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Effect of Sulfur Enriched Young Radish Kimchi on the Induction of Apoptosis in AGS Human Gastric Adenocarcinoma Cells

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Abstract

The effects of young radish (YR, *yeolmu* in Korean) on the induction of apoptosis were examined in AGS human gastric adenocarcinoma cells. The young radish kimchi (YRK) were made of YR cultivated in the soil without (Control YR kimchi: C-YRK) and with 1,818 g/m^3 sulfur (Sulfur YR kimchi: S-YRK), respectively. Methanol extracts from S-YRK exhibited higher inhibitory effect on the growth of AGS human gastric adenocarcinoma cells in a time dependent-manner compared to C-YRK at the same concentration. 4,6-diamidino-2-phenylindole (DAPI) staining showed that S-YRK induced apoptosis accompanied by the increased Bax but decreased Bcl-2 in mRNA expression. Moreover, S-YRK decreased the levels of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNA expressions. The results suggested that S-YRK cultivated in the presence of sulfur elicited stronger anticancer activity than C-YRK *in vitro*. Dietary intakes of S-YRK may be beneficial to decrease the risk of cancer.

Key words: young radish kimchi, sulfur, AGS human gastric adenocarcinoma cells, apoptosis

INTRODUCTION

Cancer is the first-leading cause of death and at least one third of all cancers are related to dietary factors. Epidemiologic studies have demonstrated that the risk of cancer is conversely related to the intake of fruits and vegetables (1,2). Cruciferous vegetables have been used for the prevention and the treatment of various cancers. Young radish (YR, *yeolmu* in Korean), a kind of the cruciferous vegetables, is abundant in vitamin A, vitamin C and other essential minerals that can prevent blood acidification (3,4). In addition, various kinds of phytochemicals including isothiocyanates are found in young radish (5). Isothiocyanates are released when chewing or macerating certain cruciferous vegetables. Sulfuraphane is an isothiocyanate that is naturally found in widely consumed vegetables (6).

The cultivation of young radish in the soil containing sulfur induced an enhanced quinone reductase activity by sulforaphane-like compounds and inhibitory effect on growth of colon cancer cells (7,8). Kim et al. suggested that the administration of extracts of young radish cultivated with sulfur suppressed pulmonary tumorigenesis, possibly due to increased activities of detoxification enzymes in the liver and lung, and partly due to cell cytotoxcity (9).

YR is mostly used to make kimchi in Korea (3,4). Some studies revealed that kimchi (baechu kimchi) extracts inhibited the survival or growth of human cancer cells (AGS gastric cancer, HT-29 colon cancer, MG-63 osteocarcinoma, HL-60 leukemia and Hep 3B liver cancer cells) in the SRB (sulforhodamin B) assay, the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and the growth inhibition test (10,11). Unfortunately, little research has been conducted on the functional properties of young radish kimchi, but it is nonetheless valuable to find the effects of YRK on the selective apoptotic effects and activation of gene expression.

In this study, the enhanced functional properties of YRK prepared with sulfur enriched YR were investigated on apototic effects and expressions of Bax and Bcl-2 genes and COX-2 and iNOS genes in AGS human gastric adenocarcinoma cells.

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MATERIALS AND METHODS

Ingredients and preparation of young radish kimchi

Young radishes (YR, *yeolmu* in Korean) were cultivated in the soil without (control-YR) and with 1,818 g/m^3 sulfur (sulfur-YR), respectively. Young radish kimchis (YRK) were prepared using control-YR and sulfur-YR, and named C-YRK and S-YRK, respectively. YR were offered by Gyeongnam Agricultural Research and Extension Services. Purchased at Bujun market in Busan, Korea were garlic, ginger, green onion, red pepper, red pepper powder, anchovy juice.

YRK were prepared based on standardized manufacturing methods (12), specifically the following recipe directions: 100 g of preserved young radish, 2.9 g crushed garlic, 1.6 g crushed ginger, 8.0 g green onion, 7.0 g red pepper, 4.2 g red pepper powder and 3.7 g anchovy juice. YRK were fermented at 5°C for 4 weeks.

Preparation of methanol extracts of samples

For optimally fermentation point (pH 4.3), YRK fermented at 5°C were freezing-dried and powdered. Twenty folds of methanol were added to the powdered samples and then extracted 2 times with shaking. The methanol extracts were evaporated and concentrated using rotary evaporator. The extracts were dissolved in dimethyl sulfoxide.

Cell culture

AGS human gastric adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Rockbille, MD, USA) and cultured in RPMI-1640 medium (Gibco Co., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% peni cillin-streptomycin (Gibco Co.) at 37°C in a humidified atmosphere containing with 5% CO_2. The medium was changed two or three times each week (13).

Growth inhibition study

For the growth inhibition analysis, 1 mL of cancer cells were plated at density of 1×10^5 cells/plate (6-well microtitre plates, Nunc) and treated with YRK supplemented medium (1.5 mg/mL) for the desired time (1~4 days). The cells were trypsinized, washed with phosphate-buffered saline (PBS), and scored with the Trypan Blue method (13).

Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI)

Untreated control and YRK treated cancer cells were harvested, washed with PBS, and fixed with 3.7% para formaldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with DAPI (Sigma) solution for 10 min at a room temperature. The cells were washed two more times with PBS and analyzed via a fluorescence microscope (Olympus BX50, Japan) (14).

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using a Trizol reagent (Invitrogen Co., CA, USA) according to the manufacture's recommendations. Total RNA was digested with RNase-free DNase (Roche, IN, USA) for 15 min at 37°C and repurified by the RNeasy kit according to the manufacture's protocol (Quiagen, CA, USA). cDNA was synthesized from 2 μg total RNA. By incubation at 37°C for 1 hr with AMV reverse transcriptase (Amersham) with random hexanucleotide according to the manufacture's instruction. The mRNA were amplified by PCR with desired primers shown in Table 1. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with cycles of denaturation at 94°C, annealing at 58°C, and extension at 72°C for 30 sec, respectively. The amplified PCR products were run in 1.0% agarose gels and visualized by EtBr, as previous described.

Statistical analysis

The data were presented as mean±SEM. Differences

Table 1. Gene-specific primers used for RT-PCR

Gene name		Sequence of primers
Bax	Sence	5'-ATG-GAC-GGG-TCC-GGG-GAG-3'
	Antisence	5'-TGG-AAG-AAG-ATG-GGC-TGA-3'
Bcl-2	Sence	5'-CAG-CTG-CAC-CTG-ACG-3'
	Antisence	5'-GCT-GGG-TAG-GTG-CAT-3'
COX-2	Sence	5'-TTC-AAA-TGA-GAT-TGT-GGG-AAA-AT-3'
	Antisence	5'-AGA-TCA-TCT-CTG-CCT-GAG-TAT-CTT-3'
iNOS	Sence	5'-AGA-GAG-ATC-CGG-TTC-ACA-3'
	Antisence	5'-CAC-AGA-GCT-GAG-GGT-ACA-3'
GAPDH	Sence	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
	Antisence	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p<0.05$. The statistical software package, SAS v9.1 (SAS Institute Inc., NC, USA), was used for the analysis.

RESULTS AND DISCUSSION

Growth inhibition by YRKs

To study whether C-YRK and S-YRK have anti-proliferative effects against AGS human gastric adenocarci noma cells, cell counting and cytotoxicity assays were conducted in cells treated with young radish kimchis using trypan blue staining. The anti-proliferative and anti-survival effects assessed by hemocytometer counts were compared (Fig. 1).

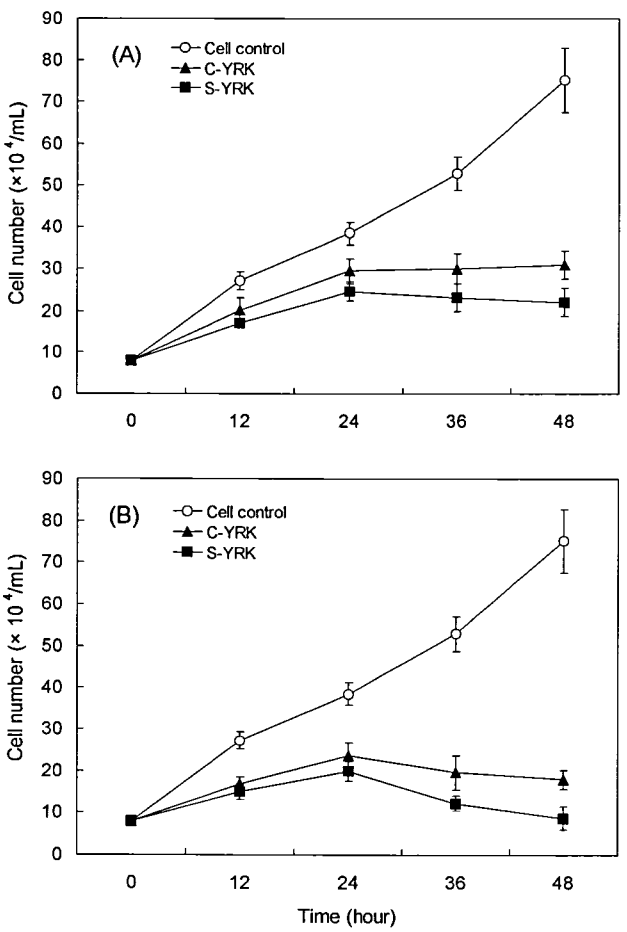

Fig. 1. Time-dependent growth inhibition by treatment of young radish kimchis in AGS human gastric adenocarcinoma cells. Cells were plated at 1×10^5 cells/60 mm plate, incubated for 24 hr and treated with (A) 1.5 mg/mL, (B) 3 mg/mL of young radish kimchis for 48 hr. The cells were trypsinized, washed with PBS and the viable cells were scored by hemocytometer counts. Each point represents the mean±SD (n=3). C-YRK: Control young radish kimchi, S-YRK: Sulfur young radish kimchi.

Cells were incubated for one day and treated with 1.5 mg/mL and 3 mg/mL YRK extracts for up to 48 hrs. Treatments of YRK showed cell growth patterns in a time-dependent manner. In lower concentration (1.5 mg/mL), after 12 hrs, there was different phase between cell control and YRKs treatment, but S-YRK showed similar phase as C-YRK. A gap of cell growth rate between control and YRKs was increased after 24 hrs, growing up gradually. The results in higher concentration (3 mg/mL) were also akin to those in lower concentration. The results demonstrated that the S-YRK was more effective on the anti-proliferative and anti-survival effects than C-YRK, and the growth inhibit effects were found after 24 hrs. In the toxicity tests, methanol extracts from the YRK exhibited higher survival rates (about 95%) at the concentrations of 0~4.0 mg/mL using the fibroblast cells (data not shown). We selected the safe range concentrations to examine inhibitory effect on the growth of AGS human gastric adenocarcinoma cells. These results suggested that preparation of kimchi using YR cultivated in the presence of sulfur could increase the inhibitory effect of S-YRK. Kim et al. (8) demonstrated that YR cultivate in the soil containing sulfur increased quinine reductase activity in Hepa 1c1c cells and isothiocyanate-like compound was analyzed by HPLC. Therefore, the higher inhibitory effect of S-YRK against the growth of AGS human gastric adenocarcinoma cells could be induced due to isothiocyanate-like compounds in YR cultivated with sulfur. Sulforaphane among isothiocyanates could be major candidate compound occurred by cultivation with sulfur. Effect of isothiocyanate on carcinogen metabolism has been known (9). Sulforaphane inhibits the reinitiation of growth and decreases the cellular viability of HT-29 human colon carcinoma cells (15).

Apoptotic cell death by YRKs

To further characterize whether the growth inhibitory activity of YRK in AGS human gastric adenocarcinoma cells was related to the induction of apoptosis, the presence of chromatin condensation was analyzed by fluorescent microscopy using the DNA-binding fluorescent dye DAPI (Fig. 2). In the absence of YRKs, AGS human gastric adenocarcinoma cells presented nuclei with homogeneous chromatin distribution. Treatment of 1.5 mg/mL YRKs induced chromatin condensation and nuclear fragmentation, suggesting the presence of apoptotic cells. Condensation and formation of apoptotic bodies, a characteristic of apoptosis, were shown in the cells cultured with S-YRK, but very few in C-YRK. These results suggested that the S-YRK was more effective on inducing the condensation and formation of apoptotic

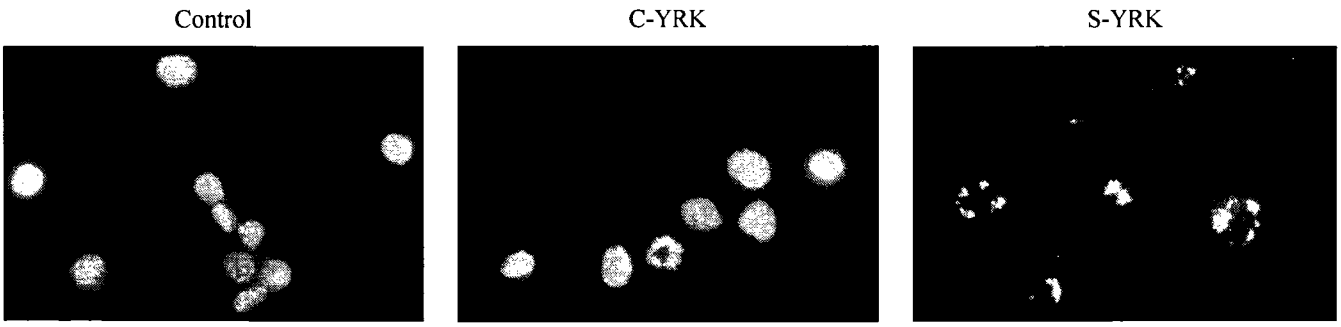

Fig. 2. Induction of apoptosis by treatment of young radish kimchi in AGS human gastric adenocarcinoma cells. Cells were incubated with young radish kimchis (1.5 mg/mL) for 48 hr and then stained with DAPI. After 10 min incubation at room temperature, the cells were washed with PBS and photographed with a fluorescence microscope using blue filter. Magnification, ×400. C-YRK: Control young radish kimchi, S-YRK: Sulfur young radish kimchi.

bodies, compared to C-YRK. Treatment with S-YRK resulted in a growth inhibition coupled with the characteristic morphological features of apoptosis. It also could be induced due to isothiocyanate-like compounds in YR cultivated with sulfur (7,8). Inducing of cancer cell death through an apoptotic pathway of sulforaphane has been known. The apoptotic pathway involves typical biochemical and ultrastructural modifications related to programmed cell death (16).

Expression of Bax and Bcl-2

To better determine which types of apoptotic pathways was induced by YRK, after 48 hrs incubation with treatment of 1.5 mg/mL YRK methanol extracts, activations of Bax and Bcl-2 gene in AGS human gastric adenocarcinoma cells were examined by RNA extraction and a reverse transcription-polymerase chain reaction (RT-PCR) (17,18). As shown in Fig. 3, in the presence of 1.5 mg/mL YRKs, the pro-apoptotic primer Bax and the anti-apoptotic primer Bcl-2 showed significant changes. Accrodingly, the results suggest that YRKs induced apoptosis in AGS human gastric adenocarcinoma cells is via a Bax dependent pathway and a Bcl-2 dependent pathway. While the decreased expression of Bcl-2 was observed, the decrease in S-YRK was much more noticeable than that in C-YRK. One of the best characterized regulators of apoptosis is the Bcl-2 family. Bcl-2 is an intracellular suppressor of apoptosis, which functions by heterodimerizing with its pro-apoptotic relative Bax (19-21). Apoptosis inducing by S-YRK in AGS human gastric adenocarcinoma cells was related with the decreased expression of the anti-apototic Bcl-2 mRNA.

Expression of COX-2 and iNOS

We further investigated whether the inhibitory effect of YRK was associated with decreased COX-2 expression and iNOS expression. After 48 hrs of incubation with treatment of 1.5 mg/mL YRK methanol extracts, the levels of COX-2 and iNOS expressions in AGS human gastric adenocarcinoma cells were examined by RNA extraction and a reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 4, in the presence of 1.5 mg/mL YRKs, mRNA expression of COX-2 was detectable in untreated control AGS cells,

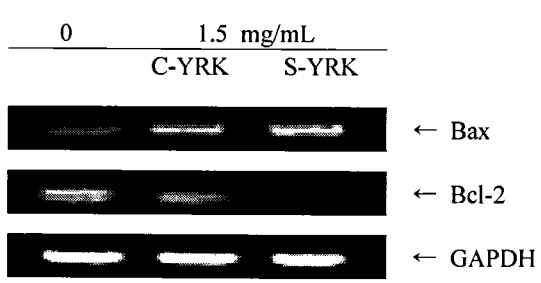

Fig. 3. Effects of young radish kimchis in the levels of Bax and Bcl-2 expressions in AGS human gastric adenocarcinoma cells. Cells were incubated with young radish kimchis for 48 hr and total RNA was isolated and RT-PCR was performed using indicated primers. The amplified PCR products were run in 1% agarose gel and visualized by EtBr staining. GAPDH was used as a house-keeping control gene. C-YRK: Control young radish kimchi, S-YRK: Sulfur young radish kimchi.

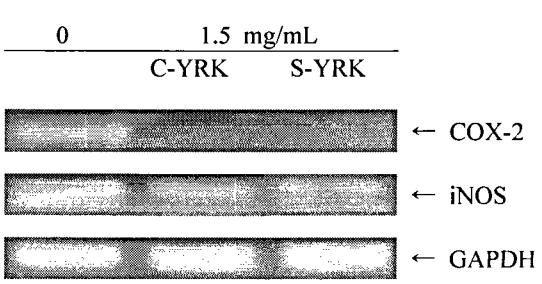

Fig. 4. Effects of young radish kimchis in the levels of COX-2 and iNOS expression in AGS human gastric adenocarcinoma cells. Cells were incubated with young radish kimchis for 48 hr and total RNA was isolated and RT-PCR was performed using indicated primers. The amplified PCR products were run in 1% agarose gel and visualized by EtBr staining. GAPDH was used as a house-keeping control gene. C-YRK: Control young radish kimchi, S-YRK: Sulfur young radish kimchi.

but undetectable after YRKs treatment and the mRNA expression of iNOS was also gradually reduced. COX-2 and iNOS are a kinds of pro-inflammatory enzyme and has been closely related with the pathophysiology in a variety of chronic diseases and cancers (22,23). Heiss et al. (24) demonstrated that sulfraphane is a selective inhibitor of iNOS targeting in study on the molecular target for sulfraphane-mediated anti-inflammatory mechanisms. Accordingly, S-YRK can be expected to contribute to the prevention of cancer risk.

The results suggested that S-YRK cultivated in the presence of sulfur can elicit stronger anticancer activity than C-YRK. Dietary intake of S-YRK may be helpful to reduce developing cancer risk.

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