment and centrifuged. After fixing, the cells were stained with DAPI solution. Stained nuclei were then observed under a fluorescent microscope using a blue filter (×400) and the counts of number of cells with apoptotic nuclear morphology were represented below the pictures. Cell viability (E) and number (F) were determined by MTT assays and hemocytometer counts of trypan blue-excluding cells, respectively. The results are from one representative experiment of three performed and show similar patterns. Each point represents the mean ± SD of three independent experiments. The significance was determined by Student’s t-test (*P < 0.05 versus untreated control).

Fig. 6. Combined treatment with SFN and TRAIL decreased cell viability in TRAIL-sensitive human leukemic U937 cells. The cells were seeded at 2 × 10⁴ cells/ml and then were treated with the indicated concentrations of SFN (A), TRAIL (B) or SFN + TRAIL (C) for 24 h. Cell viability was determined by MTT assays. Each point represents the mean ± SD of three independent experiments. The significance was determined by Student’s t-test (*P < 0.05 versus untreated control).
pathway. In particular, activated caspase-3 has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis (34). Many studies have determined that a variety of chemotherapeutic agents induce apoptosis through activation of caspasess and degradation of PARP (35,36). Our findings indicate that caspasess are critical protease mediators of apoptosis triggered by combined treatment of TRAIL and SFN.

During apoptosis, caspasess are essential for the execution of cell death in response to various stimuli (28,36). Caspase activation is regulated by various cellular proteins, including IAP (5) and Bcl-2 family proteins (4). Executioner caspasess cleave PARP and PLC-γ that are marker proteins for apoptosis (29). Although other groups have reported that TRAIL alters expression of the antiapoptotic protein, especially XIAP (31,32), we could not observe a significant downregulation of XIAP levels in the experiments. Therefore, we additionally found that combined treatment with SFN and TRAIL increased expression of the proapoptotic Bax protein and resulted in truncation of the proapoptotic Bid protein. Combined treatment also slightly downregulated the expression of antiapoptotic Bcl-2 and Bcl-XL. We also found that the combination of SFN and TRAIL caused increase of caspase activity and PARP cleavage.

Our findings are consistent with previous reports that demonstrated a linkage between MAPK, Akt and cell death in TRAIL-resistant A549 cells. Most of the signals for survival trigger growth factor receptors that activate the ERK and PI3K/Akt pathways and promote cell growth (37,38). Our results showed that combined treatment significantly downregulated the activation of ERK and Akt. These results are in opposition to other recent reports that indicated treatment with SFN alone resulted in Akt downregulation, ERK activation and the induction of G2 arrest and apoptosis human colon adenocarcinoma Caco-2 cells (20). It is possible that the disparities in expression patterns are due to combined treatment rather than to differences in cell type. This is because treatment with SFN alone induces activation of ERK- and JNK-signaling pathways for AP-1 activation in human prostate cancer PC-3 cells (21). However, contradictory findings about the regulation of JNK have also been reported. Jakubikova et al. (20) reported that SFN alone had no effect on JNK in Caco-2 cell-apoptosis. Furthermore, JNK has also been implicated in the regulation of AP-1 activity and cell death in response to treatment with SFN in PC-3 cells (21). In this study, we found that combined treatment triggers JNK activation, however, pretreatment with JNK inhibitor SP600125 (20 μM) substantially increased the cell death induced by combined treatment with SFN and TRAIL. These results show that PI3K/Akt- and ERK-signaling pathways are important for the cell death induced by combined treatment with SFN and TRAIL in A549 cells. The role of the JNK pathway in this process needs to be further investigations in order to elucidate the molecular mechanisms associated with combined treatment-induced apoptosis.

Resistance to apoptosis is a major obstacle to chemotherapeutic treatment of cancer. The ability to induce apoptosis makes SFN a potentially effective preventive and therapeutic agent to combat malignancy. Although TRAIL represents another potentially important novel anticancer agent, recent studies have shown that many cancer cells are resistant to the apoptotic effects of TRAIL. Thus, the combined treatment with SFN and TRAIL may offer a good strategy for the treatment of a variety of human cancers that are resistant to chemotherapy or TRAIL treatment alone. Taken together, the results of this study suggest that SFN sensitizes TRAIL-mediated apoptosis by upregulation of apoptotic proteins, including Bax, Bid and caspases. Expression and activation of these proteins are mediated by downregulation of ERK and Akt in the lung adenocarcinoma A549 cells. In conclusion, the use of TRAIL in combination with subtoxic doses of SFN may provide an effective therapeutic strategy for safely treating some resistant NSCLCs.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by Korean Government (MOEHRD) (KRF-2005-206-E00007) and partially by the Korea Science and Engineering Foundation through National Research Laboratory Program Grant M10500000008-06J000000810.

Conflict of Interest Statement: None declared.

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Received June 26, 2006; revised November 29, 2006; accepted December 7, 2006.