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Ethanol Extract of Fermented Soybean, Chungkookjang, Inhibits the Apoptosis of Mouse Spleen, and Thymus Cells

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Apoptosis is a step of the cell cycle which is important in the regulation of immune cell populations. Chungkookjang is a Korean traditional fermented soybean containing microorganisms, enzymes, and bioactive compounds which was used in the treatment of mouse spleen as well as thymus cells (CH1-fermented soybean containing barley, wormwood, and sea tangle; CH2-fermented soybean) and was found to exhibit substantially reduced small DNA fragmentation. An MTT assay showed that the treatment of CH1 and CH2 into the mouse splenocytes and thymocytes sharply increased their survival. Moreover, a FACS analysis also showed that CH1 and CH2 are effective at suppressing the apoptosis of splenocytes and thymocytes. The fermented soybean isoflavone concentrations, which are implicated in lowering breast and prostate cancers, lowering the risk of cardiovascular diseases, and improving bone health, were determined using Capillary Electrophoresis-Electrochemical Detection (CE-ED). The amount of Daidzein in fermented soybean significantly increased by 44-fold dramatically, compared with those in unfermented soybean. In this study, we demonstrated that ethanol extracts of Chungkookjang promote the survival of the mouse spleen and thymus cells in culture by suppressing their apoptotic death. Future studies should investigate which genes are related to apoptosis of the immune cells.

Keywords: Chungkookjang, fermented soybean, daidzein, genistein, CE, apoptosis

Cells of the immune system are in the dynamic process of proliferation and death in the development by various stimulations. Apoptosis is programmed cell death which occurs either via the TNF receptor or the mitochondria (Kissil *et al.*, 1999; Aggarwal, 2003; Newmeyer and Ferguson-Miller, 2003). Caspases are activated in apoptosis and cleave essential proteins, leading to cell death and DNA fragmentation (Kissil *et al.*, 1999; Aggarwal, 2003; Newmeyer and Ferguson-Miller, 2003).

Apoptosis is important in the regulation of various types of immune cell populations which affect the pathogenesis of inflammation, cancer, and autoimmune diseases. Some natural products including plant carbohydrates are reported to modulate the apoptosis of immune cells (Hwang *et al.*, 2003, 2005; Ji *et al.*, 2005).

Chungkookjang is a Korean traditional fermentation soybean containing microorganisms, enzymes, and diverse bioactive compounds, which are absent in unfermented soybean (Lee *et al.*, 1999). Chungkookjang is also called Natto, Tempeh, and Douchi in other Asian regions and can be prepared in a short period without using sodium chloride, which is different from the preparation of other Korean fermented soybean pastes. Excess salt in Korean diets may be associated with a high incidence of high blood pressure and stomach cancer. With this in mind, it is desirable to eat Chungkookjang which does not contain sodium chloride.

The different types of isoflavones in fermented soybean include enistein, glycetin, and daidzein and are implicated in lowering breast cancer, prostate cancer, lowering the risk of cardiovascular diseases, and improving bone health (Lee *et al.*, 1999). Chungkookjang is rich in antioxidants which may prevent oxidative injury; hence, preventing lifestyle related diseases (Lee *et al.*, 1999). Also, fermented soybean is regarded to be a healthy food, since it improves blood circulation and intestine function. However, few studies exist on the effect of fermented soybean on immune cells. In this study, we test whether ethanol extracts of Chungkookjang promote the survival of the mouse spleen and thymus cells in culture by suppressing apoptotic death of the cells. This would be the first report that fermented soybean extracts can modulate the longevity of immune cells.

Materials and Methods

Mouse and chemicals

Balb/c mice were purchased from the Korea Experimental Animal Center (Korea) and were used at 6 weeks of age. The mice were housed in polyethylene cages containing clean wood shavings and were given tap water and rodent food *ad libitum*. Most of the cell culture reagents and chemicals were purchased from the Sigma Chemical Co. (USA) except for the RNA gents Total RNA Isolation

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System which was purchased from Promega (USA).

Preparation of Chungkookjang extract

Soybean was soaked in water for 18 h, followed by autoclaving at 120°C for 30 min. The *Bacillus licheniformis* B1 culture was added onto 500 g of autoclaved soybeans, which was calculated to be a 1%. Chungkookjang fermentation was achieved by incubation at 40°C for 3 days (Lee *et al.*, 1999). The preparation of CH1 consisted of powder of barley, wormwood, sea tangle which were mixed with soybean at ratios of 7, 2.5, 0.5, 90%, respectively. In contrast, CH2 contained soybean only. Both preparations were fermented by the same strain (Yoo *et al.*, 2004).

Ethanol extraction

The powder of fermented soybean was prepared by freeze-drying (Yoo *et al.*, 2004) and was suspended in 80% ethanol (10 g/100 ml) and stored at 25°C for 24 h. The preparation was centrifuged at 4°C and 15,000×g for 30 min. The supernatant was subsequently evaporated in a vacuum and the resulting residue was freeze-dried to be made into a powder form.

Effects of CH1 and CH2 on the mouse immune cells in culture

Mouse thymocytes and splenocytes were collected from the thymus and spleen of 6 week-old female BALB/c mice. Further, the cells were incubated for 4 h in a HY/10% FBS medium supplemented with 1 mg/ml of CH1 or CH2 extract, which represents the extracts from the mixed or pure fermented soybean, respectively. Each control contained the medium plus 0.8% ethanol. The degree of apoptotic death was determined by DNA fragmentation, MTT reduction and FACS studies. The DNA fragmentation assay was performed as follows: Following the treatment with the fraction, the cell pellets were treated with solution A (10 mM EDTA, 0.5% SDS, 50 mM Tris-HCl; pH 8.0, 0.5 μg/ml proteinase K) for an hour at 50°C to dissolve the cells. After further incubation in working solution B, (10 μg/ml RNase A/50 mM Tris, pH 8.0) for an hour at 37°C to remove RNA, the samples were loaded directly onto a 1% agarose gel and the degrees of apoptosis were compared by the band intensity of small DNA fragments, which represents the extent of apoptotic degradation in chromosomal DNA. The TACS MTT Assay (R&D systems, USA) was also performed to determine the amount of live cells in the cultures by measuring the formation of formazan dye crystal according to the methods outlined by the manufacturer. Following the incubation of the cells with their extracts, the cells were treated with the MTT Reagent until the appearance of a purple precipitate. The precipitates were in turn, dissolved in the Detergent Reagent and the absorbance was measured at 595 nm to determine the degree of MTT reduction, which represents the amount of live cells. The FACS analysis was performed using annexinV and propidium iodide according to the manufacturer (BD ApoAlert AnnexinV-FITC kit, BD Biosciences, USA). After treating 1×10^6 cells with the extracts, the cells were rinsed twice with 1× binding buffer as well as staining with annexinV and propidium iodide. Following the staining procedure, the cells were analyzed using a Becton-Dickinson FACSCalibur single laser cytometer (BD, USA). The cultures treated with ethanol as vehicle were the negative controls in all experiments. The vehicle as well as the CH1 and CH2 fractions supplemented in this study, were pre-tested for the presence of bacterial endotoxins by the gel clot assay using Limulus Amebocyte Lysate as described by the manufacturer (Cape Cod Inc., USA) to confirm their endotoxin-free states. All experiments were duplicated at least once and the results were statistically evaluated using the Student's *t*-test. Statistical significance was set a priori *at* $p<0.05$.

Reagent and solution for CE

The stock solutions of daidzein and genistein (1 mg/ml) remained stable for at least a month and were prepared in ethanol and were kept at 4°C. The running buffer was 50 mM borate buffer (pH 9.2) and the stock solutions were diluted to the desired concentration with the running buffer prior to use.

Apparatus for CE

A±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China) was used as a separation voltage between the two ends of the capillary. The inlet of the capillary was positively charged, and the outlet of capillary in the detection cell was the ground. The separations were carried out in a 50 cm length of 25 μm i.d. and 360 μm o.d. fused silica capillary (Polymicro Technologies, USA).

A three-electrode electrochemical cell consisted of a laboratory-made 300 μm diameter carbon disc working electrode, a platinum auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode. The cell was used with a BAS LC-4C amperometric detector (Bioanalytical Systems Inc., USA). The detector filter was set at 0.1 Hz and the working electrode was carefully positioned opposite the outlet of the capillary with the aid of a micromanipulator (CORRECT, Japan) and arranged in a wall-jet configuration. The distance between the tip of the working electrode and the capillary outlet was adjusted to -50 μm opposed to the bore (25 μm) in the capillary, while being viewed under a microscope. The electropherograms were recorded using a LKB·REC 1 chart record (Pharmacia, Sweden) and a YS 38-1000 220 V alternate constant-voltage power supply (Shanghai Instrumental Transformer Factory, China) was employed to suppress the voltage fluctuation of the power line. The whole system was assembled in a laboratory at a constant temperature of 25°C.

Sample preparation for CE

Approximately 1.0 g of the Chungkookjang, CH2 powder was weighed and dispersed in 10 ml of 70% ethanol. The mixture was kept in a 60°C waterbath for 3 h. After cooling, the mixture was sonicated for 30 min and filtered through a filter paper. The extract was diluted using 50 mM borate buffer (pH 9.2) at a ratio of 20:1 prior to CE analysis. Because the electrokinetic injection method was used, both the stock solution and the extract of fermented soybean were diluted in running buffer to minimize the difference between the running buffer and the sample solution.

Procedures for CE

Prior to use, the carbon disc electrode was polished with emery paper and alumina powder as well as being sonicated in doubly distilled water. The capillaries used for the separation were treated before use by flushing with 0.1 M NaOH and doubly distilled water for 10 min each. Subsequently, the capillary was filled and conditioned with the running buffer for at least 10 min at a separation voltage of 15 kV between the two ends of the capillary. Moreover, the potential applied to the working electrode was +0.90 V (versus SCE). Before injection, both the anode end of the capillary and the platinum-wire anode were moved from the anode solution to the sample solution. After an injection voltage of 15 kV was applied between the two ends of the capillary for 6 s, the sample solution was introduced into the capillary. Moreover, the anode end of the capillary together with the anode was then quickly returned to the anode solution. Furthermore, a voltage of 15 kV was applied to the constant-voltage mode for CE separation. The amperometric detector was on during the injection procedures. In addition, the cathode solution in the electrochemical detection cell, the anode solution, and the sample solution were all at the same level. Moreover, the sample solutions, standard solutions, and running buffer were all filtered through a polypropylene filter (0.22 μm, Shanghai Bandao Industry Co., Ltd., China) prior to their use. The peak identification was performed by the standard-addition method.

Results

DNA fragmentation and the survival of immune cells

DNA fragmentation indicates the apoptotic death of thymocytes in culture. As shown in Fig. 1, the cells from the control culture (0.8% ethanol treatment) showed a small DNA fragment ladder, indicating that many of the cells were dying due to apoptosis. The culture treated with CH1 and CH2, however, exhibited substantially reduced small DNA fragmentation compared to the control culture. Also, it is noteworthy that a high molecular mass of DNA was conspicuously reduced in CH1 and CH2 treated cells, which indicate that CH1 and CH2 suppressed the apoptotic death of the cultured cells.

On day 6 of the culture supplemented with CH2 (1 mg/ml fermented soybean extracts), 75% of the mouse thymocytes and 61% of the mouse splenocytes were alive (Fig. 2). In contrast, only 31% of the thymocytes and 27% of the splenocytes were alive in the control culture without the extracts (Fig. 2). This demonstrates that the supplementation of cultures with fermented soybean extracts significantly increased the survival of cells.

MTT assay

The amount of live cells in the cultures was determined by measuring the formation of formazan dye crystal. Formazan is produced by the mitochondrial respiratory chain enzyme, succinate dehydrogenase, and the higher OD value indicates increased survival of cells. In thymocytes, the OD values of control (0.8% ethanol), CH1 (1 mg/ml), $CH2_1$ (1 mg/ml) were: 0.09±0.02, 0.43±0.08, 0.59±0.04, respectively (Fig. 3A). In splenocytes, the OD values of control, CH1 (1 mg/ml), $CH2_1$ (1 mg/ml) were: 0.04±0.01, 0.34±0.04, 0.55±0.02, respectively (Fig. 3B). For both splenocytes and thymocytes, we found a significant difference between control and the extract-treated cultures in the MTT reductions ($p<0.01$). Also, the treatment of both thymocytes and splenocytes with CH2, increased cellular growth proportional to the supplemented amount of fermented soybean extract (Fig. 3A and B).

FACS analysis

AnnexinV staining represents the apoptotic membrane disruption in the FACS analysis. Previous reports have demonstrated that the lower the mean fluorescence value is, the lower the apoptosis rate (Eray *et al.*, 2001). In thymocytes, the fluorescent level of the control (0.8% ethanol), CH1

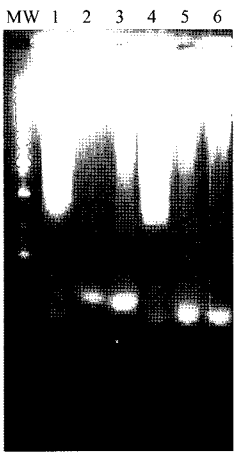

Fig. 1. The CH1 and CH2 extracts reduce the DNA fragmentation of the mouse thymocytes (lanes 1, 2, and 3) and splenocytes (lanes 4, 5, and 6) in culture. Lanes 2 and 5 represent the DNA fragmentations of the CH1 treated cultures, while lanes 3 and 6 represent those of the CH2 treated cultures. Lanes 1 and 4 represent controls. MW represents the DNA fragments from the *Hin*dIII digested λ DNA.

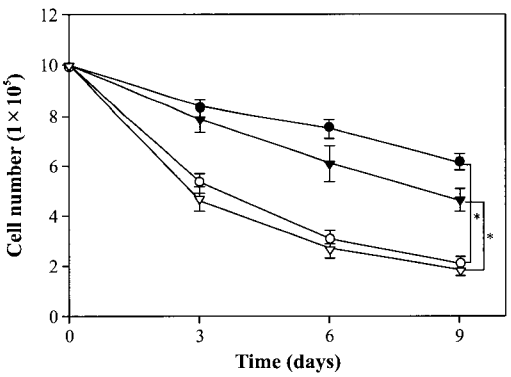

Fig. 2. Survival of the mouse thymocytes and splenocytes. The mouse thymocytes with (●) and without (○) CH2 fermented soybean extracts, and the mouse splenocytes with (▽) and without (▼) CH2 fermented soybean extracts, were cultured. The viable cells were counted in each culture. Significant decreases (*, $p<0.02$) were observed.

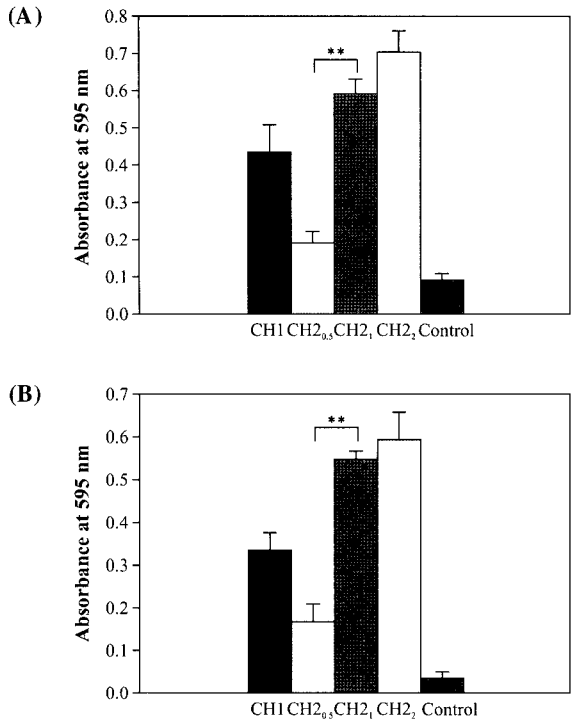

Fig. 3. MTT assay. The CH1 and CH2 extracts increase the MTT reduction of mouse thymocytes (A) and splenocytes (B) in culture. CH1, $CH2_{0.5}$, $CH2_1$, and $CH2_2$ contain 1, 0.5, 1, 2 mg/ml fermented soybean extracts, respectively. Significant increases (**, $p<0.01$) were observed.

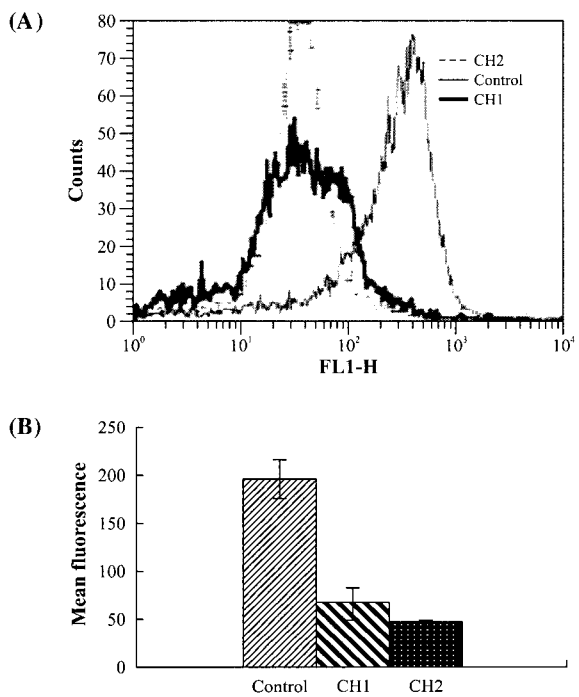

Fig. 4. FACS analysis of the mouse thymocytes treated with the CH1 and CH2 extracts using the annexinV specific antibody. Both extracts reduce the mean fluorescence of annexinV staining, indicating the reduction of apoptosis of cells in culture.

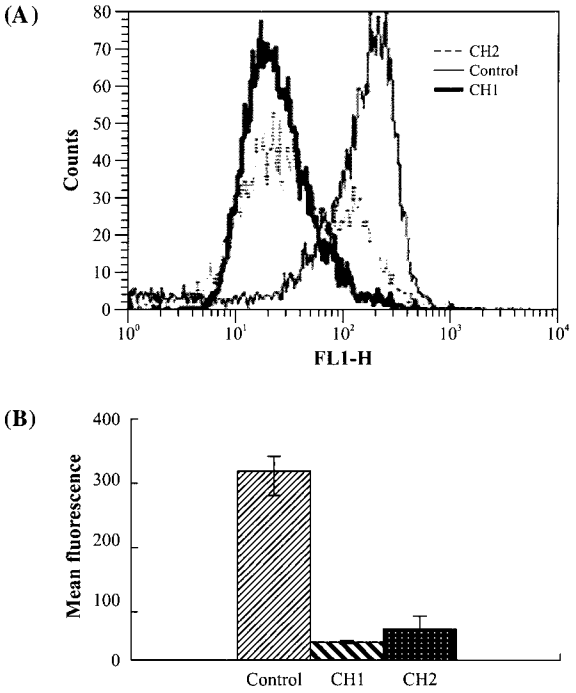

Fig. 5. FACS analysis of the mouse splenocytes with the CH1 and CH2 extracts using the annexinV specific antibody. Both extracts reduce the mean fluorescence of annexinV staining, indicating the reduction of the apoptosis of cells in culture.

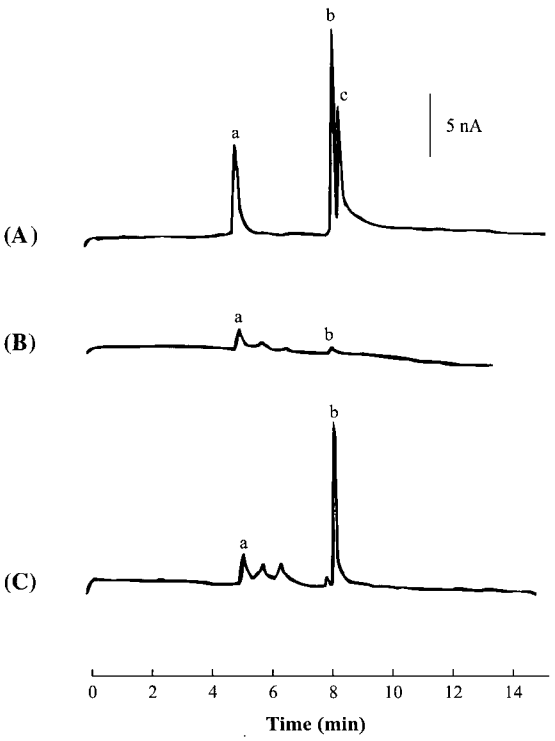

Fig. 6. (A) Electropherogram of a mixture containing 0.05 mg/ml of genistin (a), daidzein (b), and genistein (c) and typical electropherograms from a diluted extract of a sample of soybean (b) and a sample of fermented soybean (c).

Table 1. Results from assay of genistein and daidzein in soybean and fermented soybean (n=3, mg/g)[a]

Sample	Genistin	Daidzein	Genistein
Soybean	1.12 (±0.03)	0.20 (±0.01)	0
Fermented soybean	1.62 (±0.06)	8.76 (±0.36)	0

[a] Working potential is +0.90 V (versus SCE).
The data are expressed as the Mean ± Standard Deviation.

and CH2 were significantly different (192±21, 63±12, 42±2, respectively) (p<0.005) (Fig. 4). In addition, the ratio of CH1/control was 33%, whereas the CH2/control ratio was 22% (Fig. 4). In splenocytes, the fluorescent level of the control, CH1, CH2 was significantly different (317±28, 33±3, 60±21, respectively (p<0.005) (Fig. 5). Moreover, the ratio of CH1/control was 10% whereas the ratio CH2/control was 19% (Fig. 5). Thus, CH1 and CH2 are more effective in suppressing apoptosis in splenocytes than in thymocytes.

Determination of isoflavones

HPLC is a routine method in the analysis of food samples. However, it has a long analysis time, low resolution and short column lifetime due to numerous co-existent interferences. Consequently, capillary electrophoresis (CE) emerges as an important analytical separation technique because of its short analysis time, reproducibility, ultra-small sample volume, and a minimal solvent requirement (Peng and Ye, 2006). The combination of CE with electrochemical detection (ED) is highly sensitive and well selective for its electro-active constituents. CE-ED is primarily applicable to electro-active species, because the hydroxyl group in isoflavones can be readily electrochemically oxidized and can be used to determine isoflavone presence in fermented soybeans.

The amount of Daidzein (8.76±0.36 mg/g) increased by a factor of 44 in fermented soybean, compared to unfermented soybean (0.20±0.09 mg/g) (Table 1 and Fig. 6). In contrast an increase in genistein was not observed in fermented soybean (Table 1 and Fig. 6).

Discussion

Spleenocytes and thymocytes easily die in cell culture due to apoptosis. The number of live cells in culture did not increase after the addition of ethanol extract of fermented soybean from the beginning (Fig. 2). This suggests that the fraction does not have a mitogenic effect and the immune cells treated with fermented soybean extracts have a better survival rate (Fig. 2). In the MTT assay, cellular growth was proportional to the concentrations of the extracts (Fig. 3). In summary, this suggests the extracts indeed to interact with immune cells to suppress apoptotic cell death.

The question herein lies in which component of Chungkookjang, fermented soybean prevents apoptotic death of mouse spleen and thymus cells. Most of studies looked at the effect of oligosaccharides such as glucan on the apoptosis of immune cells (Hwang *et al.*, 2003, 2005; Ji *et al.*, 2005; Kim *et al.*, 2006). Enzymes of microorganisms such as β-glucanase in Chungkookjang can convert polysaccharide into oligosaccharides (Tang *et al.*, 2006). Thus, Chungkookjang may also contain oligosaccharides. It is likely that oligosacharides are more soluble in water than in ethanol. In this study, an 80% ethanol extract was used. The ethanol extract of fermented soybean is expected to contain genistein and daidzein, since isoflavones are soluble in ethanol and oligosaccharides are generally not soluble in ethanol (data not shown). As predicted, we found Chungkookjang to contain a high concentration of daidzein (Table 1 and Fig. 6). In contrast, it seems that genistein may be degraded during fermentation, since the concentration was not significantly greater than control levels. This is the first documentation of the determination of isoflavones in fermented soybean using CE-ED (capillary electrophoresis with electrochemical detection). This isoflavone may play a more important role than oligosaccarides.

Few studies exist looking at the effects of genistein and daidzein on the apoptosis of immune cells. The isoflavones may change cellular signal transduction (Aggarwal and Shishodia, 2006) and affect the apoptosis of immune cells. Usually, a high concentration of bioactive compounds was used to observe the effects. However, in this study whole extracts containing daidzein were used, which better reflects natural and physiological conditions. It is undocumented to be able to detect the immunomodulation effect of fermented soybean extracts at a physiological concentration.

Using DNA fragmentation, MTT assay, FACS analysis, we clearly demonstrated Changkookjang and its mixture to suppress the apoptosis of mouse spleen and thymus cells. Barley, wormwood, and sea tangle were added into the fermented soybean to improve flavor and taste of the fermented soybean. In the MTT assay, CH1 (fermented soybean containing barley, wormwood, and sea tangle) and CH2 (fermented soybean) showed no significant difference. However, the results of the FACS analysis of thymocytes demonstrate that CH2 is more effective at inhibiting apoptosis than CH1. In the analysis of spleenocytes we found CH1 to be more effective at suppressing apoptosis than CH2. This suggests that other materials aside from soybean may affect the apoptosis rate, depending on immune cell types. A high concentration of genistein (>20 μM) inhibits the growth of mammalian cells, whereas a low concentration stimulates their growth (Hsieh *et al.*, 1998). Furthermore, a high concentration may increase apoptosis rate by cellular toxicity. Daidzein concentration (0.32 μM) in fermented soybean is likely to contribute to cellular proliferation by inhibiting apoptosis.

To the best four knowledge, this is the first report mentioning Chungkookjang having a role in the immunomodulation of apoptosis in immune cells. Chungkookjang may relieve some disease caused by excessive apoptotic cell death.

FAS is well-known in the regulation of apoptosis (Nagata and Golstein, 1995). Down-regulations of cyclin-dependent kinase inhibitor (Ji *et al.*, 2005), up-regulation of the apop-

tosis inhibitor (Api5) and down regulation of death associated protein3 (Dap3) were also reproted. (Kim *et al.*, 2006). Future studies should investigate which genes are involved in the apoptosis of immune cells, when treated with fermented soybean, using the DNA chip analysis.

References

Aggarwal, B.B. 2003. Signaling pathways of the TNF superfamily: A double-edged sword. *Nat. Rev. Immunol.* 3, 745-756.

Aggarwal, B.B. and S. Shishodia. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.* 71, 1397-1421.

Eray, M., M. Matto, M. Kaartinen, L.C. Andersson, and J. Pelkonen. 2001. Flow cytometric analysis of apoptotic subpopulations with a combination of Annexin V-FITC, promium iodide, and SYTO 17. *Cytometry* 43, 134-142.

Hsieh, C.Y., R.C. Santell, S.Z. Haslam, and W.G. Helferich. 1998. Estrogen effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. *Cancer Res.* 58, 3833-3838.

Hwang, J.S., H.K. Chung, E.K. Bae, A.Y. Lee, H.J. Ji, D.W. Park, H.J. Jung, C.W. Cho, H.J. Choi, D.S. Lee, K.R. Lee, and H.J. Youn. 2003. The polysaccharide fraction AIP1 from *Artemisia iwayomogi* suppresses apoptotic death of the mouse spleen cells in culture. *Arch. Pharm. Res.* 26, 294-300.

Hwang, J.S., H.J. Ji, K.A. Koo, N.H. Lee, H.K. Yeo, S.W. Cheong, J.H. Park, G.S. Oh, C.S. Yoon, and H.Y. Youn. 2005. AIP1, a water-soluble fraction from *Artemisia iwayomogi*, suppresses thymocytes apoptosis in vitro and down-regulates the expression of Fas gene. *Biol. Pharm. Bull.* 28, 921-924.

Ji, H.J., H.K. Yeo, N.H. Lee, J.S. Hwang, K.A. Koo, S.W. Cheong, J.H. Park, G.S. Oh, C.S. Yoon, and H.Y. Youn. 2005. A carbohydrate fraction, AIP1, from *Artemisia iwayomogi* down-regulates Fas gene expression and suppresses apoptotic death of the thymocytes induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biotechnol. Lett.* 27, 253-257.

Kissil, J.L., O. Cohen, T. Raveh, and A. Kimchi. 1999. Structure-function analysis of an evolutionary conserved protein DAP3, which mediates TNF-alpha- and Fas-induced cell death. *EMBO J.* 18, 353-362.

Kim, K.H., Y.W. Kim, H.B. Kim, B.J. Lee, and D.S. Lee. 2006. Anti-apoptotic activity of laminarin polysaccharides and their enzymatically hydrolyzed oligosaccharides from *Laminaria japonica*. *Biotechnol. Lett.* 28, 439-446.

Lee, J.J., D.S. Lee, and H.B. Kim. 1999. Fermentation patterns of Chungkookjang and Kanjang by *Bacillus licheniformis* B1. *Kor. J. Microbiol.* 35, 296-301.

Nagata, S. and P. Golstein. 1995. The Fas death factor. *Science* 267, 1449-1456.

Newmeyer, D.D. and S. Ferguson-Miller. 2003. Mitochondria: Releasing power for life and unleashing the machineries for death. *Cell* 112, 481-490.

Peng, Y.Y. and J.N. Ye. 2006. Determination of isoflavones in red clover by capillary electrophoresis with electrochemical detection. *Fitoterapia* 77, 171-178.

Tang, D., J.H. Wang, Y. Fan, Y.L. Yang, Z.G. Tian, J. Luo, G.P. Yang, and F. Zhang. 2006. Cloning of β-1,3-1,4-glucanase gene from *Bacillus licheniformis* EGW039 (CGMCC0635) and its expression in *Escherichia coli* BL21(DE3). *Appl. Microbiol. Biotechnol.* 72, 705-712.

Yoo, H.J., D.S. Lee, and H.B. Kim. 2004. Chungkookjang fermentation of mixture of barley, wormwood, sea tangle, and soybean. *Kor. J. Microbiol.* 40, 49-53.