Sulforaphane Increases Cyclin-Dependent Kinase Inhibitor, p21 Protein in Human Oral Carcinoma Cells and Nude Mouse Animal Model to Induce G\textsubscript{2}/M Cell Cycle Arrest

Jun-Hee Kim\textsuperscript{1,*}, Ki Han Kwon\textsuperscript{2,*}, Ji-You Jung\textsuperscript{3}, Hye-Suk Han\textsuperscript{1}, Jung Hyun Shim\textsuperscript{1}, SeJun Oh\textsuperscript{1}, Kyeong-Hee Choi\textsuperscript{3}, Eun-Sun Choi\textsuperscript{1}, Ji-Ae Shin\textsuperscript{1}, Dae-Ho Leem\textsuperscript{4}, Yunjo Soh\textsuperscript{5}, Nam-Pyo Cho\textsuperscript{1,***} and Sung-Dae Cho\textsuperscript{1,***}

\textsuperscript{1}Department of Oral Pathology, School of Dentistry and Institute of Oral Bioscience, Brain Korea 21 project, Chonbuk National University, Jeonju, 561-756, Republic of Korea
\textsuperscript{2}Department of Complementary and Alternative Therapy, College of Management, Commerce and Social Welfare, Gwangju University, Gwangju, 503-703, Republic of Korea
\textsuperscript{3}Department of Companion and Laboratory Animal Science, Kongju National University, Yesan, 340-702, Republic of Korea
\textsuperscript{4}Department of Oral and Maxillofacial Surgery, School of Dentistry and Research of Clinical Medicine, Chonbuk National University, Jeonju, 561-756, Republic of Korea
\textsuperscript{5}Department of Dental Pharmacology, School of Dentistry and Institute of Oral Bioscience, Brain Korea 21 project, Chonbuk National University, Jeonju, 561-756, Republic of Korea

Received 29 June, 2009; Accepted 21 July, 2009

Summary  Previously, our group reported that sulforaphane (SFN), a naturally occurring chemopreventive agent from cruciferous vegetables, effectively inhibits the proliferation of KB and YD-10B human oral squamous carcinoma cells by causing apoptosis. In this study, treatment of 20 and 40 \textmu M of SFN for 12 h caused a cell cycle arrest in the G\textsubscript{2}/M phase. Cell cycle arrest induced by SFN was associated with a significant increase in the p21 protein level and a decrease in cyclin B expression, but there was no change in the cyclin A protein level. In addition, SFN increased the p21 promoter activity significantly. Furthermore, SFN induced p21 protein expression in a nude mouse xenograft model suggesting that SFN is a potent inducer of the p21 protein in human oral squamous carcinoma cells. These findings show that SFN is a promising candidate for molecular-targeting chemotherapy against human oral squamous cell carcinoma.

Key Words: Sulforaphane, Human Oral Squamous cell carcinoma, G\textsubscript{2}/M arrest, p21, cyclin B

Introduction

The cell cycle is highly regulated by a number of checkpoint mechanisms, that are essential for the cellular response to stress including DNA damage and abnormal mitogenic signals and the cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors are important factors that regulate the cell cycle progression. However, the checkpoint mechanism does not always work well due to the activation of CDKs
and/or inactivation of CDK inhibitors resulting in uncontrolled proliferation which is one of the causes of cancer [1].

p21 is a CDK inhibitor protein that is essential for cellular growth, differentiation and apoptosis [2]. Although p21 is rarely mutated in human tumors, a decrease in p21 protein expression contributes to the neoplastic progression of many tumors [3–17]. A number of scientific reports show that p21 is induced by DNA damage or oncogene expression, triggering irreversible cell cycle arrest, which facilitates tumor growth arrest [12–15]. For this reason, induction of the p21 protein is considered a good strategy for molecular target therapy.

The use of natural products as chemopreventive agents has been a major focus for a long time due to their possible cancer chemotherapeutic activity. Among the many naturally occurring anti-cancer agents, sulforaphane (SFN) has attracted the attention of cancer investigators. There is accumulating evidences that SFN inhibits the proliferation of cancer cells both in vitro and in vivo by causing apoptosis and/or cell cycle arrest [16–21]. Our previous study also found that SFN induced caspase-dependent apoptosis in human oral squamous carcinoma cells and a nude mouse xenograft model [22]. However, the mechanism of the cell cycle arrest induced by SFN to inhibit the proliferation of oral cancer cells is not completely understood.

This study confirmed that the treatment with SFN causes cell cycle arrest of KB and YD-10B human oral squamous carcinoma cell at the G2/M phase. In addition, it is associated with a significant increase in p21 protein expression. Moreover, SFN induced p21 protein expression in a nude mouse xenograft model confirming that SFN activates p21 protein which contributes to the potent anticancer activity of this compound.

Materials and Methods

Reagents

Sulforaphane was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for Sp1, cyclin A, cyclin B and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The p21 antibody was purchased from BD Pharmingen (San Diego, CA). Propidium Iodide was obtained by Calbiochem (San Diego, CA), and RNase A was from Sigma-Aldrich. p21 promoter reporter constructs, pWWP was provided by Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA). Reporter lysis buffer and luciferase reagent for dual luciferase assays were purchased from Promega (Madison, WI).

Cell culture and drug treatment

The human oral squamous carcinoma cell line KB was obtained from American Tissue Culture Collection (Manassas, VA). Human oral squamous carcinoma cell line YD-10B was obtained from College of Dentistry, Yonsei University (Seoul, Korea). KB Cells were maintained in Dulbecco’s modified essential medium (DMEM; Welgene, Dae-Ku, Korea) without phenol red and YD-10B cells were cultured in DMEM/F-12 media (Welgene) containing 5% fetal bovine serum (FBS) and 10 ml/L of 100X antibiotics antimycotic solution (Welgene) at 37°C in a humidified atmosphere of 5% CO2 incubator. For the treatment of cells with SFN, a stock solution of 40 mM was prepared in dimethyl sulfoxide (DMSO). An equal number of cells were seeded in 96-well, 12-well, or 6-well plates and allowed to attach until plates reached 50–60% confluence, which was usually observed 24 h after seeding. Cells were treated with vehicle (DMSO) or compounds of various concentrations diluted in DMEM without phenol red and supplemented with 2.5% FBS.

Cell proliferation assay

KB and YD-10B cells were seeded in 12-well plates and were treated with DMSO and two concentrations of the test compounds in DMEM containing 2.5% FBS for 12 h. KB and YD-10B cells were counted to evaluate the effects of SFN and the vehicle control on the number of viable cells. A cell count was carried out with a hemocytometer using equal volumes of cell suspension and 0.4% trypan blue solution (Sigma Chemical Co.). Each experiment was carried out in triplicate and results were expressed as means ± SD for each treatment group.

MTT assay

The MTT assay kit purchased from AMRESCO (Solon, OH) was used to evaluate the effect of SFN on cell proliferation in KB and YD-10B cells. Cells were seeded in 96-well plates and incubated for 24 h before the treatment with SFN. After SFN treatment for 12 h, 20 μl of the MTT solution (2 mg/ml) was added to each well and cells were incubated for 2 h at 37°C in 5% CO2. The formazan absorbance was measured at 560 nm using a microtiter plate reader (Molecular Devices).

FACS analysis for cell cycle regulation

After treatment with SFN, detached cells (floaters) were collected by centrifugation and combined with adherent cells that were released by trypsinization. Cell cycle analysis was carried out by flow cytometry; cells were fixed in 70% ethanol overnight at ~20°C. After centrifugation, ethanol was removed and the cells were subsequently stained with 0.02 mg/ml propidium iodide (PI) and subjected to DNA content analysis using a FACScan cytometer (Becton...
protein was quantified by the Bradford protein assay using 15,000 g for 20 min, supernatants were recovered, and protein was quantified by the Bradford protein assay using a reagent kit from Bio-Rad (Hercules, CA). Protein samples were size-separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels under non-reducing conditions. Separated proteins were electroblotted onto nitrocellulose membranes. The blot was blocked by incubation in a blocking buffer (5% skim milk, 10 mM Tris, pH 7.5, 10 mM NaCl and 0.1% Tween 20) for 1 h at 20°C, and then incubated with the primary antibody overnight at 4°C. Incubation with a horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibody was then carried out at room temperature (RT) for 4 h. Antibody-bound proteins were detected by the ECL Western blotting analysis system (Perkin Elmer Life and Analytical Science, Boston, MA).

Western blot analysis

Cell lysates were prepared using a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate; 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin, and 1 μg/ml aprotinin). After centrifugation of the lysate at 15,000 g for 20 min, supernatants were recovered, and protein was quantified by the Bradford protein assay using a reagent kit from Bio-Rad (Hercules, CA). Protein samples (20–60 μg) were size-separated by electrophoresis on sodium dodecylsulfate-polyacrylamide gels under non-reducing conditions. Separated proteins were electroblotted onto nitrocellulose membranes. The blot was blocked by incubation in a blocking buffer (5% skim milk, 10 mM Tris, pH 7.5, 10 mM NaCl and 0.1% Tween 20) for 1 h at 20°C, and then incubated with the primary antibody overnight at 4°C. Incubation with a horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibody was then carried out at room temperature (RT) for 4 h. Antibody-bound proteins were detected by the ECL Western blotting analysis system (Perkin Elmer Life and Analytical Science, Boston, MA).

Transfection and dual luciferase assay

KB cells were plated in 12-well plates and pWWP (0.25 g) were transfected by Lipofectamine 2000 reagent according to the manufacturer’s protocol when plates reached 50–60% confluence. After 6 h, cells were treated with complete media containing either vehicle (DMSO) or SFN for 20–22 h. Cells were then lysed with 100 μl of 1X reported lysis buffer and 30 μl of cell extract was used for luciferase assays. Results are expressed as means ± S.D. for at least three independent determinations for each treatment group.

Immunohistochemistry

For immunohistochemistry, an EnVision™ Detection Kit System (Dako, Glostrup, Denmark), which is based on the peroxidase-catalysed deposition of biotinylated tyramide was used. Briefly, 4-μm-thick sections were cut from a paraffin-embedded tissue block. Sections were then deparaffinized and hydrated by sequential immersion in xylene and grade alcohol solution. For antigen retrieval, sections were immersed in a 0.1 M citrate buffer solution at pH 6.0. After boiling twice in a microwave oven for 5 min, the slides were cooled to RT and rinsed in phosphate buffered saline (pH 7.4). The endogenous peroxidase activity was quenched by treating the specimens with 3% hydrogen peroxide for 5 min at RT. The sections were incubated with primary antibodies for 1 h at 37°C and then incubated with the secondary antibody for 30 min at RT. Staining was visualized using a diaminobenzidine (DAB) and counterstained with Mayer’s hematoxylin. The negative control was performed with the incubated antibody diluent as a substitute for the primary antibody.

Nude mouse xenograft assay

Female nude BALB/cAnNCrjBgi-nu mice (6-week-old) were purchased from Orient Ltd. (Seoul, South Korea), and maintained in accordance with Institutional Animal Care Use Committee guidelines. A xenograft was established by s.c. injection of in vitro cultured KB cells (10⁶ cells/200 ul) into the flank of mice. Mice were randomized into two groups of 5 mice/group (2 tumors/mouse). Experimental animals were treated orally with SFN (50 μg/kg) 3 times/week (Monday, Wednesday and Friday) for 21 days beginning the day of tumor cell implantation. Control mice received an equal volume of the vehicle. Tumors were measured along two diameter axis with calipers to permit calculation of tumor volume, \[ V = \pi / 6 \{ (D + d) / 2 \}^3 \], where D and d were the larger and smaller diameters, respectively. Statistical significant differences in tumor volume, wet tumor weight or body weight between control and treated mice was assessed by Student’s t tests.

Statistical analysis

Statistical significance was assessed using Student’s t tests. A value of \( p<0.05 \) compared with solvent control was considered statistically significant.

Results

Effect of sulforaphane on KB and YD-10B cell proliferation

The growth-inhibitory effect of SFN was examined initially by assessing the proliferation efficacy on KB and YD-10B human oral carcinoma cells using cell counting. As shown in Fig. 1A and B, SFN significantly reduced the viability of both cell lines. Treatment with 40 μM SFN inhibited the cell proliferation of KB and YD-10B cells by approximately 58% and 46%, respectively. The results of the cell counting assay were confirmed by estimating the number of viable cells using an MTT cell proliferation assay. This assay also showed that SFN inhibits cell viability (Fig. 1C and D). Based on these results, we confirmed that SFN has a growth-inhibitory effect on cell proliferation in KB and YD-10B cells.

Sulforaphane induces the G2/M phase cell cycle arrest

Since SFN inhibits KB cell proliferation, we analyzed the cell cycle distribution under the SFN-induced growth-inhibitory conditions using flow cytometry analysis. When the cells were treated with SFN for 12 h, cell proliferation was arrested and accumulated mainly in the G2/M phase.
Sulforaphane Increases p21 protein in Oral Cancer

Vol. 46, No. 1, 2010

(Fig. 2A). The G2/M phase arrested cells were increased from 28% and 24% in DMSO-treated KB and YD-10B cells to 54% and 35% in 40 μM of SFN-treated cells, respectively (Fig. 2B).

Sulforaphane induces CDK inhibitor, p21 protein and its transactivation

Because, the most of the cells were arrested at the G2/M phase, experiments were carried out to determine whether the SFN treatment also affects the expression level of G2/M phase cell cycle regulatory proteins. KB cells were treated with 20 μM of SFN for 12 h and the expression of these proteins (p21, cyclin A and cyclin B) were examined by western blot analysis. Fig. 3A and B showed a significant increase in the level of p21 protein expression and the level of cyclin B expression was significantly reduced by SFN whereas cyclin A was not changed. Furthermore, SFN induced the transactivation in KB cells transfected with pWWP (Fig. 3C). To find whether sp1 protein regulates p21 protein expression, we evaluated the level of sp1 protein expression. Unfortunately, sp1 protein was not changed by SFN compared to control (Fig. 3D). These results suggest that SFN modulates the expression level of the G2/M phase cell cycle regulatory proteins and its transactivation in KB oral carcinoma cells.

Sulforaphane induces p21 protein in nude mouse xenograft model

Immunohistochemistry detected the level of p21 protein from the tumor sections and they were obtained from animals in previous studies which showed that SFN inhibited oral carcinoma growth in athymic nude mice bearing KB cells as xenograft [22]. There was markedly increased immunostaining of p21 protein in oral carcinoma treated with 50 μg/kg of SFN compared to the tumors from the vehicle control (Fig. 4).

Discussion

Cruciferous or Brassica vegetables come from the Brassica genus, including broccoli, Brussels sprouts and cauliflower. They are a good source of glucosinolates and their hydrolysis products, including indoles and isothiocyanates. The dietary intake of cruciferous vegetables has been associated with a lower risk of certain types of cancers including prostate, lung and colorectal cancer in some epidemiological studies [23–27]. Hence, an examination of the relationships between cancer risk in humans and phytochemicals specifi-
cally rich in cruciferous vegetables is expected to help prevent cancer. Among the phytochemicals in these vegetables, SFN is the most characterized isothiocyanate compound. Many studies have reported that SFN is an effective agent against the proliferation of a variety of cancer cells [22, 28–30]. It was previously reported that SFN exhibited a significant inhibition on the growth of KB and YD-10B cells with caspase-dependent apoptosis [22]. In this study, it was found that early-time exposure (12 h) to SFN significantly decreases the number of viable KB and YD-10B cell growth in a dose-dependent manner (Fig. 1). On the other hand, there were no apoptotic events observed in SFN-treated cell for 12 h. Therefore, other possible mechanisms for how SFN inhibits the proliferation of oral squamous carcinoma cells were examined. Several studies on SFN reported that SFN induces G2/M cell cycle arrest in cancer cells [18, 31, 32]. Therefore, this study examined cell cycle regulation in KB and YD-10B cells and SFN induced a profound arrest in the G2/M phase (Fig. 2). These results suggest that the G2/M cell cycle arrest induced by SFN is a prerequisite for caspase-dependent apoptosis in these cell lines. However, more critical experiments will be needed to test this hypothesis.

CDK inhibitors have grown in importance and number since they were first described and they appear to be universal inhibitors of cyclin/CDK activity which functions by forming a complex with the cyclin/CDK to inhibit the growth of cancer [2, 33, 34]. p21, a CDK inhibitor also inhibits CDKs. The induction of p21 molecule causes a number of different cell lines to arrest in both G1 and G2 checkpoint. SFN was reported to induce G2/M arrest in a variety of cancer cell lines and p21 expression is associated with G2/M phase cell cycle arrest [31, 35, 36]. Our study showed that SFN induces the p21 expression and its transactivation to induce G2/M phase arrest (Fig. 3). There results are fully consistent with other studies. We also previously reported that SFN decreased tumor growth in athymic nude mice bearing KB cells as xenograft. Fig. 4 shows that p21 was also induced in the tumor sections from the same animals demonstrating comparable induction of this pro-apoptotic protein in both in vivo and in vitro tumor models. The specificity protein (Sp) family recognizes GC-rich or GT-rich DNA and regulates the promoters of several genes [37, 38]. The human p21 gene contains six Sp1 binding sites [39]. Due to close relationship between Sp1 and p21, we
evaluated Sp1 protein expression level in this system. Unfortunately, Sp1 protein was not affected by SFN indicating that Sp1 is not an upstream protein of p21 (Fig. 3D). Of several CDK inhibitors, p21 is an important mediator of cell cycle arrest regulated by tumor suppressor p53 in response to DNA damage [40]. In addition to being induced by p53, p21 is also induced by other factors in a p53-independent pathway [41, 42]. Recently, Traka et al. (2009) reported that the induction of p21 protein by SFN can be mediated by Kruppel-like factor 4 (KLF4) in colon cancer cells and tissues suggesting that KLF4 might be a possible mediator for p21 protein in this study [43]. Therefore, current studies are focused on identifying these critical proteins induced by SFN in oral squamous carcinoma cell lines to induce p21 protein.

In summary, this study demonstrates for the first time that SFN inhibit growth and induce G2/M cell cycle arrest in oral squamous carcinoma cell lines. Moreover, p21 protein plays an important role in SFN-induced cell cycle arrest. Therefore, these findings confirm that SFN is a promising...
candidate for molecular-targeting chemotherapy against human oral squamous cell carcinoma.

Acknowledgment

This paper was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund, KRF-2008-331-E00260). Also, this study was conducted in part by research funds from Gwangju University, South Korea in 2009.

References


[36] Boorman, 67

Sulforaphane Increases p21 protein in Oral Cancer

Vol. 46, No. 1, 2010