Sulforaphane enhances TRAIL-induced apoptosis through the induction of DR5 expression in human osteosarcoma cells

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Sulforaphane (SFN), a naturally occurring isothiocyanate, is an attractive agent because of its potent anticancer effects. SFN suppresses the proliferation of various cancer cells in vitro and in vivo. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is also one of the most promising candidates for cancer therapeutics owing to its ability to selectively induce apoptosis in tumor cells. In this study, we report that SFN enhances TRAIL-induced apoptosis in human osteosarcoma cells, Saos2 and MG63. The apoptosis induced by co-treatment with SFN and TRAIL was markedly blocked by a dominant negative form of the TRAIL receptor or caspase inhibitors. The combined use of SFN and TRAIL effectively induced Bid cleavage and the activation of caspases and Bid following interaction with DR5 and DR4 (15). In addition, SFN was dissolved in dimethyl sulfoxide (DMSO). Equivalent DMSO was used as a control. The maximum volume (%) of DMSO in the assays was 0.1%.

In this study, we report that SFN enhances TRAIL-induced apoptosis through the induction of DR5 expression in normal human peripheral blood mononuclear cells. Thus, combined treatment with SFN and TRAIL might be a promising therapy for osteosarcoma.

Abbreviations: DR4 and DR5, death receptors 4 and 5; DMSO, dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; SFN, sulforaphane; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Introduction

Osteosarcoma is the most common form of primary malignant bone tumor and mainly occurs in juvenile patients. The development of effective adjuvant or neoadjuvant regimen of chemotherapy has substantially improved the prognosis of patients, leading to a cure rate of 50–70% (1–4). In patients with detectable metastases at diagnosis or with unresectable tumors, the prognosis remains poor (5–9). To improve the prognosis, new antitumor agents or chemicals and new antitumor therapeutic approaches need to be developed.

Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells in vitro and in vivo with little or no toxicity toward normal cells (10,11). Therefore, TRAIL is one of the most promising new candidates for antitumor therapeutics.

Death receptor 5 (DR5), also called TRAIL-R2, Apo2 or KILLER, and death receptor 4 (DR4/TRAIL-R1) are members of the TNF-receptor family, and are receptors for TRAIL (12–14). TRAIL transmits apoptotic signaling through the cleavage and activation of caspases and Bid following interaction with DR5 and DR4 (15). In addition, DR5 is a downstream gene of the p53 tumor-suppressor gene, and is upregulated by conventional anticancer drugs such as doxorubicin and etoposide (14,16,17). Moreover, the downregulation of DR5 promotes colon tumor xenograft growth in mice and confers resistance to chemotherapeutic agents (18). Therefore, DR5 is an attractive molecular target for effective cancer therapy.

Sulforaphane (SFN), a naturally occurring member of the isothiocyanate family, is produced from cruciferous vegetables, such as broccoli (19). SFN is an effective agent in the chemoprevention of chemically induced breast (20,21), colon (22) and stomach (23) cancers in rats. In a chemotherapeutic study, SFN drastically inhibited the growth of xenografts of human prostate cancer by oral administration (24) and breast cancer by intravenous injection (25). SFN suppresses the growth of cancer cells in vitro by inhibiting cell cycle progression (25–29) and/or causing apoptosis (24,26,27) in T-cell leukemia, colon, breast and prostate cancer cells. Several studies reported that SFN induces apoptosis through the mitochondrial apoptotic pathway, via the up- or downregulation of Bax, Bak, XIAP and Bcl-2 expression (24–27,30). However, the effect via the death receptor pathway of SFN has not yet been studied. Thus, we examined the effective use of SFN via the death receptor pathway.

In this study, we show for the first time that SFN is a potent enhancer of TRAIL-induced apoptosis through the induction of DR5 expression in human osteosarcoma cells. On the other hand, SFN neither induced DR5 protein expression or enhanced TRAIL-induced apoptosis in normal human peripheral blood mononuclear cells. This result raises the possibility that the combined treatment of SFN with TRAIL might be promising for molecular-targeting chemotherapy.

Materials and methods

Reagents

Sulforaphane (SFN) and soluble recombinant human TRAIL/APO2L were purchased from LKT (St Paul, MN) and PeproTech (London, UK), respectively. SFN was dissolved in dimethyl sulfoxide (DMSO). Equivalent DMSO was used as a control. The maximum volume (%) of DMSO in the assays was 0.1%. Recombinant human DR5 (TRAIL-R2/Fc chimera, and the caspase inhibitors
zVAD-fmk, zDEVD-fmk, zIETD-fmk, zLEHD-fmk and zAEVD-fmk, were purchased from R & D Systems (Minneapolis, MN).

**Cell culture**
The human osteosarcoma cell lines Saos2 and MG63 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere of 5% CO₂. Normal human PBMC were isolated as described previously (31). The cells were seeded at a density of 1 × 10⁶ cells in a 6-well plate. Twenty-four hours after the seeding, the confluency of cells at the time of treatment was 30–40%.

**Northern blot analysis and RNase protection assay**
Northern blot analysis was performed as described previously (31) using a full-length DR5 cDNA as a probe. The RNase protection assay was also performed as described previously (31). Radio-labeled band intensities were assessed by BAS2000 (Fuji Film, Kanagawa, Japan).

**Western blot analysis**
Western blot analysis was performed as described previously (31) using rabbit polyclonal anti-DR5 antibody (1:250; Cayman Chemical, Ann Arbor, MI), anti-Bid antibody (1:1000; Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-cleaved-caspase 3 antibody (1:1000; Cell Signaling Technology), mouse monoclonal anti-caspase 8 (1:1000), anti-caspase 9 (1:1000), anti-caspase 10 (1:1000) antibodies (MBL, Nagoya, Japan) and mouse monoclonal anti-pro-caspase 3 (1:10) antibody (Immunotech, Marseille, France). Enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ) was used for detection.

**Detection of apoptosis**
To analyze apoptosis, hypodiploid DNA (Sub-G1) populations were assayed using a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) as described previously (32). For all assays, 10 000 events were counted.

For the observation of nuclear morphology, cells grown in 6-well plates were incubated with DMSO or SFN at 30 μM for 24 h. They were next fixed in methanol, incubated with 4′-diamino-2-phenylindole (DAPI) solution for 30 min in the dark, and then analyzed using a fluorescent microscope (Olympus, Tokyo, Japan) at 420 nm.

**Small interfering RNAs**
The DR5, DR4 and Green Fluorescent Protein (GFP) small interfering RNA (siRNA) sequences that were used were described previously (32,33) (synthesized by Proligo, Kyoto, Japan). GFP siRNA was used as an siRNA control. In brief, 1 day before transfection, Saos2 cells were seeded without antibiotics to a density of 30–40%. DR5, GFP and DR4 siRNAs (25 nM) were then transfected into cells using a modified oligofectamine protocol (Invitrogen, Carlsbad, CA) as described previously (32). Mock samples were treated with oligofectamine alone. Twenty-four hours after transfection, cells were treated with 30 μM SFN and/or 50 ng/ml TRAIL for 24 h and then harvested.

![Image of SFN concentrations](image-url)

**Fig. 1.** Sensitization to TRAIL-induced apoptosis by SFN in Saos2 cells. SFN markedly enhances TRAIL-induced apoptosis in Saos2 cells. Saos2 cells were treated with SFN and/or TRAIL at the indicated concentrations for 24 h. Apoptosis (Sub-G1) was determined by FACS analysis of the DNA content of propidium iodide-stained nuclei as described in Materials and methods. The experiments were repeatedly performed to confirm the results.
Results

Combined treatment with SFN and TRAIL effectively induced apoptosis in Saos2, a human osteosarcoma cell line

We investigated the effects of SFN and TRAIL in Saos2 osteosarcoma cells. As a single agent, both SFN and TRAIL weakly induced apoptosis in Saos2 cells (Figure 1), though the cell viability of Saos2 cells was moderately reduced by the treatment with SFN for 24 h in a dose-dependent manner in WST-8 assay, the modified MTT assay (data not shown). Interestingly, we found that combined treatment with SFN and TRAIL effectively induced apoptosis in Saos2 cells.
The apoptosis induced by co-treatment with SFN and TRAIL is blocked by DR5/Fc chimeric protein or caspase inhibitors in Saos2 cells

To elucidate whether the sensitization to TRAIL-induced apoptosis by SFN occurred via a specific interaction between TRAIL and its receptors, we used recombinant human DR5/Fc chimeric protein, which exerts a dominant negative effect by competing with receptors for TRAIL. As shown in Figure 2, the SFN-mediated enhancement of TRAIL-induced apoptosis was markedly blocked by the DR5/Fc chimera, indicating that the sensitization by SFN was mediated through interactions of TRAIL with its receptors. We also found that the pancaspase inhibitor zVAD-fmk, and caspases 3, 8, 9 and 10 inhibitors, efficiently blocked the apoptosis induced by co-treatment with SFN and TRAIL. These results indicate that the apoptosis induced by co-treatment with SFN and TRAIL could be blocked by inhibition of DR5 or caspases.

Combined treatment with SFN and TRAIL induces apoptosis coupled with caspase activation and Bid cleavage in Saos2 cells

To confirm the effect of SFN on the TRAIL-induced activation of caspases, we carried out western blot analysis. As shown in Figure 3A, each agent only just weakly affected Bid and the caspases. However, SFN and TRAIL together drastically cleaved Bid and reduced pro-caspases 8, 9, 10 and 3. Moreover, co-treatment with SFN and TRAIL induced active form of caspases 8, 9 and 3. We also found that the DR5/Fc chimera efficiently blocked the cleavage of Bid and the activation of these caspases. These results are consistent with the data in Figure 2. Figure 3B shows the morphological features of Saos2 cells, which exhibited characteristic features of apoptosis including chromatin condensation.

SFN upregulates DR5 expression in Saos2 cells

To elucidate the mechanism of SFN-mediated enhancement of TRAIL-induced apoptosis, we examined the expression of death receptor-related genes after SFN treatment using an RNase protection assay. As shown in Figure 4A, SFN markedly induced DR5 and RIP mRNA expression in Saos2 cells. Also, Fas expression slightly decreased and DR4 and TNF-receptor (TNFR) expression slightly increased. TRAIL expression was not detected and did not increase. To confirm the upregulation of DR5 mRNA expression, we performed northern blot analysis. As shown in Figure 4B, SFN increased DR5 mRNA expression in a dose-dependent manner. In addition, we found that SFN also induced DR5 protein expression in dose- and time-dependent manners using western blotting (Figure 4C). Furthermore, SFN also induced DR4 protein expression in a dose-dependent manner. The DR5 upregulation by SFN was not affected by TRAIL and the DR5/Fc chimera (Figure 4D).

**DR5 upregulation by SFN contributes to the enhancement of TRAIL-induced apoptosis in Saos2 cells**

We tested whether or not the upregulation of DR5 and DR4 expression by SFN has an effect on TRAIL-induced apoptosis in Saos2 cells. The expression of DR5 and DR4 protein was efficiently reduced by transiently transfected DR5 and DR4

**Fig. 3.** The effects of the combination of SFN and TRAIL on Bid and caspases with morphological changes in Saos2 cells. (A) The enhancement of TRAIL-induced Bid cleavage and caspase activation by SFN. Saos2 cells were treated with 30 μM SFN, 50 ng/ml TRAIL and/or 1 μg/ml DR5/Fc chimera for 24 h. Bid and caspases 8, 9, 10 and 3 were then assessed by western blotting. β-actin was used to ensure equal gel loading. (B) DAPI staining of Saos2 cells. Saos2 cells were untreated or treated with 30 μM SFN and/or 50 ng/ml TRAIL for 24 h, and then nuclear morphology was visualized using DAPI staining under a fluorescence microscope.
Fig. 4. SFN upregulated DR5 expression in Saos2 cells. (A) The effect of SFN on death receptor-related genes. RNase protection assay was performed as described in Materials and methods. Lane 1, 1/10 probes without RNase treatment; lane 2, RNase-protected probes after hybridization with yeast tRNA; lanes 3 and 4, RNase-protected probes after hybridization with total RNA from Saos2 cells treated with or without 30 μM SFN for 24 h. GAPDH and L32 were shown as controls. CT: equivalent DMSO as a control. (B) SFN upregulated DR5 mRNA expression. Saos2 cells were treated with SFN at the indicated concentrations for 24 h. Northern blotting was then performed as described in Materials and methods. Ethidium bromide-stained 28S and 18S ribosomal RNA were shown as controls. The relative band intensities were estimated after normalization with the band intensity of 28S ribosomal RNA. (C) SFN upregulated DR5 protein expression. Saos2 cells were treated with SFN at the indicated concentrations for 24 h or with 30 μM SFN for the indicated periods. Western blotting was then performed as described in Materials and methods. β-actin was used to ensure equal gel loading. (D) Saos2 cells were treated with 30 μM SFN, 50 ng/ml TRAIL and/or 1 μg/ml DR5/Fc chimera for 24 h. DR5 was then assessed by western blotting. β-actin was used to ensure equal gel loading.
Fig. 5. Downregulation of DR5 reduced SFN-mediated TRAIL-induced apoptosis in Saos2 cells. (A) Reduction of DR5 and DR4 protein by DR5 and DR4 siRNA. Saos2 cells were treated with DR5 siRNA, GFP siRNA, DR4 siRNA or transfection reagent oligofectamine alone (siRNA -). Twenty-four hours after transfection, cells were treated with or without 30 μM SFN for 24 h. Western blotting was then performed as described in Materials and methods. β-actin was used to ensure equal gel loading. (B) Inhibition of DR5 expression reduced the sensitization to TRAIL-induced apoptosis by SFN. Saos2 cells were treated with DR5 siRNA, GFP siRNA, DR4 siRNA or transfection reagent oligofectamine alone. Twenty-four hours after transfection, cells were treated with or without 30 μM SFN and 50 ng/ml TRAIL for 24 h. Apoptosis (Sub-G1) was determined by FACS analysis of the DNA content of propidium iodide-stained nuclei as described in Materials and methods. The experiments were repeatedly performed to confirm the results.
results suggest that the upregulation of DR5 expression accounts at least in part for the enhancement of TRAIL-induced apoptosis by SFN treatment in Saos2 cells.

**SFN upregulates DR5 expression and enhances TRAIL-induced apoptosis in MG63 cells**

Next, we tested the effect of SFN on TRAIL-induced apoptosis in another osteosarcoma cell line, MG63. As shown in Figure 6A, SFN also induced DR5 protein expression in dose- and time-dependent manners in MG63 cells. Furthermore, SFN significantly enhanced TRAIL-induced apoptosis in MG63 cells (Figure 6B). These results suggest that SFN induces the upregulation of DR5 and enhances TRAIL-induced apoptosis in other osteosarcoma cell types.

**SFN does not induce DR5 protein expression and fails to enhance TRAIL-induced apoptosis in primary non-malignant human cells**

We discovered that SFN upregulated DR5 expression and enhanced TRAIL-induced apoptosis in Saos2 and MG63 cells. Next, to examine the effect of SFN treatment on normal cells, we used normal human PBMC. As shown in Figure 7A, the level of DR5 expression in PBMC was not upregulated by SFN treatment. Furthermore, SFN did not enhance TRAIL-induced apoptosis in PBMC like malignant osteosarcoma cells (Figure 7B).

**Discussion**

To improve the prognosis of osteosarcoma, new strategies are necessary. In the search for new strategies and antitumor agents, we found that SFN is a potent enhancer of TRAIL-induced apoptosis in osteosarcoma cells. Moreover, we found that the downregulation of DR5 expression by DR5 siRNA reduced the sensitization to TRAIL-induced apoptosis by SFN (Figure 5B). This finding indicates that the upregulation of DR5 expression by SFN is at least in part responsible for the enhancement of TRAIL-induced apoptosis.

SFN has been reported to induce either a G1 arrest or a G2/M arrest in various cancer cell lines. We analyzed the data of the fluorescence activated cell sorter (FACS) analysis using the ModFit LT V2.0 software package (Verity Software, Topsham, ME). The FACS analysis revealed that a 24 h treatment with SFN at 20 μM or more increased the population of G2/M phase cells. Cells at the G2/M phase increased from 8.4% in medium alone to 11.0, 31.3, 28.7 and 29.1% by 10, 20, 30 and 40 μM SFN, respectively (Figure 1). The viability of Saos2 cells was
reduced by the treatment of SFN for 24 h caused by the cell cycle arrest. However, as a single agent, SFN weakly induced apoptosis in Saos2 cells, and the combined treatment with SFN and TRAIL effectively induced apoptosis in Saos2 cells.

Whereas the mechanism of the mitochondrial apoptotic pathway of SFN-induced apoptosis was reported previously, the mechanism of the SFN-mediated death receptor pathway was not. In this study, we determined for the first time that SFN...
together with TRAIL significantly induces apoptosis through the death receptor pathway.

In addition, SFN upregulates DR5 expression and sensitizes TRAIL-induced apoptosis in a p53-independent manner because functionally inactivated mutations of the p53 gene exist in Saos2 and MG63 cells. In osteosarcoma cells, p53 is frequently inactivated (34). Several studies have shown that conventional anti-osteosarcoma agents such as doxorubicin, cisplatin and etoposide mainly induce apoptosis in a p53-dependent manner (35,36). Therefore, the combined treatment with SFN and TRAIL may be effective for osteosarcoma with resistance to conventional agents caused by inactivated p53.

Furthermore, conventional anticancer agents that are cytotoxic in normal cells, cause side-effects and reduce the quality of life for the patient. It is necessary to develop anticancer agents with tumor-selective cytotoxicity. For this, TRAIL is promising owing to little or no toxicity against normal cells (10,11).

SFN is a food factor that is contained in vegetables. Ye et al. (37) reported the human plasma concentrations to reach only 2 μM after consuming SFN-rich broccoli sprouts. However, Hu et al. (38) reported that plasma concentration reaches 20 μM after oral administrations of SFN in rat. We used not broccoli sprouts but purified SFN as an antioxidant agent for osteosarcoma cells in this study. The human plasma concentrations might reach a concentration used in our in vitro study after the administration of purified SFN.

Our previous study showed that histone deacetylase inhibitors (HDACIs) such as trichostatin A (TSA), sodium butyrate and suberoylanilide hydroxamic acid (SAHA) induce DR5 protein and enhance TRAIL-induced apoptosis in malignant tumor cells. However, HDACIs neither induce DR5 protein or enhance TRAIL-induced apoptosis in normal human PBMC (31), thus explaining their low toxicity against normal bone marrow cells in cancer patients. In this study, we determined that SFN also does not enhance TRAIL-induced apoptosis in normal human PBMC (Figure 7), suggesting that this regimen may also expected to be safe in the clinic. Previously, Myzak et al. (39) reported an HDAC inhibitory activity of SFN. We examined a HDAC inhibitory activity of SFN by detection of acetylated histone H4 using western blot analysis in Saos2 cells. However, we did not detect increase of acetylated histone H4 after treatment with SFN (data not shown). This result suggests that SFN may induce DR5 expression through a mechanism different from the function of HDACIs in osteosarcoma Saos2 cells.

In conclusion, we showed for the first time that SFN significantly enhances TRAIL-induced apoptosis through the induction of DR5 expression in human osteosarcoma cells but not in primary non-malignant human cells. Although we need further study of chemotherapeutic effect in vivo, these results raise the possibility that the combination of SFN and TRAIL might be a clue to a promising therapy for osteosarcoma.

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