Food Sci. Technol. Res., **11** (3), 311–317, 2005

Apoptosis Induction by Vinegar Produced from Boiled Extract of Black Soybeans in Human Monoblastic Leukemia U937 Cells: Difference in Sensitivity to Cell Toxicity Compared to Normal Lymphocytes

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Received April 4, 2005; Accepted September 29, 2005

The effect of vinegar produced from the boiled extract of black soybeans on apoptosis induction in human monoblastic leukemia U937 cells was investigated. The boiled extract of black soybeans obtained from a natto (traditional Japanese fermented soybean product) factory was employed as a novel raw material for vinegar production. An ethyl acetate-soluble fraction of this vinegar (EBV fraction) was shown to strongly suppress cell proliferation of U937 cells. Fragmentation of DNA and morphological changes showing apoptotic bodies, which are characteristic of apoptotic cell death, were observed in U937 cells treated with 10 mg/ml of the EBV fraction. Flow cytometry analysis using an Annexin-V-FLUOS Staining Kit confirmed that cell death was due to apoptosis. On the other hand, the cell viability of lymphocytes separated from human blood was less affected by the EBV fraction, and fragmentation of DNA was not induced. These results suggest that the EBV fraction exerted cell toxicity via apoptosis in U937 cells but not in normal lymphocytes.

Keywords: boiled extract of black soybeans, vinegar, EBV, apoptosis, normal lymphocytes

Introduction

Black soybean (Kuro-mame in Japanese) is a type of soybean (*Glycine max* (L.) Merrill.) with a black seed coat. "Kuro-mame natto" is a traditional Japanese fermented food prepared from black soybeans. Boiled black soybeans are a waste product in the production of natto, and a natto factory discards about 1 ton/day of this extract. It has been reported that black soybean has potent physiological activity, including radical-scavenging activity (Furuta *et al.,* 2003), anti-tumor activity (Katsuzaki *et al.,* 2003; Liao *et al.,* 2001), anti-viral activity (Yamai *et al.,* 2003), and the ability to improve blood fluidity (Kikuchi *et al.,* 1999). These physiological activities would be also expected in the boiled extract of black soybeans because the same components are present.

Vinegar is traditionally used as a seasoning in Asian countries, and it also has medical uses due to its physiological function (Nanda *et al.,* 2004; Nishida *et al.,* 2000; Shimoji *et al.,* 2003). As a new product, we produced vinegar from the boiled extract of black soybeans and named it "black soybean vinegar."

Recently, several food constituents have been reported to have anti-tumor activity thought to occur through the induction of apoptosis (Hayakawa *et al.,* 2001, 2002; Katsuno *et al.,* 2001; Koyama *et al.,* 2002; Saeki *et al.,* 2002; Watanabe *et al.,* 2002), which is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and fragmentation of DNA (Arends *et al.,* 1990; Cohhen, 1993; Kerr *et al.,* 1972; Steller *et al.,* 1995). For instance, epigallocatechin-3-gallate (EGCG), a prominent component of green tea, has been shown to have apoptosis-inducing activity mediated by the mitogen activated protein kinase (MAPK) cascade (Saeki *et al.,* 2002). Also, licocoumarone purified from licorice is known to induce apoptosis via caspase-signal transduction (Watanabe *et al.,* 2002).

In this study, we investigated the anti-tumor activity of vinegar produced from black soybean boiled extract, which exhibited a growth-inhibitory effect and apoptosis induction in U937 cells, and compared the effects to those of normal lymphocytes separated from human blood.

Materials and Methods

Materials Human leukemic U937 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). RPMI1640 medium, fetal bovine serum, and antibiotics (penicillin/streptomycin/amphotericin) were purchased from Iwaki Glass (Chiba, Japan). Sodium iodide (NaI), sodium dodecyl sulfate (SDS), ethylenediamine tetraacetic acid (EDTA), and glycogen were obtained from Nacalai Tesque (Kyoto, Japan). N-sodium dodecanyl sarcosinate was obtained from Wako Chemical (Tokyo, Japan). Proteinase K and RNaseA were from Nippon Gene (Toyama, Japan). Lymphosepare was from Immuno-Biological Laboratories (Tokyo, Japan). Phytohemagglutinin-

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M (PHA-M) was from Gibco Laboratories (Grand Island, NY, USA). Cyanidine-3-glucoside was purchased from Funakoshi (Tokyo, Japan).

Laboratory scale production of black soybean vinegar The boiled extract of black soybeans (200 ml), obtained as a by-product from 0.6 kg of black soybeans, was poured into a 500-ml Erlenmeyer flask and autoclaved at 121℃ for 20 min. For the initial process of vinegar production, ethanol fermentation was carried out. Rice *koji* (malt) was added into the flask to a concentration of 13% and inoculated with *Saccharomyces cerevisiae* kyokai no. 77. The flask was then incubated at 25℃ using a static culture method for 2 days. The insoluble materials containing *S. cerevisiae* kyokai no. 77 were pelleted by centrifugation for 20 min at 400 x *g* and the supernatant was transferred to a 3-l jar fermentor (BMS03PI, ABLE, Tokyo, Japan) and inoculated with *Acetobacter aceti* NBRC 3283. Cultivation was performed at 30℃ at a rotation speed of 600 rpm with 1 volume per volume per minute (vvm) of aeration. During the cultivation, the pH was automatically maintained at 4.0 by the addition of 3N NaOH solution using a pH controller, and the concentration of dissolved oxygen (DO) was monitored using a DO sensor. Acetic acid fermentation was stopped due to lack of ethanol, at which time DO concentration reached the initial level.

Ethyl acetate extraction Black soybean vinegar produced as described above was divided into ethyl acetate- and water-soluble fractions. First, 100 ml of ethyl acetate was added to an equal amount of vinegar. After mixing for 20 min in a shaker (EYELA, Tokyo), the ethyl acetate-soluble fraction was transferred to a new shaker flask; this procedure was repeated twice. Then, the ethyl acetate- and water-soluble fractions were dried by evaporation and freeze-drying, respectively. The resulting dried powder (0.2 mg) was dissolved in 2 ml of phosphate buffered saline (PBS) to give a sample concentration of 100 mg/ml. The solution was adjusted to pH 7.0 by the addition of 1N NaOH, filtered with DISMIC-25cs (Toyo Roshi, Tokyo), and used for the assays. Ethyl acetate- and water-soluble fractions from yellow soybean vinegar, separated in the same manner, were used as a control. The sample concentration is given as mg of dried matter per ml of culture medium.

Culture and preparation of cells U937 cells were cultured in a RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) at 37℃ under 5% CO_2. For each test, U937 cells were seeded and cultured in the presence or absence of test samples. The cells were harvested by centrifugation for 10 min at 200 x *g* and washed with PBS.

Assay for growth inhibition Cells were subcultured for 12 h in 96-well plates at a density of 10^4 cells/well and then treated with different doses of test samples. After incubation for 24 h, the cell viability was determined based on a WST-8 assay using a commercial cell counting kit (Kishida Chemical, Tokyo, Japan) according to the manufacturer's instructions. The inhibitory effects of test samples on U937 cells and normal lymphocytes were evaluated by measuring absorbance at 490 nm on an ELISA reader (Nalge Nunc International K.K.), and the cell survival ratio was obtained.

Determination of DNA fragmentation DNA fragmentation analysis was performed according to the method described by Ishizawa *et al.,* (1991) with some modifications. Briefly, 4×10^5 cells were suspended in 200 μl of lysis buffer (0.05% SDS, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA), and treated with 1 mg/ml proteinase K for protein digestion, followed by incubation for 30 min at 37℃. DNA extraction buffer (300 μl; 6 M NaI, 13 mM EDTA, 0.5% sodium-N-lauroyl sarcosinate, 10 μg/ml glycogen, 26 mM Tris-HCl (pH 8.0)) was added to the solution, which was then incubated for 15 min at 60℃. The fragment DNA was then precipitated for 2 h at 4℃ in 50% isopropanol. The precipitates were pelleted by centrifugation for 10 min at 14,000 x *g,* dried, and resuspended in TE (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) buffer. The DNA solution was treated with 10 μg/ml RNaseA. Equivalent amounts of DNA (1 μg) were loaded into wells of 1.5% agarose gels, electrophoresed in 40 ml TAE buffer (Tris-acetic acid (pH 8.0) and 2 mM EDTA), stained with ethidium bromide, and imaged using a FluorImager (FASIII, Toyobo, Osaka, Japan).

Flow cytometry analysis Flow cytometry analysis to monitor apoptosis was performed with an Annexin-V-FLUOS Staining Kit. The membrane and nuclear events (Savill *et al.,* 1993; Vermes *et al.,* 1995) during apoptosis were analyzed by measuring the binding of FITC-labeled Annexin-V protein to phosphatidylserine (PS) present on the external surface of the apoptotic cell membrane, and propidium iodide (PI) to the nuclear DNA, respectively. The cells (1×10^6) were treated in the presence or absence of test samples for 12 h at 37℃ under 5% CO_2. Cells were centrifuged at 200 x *g* for 5 min, washed with PBS, and then stained with 100 μl of Annexin-V-FLUOS labeling solution (containing FITC-labeled Annexin-V and PI). After incubation for 15 min at room temperature, the cells were subjected to Cyto ACE (Jasco, Tokyo, Japan). Fluorescence was detected in fluorescence channels FL1 (488 nm excitation and 530 nm emission for FITC-labeled Annexin-V) and FL3 (488 nm excitation and 600 nm emission for PI). Data were processed using the Cytoflow-300 analysis program. The proportions of total apoptotic cells were calculated as the distribution of primary apoptotic cells (Annexin-V positive (+) and PI negative (−)) in the quadrant.

Preparation of human lymphocyte cells Diluted blood (heparinized blood/physiological saline=1: 1) was layered over 10 ml of lymphocyte separation medium in a centrifuge tube. The tube was centrifuged at 400 x *g* at room temperature for 30 min. The lymphocyte layer (interlayer of centrifuged blood) was transferred to a new centrifuge tube. The lymphocyte solution was diluted with an equal volume of PBS and centrifuged for 10 min at room temperature at 400 x *g*. The precipitates were washed twice and suspended in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/

ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml) and 2% PHA-M.

Statistical analysis All values are shown as the mean ±standard error (S.E.M). The difference between the groups was evaluated by Student's *t* test. Differences were considered to be significant for values of $p < 0.05$.

Results

Inhibitory effect of each fraction from vinegar from yellow and black soybeans on proliferation of U937 cells In the initial experiment, we studied the growth-inhibitory effect of each fraction of black soybean vinegar and yellow soybean vinegar in U937 cells using the WST-8 assay. As shown in Fig. 1A, the proliferation of U937 cells was inhibited by the ethyl acetate fractions of both types of vinegar in a concentration-dependent manner. The water-soluble fractions had no effect. From these results, it appears that the growth inhibitory effect in U 937 cells elicited by vinegar from black and yellow soybeans depends upon its ethyl acetate-soluble fraction.

Determination of apoptosis induction DNA fragmentation analysis was performed as described above. As shown in Fig. 2A, only the EBV fraction induced the fragmentation of DNA dose-dependently. The EBV fraction induced fragmentation time-dependently after 12 and 24 h (Fig. 2B). The formation of an apoptotic body, a characteristic feature of apoptosis, was also observed in U937 cells treated with 10 mg/ml of the EBV fraction for 12 h using phase contrast microscopy (Fig. 3), but it was not observed with 10 mg/ml of the EYV fraction (data not shown). Positioning of Annexin-V/PI dot plots in the quadrant was used to discriminate apoptotic vs. non-apoptotic cells using flow cytometry. U937 cells treated with 10 mg/ml of the EBV fraction for 12 h showed significantly increased Annexin-V fluorescence (Fig. 4A, b). The proportion of Annexin-V+/PI−, which shows that cells are primarily apoptotic, was 27.7% without treatment with the EBV fraction, whereas the proportion in the EBV fraction-treated cells increased to 67% (Fig. 4B). This result suggests that PS externalization-characterized apoptosis was induced by treatment with the EBV fraction. U937 cells treated with 10 mg/ml of the EYV fraction for 12 h were mostly distributed at the Annexin-V−/PI− and Annexin-V−/PI+ areas and did not exhibit increased Annexin-V fluorescence (Fig. 4A, c).

Effect of EBV fraction in normal lymphocytes To investigate the effect of the EBV fraction in healthy cells, we turned to the WST-8 assay and DNA fragmentation analysis using normal lymphocytes separated from human blood in the same manner as described above. As shown in Fig. 5A, the EBV fraction showed a lower cell toxicity against normal lymphocytes than against U937 cells. The EBV fraction had no effect on fragmentation of DNA in normal lymphocytes (Fig. 5B).

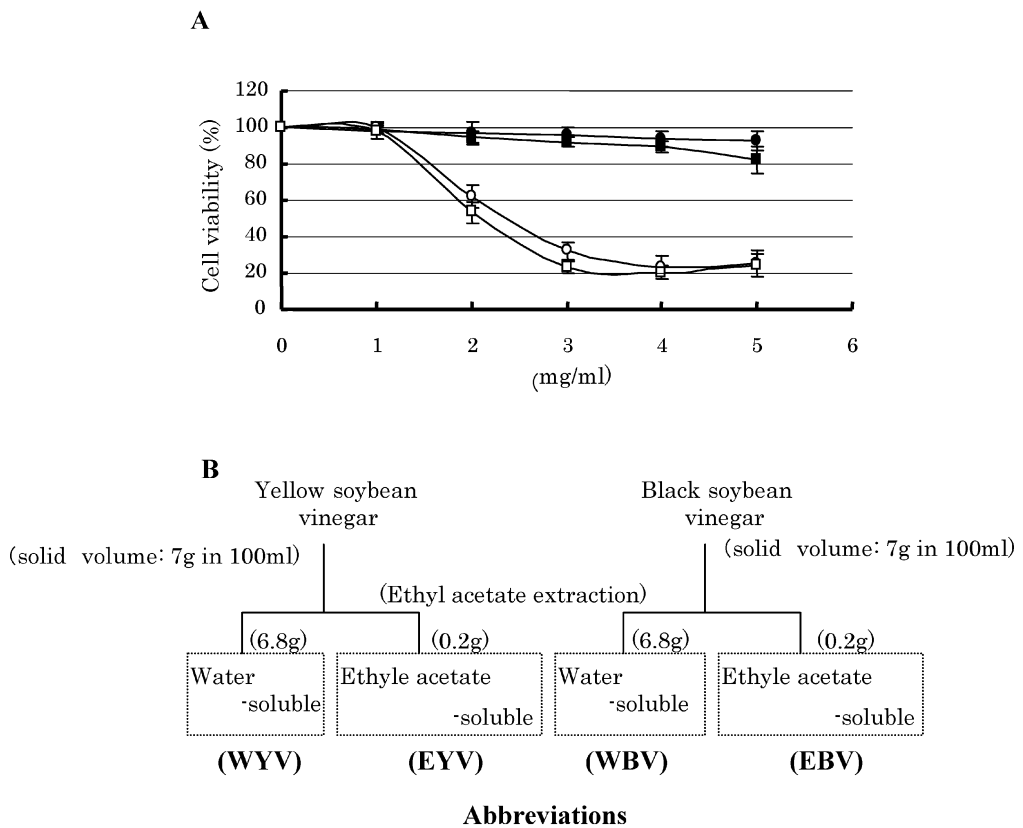

Fig. 1. Effect of each fraction from soybean vinegar on proliferation of U937 cells The cells were treated with various concentrations of the following samples for 24 h: ■, WBV; ●, WYV; □, EBV; ○, EYV. The data presented are the means (±S.E.M) of triplicate experiments (A). Abbreviations of each sample are shown in (B).

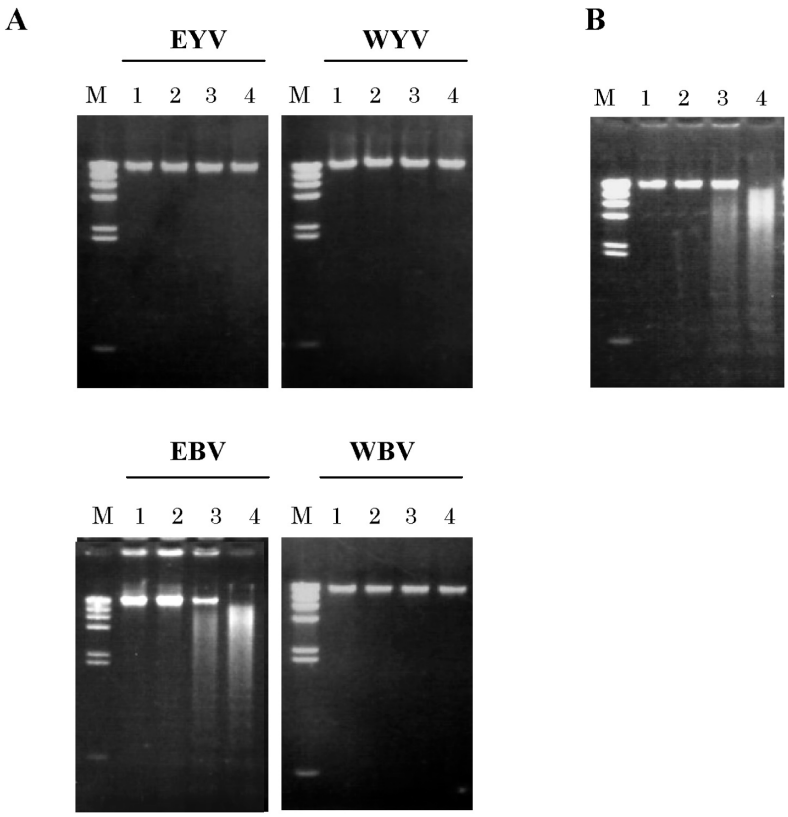

Fig. 2. Effect of each fraction from soybean vinegar on fragmentation of DNA The effect of the EBV fraction at different concentrations was examined. U937 cells were treated with the absence (lane 1) or presence of 1 mg/ml (lane 2), 5 mg/ml (lane 3), or 10 mg/ml (lane 4) of each fraction for 24 h (A). The effect of treatment with the EBV fraction for different lengths of time was examined. The cells were treated with the absence (lane 1) or presence of 10 mg/ml EBV for 4 h (lane 2), 12 h (lane 3), or 24 h (lane 4) (B). M, λDNA digested *Hind*III.

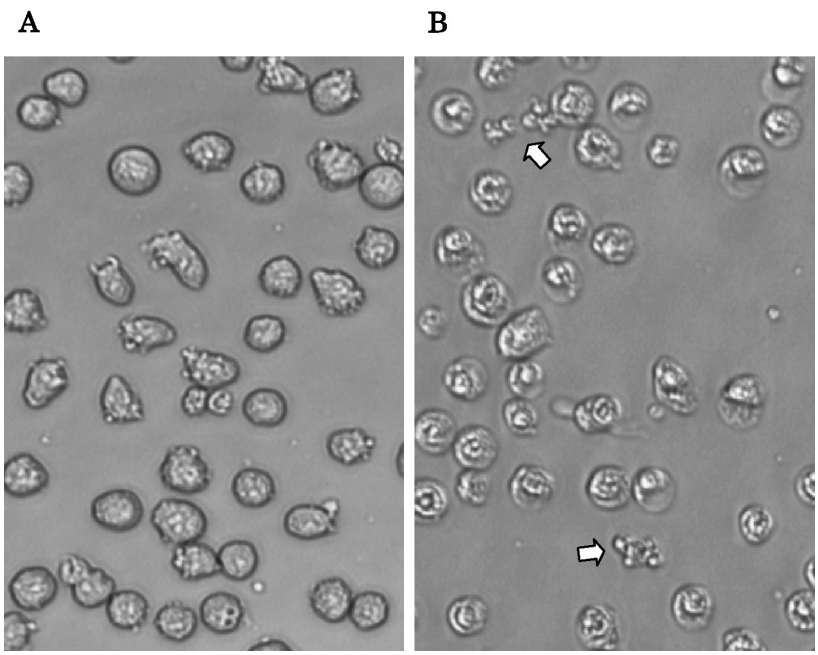

Fig. 3. Morphological changes induced by the EBV fraction U937 cells were treated in the absence (A) or presence (B) of 10 mg/ml of the EBV fraction for 24 h. Arrows indicate apoptotic bodies.

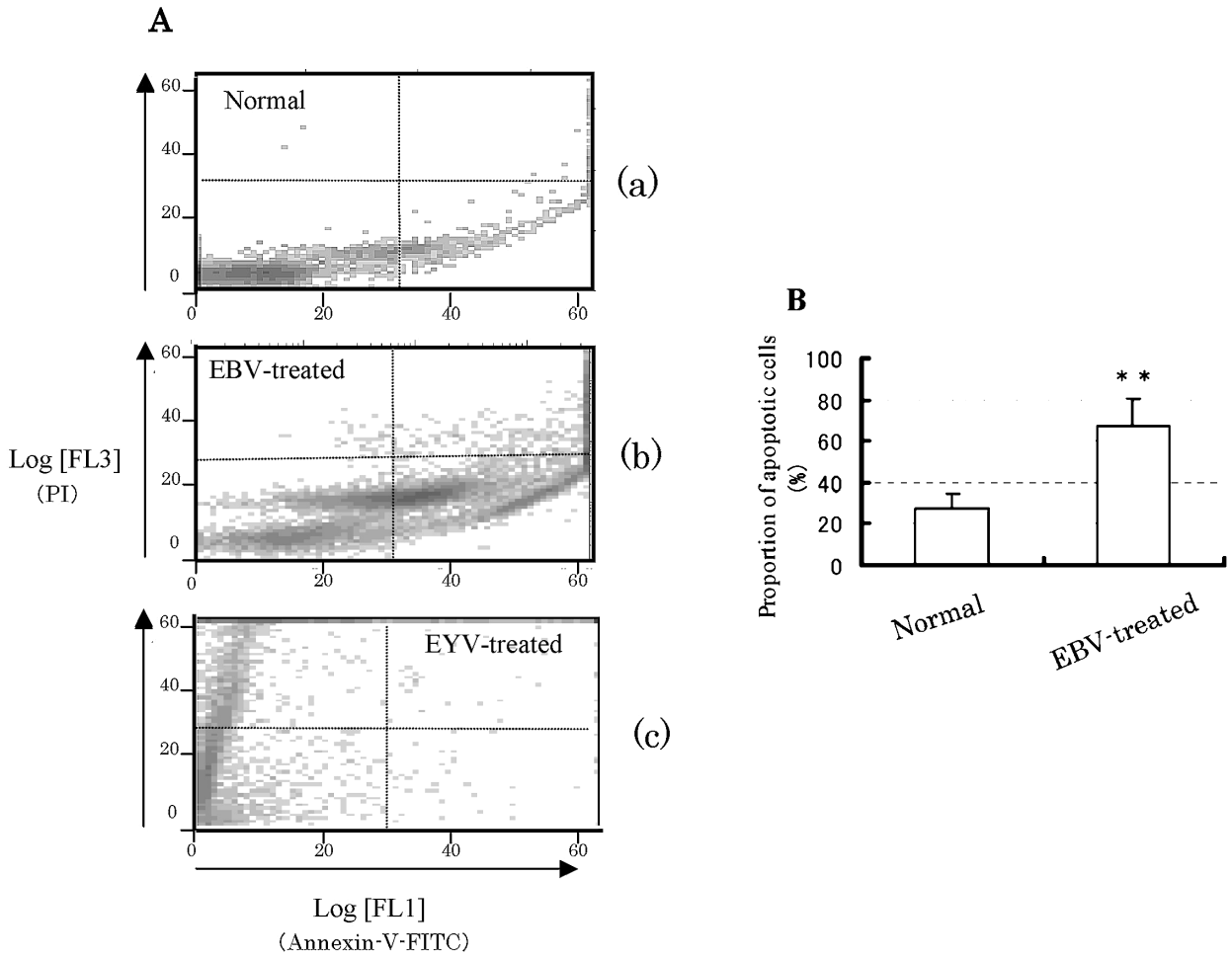

Fig. 4. Determination of apoptosis using flow cytometry The distribution of Annexin-V/PI dot plots in quadrants was used to discriminate normal (Ane−/PI−), apoptotic (Ane+/PI+), and necrotic cells (Ane+/PI+) (A). The proportions of apoptotic cells are shown by the bar diagram as the distribution of the Annexin-V+/PI− area (B). The values shown are means (±S.E.M) of triplicate experiments. $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$.

Discussion

In this study, we produced yellow and black soybean vinegar, and compared the growth-inhibitory effect and apoptosis induction in U937 cells for ethyl acetate- and water-soluble fractions of each type of vinegar. We found that the ethyl acetate-soluble fractions of both vinegars (EYV and EBV fractions) had a strong growth-inhibitory effect on U937 cells, whereas only the EBV fraction induced apoptosis via fragmentation of DNA and continuous morphological change; the EYV fraction did not induce apoptosis. From these results, we conclude that the EBV fraction contains an apoptosis-inducing compound, but the EYV fraction does not. It also seems that the inhibitory effect of EYV on proliferation of U937 cells may result from cell toxicity caused by a compound other than that responsible for apoptosis. Moreover, the EBV fraction inhibited proliferation of U937 cells significantly compared to that of normal lymphocytes, and the EBV fraction did not induce apoptosis in normal lymphocytes. It has been reported that the sensitivity of cells to toxicity and the molecular mechanism can be different for different types of cells (Hasegawa *et al.,* 2005; Juan *et al.,* 1996). In the present research, it also appears that sensitivity to toxicity differs between the U937 cells and normal lymphocytes. This difference might be due to a difference in the physiological phenotype, for instance in the expression pattern of a functional protein such as a membrane receptor, intracellular signal transduction factor, *etc.* between U937 cells and normal lymphocytes.

Potent apoptosis inducers of botanical origin could have the potential to be cancer-chemopreventive or -chemotherapeutic agents without side effects. Black soybean vinegar is promising in this regard because of its apoptosis-inducing activity and lack of effect against normal lymphocytes. Therefore, the black soybean vinegar may be suitable as a safe, healthy food.

Cyanidin-3-glucoside, an anthocyanin derived from black soybeans, was reported to induce apoptosis via fragmentation of DNA in Molt3B cells (Katsuzaki *et al.,* 2003). In a preliminary examination using high performance liquid chromatography (HPLC), the boiled extract of black soybeans contained a small amount of cyanidin-3-glucoside, and a commercially obtained equivalent could not induce fragmentation of DNA in U937 cells. We showed that,

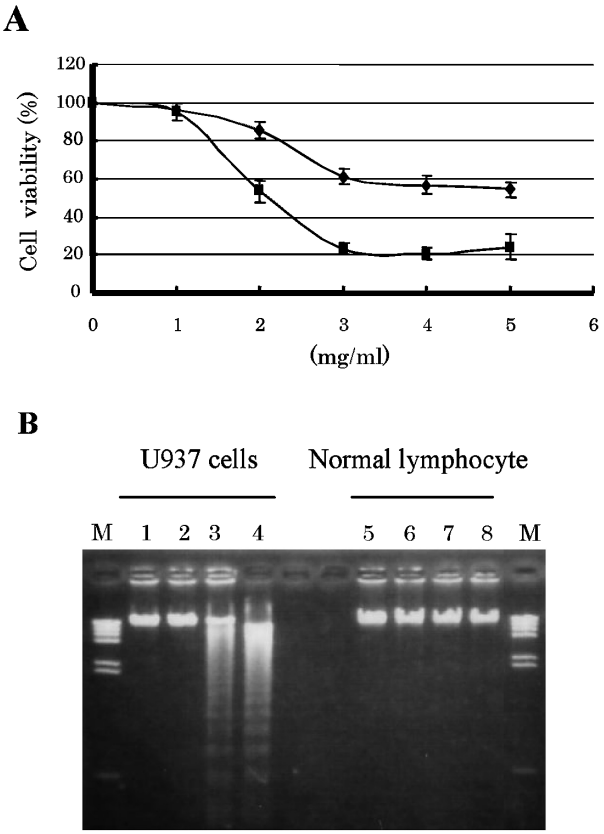

Fig. 5. Effect of EBV fraction on normal lymphocytes Cell proliferation and fragmentation of DNA were compared between normal lymphocytes and U937 cells. U937 cells and normal lymphocytes were treated with various concentrations of the EBV fraction for 24 h: ■, U937 cells; ●, normal lymphocytes. Cell viability was examined using the WST-8 assay. The data presented are the means (±S.E.M) of triplicate experiments (A). U937 cells and normal lymphocytes were treated in the absence (lane 1, 5) or presence of 1 mg/ml (lane 2, 6), 5 mg/ml (lane 3, 7), or 10 mg/ml EBV fraction (lane 4, 8). M, λDNA digested *Hind*III.

among the four fractions, only the EBV fraction could induce apoptosis. It was also confirmed that the ethyl acetate-soluble fraction of the boiled extract of black soybeans (i.e., the raw material of black soybean vinegar) induced apoptosis to a similar degree as the EBV fraction (data not shown). From these results, we suggest that the effect of the EBV fraction on apoptosis induction in U937 cells is attributable to a compound other than cyanidin-3-glucoside that is not found in yellow soybeans. Further study is required to identify this active compound.

In conclusion, we demonstrated that the EBV fraction of black-soybean vinegar has apoptosis-inducing activity in U937 cells but does not affect normal lymphocytes.

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