Induction of G_2/M arrest and apoptosis by sulforaphane in human osteosarcoma U2-OS cells

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Abstract. Sulforaphane is one of the most abundant isothiocyanates found in certain cruciferous vegetables, particularly broccoli. To date, sulforaphane has gained attention as a chemopreventive compound. The mechanism responsible for the anticancer effects of sulforaphane in osteosarcoma, however, is not clear. In this study, we demonstrate an anti-proliferative mechanism of sulforaphane in human osteosarcoma cells. The treatment of cells with sulforaphane resulted in a concentration- and time-dependent inhibition of growth and G_2/M phase arrest of the cell cycle. This effect was associated with a decrease in protein expression of cyclin A and B1 and their activating partners, cyclin-dependent kinases (CDKs) 1 and 2, with concomitant up-regulation of p21, a CDK inhibitor. Sulforaphane treatment also resulted in apoptosis as evidenced by an increase in annexin V^+/propidium $iodide^-$ (V^+/PI^-) cells, the cleavage of 116-kDa poly (ADP-ribose) polymerase (PARP) and ICAD and oligonucleosomal DNA fragmentation. Taken together, these findings indicate that the molecular mechanisms underlying sulforaphane-mediated growth inhibition in U2-OS cells may be the modulation of the cell cycle machinery and the induction of apoptosis.

Introduction

Numerous anti-proliferative agents interfere with the cell cycle machinery (1). Cell cycle transitions are largely governed by a family of cyclin-dependent kinases (CDKs). The activities of CDKs are regulated by various factors including: (i) levels of various CDKs, (ii) levels of cyclins that interact with CDKs, and (iii) levels of CDK inhibitory proteins (CDKIs). Alterations in the formation of CDK/cyclin complexes could lead to increased cell growth and proliferation, decreased cell growth, and proliferation followed by differentiation and/or cell death by apoptosis (1).

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The formation of active complexes of CDK1 or 2 with cyclin A and/or B is required for transition from the G_2 to the M phase of the cell cycle. Cyclin B1 is a key component of the G_2 to M phase transition (2). Cyclin B1 binds to CDK2 at the beginning of the G_2 phase, forming an activated cyclin B1/CDK2 complex. The activated cyclin B1/CDK2 complex then phosphorylates its downstream substrates that control the G_2 to M transition and promotes mitotic cell division (3). By contrast, CDK1 plays a crucial role in controlling the cell cycle by coordinating internal and external signals that impede proliferation at several key checkpoints (4). Among the CDKIs, p21/WAF1/CIP1 is a significant mediator of cell cycle arrest. Several studies have demonstrated that levels of p21 may be critical for determining the threshold kinase activity of various cyclin/CDK complexes, suggesting that finite levels of p21 may be critical in the regulation of cell growth (5,6).

Apoptosis plays a role in the maintenance of tissue homeostasis. Apoptosis removes damaged cells, and therefore, impaired apoptosis is thought to contribute to the development of cancer (7). Apoptosis is a physiological marker of cell death and is characterized by chromatin condensation, membrane blebbing, cell shrinkage and DNA fragmentation (8). The process is tightly regulated by the balance of pro- and anti-apoptotic proteins in the cell, which include members of the Bcl-2 family and inhibitors of apoptosis (9). Apoptosis also results from the coordinated actions of several caspase enzymes (7,9). Caspase cleaves many substrate proteins, including poly (ADP-ribose) polymerase (PARP) and structural proteins, such as actin, fodrin or lamin to generate the characteristic apoptotic morphology (10).

Advances in the understanding of the molecular events that drive tumor progression have provided an opportunity to identify effective therapeutic regimens for the treatment and management of malignant cancers. Sulforaphane is one of the most abundant isothiocyanates in certain cruciferous vegetables, particularly broccoli (11). It has been shown that sulforaphane protects against ischemia reperfusion injury (12), diabetes (13) and several major cancers (14). Initial studies on human colon and prostate cancer cells have demonstrated that sulforaphane exerts anticancer effects via the inhibition of histone deacetylase activity (15). The studies by Matsui *et al* have demonstrated that sulforaphane has anti-proliferative properties towards osteosarcoma cells via death receptor 5 expression and cell cycle arrest (16,17). This study aimed to

further investigate the detailed mechanism of sulforaphane on cell cycle arrest and apoptosis in human osteosarcoma cells. The data demonstrate that sulforaphane induces apoptosis and cell cycle arrest at the G_2/M phase through a coordinated mechanism involving the down-regulation of CDKs and cyclins and the induction of p21.

Materials and methods

Cell culture and reagents. Human osteosarcoma cell lines (U2-OS and MG63) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown at 37˚C under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Sulforaphane was purchased from LKT Laboratories (St. Paul, MN, USA). All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted.

MTT assay for cell proliferation. The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Cells were seeded in 96-well plates. Following treatment, the cells were washed twice with phosphate-buffered saline (PBS) and MTT (100 μg/100 μl PBS) was added to each well. The plates were then incubated at 37˚C for 1 h and then DMSO (100 μl) was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm with a SpectraMax Plus model (Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis. Cells ($1x10^4$) were incubated with various concentrations of sulforaphane for 24 h, washed with PBS and fixed in 70% ethanol overnight at 4˚C. The following day, cells were washed with PBS containing 0.1% BSA and incubated with 5 mg/ml of RNase A (DNase free) and 50 mg/ml propidium iodide (PI) for 90 min at 4˚C. The percentages of cells in various phases of the cell cycle were measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Annexin V staining. Apoptosis was determined by staining cells with Alexa Fluor 488 annexin V (Invitrogen, Carlsbad, CA, USA). Cells ($5x10^6$) were incubated with various concentrations of sulforaphane for 48 h, washed twice with cold PBS and resuspended in 500 μl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). The following day, cells were incubated with 100 μg/ml PI, stained with annexin V Alexa Fluor 488, and analyzed using the FACSCalibur flow cytometer (Becton-Dickinson).

Detection of DNA fragmentation by gel electrophoresis. Cell pellets ($5x10^6$) were resuspended in 500 μl lysis buffer (0.5% Triton X-100, 10 mM EDTA and 10 mM Tris-HCl, pH 8.0), incubated at room temperature for 15 min and centrifuged at 16,000 x g for 10 min. DNA was then extracted twice with phenol/chloroform (1:1), precipitated with ethanol and resuspended in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). DNA was analyzed following separation by gel electrophoresis (2% agarose).

Western blot analysis. Cells were homogenized in 100 μl ice-cold lysis buffer containing 20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin. Homogenates containing 20 μg protein were separated by SDS-PAGE using a 10% resolving gel with a 3% acrylamide stacking gel. Proteins were transferred from the polyacrylamide gel to nitrocellulose membranes, blocked with 2% bovine serum albumin, and then probed overnight with primary antibodies for PARP, ICAD, cyclins A and B1, CDKs 1 and 2 and p21 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA, USA) was used as a secondary antibody and the protein expression level was determined by analyzing the signals captured on the nitrocellulose membranes using a ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Statistical analysis of the data was performed using ANOVA and Duncan's test. Differences with a p-value of <0.05 were considered statistically significant.

Results

Sulforaphane induced cell death of human osteosarcoma cells. The MTT conversion assay was performed to determine the cytotoxic effects of sulforaphane on the human osteosarcoma cells, U2-OS and MG63. As shown in Fig. 1, sulforaphane treatment induced the cell death of U2-OS and MG63 cells in a concentration- and time-dependent manner. Treatment of U2-OS and MG63 cells with 20 μM sulforaphane for 48 h resulted in a 54.56±0.04% ($p<0.05$) and 77.36±0.74% ($p<0.01$) inhibition of cell viability, respectively.

Sulforaphane induced G_2/M arrest in U2-OS cells. Based on the cytotoxic response of osteosarcoma cells to sulforaphane, the next experiments were focused on the U2-OS cells. The effects of sulforaphane on cell cycle progression were analyzed by flow cytometry. Consistent with the effect on cell viability, 20 μM sulforaphane treatment resulted in the accumulation of U2-OS cells in the G_2/M phase (from 14.01±1.85% to 29.68±2.55%, Fig. 2).

Sulforaphane modulated protein levels of significant G_2/M phase regulators. Perturbations in cell cycle regulation have been demonstrated as one of the most common characteristics of cancer therapies (18-20). These alterations are generally associated with uncontrolled cell growth and involve down-regulation of CDK and cyclin expression or increased expression of CDKIs. Based on the data revealing that sulforaphane induces G_2/M arrest in U2-OS cells, the effect of sulforaphane on cell cycle regulatory molecules, including CDKs and cyclins, which play essential roles in the G_2/M transition phase, was assessed. The expression of cyclins A and B1 decreased following sulforaphane treatment in a concentration- and time-dependent manner (Fig. 3). Sulforaphane treatment of U2-OS cells also decreased the expression of CDKs 1 and 2 in a concentration- and time-dependent manner (Fig. 3). The down-regulation of cyclin and CDK protein levels observed following sulfora-

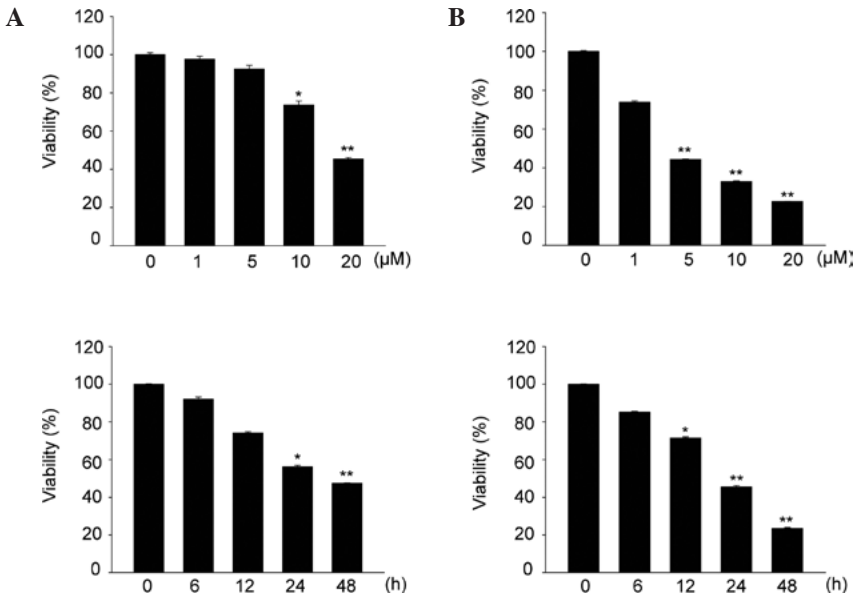

Figure 1. Cytotoxic effects of sulforaphane on human OS U2-OS and MG63 cells. (A) U2-OS and (B) MG63 cells ($1x10^4$) were treated with the indicated concentrations of sulforaphane for 48 h or with 20 μM sulforaphane for the indicated times. Cell viability was determined by the MTT assay and is presented as a calculated percentage of viable cells between sulforaphane-treated and untreated control cells. Each point represents the mean ± SEM of three determinations. *p<0.05, **p<0.01 vs. untreated control.

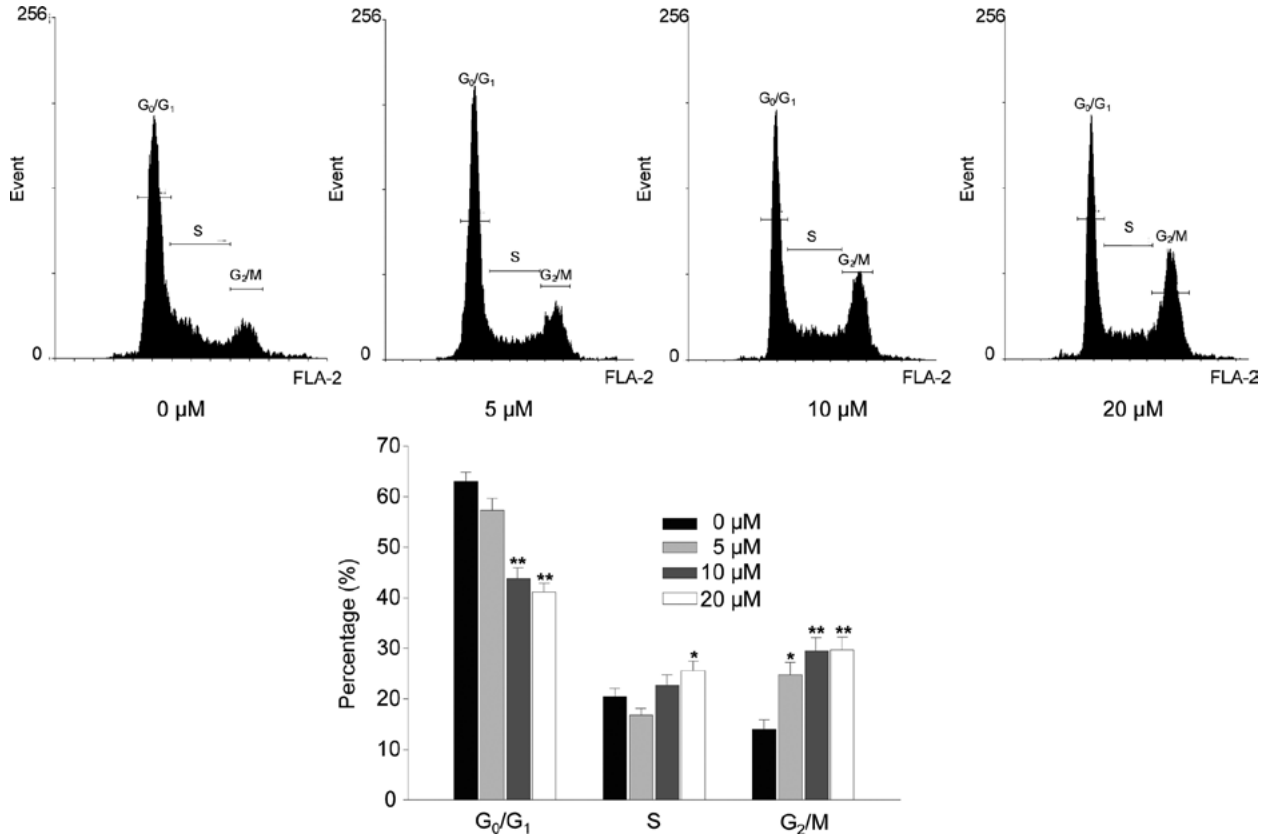

Figure 2. Effects of sulforaphane on the cell cycle pattern of U2-OS cells by flow cytometry. U2-OS cells ($1x10^4$) were treated with the indicated concentrations of sulforaphane for 24 h. Both floating and attached cells were collected and processed for an analysis of cell cycle regulation. Representative histograms of cell cycle distribution in the untreated control and sulforaphane-treated cells are shown. The data points in the graph represent the means ± SD of three independent experiments in U2-OS cells. *p<0.05, **p<0.01 vs. untreated control.

phane treatment was not due to overall changes in protein levels, as confirmed by probing the membranes with β-actin antibodies.

Since sulforaphane treatment revealed a marked decrease in the expression of CDKs and cyclins, p21 was investigated as enhanced levels of p21 have been shown to be associated with

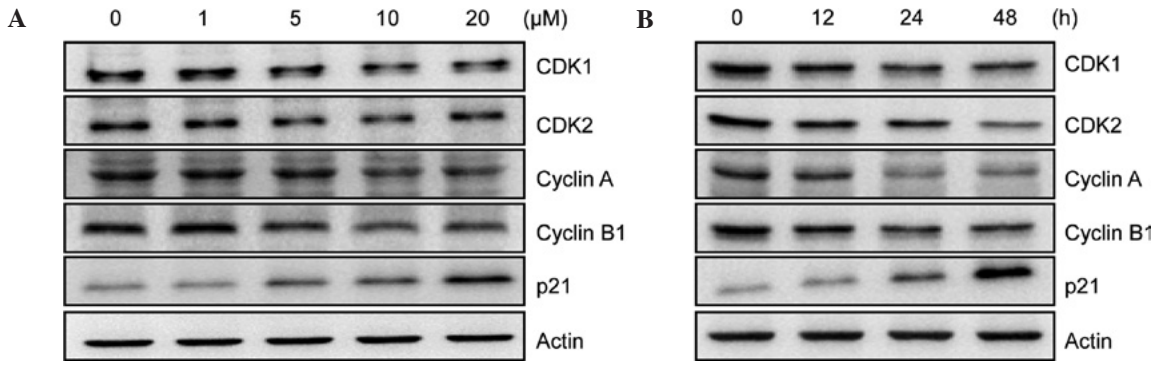

Figure 3. Concentration- and time-dependent effects of sulforaphane on cell cycle-related proteins. U2-OS cells ($1x10^6$) were treated (A) with the indicated concentrations of sulforaphane for 24 h or (B) 20 μM sulforaphane for the indicated times. Cyclins A and B1, CDKs 1 and 2, and p21 were examined by Western blotting. The figure is a representative result selected from at least three separate experiments yielding similar results.

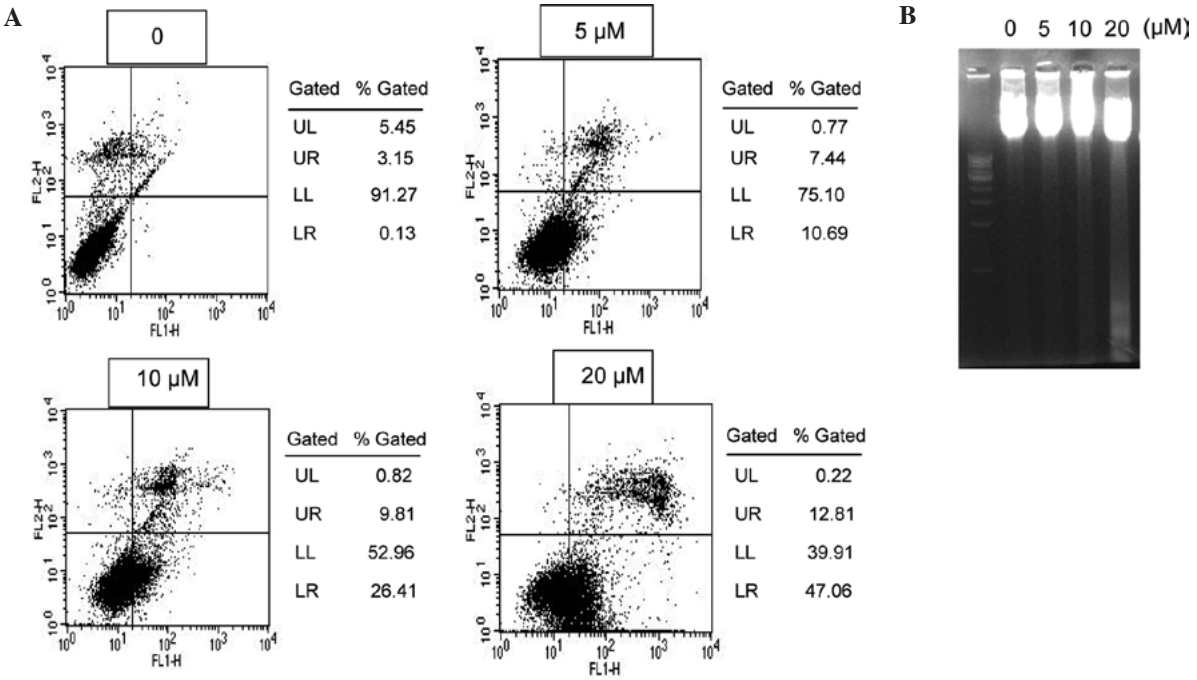

Figure 4. Induction of apoptotic cell death by sulforaphane in U2-OS cells. U2-OS cells ($5x10^6$) were incubated with the indicated concentrations of sulforaphane for 48 h and apoptosis was analyzed by (A) annexin V and PI staining or (B) a DNA fragmentation assay, as described in Materials and methods. The figure is a representative result selected from at least three separate experiments yielding similar results.

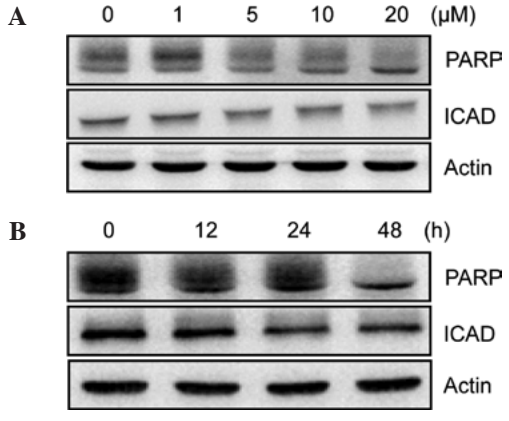

Figure 5. Proteolytic cleavage of PARP and ICAD by sulforaphane in U2-OS cells. U2-OS cells ($5x10^6$) were treated with the indicated concentrations of sulforaphane for 48 h or 20 μM sulforaphane for the indicated times. Western blot analysis of PARP and ICAD was performed as described in Materials and methods. Data are representative of three independent experiments.

G_2/M arrest (5,6). Western blot analysis revealed that sulforaphane treatment of U2-OS cells for 24 h markedly induced protein levels of p21 in a concentration- and time-dependent manner (Fig. 3).

Sulforaphane caused apoptotic death of U2-OS cells. The possibility that sulforaphane-induced apoptosis could have contributed to the potent effects associated with G_2/M arrest was investigated. Treatment with sulforaphane resulted in an increase of annexin V^+/PI^- cells, which is a typical presentation of apoptotic cells stained with annexin V (Fig. 4A). DNA extracted from U2-OS cells treated with sulforaphane for 48 h displayed a characteristic ladder pattern of discontinuous DNA fragments on agarose gel electrophoresis (Fig 4B).

To further confirm the apoptotic effects of sulforaphane, Western blot analysis was performed on extracts of U2-OS cells treated with sulforaphane. As shown in Fig. 5, treatment of

U2-OS cells with sulforaphane induced proteolytic cleavage of PARP (116 kDa), resulting in the accumulation of the 85-kDa cleavage product. Sulforaphane also increased the degradation of ICAD in a concentration- and time-dependent manner.

Discussion

Cell proliferation and differentiation are tightly regulated processes that must be precisely coordinated in order to maintain tissue homeostasis. Specifically, passage through the cell cycle is governed by a family of protein kinase complexes that are controlled, in part, by CDK and its essential activating partner, cyclin (20,21). Under normal conditions these complexes are activated at specific intervals, and through a series of events result in the progression of cells through various phases of the cell cycle, thereby ensuring normal cell growth. Any defect in this machinery may cause an alteration in cell cycle regulation that results in unwanted cell proliferation and ultimately culminates in the development of cancer (18,22). Thus, regulating cyclin and CDK activity has turned out to be the most productive strategy for the discovery and design of novel anticancer agents targeting the cell cycle.

Cyclins A and B1, along with CDK1 and 2, are the driving forces behind the G_2/M phase of the cell cycle, whereas p21 induces G_1 and G_2/M phase cell cycle arrest (5,6). Unscheduled misregulation of cyclin B1 during the cell cycle leads to uncontrolled cell growth and aberrant cell function (23). In our experiments, sulforaphane induced G_2/M arrest in a concentration-dependent manner in the U2-OS cell line. Cell cycle arrest at the G_2/M phase is observed when there is a defect in DNA damage repair, spindle attachment with centromeres and polymerization of spindle microtubules (24). Significant decreases in cyclins A and B1 and CDKs 1 and 2 with a concomitant up-regulation of p21 were observed in the sulforaphane-treated U2-OS cells using Western blot analysis. These results suggest that cell cycle arrest at the G_2/M phase is mediated by limitation of the supply of cyclins A and B1 and CDKs 1 and 2, which are essential factors regulating the passage into mitosis.

The effect of sulforaphane on cell cycle arrest prompted the next series of experiments wherein the extent of apoptosis in U2-OS cells treated with sulforaphane was determined. The results revealed that sulforaphane caused apoptotic cell death, as evidenced by an increase in annexin V^+/PI^- cells, oligonucleosomal DNA fragmentation and the degradation of PARP and ICAD. Since the induction of CDKI has been reported in anticancer agent-induced apoptosis in human leukemia and prostate cancer cells (25,26), the sulforaphane-related increase in p21 may have been responsible, in part, for the observed apoptotic cell death of U2-OS cells. In addition, the degradation of PARP suggests that caspase activation could have played a significant role in the induction of apoptosis. However, more detailed studies are required to better understand the mechanism underlying sulforaphane-induced apoptotic death in U2-OS cells.

Osteosarcoma is the most common type of bone tumor that mainly develops in adolescents and young adults. Despite recent advances in chemotherapy and wide excision of tumors, a substantial number of patients still succumb to their disease as a result of local recurrence or distant metastasis (27). The search for promising agents that could reduce the incidence and burden of cancer has become increasingly essential in recent years. Naturally occurring dietary supplements and herbs have stimulated great interest due to their chemopreventive and chemotherapeutic properties as well as their relatively non-toxic effects, low cost and availability as oral formulations (7,14). In this regard, the study focused on the effects of sulforaphane on cell proliferation in a human osteosarcoma cell line with the objective of elucidating the signaling mechanism through which sulforaphane exerts its anticancer effects. The study demonstrates that sulforaphane markedly induces G_2/M arrest and apoptosis through the induction of p21 expression and down-regulation of CDK/cyclin complexes. The data suggest the possible application of sulforaphane as a cancer preventive or therapeutic agent against osteosarcoma.

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References

1. Hartwell LH and Kastan MB: Cell cycle control and cancer. Science 266: 1821-1828, 1994.
2. Jackman M, Lindon C, Nigg EA and Pines J: Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat Cell Biol 5: 143-148, 2003.
3. Jin P, Hardy S and Morgan DO: Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. J Cell Biol 141: 875-885, 1998.
4. Morgan DO: Principles of CDK regulation. Nature 374: 131-134, 1995.
5. Martinez JD, Stratagoules ED, LaRue JM, *et al*: Different bile acids exhibit distinct biological effects: the tumor promoter deoxycholic acid induces apoptosis and the chemopreventive agent ursodeoxycholic acid inhibits cell proliferation. Nutr Cancer 31: 111-118, 1998.
6. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and Beach D: p21 is a universal inhibitor of cyclin kinases. Nature 366: 701-704, 1993.
7. Kwon KB, Park BH and Ryu DG: Chemotherapy through mitochondrial apoptosis using nutritional supplements and herbs: a brief overview. J Bioenerg Biomembr 39: 31-34, 2007.
8. Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 68: 251-306, 1980.
9. Fesik SW: Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer 5: 876-885, 2005.
10. Kurokawa M and Kornbluth S: Caspases and kinases in a death grip. Cell 138: 838-854, 2009.
11. Zhang Y, Talalay P, Cho CG and Posner GH: A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 89: 2399-2403, 1992.
12. Yoon HY, Kang NI, Lee HK, Jang KY, Park JW and Park BH: Sulforaphane protects kidneys against ischemia-reperfusion injury through induction of the Nrf2-dependent phase 2 enzyme. Biochem Pharmacol 75: 2214-2223, 2008.
13. Song MY, Kim EK, Moon WS, *et al*: Sulforaphane protects against cytokine- and streptozotocin-induced beta-cell damage by suppressing the NF-kappaB pathway. Toxicol Appl Pharmacol 235: 57-67, 2009.
14. Antosiewicz J, Ziolkowski W, Kar S, Powolny AA and Singh SV: Role of reactive oxygen intermediates in cellular responses to dietary cancer chemopreventive agents. Planta Med 74: 1570-1579, 2008.
15. Dashwood RH and Ho E: Dietary agents as histone deacetylase inhibitors: sulforaphane and structurally related isothiocyanates. Nutr Rev 66 (Suppl 1): 36-38, 2008.
16. Matsui TA, Murata H, Sakabe T, *et al*: Sulforaphane induces cell cycle arrest and apoptosis in murine osteosarcoma cells *in vitro* and inhibits tumor growth *in vivo*. Oncol Rep 18: 1263-1268, 2007.

17. Matsui TA, Sowa Y, Yoshida T, *et al*: Sulforaphane enhances TRAIL-induced apoptosis through the induction of DR5 expression in human osteosarcoma cells. Carcinogenesis 27: 1768-1777, 2006.
18. Jacks T and Weinberg RA: Cell-cycle control and its watchman. Nature 381: 643-644, 1996.
19. Shapiro GI: Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 24: 1770-1783, 2006.
20. Sherr CJ and Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9: 1149-1163, 1995.
21. Sherr CJ: Cancer cell cycles. Science 274: 1672-1677, 1996.
22. Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ and Arnold A: The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr Rev 20: 501-534, 1999.
23. Grabsch H, Lickvers K, Hansen O, *et al*: Prognostic value of cyclin B1 protein expression in colorectal cancer. Am J Clin Pathol 122: 511-516, 2004.
24. Rieder CL, Schultz A, Cole R and Sluder G: Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. J Cell Biol 127: 1301-1310, 1994.
25. Kim EK, Kwon KB, Han MJ, *et al*: Induction of G1 arrest and apoptosis by *Scutellaria barbata* in the human promyelocytic leukemia HL-60 cell line. Int J Mol Med 20: 123-128, 2007.
26. Don MJ, Chang YH, Chen KK, Ho LK and Chau YP: Induction of CDK inhibitors (p21(WAF1) and p27(Kip1)) and Bak in the beta-lapachone-induced apoptosis of human prostate cancer cells. Mol Pharmacol 59: 784-794, 2001.
27. Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A and Picci P: Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy: 15-year experience in 789 patients treated at a single institution. Cancer 106: 1154-1161, 2006.