Note

Enin Isolated from Skin of Red Grape (*Vitis vinifera*) Induces Apoptosis in Human Lymphoid Leukemia Molt 4B Cells

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We have investigated the effect of extract prepared from skin of red grape (*Vitis vinifera*) on the growth of human lymphoid leukemia Molt 4B cells. It was found that the extract obtained by extraction with butanol suppressed the growth of Molt 4B cells. Fraction IV obtained by separation with HPLC inhibited the growth of the Molt 4B cells by 86% at the concentration of 600 μ M. We determined the structure of compound contained in Fraction IV and identified it as enin, a kind of anthocyanin, by ¹H- and ¹³C-NMR. The enin-treated cells clearly exhibited morphological changes, indicating the blebbing and chromatin condensation which are characteristic of apoptosis. Characteristic oligonucleosomal-sized DNA fragments were observed in the enin-treated cells. These findings suggest that the suppression by enin of growth of Molt 4B cells results from the induction of apoptosis by this compound.

Keywords: enin, growth inhibition, apoptosis, leukemia, skin of red grape (*Vitis vinifera*)

We purified several compounds from plants and evaluated the antitumor activity of those compounds. In previous papers we demonstrated the inhibitory effects of persimmon extract (Achiwa *et al.*, 1997), oleanolic and ursolic acids isolated from leaves of loquat (Komiya *et al.*, 1998), and sesamol isolated from sesame oil (Miyahara *et al.*, 2000) on the growth of human lymphoid leukemia Molt 4B cells, respectively. Surh *et al.* (1999) reported that resveratrol, a triphenolic stilbene present in grapes, induced apoptosis in human promyelocytic leukemia (HL-60) cells.

In the present study, we obtained fractions having antiproliferative activity from the skin of red grape (*Vitis vinifera*) and determined the structures of the compounds contained in these fractions. In this report, we identify enin and gallic acid from the skin of red grape and report that the inhibitory effects of enin on the growth of human lymphoid leukemia Molt 4B cells results from the induction of apoptosis.

Materials and Methods

Chemicals All chemicals were of the highest grade available from Wako Chemicals, Kyoto

Apparatus ¹H- and ¹³C- NMR spectra were taken with JEOL, JNM- δ 500. For preparation of enin and gallic acid as described in the next item, high pressure liquid chromatography (HPLC) was performed with a JASCO 880-PV.

Isolation and identification of enin Grape skin colored powder was made from the red grape skin of *Vitis vinifera*, an expressly selected grape growing in a restricted area of Emilia in Italy. It was prepared by the aqueous extraction of the fresh deseeded marc remaining after grapes had been pressed to pro-

duce juice or wine. During the steeping process, sulfur dioxide was added as a preservative, and most of the extracted sugars were fermented to alcohol. The extract was concentrated by vacuum evaporation, during which practically all of the alcohol was removed, and clarified by slow centrifugation. A small amount of sulfur dioxide (less than 3 g/kg of grape skin colored powder) remained as residue. The resulting supernatant was transformed into a magenta-red powder through a spray-drying process, and the obtained powder (1.5 g) was dissolved in distilled water. The resulting solution is called a water-soluble fraction and is used for DNA fragmentation assay and extracted with butanol containing 0.1% trifluoroacetic acid (TFA). The butanol fraction having antiproliferative activity was separated into four fractions of I (16.1 mg), II (22.5 mg), III (15.4 mg) and IV (30.4 mg) by preparative HPLC with ODS-5 (Nomura Chemicals, Aichi) using the following mobile phases: solution A, distilled water containing 0.1% TFA; solution B, methanol containing 0.1% TFA in a three-step linear gradient starting from 100% solution A up to 100% solution B in 80 min. The flow rate was 1.5 ml/min. Chromatograms by HPLC were recorded at 280 nm (Fig. 1). Fractions I and IV were purified and identified as gallic acid and enin, respectively, by comparison of the spectral ¹H- and ¹³C-NMR data with authentic samples. Chemical structures of gallic acid and enin, and spectral ¹³C-NMR and ¹H-NMR data for Fraction I (gallic acid) and Fraction IV (enin), respectively, are shown in Fig. 2.

Cell culture Human lymphoid leukemia Molt 4B cells were obtained from Dainippon Pharmaceutical Co., Osaka, and cultivated as previously described (Hibasami *et al.*, 1989). Molt 4B cells were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) with 10% fetal calf serum, penicillin G (50 IU/ml) and streptomycin (50 μ g/ml) at 37°C in a humidified

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95% air-5% CO₂ atmosphere and passaged every 7 days. Mycoplasma testing was routinely negative.

Fig. 1. HPLC pattern of butanol fraction from red grape skin extract. Butanol fraction was separated by preparative HPLC with ODS-5 column as described in Materials and Methods.

structure of gallic acid

¹³C- and ¹H-NMR data (in CD₃OD) for F I

No.	¹³ C-NMR (ppm)	¹ H-NMR (ppm)
1	121.9	
2,6	110.2	6.99
3,5	146.4	
4	139.6	
7	170.4

structure of enin

¹³C-NMR and ¹H-NMR (in CD₃OD) for F4

No.	¹³ C-NMR (ppm)	¹ H-NMR (ppm)
1	163.0	
2	146.0	
3	136.8	8.94
4	159.1	
5	163.4	6.56
6	170.1	
7	95.4	6.86
8	157.2	
9	113.3	
1'	119.8	
2',6'	110.5	7.87
3',5'	149.7	
7',8'	57.2	3.93
4'	145.7	
1''	103.7	5.26
2''	75.0	3.54
3''	78.2	
4''	71.1	
5''	78.0	
6''	62.3	3.88

Fig. 2. Chemical structures of gallic acid and enin, and spectral ¹³C-NMR and ¹H-NMR data for F I (gallic acid) and F IV (enin), respectively.

into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) containing 2 mM EDTA.

Results and Discussion

The effect of extract obtained by extraction with butanol from

red grape skin on the growth of human lymphoid leukemia Molt 4B cells was investigated. Fraction IV obtained by HPLC inhibited the proliferation of Molt 4B cells dose-dependently. The compound contained in the fraction was determined by NMR and identified as enin, an anthocyanin present in red grape. Another

Table 1. Effects of enin on the growth of Molt 4B cells.

Compound	Concentration (μM)	Inhibition rate (%)
Enin	0	—
	200	27.8±0.8
	400	57.2±2.6 ^{a)}
	600	86.3±5.2 ^{a)}
	1000	98.1±8.9 ^{a)}

Each value is the mean±SD of triplicate experiments.
^{a)}*p*<0.01 versus the control (without enin).

Fig. 3. Morphological changes of Molt 4B cells. (A) non-treated Molt 4B cells. (B) Molt 4B cells treated with 600 μM enin. These cells were cultivated with enin for 3 days, fixed with 1% glutaraldehyde, stained with Hoechst 33258, and then observed under an epifluorescence microscope equipped with a cooled CCD camera (photometrics, P×L 1400) digital imaging system and Fuji pictography 3000.

Fig. 5. Dose-dependency of induction of apoptosis by enin in Molt 4B cells. The cells were cultivated in the presence of vehicle for 3 days (lane 1), or in the presence of 200 (lane 2), 600 (lane 3), and 1000 (lane 4) μM enin for 3 days. After the isolation of DNA from the enin-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5 containing 2 mM EDTA. Arrows indicate DNA ladders. M: λDNA digested with Hind III.

Fig. 4. (A) Dose-dependency of induction of apoptosis by water-soluble fraction from red grape skin. The cells were cultivated in the absence (lane 1), or presence of water-soluble fraction of 1 mg/ml (lane 2), 2 mg/ml (lane 3), and 4 mg/ml (lane 4). (B) Time-course of induction of apoptosis by water-soluble fraction from red grape skin. The cells were cultivated in the absence (lane 1), or presence for 1 day (lane 2), 2 days (lane 3), and 3 days (lane 4). After the isolation of DNA from the cells treated with the water-soluble fraction, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5 containing 2 mM EDTA. Arrows indicate DNA ladders. M: λDNA digested with Hind III.

Fig. 6. Time-course of induction of apoptosis by enin in Molt 4B cells. The cells were cultivated in the presence of vehicle for 2 days (lane 1), or in the presence of 600 μM enin for 1 day (lane 2) and 2 days (lane 3). After the isolation of DNA from the enin-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid, pH 7.5 containing 2 mM EDTA. Arrows indicate DNA ladders. M: λ DNA digested with Hind III.

compound contained in Fraction I was determined by the same method and identified as gallic acid. The chemical structures of enin and gallic acid are shown in Fig. 2.

Enin and gallic acid were thus isolated from the skin of red grape (*Vitis vinifera*). We investigated the effects of enin on the growth of human lymphoid Molt 4B cells. As shown in Table 1, rate of growth inhibition of these cells by enin rose as the concentration of enin was increased.

The significant growth-inhibitory activity of the water-soluble fraction and enin led us to investigate whether part of the antitumor effect was the result of apoptosis induction. Morphological change showing apoptotic bodies and fragmentation of genomic DNA into oligonucleosomal-sized fragments are characteristic of the occurrence of apoptosis. The morphology of enin-treated cells showed apoptotic bodies after 3 days of treatment with enin (Fig. 3B). As shown in Fig. 4, the amount of oligonucleosomal-sized fragments in the Molt 4B cells treated with the water-soluble fraction from red grape skin was increased as the concentration of the fraction was increased from 1 mg/ml to 4 mg/ml (A),

and as the cultivation time was increased from 1 to 3 days (B). The amount of oligonucleosomal-sized fragments in the Molt 4B cells treated with enin increased as the concentration of enin was increased from 200 to 1000 μM (Fig. 5) and as the cultivation time was increased from 1 to 2 days (Fig. 6). Thus, enin induced apoptosis in a dose- (from 200 to 1000 μM) and time-dependent (from 1 to 2 days) manner. In contrast, we observed no induction of apoptosis by 1000 μM enin in leukocytes prepared from healthy volunteers (data not shown).

The search for better cancer chemotherapeutic agents as well as foods that protect against cancer is ongoing. However, there are as yet no anticancer drugs which are free of side effects. The present study demonstrates that enin isolated from red grape skin has a significant growth inhibitory effect on human leukemia cells; this is the first evidence that enin can induce apoptosis in the Molt 4B cells. The induction of apoptosis by gallic acid in lung cancer cells was reported by Ohno *et al.* (1999). In our experiment, oligonucleosomal-sized DNA fragmentation resulted from the continuous exposure of Molt 4B cells to enin. Our findings demonstrate that enin might exert antitumor activity by triggering apoptosis.

References

- Achiwa, Y., Hibasami, H., Katsuzaki, H., Imai, K. and Komiya, T. (1997). Inhibitory effect of persimmon (*Diospyros kaki*) extract and related polyphenol compounds on growth of human lymphoid leukemia cells. *Biosci. Biotechnol. Biochem.*, **61**, 1099–1101.
- Hibasami, H., Maekawa, S., Murata, T. and Nakashima, K. (1989). Antitumor effect of a new multienzyme inhibitor of polyamine synthetic pathway, methylglyoxal-bis(cyclopentylamidino)hydrazine, against human and mouse leukemia cells. *Cancer Res.*, **49**, 2065–2068.
- Komiya, T., Achiwa, Y., Katsuzaki, H., Imai, K., Adachi, T., Yamada, T. and Hibasami, H. (1998). Effect of oleanolic- and ursolic acids isolated from loquat (*Eriobotrya*) on the growth of human lymphoid leukemia cells. *Food Sci. Technol. Res.*, **4**, 282–284.
- Miyahara, Y., Hibasami, H., Katsuzaki, H., Imai, K. and Komiya, T. (2000). Sesamol induces apoptosis in human lymphoid leukemia Molt 4B cells. *Food Sci. Technol. Res.*, **6**, 201–203.
- Mori, K., Hibasami, H., Satoh, N., Sonoda, J., Uchida, A. and Nakashima, K. (1997). Induction of apoptotic cell death in three human osteosarcoma cell lines by a polyamine synthesis inhibitor, methylglyoxal bis (cyclopentyl-amidino)hydrazine (MGBCP). *Anticancer Res.*, **17**, 2385–2390.
- Ohno, Y., Fukuda, K., Takemura, G., Toyota, M., Watanabe, M., Fujiwara, T. and Fujiwara, H. (1999). Induction of apoptosis by gallic acid in lung cancer cells. *Anti-Cancer Drugs*, **10**, 845–851.
- Okumura, K., Nogami, M., Taguchi, H., Hisamatsu, H. and Tanaka, K. (1995). The genes for the HC3 and β -type HC5 subunits of human proteasome map to chromosomes 6q27 and 7q12-p13 by fluorescence in situ hybridization. *Genomics*, **27**, 377–379.
- Surh, Y.-J., Hurh, Y.-J., Kang, J.-Y., Lee, E., Kong, G. and Lee, S.J. (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Lett.*, **140**, 1–10.