Differentiation-Inducing Effects of Small Fruit Juices on HL-60 Leukemic Cells

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Epidemiological studies indicate that high intakes of fruits and vegetables are associated with a reduced risk of cancer, and several plant-derived drugs have been developed in medical oncology. Since only a small part of the flora has been tested for any kind of bioactivity, we chose small fruits as sources of differentiation-inducing activity against HL-60 leukemic cells. We have prepared juices from various small fruits that grow mainly in the northern part of Japan. Screening of 43 samples indicated that juices of *Actinidia polygama* Maxim., *Rosa rugosa* Thunb., *Vaccinium smallii* A. Gray, and *Sorbus sambucifolia* Roem. strongly induced differentiation of HL-60 cells to monocyte/macrophage characteristics in a concentration-dependent manner as indicated by histochemical and biochemical examinations.

Keywords: *Small fruit; differentiation; HL-60*

INTRODUCTION

It is commonly accepted that cancer formation can be prevented by the consumption of certain foods (Stavric, 1994), and epidemiological studies indicate that high intakes of fruits and vegetables are associated with a reduced risk of cancer (Steinmetz and Potter, 1991). The association is generally most marked for epithelial cancer, apparently stronger for those of the digestive and respiratory tracts, and somewhat weaker for hormone-related cancer (La Vecchia and Tavani, 1998). Several plant-derived drugs have been developed in medical oncology for the following reasons. First, administration of fruits to rodents protects against chemical carcinogenesis (Katiyar and Mukhtar, 1997). Second, many seemingly unrelated compounds including flavonoids (Tanaka et al., 1997), coumarins (Tanaka et al., 1998a,b), cinnamates (Huang et al., 1995; Asanuma et al., 1994; Rao et al., 1993), and other phenolics (Tanaka et al., 1993) can protect rodents against chemical carcinogens.

Since only a small part of the flora has been tested for any kind of bioactivity, we chose small fruits as sources of differentiation-inducing activity against HL-60, a promyelocytic leukemic cell line. The HL-60 cell line (Collins et al., 1978), established from an acute myeloid leukemia patient, provides a useful model system for studying differentiation of leukemic cells. Terminal differentiation of HL-60 can be monitored by changes of morphological, biochemical, and immunological properties (Yam et al., 1971).

We have prepared juices from various small fruit plants, which grow primarily in the northern part of Japan, especially on Hokkaido Island. These juices were subjected to screening for HL-60 differentiation-inducing activity by nitro blue tetrazolium (NBT) reducing activity. Among 43 samples, juices of *Actinidia polygama* Maxim. (Japanese name, Matatabi), *Rosa rugosa* Thunb. (Japanese name, Hamanasu), *Vaccinium smallii* A. Gray (Japanese name, Obasunoki), and *Sorbus sambucifolia* Roem. (Japanese name, Takanenanakamado) demonstrated potent activity. Nonspecific esterase, specific esterase, and phagocytic activities were also examined in order to determine the direction of HL-60 differentiation.

MATERIALS AND METHODS

Fruit Samples. All fruits were harvested from trees at the Hokkaido Forestry Research Institute, Bibai, Hokkaido, Japan in July−September 1996. Fruit was homogenized in ethanol, and the ethanolic extract was filtrated, concentrated in vacuo, and dissolved in RPMI1640 medium at a concentration of 10 mg/mL as a stock solution. The composition of RPMI1640 medium is as follows (mg/L): L-arginine, 200.0; L-asparagine, 50.0; L-aspartic acid, 20.0; L-cystine, 50.0; L-glutamic acid, 20.0; L-glutamine, 300.0; glycine, 10.0; L-histidine, 15.0; hydroxy-L-proline, 20.0; L-isoleucine, 50.0; L-leucine, 50.0; L-lysine, 40.0; L-methionine, 15.0; L-phenylalanine, 15.0; L-proline, 20.0; L-serine, 30.0; L-threonine, 20.0; L-tryptophan, 5.0; L-tyrosine, 20.0; L-valine, 20.0; glutathione, 1.0; $Ca(NO_3)_2 \cdot 4H_2O$, 100; KCl, 400.0; $MgSO_4 \cdot 7H_2O$, 100.0; NaCl, 6000; $NaHCO_3$, 2000; $Na_2HPO_4 \cdot 7H_2O$, 1512; phenol red, 5.0; biotin, 0.2; folic acid, 1.0; nicotinamide, 1.0; calcium pantothenate, 0.25; pyridoxine hydrochloride, 1.0; riboflavine, 0.2; thiamine hydrochloride, 1.0; vitamin B_{12}, 0.005; choline chloride, 3.0; D-glucose, 2000; isoinositol, 35.0; *p*-aminobenzoic acid, 1.0.

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Table 1. Differentiation-Inducing Activity of Small Fruits toward HL-60

scientific name	Japanese name	extract (mg/g of fresh fruit)	NBT-reducing cells[a] (%)	phenolics (mg/10 g of extract)	anthocyanin (mg/10 g of extract)
Actinidia arguta Planch.	Kokuwa	46.5	19.3 ± 5.4	15.6	nd[b]
Actinidia arguta Planch. cv. Issaikokuwa	Issaikokuwa	17.9	7.3 ± 3.7	50.9	nd
Actinidia kolomikta Maxim.	Miyamamatatabi	49.1	49.3 ± 7.4	32.6	0.7
Actinidia polygama Maxim.	Matatabi	22.8	77.7 ± 5.0	42.0	nd
Akebia trifoliata Koidz.	Mitsuba-akebi	30.7	33.2 ± 9.4	18.3	0.8
Alonia melanocarpa	Melanocarpa	52.4	8.3 ± 1.2	1.1	6.4
Ampelopsis brevipedunculata Trautv.	Nobudou	24.8	45.3 ± 6.7		
Chaenomeles japonica Lindl.	Kusaboke	27.9	50.8 ± 7.6	73.3	nd
Cydonia oblonga Miller	Marumero	32.4	17.0 ± 7.9	24.9	nd
Elaeagnus multiflora Thunb.	Natsugumi	88.1	7.0 ± 7.1	2.6	0.1
Elaeagnus umbellata Thunb.	Akigumi	48.7	10.3 ± 9.9	6.3	nd
Gaultheria miqueliana Takeda	Shiratamanoki	93.9	30.3 ± 5.3	6.5	0.2
Lonicera caerulea (Kiritappu, Japan)	Hasukappu	71.4	33.8 ± 5.6	22.0	40.3
Lonicera caerulea (Taiki-cho, Japan)	Hasukappu	26.6	24.7 ± 4.2	35.4	49.3
Lonicera caerulea (Tomato-highbush, Japan)	Hasukappu	78.8	28.8 ± 8.5	25.8	45.3
Lonicera caerulea (Tomato-prostrate, Japan)	Hasukappu	29.6	23.7 ± 8.2	21.3	21.6
Lonicera caerulea (China)	Hasukappu	65.3	25.7 ± 8.0	17.8	29.9
Lonicera caerulea (Taisetsu, Japan)	Keyonomi	12.6	17.7 ± 0.5	30.2	65.1
Lonicera caerulea (Yokotsu-dake, Japan)	Keyonomi	61.7	49.0 ± 0	24.0	47.4
Lonicera caerulea (Bihoro-toge, Japan)	Keyonomi	31.2	19.7 ± 3.1	30.7	50.5
Lonicera morrowii A. Gray	Hyoutanboku	63.2	27.5 ± 9.2	16.8	0.5
Malus baccata var. mandshurica C. K. Schn.	Ezonokoringo	89.4	10.3 ± 4.9	16.4	0.0
Prunus salicina Lindl.	Sumomo	66.9	15.2 ± 4.1	6.3	0.7
Ribes grossularia (collected on 7/27/96)	Marusuguri	47.9	21.3 ± 6.5	7.4	0.2
Ribes grossularia (collected on 8/8/96)	Marusuguri	115.9	26.3 ± 2.5	7.0	0.1
Ribes idaeus	European Ki-ichigo	59.2	38.3 ± 10.4	8.7	0.1
Ribes japonicum Maxim.	Komagatakesuguri	50.2	10.0 ± 5.9	11.5	0.2
Ribes latifolium Jancz.	Ezosuguri	38.9	8.0 ± 2.9		
Ribes nigrum (small fruit-producing line)	Kurosuguri	36.9	65.0 ± 9.8	27.8	1.7
Ribes nigrum (large fruit-producing line)	Kurosuguri	58.8	9.3 ± 1.7	9.9	0.2
Rubus phoenicolasius Maxim. (collected on 8/8/96)	Ebigaraichigo	22.9	31.3 ± 6.8	10.0	0.1
Rubus phoenicolasius Maxim. (collected on 7/27/96)	Ebigaraichigo	41.4	29.5 ± 10.4		
Ribes rubrum	Fusasuguri	28.1	20.7 ± 9.6	12.1	6.2
Rosa rugosa Thumb.	Hamanasu	44.4	77.7 ± 9.9	26.0	0.5
Rubes mesogaeus Focke	Kuroichigo	58.6	7.3 ± 1.2	17.3	5.0
Rubus parvifolius	Nawashiroichigo	60.3	64.7 ± 10.8	30.3	8.2
Schizandra chinensis Baill.	Chosengomishi	169.4	44.8 ± 7.4	6.5	1.6
Sorbus sambucifolia M. Roemer	Takanenanakamado	92.1	65.3 ± 8.6	73.3	0.2
Taxus baccata	European ichii	108.1	14.0 ± 4.3	4.0	0.2
Vaccinium smallii A. Gray	Obasunoki	57.2	75.5 ± 5.4	50.2	91.7
Vaccinium praestans Lamb.	Iwatsutsuji	98.5	52.8 ± 5.6	2.9	0.5
Viburnum opulus var. sargentii Takeda	Kanboku	39.1	52.7 ± 7.0	157.6	3.5
Viburnum wrightii Miq.	Miyamagamazumi	17.7	16.3 ± 4.6	24.8	2.1
blank			14.5 ± 5.0		

[a] NBT-reducing assay was done at a concentration of 5 mg/mL. [b] nd, not detected.

Cell Differentiation Assay. HL-60 cells, obtained from the Riken Gene Bank (Tsukuba, Japan), were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (BioWhittaker Inc., Walkersville, MD). HL-60 cells in log phase (approximately 10^6 cells/mL) were diluted to 1.2×10^5 cells/mL and preincubated for 18 h in 24-well plates (approximately 2×10^5 cells/mL). Samples were then added. After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting morphological and biochemical differentiation.

The differentiated HL-60 phenotype is characterized by nitro blue tetrazolium (NBT) reducing, nonspecific esterase, specific esterase, and phagocytic activities. NBT reduction, nonspecific esterase, and phagocytosis are positive in HL-60 cells, which are induced to monocytes/macrophages, whereas HL-60 cells, differentiated to monocyte/granulocytes, increase NBT-reducing and specific esterase activities. For an NBT-reducing assay, a 1:1 (v/v) mixture of a cell suspension (10^6 cells/500 μL) and freshly prepared 12-*O*-tetradecanoylphorbol 13-acetate (TPA)/NBT solution (phosphate-buffered saline solution containing 1 mg/mL NBT and 1 μg/mL TPA) was incubated for 15 min at 37 °C. Cells were then smeared on a glass slide and counterstained by 0.25% (w/v) safranin O in 10% ethanol. Differentiated cells, which showed an intracellular black-blue formazan deposit, were examined by counting a minimum of 200 cells in triplicate for each experiment. For determination of nonspecific and specific esterase activities, assays by α-naphthyl acetate esterase (nonspecific acid esterase, NSE) and by naphthyl AS-D chloroacetate esterase (specific acid esterase, SE) were done using cytochemical kits from Sigma Chemical Co. (91-A and 91-C, St. Louis, MO). Differentiated cells were examined by counting a minimum of 200 cells in triplicate for each experiment.

Phagocytic activity was assayed by using polystyrene latex particles (average diameter, 0.81 μm, Difco Lab., Detroit, MI) that were suspended in RPMI1640 medium at a concentration of 10^9 particles/mL. Treated HL-60 cells were washed, suspended in RPMI1640 containing 20% AB serum (prepared from human type AB plasma, BioWhittaker, Inc., Walkersville, MD) at a final concentration of 2×10^6 cells/mL. A 1:1 mixture of the latex particle and HL-60 cell suspensions was incubated for 4 h at 37 °C. Phagocytic activity was determined by counting cells that ingested the latex particles with a hemacytometer using a minimum of 200 cells.

Cell Proliferation Assay. The level of cell proliferation was measured by using Alamar Blue (Biosource International, Lewisville, TX), an oxidation−reduction indicator. The level of proliferation was measured for HL-60 cells grown in 96-

well microtiter plates. Inhibition of cellular proliferation (% of untreated control) was calculated with the following equation:

$$\text{inhibition (\%)} = 100 - \frac{[(A_{570} - A_{595}) \text{ of test agent dilution}] - [(A_{570} - A_{595}) \text{ of blank}]}{[(A_{570} - A_{595}) \text{ of positive growth control}] - [(A_{570} - A_{595}) \text{ of blank}]} \times 100 \quad (1)$$

where A_{570} and A_{595} are the absorbances at 570 and 595 nm, respectively.

Total Phenolics. The total phenolics were determined with Folin−Ciocalteu reagent primarily according to the method described in the literature (Slinkard and Singleton, 1977; Prior et al., 1998). We used 100 μL of 1/10 or 1/20 diluted sample or 50, 40, 30, 20, and 10 mg/L and a 0-blank of standard series of gallic acid solutions plus 500 μL of 1/10 diluted Folin−Ciocalteu stock reagent, followed after 5 min by the addition of 400 μL of 7.5% (w/v) Na_2CO_3 solution. After 2 h at room temperature, the absorbance at 765 nm was read. Results were expressed as milligrams of gallic acid equivalent/10 g of ethanol extract.

Total Anthocyanin. The total anthocyanin was estimated by a pH differential method (Cheng and Breen, 1991). Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and pH 4.5, using $A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$ and a molar extinction coefficient of cyanidin-3-glucoside of 29 600. Results were expressed as milligrams of cyanidin 3-glucoside equivalent/10 g of ethanol extract.

Statistical Analysis. One-way ANOVA, according to the Tukey−Kramer honestly significant difference test (Ludbrook, 1991), was applied to the variables that could contribute to find significant effects on HL-60 cell growth and differentiation. The statistical significance at $p = 0.05$ was estimated by the actual absolute difference in the means minus the least significant difference, which is the difference that would be significant.

RESULTS

Table 1 summarizes the effects of small fruit juices on the induction of HL-60 cell differentiation at a concentration of 5 mg/mL. Among 43 samples, juices of *Actinidia polygama* Maxim. (Japanese name, Matatabi), *Ribes nigrum* (Japanese name, Kurosuguri, the small-fruit-producing line), *Rosa rugosa* (Japanese name, Hamanasu), *Rubus parvifolius* (Japanese name, Nawashiroichigo), *Sorbus sambucifolia* M. Roem. (Japanese name, Takanenanakamado), and *Vaccinium smallii* Roem. (Japanese name, Obasunoki) demonstrated potent differentiation-inducing activity toward HL-60. The geographic growth location of *Lonicera caerulea* var. emphyllocalyx Nakai (Japanese name, Keyonomi) was indicated to have some influence on HL-60 differentiation-inducing activity (range 17.7 ± 0.5−49.0 ± 0%). *L. caerulea* grown at Mt. Yokotsu-dake had a most potent activity among *L. caerulea* samples examined in this study. Table 1 also shows that morphology of fruits had an influence on the activity of *Ribes nigrum* L. (Japanese name, Kurosuguri). The juice prepared from the small-fruit-producing line had a potent differentiation-inducing activity (65.0 ± 9.8%), whereas the large-fruit-producing line did not show any activity (9.3 ± 1.7%).

The relationship between NBT-reducing activity and the total phenolics or total anthocyanin content is shown in Figures 1 and 2. A positive correlation was observed between NBT-reducing activity and the total phenolic or total anthocyanin content. The correlation coefficient was much higher between NBT-reducing activity and the total phenolics ($r = 0.4292$) as compared to NBT-reducing activity and the total anthocyanin ($r = 0.1352$).

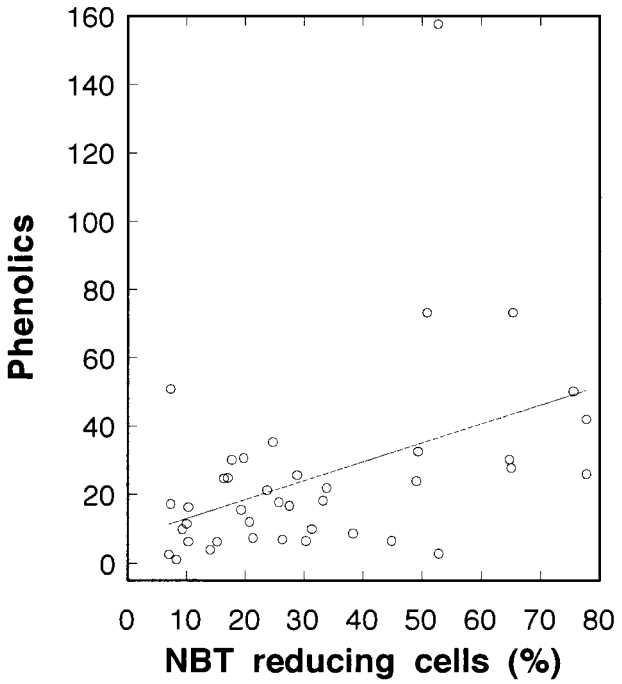

Figure 1. Relationship between the total phenolics content and NBT-reducing activity.

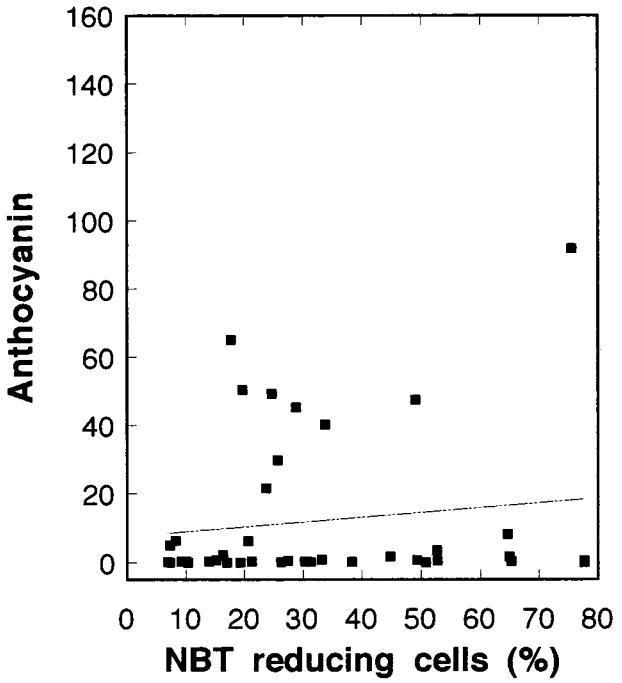

Figure 2. Relationship between the total anthocyanin content and NBT-reducing activity.

Concentration−response effects of *A. polygama* (panel A), *R. rugosa* (panel B), *V. smallii* (panel C), and *S. sambucifolia* (panel D) on induction of HL-60 differentiation is shown in Figure 3. The differentiation-inducing activity was monitored by NBT-reducing activity, specific and nonspecific esterase activities, and phagocytic activity to determine the direction of HL-60 cellular differentiation. These juices appeared to induce monocyte/macrophage characteristics since HL-60 cells treated with these compounds showed NBT-reducing activity, nonspecific esterase activity, and phagocytic activity in a concentration-dependent manner, whereas they did not express any naphthyl AS-D chloroacetate

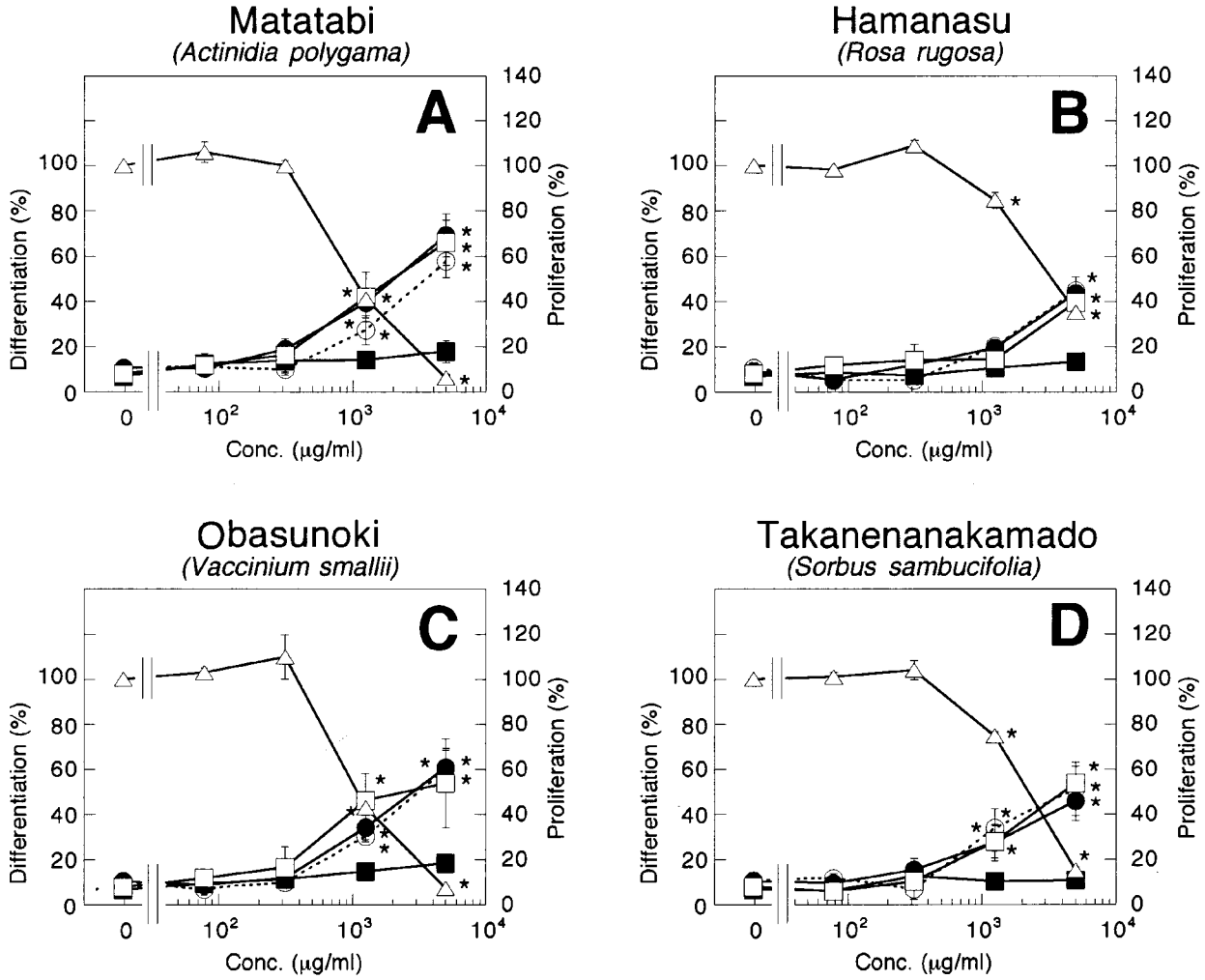

Figure 3. Concentration−response of the juices of *Actinidia polygama* Maxim. (A), *Rosa rugosa* Thunb. (B), *Vaccinium smallii* A. Gray (C), and *Sorbus sambucifolia* Roem. (D) on HL-60 differentiation-inducing activity. Nitro blue tetrazolium reducing activity (●), nonspecific esterase activity (○), specific esterase activity (■), phagocytic activity (□), and cellular proliferation (□). Each point represents the mean of triplicate experiments. Vertical bars indicate standard deviations. Asterisks indicate significant difference ($P < 0.05$) after assessment by one-way ANOVA (Tukey−Kramer honestly significant difference test).

esterase activity. Incubation of HL-60 cells with these samples also induced cellular adhesion to the surfaces. These three assays correlated each other; these samples induced similar percentages of monocyte/macrophage cells by all assays (Figure 3). Following treatment with *A. polygama*, *R. rugosa*, and *V. smallii*, approximately 60% of HL-60 cells were differentiated at a concentration of 5×10^3 μg/mL. *S. sambucifolia* was less active than these three samples, inducing 50% of HL-60 to be differentiated at the same concentration.

There was a decrease in cellular proliferation, indicating that these samples suppressed HL-60 cell growth. After a 4-day treatment, *A. polygama*, *R. rugosa*, *V. smallii*, and *S. sambucifolia* at 5×10^3 μg/mL inhibited proliferation 94, 65, 93, and 51%, respectively.

DISCUSSION

This study examined 43 juices from small fruits, growing mainly in the northern part of Japan, for their differentiation-inducing activity against HL-60. Among them, juices of *Actinidia polygama* Maxim. (Japanese name, Matatabi), *Ribes nigrum* L. (Japanese name, Kurosuguri, the small-fruit-producing line), *Rosa rugosa* (Japanese name, Hamanasu), *Rubus parvifolius* (Japanese name, Nawashiroichigo), *Sorbus sambucifolia* M. Roem. (Japanese name, Takanenanakamado), and *Vaccinium smallii* Roem. (Japanese name, Obasunoki) demonstrated marked activity on HL-60 differentiation.

Induction of differentiation of HL-60 is commonly accepted to have a potentially therapeutic importance (Breitman et al., 1980; Honma et al., 1980; Tanaka et al., 1982; Koeffler et al., 1984; Harris et al., 1985; Reiss et al., 1986; Zhou et al., 1989; Degos, 1990). Cell growth inhibition by cytotoxic agents originates directly from slowing the cellular proliferation, whereas decrease of cellular replication by inducers of HL-60 differentiation results primarily from maturation-related growth arrest (Reiss et al., 1986). For example, retinoic acid, an effective inducer of HL-60 differentiation, is clinically effective against acute promyelocytic leukemia (Menger et al., 1988). Retinoic acid also exerts its inhibitory activity toward small cell lung cancer (SCLC) by up-regulation of L-myc gene expression through stimulation of transcript initiation, and it is suggested that the biological effects of retinoic acid in SCLC cells may be mediated through the retinoic acid-receptor β-dependent pathway (Ou et al., 1996). However, these therapeutic actions are short-lived and accompanied by a variety of side effects (Kamm, 1982; Orfanos et al., 1987). Thus,

it is desirable to discover more potent inducers of HL-60 differentiation with fewer side effects.

Fruits are good sources of anticancer substances, and considerable attention has been focused on vitamin C, vitamin E, and β-carotene contents of fruits. However, fruits may also contain many other substances that have anticancer activity with fewer side effects. Seeking additional substances from plants, which exhibit activity against cancer, has been intensified recently since only a small part only of the flora has been tested for any kind of bioactivity (Booth, 1987). The beneficial effects of fruits may be due, in part, to flavonoids, coumarins, cinnamates, and other phenolics. These compounds are present in high concentrations in human foods derived from fruits and vegetables, and they have been shown to have anticancer activity.

An extract of the fruit of *Actinidia polygama* (Japanese name, Matatabi) demonstrated the most potent HL-60 differentiation-inducing activity among small fruit samples examined in this study, and *A. kolomikata* Maxim. (Japanese name, Miyamamatatabi) showed moderate activity. Members of the genus *Actinidia* are frequently used as an herbal medicine, and *A. deliciosa* (kiwifruit) is a horticultural crop. *A. chinensis* has been reported to have antimutagenic activity (Lee and Lin, 1988), and a new polysaccharide having antitumor effects on murine tumors has been isolated from the root of *A. chinensis* (Lin, 1988).

Vaccinium fruits have been reported to have anticancer activity (Bomser et al., 1996). The proanthocyanidin fraction exhibits anticarcinogenic activity as evaluated by in vitro screening test. The crude extract of *Vaccinium* fruits shows scavenging activity toward chemically generated superoxide radicals and an inhibitory activity against xanthine oxidase (Costantino et al., 1992).

Figure 1 and Table 1 show that the HL-60 differentiation inducing activity of various small fruits could partly be accounted for by the total phenolic content. Samples, which contained relatively high content of phenolics, namely *A. polygama*, *Rosa rugosa*, *Rubus parvifolius*, *S. sambucifolia*, and *V. smallii*, demonstrated potent HL-60 differentiating activity. The results of phenolic assay shows the difference of the total phenolic content between the small-fruit-producing and large-fruit-producing lines of *Ribes nigrum*, thus suggesting the possible role of phenolics for the NBT-reducing activity. On the other hand, the total phenolic or total anthocyanin content, however, could not explain the difference of NBT-reducing activity of *Lonicera caerulea* var. emphyllocalyx Nakai (Japanese name, Keyonomi), which had been originally collected at different sites.

In conclusion, with this screening procedure several fruit juices from small fruits growing in northern part of Japan exhibit some biological activity. A massive screening for anticancer substances in foods may lead to discovery and development of new agents suitable for differentiation therapy of leukemia and other types of cancer with fewer side effects.

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