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Effectiveness of sulforaphane as a radiosensitizer for murine osteosarcoma cells

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Abstract. Sulforaphane (SFN), a naturally occurring member of the isothiocyanate family, is effective against various types of malignant tumor cells. We studied whether the combination of SFN and radiation would be more effective against osteosarcoma cells when compared to these treatments alone. LM8 murine osteosarcoma cells were cultured with various concentrations of SFN for 24 h and/or 2 Gy X-irradiation. The effects of individual and combination treatments on the number of cells, the cell cycle, cell proliferation-related factors and apoptosis were analyzed. The combination of SFN plus radiation had significantly greater antitumor effects than either treatment alone. Exposure to SFN increased the population of cells in the G_2/M phase. Combination treatment resulted in a higher percentage of cells being in sub-G_1 than did SFN alone. In addition, the combination of SFN and radiation effectively induced nuclear fragmentation and apoptotic bodies, as shown by DAPI staining. The combination of SFN and 2 Gy radiation increased the cleavage and activation of caspase-3 compared with SFN or radiation alone, as shown by western blotting. Although radiation alone increased the phosphorylation of ERK and Akt proteins, the combination of SFN and radiation induced suppression of ERK and Akt phosphorylation when compared with radiation alone. We found that SFN enhanced the radiosensitivity of LM8 murine osteosarcoma cells by inducing apoptosis through G_2/M-phase arrest and by inhibiting ERK and Akt activation. These findings suggest that SFN can be used as a radiosensitizer for osteosarcomas.

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Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. Standard treatment consists of multi-agent neoadjuvant chemotherapy, radical excision of the tumor and adjuvant chemotherapy (1,2). However, many patients still succumb to the disease as a result of tumor metastasis and relapse (3,4). The chemoresistance of tumor cells is one of the most prevalent causes of therapeutic failure (5,6). Although patients with chemoresistant cells require palliative treatment such as radiotherapy, osteosarcomas are considered to be radioresistant tumors (7,8), necessitating the combination of chemotherapy and radiotherapy for these patients (8,9).

In general, tumor cells are the most radiosensitive during G_2/M phase of the cell cycle and the most radioresistant in S phase (10,11). Radiotherapy has been shown to activate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways, which regulate cell proliferation and apoptosis. Inhibition of these pathways has been reported to enhance radiosensitivity of cells (12,13).

New osteosarcoma treatment regimens have been investigated, including many clinical trials of novel agents, among which is sulforaphane (SFN) (14), a naturally occurring member of the isothiocyanate family produced by cruciferous vegetables such as broccoli (15). SFN has been shown to suppress the growth of T-cell leukemia, colon, breast and prostate cancer cells *in vitro* by inhibiting cell cycle progression (16-20) and/or causing apoptosis (17,18). We previously reported that SFN inhibited the proliferation of cultured murine osteosarcoma LM8 cells i) by inducing G_2/M phase arrest, as shown by the appearance of cells with sub-G_1 DNA content; and ii) by inducing apoptosis, as shown by the cleavage and activation of caspase-3 (21). In addition, SFN inhibited the activation of the PI3K/Akt and MAPK pathways in pancreatic and prostate cancer cells (22,23).

Our findings that SFN induced G2/M-phase arrest and inhibited the PI3K/Akt and MAPK pathways, suggest that SFN may enhance the radiosensitivity of LM8 cells. Moreover, the combination of SFN and radiotherapy may

further inhibit cell growth, thereby allowing a decrease in the doses of both drug and irradiation to safer levels than when used alone, ensuring a lower incidence and grade of side effects. Although SFN has been found to promote the radiosensitization of cancer cells (24,25), the combined effects of SFN and radiation in osteosarcoma cells have not been studied. We, therefore, analyzed the effects of SFN and radiation on LM8 cells, including their effects on cell cycle, the inhibition of the MAPK and PI3K/Akt pathways and the induction of apoptosis.

Materials and methods

Reagents. Sulforaphane (SFN) was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), and was dissolved in dimethyl sulfoxide (DMSO); equivalent volumes of DMSO were used as controls. The maximum percentage of DMSO in the assays was 0.1%.

X-ray irradiation. Cultured cells were irradiated with 2 Gy X-rays using Softex M-150WE (Softex Co., Ltd., Tokyo, Japan). The cells were placed 1 cm from the focus and the irradiation rate was 0.5 Gy/min in air.

Cell culture. The LM8 murine osteosarcoma cell line was established from the murine Dunn osteosarcoma cell line and exhibit high metastatic potential to the lungs (27). LM8 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) containing antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin) and incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Concurrent exposure to SFN and radiation

Effects on cell growth. LM8 cells were cultivated in 6-well plates at $2x10^4$ cells/well in 2 ml medium for 24 h, followed by incubation with various concentrations of SFN for 24 h and/or X-irradiation at 2 Gy. After 24 or 48 h, the number of viable cells was counted using a trypan blue dye exclusion test. The data are presented as the means ± standard deviation (SD) of at least three independent experiments.

Analysis of cell cycle progression. To assess the effects of SFN alone, radiation alone or the two treatments together on the cell cycle, LM8 cells were cultivated in 6-well plates at $2x10^4$ cells/well and exposed to various doses of SFN and/or irradiation for 24 h. After 48 h, the cells were stained with propidium iodide (Sigma Aldrich, St. Louis, MO, USA), and the stained nuclei were analyzed by flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA). DNA histograms were created using CellQuest software for Apple Macintosh (Becton-Dickinson). For all assays 10,000 events were counted, with each assay performed in triplicate.

Western blot analysis. LM8 cells were plated in 6-well culture plates at $2.0x10^4$ cells/well and incubated for 24 h, followed by incubation with 20 μM SFN for 24 h and/or radiation at 2 Gy. After 1 and 48 h, the cells were washed twice with PBS and lysed with RIPA buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 40 mM NaF, and protease inhibitor cocktail (Sigma Aldrich)]. The lysates were centrifuged at 15,000 rpm for 20 min; the supernatant lysate was incubated in sample buffer [0.0625 M Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 5% 2-ME] at 95˚C for 5 min; and the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto nitrocellulose membranes (Amersham Biosciences, Tokyo, Japan). The membranes were incubated in 5% (wt/vol) non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) [25 mM Tris HCl (pH 7.8), 140 mM NaCl, 0.1% (vol/vol) Tween 20] and incubated overnight with the following antibodies (each from Cell Signaling Technology, Beverly, MA, USA, and diluted 1:1,000 in TBST): extracellular signal-regulated kinase (ERK1/2), phosphorylated ERK1/2 (pERK1/2), Akt, phosphorylated Akt (p-Akt), caspase-3, cleaved caspase-3 and GAPDH. The membranes were washed thoroughly with TBST, incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or -rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:5,000 in TBST, and developed with enhanced chemiluminescence kits (Amersham Biosciences).

Analysis of nuclear morphology. LM8 cells treated with SFN and/or radiation under appropriate conditions were cultured for 48 h, fixed with 2% paraformaldehyde in PBS for 10 min, and stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) (Nacalai Tesque, Inc., Kyoto, Japan) at 4˚C in the dark. For fluorescence microscopy, cells were cytospun onto slides and examined using a fluorescence microscope Eclipse 1000 (Nikon, Tokyo, Japan) with UV illumination. Apoptotic cells were identified on the basis of characteristic changes, including nuclear condensation, fragmentation and apoptotic bodies.

Statistical analysis. All data are represented as the means ± SD. Statistical significance was determined using Student's t-tests. $P<0.05$ was considered to indicate a statistically significant result.

Results

Growth inhibitory effects of combination therapy in murine osteosarcoma LM8 cells. The combination of SFN and radiation treatment produced significantly greater antitumor effects on the LM8 osteosarcoma cells than either treatment alone (Fig. 1). Stronger combined effects were observed 48 h after treatment of SFN and radiation than effects obtained after 24 h.

Combined effects of SFN and radiation on the distribution of the cell cycle. To determine the effects of SFN and radiation on cell cycle progression in LM8 cells, the DNA content of their nuclei was assessed by flow cytometry. Exposure to SFN for 72 h dose-dependently increased the population of cells in the G_2/M phase (Fig. 2A). Following exposure to SFN plus 2 Gy radiation, the numbers of cells in the G_2/M phase (Fig. 2A) and in sub-G_1 (Fig. 2B) were greater than these values after exposure to SFN alone.

Combined effects of SFN and radiation on apoptosis of LM8 cells. Nuclear fragmentation and apoptotic bodies characteristic of apoptosis were observed with DAPI staining in LM8

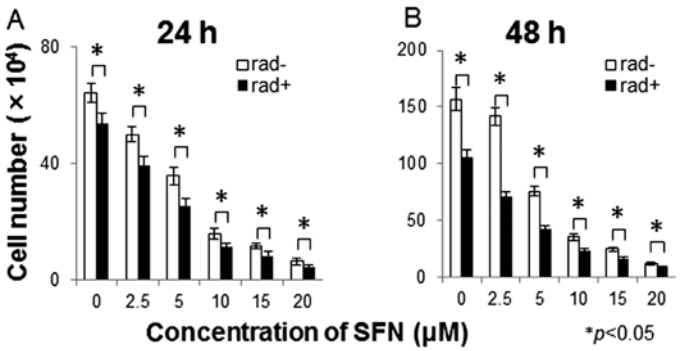

Figure 1. Effect of combination therapy on the growth of murine osteosarcoma LM8 cells. Twenty-four hours after seeding, LM8 cells were incubated with 0, 2.5, 5, 10, 15 and 20 μM SFN for 24 h, followed by exposure to 2 Gy X-irradiation (black bars, rad+) or its absence (white bars, rad-). The numbers of viable cells were counted after (A) 24 h and (B) 48 h using a trypan blue dye exclusion test. Shown are the means ± SD (bars) of at least three independent experiments.

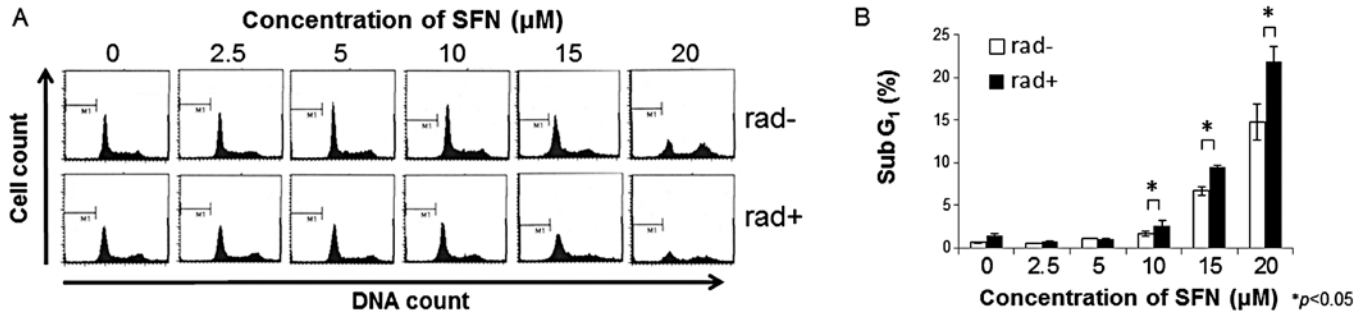

Figure 2. Effects of SFN plus radiation on the cell cycle and the proportion of cells in sub-G_1. (A) Cell cycle analysis following combined treatment with SFN plus radiation. Twenty-four hours after seeding, LM8 cells were treated with 0, 2.5, 5, 10, 15 and 20 μM SFN for 24 h, followed by treatment with (rad+) or without (rad-) 2 Gy X-irradiation. After 48 h, the DNA content of propidium iodide-stained nuclei was analyzed by FACSCalibur flow cytometry, as described in Materials and methods. (B) Percentage of cells in Sub-G_1. LM8 cells were treated with the indicated concentrations of SFN in the presence (black bars, rad+) or absence (white bars, rad-) of 2 Gy X-irradiation, and the cells were analyzed by FACSCalibur flow cytometry. Data are shown as means (bars, SD) (n=3). *P<0.05.

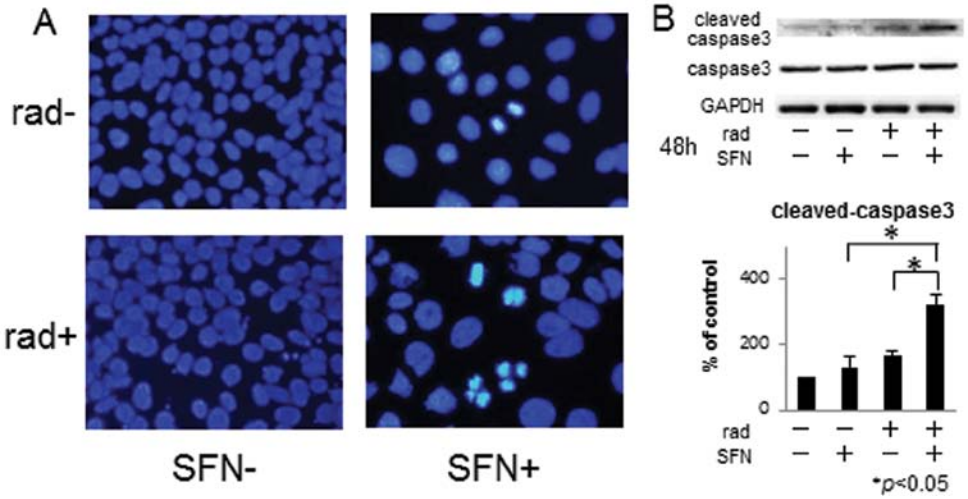

Figure 3. Combined effect of SFN and radiation on cellular apoptosis. (A) DAPI staining of LM8 cells. LM8 cells were treated with (SFN+) or without (SFN-) 20 μM SFN, followed by culture in the presence (rad+) or absence (rad-) of 2 Gy X-irradiation. The cells were subsequently stained with DAPI staining and examined by fluorescence microscopy. (B) Effects of SFN and radiation on caspase-3 activation. LM8 cells were treated with (SFN+) or without (SFN-) 20 μM SFN, followed by culture in the presence (rad+) or absence (rad-) of 2 Gy X-irradiation, and caspase-3 expression was assessed by western blotting. Subsequent incubation with an anti-GAPDH antibody was used as a loading control. Data are shown as means (bars, SD) (n=3). *P<0.05.

cells treated with 20 μM SFN for 48 h plus 2 Gy X-irradiation for 48 h (Fig. 3A), and were more frequently observed than in cells treated with SFN alone. In addition, western blotting showed an increase in the amount of activated caspase-3 in

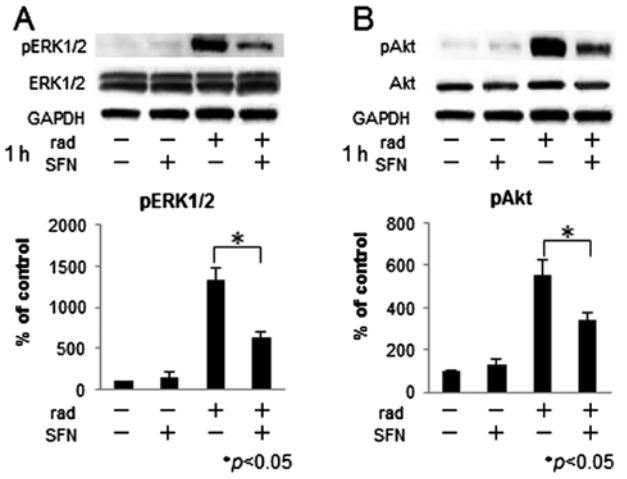

Figure 4. Effects of SFN and radiation on the phosphorylation of ERK and Akt. LM8 cells were treated with 20 μM SFN and/or 2 Gy X-irradiation. After 1 h, the expression levels of ERK, phosphorylated ERK, Akt and phosphorylated Akt proteins were assessed by western blotting. An anti-GAPDH antibody was used to confirm equal gel loading. Data are shown as means (bars, SD) (n=3). *P<0.05.

cells treated with SFN plus irradiation when compared with that in cells treated with SFN alone (Fig. 3B).

Combined effects of SFN and radiation on the phosphorylation of ERK and Akt. To assess the effect of SFN and radiation on the phosphorylation of ERK and Akt, LM8 cells were treated with 20 μM SFN and 2 Gy X-irradiation for 1 h, and the expression levels of ERK, phosphorylated ERK, Akt and phosphorylated Akt protein were evaluated by western blotting (Fig. 4). We found that X-irradiation alone increased the expression of phosphorylated ERK and Akt proteins, whereas the levels of phosphorylation were lower in cells treated with both SFN and X-irradiation than in cells treated with X-irradiation alone.

Discussion

SFN, first identified in broccoli sprouts in 1992 (15), is a cancer chemopreventive agent that suppresses the growth of osteosarcoma cells and other malignant tumors. It is already being assessed in clinical trials, including a phase II trial in patients with prostate cancer. We previously reported that intraperitoneal administration of SFN significantly inhibited the growth of LM8 xenografts to less than 30% of the controls in a murine tumor model, without causing any toxicity (21).

Cell cycle arrest and apoptosis are considered to be most important among the suggested mechanisms of action of SFN. SFN has been reported to induce G_2/M arrest and apoptosis in human osteosarcoma U2-OS cells (28), as well as to induce growth arrest and upregulate the expression of $p21^{WAF1/CIP1}$ protein in a p53-independent manner in human osteosarcoma MG63 cells (14). Moreover, SFN inhibited the growth of LM8 cells i) by causing G_2/M-phase arrest, as shown by the appearance of cells with sub-G_1 DNA content; and ii) by inducing apoptosis, as shown by the cleavage and activation of caspase-3 (21). In addition, SFN was found to inhibit the phosphorylation of Akt and ERK and to regulate apoptosis and cell proliferation. In pancreatic cancer cells, SFN was shown to induce apoptosis through the inhibition of both the PI3K/Akt and MEK/ERK pathways (22).

Following oral administration of the effective dose of SFN to rats, its maximum plasma concentration was 20 μM (28). However, it was found that in humans the maximum plasma concentrations were only 2 μM after oral intake of SFN-rich broccoli sprouts (29). Therefore, we studied whether or not the effects of SFN can be enhanced when combined with X-irradiation.

Radiotherapy has long been used to treat malignant tumors. In the treatment of osteosarcoma, however, standard treatment consists of neoadjuvant chemotherapy, surgical excision and adjuvant chemotherapy. The use of radiotherapy has been limited to patients in poor general condition and those with unresectable tumors (30). In general, cells are most radiosensitive during the G_2/M phase and most radioresistant during the S phase (10,11). Agents that induce cell cycle arrest in the G_2/M phase have thus exhibited potent radiosensitivity *in vitro* and *in vivo* (31-34). Inhibition of WEE1 kinase has been reported to abrogate G_2 arrest and may sensitize OS cells to irradiation-induced cell death (34). In contrast, radioresistance may be due to radiation-induced activation of ERK and Akt, resulting in the dynamic and rapid adaptation of tumor cells to maintain growth and viability (12). Thus, inhibition of ERK and Akt activation may enhance the radiosensitivity of tumors (13,35).

SFN has been reported to enhance the radiosensitivity of HeLa human cervical carcinoma cells *in vitro* and *in vivo* by inhibiting the repair of DNA double-strand breaks (DSB), through the inhibition of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and RAD51 (24). Moreover, a combination of SFN and radiation was found to decrease clonogenic survival in 4 human cancer cell lines derived from head and neck squamous cell carcinomas, in which apoptosis is not regulated through Akt or the Mcl-1 protein (25).

We found that either SFN alone or radiation alone significantly and dose-dependently inhibited the growth of LM8 cells, whereas the combination of SFN and irradiation further enhanced the growth inhibitory effects. However, the precise synergistic mechanism of action of SFN and radiation is currently unknown. We, therefore, investigated the mechanisms involved when SFN and radiation were combined. Incubation of LM8 cells with SFN alone dose-dependently increased the number of cells in the G_2/M phase and in sub-G_1, as previously described (21,27). Although radiation alone had no effect on the cell cycle, the combination of SFN and irradiation significantly increased the number of cells in sub-G_1. These findings suggest that combination treatment may induce apoptosis more efficiently. Indeed, we found that combination treatment increased the number of cells showing nuclear fragmentation and apoptotic bodies and the expression of activated caspase-3. Thus, the SFN-induced death of LM8 cells is considered to be apoptotic.

We also studied whether the combination of SFN and radiation activates the pathway of ERK and Akt. It turned out that SFN inhibited the radiation-induced phosphorylation of ERK and Akt, suggesting that SFN enhanced the radiosensitivity of LM8 cells. These results were similar to previous findings, although the induction of apoptosis by SFN and radiation was regulated through Akt in head and

neck squamous cell carcinoma cell lines (25). It is known that squamous cell carcinomas are considered radioreactive, whereas osteosarcomas are not. It could thus be argued that tumor cell-intrinsic properties in terms of radiosensitivity may predispose to the difference between our results and those by Kotowski *et al* (25).

In conclusion, we found that SFN enhanced the radiosensitivity of murine osteosarcoma LM8 cells by inducing apoptosis through G2/M-phase arrest and inhibiting ERK and Akt activation. Thus, combined treatment with SFN and radiotherapy may be useful in enhancing the antitumor effects of SFN alone. We would propose a novel therapeutic regimen for patients with osteosarcoma in which SFN and radiation are combined.

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