Sulforaphane inhibits proliferation by causing cell cycle arrest at the G_2/M phase in rabbit articular chondrocytes

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Abstract. Sulforaphane (SFN), a natural compound extracted from cruciferous vegetables, exhibits potent anticancer activity in various types of tumor cells. However, the effect of SFN on the proliferation of chondrocytes is not well understood. In the present study, we addressed the mechanism of action by which SFN suppresses proliferation. We demonstrate that SFN causes an irreversible arrest in cell proliferation, as determined by trypan blue dye exclusion assay and flow cytometric analysis. SFN induced cell cycle arrest at the G_2/M phase by downregulation of cyclin B, cdc2 and cdc25c and upregulation of $p21^{WAF1/CIP1}$ and p53 in a dose- and time-dependent manner, as determined by western blot analysis. Our data suggest that SFN regulates cell cycle arrest at the G_2/M phase in rabbit articular chondrocytes.

Introduction

Maintenance of articular cartilage integrity and its ability to react to mechanical loads and injury requires a properly orchestrated response of chondrocytes to cellular signals generated by growth factors, the extracellular matrix and cytokines (1). Under physiological conditions, programmed cell death in cartilage is uncommon, owing to maintenance of metabolic homeostasis and chondrocyte adhesion to extracellular matrix proteins (2).

Sulforaphane [SFN; 1-isothiocyanato-4-(methyl-sulfinyl) butane; Fig. 1] is an isothiocyanate identified in broccoli and other cruciferous vegetables. SFN forms following hydrolysis of the glucosinolate compound glucoraphanin by thioglucoside glucohydrolase (3,4). The compound has been revealed to be a naturally occurring cancer chemopreventive agent in animal models (5). Previous studies using animals have demonstrated the protective effect of SFN with regard to chemically induced carcinogenesis in various organs, including lung, esophagus, liver, mammary gland, colon (6,7) and stomach (8). The chemopreventive activity of SFN has been associated with various effects. However, the mechanism by which SFN exerts its effects on chondrocytes is not fully understood.

SFN has been demonstrated to inhibit neoplastic cell proliferation, block cell cycle progression at G_2/M, cause apoptosis and modulate signal transduction pathways, suggesting that it may be an effective inhibitor of neoplastic cell proliferation and cancer promotion/progression (4,9).

Generally, mammalian cells respond to DNA-damaging agents by activating cell cycle checkpoints, resulting in a delay of cell cycle progression until errors have been corrected (10). Cell cycle arrest at the G_2/M phase prevents cells from completing the cell cycle and proliferating (11). The phase is regulated by the sequential activation and deactivation of cdc family protein and cyclin complexes, including the cdc2/cyclin B complex.

Cyclin-dependent kinases (CDKs), cyclins and CDK inhibitors (CDKIs) are key molecules that play significant roles in cell cycle progression (12). $p21^{WAF1/CIP1}$ is a CDKI protein essential for cellular growth, differentiation and apoptosis (13). Therefore, induction of cell cycle arrest and apoptosis by chemotherapeutic agents is an effective approach to the inhibition of uncontrolled cell proliferation and survival in chondrocytes. We confirm that SFN induces cell cycle arrest at the G_2/M phase via the $p21^{WAF1/CIP1}$ and p53 pathways in rabbit articular chondrocytes. The present study sought to define the signal transduction pathways involved in the disruption of cartilage homeostasis, with the aim of identifying new therapeutic strategies for the prevention of cartilage destruction.

Materials and methods

Cell culture. Rabbit articular chondrocytes were isolated from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion. Cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 U/mg solid; Sigma, St. Louis, MO, USA) in DMEM (Gibco-BRL, Gaithersburg, MD, USA). Individual cells were suspended in DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL), 50 g/ml streptomycin (USB, Staufen, Germany)and 50 U/ml penicillin (Sigma). Following this, cells were plated on culture dishes at a

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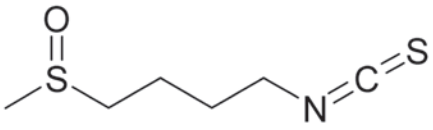

Figure 1. Structure of sulforaphane (SFN; molecular weight, 177.29; molecular formula, $C_6H_{11}NOS_2$).

density of $5x10^4$ cells/cm^2. Seeding medium was changed every 2 days, and cells reached confluence in ~5 days. The 3.5-day cell cultures were treated with SFN. SFN was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). The study was approved by the ethics committee of Kongju National University, Gongju, Republic of Korea.

Western blot analysis. Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40 and 0.1% sodium dodecylsulfate (SDS) supplemented with protease inhibitors (10 g/ml leupeptin, 10 g/ml pepstatin A, 10 g/ml aprotinin and 1 mM AEBSF) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). Lysates were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 5% non-fat dry milk in Tris-buffered saline. Expression levels of cdc2, cyclin B, cdc25c, p53, p21 and β-actin were detected using antibodies purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Blots were developed using a peroxidase-conjugated secondary antibody and imaged using an ImageQuant LAS 4000 system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Quantification of protein expression was performed using densitometric analysis (Image J).

Cell cycle distribution by FACS analysis. Cell cycle distribution was assessed by staining DNA content with propidium iodide. Briefly, chondrocytes were plated at a density of $2x10^4$ cells/cm^2 and incubated for 24 h. Fresh media containing SFN were applied to culture dishes and further incubated for 24 h. Following incubation, cells were harvested and fixed with 70% ethanol in PBS overnight. Fixed cells were incubated with RNase A (50 μg/ml) for 25 min, prior to staining nucleic acid with propidium iodide (50 μg/ml) for 5 min. The DNA content of $5x10^4$ cells/group was analyzed by flow cytometry (Partec GmbH, Münster, Germany) and the results presented as histograms of DNA content. Quantification of the distinct cell cycle phases was calculated using FloMax analysis software (Partec GmbH).

Trypan blue dye exclusion assay. The effect of SFN on the survival/proliferation of rabbit articular chondrocytes was determined by trypan blue dye exclusion assay. Cells were collected by trypsinization, separated into single cell suspensions in culture medium and inoculated into culture dishes at densities of $5x10^4$ cells/cm^2. Following culture for 24 h, cells were exposed to increasing concentrations of SFN or DMSO for 48 h, followed by trypsinization of cells adherent to the culture dishes and cell count anaysis by hemocytometer. Each experiment was performed in triplicate. Results were reported as an average of at least three separate experiments.

Data analyses and statistics. Results are expressed as the mean ± SE, calculated from the specified number of determinations. A Student's t-test was used to compare individual treatments with their respective control value. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SFN suppresses proliferation of rabbit articular chondrocytes. Phase contrast microscopy (x400; Fig. 2A) demonstrated that treatment of chondrocytes for 24 h with an increasing concentration of SFN, up to 50 μM, inhibited cell prolifera-

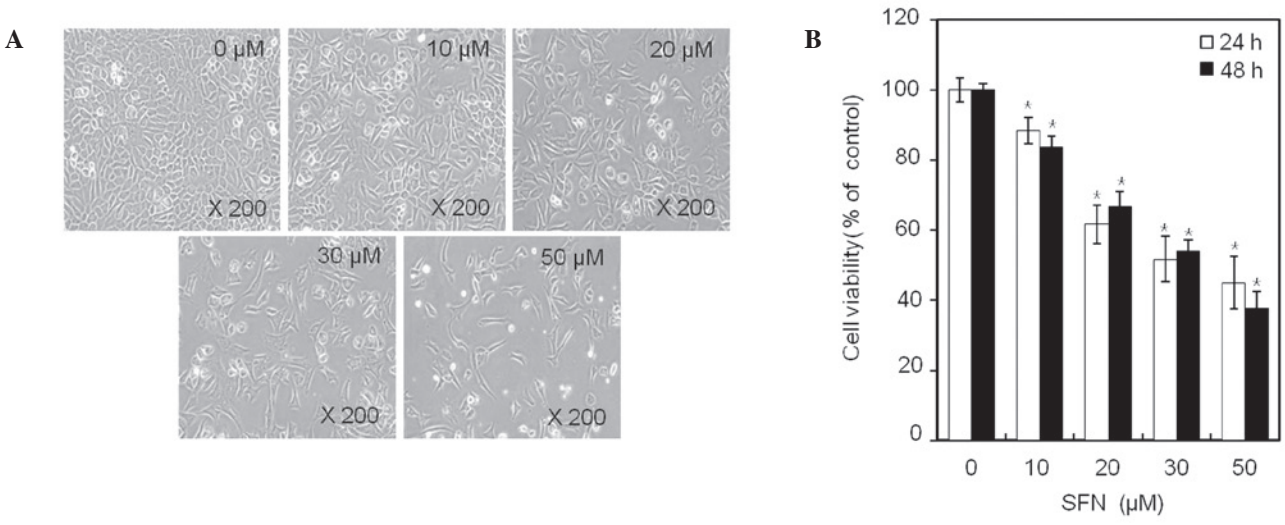

Figure 2. Effect of SFN on cell proliferation. (A) Articular chondrocytes were untreated, or treated with indicated concentrations of SFN for 24 h. Images were captured using a phase contrast microscope (magnification, x200; n=3). (B) Chondrocytes were untreated, or treated with increasing concentrations of SFN for 24 or 48 h. Cell viability was measured using trypan blue exclusion assay. (A and B) Data presented are representative of typical results obtained, and (B) include mean values with standard deviation. *$P<0.05$, compared with untreated cells (n=4). SFN, sulforaphane.

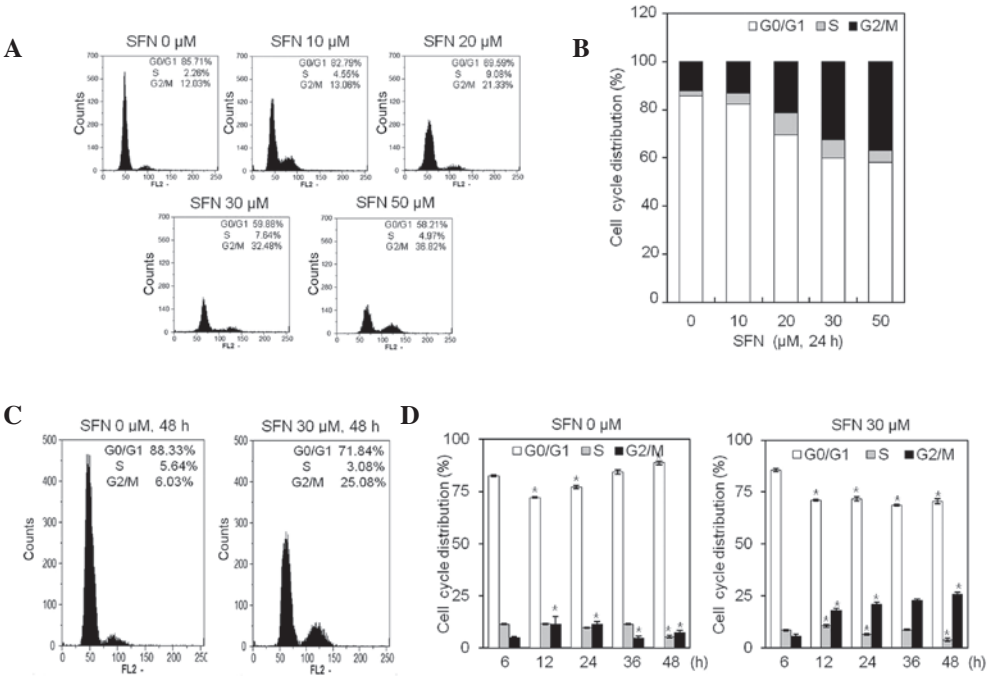

Figure 3. Effect of SFN on cell cycle distribution. (A and B) Primary articular chondrocytes were untreated, or treated with various concentrations of SFN for 24 h. (C and D) Cells were untreated, or treated with 30 μM SFN for 48 h. (A-D) DNA was stained with propidium iodide and % of cells in phases G_0/G_1 (white), S (gray) and G_2/M (black) were analyzed by flow cytometry. Data presented are representative of typical results obtained (n=3). (D) $^*P<0.05$, compared with untreated cells. SFN, sulforaphane.

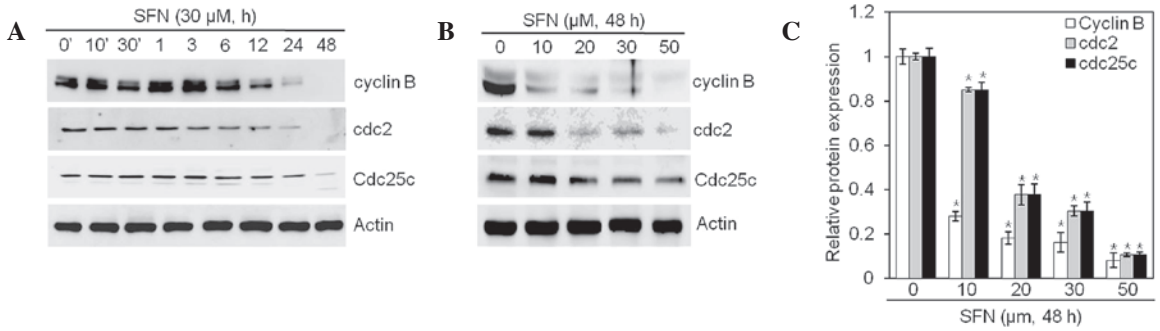

Figure 4. Effect of SFN on expression level of cyclin B, cdc2 and cdc25c. (A) Chondrocytes were untreated, or treated with 30 μM SFN for specified time periods. (B) Rabbit chondrocytes were untreated, or treated with the indicated concentrations of SFN for 24 h. (A and B) Expression levels of cyclin B, cdc2, cdc25c and β-actin were determined by western blot analysis. Expression of β-actin was used as loading control. (C) Quantification of the protein contents of cyclin B, cdc2 and cdc25c was performed using densitometric analysis. Data presented are representative of typical results obtained (n=3). $^*P<0.05$, compared with untreated cells. SFN, sulforaphane.

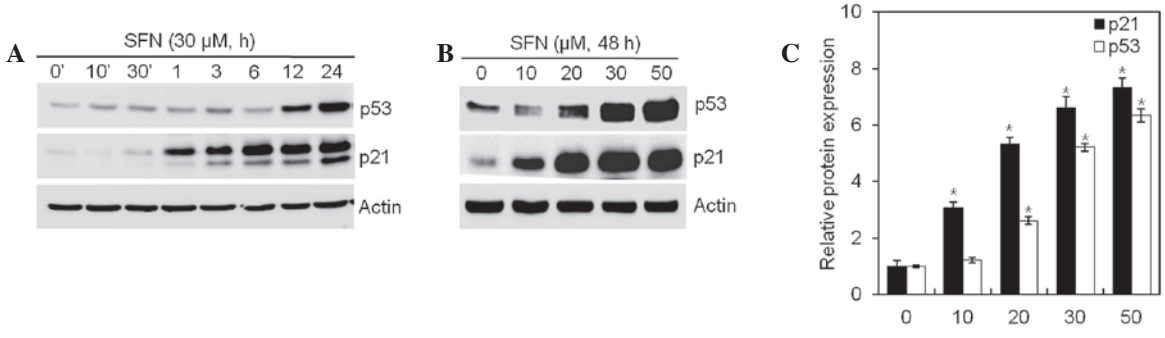

Figure 5. Effect of SFN on expression levels of p53 and $p21^{WAF1/CIP1}$. (A) Chondrocytes were untreated, or treated with 30 μM SFN for specified time periods. (B) Chondrocytes were untreated, or treated with the indicated concentrations of SFN for 24 h. (A,B) Expression of p53 and $p21^{WAF1/CIP1}$ were determined by western blot analysis. Expression of β-actin was used as loading control. (C) Quantification of the protein contents of p53 and $p21^{WAF1/CIP1}$ by densitometric analysis. Data presented are representative of typical results obtained (n=3). $^*P<0.05$, compared with untreated cells. SFN, sulforaphane.

tion in a dose-dependent manner. Within 48 h, SFN treatment decreased the number of cells attached to the plating surface and the total number of cells present in culture. Treatment with 50 μM SFN for 24 or 48 h inhibited cell proliferation of chondrocytes by 45 and 38%, respectively (Fig. 2B). These results demonstrate that SFN has a growth inhibitory effect on cell proliferation in rabbit articular chondrocytes (Fig. 2).

SFN causes cell cycle arrest at G_2/M phase. Since SFN inhibits cell proliferation of chondrocytes, we investigated the effects of SFN on cell cycle distribution by flow cytometric analysis. As demonstrated in Fig. 3A, FACS analysis revealed that exposure to SFN for 24 h increased the population of G_2/M phase cells in a dose-dependent manner. Chondrocytes at the G_2/M phase increased from 12.03% (medium alone) to 36.82%, by treatment with 50 μM SFN (Fig. 3A and B). Exposure of chondrocytes to a growth suppressive concentration of 30 μM SFN for 48 h resulted in accumulation of cells in the G_2/M phase, and was accompanied by a decrease in cells in the G_0/G_1 phase (Fig. 3C and D). Compared with the control, the percentage of cells in G_2/M phase was increased by ~4-fold, upon treatment with 30 μM SFN for 48 h (Fig. 3C and D). These results suggest that SFN is effective in suppressing chondrocyte proliferation through induction of cell cycle arrest at the G_2/M phase (Fig. 3). Inhibition of growth was not accompanied by an increase of apoptosis at the G_0/G_1 phase, as determined by FACS analysis (Fig. 3).

SFN inhibits expression of cyclin B1/cdc2 and cdc25c. To determine the molecular mechanisms by which SFN induces G_2/M phase arrest, we examined cell cycle-regulatory proteins involved in the G_2/M phase. Western blot analysis was performed, using antibodies against cell cycle-related proteins (cyclin B1, cdc2 and cdc25c; Fig. 4). SFN treatment resulted in substantial reductions in levels of cyclin B1, cdc2 and cdc25c proteins (Fig. 4A and B). Levels of cyclin 1 and cdc2 decreased faster than levels of cdc25c (Fig. 4A). Quantification of protein content identified a significant reduction of cyclin B1, cdc2 and cdc25c in a dose- and time-dependent manner (Fig. 4C). Collectively, these data indicate that treatment of articular chondrocyte cells with SFN induces expression changes in cell cycle regulators, resulting in subsequent G_2/M phase arrest.

SFN increases $p21^{WAF1/CIP1}$ expression through a p53-dependent pathway. SFN inhibited cell proliferation and induced G_2/M phase cell cycle arrest, observable by flow cytometry analysis. Following DNA damage, p53 activates $p21^{WAF1/CIP1}$ and causes G_2/M phase cell cycle arrest. Expression of $p21^{WAF1/CIP1}$ and p53 in articular chondrocytes exposed to SFN, was examined by western blot analysis. SFN increased expression of $p21^{WAF1/CIP1}$ and p53 in a dose- and time-independent manner (Fig. 5). These data indicate that SFN-mediated G_2/M phase arrest is regulated by the p53/$p21^{WAF1/CIP1}$-dependent pathway.

Discussion

Cruciferous or brassica vegetables come from the brassica genus and include broccoli, brussel sprout and cauliflower. These vegetables are a good source of glucosinolates, and their hydrolysis products include indoles and isothiocyanates. SFN is an isothiocyanate associated with chemopreventive activity, linked to inhibition of cell growth and disruption of microtubule polymerization (4). In the present study, SFN inhibited the growth of articular chondrocytes, as determined by trypan blue exclusion assay (Fig. 2B).

Various checkpoint mechanisms function to regulate the cell cycle, ensuring appropriate cellular response to external stresses, including abnormal mitogenic signaling (14).

In an effort to elucidate the mechanism by which SFN treament results in the inhibition of cell growth, its effects on cell cycle progression were assessed. The present study demonstrated that SFN induces a 27% decrease of G_0/G_1 phase and a 24% increase of G_2/M phase, following 24 h treatment with 50 μM SFN (Fig. 3A and B). Several studies on SFN have reported that SFN induces cell cycle arrest at the G_2/M phase in cancer cells (15-17). To the best of our knowledge, activation of cdc2, triggered by a positive feedback loop at the end of the G_2 phase, is the key event that initiates mitotic entry (18,19). The present study revealed that SFN treatment, resulting in G_2/M cell cycle arrest in articular chondrocytes, was correlated with decreased levels of cyclin B1/cdc2 and cdc25c (Fig. 4).

Cell cycle progression is mediated by various CDKs whose activities are regulated by CDKIs, including $p21^{WAF1/CIP1}$. The tumor suppressor gene p53 also regulates inhibition of cell growth (20), and expression of p53 has been revealed to cause a limited arrest in the G_1 phase (21). However, other studies have demonstrated that p53 leads to G_2/M phase progression in the rat cell line REF52, and a human ovarian cancer cell line (22,23). p53 directly stimulates expression of $p21^{WAF1/CIP1}$ to promote cell cycle inhibition. $p21^{WAF1/CIP1}$ competently blocks instigation of early mitotic progression by inhibiting activation of cyclin B and cdc2 (24). The present study suggests that activation of p21 and p53 with SFN may regulate G_2/M phase arrest, by inhibiting formation of cdc2/cyclin B complexes (Figs. 4 and 5). Cdc25c is a major phosphatase which activates cdc2 by dephosphorylatyion, enabling formation of the cdc2/cyclin B complex. These formations result in mitotic transition of cells (25).

In conclusion, our results demonstrate that SFN inhibits cell growth and induces cell cycle arrest in rabbit articular chondrocytes.

Acknowledgements

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