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The Polysaccharide Fraction AIP1 from *Artemisia iwayomogi* Suppresses Apoptotic Death of the Mouse Spleen Cells in Culture

Jung Suk Hwang, Hye Kyung Chung, Eun Kyong Bae, Ah Young Lee, Hee Jung Ji, Dong Woon Park, Hwa Jin Jung, Chung Won Cho, Hyun Ju Choi[1], Dong Seok Lee[1], Kang Ro Lee[2], and Hyun Joo Youn

Department of Microbiology and School of Biotechnology & Biomedical Science, [1]Department of Medical Technology, Inje University, Gimhae 621-749, Korea, and [2]Department of Pharmacy, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

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A polysaccharide fraction, AIP1, purified from *Artemisia iwayomogi* was shown to have immunomodulating and anti-tumor activities in mice. In order to determine how the AIP1 fraction exhibits the immunomodulating activity, the effect of the fraction on the apoptosis of mouse spleen cells was investigated. Treatment of the mouse spleen cells with the AIP1 fraction resulted in the suppression of apoptotic death and an extension of cell survival in culture, indicating that the fraction might modulate the death of spleen cells. Treatment of the mice with the AIP1 fraction *in vivo* also resulted in less apoptosis of the spleen cells, which indicates the physiological relevance of the anti-apoptosis effect of the fraction *in vitro*. A mouse gene array was used to determine the profile of the gene expression change showing a pattern of up- and down-regulated genes by the AIP1 treatment. This study provides preliminary information regarding the immunomodulatory mechanism of the AIP1 fraction.

Key words: Polysaccharide, *Artemisia iwayomogi*, Apoptosis, Genomics

INTRODUCTION

It is well known that a large number of plant carbohydrates exhibit various biological activities including immunomodulatory activity (Srivastava and Kulshreshtha, 1989). Immune system cells always proliferate or die throughout the developmental process or by various stimulations, and plant polysaccharides might be involved in this process by modulating the function of certain immune cell populations. Han *et al.* (2001) reported that a polysaccharide from *Platycodon grandiflorum* selectively acted on B cells and macrophages to present its immunomodulatory effect. The *Artemisia iwayomogi* plant has been used traditionally in Korea to treat various inflammatory diseases, particularly hepatitis (Koo *et al.*, 1994). Programmed cell death or apoptosis is an important process in the homeostatic regulation of many types of immune cell populations and plays a role in regulating the immune responses. It also functions in the pathogenesis of a variety of disorders including cancer and autoimmune diseases (Lindsten *et al.*, 2000), prompting an interest into the agents that modulate the apoptosis process including natural products. Recently, some natural products have been shown to modulate the apoptosis of immune cells, demonstrating ways of identifying the novel compounds using the modulation of cell-death or cell-survival (Kuo *et al.*, 2000).

A previous study reported that the AIP1 fraction, a fraction of water soluble polysaccharides of smaller than 2 kDa purified from *Artemisia iwayomogi*, increased antibody production and suppressed transplanted tumor cell growth (Koo *et al.*, 1994). It was also suggested that the AIP1 fraction might be involved in the survival of immune cells either by suppressing apoptotic death or by stimulating cell proliferation. This study found that the AIP1 fraction interacts with mouse spleen cells and promotes their survival by suppressing the apoptotic death of the cells both *in vitro* and *in vivo*. The gene expression profile change of the cultured spleen cells as a result of the AIP1 treatment was also examined by a functional genomics study using a mouse gene array. The implication of the change is also discussed.

Correspondence to: Hyun Joo Youn, Department of Microbiology and School of Biotechnology & Biomedical Science, Inje University, Gimhae, Gyungnam 621-749, S. Korea
E-mail: mbhjyoun@ijnc.inje.ac.kr

MATERIALS AND METHODS

Mice and chemicals

BALB/c mice were purchased from the Korea Experimental Anima Center (Seoul, Korea) and were used at 6 weeks of age. The mice were housed in polyethylene cages containing clean wood shavings and were provided with rodent chow and tap water *ad libitum*. They were kept in the room maintaining a constant temperature and humidity as well as a 12 hours light and dark cycle. Most of the cell culture reagents and chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The Atlas Mouse cDNA Expression Array and Atlas Pure Total RNA Labeling System were obtained from Clonetech Lab Inc. (Palo Alto, CA, USA) and RNAgents The total RNA Isolation System was purchased from Promega (Madison, WI, USA). TACS MTT Assay was purchased from R&D systems (Minneapolis, MN, USA).

Purification of the AIP1 fraction

The crude polysaccharide extract was prepared by percolating the dried leaves of *Artemisia iwayomogi* in high-pressure steam water for 3 hours. After concentrating the crude polysaccharide extract, it was size-fractionated with a Sephadex G-50 column chromatography using distilled water. The fractions containing the carbohydrates were determined using a modified phenol sulfuric acid method (Taylor, 1995), producing a minor peak around 50 kDa and a major peak smaller than 2 kDa. The fractions around 2 kDa were pooled and used as the AIP1 fraction (Koo *et al.*, 1994).

Preparation of mouse spleen and bone marrow cells and peritoneal macrophages

The mouse spleen and bone marrow cells were collected from the corresponding tissues of 6 week-old female BALB/c mice, as described by Mishell and Shiigi (1980). The cells from the spleen were subsequently treated with an ammonium chloride solution to remove the red blood cells. The macrophages were isolated from the mouse peritoneum, as described by Hudson and Hay (1989). Briefly, 6 week-old female BALB/c mice were treated with an intra-peritoneal injection of a 1% starch solution for 3 days prior to sacrifice. The peritoneal macrophages were collected from the peritoneal exudates and examined morphologically using the neutral red and Wright staining methods (Strober, 1992). Approximately 70% of the cells were identified to be macrophage type cells.

Effects of the AIP1 fraction on the apoptosis of the cells *in vitro*

After incubating 1×10^6 cells from each tissue in DMEM/10% FCS medium supplemented with 2 mg/mL of the AIP1 fraction for 4 hours, the degrees of apoptosis were determined by the DNA fragmentation assay (Kroemer *et al.*, 1997). Briefly, the cells from the culture were treated with 20 μL of a working solution A (10 mM EDTA/0.5% SDS/50 mM Tris pH 8.0/0.5 μg/mL proteinase K) for one hour at 50°C. After incubation in 10 μL of working solution B (10 μg/mL RNase A/50 mM Tris pH 8.0) for one hour at 37°C to remove the RNA, the samples were loaded onto 1% agarose gel and the degree of apoptosis was compared by the band intensity of the small DNA fragments representing the apoptotic degradation of the chromosomal DNA. Since this procedure presented the intact chromosomal DNA as well as small DNA fragments, the relative integrity of the DNA samples and experimental variation can be easily monitored. The apoptosis of the peritoneal macrophages was also determined, as described above, except that the cells were treated with 0.5, 1.0, and 2.0 mg/mL of the AIP1. The cultures supplemented with distilled water instead of the AIP1 fraction were employed as the negative controls. All experiments were performed in duplicate and repeated at least twice.

The MTT assay was also used to determine the degree of cell death in the cultures by measuring the formation of formazan dye crystals, as described by the manufacturer. Following the incubation of 10^6 spleen cells in the medium supplemented with either AIP1 or distilled water for 4 hours, the cells were treated with the MTT Reagent for 6 hours until purple precipitates were observed. The precipitates were dissolved using a Detergent Reagent and their absorbance was measured at 595 nm to determine the degree of MTT reduction representing the quantity of live cells. All experiments were performed in triplicate and repeated twice. The results were statistically evaluated using a Student's *t*-test ($p<0.05$).

In order to confirm their endotoxin free states, the distilled water and the AIP1 fraction supplemented in the cultures were tested for the presence of a bacterial endotoxin by a gel clot assay using Limulus Amebocyte Lysate, as described by the manufacturer (Cape Cod Inc., MA, USA).

Effects of the AIP1 fraction on the apoptosis of the cells *in vivo*

In order to examine the effects of the AIP1 fraction on the apoptosis of the spleen cells *in vivo*, 4 mg of the AIP1 fraction (0.16 mg/g bodyweight) was injected into the peritoneum of 4 week-old female BALB/c mice for 5 consecutive days prior to sacrifice. Following the isolation of the cells from the spleen and bone marrow, the cells from the AIP1 treated mice were incubated in DMEM/10% FCS medium for 4 hours without the AIP1 fraction and the degree of DNA fragmentation was determined. The BALB/c

mice injected with distilled water instead of the AIP1 fraction were used as the control.

Gene array of the spleen cells in culture

Approximately, 3×10^8 spleen cells from the 6 week-old female BALB/c mice were incubated in the DMEM/10% FCS medium supplemented with 2 mg/mL of the AIP1 fraction for 4 hours, and then subjected to total RNA isolation using a RNAgents Total RNA Isolation System (Promega) according to the manufacturer's instructions. Following treatment of each RNA sample with DNaseI, the RNA was used for the radioactive cDNA probe synthesis using a Total RNA Labeling System (Clonetech) and [α-^{32}P] dATP according to the manufacturer's. The labeled cDNA probes of more than 10^6 cpm were used for the hybridization with the Atlas Mouse cDNA Expression Array where the cDNA fragments from 588 different genes had been attached in duplicate to a positively charged nylon membrane. The membranes were pre-hybridized with the hybridization solution containing salmon sperm DNA at 68 °C for more than 4 hours and then hybridized with denatured probes at 68°C for 16 hours in a rotating hybridization chamber (Robbins Scientific, Sunnyvale, CA, USA). The membranes were washed twice with 2XSSC/1%SDS and 0.1XSSC/0.5%SDS at 68°C. Following exposure on X-ray film, the image was analyzed using an Alpha Imager 1220 image analysis system (Alpha Innotech Cooperation, San Leandro, CA, USA). The densitometric value for each gene of interest was corrected for the background and non-specific hybridization. In order to compare gene expression profile, the array using the total RNA from the cells treated with distilled water was used as the AIP1 untreated control. The signals from the positive (glyceraldehyde 3 phosphate dehydrogenase, hypoxanthine guanine phosphoribosyl transferase and myosine 1 α genes) and negative (M13 mp18 and pUC18 DNA's) control spots were averaged to normalize the signals from the AIP1 treated and untreated blots.

RESULTS AND DISCUSSIONS

Effects of the AIP1 fraction on spleen cells *in vitro* and *in vivo*

The AIP1 fraction was purified from the water-soluble crude polysaccharide extract from *Artemisia iwayomogi* using Sephadex G-50 column chromatography, as the polysaccharides smaller than 2 kDa. HPLC analysis revealed that the fraction was composed of one monosaccharide and several oligosaccharide fractions. The mice pretreated with the fraction exhibited augmented antibody production and improved the survival of the mice against the transplanted tumor cells (Koo *et al.*, 1994), revealing immunomodulating activity of the fraction. To determine how the AIP1 fraction presented those activities, the degrees of the apoptotic death of the spleen cells in the culture were measured using DNA fragmentation and MTT reduction assays. For the *in vitro* study, the bone marrow and spleen cells from the BALB/c mice were incubated in the DMEM/10% FCS medium supplemented either with or without the AIP1 fraction and the degrees of DNA fragmentation were compared. Although a small amount of DNA fragmentation could be observed in the bone marrow cells in either the AIP1 treated or untreated cultures, the spleen cells exhibited significant suppression of DNA fragmentation by the treatment with the AIP1 fraction (Fig. 1, lanes 1-4). This suggests that the AIP1 fraction could suppress the apoptotic death of the spleen cells in culture. In order to confirm the *in vitro* anti-apoptosis effect of the AIP1 fraction *in vivo*, the BALB/c mice were pretreated with the AIP1 fraction for 5 consecutive days by a peritoneal injection of the fraction and the degrees of apoptosis in the spleen or bone marrow cells from the mice were examined. The spleen cells from the AIP1 pretreated mice exhibited much lower DNA fragmentation compared to that of the cells from the untreated control mice while the bone marrow cells did not (Fig. 1, lanes 5-8), indicating the physiological relevance of the *in vitro* anti-apoptosis activity of the AIP1 fraction to an *in vivo* system. The degrees of cell death by the AIP1 treatment were also determined quantitatively using a MTT reduction assay. The supplementation of the spleen cell culture with the AIP1 fraction significantly reduced the cell death induced by the *in vitro* cultivation as more formazan precipitates were observed in the AIP1

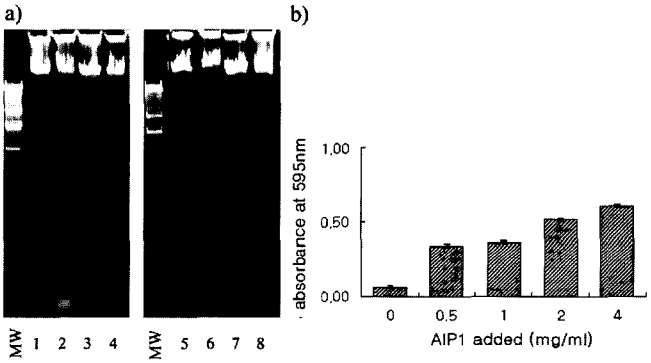

Fig. 1. The AIP1 fraction reduces apoptotic death of mouse spleen cells. a) Treatments with the AIP1 fraction of the spleen cells *in vitro* (lanes 1-4) and *in vivo* (lanes 5-8) reduce DNA fragmentation of the cells in culture. Lanes 1, 2, 5 and 6 show the DNA fragmentations of the spleen cells while lanes 3, 4, 7 and 8 represent those of bone marrow cells. Lanes 1, 3, 5 and 7 are the cells treated with the AIP1 fraction and lanes 2, 4, 6 and 8 are the untreated cells. MW represent the DNA fragments from the HindIII digested λ DNA. b) Treatments with the AIP1 fraction of the spleen cells *in vitro* reduce the degree of cell death in a dose dependent manner. More MTT reduction can be seen in the cultures treated with increasing amounts of the AIP1 fraction, indicating more cells survived in the AIP1 treated cultures.

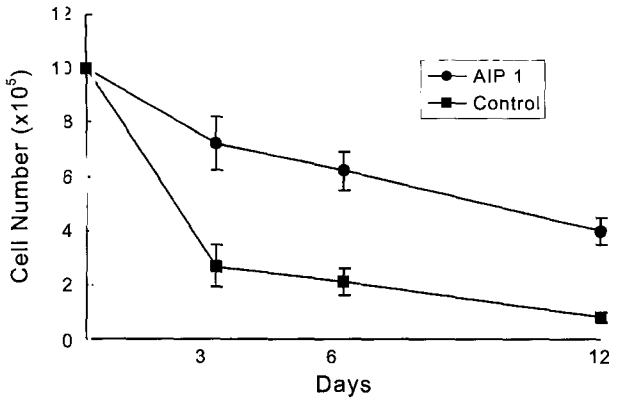

Fig. 2. Survival of the mouse spleen cells in the culture treated with the AIP1 fraction. The cells in the culture were grown in the medium supplemented with the 2 mg/mL AIP1 fraction (black circles) for up to 12 days with a changing of the medium every three days, and the viable cells in the cultures were counted. The line with the black rectangles represents the number of cells in the untreated control.

treated cultures. As shown in the Fig. 1b, more MTT reduction could be seen in the cultures treated with increasing amounts of the AIP1 fraction, which indicates that the AIP1 fraction influences spleen cell survival in a dose dependent manner. The anti-apoptosis activity of the AIP1 fraction also appeared to influence the survival of the spleen cells in culture, as more cells had survived in the AIP1 treated cultures than in the untreated controls for up to 12 days (Fig. 2), supporting the hypothesis that the AIP1 fraction may present its immunomodulating activity by regulating the survival of the mouse spleen cells *in vivo*. Considering the function of the secondary lymphoid organ such as the spleen, it appears that the immunomodulating effect of the AIP1 fraction results from the suppression of apoptotic death of mature immune cells.

Gene expression profile change by the AIP1 treatment

In order to obtain preliminary information on the anti-apoptotic mechanism of the AIP1 fraction, a gene array technology was used to screen the genes whose expressions were altered by the AIP1 treatment. A mouse gene array containing a panel of 588 cDNAs for the growth factors, cytokines and other molecules involved in signal transduction and apoptosis was used, and the changes in gene expression were probed using the cDNAs either from the AIP1 treated or untreated spleen cell culture. Among the numerous genes whose expression were changed by the AIP1 treatment, 8 genes exhibited no less than a 2-fold up-regulation while 14 genes showed a greater than 3-fold down-regulation (Table I) compared to the normalized expression of the genes. Although the effect of AIP1 comes from various cell populations in the mouse spleen, and interpreting a gene expression profile change might be confusing, a careful study of the expression profile of the changed genes should provide valuable information regarding the anti-apoptosis mechanism of the fraction.

Table I. The genes up- and down-regulated by the treatment of AIP1 fraction

Gene	Fold of expression
c-Fos proto oncogene	+5
NR4A1 (Nur77)	+4
laminin receptor 1 (RPSA)	+3
HSP90	+3
Cek5 receptor ligand	+3
Cathepsins B	+2
CD2	+2
PAX5 (BASP)	+2
CCR2 chemokine receptor	-10
oxidative stress-induced protein (OSI)	-10
CXCR2 chemokine receptor	-10
PDGF receptor beta (PDGFRB)	-10
endoplasmic reticulum stress protein 72 (ERP72)	- 5
endothelin receptor type B (EDNRB)	- 5
TGFβ1	- 5
low affinity IgG Fc receptor II beta (FCGR2B)	- 4
macrophage colony stimulating factor I receptor (CSF1R)	- 4
nuclear factor erythroid 2 related factor 2 (NFE2L2)	- 3
c-Cbl proto oncogene	- 3
STAT1	- 3
protein kinase C inhibitor 1 (KCIP1, 14-3-3 eta protein)	- 3
tumor necrosis factor receptor 2 (TNFR2)	- 3

* The fold of expression represents the expression level of a gene compared to that of the control culture.
* "+" and "-" represent increase and decrease of expression by the AIP1 treatment, respectively.

The expression change of some genes suggests that AIP1 might directly interact with the spleen lymphocytes, a major population of the spleen, to present its anti-apoptosis activity. The up-regulated genes include the genes involved in the signal transduction pathway including the signal transducers and the cell surface receptors and their ligands (Table I). Although the relevance of the upregulation of the NR4A1, Cek5 receptor ligand and the cathepsin B genes is unclear, other genes appear to be more directly involved in the proliferation or survival of the spleen cells. The gene most up-regulated is the c-Fos constituting the AP-1 transcription factor. Considering that AP-1 binds to the regulatory regions of the lymphocyte specific genes and is up-regulated during cell proliferation (Takayanagi *et al.*, 2002) while it is down-regulated in apoptosis induction (Macian *et al.*, 2002), it can be hypothesized that the up-regulation of the gene is more directly related to the

suppression of apoptosis. The laminin receptor 1 and CD2 are involved in the survival or proliferation of some T cells (Suarez *et al.*, 2002), and both Pax5 and Hsp90 inhibit the apoptosis of B cells and leukemic cells (Emelyanov *et al.*, 2002; Blagosklonny, 2002). This supports the proposal that the AIP1 fraction may interact with the spleen lymphocytes to block the death of these cells. Among the scores of the down-regulated genes (Table I), some of the genes appeared to be directly involved in the apoptotic death of the spleen cells. Since OSI promotes cell death by lethal oxidant damage (Choi *et al.*, 2002) and TNFR2 and CCR2 promote lymphocyte apoptosis (Cusson *et al.*, 2002; Dupuis *et al.*, 2001), the down-regulation of the genes is linked to the protection of the spleen cells from apoptosis. Both TGFβ1 and FCGR2B were involved in suppressing T and B cell activation (Takayama *et al.*, 2002; Rudge *et al.*, 2002), and the ERP72, NFE2L2 and KCIP1 genes also promote the apoptotic death of various cell types (Kim *et al.*, 2002; Argiles *et al.*, 2002; Yaffe, 2002). This suggests that the down-regulation of these genes is also implicated in the suppression of the spleen cell apoptosis by the AIP1 treatment.

The gene array study might provide a novel insight regarding the suppression of spleen cell death by the AIP1 treatment. Among the genes whose expression levels were altered by the AIP1 treatment, a dozen of genes appeared to be involved in the proliferation or apoptosis of the inflammatory cells such as macrophages. This observation suggests that the suppression of the apoptosis of spleen cells may occur indirectly from the inactivation of these inflammatory cells since they are also known to induce the apoptotic cell death of lymphocytes (Zhu *et al.*, 2002; Lazzarino *et al.*, 2001). The C-Fos gene, whose expression level was dramatically increased by the AIP1 treatment, suppressed the macrophage function (Xu *et al.*, 2002) and might be related to this assumption. The down-regulation of the expression levels of the TGFβ1, CSFR1, CCR2, CXCR2 and PDGFR genes also supports this notion since these genes are involved in macrophages activation (Vujaskovic *et al.*, 2002; Dai *et al.*, 2002; Tylaska *et al.*, 2002; Glynn *et al.*, 2002; Iihara *et al.*, 1996). Therefore, the down-regulation of these genes might compromise the macrophage activity. The hypothesis that the inhibition of macrophage activation suppresses spleen cell death is also supported by the down-regulation of the FCGR2B, c-Cbl and STAT1 genes because these genes are known to be involved in the proliferation and accumulation of macrophages (Aittomaki *et al.*, 2002; Bisson *et al.*, 2002). Though the idea of macrophage involvement requires additional study and verification using cell separation and northern blotting analyses, the suggestion attracts some interest regarding to the facts that the *Artemisia iwayomogi* plant has been used traditionally

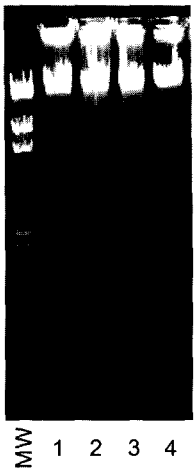

Fig. 3. Effect of the AIP1 fraction on the apoptosis of the mouse peritoneal macrophages. Lanes 1, 2 and 3 represent the DNA fragmentations of the peritoneal macrophages treated with the 0.5, 1 and 2 mg/mL AIP1 while lane 4 shows the fragmentation of the untreated control. MW represents the DNA fragments from the HindIII digested λ DNA.

in Korea to treat various inflammatory diseases, particularly hepatitis (Koo *et al.*, 1994) and that macrophages play important roles in an immune dysfunction and liver disease progression (Nakamoto *et al.*, 2001). Recently, we investigated the effect of the AIP1 fraction on mouse peritoneal macrophages in cultures and found that the macrophages were indeed vulnerable to the apoptotic death by the AIP1 treatment (Fig. 3), which would support the idea that the AIP1 fraction may suppress the activity of the macrophages thereby protecting the splenic lymphocytes from apoptosis.

This study showed that apoptotic death of the spleen cells was suppressed by the treatment with the AIP1 fraction purified from *Artemisia iwayomogi,* and also presented the changes in the gene expression pattern by this treatment. Although some of the data generated from this study is largely descriptive, the overview obtained from this study is a useful baseline for further functional studies since the analysis of the gene expression change has produced a novel insight into the anti-apoptotic mechanism of the AIP1 fraction such as the involvement of macrophages. These results are valuable not only just for a better understanding of the function of the fraction but for the development of novel immunomodulating agents.

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