Effect of Small Black Soybean Fraction on the T cell-mediated Immune Responses in vivo and Proliferation of Leukemia Cells in vitro

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Abstract − We investigated effect of small black soybean fraction (SBSF) T cell-mediated responses for tumor surveillance and proliferation in leukemia cells in vitro. Each SBSF butanol fraction (SBSFBu) and SBSF chloroform fraction (SBSFCh) was administered p.o. once a day for 21 days in BALB/c mice and then levels of serum cytokines and subpopulation of lymphocytes were measured. Moreover, SBSF fraction was treated into the cultured various cell lines for proliferation in leukemia cell lines, NO production by RAW264.7 cells, and expression of p53 gene in U937 leukemia cells. These results showed that SBSFBu increased levels of serum IL-4 but not IL-2 and IFN-γ, and increased expression of CD4+ T cells and CD8+ T cells in splenocytes in vivo, while SBSFCh increased levels of serum IL-2 and IFN-γ but decreased IL-4, and increased CD8+ T cells but not CD4+ T cells. Moreover, both of SBSFBu and SBSFCh inhibited proliferation of HL60, U937, and L1210 leukemia cell lines in a dose-dependent manner, up-regulated NO production by RAW264.7 cells in a dose-dependent manner, and enhanced expression of p53 gene in U937 leukemia cells. Our findings indicate that SBSFBu and SBSFCh may enhance T cell-dependent immune responses, and that both of SBSFBu and SBSFCh may inhibit proliferation of leukemia cells by up-regulation of NO production and expression of p53 gene.

Keywords − Glycine max, lymphokines, CD4+, CD8+, cell proliferation, NO, p53

Introduction

Black soybean has been used as a health food in China. Black soybean has been reported to have antioxidant activity (Yang et al., 1999; Furuta et al., 2003) and to increase estrogenic effects making menopause related conditions ease (Zhao and Lou, 2006). Black soybean increased antiviral activity against a human respiratory illness virus (Yamai et al., 2003) and polysaccharide of black soybean promotes myelopoiesis activity in the bone marrow, stimulates production of various hematopoietic growth factors from spleen cells, and reconstitutes bone marrow that has been myelo suppressed by irradiation and 5-FU (Liao et al., 2005). A polysaccharide component from black soybean activated the immune response of mononuclear cells and inhibited proliferation in human leukemic U937 cells (Liao et al., 2001).

The aim of this experiment is to study the effects of small black soybean solvent fractions on the T cell-mediated responses for tumor surveillance and antiproliferation in leukemia cells. Our results demonstrated that chloroform-soluble fraction increased IL-2 and IFN-γ leading to Th1 responses for tumor surveillance but butanol-soluble fraction increased IL-4 toward Th2 responses, and that both of butanol- and chloroform-soluble fractions induced inhibition of proliferation in leukemia cells, upregulation of NO production by RAW264.7 cells, and increased expression of p53 gene in leukemia cells.

Experimental

Animals − Male BALB/c mice at 5-6 weeks of age were purchased from Damul Science (Dajeon, Korea) and maintained with the standard rodent chow and water available ad libitum.

Cell lines − Human acute promyelocytic (HL60), human histiocytic (U937), and mouse lymphocytic (L1210) leukemia cell lines were provided by Korea Cell Line Bank (Seoul, Korea).
Soybean material extraction and administration — The crude extract fractions were obtained from small black soybean of *G. max* (the 2003 product; Insil, Jeonbuk, Korea) using chloroform and *n*-butanol. The extracts were dried and quantified for the total amount of crude extract. A stock solution was prepared at 100 µg/mL in dimethyl sulfoxide (DMSO; Sigma) and was further diluted with RPMI 1640 immediately before treatment of the cells to achieve variously indicated concentrations (mg/mL). Each *n*-butanol-soluble fraction of small black soybean (SBSFBu) or chloroform-soluble fraction of small black soybean fraction (SBSFCh) was administered consecutively p.o. once a day for 21 days in BALB/c mice.

Serum preparation — Mice that administered SBSFBu or SBSFCh for 21 days were sacrificed and then serum was collected by heart puncture. The serum was stored at −70 °C for cytokine assay.

Preparation of lymphoid cells — Splenocyte suspensions were prepared from mice administered SBSFBu or SBSFCh for 21 days using Hanks’ balanced salt solution (HBSS; Gibco Co., Grand Island, N.Y., USA.). Erythrocytes in the single cell suspensions were lysed by brief treatment with sterile red blood cell lysing buffer solution (Sigma). Subsequently, the cells were washed with HBSS and resuspended into a suspension of 1 × 10^7 cells/mL with RPMI 1640 complete medium supplemented with 10% fetal bovine serum (FBS) and penicillin (10 U/mL)-streptomycin (10 µg/mL).

Cytokine assay — The levels of serum IL-2, IL-4, and IFN-γ were determined using ELISA with cytokine monoclonal antibodies (BD Biosciences Pharmingen, U.S.A.). All measurements were carried out in duplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA reader (Molecular Devices Co., Ltd., Sunnyville, CA). The lower limit of sensitivity for each of the ELISA was equal to or smaller than 5 pg/mL.

Flow cytometry analysis — Splenocytes (1 × 10^6 cells/mL) from mice administered SBSFBu or SBSFCh were preincubated with anti-Fc receptor monoclonal antibody (MAb) 2.4G2. The cells (1.0 × 10^6 cells/0.1 mL) were directly stained with phycoerythrin (PE)/fluorescein isothiocyanate (FITC) conjugated anti-B220/Thyl or anti-CD4/CD8 antibody (Pharmingen, San Diego, CA, USA). The cells were incubated for 30 min in the dark, washed, and fixed with 1% paraformaldehyde until analysis. Cells were acquired (5,000 events per group) in the lymphocyte gate and analyzed for two-parameter immunofluorescence using flow cytometry (Coulter, EPICS/ML).

Cell proliferation — The cultured various leukemia cell lines HL60, U937, and L1210 at various concentrations of SBSFBu or SBSFCh were plated into a 96-well plate at a density of 5 × 10^4 cells/well. Cell proliferation was measured by the microculture tetrazolium (MTT) method. The cells were resuspended in a 100 µL complete RPMI 1640 medium at 1 × 10^4 cells/mL after verifying cell viability by a trypan blue dye exclusion assay. One hundred µL of cell suspension were distributed into each well of a 96-well plate. After the treated cells were incubated for 44 h, 50 µL MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} (1 mg/mL, Sigma) was added into each well. The plates were incubated for an additional 4 h. To dissolve formazan, 150 µL DMSO was added and the absorbance values of each well at 570 nm were measured using an ELISA reader (Molecular Devices, Sunnyville, CA). Cell proliferation in non-stimulated cells from normal mice was used as a negative control. Cell proliferation rates (PR) were calculated as follows:

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PR(\%) = \left(\frac{\text{absorbance of the cell wells with/without mitogen}}{\text{absorbance of the normal control cell wells without mitogen}}\right) \times 100\%
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NO production — RAW264.7 macrophage cell line (1 × 10^6 cells/mL) were treated by various concentrations of SBSFBu or SBSFCh and then cultured in complete RPMI 1640 medium for 24 h in the presence or absence of LPS 10 µg/mL (Sigma Chemical Co., St. Louise, MO) at 37°C, 5% CO₂ incubation. The cell supernatants were harvested and NO production was measured at 570 nm using ELISA reader. Nitric oxide standard curve was measured with NaN₃.

RT-PCR — U937 leukemia cells were treated by SBSFBu or SBSFCh fraction (each 1 × 10^−5 mg/mL) and incubated for 24 h at 37°C. Total RNA was isolated from the samples using RNA extract kit (Sigma, Saint Louis, USA). The sequences of the primers for RT-PCR were identical to the sense (from bp number 121 to 142, 5'-ATGGAGGAGCCGCAGTCAGATC-3') or antisense (from bp number 1,281 to 1,302, 5'-CTGATGATCTCCGGAGT-3') sequences of p53 cDNA. RT-PCR was performed using the Two Step RNA PCR kit (Takara, Japan) with the primers and 100 ng of total RNA. RT-PCR products were identified by 1% (w/v) agarose gel electrophoresis.

Statistical analysis — Data from an individual experiment were described as a mean ± standard error. All statistical analyses were performed on a statistical analysis system (SAS) program, and significant difference between mean