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Selective Cytotoxic Effects of Doenjang (Korean Soybean Paste) Fermented with *Bacillus* Strains on Human Liver Cell Lines

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Abstract This report compares the selective cytotoxic effects of Doenjang fermented by various *Bacillus* strains (*Bacillus* sp. SS9, SSA3, and PM3) on human liver cell lines with that of conventional Doenjang (DTY, DTG, and DTK) and commercial Doenjang (DCM, DCD, and DCS). To investigate selective cytotoxic effects of Doenjang extracts, the cell density of HepG2 (Hepatocellular carcinoma) and CCL-13 (cells derived from human normal liver) was estimated after addition of the extracts by using a viable cell counting method. The maximum selectivity ratio (IC_{50} value against CCL-13/IC_{50} value aganist HepG2) was observed by PM3 (extracts of Doenjang fermented with *Bacillus* sp. PM3). As for morphological changes shown by the addition of PM3 into HepG2 and CCL-13 cultures, HepG2 was significantly disrupted, however, CCL-13 was not affected. Also, the growth rate of HepG2 was decreased significantly by the addition of PM3. Consequently, PM3 showed a more detrimental effect on HepG2 than that on CCL-13.

Key words: Doenjang, cytotoxicity, selectivity ratio

Doenjang is a traditional fermented soybean product and an important nutrient source in Korea. Many researchers have studied Doenjang in relation to its nutritional aspects, such as protein, lipid, and carbohydrate concentration. But now, many researchers are focusing on its health aspect after the knowledge of biological activity from various traditional foods, especially, Doenjang (Korean soybean paste). Thus, its extracts were studied in various physiological fields such as its anticancer [1], antimutagen [5, 15, 20], antioxidant [8, 11], antihypertension [6, 17, 21] properties and lowering of cholesterol [3, 4]. Among them, most of the study is focused on decreasing cancer occurrence and the growth of cancer cells. Several studies attributed the anticarcinogenic effect of soybeans to protease inhibitors [7, 18], a phenol compound [2, 14], phytic acid [15, 16], and soybean isoflavones. These biologically active materials may overcome the existing chemotherapy for cancer which cause harmful effects on cancer cells as well as normal cell.

However, we encountered some drawbacks for the research of the biological activity of Doenjang. Doenjang is a fermented product. Hence, many microorganisms, such as *Aspergillus*, *Penicillium*, *Bacillus*, and so on, engage in the process of fermentation [12], and each offers a different quality and biological activity. Therefore, we isolated *Bacillus* sp. PM3 from Doenjang, and *Bacillus* sp. SS9 and SSA3 from Ganjang (traditional Korean soy sauce). Each strain can individually ferment soybean and the resulting product, Doenjang, has a characteristic flavor depending on the fermenting strain. Doenjang was manufactured as follows: cooked soybean were precultured at 35°C for 3 days by each strain and 12% of NaCl in water was added into the precultured soybean. Then, it was fermented for 2 months at room temperature [unpublished data]. *Bacillus* sp. SSA3 produced novel pigments, which have inhibitory action on aflatoxin B_1 [9, 10] and human liver cancer cells (HepG2) [unpublished data].

Generally, the anticancer effect of biologically active materials is focused on the inhibition of cancer cells only. This causes the misreading of the results for *in vitro* anticancer effects because there may also be a probability of inhibiting non-carcinogenic cell growth. Therefore, it is important to investigate biologically materials which inhibit the growth of cancer cells selectively.

In this report, Doenjang was manufactured by a few *Bacillus* species, and their extracts were investigated for *in vitro* selective cytotoxic effects between HepG2 and CCL-13. The results were compared with those of traditional and commercial Doenjangs.

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MATERIALS AND METHODS

Preparation of Doenjang

Korean soybeans were fermented with different types of *Bacillus* strains (*Bacillus* sp. SS9 (SS9) SSA3, (SSA3), PM3 (PM3)). Traditional home-made Doenjangs were collected from Kuanmoondong, Yosu (DTY), Kwangmudong, Yosu (DTG), and Kongwhadong, Yosu (DTK), Korea. Commercial Doenjang (DCM, DCD, and DCS) was purchased from the A, B, C company. The words in parentheses are abbreviation of each extracts.

Preparation of Extracts

Doenjang was extracted with 10-fold volume of hot water (100°C) for 30 min. The extracts were filtered with Whatman No. 4 filter paper and each extract was centrifuged at 12,000 rpm for 15 min. The supernatant was dialyzed, lyophilized, and then kept at -20°C.

Cell Culture

Cell lines used were as follows: HepG2 (cells derived from Hepatocellular carcinoma, ATCC No. HB-8065) and CCL-13 (cells derived from normal human liver, ATCC No. CCL-13). Cells were grown as a monolayer in DMEM (Dulbecco's Modified Eagle's Medium, Sigma Co., U.S.A.) supplemented with 5% FBS (Fetal Bovine Serum, Gibco Co., U.S.A.). Cells were maintained in a 5% CO_2 incubator (VS-9011C, Vision Scientific Co., Korea) at 37°C.

Cytotoxicity Assay

Adherent cells were dispersed to single cells by treatment with trypsin-EDTA solution and were dropped into tissue culture dishes (10×35 mm, Falcon Co., U.S.A.). Each dish contained 1×10^5 cells in 2 ml medium and were cultured for 24 h. After removing medium, various concentrations of Doenjang extracts with fresh media were added into the culture dishes. They were incubated at 37°C with 5% CO_2 for 72 h. The experiment was carried out in duplicate. After 72 h incubation, the medium was removed from the dish and the surface of the dish was rinsed twice with PBS (phosphate buffered saline). Then, the cells were trypsinized, stained with 0.4% of trypan blue, and counted with a hemocytometer [19].

Morphology of Cells

To observe the morphology of HepG2 and CCL-13, 1×10^5 cells/ml were inoculated into a tissue culture dish and allowed to attach onto the dish for 24 h. Doenjang extract (*Bacillus* sp. PM3, 1 mg/ml) in culture medium was added into the dish. After 72 h incubation, the medium was removed. Cells were washed twice with PBS. To stain the cells, they were treated with PBS solution containing 0.5% crystal violet and 40% ethanol. After staining, cells were washed with PBS, and photographed using an automatic photomicrographic system (Olympus, PM-20, Japan).

Measurements of Cell Growth

Hepatocellular carcinoma (HepG2) and monotypic hepatocyte (CCL-13) were inoculated into tissue culture dishes with medium. Doenjang extracts were added into the culture dish and cells were incubated for 8 days with occasional medium changes (100% exchange with fresh medium containing extract). The growth of cells in normal medium and medium containing Doenjang extract was estimated by using a hemocytometer.

RESULTS AND DISCUSSION

Selective Cytotoxic Effects

Selective cytotoxic effects of Doenjang fermented with various *Bacillus* species on human liver cell lines were compared with that of conventional and commercial Doenjang. Figure 1 shows the cytotoxic effect of Doenjang (fermented with *Bacillus* sp.) extracts. In the case of HepG2, PM3 and SS9 showed higher inhibitory activity below

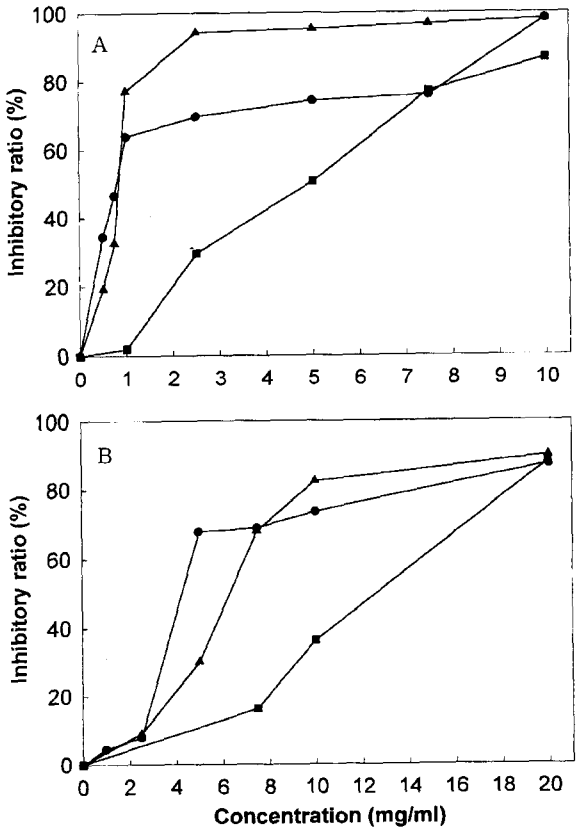

Fig. 1. Cytotoxic effect of Doenjang (fermented with *Bacillus* sp.) extracts on HepG2 (A) and CCL-13 (B).
▲, SS9; ■, SSA3; ●, PM3.

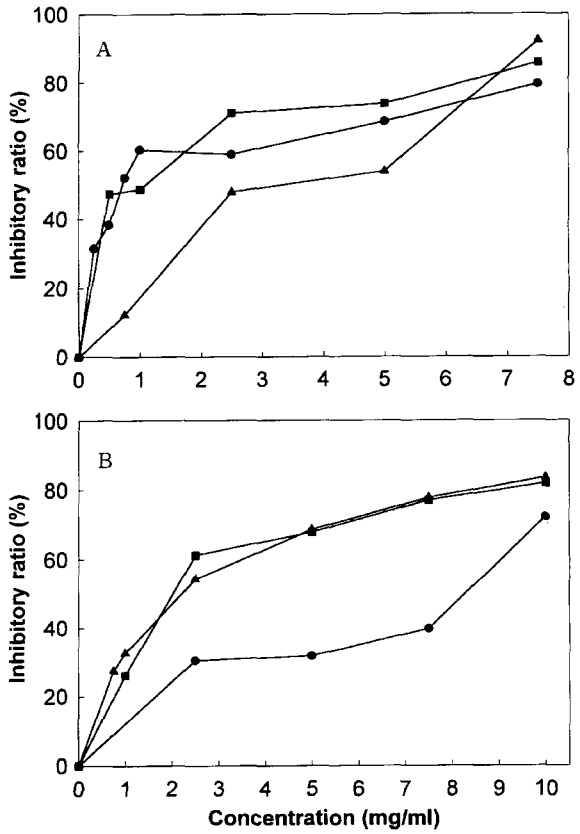

Fig. 2. Cytotoxic effect of conventional Doenjang extracts on HepG2 (A) and CCL-13 (B).
●, DTY; ▲, DTG; ■, DTK.

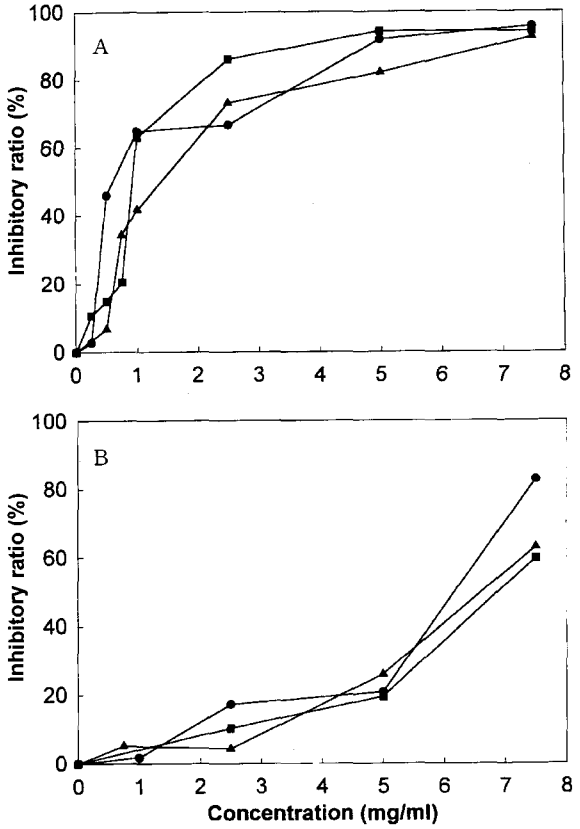

Fig. 3. Cytotoxic effect of commercial Doenjang extracts on HepG2 (A) and CCL-13 (B).
●, DCM; ▲, DCD; ■, DCS.

1 mg/ml. However, CCL-13 showed significant viability below 3 mg/ml. Consequently, extracts of Doenjang appeared to have selectivity between cancer and non-carcinogenic cells. This is very important, because defined material from any extract should have low toxicity for non-carcinogenic cells and high toxicity against cancer cells. Therefore, conventional Doenjangs (DTY, DTG, DTK) were collected for comparison with Doenjang fermented by various *Bacillus* strains.

The selective cytotoxic effect of conventional Doenjang (DTY, DTG, and DTK) extracts against HepG2 and CCL-13 is shown in Fig. 2. DTY and DTK also showed high inhibitory effects against HepG2 cells and low inhibitory effects against CCL-13. However, the cytotoxicity of conventional Doenjang extracts for CCL-13 was higher than that of SS9, SSA3, and PM3. This implies that selective cytotoxicity of Doenjang fermented with *Bacillus* strains for cancer cells is higher than that of conventional Doenjang. We also investigated the cytotoxic effect of commercial Doenjang (DCM, DCD, DCS) against HepG2 and CCL-13 (Fig. 3). At 2 mg/ml of DCM, DCD, and DCS 60-80% inhibitory effects were observed on HepG2, but the inhibitory effect on CCL-13 was below 13%.

Overall results are presented with IC_{50} values in Table 1. IC_{50} was defined as the concentration of the extracts required to inhibit cell viability by 50%. Also, selectivity

Table 1. Anticancer activity and selectivity ratio of various Doenjang extracts.

Cell line	extract	Cytotoxicity (IC_{50}, mg/ml)								
		Doenjang fermented with *Bacillus* strains			Conventional Doenjang			Commercial Doenjang		
		SS9	SSA3	PM3	DTY	DTG	DTK	DCM	DCD	DCS
HepG2	Hot-water extract	0.8	5.0	0.2	0.7	3.2	1.1	0.6	1.4	0.9
CCL-13	Hot-water extract	6.1	12.7	4.1	8.2	2.1	1.9	6.1	6.7	6.9
Selectivity ratio[a]		7.6	2.5	20.5	11.7	-	1.7	10.2	4.8	7.7

[a]Selectivity ratio=CCL-13 IC_{50}/Hep G2 IC_{50}.

ratio was estimated by the ratio of the IC_{50} value against CCL-13 versus the IC_{50} value against HepG2. In this result, PM3 showed the highest selective cytotoxicity among them. This means that a certain material in PM3 may offer a possibility to selectively kill cancer cells. Lim *et al.* [12] reported that the concentration of linoleic acid required to inhibit non-carcinogenic cell growth by 50% was 0.05%, whereas that of linoleic acid required to inhibit cancerous cell growth by 50% was 0.005% (selectivity ratio about 10). In our result, PM3 and DCM showed higher selectivity ratios than 10. Consequently, Doenjang fermented with *Bacillus* sp. PM3 appeared to be a good biologically active food for human diet.

Changes of Morphology

The morphological change of cells in normal medium and medium containing PM3 was observed with an inverted microscope. PM3 (1 mg/ml) was added to HepG2 and CCL-13 cells. In the case of CCL-13, PM3 did not show cell disruption (Fig. 5). However, HepG2 cells did not grow and thoroughly lysed (Fig. 4). We cannot explain the reason of selective cytotoxicity for cancer cells. However, the reason might be due to apoptosis or membrane disruption. So, apoptosis was examined for PM3, but no significant result was observed (data not shown). Further work on membrane disruption and other changes are in progress.

Effect on Cell Growth

To know the adaptation of cancer cells to PM3, continuous cell growth was investigated under the medium containing PM3. Growth curves of the two cell lines are shown in Fig. 6. The growth of CCL-13 in normal medium is similar to the growth of CCL-13 in medium containing PM3. However, the growth of HepG2 in medium containing PM3 was significantly inhibited. Until the 6th day, HepG2 cell showed a lag phase. It indicates that PM3 did not cause adaptation of cancer cells. In addition to the above results, further study will be focused on the identification of the active compound and structure from PM3 and also on the mechanism of the selective anticancer effect.

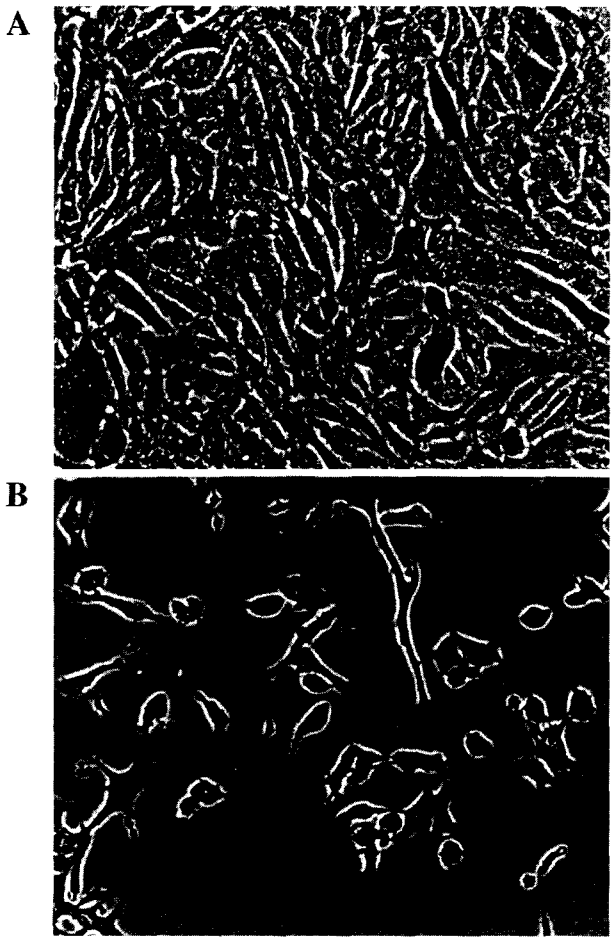

Fig. 4. Morphology of HepG2 grown in normal medium (A) and in medium containing PM3 (1 mg/ml, B).

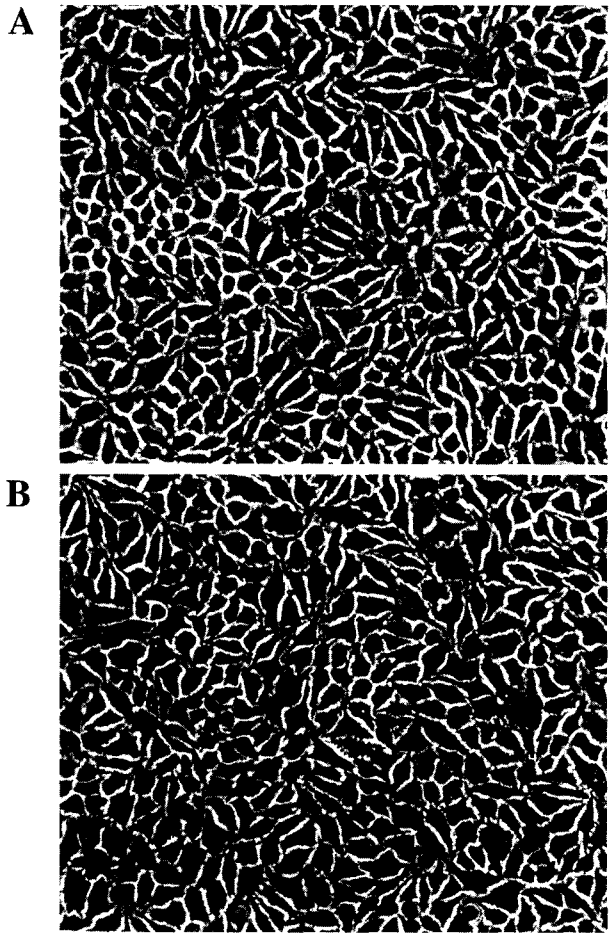

Fig. 5. Morphology of CCL-13 grown in normal medium (A) and in medium containing PM3 (1 mg/ml, B).

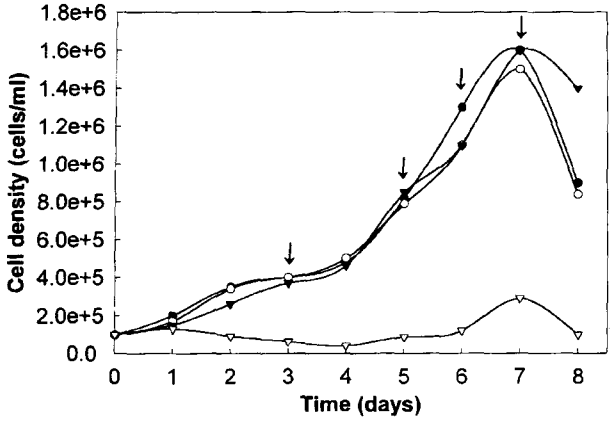

Fig. 6. Effects of Doenjang extracts on the growth of CCL-13 and HepG2 cell lines.
●, CCL-13 grown in normal medium; ○, CCL-13 grown in medium containing PM3 (1 mg/ml); ▼, HepG2 grown in normal medium; ▽, HepG2 grown in medium containing PM3 (1 mg/ml); ↓, Changed with fresh medium.

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