Induction of apoptosis in human leukemia U937 cells by anthocyanins through down-regulation of Bcl-2 and activation of caspases

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Abstract. Anthocyanins are a class of flavonoids, widely spread throughout the plant kingdom, that exhibit important anti-oxidant and anti-inflammatory actions as well as chemotherapeutic effects. However, little is known concerning the molecular mechanisms by which these activities are exerted. In this study, we investigated the anthocyanins isolated from *Vitis coignetiae* Pulliat for their potential anti-proliferative and apoptotic effects on human leukemia U937 cells. It was found that these anthocyanins inhibit cell viability and induce apoptotic cell death of U937 cells in a dose-dependent manner, as measured by hemocytometer counts, by alteration in the mitochondrial membrane potential, by increases in sub-G1 populations and by DNA ladder formation. Apoptosis of U937 cells by anthocyanins was associated with modulation of expression of Bcl-2 and IAP family members. Consequently, anthocyanin treatment induced proteolytic activation of caspase-3, -8 and -9, and a concomitant degradation of poly(ADP-ribose) polymerase. However, anthocyanin-induced growth inhibition and apoptosis were significantly attenuated in Bcl-2 overexpressing U937 cells. Furthermore, z-DEVD-fmk, a caspase-3 specific inhibitor, blocked apoptosis and increased the survival of anthocyanin-treated U937 cells. Taken together, these results show that Bcl-2 and caspases are key regulators of apoptosis in response to anthocyanins in human leukemia U937 cells.

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Introduction

Apoptosis (programmed cell death) is an active form of cell death that plays a crucial role in the normal development and differentiation of multicellular organisms. It is charac-terized by a highly stereotypical series of morphological and biological changes, such as cytoplasmic shrinkage, blebbing of the plasma membrane, chromatin condensation and DNA degradation (1,2). Apoptosis is a fundamental cellular activity that is essential for maintaining the physiological balance of an organism. It is involved in the immune defense machinery and plays an important role as a protective mechanism against carcinogenesis by eliminating damaged cells or preventing the abnormal proliferation of cells (3). In general, depending on the cell type or trigger, apoptosis can be initiated by an extrinsic pathway or an intrinsic pathway. In the former case, plasma membrane death receptors are involved and the apoptosis signal is provided by the interaction between the ligand and death receptor. The intrinsic pathway can be triggered by changed in mitochondrial integrity by a broad range of physical and chemical stimuli (4,5). Besides the morphological and biological changes, many other biomarkers and events can be used for precise determination of the type of apoptosis.

Phytochemicals have recently received much attention as potential chemopreventive and chemotherapeutic agents.

Among these naturally coccurring plat compounds, much attention has been given to those with established antioxidant activities and which show less cellular toxicity (6-9). These substances have shown much promise for cancer prevention and treatment in preclinical models and clinical trials (10,11). Anthocyanins are a class of naturally occurring phenolic compounds named flavonoids that impart color to fruits, vegetables, and other plants (12). Recent reports have evidenced the importance of anthocyanins as dietary antioxidants for prevention of oxidative damage (12-15). Anthocyanins possess known pharmacological properties, including scavenging effects on activated carcinogens and mutagens and effects on cell cycle regulation, and have been used for therapeutic purposes (12-17). Although anthocyanins have attracted much attention for their possible benefits, little is known regarding their anti-cancer action. Furthermore, how the known molecular mechanisms and signaling pathways initiated by anthocyanins may relate to apoptosis is poorly understood.

In the present study, we examined the anti-proliferative activity of anthocyanins isolated from *Vitis coignetiae* Pulliat along with their effects on the apoptosis of human monocytic leukemia U937 cells. Furthermore, the levels of several important genes that are strongly associated with the signal transduction pathway of apoptosis were assayed in order to establish the potential anti-cancer mechanism of anthocyanins as chemopreventive agents.

Materials and methods

Cell culture and viability assay. The human leukemia cell line U937 was obtained from the American Type Culture Collection (Rockville, MD), and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD) at 37˚C and 5% CO_2. Anthocyanins isolated from *V. coignetiae* Pulliat were a generous gift from Dr S.C. Shin (Department of Chemistry, Gyeongsang National University, Korea) and 100 mg/ml concentration stock solution was made by dissolving anthocyanins in distilled water. The composition of the anthocyanin mixture was as follows: delphinidin-3,5-diglucoside:cyanidin-3,5-diglucoside:petunidin-3,5-diglucoside:delphinidin-3-glucoside:malvdin-3,5-diglucoside:peonidin-3,5-diglucoside:cyanidin-3-glucoside:petunidin-3-glucoside:peonidin-3-glucoside:malvidin-3-glucoside = 27:63:8.27:1:2.21:6.7:1.25:5.72:1.25. For the cell viability assay, the cells were seeded onto 24-well plates at a concentration of $1x10^5$ cells/ml and treated with anthocyanins for 48 h. The number of surviving cells was then counted using the trypan-blue exclusion method.

Nuclear staining with DAPI. After the anthocyanin treatment, the cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS, and stained with a 4,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., St. Louis, MO) solution for 10 min at room temperature. The cells were then washed twice with PBS and analyzed by fluorescence microscopy (Carl Zeiss, Germany).

Agarose gel electrophoresis for DNA fragmentation assay. The cells were lysed in a buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. The lysates were vortexed, then cleared by centrifugation at 10,000 g for 20 min. The DNA in the supernatant was extracted using a 25:24:1 (v/v/v) equal volume of neutral phenol:chloroform:isoamyl alcohol (Sigma) and analyzed electrophoretically on 1.2% agarose gel containing 0.1 μg/ml ethidium bromide (EtBr, Sigma).

Flow cytometry analysis for measurement of sub-G1 phase. The DNA content of the cells was measured using a DNA staining kit (CycleTest™ Plus Kit, Becton-Dickinson, San Jose, CA). Propidium iodide (PI)-stained nuclear fractions were obtained using the instructions provided in the kit. Flow cytometric analyses were carried out using a FACS flow cytometer (FACSCalibur, Becton-Dikinson) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence (18).

RNA extraction and reverse transcription-PCR. Total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA) and primed with random hexamers to synthesize the complementary DNA using AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out using a Mastercycler (Eppendorf, Hamburg, Germany) with the primers shown in Table I. The following conditions were used for the PCR reactions: 1X (94˚C for 3 min); 35X (94˚C for 45 sec; 58˚C for 45 sec; and 72˚C for 1 min) and 1X (72˚C for 10 min). The amplification products obtained by PCR were separated electrophoretically on 1% agarose gel and visualized by EtBr staining (19).

Gel electrophoresis and Western blot analysis. The cells were harvested, lysed, and the protein concentrations were quantified using a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA), according to the procedure reported by the manufacturer. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electroblotting. The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham). Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Calbiochem (Cambridge, MA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Assay of caspase-3, -8 and -9 activity. The enzymatic activity of the caspases induced by anthocyanins was assayed using colorimetric assay kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Briefly, the cells were lysed in a lysis buffer for 30 min on an ice bath. The lysed cells were centrifuged at 12, 000 g for 10 min, and 100 μg of the protein was incubated with 50 μl of a reaction buffer and 5 μl of the colorimetric tetrapeptides, Asp-Glu-Val-Asp

Table 1. Gene-specific primers for RT-PCR.

Name		Sequence of primers
Bax		
	Sense	5'-ATG-GAC-GGG-TCC-GGG-GAG-3'
	Antisense	5'-TGG-AAG-AAG-ATG-GGC-TGA-3'
Bcl-2		
	Sense	5'-CAG-CTG-CAC-CTG-ACG-3'
	Antisense	5'-GCT-GGG-TAG-GTG-CAT-3'
GAPDH		
	Sense	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
	Antisense	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

(DEVD)-p-nitroaniline (pNA) for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8 and Leu-Glu-His-Asp (LEHD)-pNA for caspase-9, respectively, at 37˚C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Measurement of loss of mitochondrial membrane potential (MMP). To measure the MMP ($\Delta\Psi_m$), the dual-emission potential-sensitive probe, 5,5 V, 6,6 V-tetrachloro-1,1 V,3,3 V-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma), was used. JC-1 is a green compound which forms red-fluorescent aggregates in response to the membrane potential of energized mitochondria. The ratio of JC-1 depends only on the membrane potential, with a decrease being indicative of membrane depolarization. Briefly, the cells were harvested, loaded with 2 μg/ml JC-1 at 37˚C for 20 min and then analyzed using a FACS flow cytometer.

Statistical analysis. The data are presented as means ± SD. Significant differences among the groups were determined using the unpaired Student's t-test. A $p<0.05$ was accepted as being statistically significant. All the figures shown in this article were obtained from at least two or three independent experiments.

Results

Anthocyanins inhibit cell viability and induce apoptosis in U937. The anthocyanin-induced cell cytotoxicity was determined from the effects of different anthocyanin concentrations on U937 cell viability. The data showed that a treatment with anthocyanins for 48 h decreased the viability of U937 cells in a concentration-dependent manner (Fig. 1A). Further experiments were carried out to determine if this inhibitory effect of anthocyanins is the result of apoptotic cell death. By morphological analyses, nuclei with chromatin condensation and the presence of apoptotic bodies were observed in a concentration-dependent manner in the cells cultured with anthocyanins, while very few were observed in the control culture (Fig. 1B). The next experiments were carried out to determine if anthocyanins induce DNA fragmentation in U937 cells. As shown in Fig. 1C, increasing concentration of anthocyanins induced a progressive accumulation of fragmented DNA, which appeared as a typical ladder pattern of DNA fragmentation due to internucleosomal cleavage associated with apoptosis. Therefore, flow cytometry analysis was used to determine the magnitude of apoptosis elicited by anthocyanins. In Fig. 1D, the addition of anthocyanins is seen to result in the increased appearance of cells in the sub-G1 phase, similar to the results observed for anthocyanin-induced loss of cell viability, which strongly suggests that U937 cells undergo apoptosis after exposure to anthocyanins.

Effects of anthocyanins on the expression of Bcl-2 and IAP family members, and levels of MMP in U937 cells. As a first step, the apoptotic cascades in U937 cells were examined by exposing the cells to anthocyanins and then examining the levels of Bcl-2 and IAP family members. RT-PCR and Western immunoblotting data showed that the levels of anti-apoptotic Bcl-2 expression were down-regulated in response to anthocyanin treatment. In the case of the pro-apoptotic protein Bax, there was a concentration-dependent up-regulation observed in U937 cells treated with anthocyanins (Fig. 2). Furthermore, the levels of IAP family proteins such as XIAP, cIAP-1 and cIAP-2 were down-regulated in anthocyanin-treated cells. In order to determine whether anthocyanin-induced apoptosis is associated with mitochondrial dysfunction, we next investigated the protein levels of truncated Bid (tBid), a BH3-only proapoptotic member of the Bcl-2 family. As shown in Fig. 2B, Western blot analyses revealed that anthocyanin treatment significantly induced the cleavage of Bid in a concentration-dependent manner. We further investigated mitochondrial function following anthocyanin-induced apoptosis by measuring JC-1 dye retention. As shown in Fig. 3, exposing cells to anthocyanins significantly reduced their MMP ($\Delta\Psi_m$) level in a concentration-dependent manner as evidenced by the increased depolarization of the MMP with increasing anthocyanin concentration. These results suggest that anthocyanin exposure increases mitochondrial dysfunction, which may lead to cellular apoptosis.

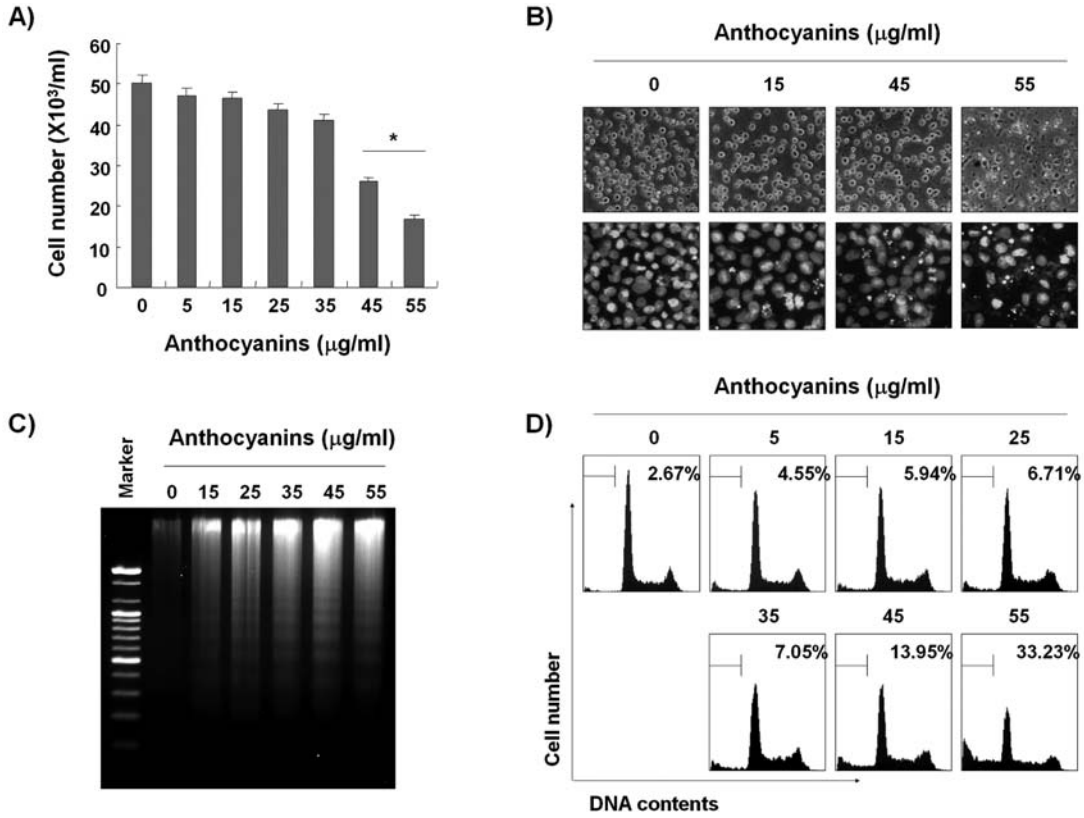
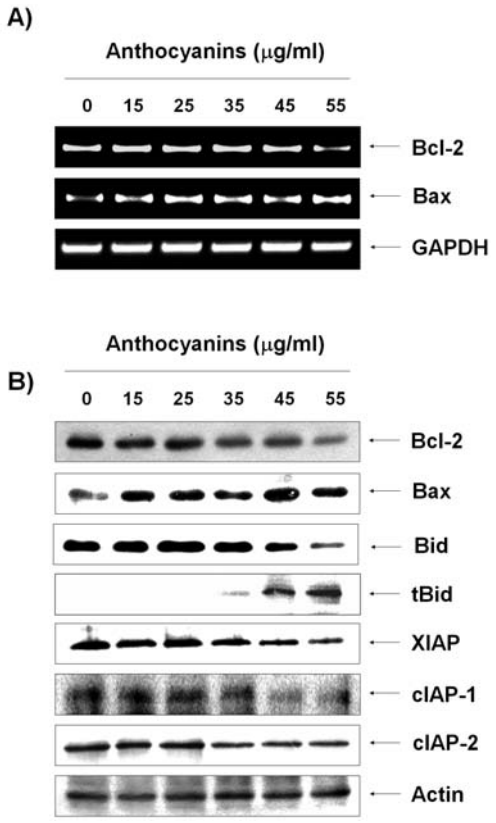


Figure 1. Inhibition of cell viability and induction of apoptosis in human leukemia U937 cells by anthocyanin treatment. U937 cells were seeded at $1x10^5$ cells/ml and then treated with the indicated concentrations of anthocyanins for 48 h. (A) Cell viability was determined by hemocytometer counts of trypan-blue excluding cells. Data are expressed as mean ± SD of three independent experiments. The significance was determined by Student's t-test (*p<0.05 vs. untreated control). (B) After treatment with anthocyanins for 48 h, the cells were examined under light microscopy (magnification x200) or fixed and stained with DAPI. The nuclear morphology was then photographed under fluorescence using a blue filter (magnification x400). (C) For the analysis of DNA fragmentation, genomic DNA was extracted and analyzed on 1.2% agarose gels. Marker indicates a size marker of the DNA ladder. (D) To quantify the degree of apoptosis induced by anthocyanins, the cells were evaluated for sub-G1 DNA content using a flow cytometer. Data are expressed as mean ± SD of three independent experiments. For statistical analysis, t-test was performed (*p<0.05).



Effect of anthocyanins on caspase activation. To determine if anthocyanin-induced apoptosis is associated with the activation of caspases, the expression and activity of caspase-3, -8 and -9 in the anthocyanin-treated cells were examined. Western blot analyses revealed that anthocyanin treatment significantly decreased the expression of pro-caspase-3, -8 and -9 in a time-dependent manner (Fig. 4A). The activation of caspase often leads to the proteolytic cleavage of several target proteins, such as poly(ADP-ribose) polymerase (PARP), which is a downstream target of activated caspase-3. Subsequent Western blot analysis showed the progressive proteolytic cleavage of PARP in U937 cells after anthocyanin treatment.

Figure 2. The effects of anthocyanins on the expression of Bcl-2 and IAP family members in U937 cells. (A) The cells were incubated with different concentrations of anthocyanins for 48 h, and the total RNA was isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with the indicated primers and the reaction products were subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. GAPDH was used as the internal control. (B) The cells grown under the same conditions as (A) were lysed and equal amounts of proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by ECL. Actin was used as the internal control.

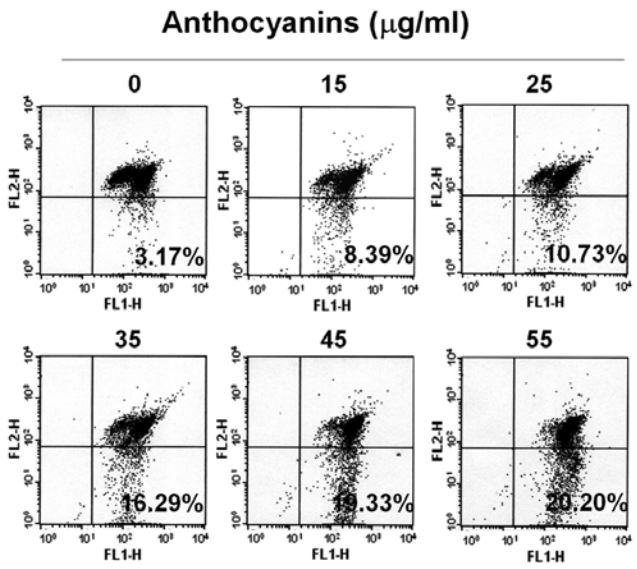


Figure 3. Loss of MMP in U937 cells by anthocyanin treatment. After 48-h incubation with the indicated concentrations of anthocyanins, the cells were stained with JC-1 for 20 min at 37˚C. The mean JC-1 fluorescence intensity was then detected using a flow cytometer. Data represent the means of two independent experiments.

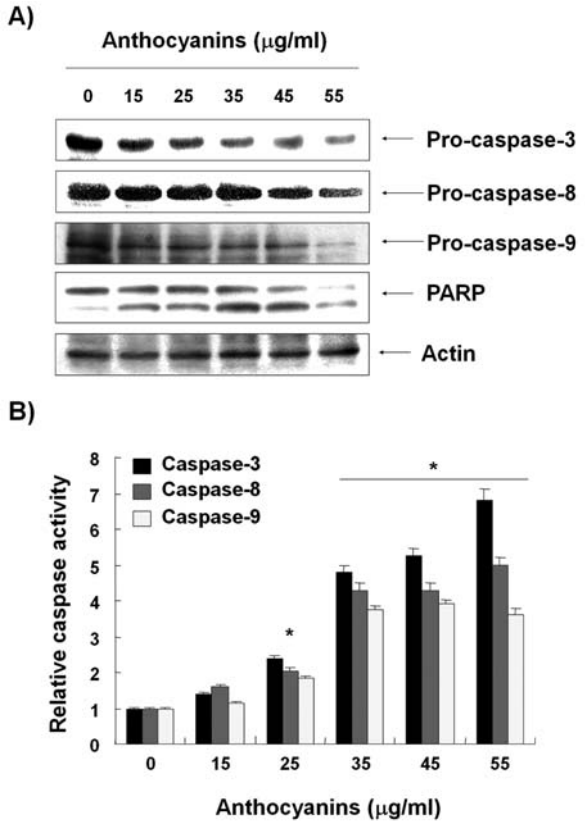


Figure 4. Activation of caspases in U937 cells by anthocyanins. (A) The cells were incubated with different concentrations of anthocyanins for 48 h, lysed and the cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) The cell lysates from the cells treated with anthocyanins for 48 h were assayed for *in vitro* caspase-3, -8 and -9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, as substrates. The released fluorescent products were measured. Data are expressed as mean ± SD of three independent experiments. The significance was determined by Student's t-test (*p<0.05 vs. untreated control).

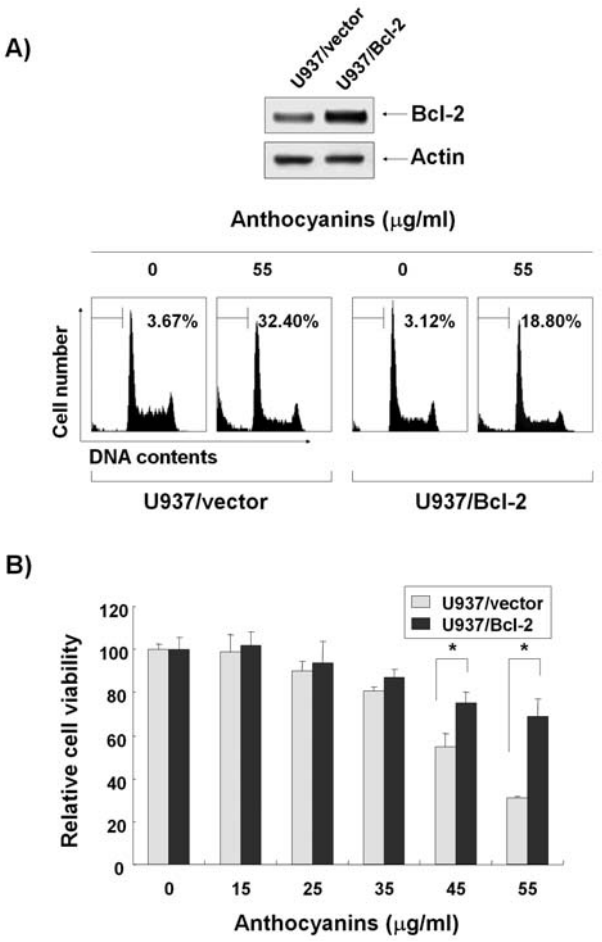


Figure 5. Effects of Bcl-2 overexpression in U937 cells on the apoptosis induction and growth inhibition by anthocyanins. (A) U937/vector or U937/Bcl-2 cells were treated with 55 mg/ml of anthocyanins for 48 h, and evaluated for sub-G1 DNA content using a flow cytometer. Data are reported as mean ± SD of two independent experiments. (B) The cells grown under the same conditions as (A) were evaluated for cell viability assay by hemocytometer counting. Data are expressed as mean ± SD of three independent experiments. The significance was determined by Student's t-test (*p<0.05 vs. untreated control).

Next, cell lysates containing equal amounts of total protein from cells treated with anthocyanins were assayed for *in vitro* caspase activity. As shown in Fig. 4B, treatment with anthocyanins significantly increased activity of these caspases. These results indicate that the activation of caspases may be a key step in the anthocyanin-induced apoptotic pathway in U937 cells.

Inhibition of anthocyanin-induced apoptosis by overexpression of Bcl-2 in U937 cells. Since Bcl-2 is a well-known anti-apoptotic protein and since anthocyanins down-regulated Bcl-2 expression, the effect of high intracellular levels of Bcl-2 on anthocyanin-induced apoptosis was evaluated by comparing U937/vector with U937/Bcl-2 cells that constitutively express high levels of Bcl-2. The Bcl-2 expression level in U937/Bcl-2 cells was approximately four times higher than that in the Bcl-2 in U937/vector cells (Fig. 5A). As shown in Fig. 5A, Bcl-2 overexpression was found to significantly block the anthocyanin-induced increase in cells that were in the sub-G1 population when compared to U937/vector cells. In addition, ectopic Bcl-2 overexpression was found to markedly inhibit

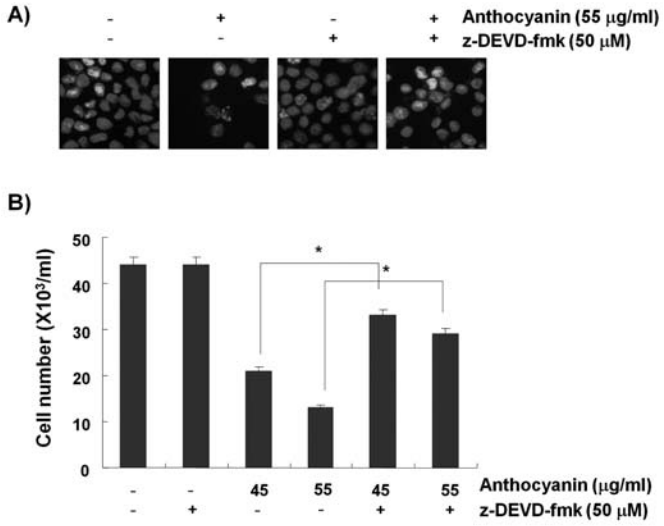


Figure 6. Inhibition of anthocyanin-induced apoptosis in U937 cells by a caspase-3 inhibitor. (A) U937 cells were pretreated for 1 h with or without z-DEVD-fmk (50 μM), and then treated with 55 mg/ml of anthocyanins for additional 48 h. The cells were stained with DAPI for 10 min and photographed with a fluorescence microscope using a blue filter (magnification x400). (B) In a parallel experiment, the degree of growth inhibition was determined by hemocytometer counting. Each point represents the mean ± SD of three independent experiments. The significance was determined by Student's t-test ($^*p<0.05$ vs. untreated control).

the reduction in cell viability relative seen in response to anthocyanins in the U937/vector cells (Fig. 5B), suggesting that Bcl-2 overexpression may effectively attenuate anthocyanin-induced apoptosis.

Inhibition of anthocyanin-induced apoptosis by a caspase-3 inhibitor. In order to show that the activation of caspase-3 is a key step in the anthocyanin-induced apoptotic pathway, U937 cells were pretreated with z-DEVD-fmk (50 μM), a cell-permeable caspase-3 inhibitor, for 1 h, followed by a treatment with 55 mg/ml of anthocyanin for 48 h. The blockade of caspase-3 activity by z-DEVD-fmk pre-treatment significantly prevented not only the anthocyanin-induced chromatin condensation but also the decrease in cell viability (Fig. 6). These results clearly show that anthocyanin-induced apoptosis is associated with the activation of caspase-3.

Discussion

Recently, many biological effects of anthocyanins, including anti-oxidant, anti-inflammatory, immune-modulating, and chemopreventive activities have been described, all of which suggest that anthocyanins might be effective natural components for cancer chemoprevention (12-17). While a cancer cell killing mechanism for anthocyanins has been suggested (20-24), the precise cell death mechanism induced by anthocyanins is not completely understood. Therefore, this study investigated the effects of anthocyanins on the growth of a human leukemia U937 cell line to examine the mechanisms of its anti-proliferative pathway. Our data indicated that anthocyanin treatment resulted in a reduction in cell viability and the induction of apoptosis in these cells in a concentration-dependent manner. The induction of apoptosis by anthocyanins was confirmed by characteristic morphological changes, chromatin condensation, protein and gene expression changes and mitochondrial response.

Apoptosis is an endogenous programmed cell death that can be triggered by various stimuli, including death receptor-mediated signaling (extrinsic pathway) and intracellular stresses (intrinsic pathway). It is widely accepted that alterations to mitochondrial structure and function, as well as caspase activation, play important roles in apoptosis (4,5). To gain insights into the mechanism of anthocyanin-induced apoptosis, we investigated both the catalytic activity of caspases and mitochondrial dysfunction and demonstrated that anthocyanins increased the enzymatic activity of both extrinsic and intrinsic caspase cascades, including caspase-8 and -9 (Fig. 4). Mitochondria are important regulators of extrinsic as well as intrinsic apoptosis pathways, and they undergo a series of sequential changes during apoptosis. A loss of MMP, together with a permeability transition of membrane pores, are early events in the apoptotic cascades that result in mitochondrial swelling and disruptions of the outer mitochondrial membrane. Mitochondrial function is controlled by several factors, such as the pro- and anti-apoptotic members of the Bcl-2 family. In addition, the tBid can translocate to mitochondria and bind to Bax, leading to a conformational change of pro-apoptotic Bax mRNA and protein, and to the activation of caspase-9 (4,5). In the present study, anthocyanins resulted in mitochondrial dysfunction, as evidenced by the loss of MMP observed with JC-1 staining (Fig. 3). Furthermore, anthocyanin treatment was found to increase the levels of Bax and to decrease those of anti-apoptotic Bcl-2. These changes may have increased mitochondrial depolarization, leading to the activation of caspase-9, which would indicate that the anthocyanins may increase Bax/Bcl-2 to induce the mitochondrial dysfunction that leads to apoptosis in U937 cells. These results demonstrate that anthocyanin treatment triggers apoptosis through activation of the intrinsic caspase pathway, in addition to the extrinsic pathway.

Activation of the mitochondrial pathway leads to the release of pro-apoptotic factors, which remove the IAP blockage of caspase activation. The IAP family functions by binding to and inhibiting several caspases (25,26). Caspase signaling is initiated and propagated by proteolytic autocatalysis, as well as the cleavage of downstream caspases and substrates (3,5). Among these, caspase-3 is one of the key executioners of apoptosis, because it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as PARP, a protein important for cell viability, but which also serves as a marker of apoptosis when cleaved (27). Further studies have shown that exposure of U937 cells to anthocyanins caused a down-regulation of IAP family proteins such as xIAP, cIAP-1, and cIAP-2, and an activation of caspase-3 and the concomitant degradation of PARP (Fig. 4). The present results indicate that anthocyanins increase mitochondrial dysfunction, which, in turn, results in the activation of caspase-9, leading to the activation of caspase-3, also associated with inhibition of IAP family protein functions. Under the same conditions, a specific caspase-3 inhibitor, z-DEVD-fmk, was able to prevent anthocyanin-induced apoptosis by blocking apoptotic body

formation (Fig. 6). Furthermore, it was shown that Bcl-2 overexpression significantly attenuated anthocyanin-induced growth inhibition and apoptosis in U937 cells (Fig. 5). These data suggest that anthocyanin-induced apoptosis was caused by caspase-3-dependent cell death, and that Bcl-2 plays an important role in anthocyanin-induced apoptosis in these cells.

In conclusion, we have demonstrated that apoptosis is significantly induced in human leukemia U937 cells exposed to anthocyanins isolated from *V. coignetiae* Pulliat. The present data indicate that anthocyanins activated initiator and effector caspases, such as caspase-8, -9, and -3, and induced sequential cleavage of caspase substrates, such as PARP, which is required for apoptosis induction. These observations further demonstrate the importance of a mitochondrial amplification step in anthocyanin-induced apoptosis in U937 cells. Although further studies are needed to examine the mechanisms in detail, this study further supports the contention that anthocyanins might be promising molecules for use in cancer chemoprevention or chemotherapy.

Acknowledgments

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