Sulforaphane induces cell cycle arrest and apoptosis in murine osteosarcoma cells *in vitro* and inhibits tumor growth *in vivo*

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Abstract. Sulforaphane (SFN), a naturally occurring isothiocyanate, is an attractive agent due to its potent anticancer effects. SFN suppresses the proliferation of various cancer cells *in vitro* and *in vivo*. In this study, we report that SFN inhibited the proliferation of cultured murine osteosarcoma LM8 cells. Twenty micromolar SFN completely inhibited the growth of LM8 cells and caused G_2/M-phase arrest. SFN induced the expression of $p21^{WAF1/CIP1}$ protein causing the cell cycle arrest in a dose-dependent manner. SFN induced apoptosis which was characterized by the appearance of cells with sub-G_1 DNA content and the cleavage and activation of caspase-3. We showed that SFN induced the growth arrest and up-regulated the expression of $p21^{WAF1/CIP1}$ protein in a p53-independent manner in human osteosarcoma MG63 cells. We found that intraperitoneal administration of SFN (1 or 2 mg, 5 times/week) significantly inhibited the growth of LM8 xenografts to <30% of the controls in a preclinical animal model without causing any toxicity. In osteosarcoma cells, our findings provide *in vivo* evidence for the efficacy of SFN against the advanced growth of tumor. We showed that SFN induces cell cycle arrest and apoptosis in osteosarcoma cells and inhibits tumor xenograft growth. Furthermore, SFN is a potent inducer of $p21^{WAF1/CIP1}$ in osteosarcoma cells. These results raise the possibility that SFN may be a promising candidate for molecular-targeting chemotherapy against osteosarcoma.

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Abbreviations: SFN, sulforaphane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: osteosarcoma, sulforaphane, $p21^{WAF1/CIP1}$, cell cycle arrest, apoptosis, xenografts

Introduction

Osteosarcoma is a high-grade malignant bone tumor that mainly occurs in juvenile patients. Although the prognosis of these patients have improved substantially through the development of effective adjuvant or neoadjuvant regimens of chemotherapy (1-4), >20% of patients still die as a result of tumor metastasis and unresectable tumor (5-10). One of the most serious causes of therapeutic failure is the resistance of the tumor cells to chemotherapeutic agents (11,12). To overcome the drug resistance, identification of novel anti-tumor agents or chemicals and the development of new anti-tumor therapeutic approaches are urgently required.

One of the causes of cancer is uncontrolled proliferation due to the loss of the checkpoint control associated with the activation of cyclin-dependent kinases (CDKs) responsible for cell cycle progression (13). CDKs, cyclins and CDK inhibitors (CDKIs) are key molecules that play important roles in cell cycle progression (14). $p21^{WAF1/CIP1}$ is a member of the CDKI family and induces G_1- and G_2/M-phase cell cycle arrest (15-18). $p21^{WAF1/CIP1}$ induces differentiation of both normal and transformed cells and suppresses the growth of malignant cells *in vitro* and *in vivo* (19,20). Therefore, $p21^{WAF1/CIP1}$ is an attractive molecular target which suppresses cell growth in malignant tumor cells and $p21^{WAF1/CIP1}$-inducing agents may be effective for the chemotherapy of poor prognostic osteosarcoma.

Furthermore, cancer cells acquire alternations for enhanced survival and become apoptosis-resistant to anticancer therapies (21). Therefore, the induction of the cell cycle arrest and apoptosis by chemotherapeutic agents can be an effective approach to inhibiting uncontrolled cell proliferation and survival in malignant tumor cells.

Sulforaphane (SFN), a naturally occurring member of the isothiocyanate family, is produced from cruciferous vegetables, such as broccoli (22). SFN is an effective agent in the

chemoprevention of chemically-induced breast (23,24), colon (25) and stomach (26) cancers in rats. In a chemotherapeutic study, SFN drastically inhibited the growth of xenografts of human prostate cancer by oral administration (27) and breast cancer by intravenous injection (28). SFN suppresses the growth of cancer cells *in vitro* by inhibiting cell cycle progression (28-32) and/or causing apoptosis (27,29,30) in T-cell leukemia, colon, breast and prostate cancer cells. In addition, it was reported that SFN induces $p21^{WAF1/CIP1}$ and G_1- and G_2/M-phase cell cycle arrest in human colon cancer cells. We previously reported that SFN up-regulates DR5 expression and the combined treatment with SFN and TRAIL-induced apoptosis in human osteosarcoma cells (33). However, in osteosarcoma cells, the anti-tumor effects of SFN were not reported *in vivo*.

In this study, we confirmed that SFN causes cell cycle arrest and apoptosis *in vitro*. Furthermore, we investigated the anti-tumor activity of SFN against osteosarcoma cells *in vivo*.

Materials and methods

Reagents. Sulforaphane (SFN) was purchased from LKT (St. Paul, MN). SFN was dissolved in DMSO. Equivalent amounts of DMSO were used as controls. The maximum volume (%) of DMSO in the assays was 0.1%.

Cell culture. We used a human osteosarcoma cell line (MG63) and a murine osteosarcoma cell line (LM8), which was established from the murine Dunn osteosarcoma cell line and has high metastatic potential to the lungs (34). LM8 cells and human osteosarcoma MG63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Cell growth study. For the cell growth study, LM8 cells and MG63 cells were seeded at a density of $1x10^4$ cells in a 12-well plate. Twenty-four hours after the seeding, SFN was added at various concentrations. From 24 to 48 h after the treatment, the number of viable cells were counted using a trypan blue dye exclusion test. The data are presented as the mean ± SD of at least three independent experiments.

Analysis of cell cycle progression. Unsynchronized cells were exposed to SFN for 24 h and harvested from culture dishes. After washing with PBS, the cells were suspended in PBS containing 0.1% Triton X-100, treated with RNase A and the nuclei were stained with propidium iodide (PI). DNA content was measured using a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). For all assays, 10,000 events were counted. The ModFit LT V2.0 software package (Verity Software, Topsham, ME) was used to analyse the data.

Western blot analysis. A protein extraction and Western blot analysis was performed as previously described (35), using a rabbit polyclonal anti-$p21^{WAF1/CIP1}$ antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal anti-GAPDH (1:1000) antibody (Immunotech, Marseille, France) and a mouse monoclonal anti-pro-caspase-3 (1:10) antibody (Immunotech, Marseille, France). Enhanced chemiluminescence (GE Science, Piscataway, NJ) was used for detection.

Detection of apoptosis. To analyse apoptosis, hypodiploid DNA (Sub-G1) populations were assayed using a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson) as previously described (36). For all assays, 10,000 events were counted and carried out in triplicate. The data were analysed using the Student's t-test. Differences were considered to be statistically significant from the controls for $p<0.05$.

For the observation of nuclear morphology, cells grown in six-well plates were incubated with DMSO or SFN at 20 μM for 48 h. The cells were then fixed in methanol, incubated with 4'-diamino-2-phenylindole (DAPI) solution for 30 min in the dark and then analysed using a fluorescence microscope (Olympus, Tokyo, Japan) at 420 nm.

Xenograft assay. Male Balb/C mice (Oriental Bio Service, Kyoto, Japan) were maintained according to the Institutional Animal Care Use Committee guidelines. LM8 cells were mixed in PBS and a suspension containing 10^7 LM8 cells was administered to the right flank of mice via an s.c. injection. Mice were randomized into three groups of 5 mice/group. Twenty-four hours later, intraperitoneal injections of SFN (1 or 2 mg in 0.1 ml PBS) were performed 5 times/week. Control mice received an equal volume of the vehicle. Tumor volume was determined as previously described (37). Statistically significant differences in tumor volume between the control and the treated mice were assessed by the Student's t-test.

Results

SFN inhibited the growth of murine osteosarcoma LM8 cells. We investigated the effects of SFN on the growth of murine osteosarcoma LM8 cells. Fig. 1 shows the growth of LM8 cells in the presence of various concentrations of SFN. A dose-dependent inhibition of cell growth was observed at concentrations of 5 μM or more. Forty-eight hours after the addition of SFN, the growth of LM8 cells was inhibited to 71.3, 18.4 and 4.4% of the control level by 5, 10 and 20 μM SFN, respectively (Fig. 1).

SFN arrested LM8 cells at the G_2/M phase in the cell cycle progression and up-regulated $p21^{WAF1/CIP1}$ expression. To elucidate the effect of SFN on the cell cycle progression of LM8 cells, the DNA content of nuclei of LM8 cells was measured by flow cytometric analysis. As shown in Fig. 2A, the FACS analysis revealed that a 24 h exposure to SFN increased the population of G_2/M phase cells in a dose-dependent manner. LM8 cells at the G_2/M phase increased from 13.3% in medium alone to 33.0% by treatment with 20 μM SFN.

We examined the expression of the $p21^{WAF1/CIP1}$ protein after SFN treatment. In LM8 cells, as shown in Fig. 2B, we found that SFN increased the $p21^{WAF1/CIP1}$ protein expression in a dose-dependent manner using Western blotting.

SFN effectively induced apoptosis in LM8 cells. We investigated that SFN induces apoptosis in LM8 cells. Treatment

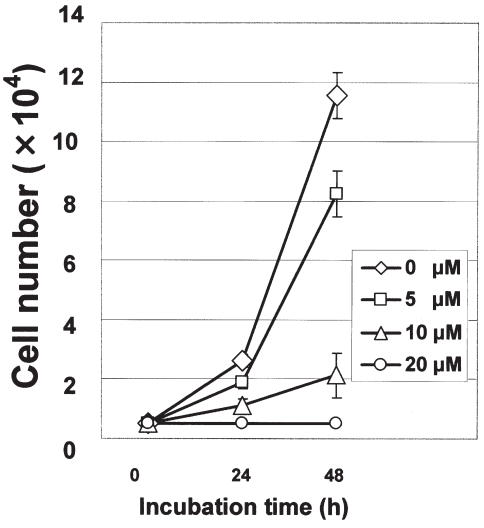

Figure 1. Effect of SFN on the growth of LM8. Twenty-four hours after seeding of LM8 cells, SFN at 5 (□), 10 (△), or 20 (○) μM was added and the cell number was compared with a control culture with equivalent DMSO (◇) by counting the cells using a trypan blue dye exclusion test. The values shown are means (bars, SD) (n=3).

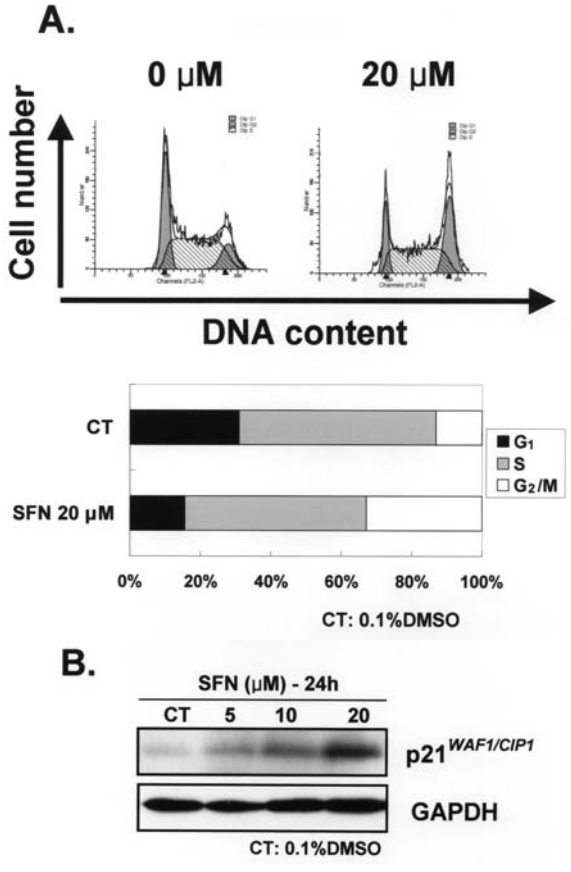

Figure 2. The effect of SFN on the cell cycle progression and up-regulation of p21$^{WAF1/CIP1}$ expression by SFN in LM8 cells. (A) LM8 cells were treated with or without 20 μM SFN at the indicated concentrations for 24 h. The DNA content of propidium iodide-stained nuclei was analysed by FACSCalibur flow cytometry as described in Materials and methods. The experiments were repeated to confirm the results. The percentage of cells in phases G_1 (black), S (gray) and G_2/M (white) was determined. (B) SFN up-regulated the p21$^{WAF1/CIP1}$ protein expression. LM8 cells were treated with SFN at the indicated concentrations for 24 h. Western blotting was then performed as described in Materials and methods. An anti-GAPDH antibody was used to confirm equal gel loading.

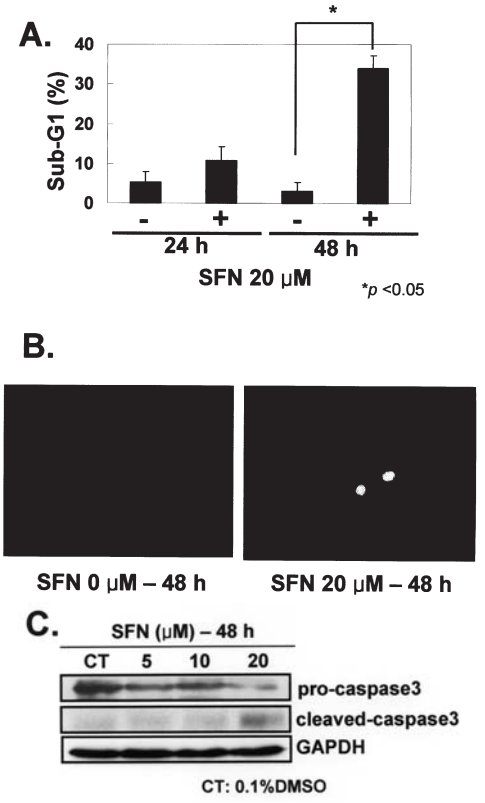

Figure 3. SFN induced apoptosis in LM8 cells. (A) LM8 cells were treated with or without 20 μM SFN for the indicated periods. Apoptosis (Sub-G1) was determined by FACS analysis of the DNA content of propidium iodide-stained nuclei as described in Materials and methods. Data are shown as means (bars, SD) (n=3).*p<0.05. (B) DAPI staining of LM8 cells. LM8 cells were treated with or without 20 μM SFN for 48 h and then nuclear morphology was visualized using DAPI staining using a fluorescence microscope. (C) Caspase-3 was activated by SFN. LM8 cells were treated with SFN, at the indicated concentrations for 48 h. Caspase-3 was then assessed by Western blotting. An anti-GAPDH antibody was used to confirm equal gel loading.

with 20 μM SFN for 24 h weakly induced apoptosis in LM8 cells. However, treatment with 20 μM SFN for 48 h effectively induced apoptosis in LM8 cells (Fig. 3A).

Fig. 3B shows the morphological features of LM8 cells that exhibited the characteristic features of apoptosis including chromatin condensation.

To confirm the effect of SFN on the activation of caspases, we carried out a Western blot analysis. As shown in Fig. 3C, SFN caused the cleavage and activation of caspase-3. These results indicate that the SFN-mediated cell death occurred in a caspase-dependent manner.

SFN induced a G_2/M phase cell cycle arrest and up-regulated p21$^{WAF1/CIP1}$ expression in human osteosarcoma MG63 cells in a p53-independent manner. We investigated the effects of SFN on the growth and the cell cycle of human osteosarcoma MG63 cells. Fig. 4A shows that a dose-dependent inhibition of the cell growth was observed at concentrations of 5 μM or more. FACS analysis revealed that a 24 h exposure to SFN increased the population of G_2/M phase cells in a dose-dependent manner (Fig. 4B). We examined the expression of the p21$^{WAF1/CIP1}$ protein after SFN treatment and found that the SFN increased the p21$^{WAF1/CIP1}$ protein expression in human osteosarcoma MG63 cells in a dose-dependent manner using

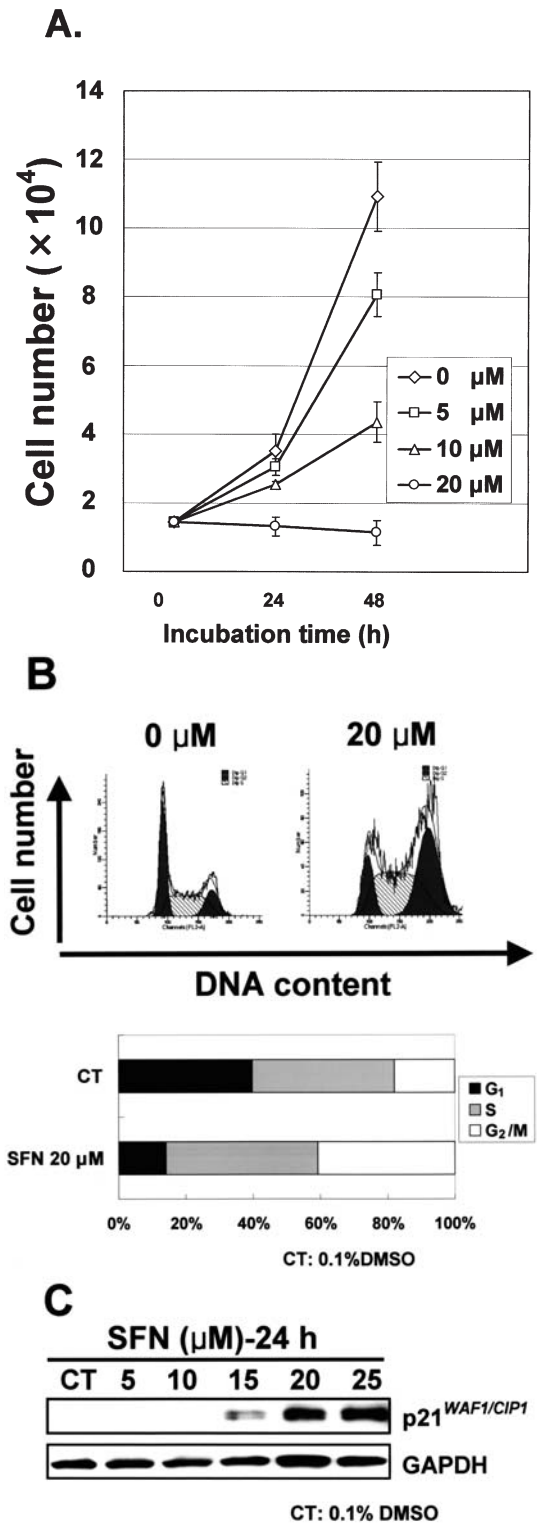

Figure 4. p21$^{WAF1/CIP1}$ induction by SFN in a p53-independent manner in human osteosarcoma MG63 cells. (A) Twenty-four hours after seeding of MG63 cells, SFN at 5 (□), 10 (△), 20 (○) μM was added and the cell number was compared with a control culture with equivalent DMSO (◇) by counting the cells using a trypan blue dye exclusion test. The values shown are means (bars, SD) (n=3). (B) MG63 cells were treated with or without 20 μM SFN for 24 h. The DNA content of propidium iodide-stained nuclei was analysed by FACSCalibur flow cytometry as described in Materials and methods. The experiments were repeated to confirm the results. The cell percentage in phases G_1 (black), S (gray) and G_2/M (white) was determined. (B) SFN up-regulated p21$^{WAF1/CIP1}$ protein expression in a p53-independent manner. MG63 cells were treated with SFN at the indicated concentrations for 24 h. Western blotting was then performed as described in Materials and methods. An anti-GAPDH antibody was used to confirm equal gel loading.

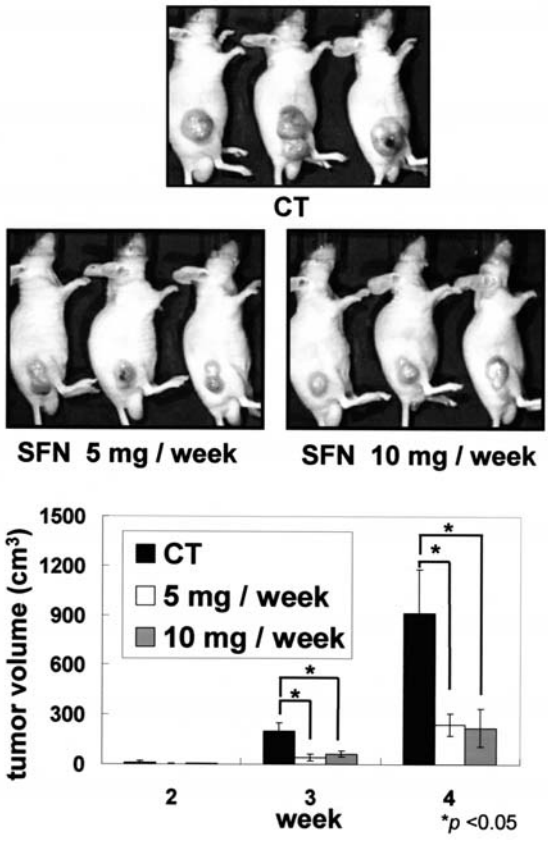

Figure 5. Intraperitoneal administration of SFN inhibits LM8 tumor xenograft growth in syngeneic Balb/C mice. Each mouse was implanted with ten million LM8 cells mixed in PBS on the right flank via a subcutaneous injection. After 24 h, mice were treated with intraperitoneal injections of saline (control group) or SFN (1 or 2 mg in 0.1 ml PBS) for 5 days/week for 4 weeks. Mean tumor volume/mouse (mm^3) was significantly reduced in SFN-treated mice, as compared with controls. Statistical significance of different tumor volume or body weight between the control and the treated mice was assessed by the Student's t-test.

Western blotting (Fig. 4C). These findings showed that SFN induced a p21$^{WAF1/CIP1}$ protein expression through a p53-independent pathway because p53 is inactivated in MG63 cells.

Intraperitoneal administration of SFN inhibits growth of LM8 xenografts in vivo. Prior to clinical trials, it is important that the *in vivo* efficacy of potential anticancer agents is determined in an animal model. Therefore, we performed an *in vivo* study to determine whether SFN administration inhibits the growth of LM8 xenografts in nude mice. As shown in Fig. 5, SFN treatment caused a significant inhibition of LM8 xenograft growth. Four weeks after starting therapy, LM8 xenografts of BALB/C mice injected i.p. with SFN (5 mg/week) were smaller (73.8% less in mass) than vehicle-treated controls (Fig. 5). Similarly, the average tumor volumes in SFN (10 mg week) -treated mice were 75.8% lower than those of control mice. No remarkable signs of toxicity were observed following SFN administration.

Discussion

Previous studies revealed that SFN is a potent inhibitor of chemically-induced cancer in animals (23-26). Some studies

reported that SFN effectively suppressed cancer xenografts in mice (27,28). These reports indicated that SFN may be an attractive compound for molecular-targeting chemotherapy or chemoprevention for malignant tumors. The main objective of the present study was to evaluate anti-tumor efficacy and the mechanisms of SFN in osteosarcoma cells in the culture and to translate the *in vitro* findings in to an *in vivo* preclinical osteosarcoma model.

Our study revealed that SFN induces cell growth inhibition via cell cycle arrest specifically at the G_2/M phase in murine osteosarcoma LM8 cells in culture studies. SFN was reported to induce either a G_1 arrest or a G_2/M arrest in various cancer cell lines. Some studies reported that SFN induces G_1- and G_2/M-phase cell cycle arrest through the induction of the $p21^{WAF1/CIP1}$ expression (31,38). In this study, we confirmed that SFN induced the $p21^{WAF1/CIP1}$ expression and G_2/M-phase cell cycle arrest in osteosarcoma cells.

We also found that SFN induces cell cycle arrest in human osteosarcoma MG63 cells through the p53-independent activation of $p21^{WAF1/CIP1}$. Recent studies reported that conventional anti-osteosarcoma agents such as doxorubicin, cisplatin and etoposide have anti-tumor effects mainly in a p53-dependent manner (39,40). Therefore, the p53-independent pathway of the $p21^{WAF1/CIP1}$ induction by SFN may be effective for the chemotherapy of osteosarcoma with resistance to conventional agents due to the inactivated p53.

Several studies reported that SFN induces apoptosis through the mitochondrial apoptotic pathway, via the up- or down-regulation of Bax, Bak, XIAP and Bcl-2 expression (27-30,41). In addition, recent reports revealed the death receptor pathway of SFN (33,42). In these reports, caspase-3 was activated by the mitochondrial and death receptor apoptotic pathways. In this study, SFN-induced cell death was apoptotic and accompanied by caspase-3 activation in LM8 cells. We reported that SFN up-regulates DR5 expression and sensitizes TRAIL-induced apoptosis in human osteosarcoma cells (33). Therefore, rather than as a single agent, the combined treatment using SFN with TRAIL and/or other anti-tumor agents may be more effective for chemotherapy against osteosarcoma.

Based on the encouraging *in vitro* anti-tumor efficacy of SFN against osteosarcoma, we found that the intraperitoneal administration of SFN (5 and 10 mg/week) significantly retarded the growth of LM8 xenografts to <30% controls in a preclinical animal model without causing any toxicity. Singh *et al* reported that oral administration of SFN (5.6 μmol, 3 times/week) significantly inhibited the growth of xenografts of human prostate cancer to ~50% of the mass of vehicle-treated controls (27). Jackson and Singletary reported that daily intravenous injection of SFN (15 nmol/day for 13 days) significantly affected smaller xenografts of human breast cancer to ~40% of the mass of vehicle-treated controls (28). In osteosarcoma cells, our findings in this study provide *in vivo* evidence for the efficacy of SFN against the advanced growth of tumors.

SFN is a food factor contained in vegetables. Ye *et al* reported the human plasma concentrations to reach only 2 μM after consuming SFN-rich broccoli sprouts (43). However, Hu *et al* reported that plasma concentrations reached 20 μM after oral administration of SFN in rats (44). After the administration of purified SFN, the murine plasma concentrations in this *in vivo* study may reach a concentration used in our *in vitro* study. In the clinic, we will use purified SFN as an anti-tumor agent for osteosarcoma cells.

Recently, a histone deacetylase (HDAC) inhibitory activity of SFN was reported (45,46). Our previous study showed that histone deacetylase inhibitors (HDACIs) such as trichostatin A (TSA), sodium butyrate and suberoylanilide hydoxamic acid (SAHA) induce the $p21^{WAF1/CIP1}$ protein in malignant tumor cells. Though we examined an HDAC inhibitory activity of SFN by the detection of acetylated histone H4 using Western blot analysis in MG63 cells, we did not detect an increase of acetylated histone H4 after treatment with SFN (data not shown). This result suggests that SFN may induce the $p21^{WAF1/CIP1}$ expression through a mechanism different from the function of HDACIs in osteosarcoma MG63 cells.

In conclusion, our results showed that SFN inhibits cell growth and induces cell cycle arrest and apoptosis in murine osteosarcoma cells. Furthermore, findings in xenograft studies translate the anti-tumor effects in a preclinical osteosarcoma model. These results raise the possibility that treatment with SFN is promising for the chemotherapy of osteosarcoma.

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