RESEARCH COMMUNICATION

Stimulation of Dendritic Cell Maturation and Induction of Apoptosis in Leukemia Cells by a Heat-stable Extract from Azuki bean (*Vigna angularis*), a Promising Immunopotentiating Food and Dietary Supplement for Cancer Prevention

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Abstract

Non-toxic stimulation of dendritic cells (DCs), which are central immunomodulators, may aid the prevention of cancer. Furthermore, induction of apoptosis in cancer cells by anticancer agents contributes to the induction of DC maturation. We previously reported that extracts from *Pinus parviflora* Sieb. et Zucc pine cone and Mucuna seed induce differentiation of mouse bone marrow cells into mature dendritic cells and also induce apoptosis in various human cancer cell lines. In the present study, we screened 31 kinds of edible beans with biological activity similar to that of extracts from pine cone and *Mucuna* and found that the heat-stable extract from azuki bean (*Vigna angula*) stimulated differentiation of bone marrow cells into immature DCs with the greatest efficacy. The level of IL-6 produced by sequential treatment of DCs with azuki extract and lipopolysaccharide was the highest among the examined beans. Azuki extract also inhibited the growth of human leukemia U937 cells, leading to induction of apoptosis. These results suggest that azuki bean and its extract are immunopotentiating foods that can be used as a dietary supplement for cancer prevention and immunotherapy.

Keywords: Azuki bean - dendritic cells - immunopotentiation - cancer prevention

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Introduction

Accumulating evidence indicates that stimulation of anti-cancer immune responses may contribute to the prevention of cancer and also to its management after conventional radiotherapy and chemotherapy (Zitvogel et al., 2008). Dendritic cells (DCs) are central immunomodulators (Banchereau and Steinman, 1998; Jeras et al., 2005), and mature DCs are critical for a potent immune response against cancer cells following radiotherapy and chemotherapy (Vicari et al., 2002; Tesniere et al., 2008). DCs engulf apoptotic tumor cells through recognition of apoptotic characteristics of cancer cells induced by γ-irradiation or treatment with anti-cancer agents such as doxorubicin and mitoxantrone, leading to maturation of DCs and a potent *in vivo* immune response (Obeid et al., 2007; Apetoh et al., 2008). These findings suggest that combined administration of an inducer of apoptosis in cancer cells and chemical agents with strong differentiation and maturation-inducing activity for DCs might be useful for preventing and/or curing cancer.

We previously reported that an extract (polyphenylpropanoid polysaccharide complex; PPC) from *Pinus parviflora* Sieb. et Zucc pine cone and an extract from *Mucuna pruviens* var. utilis seed induce differentiation of human mononuclear cells into DCs *in vitro*, and these extracts have also been shown to induce apoptosis in human cancer cells (An et al., 2010; Kurokawa et al., 2011). PPC is now commercially available in the United States as a supplement for activating immune cells. Although *Mucuna* is cultivated in tropical and subtropical regions such as India, south-east Asia, and Brazil, the seed coat is so thick that extended boiling is needed for cooking, and it has not been cultivated as a dietary food in Japan. In the present study, therefore, we screened an additional 31 kinds of edible beans containing components with biological activity similar to that of PPC and Mucuna extract. Results revealed that beans such as azuki bean (*Vigna angularis*), black bean (*Glycine max* (L.) Merr.), and flower bean and kougenn bean (both *Phaseolus coccineus* L.) contain differentiation/maturation-inducing activity for DCs and apoptosis-inducing activity for human leukemia U937 cells. The DC differentiation/maturation-inducing activity of azuki beans was the highest among the examined beans.

Azuki beans have been consumed as a popular food in East Asia for many centuries. In addition, azuki beans have been used in Chinese traditional medicine for treatment of edema and thiamine deficiency. In Japan, various kinds of traditional confectionaries such as youkan (azuki jam cake) and manju (boiled azuki beans) are prepared by boiling azuki beans. The present paper indicates the

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possibility that azuki beans and dietary supplements prepared from them might be useful for cancer prevention and immunotherapy.

Materials and Methods

Reagents and Mice

Beans were purchased from the market located at Honcho 6, Niigata city in Japan. A subspecies of *Mucuna* (*Mucuna hassjoo* sieb) was obtained from the Niigata Agricultural Research Institute. RPMI 1640 medium was purchased from Gibco Laboratories (Grand Island, NY). Murine recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF) was provided by PeproTech EC (London, UK). Trypan blue (0.4 %) was from MP Biomedicals, Inc. (Solon, OH). Mouse IL-6 (interleukin-6) and TNF-α ELISA Ready-SET-Go were from eBioscience, Inc (Boston, MA). 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), phenazine methosulfate (PMS), Sephadex G-50, and lipopolysaccharide (LPS; from *E. coli* 0111:B4) were provided by Sigma (Saint Louis, MO). *Phellinus linteus* powder was purchased from NIHONDO Co. Ltd. (Tokyo, Japan). Male C57BL/6 mice, 6 weeks old, were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). The study was approved by the Ethics Committee of Niigata University of Pharmacy and Applied Life Sciences.

Extraction procedure

Extracts of beans and the mushroom *Phellinus linteus* were prepared by the same methods used for the preparation of *Mucuna* extract (Kurokawa et al., 2010). Briefly, beans (50 g) were ground with a mixer and mixed with 0.1 M NaOH solution (500 mL) for 10 min and extracted at 121 °C in an autoclave. After the mixture had cooled to room temperature, it was centrifuged at 3,000 × g for 5 min, and the pellet was removed. The supernatant was neutralized to pH 7 with HCl and the solution was centrifuged at 10,000 rpm for 10 min. The resulting supernatant was further centrifuged at 40,000 rpm for 30 min. The obtained supernatant was sterilized by filtration (pore size, 0.22 microns).

Cell culture

For preparation of bone marrow (BM)-derived DCs, the femurs and tibiae of C57BL/6 mice were placed in a 90 mm dish with RPMI 1640 on ice after removing muscle with scissors and forceps. The ends of the bones were cut with scissors and the marrow was flushed out using 2 mL RPMI-1640 with a syringe fitted with a 21 gauge needle. The BM cells were centrifuged once and suspended in ACK buffer (155 mM NH_4Cl, pH 7.2–7.4; 10 mM $KHCO_3$; and 0.125 mM EDTA) at room temperature for 3 min to hemolyse red blood cells. The cells were then washed, centrifuged, and suspended in RPMI 1640 containing 10% fetal bovine serum (FBS). After passing the cells through a 40-μm nylon mesh, 1×10^6 cells in a volume of 1 mL were transferred to a 50-mm dish and cultured with 3 mL medium at 37 °C in humidified air (5% CO_2). BM cells were cultured in the presence of GM-CSF (20 ng/mL) for 7 days, and 1 μg/mL of LPS was added and incubated for a further 3 days to induce DC maturation. U937 cells were provided by the Japanese Cell Research Resources Bank and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in humidified air (5% CO_2).

Assays of cytokine production from mouse BM cells following treatment with azuki extract

BM cells (1×10^6/mL) were cultured in the presence of azuki extract, instead of GM-CSF, for 7 days, and 1 μg/mL of LPS was added and the cells were further incubated for 3 day to induce DC maturation. Levels of cytokines IL-6, IL-12, and TNF-α were examined using an ELISA Kit (eBioscience Inc., Boston, MA) according to the manufacturer's instructions.

Assays of cell proliferation and induction of apoptosis

U937 cells were seeded in 24-well plates along with various concentrations of azuki extract (0–100 μg/mL) and cultured for 72 h. The cells were then mixed with an equal volume of 0.4% trypan blue solution (Kaufmann, 1989). The percentage of viable cells was determined by counting the proportion of cells unstained by trypan blue. Apoptotic cells were identified under a fluorescence microscope as cells with condensed and fragmented nuclei after staining with Hoechst 33258. Apoptosis was also assessed by measuring the formation of mono- and oligonucleosomes using a Cell-Death detection ELISAPLUS kit (Boehringer Mannheim, Mannheim, Germany).

High porosity polymer MCI gel CHP20 chromatography Extract of azuki beans (500 g) was extracted with an aqueous alkaline solution (3.5 L) as described above, and the extract (168 g in 120 mL) was applied to a column of high porosity polymer MCI gel CHP20 (15 × 100 cm) equilibrated with distilled water and eluted stepwise with 10%, 20%, 50%, and 100% methanol at room temperature at a flow rate of 10 mL/h. Absorbance of the eluted solution was monitored at 275 nm, and fractions were collected until absorbance was reduced to a low and constant level. The eluate was concentrated by rotary evaporation. Each fraction was assayed for growth inhibition of U937 cells and for the production of IL-6 from BM cells, as described above.

Results

Differentiation of DC proceeded in two-steps (Banchereau and Steinman, 1998; Jeras et al., 2005). BM cells were induced to differentiate into immature DCs by cultivation in the presence of GM-CSF and IL-4. Then, stimulation of immature DCs with a microbial component (LPS) or an inflammatory cytokine (TNF-α) led to their differentiation into mature DCs (Banchereau and Steinman, 1998; Jeras et al., 2005). We then examined whether extracts from each of the 31 kinds of beans, which constituted all types commercially available, promoted differentiation of BM cells into immature DCs as we had previously observed for Mucuna extract (Kurokawa et al., 2010). As shown in Figure 1, sequential treatment of BM cells with increasing concentrations of azuki extract up to 300 μg extract/mL, then with LPS, produced increasing amounts of IL-6. As shown in Table 1, the level of IL-6

produced by sequential treatment with 50 μg/mL of azuki extract and LPS was the highest among the examined bean extracts using identical concentrations. Treatment with extracts from kougenn bean and flower bean, then with LPS, also produced significant amounts of IL-6. Although treatment with black bean of *Glycine max* (L.) Merr. and then with LPS also produced significant amounts of IL-6, extracts from other members of *Glycine max* (L.) Merr. such as soybean, as well as those from 26 other kinds of bean, failed to produce detectable levels of IL-6.

When BM cells were cultured for 7 days in the presence of GM-CSF and further incubated in the presence of various concentrations of azuki extract for 3 days, virtually no IL-6 was secreted into the culture media (data not shown). This result indicates that the immunological activity of azuki extract is different from that of LPS, which stimulates the maturation of DCs from immature DCs (Kurokawa et al., 2010).

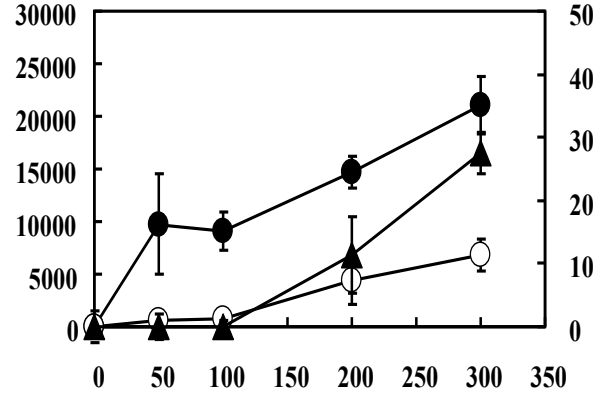

Figure 1. Effects of Extracts from Azuki Bean (*Vigna angularis*) on the Induction of Factor Secretion by BM Cells. BM cells were cultured in medium containing various concentrations of azuki extract for 7 days and treated with 1 μg/mL of LPS. After culturing for a further 3 days, the levels of IL-6 (●), TNF-α (❍), and IL-12 (▲) secreted into the culture medium were determined by ELISA. Data are expressed as mean ± SD (n = 3).

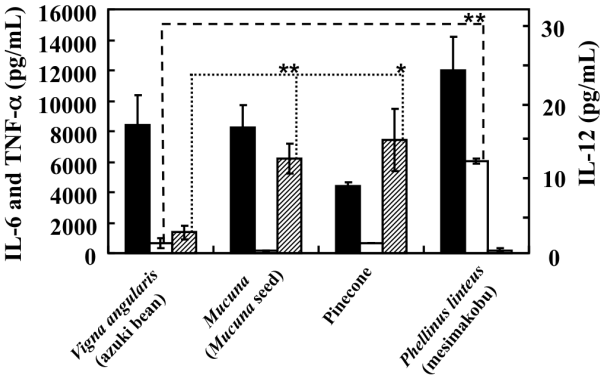

Figure 2. Comparison of Cytokines Produced by BM Cells Treated with Extracts from Azuki Beans, *Mucuna* Seeds, Pine Cones, and Meshimakobu (*Phellinus linteus*). BM cells were cultured in the presence of azuki extract (100 μg/mL), *Mucuna* extract (50 μg/mL), pine cone extract (40 μg/mL), or mesimakobu extract (50 μg/mL) for 7 days, and then LPS (1 μg/mL) was added. After culturing for a further 3 days, the levels of IL-6 (black column), TNF-α (white column), and IL-12 (hatched column) secreted into the culture medium were determined. Data are expressed as mean ± SD (n = 3). Asterisks indicate statistically significant differences (*p<0.02; ** p<0.001).

Table 1. Effect of Extracts from Various Beans on Induction of IL-6 Secretion by BM Cells and Growth Inhibition of Human Leukemia U937 Cells

Botanical name	Common name	IL-6 (pg/mL)	IC_{50}
Vigna angularis			
	Azuki bean	9737+751	4.7 +1.5
Mucuna hassjoo sieb			
	Mucuna	8750+286	2.7 +1.1
Phaseolus coccineus L			
	Kougenn bean	2405+389	3.3 +0.8
	Flower bean	1386+130	3.2 +0.8
Glycine max (L.) Merr.			
	Black bean	2558+349	8.4 +0.5
	Soybean	ND	ND

*BM cells were cultured in a medium containing 50 μg/mL of the extracts from various beans, and the levels of IL-6 secreted into the culture medium were determined by ELISA. Data are expressed as mean ± SD (n = 3). ND: not detectable. No secretion of IL-6 was detected following treatment with extracts from the following species: an additional 5 varieties of *Glycine max* (L.) Merr., 12 varieties of *Phaseolus vulgaris* L, 3 varieties of *Pisum sativum* L, 2 varieties of *Canavalia gladiata* DC, groundnuts (*Arachis hypogaea* L), hiyoko bean (*Cicer arietinum* L), sora bean (*Vicia* L), and lentil bean (*Lens cultinaris*). IC_{50} is the concentration for 50% inhibition of growth of leukemia U937 cells treated with the extracts from various beans. Data are expressed as mean ± SD (n = 3).

Next, we examined whether other markers associated with mature DCs, such as TNF-α and IL-12, might be produced by sequential treatment with azuki extract and LPS. Such sequential treatment of BM cells with increasing concentrations of azuki extract and LPS stimulated the production of increasing levels of TNF-α and IL-12 (Figure 1).

Production of cytokines induced by azuki extract was compared with that induced by other DC stimulants. As shown in Figure 2, the levels of IL-12 produced by the sequential treatment of BM cells with azuki extract and LPS were significantly lower than those observed following treatment with *Mucuna* or pine cone extracts. In addition, the ratio of IL-12 to IL-6 in response to azuki extract treatment was lower than that obtained by treatment with *Mucuna* or pine cone extracts. The levels of TNF-α produced by sequential treatment with azuki extract and LPS were significantly lower than those produced by treatment with the mushroom meshimakobu (*Phellinus linteus*), which induces functional maturation of DCs *in vitro* and *in vivo* (Park et al., 2003; Matsuba et al., 2008). Practically no IL-12 was produced following sequential treatment of BM cells with meshimakobu extract and LPS. These results suggest that sequential treatment with azuki extract and LPS stimulates DCs via a different mechanism than treatment with extracts from *Mucuna*, pine cone, or meshimakobu.

A low concentration of azuki extract markedly inhibited the growth of human leukemia U937 cells as determined by trypan blue staining (Figure 3). The IC50 value was calculated as 4.7±1.5 μg/mL (Table 1). As shown in Table 1, extracts from black bean, kougenn

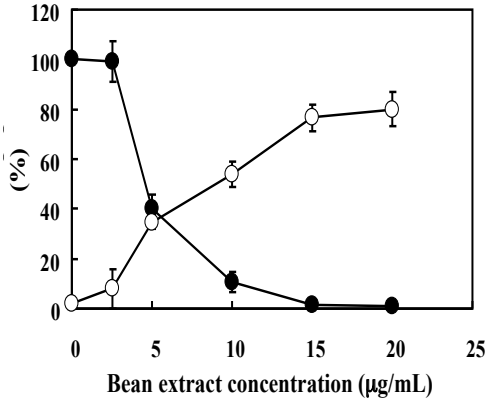

Figure 3. Growth Inhibitory and Apoptosis-inducing Effects of Azuki Bean Extract on Human Leukemia U937 Cells. U937 cells were treated with azuki extract at various concentrations for 72 h, and the percentage of viable cells (●) and apoptotic cells (❍) were determined. Data are expressed as mean ± SD (n = 3).

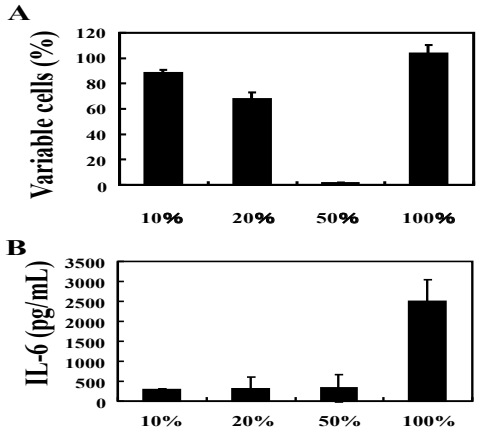

Figure 4. Fractionation of Azuki Extract by Column Chromatography and Biological Activity of Separated Fractions. (A) Each methanol fraction (10 μg in 1 μL methanol) was added to 1 mL of culture medium, and U937 cells were cultured for 72 h. The growth inhibitory effect was measured by counting viable cells. Data are expressed as mean ± SD (n = 3). (B) Each methanol fraction (10 μg in 1 μL methanol) was added to 1 mL of the culture medium of BM cells, and then LPS (1 μg/mL) was added. After culturing for 3 days, the level of IL-6 in the culture medium was analyzed by ELISA. Data are expressed as mean ± SD (n = 3).

bean, flower bean, and meshimakobu had marked growth inhibitory activity for U937 cells, similar to that produced by Mucuna extract. The extracts from beans other than azuki bean, black bean, kougenn bean, and flower bean showed no or very weak growth inhibitory activity for U937 cells, so their IC_{50} values could not be calculated.

Growth inhibition of cancer cells by chemotherapeutic anticancer agents generally induces apoptosis. As shown in Figure 3, the treatment of U937 cells with azuki extract induced apoptosis in a dose-dependent manner. Approximately 80% of U937 cells exhibited fragmented and condensed nuclei, as determined by Hoechst 33258 staining, following treatment with 20 μg/mL of azuki extract for 72 h (Fig. 3). Neither maturation-inducing activity for DCs nor apoptosis-inducing activity for leukemia U937 cells in response to azuki extract was changed by heating at 100°C for 3 min, indicating that azuki extract is heat-stable.

As shown in Figure 4, the growth of U937 cells was almost completely inhibited by the azuki extract fraction eluted with 50% methanol, while the fractions eluted with 10%, 20%, and 100% methanol had very little or practically no inhibitory effect.

When BM cells were cultured for 7 days in the presence of fractions eluted with 10%, 20%, 50%, and 100% methanol and further cultured in the presence of LPS, marked IL-6 inducing activity was observed only in cells treated with the fraction eluted with 100% methanol (Figure 4B).

Discussion

We screened various edible beans, the extracts of which exhibit both DC differentiation/maturation-inducing activity and apoptosis-inducing activity for human leukemia U937 cell lines. We found that the extract from azuki bean had the greatest DC differentiation/maturation-inducing activity among 31 kinds of beans examined. In addition to its DC differentiation/maturation activity, azuki extract also induced apoptosis in human leukemia U937 cells. The results obtained from column chromatography, as shown in Figure 4, indicate that azuki extract contains two components; one with DC differentiation/maturation-inducing activity and the other with apoptosis-inducing activity for leukemia U937 cells. The component which induced differentiation from BM cells to immature DCs was eluted with 100% methanol, while the apoptosis-inducing component for leukemia U937 cells was eluted with 50% methanol. Since engulfment of apoptotic cancer cells stimulates DCs (Apetoh et al., 2008; Obeid et al., 2007), it is possible that these two components of azuki extract have additive or synergistic immunopotentiating effects against cancer cells *in vivo*. Itoh et al. demonstrated that a hot-water extract of azuki suppressed the growth of human stomach cancer cells *in vitro* and was effective for reducing murine stomach cancer induced with benzo-α-pyrene *in vivo* (Itoh et al., 2004). Further investigation of the immunopotentiating and chemopreventive activity of azuki extract in animal cancer models may be warranted.

Mushroom polysaccharides have been widely used as immunostimulants in Asia and as tumor immunotherapy agents in Japan (Borchers et al., 2004). As shown in Figure 2, azuki extract stimulated DCs in a different manner to meshimakobu or pine cone extracts. Thus, the additive or synergistic effects on immune response might be induced by combining these immunopotentiating agents. A major advantage of azuki extract is that it can be cultivated on a larger scale and at a lower cost than mushrooms, and it is also consumed daily across Asia. Thus, azuki beans and azuki extract may be promising immunopotentiating foods, dietary supplements, and adjuvants for cancer prevention and immunotherapy.

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