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Inhibitory Effect of *Doenjang* (fermented Korean soy paste) Extracts and Linoleic Acid on the Growth of Human Cancer Cell Lines

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Abstract

The inhibitory effects of *doenjang* extracts and linoleic acid (LA) which was identified as one of the active compounds in *doenjang* on the growth of human cancer cells were studied, comparing to the actions on normal cells. Methanol extract and hexane fraction from *doenjang* exhibited the strong growth inhibitory effect on HT-29 human colon carcinoma cells. Inhibitory effects of chloroform, ethyl acetate, butanol and aqueous fractions on the cancer cells were observed, moderately or weakly. When cell counts of SNU-C_1 human colon carcinoma cells were determined daily for 6 days, the inhibitory effect of hexane fraction on this cell line was higher than that of the methanol extract from *doenjang*. LA completely suppressed the growth of SNU-C_1 cells after 4 days, while conjugated linoleic acid (CLA) resulted in 98% inhibition after 6 days. With the addition of LA and other free fatty acids such as stearic acid, oleic acid, linolenic acid and γ-linolenic acid (γ-LnA) to the culture system, the growth of HT-29 cells and SNU-C_1 cells was greatly suppressed after 6 days. Inhibitory effects of LA and γ-LnA on the growth of these cells were stronger than other fatty acids. On the growth of AZ-521 human gastric carcinoma cells, LA and CLA completely suppressed the growth of the cells after 4 days and 3 days, respectively. At the level of 0.001% ~0.01% of LA, there was no cytotoxic effect on normal rat kidney cells and normal intestine human cells. These results showed that LA, a major active compound of *doenjang*, had strong inhibitory effects on the growth of human cancer cells without damaging normal cells.

Key words: *doenjang*, linoleic acid, human cancer cells

INTRODUCTION

Doenjang (fermented Korean soy paste) is one of the most important fermented foods in Korea. A concern had been centered on *doenjang* because of the possible contamination of mycotoxins, particularly aflatoxin during the manufacturing process since *doenjang* is naturally fermented (1). Crane et al. (2) reported that the high incidence of stomach cancer in Koreans was probably due to the aflatoxin contaminated *doenjang*. However, the toxins were degraded almost completely during the long ripening period due to some factors such as NH_3 production, melanoidin color formation, charcoal addition, etc (3,4). *Doenjang* has already been demonstrated to exhibit antimutagenic and anticancer activities (5-13). Trypsin inhibitor, isoflavone, phytic acid, saponin, lignin, vitamin E and unsaturated fatty acid in *doenjang* are believed to be compounds which have antimutagenic and anticancer effects (14-18). β-Sitosterol-glycoside, genistein and linoleic acid were tentatively identified as active compounds in *doenjang* (19,20).

As the amount of triglycerides decreases, the level of free fatty acids, especially, linoleic acid increases during soybean fermentation (21). Several studies have shown that some fats rich in linoleic acid, a precursor of arachidonic acid and prostanoids, enhanced the carcinogenic effect of chemicals (22,23). High fat diets containing corn oil, safflower oil, beef fat or lard were reported to increase the chemically induced colon tumors, whereas fish oil diets rich in n-3 polyunsaturated fatty acids (PUFAs) such as EPA or DHA inhibited the development of colon tumors (24). However, it was reported that linoleic acid and its metabolites not only inhibited their growth but selectively killed human breast, lung and prostate cancer cells without damaging normal fibroblasts and animal kidney cells (25). Norman et al. (22) also reported that the essential fatty acids, linoleic acid, α-linolenic acid and several of their metabolites suppressed the proliferation rate of cancer cells. Ha et al. (26,27) reported that isomeric derivatives of linoleic acid which was isolated from grilled ground beef was effective in partially inhibiting the initiation of mouse epidermal carcinogenesis by 7,12-dimethylbenz(a)anthracene and forestomach tumorogenesis induced by benzo(a)pyrene.

Therefore, in this study, the inhibitory effects of *doenjang* extracts and linoleic acid (LA) which was identified as one of the active compounds in *doenjang* on the growth of human cancer cells were studied, compared to the actions on normal cells.

MATERIALS AND METHODS

Preparation of samples

Preparations of methanol extract from *doenjang*

Doenjang was obtained from Hwayoung Co. (Pusan, Ko-

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rea). The *doenjang* was fermented by *Aspergillus oryzae* and *Bacillus subtilis* according to traditional method. *Doenjang* was freeze dried, powdered and extracted with methanol (1:10 g/v), three times, by shaking for 8 hours. Each extract was separated by centrifugation at 10,000 rpm for 10 min. The methanol extract was evaporated using a rotary vacuum evaporator (Buchi RE 121, Switzerland), concentrated, then transferred to vials, and dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for the test.

Solvent fractionation of the methanol extract

Methanol extract of *doenjang* was fractionated by sequential extractions using hexane, chloroform, ethyl acetate, butanol. Each extraction was repeated for 3 times and dissolved in DMSO for the test.

Free fatty acids

Linoleic acid (LA), linolenic acid (LnA), γ-linolenic acid (γ-LnA), oleic acid (OA) and stearic acid (SA) of 99% purity were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). CLA (isomeric derivative of linoleic acid containing a conjugated double-bond system) was obtained from Dr. Y. L. Ha (Department of Agricultural Chemistry, Kyungsang National University) and dissolved in DMSO.

Growth inhibition test

Dulbeco's modified Eagle's medium (DMEM), fetal calf serum (FCS), 0.05% trypsin-0.02% EDTA, and 100units/ml penicillin-streptomycin were purchased from GIBCO Co. (Gaithersburg, MD, USA). CO_2 incubator (Sanyo, model MCO96, Japan) was used for cell culture. HT-29 human colon cancer cells and SNU-C_1 human colon carcinoma cells were obtained from Korea Cell Line Bank (Medical School, Seoul National University) and AZ-521 gastric cancer cells were obtained from Japanese Cancer Research Resources (JCRB)-Cell Bank (Tokyo, Japan). Normal rat kidney (NRK) and normal intestine human cells (NIH) were obtained from Dr. H.S. Kim (Medical School, Pusan National University). Cancer cells (HT-29, SNU-C_1 and AZ-521) and normal cells (NRK and NIH) were cultured in DMEM supplemented with 100 units/ml of penicillin-streptomycin and 10% FCS in 5% CO_2 incubator. Media were changed twice or three times every week. After six or seven days (eight or nine days), cultured cancer cells (or normal cells) were washed with phosphate buffered saline (PBS). The cells were harvested after trypsin-0.02% EDTA treatment followed by centrifugation. The cell suspension cells (2×10^4 cells/ml) were seeded in 24-well plates and incubated in 5% CO_2 incubator at 37°C for 24 hrs. The media supplemented with samples were changed every two days. In 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 (28).

RESULTS AND DISCUSSION

The methanol extract and various fractions from the methanol extract of *doenjang* inhibited the growth of HT-29 human colon cancer cells after 6 days of incubation (Fig. 1). Among the fractions of *doenjang*, hexane fraction showed the highest inhibitory effect on the growth of HT-29 cells, where 93%, 98% of inhibition were observed with the addition of 0.025%, 0.05%, respectively. We previously reported that haxane fraction of *doenjang* had the highest inhibitory effect on AFB_1-induced mutagenicity in *Salmonella typhimurium* strains of TA98 and TA100 (1). Linoleic acid (LA) was identified as one of the antimutagenic compounds from the hexane fraction of *doenjang* (19). *Doenjang* methanol extract showed the highest inhibitory effect on several different cancer cell lines among other soybean fermented foods and original raw materials (10). Park (8) indicated that the hexane extracts of *doenjang* inhibited the cyclin B_1 formation and synthesis of mRNA of cyclin B_1 in MCF-7 human breast carcinoma cells, suggesting the extract arrested the cell cycle progression. We also demonstrated that the solid tumor formation was significantly inhibited when 5 mg/kg of hexane, methanol and boiling extracts from *doenjang* were administered to the Balb/c mice (11).

To determine the inhibitory effect of LA on the growth of human cancer cells, the inhibitory effects of LA and active fraction of *doenjang* (methanol extract and hexane fraction) on the growth of SNU-C_1 human colon carcinoma cells were studied as compared to CLA (isomeric derivative of linoleic acid containing a conjugated double bond system) which has been recently reported as an anticancer and antitumor compound (26,27,29). The hexane fraction from the methanol extract of *doenjang* was more effective than the methanol extract from *doenjang* on the growth inhibition of SNU-C_1 cells (Fig. 2). LA completely suppressed the growth of SNU-C_1 cells after 4 days, while conjugated linoleic acid (CLA) caused 98% inhi-

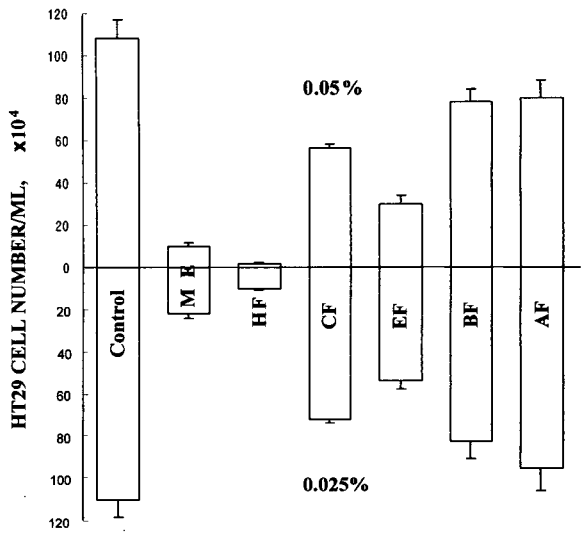

Fig. 1. Growth inhibitory effects of the extracted fractions from *doenjang* on the growth of HT-29 human colon carcinoma cells after 6 days of incubation (FCS 10%); ME (methanol extract), HF (hexane fraction), CF (chloroform fraction), EF (ethyl acetate fraction), BF (butanol fraction), AF (aqueous fraction).

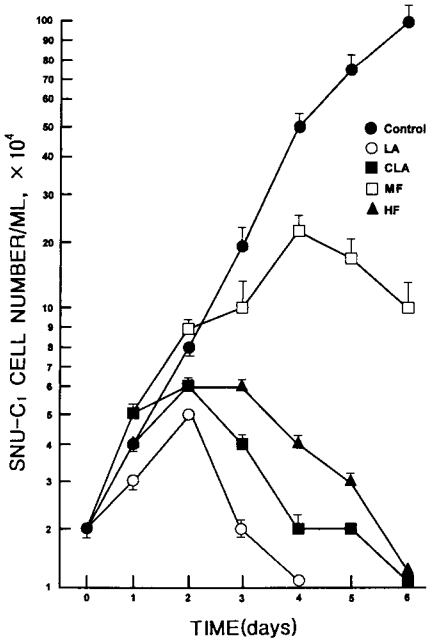

Fig. 2. Growth curves of SNU-C_1 human colon carcinoma cells in the presence of linoleic acid (LA), conjugated linoleic acid (CLA), methanol fraction (MF) and hexane fraction (HF) of *doenjang*.

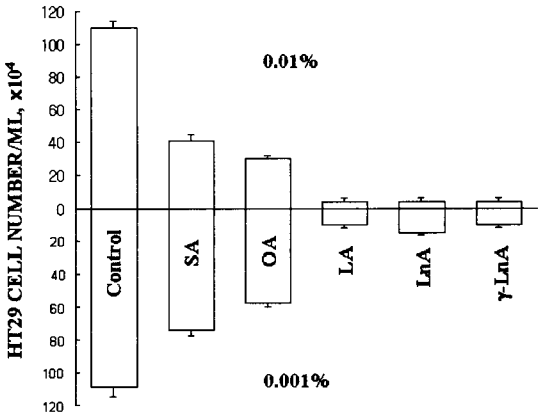

Fig. 3. Growth inhibitory effect of various free fatty acids on HT-29 human colon carcinoma cells after 6 days of incubation. The concentration of FCS used was 10%. SA: Stearic acid, OA: Oleic acid, LA: Linoleic acid, LnA: Linolenic acid, γ-LnA: γ-Linolenic acid.

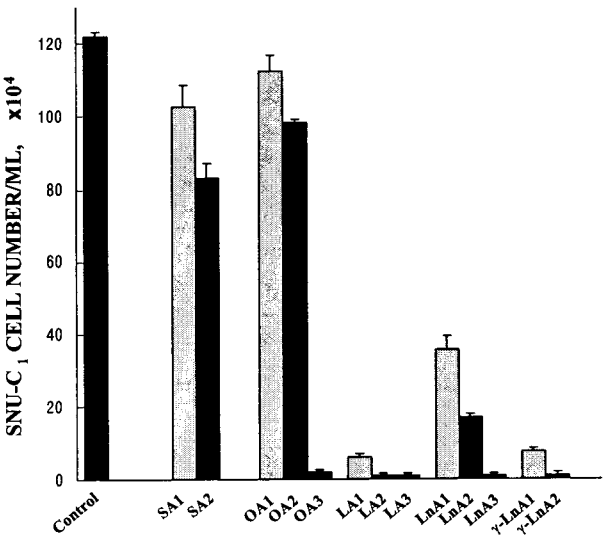

Fig. 4. Growth inhibitory effect of various free fatty acids on SNU-C_1 human colon carcinoma cells after 6 days of incubation (FCS 10%); SA (Stearic acid), OA (Oleic acid), LA (Linoleic acid), LnA (Linolenic acid), γ-LnA (γ-Linolenic acid). 1, 2 and 3 beside abbreviations of fatty acids are presented the concentrations of 0.001%, 0.01% and 0.1%, respectively.

bition after 6 days. These results showed that LA, a major active compound of the hexane fraction from *doenjang*, had a strong inhibitory effect on the growth of SNU-C_1 cells.

It has been reported that the content of free fatty acids increased by hydrolysis of triglycerides during the fermentation process of soybean *meju* for *doenjang* (30,31). The composition of the fatty acids in crude lipid of soaked soybean was 57.2% of LA, 20.9 of oleic acid and 7.3% of linolenic acid (30).

With the addition of LA and other free fatty acids such as stearic acid (SA), oleic aicd (OA), linolenic acid (LnA) and γ-linolenic acid (γ-LnA) to the cell culture system, the growth of the HT-29 cells was differently inhibited after 6 days of incubation (Fig. 3). LA, LnA and γ-LnA had stronger Inhibitory effects on the growth of HT-29 cells than SA and OA. Even at low concentration of 0.001%, LA and γ-LnA inhibited the growth of HT-29 cells more than 90%. LA, LnA and γ-LnA also exhibited inhibitory effects on the growth of SNU-C_1 colon cancer cells (Fig. 4). The treatment of 0.001% of LA and γ-LnA suppressed SNU-C_1 cells by 95% and 93%, respectively, however the viability of the cells was not as severely affected by SA and OA. The trends of inhibitory effects of LA and other free fatty acids on SNU-C_1 cells were similar to those on HT-29 cells.

The above data suggest that LA has strong inhibitory effects on the growth of human colon cancer cell lines. These resuts are in good agreement with a report indicating that LA inhibited the growth of malignant human colon adenocarcinoma cells (32). Nakahara (33) observed increases in the resistance of mice to several transplantable tumors following the injection of unsaturated fatty acid, such as OA, LA and LnA. Tolnai and Morgan (34) also showed that unsaturated fatty acid, especially LA and LnA had antitumor activity *in vitro* against three different mouse ascites tumors.

In order to confirm the growth inhibitory effects of the other human cancer cells by LA, LA and CLA were administered to the culture system of AZ-521 human gastric cancer cells. LA and CLA totally suppressed the growth of AZ-521 cells after 4 days and 3 days, respectively during 6 days of incubation at 37°C (Fig. 5). In a previous study (35), we observed the growth inhibitory effect and changes in membrane phospholipid fatty acid composition on MG-63 and AZ-521 human cancer cells by linoleic acid.

Zhu et al. (36) reported that LA significantly prolonged

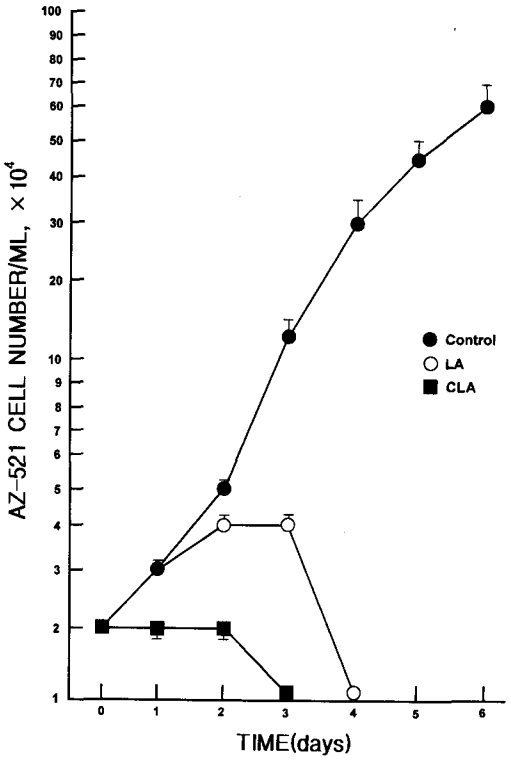

Fig. 5. Growth curves of human gastric cancer cells of AZ-521 in the presence of linoleic acid (LA) and conjugated linoleic acid (CLA).

the life span of Ehrlich ascites carcinoma-bearing mice and inhibited the growth of Ehrlich solid carcinoma in mice compared to the findings in untreated control mice. Besides, it was also reported that LA enhanced the phagocytic activity and NBT reduction of peritoneal phagocyte of mice (37).

In an effort to clarify whether LA was generally cytotoxic or whether its action was limited to the cancer cells, inhibitory effects of LA on the growth of normal cells such as normal rat kidney (NRK) cells and normal intestine human (NIH) cells were studied. As shown in Table 1, LA was not cytotoxic on NRK and NIH normal cells at the levels of 0.001~0.01%. Begin et al. (25) also reported that linoleic acid inhibited the growth of various cancer cells without damaging normal cells.

Table 1. Effect of various concentrations of linoleic acid on the growth of NRK normal cells (rat kidney fibroblast) and NIH normal cells after 6 days of incubation[1)]

	Number of cells ($\times 10^4$/ml)	
Concentration (%)	NRK	NIH
1.0	1±1	1±1
0.1	5±2	2±1
0.05	21±8	14±3
0.01	51±8	85±13
0.005	54±8	117±19
0.001	55±9	129±16
Control	64±7	139±14

[1)]The plated original cell number was 2×10^4/ml.

With these results, it can be concluded that *doenjang* extract decreased the growth of human cancer cells and the inhibitory effect of *doenjang* on human cancer cells was primarily due to the presence of free fatty acid of LA and LnA originated from the soybean fermentation. LA also showed strong inhibitory effects on the growth of several cancer cells. To elucidate the anticancer function of LA, further study on *in vivo* effects and its mechanism is needed.

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