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Induction of Apoptosis by the Anthocyanidins through Regulation of Bcl-2 Gene and Activation of c-Jun N-Terminal Kinase Cascade in Hepatoma Cells

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Anthocyanidins that are reddish pigments widely distributed in fruit and vegetables have been reported to possess antioxidant and anticancer activities. To understand the molecular basis of the putative anticancer activity of anthocyanidins, we investigated the antiproliferation effects of anthocyanidins in human hepatoma cell lines. Delphinidin, cyanidin, and malvidin exhibited strong growth inhibitory effects against human hepatoma $HepG_2$, but were less effective against Hep3B. According to the appearance of the caspase-3 fragments and stimulated proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) in time-dependent studies, delphinidin induced apoptotic cell death characterized by internucleosomal DNA fragmentation and caused a rapid induction of caspase-3 activity. RT-PCR and Western blot data revealed that delphinidin stimulated an increase in the c-Jun and JNK phosphorylation expression at mRNA and protein levels, respectively. Moreover, delphinidin-induced apoptotic cell death was accompanied by up-regulation of Bax and down-regulation of Bcl-2 protein. Dephinidin-induced DNA fragmentation was blocked by *N*-acetyl-L-cysteine and catalase, suggesting that the death signaling was triggered by oxidative stress. Our experiments provide evidence that delphinidin is an effective apoptosis inducer in $HepG_2$ cells through regulation of Bcl-2 family moleculars and activation of c-Jun N-terminal kinase cascade. The results suggest that induction of apoptosis by anthocyanidins is a pivotal mechanism of their cancer chemopreventive functions.

KEYWORDS: Anthocyanidin; apoptosis; $HepG_2$ cell; JNK pathway; Bcl-2 family; caspase-3

INTRODUCTION

Hepatocellular carcinoma is one of the most common malignant tumors worldwide and may be the most common fatal cancer (*1*). An epidemiology study showed that one-third of Asian hepatoma cases occurred in China (*2*). Because of metastasis, cirrhosis, and other pathological changes in the liver parenchyma, surgical resection is frequently limited. Therefore, the development of chemotherapeutic/chemopreventive agents for hepatocellular cancer is important for reducing the mortality caused by this disease.

A high consumption of natural dietary polyphenolic compounds might reduce the risk of cardiovascular disease and also protect against many types of cancer. The mechanisms of these beneficial effects are not well-known although there is evidence that polyphenolic compounds found in grapes, wine, berry, and various fruits may provide some benefits (*3*). Anthocyanins are natural colorants belonging to the flavonoid family that is widely spread in foods of vegetal origin (*4*). Many physiological functions of anthocyanins have been discussed both in vitro and in vivo, such as: maintenance of normal vascular permeability (*5*), vasoprotective and antiinflammatory properties (*6*), antiangiogenesis (*7*), and anticancer activity (*8*). Animal studies have also demonstrated that feeding with anthocyanin-rich extract protected against *tert*-butyl hydroperoxide-induced hepatic toxicity (*9*) and decreased lipid peroxidation and DNA damage in vitamin E-depleted rats (*10*). More recently, anthocyanins have been shown to be an effective chemopreventive agent against 1,2-dimethylhydrazine (DMH)- and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary carcinogenesis in rats (*11*). In addition, anthocyanins can be directly absorbed and distributed to the blood (*12*) and be incorporated in cell cultures, both in the plasma membrane and in the cytosol (*13*). Extensive studies indicated that anthocyanins have strong free radical scavenging and antioxidant activities (*14*−*16*), suggesting that they played an important role in preventing mutagenesis and carcinogenesis (*17*).

Anthocyanins also showed inhibitory effects on the growth of some cancer cells (*18*). A recent study has reported (*19*) that the bilberry extract and the anthocyanidins inhibited the growth of cancer cells through the induction of apoptosis. However, few studies have investigated the application of anthocyanidins to the treatment of hepatoma; thus, the objective of the present study was to determine whether anthocyanidins would be useful in the prevention of human hepatoma carcinogenesis. In the present study, two human hepatoma cells, Hep3B and $HepG_2$

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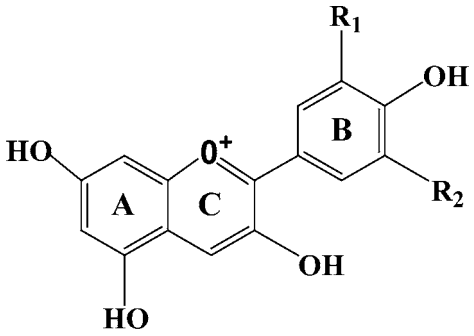

Name	R1	R2
Peonidin	OCH3	H
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH3	OCH3

Figure 1. Chemical structure of anthocyanidins.

of different characteristics, were chosen for study. Hep3B cells expressed hepatitis virus B antigen and produced mostly plasma major proteins; $HepG_2$ cells did not contain hepatitis viral genome and produced mostly receptors of insulin as well as insulin-like growth factor II (*20*). A tumorigenicity model, in which tumors were formed as Hep3B cells were injected into nude mice, was also investigated for exploring in vivo protection (*21*).

Apoptosis is one of the major mechanisms of cancer suppression. It is a highly regulated process that involves activation of a serious of molecular events, leading to cell death that is characterized by cellular morphological change, chromatin condensation, and apoptotic bodies which are associated with DNA cleavage into ladders (*22*). Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs. Many therapeutic agents for cancer, such as cisplatin (*23*), paclitaxel (*24*), isothiocyanate (*25*), and adriamycin (*26*), have been reported to eliminate tumor cells by inducing apoptotic cell death. In addition, some flavonoids, such as quercetin, apigenin, and phloretin, also inhibited cancer cell growth through the induction of apoptosis (*27*). Consequently, understanding the mechanism of apoptosis has important implications in the prevention and treatment of many diseases.

Since peonidin, pelargonidin, cyanidin, delphinidin, and malvidin are represented by the aglycons of most anthocyanidins in plants (**Figure 1**), they are used to investigate the mechanism of the anticancer activities of anthocyanidins in the present study. We first examined the antiproliferative effects of five typical anthocyanidins in two human hepatoma cell lines, Hep3B and $HepG_2$. In addition, the molecular mechanisms of the apoptotic effects induced by delphinidin were determined.

MATERIALS AND METHODS

Cell Culture and Chemicals. Human hepatoma cells (Hep3B and $HepG_2$ cells were obtained form the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (GIBCO BRL, Grand Island, NY), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.37% (w/v) $NaHCO_3$, 0.1 mM NEAA, 1 mM sodium pyruvate, and 0.03% L-glutamine at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was renewed each day. Cells were weekly detached for transfer with 0.1% trypsin and 10 μM EDTA in phosphate-buffered saline (PBS). Dubecco's Modified Eagle Medium, fetal bovine serum (FBS), and trypsin−EDTA (T/E) were obtained from Gibco BRL Co. (Grand Island, NY). Anthocyanidins (peonidin chloride, pelargonidin chloride, cyanidin chloride, delphinidin chloride, and malvidin chloride) purified by HPLC were obtained from Extrasynthese (Genay, France). A lactate dehydrogenase optimized kit DG-1340-K, *N*-acetylcysteine (NAC), catalase, 2′-7′-dichlorofluorescein diacetate (DCFH-DA) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), and the rabbit antirabbit IgG polyclonal antibody conjugated to peroxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-JNK, anti-phospho-JNK, anti-capsase-3, anti-poly (ADP-ribose) polymerase, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2 and anti-Bax antibodies were obtained from Pharmingen (San Diego, CA). Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Polyvinyl difluoride (PVDF) membrane for Western blotting was obtained from Millipore (Bedford, MA). TRIzol RNA isolation kit was obtained from Life Technologies (Rockville, MD). Primers for RT-PCR, dNTP, reverse transcriptase, and Taq polymerase were obtained Gibco BRL (Cergy Pontoise, France). All other chemicals used were of the highest pure grade available.

Cell Proliferation Assays. The cell proliferation and cytotoxicity were determined with the MTT and LDH leakage assays. Hep3B and $HepG_2$ cells were seeded onto 96-well plates at a concentration of 1×10^6 cells/well in DMEM plus 10% FBS. Cells were incubated with different concentrations of anthocyanidins for 24 h. Dye solution (10 μL) specific for the MTT assay was added to each well for additional 4 h incubation at 37°C. After the addition of dimethyl sulfoxide (100 μL/well), the absorbance at 570 nm (formation of formazan) and 630 nm (reference) were recorded with a FLUOstar Galaxy plate reader (BMG Lab Technology, GmbH, Offenburg, Germany). The percent viability of the treated cells was calculated as follows: $(A_{570\ nm} - A_{630\ nm})_{sample}/(A_{570\ nm} - A_{630\ nm})_{control} \times 100$. Lactate dehydrogenase (LDH) leakage was measured as an another index of cytotoxicity. $HepG_2$ cells were seeded onto 12-well plates at a concentration of 1×10^6 cells/well in DMEM plus 10% FBS. Cells were incubated with various of anthocyanidins for 24 h and then analyzed for LDH leakage into the culture media by using the commercial kits (Sigma Chemical Co.). The total LDH activity was determined after cells were thoroughly disrupted by sonication. The percentage of LDH leakage was then calculated to determine membrane integrity. The LDH leakage was expressed as a percentage of total activity: (activity in the medium)/(activity in the medium + activity of the cells) × 100.

DNA Fragmentation Analysis. $HepG_2$ cells (2×10^6 cells/mL) under different treatments were collected, washed with PBS twice, and then lysed in 100 mL of lysis buffer (50 mM Tris, pH 8.1; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/mL prteinase K) for 3 h at 56 °C and treated with RNase A (0.5 μg/mL) for another hour at 37 °C. The DNA was extracted by phenol/chloroform/isoamyl (v/v/v, 25:24:1) before loading and analyzed by 1.8% agarose gel electrophoresis. The agarose gels were run at 50 V for 90 min in Tris-borate/EDTA electrophoresis buffer (TBE). Approximate 30 μg of DNA was loaded in each well and visualized under UV light and photographed.

RT-PCR for c-Jun. RT-PCR was performed to determine the level of c-Jun gene expression. $HepG_2$ cells (1×10^6 in 10 mL medium) were plated in 100-mm tissue culture dishes. After preincubation for 24 h, apoptosis induction was carried out for a time-course by delphinidin in 0.05% DMSO as described in the DNA fragmentation assays. Cellular RNA was extracted with a TRIzol RNA isolation kit (Life Technologies, Rockville, MD). The 290-bp target in c-Jun cDNA was amplified by using the sense primer (5′-CAACATGCTCAGGGAACAGG) at positions 261−280 and antisense primer (5′-GGTCCATGCA- GTTCTTGGTC) at positions 531−550. As a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was amplified by using the sense primer (5′GACCCCTTCATTGACCTCAAC) at position 143−162 and anti-sense primer (5′-CATACCAGGAAATGAGCTTG) at positions 965−984. Briefly, from each sample, 250 ng

of RNA was reverse-transcribed using 200 units of Superscript II reverse transcriptase, 20 units of RNase inhibitor, 0.6 mM of dNTP, and 0.5 μg/μL of oligo (dT)12−18. Then, PCR analyses were performed on the aliquots of the cDNA preparations to detect c-Jun and G3PDH (as an internal standard) gene expression using the FailSafe PCR system (Epicenter Technologies). The reactions were carried out in a volume of 50 μL containing (final concentration) 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MnCl_2$, 0.2 mM dNTP, 2 units of Taq DNA polymerase, and 50 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, 30 cycles of amplification (at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min) were performed, followed by a 7 min extension at 72 °C.

Analysis of PCR Products. A 10 μL aliquot from each PCR reaction was electrophoresed in a 1.8% agarose gel containing 0.2 μg/mL ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer linked to a computer analysis system. We normalized the c-Jun signal relative to the corresponding G3PDH signal from the same sample and expressed the data as the c-Jun/G3PDH ratio.

Western Blot Analysis. The cytosolic proteins were isolated from human hepatoma $HepG_2$ cells (1×10^6 cells/mL) after treatment with 100 μM delphindin for 0, 3, 6, 12, and 24 h. The total proteins were extracted by adding 500 μL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by Bradford assay (*28*) with bovine serum albumin as a standard. Total cytosolic extracts (250 μg of protein) were separated on 8% SDS-polyacrylamide minigels for PARP detection and 12% SDS-polyacrylamide minigels for caspase-3, Bcl-2 family, JNK, and β-actin protein detection, and then transferred to Immobilon polyvinylidene difluoride membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in 5% bovine serum albumin solution for 1 h at room temperature and then incubated with overnight at 4 °C with indicated primary antibodies (1:1000 dilution). After hybridization with primary antibodies, the membrane was washed with TBST (Tris buffered saline Tween-20) three times, incubated with HRP-labeled secondary antibody for 45 min at room temperature, and washed with TBST three times. Final detection was performed with ECL (Enhance Chemiluminescence) western blotting reagents (Amersham Pharmacia Biotech). Densitometric measurements of the bands in Western blot analysis were performed using the Image Acquisition and analysis Software (Cambridge, UK).

Measurement of Caspase-3 Activity. After treatment with delphinidin, cells were collected and washed with PBS and lysed in lysis buffer (1% Triton X-100, 0.32M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8, 2 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin) for 20 min at 4 °C followed by centrifugation (10000*g*) for 30 min. Caspase-3 activity was assayed in 50 μL reaction mixtures with fluorogenic report substrate peptide specific for caspase-3. The substrate peptide (200 μM) was incubated at 37 °C with cytosolic extracts (50 μg of total protein) in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, 0.1% 3-[(3-chloamidopropyldimethylammonio)-1-propanesulfonate]. Fluorescence was measured after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a FLUOstar galaxy fluorescence plate reader (BMG LabTechologies, GmbH, Offenburg, Germany).

Measurement of Intracellular Reactive Oxygen Species. Intracellular reactive oxygen species (ROS) were estimated by using a fluorescent probe, DCFH-DA. DCFH-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is believed to parallel the amount of ROS formed intracellularly. At the end of incubation, cells (10^6 cells/mL) were collected and resuspended with PBS. An aliquot of the suspension (195 μL) was loaded into a 96-well plate, and then 5 μL of DCFH-DA was added (final concentration 20 μM). The DCF fluorescence intensity was detected at different time intervals using a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies GmbH Inc., Offenburg, Germany) with an excitation wavelength at 485 nm and emission wavelength at 530 nm.

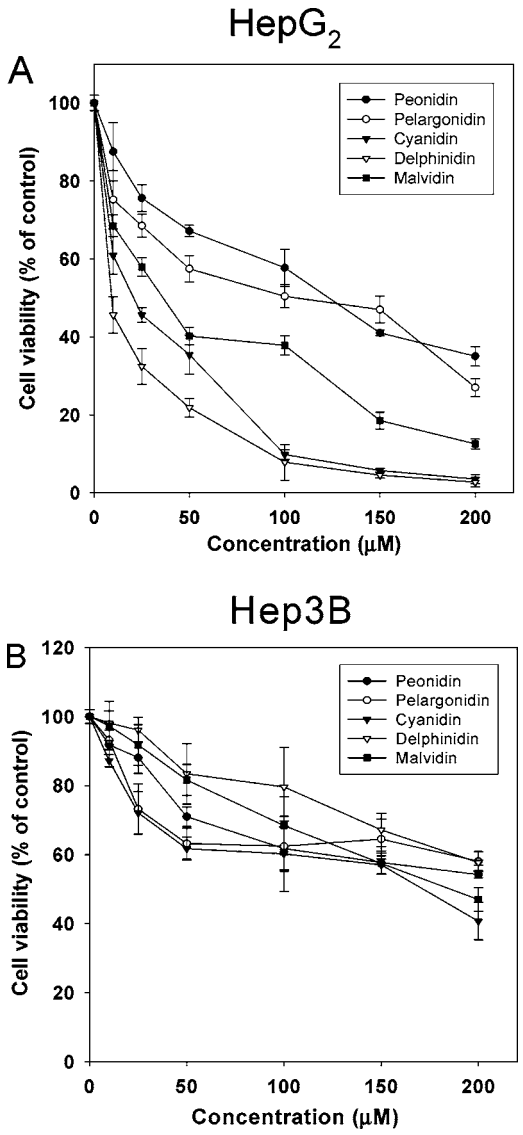

Figure 2. Effects of anthocyanidins on cell viability. (A) $HepG_2$ and (B) Hep3B cells were treated with anthocyanidins for 24 h, and cell viabilities were then determined by MTT assay. Each data point represents mean ± SD of three experiments.

Statistical Analysis. Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± SD. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered significant with the P values <0.05.

RESULTS

Effect of Anthocyanidins on Cell Viability. The chemical structures of anthocyanidins are illustrated in **Figure 1**. We first tested the effect of five representative anthocyanidins on cell viability by MTT assay. Human hepatoma cell lines, Hep3B and $HepG_2$, were treated with different concentrations of anthocyanidins. As shown in **Figure 2**A, cyanidin, delphinidin, and malvidin showed concentration-dependent growth inhibition on $HepG_2$ cells. The estimated IC_{50} values (concentration causing 50% cell death) of cyanidin, delphinidin, and malvidin were 18.4, 10.8, and 50.4 μM, respectively. Peonidin and

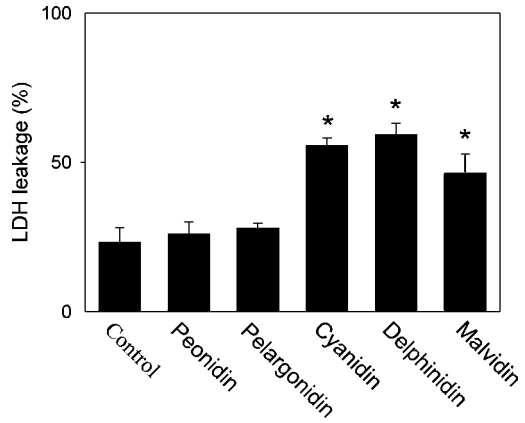

Figure 3. LDH leakage in $HepG_2$ cells treated with anthocyanidins. $HepG_2$ cells were incubated with and without anthocyanidins for 24 h. LDH leakage was determined. The concentration of each anthocyanidin was 100 μM. Each column represents the mean ± SD of three experiments. *$P < 0.05$ significantly different to control.

pelargonidin displayed approximately the same extent of inhibition on cell viability, having IC_{50} values of 129.2 and 108.4 μM, respectively. The Hep3B cells appeared to be less susceptible to the inhibitory effect of anthocyanidins. Among the five anthocyanidins, the IC_{50} values of peonidin, pelargonidin, cyanidin, delphinidin, and malvidin were 268.3, 288.7, 168.5, 210.3, and 182.7 μM, respectively (**Figure 2B**). The other anthocyanins glycosides did not show cytotoxicity (data not shown). With the MTT assay, the cell viabilities of cyaniding- and delphinidin-treated $HepG_2$ cells were markedly decreased at the concentration of 100 μM (**Figure 2A**). The LDH leakages after treatment with 100 μM of each anthocyanidin in $HepG_2$ cells are shown in **Figure 3**. Cyanidin, delphinidin, and malvidin caused significant LDH leakage ($p < 0.05$) as compared with the control.

DNA Fragmentation Induced by Anthocyanidins in $HepG_2$ Cells. As a biochemical hallmark of apoptotic cell death, DNA fragmentation was determined. To investigate the ability of anthocyanidins to induce apoptosis of cancer cells, $HepG_2$ cells were treated with all five anthocyanidins at 100 μM for 24 h. After treatment with 100 μM of various anthocyanidins or 0.05% DMSO (as control) for 24 h, the genomic DNA from cells was subjected to 1.8% agaorse gel elctrophoresis. As shown in **Figure 4**A, the intact genomic DNA was found in control. Cyanidin, delphinidin, and malvidin induced apoptosis in $HepG_2$ cells detected by DNA fragmentation. In contrast, peonidin and pelargonidin did not induce DNA fragmentation (**Figure 4A**). Next, we used delphinidin, the strongest inducer for concentration− and time−response experiments. As shown in **Figure 4B**, DNA fragmentation was observed at 24 h in a dose-dependent manner with 80−200 μM, and at 100 μM in a time-dependent manner for 6−48 h (**Figure 4C**). The efficacious induction for apoptosis was observed at 100 μM for 24 h, suggesting that the cell death induced by the anthocynidins treatment was mainly caused by apoptosis.

Delphinidin Induced the Caspase-3 Activation. Caspase-3 has been shown to play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli. To monitor the enzymatic activity of caspase-3 during delphinidin induce apoptosis, we used the specific fluorogenic peptide substrate (Ac-DEVD-MCA) for the detection of caspase-3 activity. As illustrated in **Figure 5**, delphinidin (100 μM) induced a dramatic increase of DEVD-specific caspase activity in treated $HepG_2$ cells. The induction of DEVD-specific activity was rapid. As described previously, the cell death response induced by anthocyanidins was mainly time-dependent (**Figure 4C**), so we examined the time dependence of caspase-3 activation as well. $HepG_2$ cells were treated with 100 μM of delphinidin for different hours. Cytosolic proteins were extracted and assayed for caspase-3 activity by incubation with a fluorogenic peptide substrate (Ac-DEVD-MCA). As shown in **Figure 5A**, in $HepG_2$ cells, delphinidin treatment caused a significant time-dependent increase in the caspase-3 proteolytic activity ($p < 0.05$). Additional evidence of caspase-3 activation is the proteolysis of procaspase-3 into small active fragments and cleavage of PARP. As shown in **Figure 5B**, delphinidin induced PARP cleavage, in evidence by the accumulation of an 85 kDa fragment compared to the 115 kDa intact protein. Furthermore, delphinidin treatment caused the degradation of 32 kDa procaspase-3 which generated 20/11- or 17/11-kDa fragment in a time-dependent manner (**Figure 5B**) that corresponded to those at which delphinidin induces DNA fragmentation (**Figure 4C**). The control protein β-actin showed no change in the experiments. These results indicate that cyanidin-induced $HepG_2$ cell apoptosis involves a caspase-3 activation.

Increase of c-Jun mRNA by Delphinidin. c-Jun signal is a inducible transcription factor whose activity is modulated by phosphorylation at the transactivation domain by JNK. Activation of c-Jun regulates transcription of several gene involved in apoptosis, we examined the change of c-Jun mRNA levels in the $HepG_2$ cells treated with delphinidin by RT-PCR technique. When the cells treated with 100 μM delphinidin, the level of c-Jun mRNA was increased in a time-dependent manner (**Figure 6**). By the same treatment, the levels of G3PDH mRNA expression were not affected significantly, suggesting the specific increase of c-Jun expression by delphinidin. Comparing with the data of DNA fragmentation in **Figure 4C**, delphinidin induced c-Jun expression and DNA fragmentation in a time-corresponding manner, suggesting that delphinidin-increased c-Jun expression, which seemed to precede the execution of apoptosis.

JNK Is Activated by Delphinidin. Since delphinidin activates c-Jun but JNK activates and/or phosphorylates c-Jun, the effect of delphinidin on JNK activation is worthy of investigation. The activation state of JNK pathway in the cell lysates were determined by immunoblotting assay. Using an antibody specifically directed against active JNK, we found that dephinidin at 100 μM gradually increased JNK phosphorylation levels throughout the 24 h of treatment (**Figure 7**). The increases resulted from phosphorylation, as the total JNK levels did not increase significantly during treatment with delphinidin. Phosphorylated JNK level was increased 5.0-fold of the control values after a 24 h treatment. The appearances of JNK phosphorylation and c-Jun expression by delphinidin were earlier than that of DNA fragmentation. Thus, delphinidin-induced apoptosis might be through the JNK activation pathway.

Generation of ROS by Delphinidin in $HepG_2$ Cells. 2′,7′-Dichlorofluorescin-diacetate (DCFH-DA) was used as a probe for ROS measurement. To explore the role of ROS in the mechanism of the actions of anthocyanidins, we examined whether the anthocyanidins increased the level of intracellular ROS in $HepG_2$ cells using DCF fluorescence. As shown in **Figure 8**C, cyanidin, delphinidin, and malvidin increased intracellular ROS in $HepG_2$ cells detected by a FLUOstar galaxy fluorescence plate reader. In contrast, peonidin and pelargonidin slightly increased the level of intracellular ROS at the concentration of 100 μM (**Figure 8C**). Delphinidin treatment greatly increased the intensity of DCF fluorescence in $HepG_2$ cells,

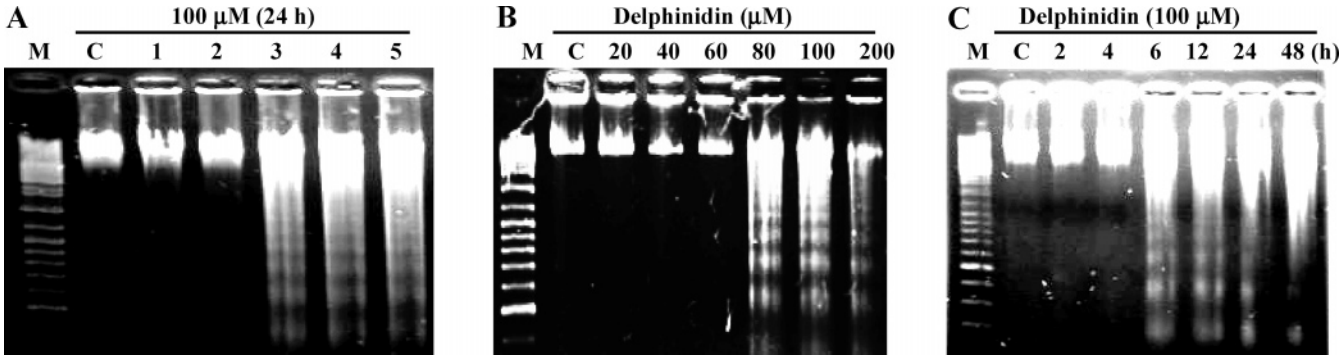

Figure 4. Induction of DNA fragmentation by anthocyanidins in $HepG_2$ cells. (A) Cells were treated with various anthocyanidins (100 μM) for 24 h, and DNA fragmentation was analyzed by electrophoresis in 1.8% agarose gel. (B) A dose-dependence of DNA fragmentation, cells were treated with different concentrations of delphinidin for 24 h. (C) Cells were treated with 100 μM delphinidin for indicated time periods. M, DNA ladder marker; C, 0.05% DMSO; 1, peonidin; 2, pelargonidin; 3, delphinidin; 4, cyanidin; 5, malvidin. Data shown are representative of three independent experiments.

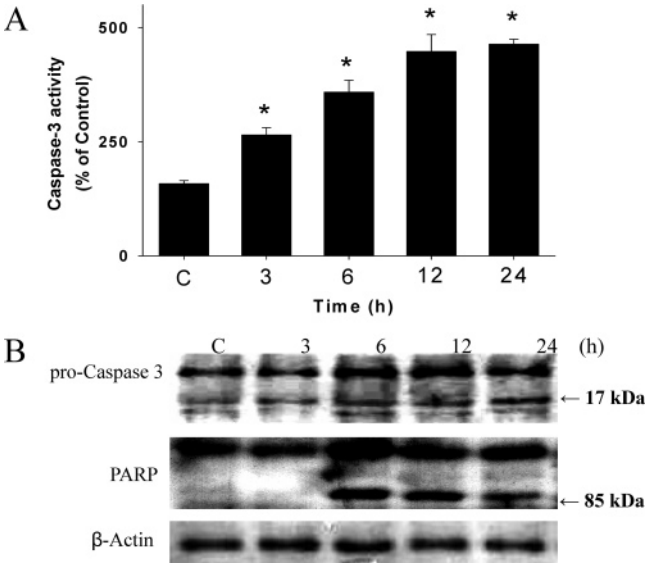

Figure 5. Activation of caspase-3 and cleavage of PARP during delphinidin-induced apoptosis. $HepG_2$ cells were incubated with 100 μM delphinidin for various time periods (0, 3, 6, 12, and 24 h). Cytosolic fraction of cells was analyzed for (A) caspase-3 activity by proteolytic fluorogenic substrates. Ac-DEVD-pNA was used as the substrate for caspase-3, and the cleavage of peptide was monitored at 405 nm. The caspase-3 activity of control cells was set to 100%, and the relative changes in the activity were shown. Each data represents the mean and standard deviation (*P < 0.05 significantly different to control). (B) Cells were treated with delphinidin at 100 μM for indicated periods. Total cell lysates extracted were analyzed for the proteolytic cleavage of PARP and caspase-3 by Western blot.

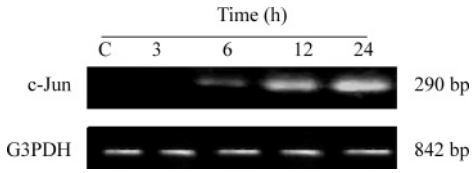

Figure 6. Delphinidin-induced c-Jun mRNA expression. A time-dependence of c-Jun mRNA induction. The cells were exposed to 100 μM delphinidin for the indicated times. Expression of G3PDH gene was used as a control. Cellular RNA was extracted with invitrogen RNA isolation kit and analyzed by RT-PCR. The RT-PCR products were separated on 1.8% agarose gel and digitally imaged after staining with ethidium bromide.

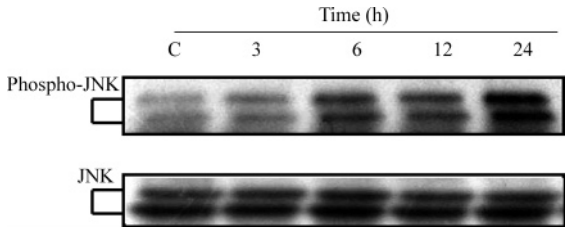

Figure 7. Delphinidin stimulates JNK phosphorylation. $HepG_2$ cells were treated with 100 μM delphinidin for the indicated times. Lysate protein of 250 μg was applied on 12% SDS–PAGE. The proteins of JNK and phosphorylated JNK were detected with corresponding specific antibodies, and visualized by chemiluminescence ECL kit.

which indicated an increase in the level of intracellular ROS. The level of ROS was increased in a dose-dependent manner (**Figure 8A**). A significant increase of ROS was observed at 20 min after the treatment with delphinidin and the level of ROS increased in a time-dependent manner (**Figure 8B**). A maximum amount of intracellular ROS production was observed with the treatment of delphinidin at 100 μM for 30 min. These data indicated that the increment of intracellular ROS might play a role as an early mediator in anthocyanidin-induced apoptosis.

Preventive Effects of Free Radical Scavengers on Delphinidin-Induced Apoptotic Responses. To examine whether the generation of intracellular ROS was a crucial step in delphinidin-induced apoptosis, we investigated the preventive effects of free radical scavengers on delphinidin-induced apoptosis. The cells were pretreated with free radical scavengers including *N*-acetyl-L-cysteine (NAC) and catalase (CAT) for 1 h at different concentrations and then exposed to 100 μM delphinidin for 24 h to determine DNA fragmentation. As shown in **Figure 9**, *N*-acetyl-L-cysteine and catalase at 5 mM and 100 U/mL, respectively, completely inhibited delphinidin-induced DNA fragmentation. Both free radical scavengers could protect against delphinidin-induced DNA fragmentation, suggesting that delphinidin-induced apoptosis involved the activation of reactive oxygen species, which could be blocked by free radical scavengers including *N*-acetyl-L-cysteine and catalase.

Regulation of Bcl-2 Family Proteins during Delphinidin-Induced Apoptosis. Since Bax and Bcl-2 play a crucial role in apoptosis, we next studied the time-dependent effects of delphinidin on the constitutive protein levels of Bax and Bcl-2 in $HepG_2$ cells. The Western blot analysis exhibited a significant increase in the protein expression of Bax at a concentration of 100 μM delphinidin, which was observed up to 12 h posttreatment compared to the basal levels (**Figure 10**A). In contrast,

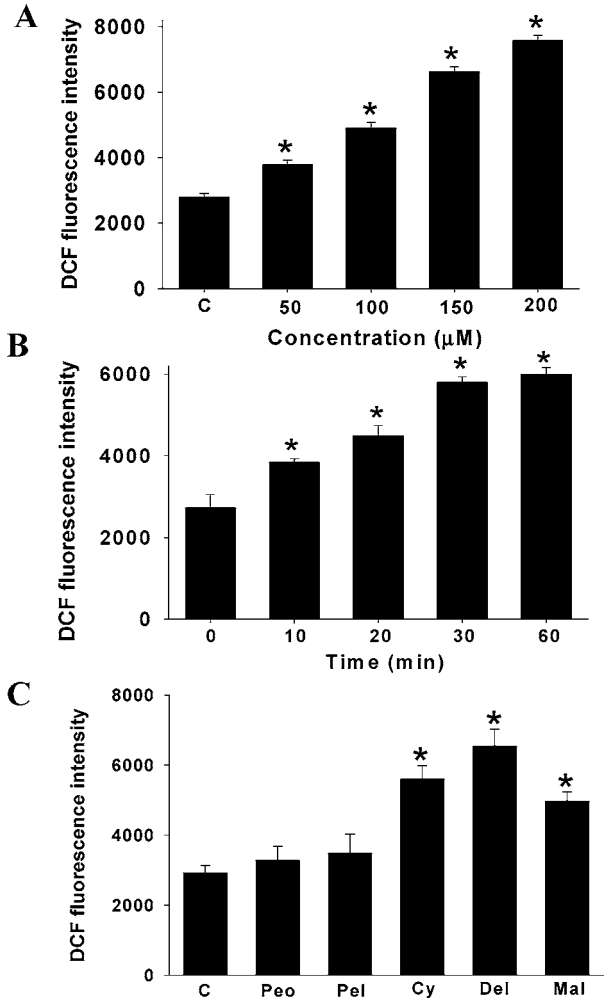

Figure 8. Generation of ROS by anthocyanidins in $HepG_2$ cells. (A) A dose-dependent production of ROS by delphinidin. Cells were exposed to the indicated concentrations of delphinidin for 30 min. (B) A time-course of the production of ROS by delphinidin. Cells were exposed to 100 μM delphinidin for the indicated times. (C) The production of ROS in anthocyanidins-treated cells. Cells were exposed to 100 μM of 0.05% DMSO (control), peonidin (Peo), pelargonidin (Pel), cyanidin (Cy), delphinidin (Del), and malvidin (Mal) for 30 min. The DCF fluorescence intensity was detected at different time intervals using a FLUOstar galaxy fluorescence plate reader with an excitation wavelength of 485 nm and emission wavelength of 530 nm. Data from three independent experiments are presented as mean ± SD. Statistically significant differences from the control are indicated as $*p < 0.05$.

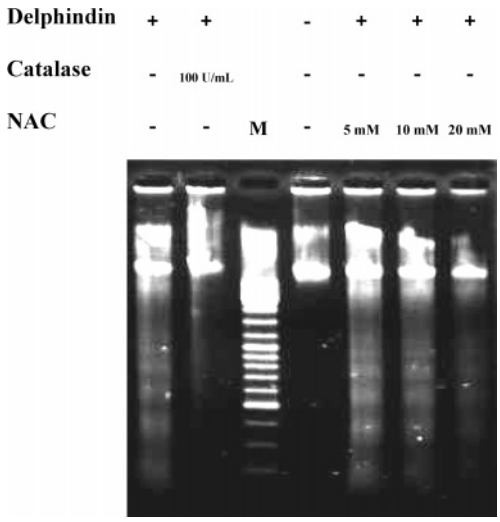

Figure 9. Suppression of delphinidin-induced DNA fragmentation by catalase and *N*-acetyl-L-cysteine. $HepG_2$ cells were pretreated for 1 h with NAC, (5, 10, 20 mM) and 100 U/mL catalase and then challenged with 100 μM delphinidin for 24 h, and agarose gel analysis of DNA fragmentation was performed. This experiment was repeated three times with similar results. M, DNA ladder marker.

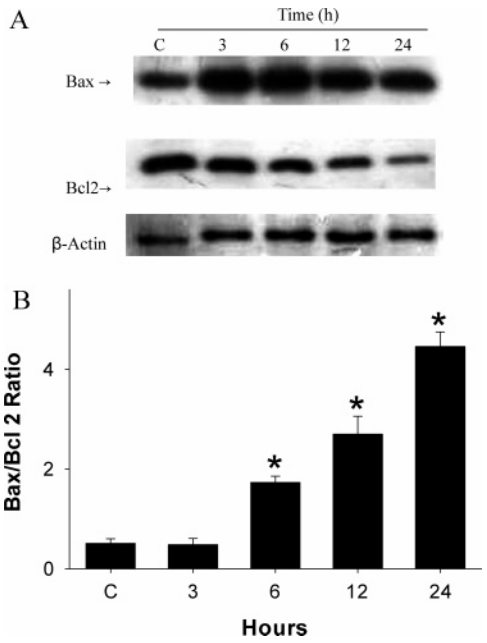

Figure 10. (A) Effect of delphinidin on protein expression of Bax and Bcl-2 in $HepG_2$ cells. The cells were treated with vehicle (0.05% DMSO) only or delphinidin (100 μM) for specified time and then harvested. (B) Time-dependent effect of delphinidin on Bax/Bcl-2 ratio. Data from three independent experiments are presented as mean ± SD. Statistically significant differences from the control are indicated as $*p < 0.05$.

the protein expression of Bcl-2 was significantly decreased by delphinidin at 100 μM. Similar time-dependent manner decrease in Bcl-2 protein expression with 100 μM delphinidin was also observed for 6, 12, and 24 h (**Figure 10A**). A significant time-dependent shift in the ratio of Bax and Bcl-2 was observed after the delphinidin treatment, indicating the induction of apoptotic process (**Figure 10B**).

DISCUSSION

Apoptosis is a type of physiological cell death accompanied by a specialized cellular mechanism and some distinctive morphological changes. Recently, apoptosis was suggested as an underlying mechanism of various anticancer and chemopreventive agents (*29*). Anthocyanins are a subclass of polyphenols that showed high chemopreventive activity in animal model systems (*10, 30*). Previous study indicated that anthocyanins exerted the remarkable cytotoxic effects in vitro on malignant human cultured cells (*18*). Thus, studies devoted to clarify the mechanisms of its action appeared to be of remarkable importance. In the present study, five typical anthocyanidins were used to investigate the potency of apoptosis induction in two human hepatoma cell lines, $HepG_2$ and Hep3B. Our data revealed that anthocyanidins possessed growth-inhibitory capability against a human hepatoma cell line, $HepG_2$ cells. The anthocyanidins at a concentration of 100 μM significantly affected the viability of $HepG_2$ cells, suggesting that the observed growth inhibition was caused by a cytotoxic effect, rather

than a cytostatic effect of anthocyanidins. It is interesting that anthocyanidins induced death in human hepatoma cells, while normal human liver cell line (chang liver cells) was completely resistant to the cytotoxic activity at the high concentration (500 μM) of anthocyanidins treatment (data not shown). Comparing anthocyanidins with anthocyanins indicated that it was the anthocyanidins, mainly cyanidin, delphinidin, and malvidin, caused strong growth inhibition in human cancer cell line $HepG_2$. The aglycoside of anthocyanins, including cyanidin 3-glucoside, peonidin 3-glucoside, pelargonidin 3-glucoside, and malvidin 3-glucoside, had lower inhibitory activities (data not shown). Hep3B cells appeared to be less susceptible to inhibition by these anthocyanidins. This is in a good agreement with the previous work of Kamei et al. (*18*), showing that anthocyanins, extracted from flower petals, were significantly suppressed the growth of the cultured cells derived from human carcinomas of the promyelocytic leukemia (HL-60 cells). Koide et al. (*31*) also reported that hydrolyzed anthocyanins from grape rinds and red rice were effective on the suppression of HCT-15 cells growth. Our results indicated that aglycon showed in general higher growth inhibition than those of the glycosides. However, the mechanisms involved in these observations remained unclear. On the basis of the estimated IC_{50} values for $HepG_2$ cells, we used delphinidin, which showed the most potent inhibition, to investigate the mechanism of apoptosis induced by the delphinidin in $HepG_2$ cells.

The induction of apoptosis will stimulate endonuclease that involves double-strand DNA breaking into oligonucleosome length fragments, resulting in a typical ladder in DNA electrophoresis and is one of the hallmarks of apoptotic cell death. As evidenced by DNA fragmentation, it appears that apoptosis is the main mechanism for cell killing in the presence of delphinidin. The apoptosis-inducing effect of delphinidin in $HepG_2$ cells appeared in a concentration- and time-dependent manners. This efficacy of delphinidin was found to be similar to their cytotoxic activities in $HepG_2$ cells. Similar to previous reports (*32*, *33*), our observations suggested that treatment with delphinidin induced the apoptotic cell death in human hepatoma cells. Multiple lines of evidence indicated that apoptosis could be triggered by the activation of a set of death effector cysteine proteases called caspases with specificity for Asp-X bonds, and their activations played important roles during apoptosis. In most of the apoptotic processes, caspase-3 has been shown to play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli (*34*). We next examined whether the caspase-3 protease was involved in the delphindin-induced cell death response. Delphinidin treatment induced the proteolytic processing of caspase-3 precursor in a time-dependent manner. Caspase-3 is a cysteine protease that exists as an inactive zymogen in cells as well as becomes activated by some sequential proteolytic events that cleave the 32 kDa precursor at aspartic acid residues to generate an active heterodimer composed of 20 and 12 kDa subunits (*35*). Furthermore, activation of caspase-3 leads to the cleavage a number of proteins, including poly (ADP-ribose) polymerase (PARP). Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis. Koide et al. (*31*) have found that anthocyanin fractions from red soybeans and red beans inhibited the growth of HCT-15 human colon carcinoma cells in vitro and prolonged the survival of Balb/C mice bearing Meth/A tumor cells. In addition, delphnidin, isolated from the bilberry extract induced apoptotic cell bodies and nucleosomal DNA fragmentation in HL-60 cells (*19*). The results presented in the present study comprised the first report to show that delphinidin inhibits the growth of human hepatoma cells and induces apoptosis, which is associated with the proteolytic degradation of PARP via the activation of caspase-3.

Some previous studies indicated that several signaling molecules such as MAPK, JNK, and p38, are involved in apoptotic process (*36*). It was also found that c-Jun N-terminal kinases (JNKs; also named stress-activated protein kinase) were involved in cellular responses to various extracellular stimuli (*37*). The JNK subfamily, including JNK1, JNK2, and JNK3 in various isoforms, is a member of the mitogen-activated protein kinase family. It has been known that JNK pathway activation is required for the apoptosis induced by ultraviolet light (UV), γ-irradiation, heat shock, proinflammatory cytokines, and different DNA damaging agents such as cisplatinum and alkylating agents (*38*). Genetic studies in animals also supported the role of JNK in an apoptotic death program. The *jnk1/jnk2*-deficient mice having severe dysregulation of apoptosis in the brain were embryonically death (*39*). One major target of the JNK pathway is the transcription factor c-Jun, which is regulated at both transcriptional and posttranscriptioanl levels by activated JNK. Moreover, the activation of this pathway leads to the phosphorylation of the c-Jun protooncogene in its N-terminal activation domain at serine residues 63 and 73 (*40*). Meiers et al. (*41*) have found that delphinidin inhibited the growth of human tumor cell line by shutting off the epidermal growth-factor receptor downstream signaling cascade. In addition, delphinidin has been recently reported to inhibit endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2 (*33*). In this study, we observed a rapid and persistent phosphorylation of JNK. c-Jun expression in a time-corresponding manner was observed after JNK phosphorylation, suggesting that the activation of JNK pathway was associated with delphinidin-induced apoptosis. Reactive oxygen species have been demonstrated to play an essential role in apoptosis induced by the anticancer agent tamoxifen in $HepG_2$ cells (*42*). Recent studies have reported that the generation of reactive oxygen species and disruption of redox homeostasis could induce apoptosis. Therefore, we investigated the role of reactive oxygen species as a upstream signal to induce apoptosis in delphinidin-treated $HepG_2$ cells. Numerous investigations have indicated the involvement of reactive oxygen spices in apoptosis induced by various stimuli (*43*). It is known that rutinoside, a flavonoid compound, induces the generation of reactive oxygen species in cell-free and intact cells (*44*). The generation of reactive oxygen species by rutinoside treatment is associated with a chemical reaction between the hydroxyl functioned groups in its chemical structure. In our study, the dose- and time-dependent generations of reactive oxygen species by delphinidin treatment were observed in $HepG_2$ cells. These data suggested that reactive oxygen species might be present in delphinidin-induced cells. Moreover, the free radical scanvengers including *N*-acetyl-L-cysteine and catalase can protect cells against the dephinidin-induced DNA fragmentation apoptosis. *N*-Acetyl-L-cysteine is a free radical scavenger and glutathione precursor, which can protect cells against oxidative damage. Catalase is an antioxidative enzyme, which converts H_2O_2 to H_2O and O_2 and protects cells from reactive oxygen species-mediated damages. In fact, the production of reactive oxygen species in anthocyanidin-treated cells was directly measured by using DCFH-DA, suggesting that apoptosis induced by delphinidin is dependent on ROS production.

Members of the Bcl-2 family of proteins are critical regulars of the apoptotic pathways. Additionally, Bcl-2 family proteins

also have been demonstrated to be involved in the process of apoptosis, pro-apoptotic, and anti-apoptotic. Bcl-2 family proteins including Bax, Bak, and Bcl-Xs for pro-apoptosis and Bcl-2, Bcl-XL, and Mcl-1 for anti-apoptosis have been identified. Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins participated in the process of apoptosis (*45*). The intermediate signaling moiety between JNK activation and cytochrome *c* release might be linked to Bax protein, as activated JNK failed to induce apoptosis in cells deficient in members of the proapoptotic Bax subfamily of Bcl-2-related proteins; furthermore, in JNK-deficient cells, stress signals failed to activate Bax, induced cytochrome *c* release, and caused cell death (*46*). Further, overexpression of anti-apoptotic Bcl-2 proteins protected cells from apoptosis induced by stimulants (*47*). Bcl-2 has been shown to form a heterodimer with the pro-apoptotic member Bax and might thereby neutralize its pro-apoptotic effects. Therefore, alterations in the levels of Bax and Bcl-2, i.e., the ratio of Bax/Bcl-2, are decisive factors and played an important role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. In our study, a decrease in Bcl-2 expression was observed in delphinidin-treated cells. Importantly, the protein expression of Bax, however, was up-regulated in delphinidin-treated cells after the treatment up to 24 h; hence, the ratio of Bax to Bcl-2 was altered in favor of apoptosis. Our results suggested that the up-regulation of Bax and the down-modulation of Bcl-2 might be another molecular mechanism through which delphinidin induced apoptosis in $HepG_2$ cells.

Recent studies concerning bioavailability of polyphenols are in agreement with their potential therapeutic effects. Anthocyanin pigments are responsible for the red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers, and possess antiinflammatory (*6*), antiangiogenesis (*7*), anticancer (*8*), and antioxidant (*14*−*16*) activities. They have long been the subject of investigation by botanists and plant physiologists because of their roles as pollination attractants and phytoprotective agents. Foods such as blueberries are often promoted as dietary sources of anthocyanins (2.67 ± 0.097 μmol/g) (*48*). Moreover, oral intake of anthocyanins from elderberry could be incorporated into human plasma with their structures maintained (*49*). Data presented here indicated that anthocyanidins, in the concentration range of 100−200 μM, had the apoptotic activity in human hepatoma $HepG_2$ cells. The range of concentrations used in the present study is consistent with many other studies (*18*, *31*, *41*) on the antitumor effect of anthocyanins in culture cells. The maximum concentration of 200 μM anthocyanidins adopted in this study, corresponding to 6.8 mg/100 mL, is within the concentration range of anthocyanins contained in blackberry juice used in a recent study regarding absorption and excretion of anthocyanins in humans (*50*). These data with other dietary consumption reports suggest that a possibility of moderate consumption of anthocyanins through the intake of the product such as blackberry extracts may be linked with cancer prevention. A structurally related activity study showed that the antioxidant activity increased with an increasing number of hydroxyl substituents present on the B-ring of anthocyanidins. However, substitution by methoxyl groups diminished the antioxidant activity of the anthocyanidins (*51*). In the present study, five typical anthocyanidins, including peonidin, pelargonidin, cyanidin, delphinidin, and malvidin, were used to study their apoptotic effects on hepatoma cells. These five anthocyanidins have different numbers of hydroxyl substituents present on the B-ring. The number of hydroxyl substituents on the B-ring for delphinidin, cyanidin, and malvidin are 3, 2, and 1, respectively. Results of the present study demonstrated that delphinidin, but not cyanidin and malvidin, showed potent apoptosis-inducing activity in $HepG_2$ cells. We proposed that the number of hydroxyl substituents on the B-ring is important for the apoptosis-inducing activity of anthocyanidin.

In conclusion, this study showed that anthocyanidins could induce apoptosis in $HepG_2$ cells. Dephinidin, the strongest inducer of anthocyanidins tested, might generate an oxidant signal to stimulate JNK pathway activation, caspase-3 activation, and DNA fragmentation, leading to the decreased cell viability. Free radical scavengers, including *N*-acetyl-L-cysteine and catalase, effectively inhibited apoptosis induced by delphinidin and further blocked reactive oxygen species generation. Furthermore, these effects were found to be correlated with a shift in Bax/Bcl-2 ratio more toward apoptosis. Our result is the first evidence that delphinidin might trigger an apoptotic death program involvement of JNK signaling pathway and regulation of Bcl-2 family proteins. Taken together, our study provides evidence that anthocyanidins might be useful in the chemopreventive treatment of human hepatoma.

ABBREVIATIONS USED

JNK, c-Jun N-terminal kinase

ROS, reactive oxygen species

PARP, poly (ADP-ribose) polymerase

G3PDH, glyceraldehydes 3 phospho-dehydrogenase

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NAC, *N*-acetyl-L-cysteine

LDH, lactate dehydrogenase

AP-1, activator protein-1

DCFH-DA, 2′,7′-dichlorofluorescein diacetate

MAPK, mitogen-activated protein kinase

ERK, extracellular signal-regulated kinase

LITERATURE CITED

(1) El-Serag, H. B.; Mason, A. C. Rising incidence of hepatocellular carcinoma in the United States. *N. Engl. J. Med.* **1999,** 340, 745−750

(2) Schafer, D. F.; Sorrell, M. F. Hepatocellular carcinoma. *Lancet* **1999,** 53, 1253−1257.

(3) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, 56, 317−333.

(4) Middleton, J. E.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, 52, 673−751.

(5) Detre, Z.; Jellinek, H.; Miskulin, M.; Robert, A. M. Studies on vascular permeability in hypertension: action of anthocyanosides. *Clin. Physiol. Biochem.* **1986**, 4, 143−149.

(6) Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y. C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, 62, 802−805.

(7) Atalay, M.; Gordillo, G.; Roy, S.; Rovin, B.; Bagchi, D.; Bagchi, M.; Sen, C. K. Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS lett.* **2003**, 544, 252−257.

(8) Bomser, J. In vitro anticancer activity of fruit extracts from *Vaccinium species*. *Planta Med.* **1996**, 62, 212−216.

(9) Wang, C. J.; Wang, J. M.; Lin, W. L.; Chu, C. Y.; Chou, F. P.; Tseng, T. H. Protective effects of *Hibucus* anthocyanins against *tert*-butyl-hydroperoxide-induced hepatic toxicity in rat. *Food Chem. Toxicol.* **1999**, *38*, 411−416.

(10) Ramirez-Tortosa, C.; Andersen, O. M.; Gardner, P. T.; Morrice, P. C.; Wood, S. G.; Duthie, S. J.; Collins, A. R.; Duthie, G. G. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radic. Biol. Med.* **2001**, 31, 1033−1037.

(11) Hagiwara, A.; Yoshino, H.; Ichihara, T.; Kawabe, M.; Tamano, S.; Aoki, H.; Koda, T.; Nakamura, M.; Imaida, K.; Ito, N.; Shirai, T.; Prevention by natural food anthocyanins, purple sweet potato color and red cabbage color, of 2-amino-1- methyl-6- phenylimidazo [4,5-b] pyridine (PhIP)-associated colorectal carcinogenesis in rats initiated with 1,2-dimethylhydrazine. *J. Toxicol. Sci.* **2002**, 27, 57−68.

(12) Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats. *FEBS Lett.* **1999**, 449, 179−182.

(13) Youdim, K. A.; Martin, A.; Joseph, J. A. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radic. Biol. Med.* **2000**, 29, 51−60.

(14) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, 45, 304−309.

(15) Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y. C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, 64, 294−296.

(16) Tsuda, T.; Shiga, K.; Ohshima, K.; Kawakishi, S.; Osawa, T. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris L. Biochem. Pharmacol.* **1996**, 52, 1033−1039.

(17) Omenn, G. S. What accounts for the association of vegetables and fruits with lower incidence of cancers and coronary heart diseases. *Ann. Epidemiol.* **1995**, 5, 333−335.

(18) Kamei, H.; Kojima, T.; Hasegawa, W.; Koide, T.; Umeda, T.; Yukawa, T.; Terebe, K. Suppression of tumor cell growth by anthocyanins *in vitro*. *Cancer Invest.* **1995**, 13, 590−594.

(19) Katsube, N.; Iwashita, K.; Tsushida, T.; Yamaki, K.; Kobori, M. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* **2003**, 51, 68−75.

(20) Knowles, B. B.; Howe, C. C.; Aden, D. P. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **1980**, 209, 497−499.

(21) Chang, C.; Lin, Y.; O-Lee, T. W.; Chou, C. K.; Lee, T. S. Induction of plasma protein secretion in a newly established human hepatoma cell line. *Mol. Cell Biol.* **1983**, 3, 1133−1137.

(22) Johnstone, R. W.; Ruefli, A. A.; Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* **2002**, 108, 153−164.

(23) Sanchez-Perez, I.; Murguia, J. R.; Perona, R. Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene* **1998**, 16, 533−540.

(24) Lee, L. F.; Li, G.; Templeton, D. J., Ting, J. P. Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). *J. Biol. Chem.* **1998,** 273, 28253−28260.

(25) Chen, Y. R.; Wang, W.; Kong, A. N.; Tan, T. H. Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates. *J. Biol. Chem.* **1998**, 273, 1769−1775.

(26) Yu, R.; Shtil, A. A.; Tan, T. H.; Roninson, I. B.; Kong, A. N. Adriamycin activates c-Jun N-terminal kinase in human leukemia cells: a relevance to apoptosis. *Cancer Lett.* **1996**, 107, 73−81.

(27) Wang, I. K.; Lin, S. Y.; Lin, J. K. Induction of apoptosis by apigenin and related flavonoids through cytochrome *c* release and activation of caspase-9 and caspase-3 in leukemia HL-60 cells. *Eur. J. Cancer* **1999**, 35, 1517−1525.

(28) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biohcem.* **1976,** 72, 248−254.

(29) Bursch, W.; Kleine, L.; Tenniswood, M. The biochemistry of cell death by apoptosis. *Biochem. Cell Biol.* **1990**, 68, 1071−1074.

(30) Tsuda, T.; Horio, F.; Kitoh, J.; Osawa, T. Protective effects of dietary cyanidin 3-O-β-D-glucoside on liver ischemia-reperfusion injury in rats. *Arch. Biochem. Biophys.* **1999**, 368: 361−366.

(31) Koide, T.; Kamei, H.; Hashimoto, Y.; Kojima, T.; Hasegawa, M. Antitumor effect of hydrolyzed anthocyanin from grape rinds and red rice. *Cancer Chemother. Pharmacol.* **1996**, 11, 273−277.

(32) Favot, L.; Martin, S.; Keravis, T.; Andriantsitohaiha, R.; Lugnier, C. Involvement of cyclin-dependent pathway in the inhibitory effect of delphinidin on angiogenesis. *Cardiovasc. Res.* **2003**, 59, 479−487.

(33) Martin, S.; Favot, L.; Matz, R.; Lugnier, C.; Andriantsitohaina, R. Delphinidin inhibits endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2. *Biochem. Pharmacol.* **2003**, 65, 669−675.

(34) Thornberry, N. A.; Lazebnik, Y. Caspases: enemies within. *Science* **1998**, 281, 1312−1316.

(35) Nicholson, D. W.; Ali, A.; Thornberry, N. A.; Vaillancourt, J. P.; Ding, C. K.; Gallant, M.; Gareau, Y.; Griffin, P. R.; Labelle, M. L.; Munday, N. A.; Raju, S. M.; Smulson, M. E.; Yamin, T. T.; Yu, V. L.; Miller, D. K. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **1995**, 376, 37−43.

(36) Cross, T. G.; Scheel-Toellner, D.; Henriquez, N. V.; Deacon, E.; Salmon, M.; Lord, J. M. Serine/threonine protein kinases and apoptosis. *Exp. Cell Res.* **2000**, 256, 34−41.

(37) Franklin, R. A.; McCubrey, J. A. Kinase: positive and negative regulators of apoptosis. *Leukemia* **2002**, 14, 2019−2034.

(38) Chen, Y. R.; Tan, T. H. The c-Jun N-terminal kinase pathway and apoptotic signaling. *Int. J. Oncol.* **2000**, 16, 651−662.

(39) Kuan, C. Y.; Yang, D. D.; Samanta, D. R.; Davis, R. J.; Rakic, P.; Flavell, R. A. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron*. **1999**, 22, 667−676.

(40) Behrens, A., Sibilia, M. and Wagner, E. F. Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.* **1999**, 21, 326−329.

(41) Meiers, S.; Kemeny, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.* **2001**, 49, 958−962.

(42) Lee, Y. S.; Kang, Y. S.; Lee, S. H.; Kim, J. A. Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in $HepG_2$ human hepatoblastoma cells. *Cell Death Differ.* **2000**, 7, 925−932.

(43) Hildeman, D. A.; Mitchell, T.; Teague, T. K.; Henson, P.; Day, B. J.; Kappler, J.; Marrack, P. C. Reactive oxygen species regulate activation-induced T cell apoptosis. *Immunity* **1999**, 10, 735−744.

(44) Chen, Y. C.; Shen, S. C.; Lin, H. Y. Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. *Biochem. Pharmacol.* **2003**, 66, 1139−1150.

(45) Gross, A.; McDonnell, J. M.; Korsmeyer, S. J. Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev.* **1999**, 13, 1899−1911.

(46) Lei, K.; Nimnual, A.; Zong, W. X.; Kennedy, N. J.; Flavell, R. A.; Thompson, C. B.; Bar-Sagi, D.; David, R. J. The Bax subfamily of Bcl-2-related proteins is essential for apoptotic signal transduction by c-Jun N-terminal kinase. *Mol. Cell Biol.* **2002**, 22, 4929−4942.

(47) Rokhlin, O. W.; Guseva, N.; Tagiyev, A.; Knudson, C. M.; Cohen, M. B. Bcl-2 oncoprotein protects the human prostatic carcinoma cell line PC3 from TRAIL-mediated apoptosis. *Oncogene* **2001**, 20, 2836−2843.

(48) Kalt, W.; Forney, C. F.; Martin, A.; Prior, R. L. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* **1999**, *47*, 4638−4644.

(49) Milbury, P. E.; Cao, G.; Prior, R. L.; Blumberg, J. Bioavailablility of elderberry anthocyanins. *Mech. Aging Dev.* **2002**, 123, 997−1006.
(50) Nielsen, I.; Dragsted, L. O.; Ravn-Haren, G.; Freese, R.; Rasmussen, S. E. Absorption and excretion of black current anthocyanins in humans and wantanbe heritable hyperlilidemic rabbits. *J. Agric. Food Chem.* **2003**, 51, 2819−2820.
(51) Seeram, N. P.; Nair, M. G. Inhibition of lipid peroxidation and structure−activity related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *J. Agric. Food Chem.* **2002**, 50, 5308−5312.

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