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Anticancer Effects of Leek *Kimchi* on Human Cancer Cells

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Abstract

The anticancer effects of leek (*buchu* in Korean) *kimchi* were evaluated in the human cancer cells: AGS gastric adenocarcinoma cells, HT-29 human colon adenocarcinoma cells and HL-60 leukemia cells. The leek *kimchi* (fermented for 6 days at 15°C) was fractionated into 7 groups: methanol extract, hexane extract, methanol soluble extract (MSE), dichloromethane (DCM) fraction (fr.), ethyl acetate fr., butanol fr. and aqueous fr. Most of the leek *kimchi* fractions inhibited the growth of AGS and HT-29 cancer cells in a dose dependent manner. In particular, the DCM fr. showed the highest inhibitory effect among the fractions. Treatment with the DCM fr. (0.1 mg/mL) reduced the survival rates of AGS and HT-29 cancer cells to 19% and 37% of the controls, respectively. Moreover the DCM fr. of the leek *kimchi* arrested G2/M phase in the cell cycle and induced apoptosis in HL-60 human promyelocytic leukemia cells. These results indicate that the leek *kimchi* exerted an anticancer effect on those human cancer cells, and that the DCM fr. arrested G2/M phase in the cell cycle and induced apoptosis in the leukemia cells.

Key words: leek *kimchi*, human cancer cells, anticancer, cell cycle arrest, apoptosis

INTRODUCTION

Kimchi is a Korean traditional fermented vegetable food. There are many types of *kimchi* depending on the ingredients and preparation methods used (1). Leek (*Allium tuberosum* L., *buchu* in Korean) *kimchi* is a traditional regional *kimchi* commonly consumed in Kyungsang province, Korea. Leek *kimchi* is prepared with large quantities of red pepper powder and pickled anchovy, making it a popular side dish because of the unique flavor of leek and hot taste (2-4). Leeks, the major ingredient for leek *kimchi*, have long been used as a medicinal food for the treatment of abdominal pain, diarrhea, hematemesis, snakebite and asthma in folk remedies (5). Leek belongs to the *Allium* genus, which are characterized by large amounts of thiosulfinates and organosulfur compounds and are rich in vitamin A, B_1, and C (6-9). Leek also contains high levels of flavonoids (10,11) which have antimutagenic and anticancer effects *in vitro* and *in vivo* (12,13). Furthermore, several studies have indicated that high consumption of leek was associated with a reduced risk for colorectal cancer (14-16). In a case-controlled study in Japan and Hawaii, high consumption of Japanese leek was associated with a decreased risk for colorectal cancer as well as for the subset of cancers in the lower part of the rectum (14,15).

The most popular *kimchi* in Korea is Korean cabbage (*baechu*) *kimchi*, for which antimutagenic and anticancer activities have already been demonstrated (17,18). It was also reported that the optimally ripened *kimchi* was more effective than freshly prepared *kimchi* (19). It has been postulated that vitamin C, β-carotene, dietary fibers, lactic acid bacteria, β-sitosterol, and other phytochemicals are responsible for such putative activities (20-23). In particular, the phytochemicals in the primary vegetables may play a significant role in the antimutagenic and anticancer activities of *kimchi* (24). Although leek *kimchi* has abundant phytochemicals such as vitamins, flavonoids, and organosulfur compounds, the protective action of leek *kimchi* against cancer has been poorly elucidated until now.

In this study, anticancer effects of optimally ripened leek *kimchi* (pH 4.3) in the human cancer cells: AGS gastric adenocarcinoma cells, HT-29 human colon adenocarcinoma cells and HL-60 leukemia cells were studied. The effects of an active fraction (DCM fr.) from the leek *kimchi* on cell cycle progression and programmed cell death in HL-60 human leukemia cells were also investigated.

MATERIALS AND METHODS

Preparations of leek *kimchi*

The leek *kimchi* was prepared by cutting the leeks into 2 pieces, soaking them in 20% salt solution for 20 min

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at room temperature, and rinsing twice with tap water. The ingredient ratio of leek *kimchi* was as follows: 9.0 parts red pepper powder, 5.0 garlic, 2.0 ginger, 13.0 fermented anchovy juice, 2.0 sugar and 13.0 glutinous rice paste in 100 parts salted leek. The final salt concentration of the leek *kimchi* was 2.3%. Leek (cultivated from Kimhae, Korea), garlic, radish, green onion, ginger, red pepper powder (Youngyang, Kyungbuk, Korea), fermented anchovy juice (Daesang Co., Chungnam, Korea), salt (Guwoon salt, Sannaedle Co., Jeonnam, Korea), sugar and glutinous rice powder were purchased from Bujeon market in Busan, Korea. The prepared leek *kimchi* was put into pint jars, the lid closed tightly, and allowed to ferment at 15°C for 6 days. After fermentation (pH 4.3), leek *kimchi* was freeze dried and powdered.

Extraction and fractionation of leek *kimchi*

Freeze dried and powdered leek *kimchi* was extracted with methanol (MeOH, 20-fold) three times by shaking for 8 hrs and decanting the MeOH extract. The powdered leek *kimchi* (4 kg) was also extracted with hexane (40 L), and the hexane extract (180 g) saved. The defatted residues were again extracted with MeOH by the previous method, and concentrated to obtain the MeOH soluble extract (MSE, 800 g). The MSE was fractionated into dichloromethane (DCM) fraction (fr., 96 g) and aqueous layer using DCM-MeOH-H_2O (10:1:9, v/v/v). Further fractionation of the aqueous phase with ethyl acetate (EA) resulted in EA fr. (16 g) and aqueous layer fractionated into butanol fr. (144 g) and aqueous fr. (504 g). Each extract and fraction was dried by with a rotary vacuum evaporator (Buchi 011 & 461, Switzerland) and dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for use (23).

Cell culture and growth inhibition test

Dulbeco's modified Eagle's medium (DMEM), fetal calf serum (FCS), trypsin, EDTA, and 100 units/mL penicillin-streptomycin were purchased from GIBCO Co. (NY, USA). AGS human gastric cancer cells, HT-29 human colon cancer cells and HL-60 human leukemia cells were obtained from Korea Cell Line Bank (Seoul, Korea). These cancer cells were cultured in DMEM supplemented with 100 units/mL of penicillin-streptomycin and 10% FCS in a 5% CO_2 incubator (Sanyo, model MCO96, Japan). Media were changed 3 times a week. After 6 days, the cultured cancer cells were washed with phosphate buffered saline (PBS). The cells were harvested after trypsin-0.02% EDTA treatment followed by centrifugation. The cell suspensions (2×10^4 cells/mL) were seeded in 24-well plates and incubated in a 5% CO_2 incubator at 37°C for 24 hrs. The media supplemented with samples were changed every 2 days. In the control experiment, the cells were treated with DMSO. After 6 days, the cells were washed with PBS, treated with trypsin-EDTA, and then counted by hemocytometer (25).

DNA flow cytometric analysis

The cells were harvested after trypsin-0.02% EDTA treatment followed by centrifugation, pelleted by low speed centrifugation, resuspended in 200 mL of citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% Me_2SO, pH 7.6), and frozen at -80°C. Before staining, cells were thawed quickly and treated with RNase A (0.1 mg/mL). Nuclei were stained with propidium iodide (PI). All solutions were prepared in a stock solution containing 3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine×4 HCl, 0.5 mM Tris-base, pH 7.6 (26,27). DNA content in each cell nucleus was determined in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

DAPI staining

Cells were grown in complete DMEM. Harvested cells were washed once with PBS, resuspended in PBS containing 0.1% Triton X 100 (to induce holes in the cells membrane and increase permeability), and incubated for 10 min on ice. These cells were spun down and resuspended at 5000 cells/mL in 4% PBS buffered paraformaldehyde solution containing 10 μL/mL 4, 6-diamidino-2-phenylindole (DAPI, Sigma Co., USA). Ten μL of this suspension were placed on a glass slide and covered with a cover slip. The morphology of the nuclei was observed by fluorescence microscopy (Olympus BH Series, Japan) at excitation wavelength of 350 nm. Nuclei were considered to have the normal phenotype when glowing brightly and homogenously. Apoptotic nuclei were identified by condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies (28).

Statistical analysis

Data analyses were performed using SAS 6.0 (SAS, Cary, NC USA). Analysis of variance was used to determine significance of differences among groups; Duncan's multiple range test was used for post hoc comparisons if significant group differences were found.

RESULTS AND DISCUSSION

We have recently reported that leek *kimchi* has stronger antimutagenic and *in vitro* anticancer properties than Korean cabbage (*baechu*) *kimchi*. Fresh leek also exhibited higher antimutagenic and antiproliferative properties than Korean cabbage by 1.5~2 folds (24). In our previous study, the anti-carcinogenic effect of leek *kimchi* was a result of MCA-mediated cytotoxicity and neoplastic transformation in C3H/10T1/2 cells (29). In an effort to identify the anticancer active compounds from the leek *kimchi*, the optimally ripened leek *kimchi* was fractionated into 7 groups, MeOH extract, hexane extract, MSE, DCM fr., EA fr., bu-

tanol fr. and aqueous fr.. As shown in Fig. 1, all the leek *kimchi* fractions inhibited the growth of AGS human gastric adenocarcinoma cells. As the concentrations were increased, the growth of the AGS cells were increasingly retarded in a dose dependent manner. In particular the DCM fr. of the leek *kimchi* significantly inhibited the growth of AGS cells. When treated with DCM fr. (0.1 mg/mL), survival of AGS cells was reduced to 11×10^4 cells/mL compared to the 58×10^4 cells/mL of the control. Besides DCM fr. of the leek *kimchi*, MSE and butanol fractions also exhibited considerable antiproliferative effect against the AGS cells, whereas the aqueous fr. only slightly inhibited the growth of the AGS cells. The leek *kimchi* fractions also inhibited the growth of the HT-29 human colon adenocarcinoma cells (Fig. 2). Most of the fractions exhibited weaker antiproliferative effect against HT-29 cells than AGS cells. Again the growth of HT-29 cells was inhibited by the leek *kimchi* fractions in a dose dependent manner. The DCM fr. of the leek *kimchi* again showed the highest cytotoxicity against the HT-29 cells, where 65% growth inhibition was observed with the addition of 0.1 mg/mL. These results indicate that the DCM fr. of leek *kimchi* may contain major active compound(s) that decrease the growth of the AGS cells and the HT-29 cells. We previously identified 2-hydroxy-1-(hydroxy-methyl) ethyl hexadecanoic acid as one of the major anti-proliferative active compounds present in DCM fr. by GC-MS (30). It was also reported that leek contains sulfides (31), linalool (32), and flavonoid glycosides (10,11). Thiosulfinates containing both methyl and 1-propyl groups were identified from dichlo-

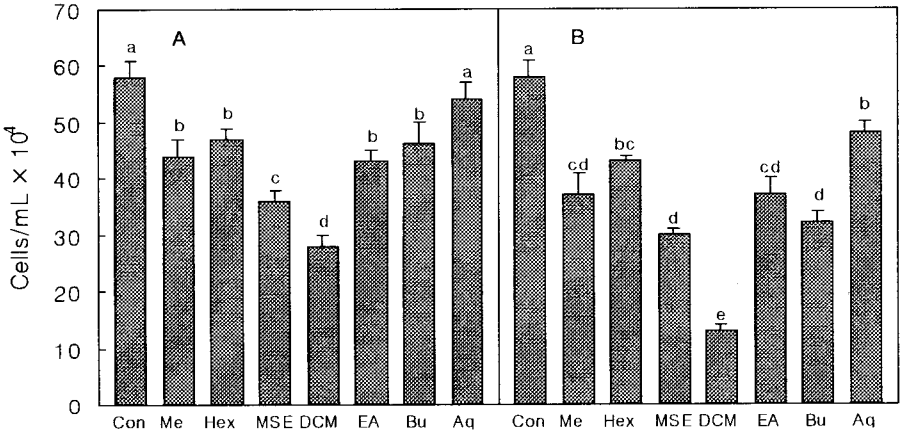

Fig. 1. Inhibitory effect of fractionated samples from leek *kimchi* on the growth of AGS human gastric adenocarcinoma cells after 6 days of incubation at 37°C. Con: control, Me: MeOH extract, Hex: hexane extract, MSE: MeOH soluble extract, DCM: dichloromethane fr., EA: ethly acetate fr., Bu: butanol fr., Aq: aqueous fr.
A: 0.05 mg/mL treated, B: 0.1 mg/mL treated.
[a~e]Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

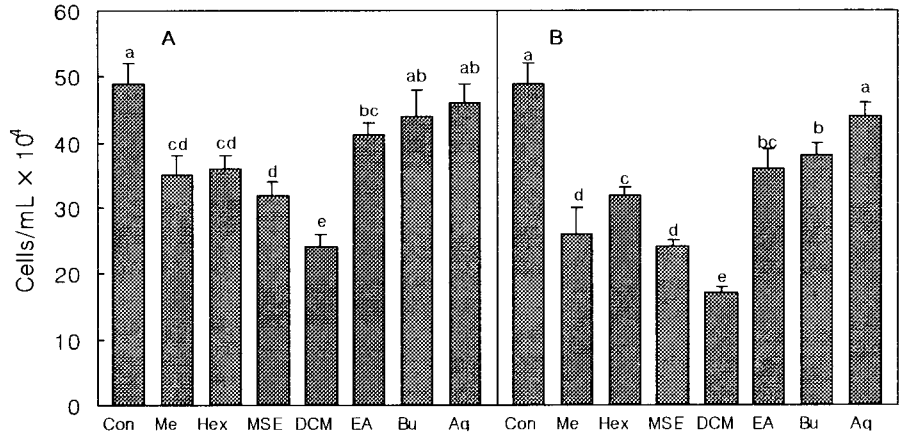

Fig. 2. Inhibitory effect of fractionated samples from leek *kimchi* on the growth of HT-29 human colon adenocarcinoma cells after 6 days of incubation at 37°C. Con: control, Me: MeOH extract, Hex: hexane extract, MSE: MeOH soluble extract, DCM: dichloromethane fr., EA: ethly acetate fr., Bu: butanol fr., Aq: aqueous fr.
A: 0.05 mg/mL treated, B: 0.1 mg/mL treated.
[a~e]Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

romethane extract of leeks by HPLC (7). β-Sitosterol and β-sitosterol-3-β-D-glucopyranoside were tentatively isolated from the $CHCl_3$ fraction of leek (33). These results suggest that the antiproliferative effect of leek *kimchi* might be due to flavonoids, fatty acid, thiosulfinates, terpenoides and sterol, and other compounds in the DCM fr.

Understanding the mechanisms that control the cancer cell numbers, including both cell cycle arrest and apoptosis, holds promise for successful cancer therapy (34,35). To elucidate the possible mechanisms of the DCM fr.-induced growth inhibition, we further investigated whether the DCM fr. of the leek *kimchi* affected the cell cycle progression of human leukemia HL-60 cells. Regulation of cell proliferation is a complex process involving the regulated expression and/or modification of discrete gene products, which control transition between different stages of the cell cycle (36,37). As shown in Table 1, flow cytometric data of DCM fr.-treated cells revealed a cell cycle block at G2/M transition phase. A concentration-dependent decrease in the percentage of cells in the G1 phase was observed in DCM fr.-treated cells. The decrease in G1 phase cells, resulting from DCM fr.-treatment, was complemented by an accumulation of cells in the G2/M phase of the cell cycle. After only 12 hrs treatment with DCM fr. (250 μg/mL), the percentage of the cells in the G2/M phase was increased by 1.7 fold compared to the control. These results were highly correlated with the growth inhibitory effect of the DCM fr., suggesting that the antiproliferative effects of the DCM fr. are a result of the block during the G2/M phase and that such cells could not enter G1 phase.

Table 1. The effect of DCM fr. from leek *kimchi* on cell cycle distribution in HL-60 human promyelocytic leukemia cells after 12 hrs of incubation at 37°C

Sample	Concentration (μg/mL)	% of cells		
		G1	S	G2/M
Control		31.49	57.75	10.76
DCM fr.	50	30.12	56.06	13.82
	125	26.48	58.22	15.30
	250	23.96	58.60	17.45

Exponentially growing cells were treated at 0 time with DCM fr. of leek *kimchi*. Cells were stained with PI and analyzed by flow cytometry after 12 hrs treatment with DCM fr.

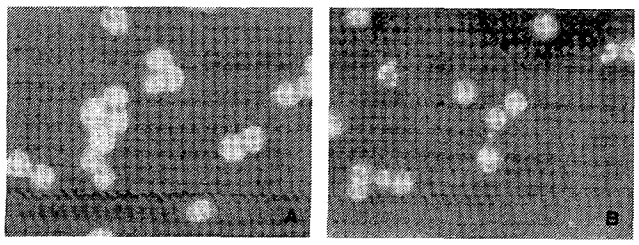

Fig. 3. Appearance of apoptotic body in HL-60 human promyelocytic leukemia cells treated with DCM fr. from leek *kimchi* after 48 hrs of incubation at 37°C (×400).
A: Control, B: DCM fr. (0.1 mg/mL) treated.

Apoptosis has been shown to be important in a number of physiological processes such as embryonal development, immune regulation, and tissue homeostasis. It is characterized by chromatin condensation, cell shrinkage, nuclear fragmentation and formation of membrane bound apoptotic bodies. Hallmark changes of chromatin condensation and nuclear fragmentation are readily visible by DAPI staining (38,39). The DCM fr. of the leek *kimchi* induced apoptosis as shown in Fig. 3, which was observed by direct visualization of morphological nuclear changes. These results suggested that the anticancer effects of the DCM fr. were also related to the induction of the apoptosis, along with the cell cycle arrest at the G2/M transition phase.

It can be concluded that leek *kimchi* fractions decreased the growth of human cancer cells, and that the inhibition was primarily due to some active compounds in the DCM fr. Two anticancer mechanisms of the DCM fr. that were identified were: blocking cell cycle progression at G2/M phase, and inducing apoptosis in the cancer cells. Further studies are needed to identify active compounds of leek *kimchi* and elucidate their precise molecular mechanisms.

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