

Doenjang Extract Has Anticancer Activity and Induces Apoptosis in AGS Human Gastric Adenocarcinoma

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Abstract

The anticancer and apoptotic effect of chloroform extract from 24 month-fermented doenjang were investigated in AGS human gastric adenocarcinoma cells. The chloroform extract of 24 month-fermented doenjang inhibited the AGS gastric cancer cell growth in a dose-dependent manner. It has been confirmed by observing the cell distribution under inverted microscope. Approximately, 48 hour treatment of 100 µg/mL doenjang extract inhibited AGS cancer cell growth by 76.7%, respectively. The growth inhibition may be caused by apoptosis of AGS cancer cells after 48 hour treatment of 24 month-fermented doenjang extract. It has been demonstrated by cell cycle arrest that revealed the shift from G₂ + M to G₀ + G₁ phase and the formation of apoptotic bodies. The fermentation period play a critical role in cell cycle arrest, in which 24 month-fermented doenjang extract was more effective than 12 month-fermented doenjang extract. The treatment of 24 month-fermented doenjang extract for 48 hours has induced intercellular Bax and decreased Bcl-2 level, indicating that it may regulate the expression level of Bax/Bcl-2 proteins. Thus, 24 month-fermented doenjang extract seems to have anticancer effect *via* cancer cell growth inhibition induced by apoptosis process.

Key words: doenjang, anticancer, AGS adenocarcinoma, growth inhibition, apoptosis

INTRODUCTION

Doenjang (fermented soypaste) is one of the most important traditional fermented foods and widely consumed in Korea. Although doenjang once had been suspected of being contaminated with aflatoxins from the growth of *Aspergillus flavus* due to the traditional natural fermentation, it was suggested that the aflatoxins can be destroyed during the fermentation process (1). The main components of detoxifying aflatoxin include NH₃, sunlight, mixed culture conditions, melanoidin formation, and addition of charcoal (2,3). Thus, deonjang may not have any significant harmful effect, despite of the possible contamination by aflatoxins.

It was reported that some of the components of soybean, especially trypsin inhibitor, isoflavones, saponin and phytic acids, showed anticancer functions (4-6). The cooked soybeans showed less inhibition of mutagenicity than raw soybeans, probably due to the destruction of the trypsin inhibitor by heat treatment (6), but the fermented soybeans (doenjang) was the most effective ($p < 0.05$). Thus, the fermented soybeans may have different components from raw soybeans, which could be driven

by chemical or biochemical reactions during fermentation process. For instance, it was reported that high consumption of miso (Japanese soypaste) decreased the rate of death from the incidence of stomach cancer (7). It was also reported that strong antimutagenic activity against aflatoxin B₁ (AFB₁) was observed by treatment with methanol extract of doenjang (8). And, in this article, author has suggested the possibility of which fermentation period may affect the antimutagenic activity. Moreover, long-term fermented doenjang extract showed higher antimutagenic effect on Ames reverse mutation test and SOS chromotest. Thus, to determine the action mode of long-term fermented doenjang extract on cancer cells, the anticancer activity and apoptotic effect of 24 month-fermented doenjang extract were investigated in employing AGS human gastric adenocarcinoma cell line.

MATERIALS AND METHODS

Sample

Doenjang prepared with different fermentation periods of 12 and 24 months were obtained from Alalee Food Co. (Koryung, Kyungbuk). Doenjang samples (100 g)

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were freeze dried (45~50 g) and powdered. 20-Fold chloroform-methanol (2:1 v/v) was added to each powdered sample, filtered to remove residue and extracted by separate funnel. The chloroform extracts were evaporated using a vacuum evaporator, concentrated, and then dissolved in ethanol.

AGS cell culture and growth inhibition

The AGS human gastric adenocarcinoma cell line (ATCC CRL-1739) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in DMEM (Whitaker, USA) supplemented with 10% heat-inactivated (55°C, 30 min) fetal bovine serum (FBS, Whitaker, USA), 2 mM L-glutamine (Sigma, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (BioWhitaker, USA) at 37°C in an atmosphere of 5% CO₂. For growth inhibition test, 2×10^5 cells/mL of AGS adenocarcinoma was attached on 24-well plate and incubated for 24 hours to settle down the cells on the plate. The different concentration of methanol extract of doenjang fermented for 24 months, generously donated from Dr. KY Park (Pusan National University, Korea), was treated with AGS cells and incubated at 37°C in an atmosphere of 5% CO₂. After 48 hour incubation, 0.05% trypsin-EDTA was treated and number of cells were determined by counting on the hemocytometer under light microscope (Olympus, Japan). In addition, growth inhibition of AGS cells after treatment of doenjang extract for 48 hours was photographed to image the cells under inverted microscope (Olympus, Japan).

Flow cytometry analysis for cell cycle arrest

To determine the effect of methanol extract of doenjang on the distribution of cell cycle, AGS cells were treated with doenjang extract for 48 hours, collected by trypsinization, washed with cold PBS and resuspended in PBS. DNA contents of cells were measured using a DNA staining kit (CycleTest Plus DNA reagent kit, Becton Dickinson, Heidelberg, Germany). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit manual. Data were acquired using CellQuest Software with a FACS calibur flow cytometry (Becton Dickinson) system using 20,000 cells per analysis. Cell cycle distributions were calculated using Modifit LT 2.0 software (Verity Software House, Topsham, ME, USA).

Fluorescent detection of apoptotic nucleus

Untreated control and doenjang extract-treated AGS cells after 48 hour incubation were harvested, washed with PBS and fixed with 3.7% paraformaldehyde (Sigma, USA) in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6-

diamidino-2-phenylindole (DAPI, Sigma, USA) solution for 10 min at room temperature. The cells were washed twice with PBS and analyzed via a fluorescence microscope (Carl Zeiss, Germany).

RNA extraction and RT-PCR

Total RNA was isolated using a Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) following the manufacture's manual. Total RNA was digested with RNase-free DNase (Roche, Indianapolis, IN, USA) for 15 min at 37°C and repurified by the RNeasy kit according to the manufacture's protocol (Quiagen, La Jolla, CA, USA). cDNA was synthesized from 2 µg total RNA by incubation at 37°C for 1 hour with AMV reverse transcriptase (Amersham) with random hexanucleotides. The reaction mixture was subjected to polymerase chain reaction (RT-PCR) to amplify sequences to desired primers. Amplification was performed in a mastercycler (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 1.5% agarose gels and visualized by EtBr under UV light.

Primer sets for PCR:

Bax	sense	5'-ATGGACGGGTCCGGGGAG-3'
	antisense	5'-TGGAAGAAGATGGGCTGA-3'
Bcl-2	sense	5'-CAGCTGCACCTGACG-3'
	antisense	5'-GCTGGGTAGGTGCAT-3'
GAPDH	sense	5'-CGGAGTCAACGCATTTGGTCGTAT-3'
	antisense	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

Statistical analysis

For growth inhibition test, statistically significant differences between groups were determined with Student's *t*-test. In all analyses, a value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Growth inhibition of methanol extract of doenjang fermented for 24 months

Park et al. (8) suggested that doenjang may have anticancer activity on certain types of cancer cells. The author also implied that fermentation period may play a critical role in anticancer activity of doenjang. In unpublished laboratory data, prolonging the fermentation period when making doenjang increases its chemopreventive effects. Two year fermented doenjang showed higher antimutagenic, antitumor and antimetastatic activities compared to 3, 6 and 12 month fermented doenjangs. Thus, this study has mostly focused on growth inhibition of AGS cancer cells by treatment of doenjang extract fermented for 24 months. The chloroform extract

of 24 month-fermented doenjang has significantly inhibited the growth rate of AGS cancer cells in a dose-dependent manner (Fig. 1). With 100 $\mu\text{g/mL}$ doenjang extract treatment, the growth of AGS cancer cells was inhibited by 76.7%. The observation of AGS cancer cells under inverted microscope supported the idea of anticancer effect of doenjang extract (Fig. 2), which is consistent with the results of growth inhibition. Our data also support the fact that doenjang extract inhibited tumor development in sarcoma-180 transplanted mice (9) although tested system was different each other. At this point, our data could not suggest that some compounds of fermented doenjang show the growth inhibition. However, we are assured that, at least, effect of osmotic pressure by NaCl could be excluded because NaCl was removed during extraction process.

Apoptosis induced by methanol extract of doenjang fermented for 24 months

Apoptosis is referred as a programmed cell death that

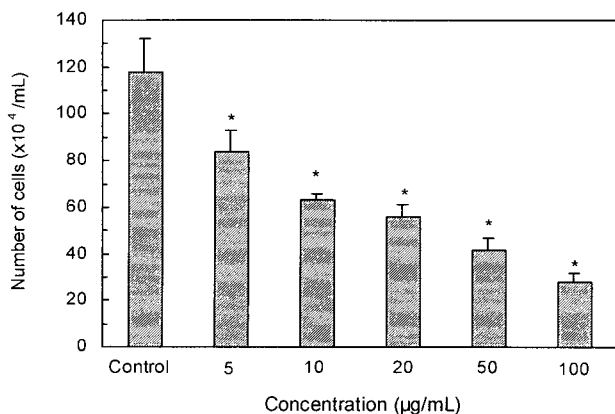


Fig. 1. Inhibitory effect of chloroform extract of 24 month-fermented doenjang extract on the growth of AGS human gastric adenocarcinoma cells. *Indicates the significant difference, compared to control at $p < 0.05$.

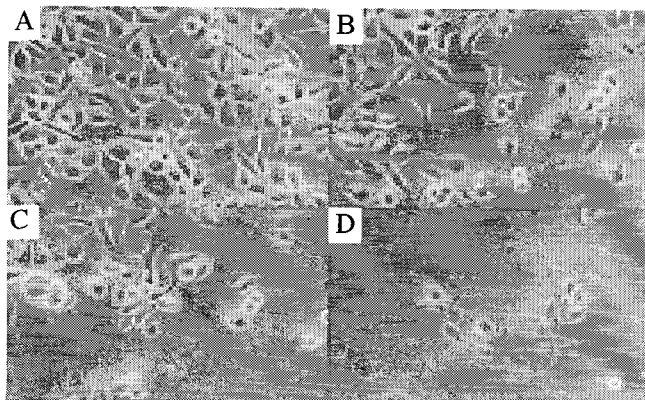
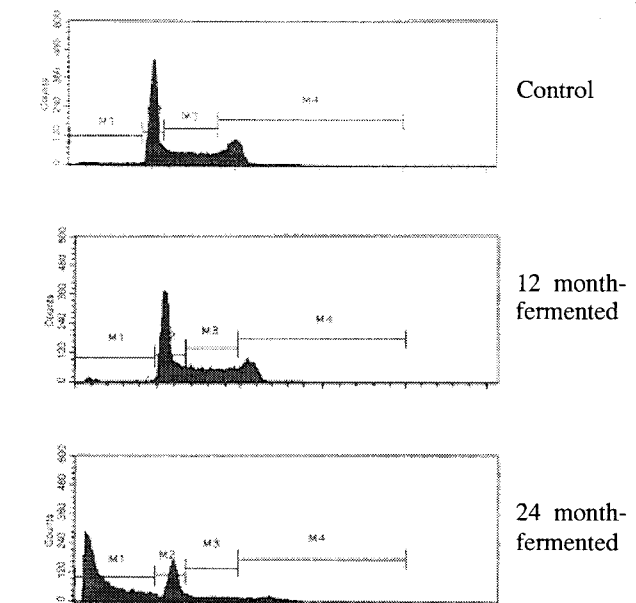


Fig. 2. The growth inhibition of 24 month-fermented doenjang extracts on AGS gastric cancer cells. The cells were plated onto each well with 2×10^5 cells/mL. Cell morphology was photographed by inverted microscope. A, control; B, 20 $\mu\text{g/mL}$; C, 50 $\mu\text{g/mL}$; D, 100 $\mu\text{g/mL}$ doenjang extracts.

acts as one of defense mechanisms in human body. And it may also play a critical role in cancer cell death without provoking the inflammation reaction. Apoptosis is complex process characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, cell cycle arrest, and formation of apoptotic bodies (10,11). These phenomena were induced via several physiological or pathological mediators such as oxidative stress, expression of ligands and receptors (TRAIL, TNF, Fas, FasL), activation of caspase pathway, and reduction of antiapoptotic proteins (Bcl-2, Bcl-xL, p35). In this study, 24 month-fermented doenjang extract shifted cell cycle from $G_2 + M$ to $G_0 + G_1$ phase, indicating that cell division or mitosis was blocked in AGS cancer cells (Fig. 3). Interestingly, 12 month-fermented doenjang extract did not show any shift of cell cycle, compared to control, supporting that fermentation period in manufacturing doenjang may play a critical role in anticancer activity. In consistency with cell cycle data, 24 month-fermented doenjang induced the apoptotic bodies in AGS cancer cells (Fig. 4). These data indicate that 24 month-



	% of cells			
	Sub-G1	G1	S	G2/M
Control	1.63	48.01	27.74	23.26
12 month	2.78	50.90	29.15	17.65
24 month	63.76	22.98	8.03	5.46

Fig. 3. DNA-fluorescence histogram of AGS human gastric carcinoma cells after 12 and 24 month-fermented doenjang extract treatment. DNA flow cytometric cell cycle analysis was performed comparing untreated controls with cells treated with non-fermented (non-ripen) doenjang extract. After treatment with doenjang extract for 48 hour, cells were collected and stained with PI, and then flow cytometric cell cycle analysis was performed.

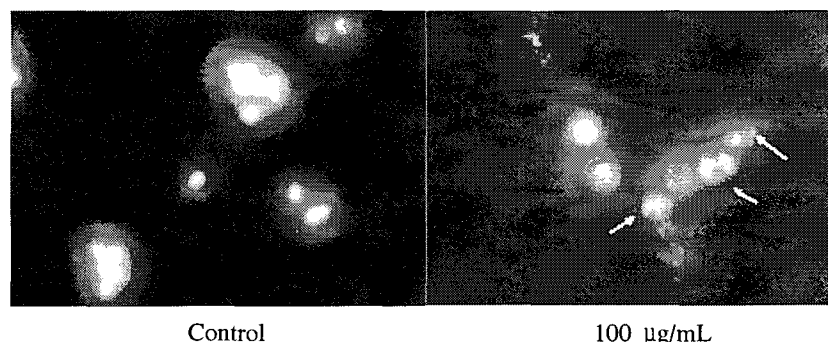


Fig. 4. Appearance of apoptotic bodies in AGS human gastric adenocarcinoma cells treated with 24 month-fermented doenjang extract after 48 hours. The untreated cells and doenjang extract-treated cells for 48 hours were harvested and spun down. After fixing, the cells were stained with DAPI solution. Stained nuclei were then observed under fluorescent microscope using blue filter.

fermented doenjang extract has blocked mitosis and rendered the cell cycle to be arrested in $G_0 + G_1$ phase where protein and RNA synthesis mainly occur instead of DNA replication. This point of view is in accordance with growth inhibition of AGS cancer cells after treatment of 24 month-fermented doenjang extract. And also apoptotic proteins such as Bax may be expressed in $G_0 + G_1$ phase. Bcl-2 is a potent antiapoptotic protein which was first cloned from a human follicular lymphoma (12). The protein has been reported to reside in mitochondrial, ER, and nuclear membranes (13). It has been suggested that Bcl-2 inhibits cell death by reducing the generation of reactive oxidants, thus preventing critical intracellular oxidations that are requisite for the completion of the apoptotic program (14). However, the apoptotic protein, Bax, promotes apoptosis by blocking the activity of Bcl-2, which eventually leads the cancer cells to death. In our study, 24 month-fermented doenjang extract has induced Bax protein expression, but reduced intracellular level of Bcl-2 protein (Fig. 5), indicating that doenjang extract causes apoptosis by regulating Bax/Bcl-2 cellular levels.

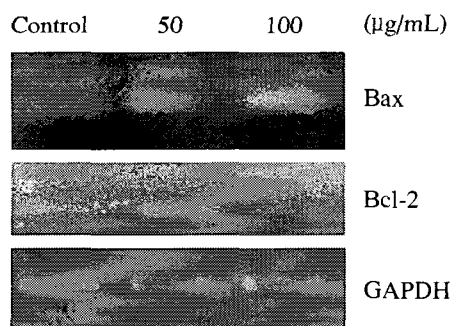


Fig. 5. Induction of Bax and inhibition of Bcl-2 gene expression after 24 month-fermented doenjang extract treatment with AGS cancer cells. Total RNA was isolated and RT-PCR was performed using indicated primers. The amplified PCR products were run in a 2% agarose gel and visualized by EtBr staining. GAPDH was used as a house-keeping control gene.

In conclusion, the chloroform extract of 24 month-fermented doenjang inhibited the AGS gastric cancer cell growth in dose-dependent manner. The growth inhibition may be caused by apoptosis of AGS cancer cells after 48 hour treatment of 24 month-fermented doenjang extract. It has been demonstrated by cell cycle arrest that revealed the shift from $G_2 + M$ to $G_0 + G_1$ phase, the formation of apoptotic bodies, and regulation of Bax/Bcl-2 proteins. From our data, we can not conclude that some components of fermented doenjang are active for apoptosis. But we might have an insight from the previous reports concerning antimutagenicity effect of doenjang extract, indicating that genistein, linoleic acid, β -sitosterol, soyasaponin, α -tocopherol, genistin, phytic acid, and trypsin inhibitor were the active compounds and, among them, genistein and linoleic acid were the most effective antimutagenic compounds (8). Thus, the biochemical change of active compounds during doenjang fermentation seems to be responsible for apoptosis process in AGS cancer cells.

ACKNOWLEDGEMENTS

This research was supported by the RRC program of MOST and ITEP in Korea.

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(Received March 21, 2005; Accepted May 10, 2005)