Induction of Apoptosis with an Extract of *Actinidia polygama* Fruit in the Promyelocytic Leukemia Cell Line HL-60

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Common small fruit produced naturally in Japan is attracted as healthy food. We studied on inhibitory activity of several small fruit extracts on growth of cancer cells to look for a candidate of healthy food or low toxic anti-cancer drugs. Among the fruit extracts examined, *Actinidia polygama* (Matatabi) extract had dose-dependent potent inhibitory activity against the growth of a promyelocytic leukemia cell line, HL-60, but was less effective on a colon cancer cell line, LS-174T, or normal human dermal fibroblast cells. We examined a part of the responsible mechanism of growth inhibition of HL-60 cells. Active agents in *A. polygama* fruit were heat-stable and small molecular weight of less than 1000. HL-60 cells incubated with an extract of *A. polygama* fruit induced apoptosis, which was detected by electrophoretic DNA fragmentation, morphological chromatin condensation and the TUNEL method with flow cytometry.

Key words —— *Actinidia polygama*, apoptosis, HL-60, inhibition

INTRODUCTION

Epidemiological studies show that some types of cancer are related to dietary habits. People who take in a large amount of fruit or vegetables have low risk of cancer.[1)] Japanese common small fruit is attracted as healthy food or low toxic anti-cancer drugs. Previously, Yoshizawa *et al*. showed that several Japanese small fruit extracts had anti-cancer effect on leukemia cells with induction of differentiation or inhibition of proliferation.[2,3)] Many researchers have tried to find low toxic anti-cancer agents in plant extracts. Various plant extracts containing coumarins,[4)] flavonoids,[5)] acridone alkaloids[6)] or diterpenes[7)] have been shown to have an anti-cancer effect. Chemical structure studies on plant-derived anti-cancer agents are progressing; however the precise mechanism of the plant-derived agents on the inhibition of cancer cell growth is still not completely understood.

We examined the inhibitory effects of several small fruit extracts on the proliferation of a promyelocytic leukemia cell line, HL-60, and selected *Actinidia polygama* to study the inhibitory mechanism on cancer cells. This paper concludes that heat-stable agents from an extract of *A. polygama* fruit have potent inhibitory activity on HL-60 cell growth and that the inhibition is due to the induction of apoptosis.

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MATERIALS AND METHODS

Fruit Extract —— All small fruits were harvested in Akita Prefecture, Japan. Fresh fruit of 10 g was homogenized with 30 ml of PBS. An extract obtained through filtration with No. 2 filter paper was frozen at –30°C as stock solutions and used for experiments after centrifugation and sterilization through 0.22 μm filter membranes (Millex-GV, Millipore, Japan). We defined concentration of those sterilized extracts of various fruits as original concentration. The original concentration of *A. polygama* fruit (No. 1) was 32 mg dry weight/ml.

Cells —— HL-60 cell line, human promyelocytic leukemia cells, was originally provided to us by Institute for Fermentation, Osaka (Japan) and maintained in our laboratory. HL-60 cells were cultured

with RPMI-1640 medium (with L-glutamine, Sigma, U.K.) supplemented with 10% fetal bovine serum (FBS, Cansera International, Canada, U.S.A.), 100 unit/ml penicillin and 100 μg/ml streptomycin (Gibco Oriental, Japan). LS174T cell line, human colon tumor cells, and normal human dermal fibroblast cells were obtained from Dainippon Seiyaku Co. (Osaka, Japan). LS174T cells were cultured with 10% FBS-MEM (Sigma, U.K.) supplemented with 1% non-essential amino acids (Gibco Oriental), 100 unit/ml penicillin and 100 μg/ml streptomycin. Normal human dermal fibroblast cells were cultured with the CS-C Complete Medium (4Z0-500) obtained from Dainippon Seiyaku Co. MC/9 cells, murine mast cell clone from fetal liver, were obtained from American Type Culture Collection (VA, U.S.A.) and cultured with 10% FBS-RPMI-1640 supplemented with 150 unit/ml rIL-3,[8] 1% non essential amino acids, 100 unit/ml penicillin and 100 μg/ml streptomycin.

Cell Proliferation Assay —— Cell proliferation was detected by the colorimetric assay[9] modified. Sterile samples of fruit extract (0.02 ml) diluted with PBS were placed in wells of a 96-well plate in quartet. More than eight of control wells were placed with PBS only. Cells suspended in the culture medium (0.2 ml of 6–8 $\times$ 10^4/ml) were added to the wells. The plate was incubated at 37°C for 3 days in 5% CO_2. And then, 0.02 ml of sterile MTT solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, 5 mg/ml in PBS), was added to the wells. The plate was incubated for 4 hr in a CO_2 incubator and centrifuged at 400 *g* for 10 min. After removing 0.15 ml of supernatant from each well, 0.15 ml of PBS was added to the wells to wash cells. The plate was shaken for 10 min on a plate mixer and centrifuged. After 0.15 ml of supernatant was removed, 0.15 ml of 0.04 N HCl in isopropanol was added to the wells and the plate was shaken for 15 min. Absorbance at 570 nm was measured with the microplate reader Model 550 (Bio-Rad, Japan). Percent inhibition of proliferation was calculated as follows: %Inhibition = [1 - (A_{570} of sample - A_{570} of blank)/(A_{570} of control - A_{570} of blank)] $\times$ 100. The control represents wells with cells and PBS, and blank represents wells with buffer only.

Stability and Molecular Weight of Active Agents —— An extract (0.2 ml) of *A. polygama* fruit was heated at 37°C or 98°C for 30 min. A control sample was kept on ice during the time. Inhibitory activity of all samples was assayed with HL-60 cells. Another part of *A. polygama* fruit-extract (2 ml) was applied on a prepacked Sephadex G-25 column (PD-10, Pharmacia) according to the directions for use and separated into high and low molecular weight fractions. Activity of the two fractions was measured.

Detection of Apoptosis —— A suspension (2 ml) of HL-60 cells (2.5 $\times$ 10^5 cells/ml) in the culture medium was mixed with 0.2 ml of different concentrations of *A. polygama* fruit-extract in a well of a 24-well plate and incubated at 37°C for 2 days in 5% CO_2. After centrifugation in a tube, cells were treated with 0.1 ml of 2% glutaraldehyde at room temperature for 1 hr. Cells were washed, suspended in 0.02 ml of PBS, and stained with 1 μl of Hoechst 33342 (1 mg/ml, Sigma). Morphological change with apoptosis was observed with a fluorescence microscopy (Nikon, Eclipse E800, Japan) with excitation at 365 nm and emission at 400 nm. In the other experiments, DNA was extracted from HL-60 cells that were incubated with *A. polygama* fruit-extract as described above. Briefly, 10^6 cells were mixed with 0.1 ml of lysis buffer containing 0.5% Triton X-100, 10 mM Tris and 10 mM EDTA, pH 7.4, at 4°C for 10 min. Supernatant obtained by centrifugation was treated with a final 0.2 mg/ml of DNase-free RNase (Wako Nippon Gene, Tokyo, Japan) at 37°C for 1 hr, and then treated with a final 0.4 mg/ml of proteinase K (Takara Co., Otsu, Japan) for additional 1 hr. The solution (0.1 ml) was mixed with 0.02 ml of 5 M NaCl and 0.12 ml of isopropanol and kept at –30°C overnight. After centrifugation, precipitated DNA was dissolved in 0.01 M Tris–EDTA buffer (pH 8.0) and applied to 2% agarose gel electrophoresis with a 0.09 M Tris-borate-0.002 M EDTA buffer. DNA ladder was stained with ethidium bromide and pictured with the imaging analyzer LAS-1000plus (Fujifilm, Japan). DNA preparation by this method contains fragmented DNA mainly, but little high molecular DNA. Apoptosis of HL-60 cells was further detected by the terminal transferase uridyl nick end labeling (TUNEL) method. Cells incubated with *A. polygama* fruit-extract were treated with the TUNEL reagents according to the kit directions (In Situ Cell Death Detection Kit, Fluorescein, Boehringer Mannheim, Tokyo, Japan). Level of apoptosis was analyzed by a flow cytometry system (FACSCalibur, Beckton Dickinson, U.S.A.).

Table 1. Names of Small Fruit Plant Examined

No.	Plant family	Scientific name	Japanese name
1	Actinidiaceae	*Actinidia polygama* Maxim.	Matatabi (Akita)
2		*Actinidia polygama* Maxim.	Matatabi (Hokkaido)
3		*Actinidia polygama* Maxim.	Matatabi (Mokutenryo)
4	Ericaceae	*Vaccinium corymbosum* Linn.	Blueberry
5		*Vaccinium oldhamii* Miq.	Natsuhaze
6	Hydrangeaceae	*Ribes grossularia* Linn.	Marusuguri (ripe)
7		*Ribes grossularia* Linn.	Marusuguri (half-ripe)
8		*Ribes grossularia* Linn.	Marusuguri (unripe)
9		*Ribes japonicum* Maxim.	Komagatakesuguri
10		*Ribes latifolium* Janczewski	Ezosuguri
11		*Ribes nigrum* Linn.	Kurosuguri
12		*Ribes rubrum* Linn.	Fusasuguri
13		*Ribes sachalinense* Nakai	Togasuguri
14	Rosaceae	*Pruna armeniaca* Linn.	Anzu
15		*Pruna salicina* Lindl	Sumomo
16	Vitaceae	*Vitis vinifera*	Kyoho

RESULTS

Effect of Small Fruit Extracts on Growth of HL-60 Cells

We examined the effect of several small fruit extracts on the growth of HL-60 cells by the MTT method. The names of small fruit examined in this paper are summarized in Table 1. Fruit extracts from *A. polygama*, *V. oldhamii*, *R. japonicum*, and *R. sachalinense* showed potent inhibitory activity on growth of HL-60 cells (Fig. 1). We selected *A. polygama* fruit to study mechanism on growth inhibition of cancer cells because we had preliminary information about the inhibitory activity of the fruit extract and *A. polygama* fruit was easily available in our region. We used *A. polygama* fruit No. 1 (Table 1) in all the following experiments.

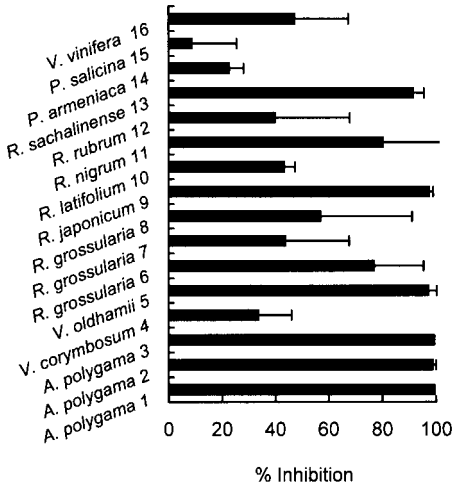

Fig. 1. Inhibitory Activity of Several Small Fruit Extracts on HL-60 Cell Growth

%Inhibition of cell growth with small fruit extracts of the original concentration (10 g fruits/30 ml) was measured. The original concentration of *A. polygama* fruit (No. 1) was 32 mg dry weight/ml. Mean ± S.D. values of four samples are shown.

Effect of an Extract of *A. polygama* Fruit on Cell Growth

We first examined the dose dependency of the inhibitory effect of *A. polygama* fruit-extract on HL-60 cell growth. Serially dilution of *A. polygama* fruit-extract decreased its inhibitory activity against HL-60 cells. Furthermore, three-day treatment of cells showed higher inhibition than one-day treatment especially at 0.7 mg/ml of the extract (Fig. 2). Therefore, an extract of *A. polygama* fruit had inhibitory effect on growth of HL-60 cells, dose- and time-dependently.

Inhibitory activity of *A. polygama* fruit-extract on cell growth was examined in some other cells. The inhibitory activity was weaker in the human colon tumor cells LS174T, human normal dermal fibroblasts, and the murine mast cell clone MC/9 than in HL-60 cells (Fig. 3A–3C). While an extract of *A. polygama* fruit induced a 94–99% inhibition of HL-60 cell growth at 1.4 mg/ml concentration, however, induced only 18% inhibition in LS174T cells, 30% inhibition in normal dermal fibroblasts, and 35% inhibition in MC/9 mast cells at the same concentration. In contrast, an extract of *A. polygama*

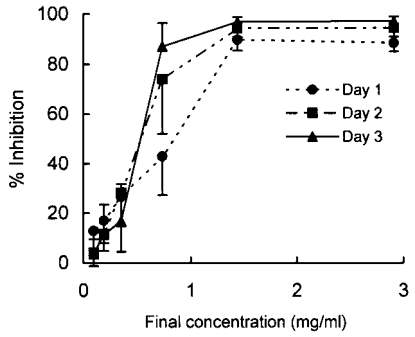

Fig. 2. Dose and Time Dependency of Inhibitory Activity of *A. polygama* Fruit-Extract on HL-60 Cells

HL-60 cells were incubated with various concentrations of extract for 1, 2 or 3 days. %Inhibition of cell growth of four samples (mean ± S.D.) is shown.

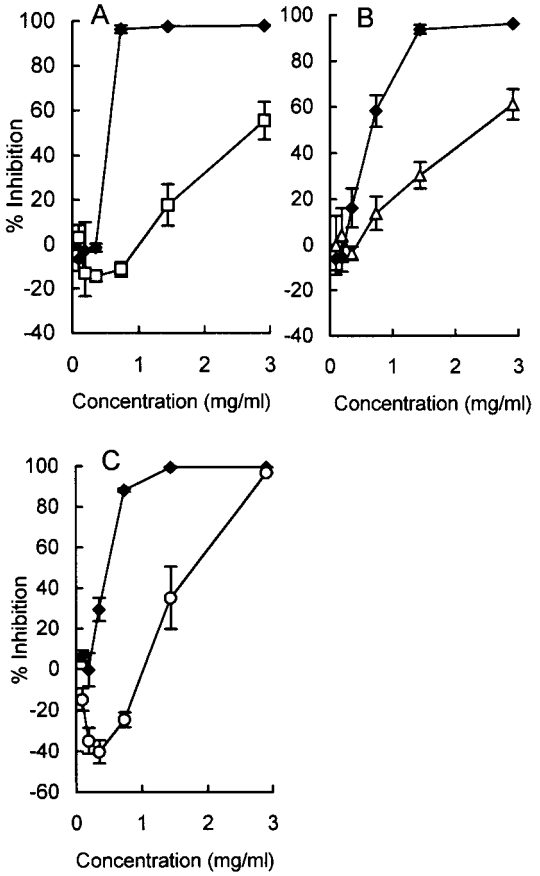

Fig. 3. Growth Inhibitory Activity of *A. polygama* Fruit-Extract on Some Cell Lines

A) HL-60 (solid mark) or LS174T cells (open square) were incubated with various concentrations of *A. polygama* fruit-extract for 4 days. B) HL-60 (solid mark) or normal human dermal fibroblasts (open triangle) were incubated with various concentrations of *A. polygama* fruit-extract for 3 days. C) HL-60 (solid mark) or MC/9 (open circle) were incubated with *A. polygama* fruit-extract for 3 days. %Inhibition of cell growth of four samples (mean ± S.D.) was measured in each experiment.

Table 2. Heat Stability of *A. polygama* Fruit-Extracts on Inhibition of HL-60 Cell Growth

Temperature treated (°C)	% Inhibition on cell growth
0	82.3 ± 8.4
37	72.6 ± 13.1
98	96.2 ± 8.6

A. polygama fruit extract was heated at 37°C or 98°C for 30 min. A control sample was kept on ice during the time. Inhibitory activity of the samples was assayed with HL-60 cells by three-day incubation. Mean % inhibition ± S.D. in triplicate is shown.

fruit stimulated growth of MC/9 cells but not an inhibition at low concentration.

Stability and Molecular Weight of Active Agents in *A. polygama* Fruit-Extract

Many of plant-derived active agents that modulate cell growth are small molecular organic compounds. *A. polygama* fruit-extract was heated and its activity was assayed with HL-60 cells. The inhibitory activity was still detected after heating up to 98°C (Table 2). An extract of *A. polygama* fruit was separated into high and low molecular weight fractions on a prepacked Sephadex G-25 column. The percent of inhibition against HL-60 cell growth was 86.1 ± 6.4 and 7.4 ± 2.6 in a low molecular weight fraction and a high molecular weight fraction, respectively. These results show that the active components in *A. polygama* fruit-extract are heat-stable and the apparent molecular weight is less than 1000.

Induction of Apoptosis in HL-60 Cells

We studied the inhibitory mechanism of *A. polygama* fruit-extract on HL-60 cell growth to see if it was apoptosis. HL-60 cells were incubated with *A. polygama* fruit-extract for one or two days to examine their DNA fragmentation and morphological change. Incubation of HL-60 cells with *A. polygama* fruit-extract showed typical DNA ladder by electrophoresis on day 1 (Fig. 4) and typical apoptotic morphology with chromatin condensation on days 2 (Fig. 5). Induction of apoptosis in HL-60 cells with *A. polygama* fruit-extract was further confirmed by the TUNEL method (Table 3).

DISCUSSION

A. polygama is a well-known plant that induces abnormal excited behavior in felines such as cats or

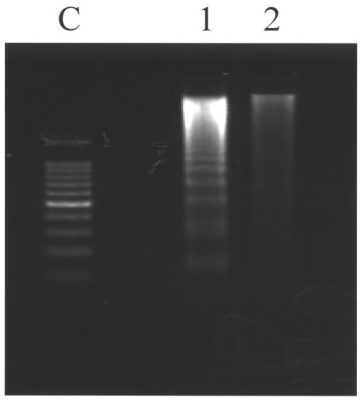

Fig. 4. DNA Fragmentation of HL-60 Cells Treated with *A. polygama* Fruit-Extract

HL-60 cells were incubated with a final 9% of *A. polygama* fruit-extract for one day. DNA was extracted from cells and applied to agarose gel electrophoresis. Lanes 1 and 2 show HL-60 cells treated with *A. polygama* fruit-extract and HL-60 cells with PBS, respectively. The left lane C represents a DNA marker.

lions. *A. polygama* fruit that is parasitized with a small fly, *Pseudosphondylia matatabi*, and formed galls (Mokutenryo) are often used as a herbal medicine having analgesic or antasthenic effect in traditional Chinese medicine. The effective compounds in *A. polygama* fruit to induce abnormal excited behavior in felines were identified as some types of lactones.[10] However, inhibitory effect on cell growth has not been studied in an extract from *A. polygama* fruit. We found that an extract of *A. polygama* fruit had potent inhibitory activity on the growth of the leukemia cell line HL-60 but was less effective on the colon cancer cell line LS-174T, and that the growth inhibition on HL-60 cells was due to induction of apoptosis. Cell growth inhibitory agents in an extract of *A. polygama* fruit were heat-stable. The lactones that induce abnormal excited behavior in felines, however, are volatile. The cell growth inhibitory agents in *A. polygama* fruit, therefore, will be different from the volatile lactones.

The apoptosis of HL-60 cells is induced *in vitro* with many agents derived from plants such as *Uncaria tomentosa*,[11] *Salvia miltiorrhiza*,[12] *Lithospermum erythrorhizon*,[13] and *Sophora flavescens*.[14] The mechanism of the apoptosis induced in HL-60 cells with plant agents can be explained in various ways.[15] Resveratrol, a polyphenol agent contained in grapes, induces apoptosis in HL-60 cells through CD95-signaling.[16] Apoptosis of HL-60 is also induced caspase-dependently[12,13] or through cyclic AMP-dependent activation of protein kinase A.[17] So far, we have little information on the mechanism of apoptosis induced in HL-60 cells with *A. polygama* fruit-extract. To investigate the mechanism further, identification of the active compounds in *A. polygama* fruit is necessary. We are now trying to identify the chemical structures of the active compounds in *A. polygama* fruit-extract.

An extract of *A. polygama* fruit inhibited the growth of HL-60 cells strongly but was less effective on LS174T cells at 1–2 mg/ml concentration. *A. polygama* fruit-extract may contain powerful

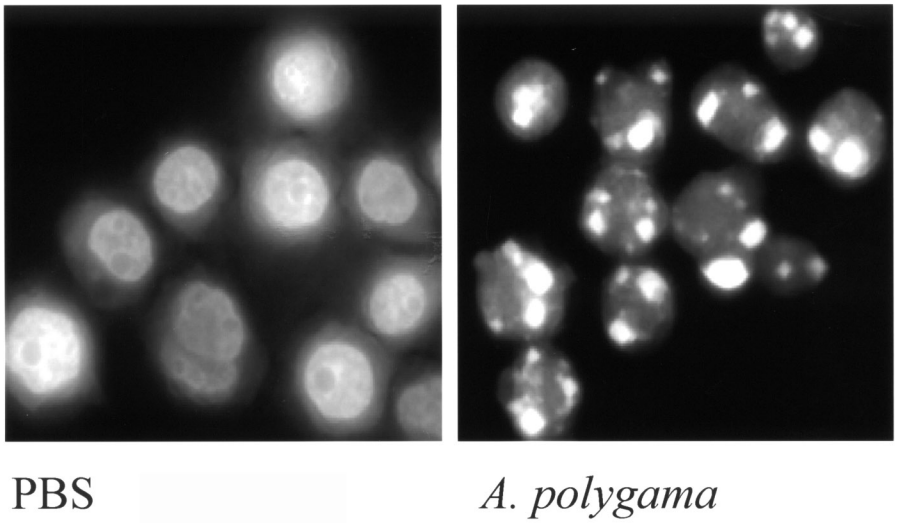

Fig. 5. Morphological Change of HL-60 Cells Treated with *A. polygama* Fruit-Extract

HL-60 cells were incubated with a final 9% of *A. polygama* fruit-extract for 2 days. Cells were fixed with glutaraldehyde and stained with Hoechst 33342. Morphology of cells was observed with a fluorescence microscopy. Left: HL-60 cells treated with PBS and right: HL-60 cells treated with *A. polygama* fruit-extract (× 400).

Table 3. Apoptosis of HL-60 Cells Detected by the TUNEL Method with Flow Cytometry

Treatment of cells	Terminal transferase	Fluorescence increased (%)	Difference (%)
A. polygama	+	49.8	49.8
	−	0.04	
Control	+	0.28	0.2
	−	0.04	

HL-60 cells were incubated with *A. polygama* fruit-extract or PBS control for one day. The TUNEL method was carried out with or without terminal deoxyribonucleotidyl transferase according to the kit directions. The labeled fluorescence on 3′-terminal was measured with flow cytometry.

agents that kill leukemia cells. On the other hand, *A. polygama* fruit-extract at low concentration stimulated LS174T cell growth slightly and stimulated growth of MC9 cells considerably. These results suggest that *A. polygama* fruit-extract may contain other agents that stimulate cell growth as well as those that inhibit leukemia cell growth. The effect of agents stimulating cell growth will be overcome with the effect of other agents inhibiting cell growth at an adequate dose. We did not adjust the osmotic pressure of cell proliferation assay solution. But, the inhibitory activity of *A. polygama* fruit-extract is not due to effects of osmotic pressure because the same concentration of the extract, 1 mg/ml, shows different inhibitory activity against HL-60, LS174T and MC9 cells (Fig. 3).

Some extracts from small fruits harvested in northern Japan showed potent inhibitory activity on HL-60 cell growth. Inhibitory agents assayed with HL-60 cells in those extracts will have different characteristics. An extract of *A. polygama* fruit had weak (Fig. 3B) or no inhibitory activity on normal human cells,[3] but an extract from *Ribes nigrum* fruit inhibited the growth of normal dermal fibroblast cells as well as the growth of HL-60 cells (data not shown). As to effective herbal medicines on cancer cells, *A. polygama* fruit-extract seems to be better than *Ribes nigrum* fruit-extract. Plant agents derived from common fruit are good candidates for healthy food or low toxic anti-cancer drugs.

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