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Protective Effect of Anthocyanins from Black Soybean Seed Coats on UVB-Induced Apoptotic Cell Death in Vitro and in Vivo

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UVB radiation proves to be one of the most relevant environmental risks because of its hazardous effects, such as premature skin aging and especially skin photocarcinogenesis. Anthocyanins, water-soluble pigments present in plants, are known to be powerful antioxidants that help protect plants from UV damage. In this study, we aimed at investigating the protective effect of anthocyanins from black soybean [*Glycine max* (L.) Merr] seed coats on UVB-induced apoptosis, and furthermore, we investigated the molecular mechanism responsible for regulation of apoptosis in vitro and in vivo. Pretreatment with anthocyanins reduced UVB-induced reactive oxygen species levels and inhibited UVB-induced apoptotic cell death through the prevention of caspase-3 pathway activation and reduction of proapoptotic Bax protein levels. UVB irradiation induced apoptotic cell death, which was inhibited by topical application of anthocyanins in hairless mice. It is concluded that anthocyanins from the seed coat of black soybeans may be useful compounds to modulate UVB-induced photoaging.

KEYWORDS: Anthocyanins; apoptosis; caspase-3; Bcl-2; Bax; ROS; UVB

INTRODUCTION

Phototherapy with ultraviolet-B (UVB) has been clinically applied to treat a variety of skin diseases, including psoriasis, because of its suppressive effects on cell-mediated immune responses and cell proliferation. However, UV irradiation accounts for most of the harmful biological effects associated with sunlight, including skin aging and malignant transformations in mammals (*1, 2*). In addition, exposure of keratinocytes to UVB leads to the expression of several genes involved in cell cycle arrest, DNA repair, and /or apoptosis. UVB can cause direct biological damage or indirect damage via the production of reactive oxygen species (ROS). UV light accounts for most of the harmful biological effects via production of ROS. In healthy aerobic organisms, the production of ROS and other free radicals such as RNS is approximately balanced by antioxidant defense systems; however, excessive ROS production causes a serious imbalance between production of ROS/reactive nitrogen species (RNS) and antioxidant defense. As a result, these situations cause oxidative damage to DNA, proteins, and lipids, and oxidative stress caused by UV contributes to inflammation, apoptosis, and carcinogenesis. Low-level exposure increases the synthesis of vitamin D, which protects from genetic damage and carcinogenesis (*3*); however, high-level exposure causes the formation of sunburn cells, which die from apoptosis. Thus, antioxidants such as *N*-acetyl-L-cysteine have been shown to effectively reduce inflammation, apoptosis, and carcinogenesis by decreasing oxidative stress (*4*).

Anthocyanins are polyphenols responsible for many fruit and floral colors and have a basic skeleton made up of 2-phenylbenzopyrylium or flavylium glycoside. These pigments act as powerful antioxidants that help to protect the plant from UV damage. Anthocyanins are abundantly present in chokeberry, cherry, aubergine, blue grape, *Vaccinium*, and red cabbage as well as the Usambara-violet. In addition, it is known that anthocyanins are especially abundant in the epidermis palisade layer of the black soybean seed coat (*4*−*9*). So far, five anthocyanins (delphinidin, pelargonidin, cyaniding, peonidine, and malvidin) are well-documented in red grapes. In contrast,

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three main anthocyanins, that is, cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside, have been characterized in black soybean seed coats (*5, 7, 9*). To date, 17 naturally occurring anthocyanins are known. Anthocyanins have been shown to have high antioxidant activity, which may be partially responsible for their anticarcinogenic activity (*10*). Recently, we have reported that anthocyanins reduced ischemic reperfusion (I/R)-induced myocardial injury in vivo and attenuated adhesion molecules expression, such as intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1, as well as inflammatory gene expression such as COX-2, which is known to be responsible for I/R injury (*11*). However, the effect of anthocyanins from soybean seed coat on UV-induced oxidative stress and apoptosis and the molecular mechanisms of their protective effects are not yet known. Thus, the aims of this study were to (i) investigate the protective effect of anthocyanins from black soybean (*Glycine max* (L.) Merr) on UVB-induced apoptosis and (ii) investigate the molecular mechanism for regulation of apoptosis by anthocyanins in a human keratinocyte HaCaT cell line.

MATERIALS AND METHODS

Materials and Chemicals. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were obtained from Gibco BRL (Rockville, MD). The anti-Bax and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals including anticleaved caspase-3 (CPP20) antibody were supplied by Sigma (St. Louis, MO).

Plant Materials. Soybean [*G. max* (L.) Merr.] accessions with black seed coat were planted at the experimental field of Gyeongsang National University (Korea) in May 2004. Plots were harvested when the plants in the plot reached maturity. Harvested seeds were air-dried to a seed moisture content of about 8.0%. The dried seeds were stored at 4 °C until they were used. The seed coats of soybean accessions were peeled manually.

Anthocyanins Extraction and Purification. The seed coats of soybean accessions (200 g) were extracted for 24 h at 4 °C with methanol. The extraction was repeated three times. After concentration under reduced pressure, the extract was diluted to a total volume of 200 mL and partitioned against ethyl acetate (3 × 200 mL). The solution containing anthocyanins was concentrated to 100 mL. The solution was subjected to an Amberlite XAD-7 (Aldrich, St. Louis, MO) column and washed with deionized water and eluted with methanol containing 1% HCl. The solvent was vaporized under reduced pressure, and the purple sticky solids were dissolved in 50 mL of 30% aqueous methanol containing 1% HCl. The solution was applied to a column packed with Sephadex LH-20 (Amersham Biosciences, Sweden) and eluted using 30% aqueous methanol containing 1% HCl. Cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside were isolated from seed coats of black soybean and used as an anthocyanin source. The purity and the compositions of anthocyanins were analyzed using high-performance liquid chromatography. Anthocyanins consisted of cyanidin-3-glucoside (72%), delphinidin-3-glucoside (20%), and petunidin-3-glucoside (6%).

Animals. Eight to ten week old adult female hairless mice (HR-1) were provided by Japan SLC Inc. (Shizuoka, Japan). All experiments were performed in compliance with institutional guidelines set by the Institutional Animal Care and Use Committee at the Gyeongsang National University (Republic of Korea).

Cell Culture. The immortalized human keratinocyte cell line, HaCaT, was kindly provided by Professor N. Fusenig (German Cancer Research, Germany). The cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were then seeded at a density of 1×10^6 cells/100 mm dishes. After 48 h, the cells were washed with serum-free medium and replaced with media without FBS for 12 h prior to anthocyanins treatment. After starvation time, cells were pretreated with anthocyanins in different doses for 24 h, washed three times with PBS, and then stimulated with UVB in PBS. After stimulation, cells were incubated with or without anthocyanins for 1 (ROS detection) or 24 h (cell viability or apoptosis detection). Cells were used for experiments until passage number 20.

UV Irradiation to HaCaT. UVB irradiation was performed on serum-starved monolayer cultures utilizing a FS20 Lamp (National Biological, Twinsburg, OH). The UVB source was a bank of two FS20 lamps emitting a continuous spectrum from 270 to 390 nm, with a peak emission at 313 nm; approximately 65% of the radiation was within the UVB (280−320 nm) wavelength. The irradiance of the single bulb averaged 6.25 J/m^2/s at a tube-to-target distance of 8 cm, and the irradiance of the bank of two bulbs averaged 12 J/m^2/s, as measured by a spectroradiometer (model IL1700A; International Light, Inc., Newburyport, MA). The cells were irradiated with UVB in a minimum amount of PBS (about 3 mL). After UVB irradiation, the cells were cultured in serum-free medium for the indicated time with or without anthocyanins or other drugs.

UVB Irradiation of Animals and Preparation of Skin Samples. Fifteen hairless mice were divided into five groups of three animals each and exposed to UVB irradiation (2.4 kJ/m^2). UVB irradiation to animals was performed as described by Kim et al. (*12*). Immediately after UVB irradiation, anthocyanins (at a dose of 50 or 100 mg/kg in sterile water, 100 μL) were topically applied to the mice. Twenty-four hours later, mice were anesthetized (pentobarbital sodium, 50 mg/kg) and killed by exsanguinations from the retro-orbital plexus. The dorsal skin (ca. 1 cm × 2 cm) was excised from each mouse and cut into two pieces. One piece was immediately fixed in 10% neutral buffered formaldehyde for paraffin embedding. The other piece was homogenized and subjected to Western blotting.

Assessment of Cell Viability. To evaluate cell survival after different treatments, 10 μL of trypan blue solution was added to 10 μL of cells collected from each plate, and the cells were incubated for 2 min. Unstained live cells were counted on a hemocytometer.

Western Blot Analysis. Cells were harvested and lysed with buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), and protease inhibitors. Skin samples were also harvested and lysed with lysis buffer. Then, protein was taken from the supernatant after centrifugation at 10000 rpm for 15 min. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL). Thirty micrograms of total protein was electrophoresed on a 12% polyacrylamide gel. The separated proteins were transferred to polyvinylidene fluoride membrane (Amersham) for 2 h at 20 mA with a SD Semidry Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. The membranes were then incubated with primary antibodies (anti-Bax, anti-Bcl-2, and anti-cleaved caspase-3 antibodies) at a concentration of 1:500 in 5% skim milk TBS-T overnight at 4 °C. Bound antibody was detected by horseradish peroxidase-conjugated secondary antibodies, and signals were detected by the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

TdT-Mediated dUTP Nick End Labeling (TUNEL) Assay. A TUNEL assay was performed by an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol, with a minor modification.

Measurement of Intracellular ROS. Intracellular ROS production was measured by 2′,7′-dichlorofluorescein fluorescence using confocal laser scanning microscopy. Dishes of subconfluent cells were washed and incubated in the dark for 30 min in the presence of 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Culture dishes were transferred to an inverted microscope with a confocal imaging system (Olympus, Tokyo, Japan), and ROS generation was detected by oxidation of DCFH using a fluorescein isothiocyanate (FITC) filter set. Pixel images were collected by single rapid scans. Fluorescent images were analyzed using SigmaGel 1.0 (Jandel Scientific, Germany). Analyses were repeated three times over the same region, and the results are the means of three independent experiments.

Flow Cytometric Analysis. FACS analysis was performed using a slight modification of the method reported by Telford et al. (*13*). Briefly,

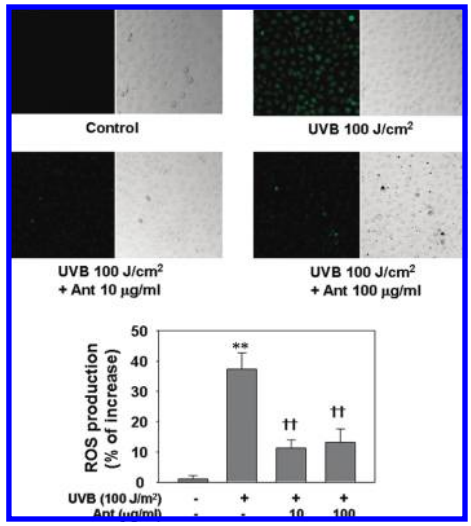

Figure 1. Effect of anthocyanins on ROS production induced by UVB in HaCaT. Cells were pretreated with anthocyanins (10 or 100 μg/mL) for 24 h and then irradiated with UVB (100 J/m^2). After 1 h of incubation with/without anthocyanins, ROS production by confocal microscopy (bright green) analysis was performed as described in the Materials and Methods. The experiments were performed three times ($n = 3$). $^{**}P < 0.01$, significance as compared with the control; $^{\dagger\dagger}P < 0.01$, significance as compared with UVB irradiation.

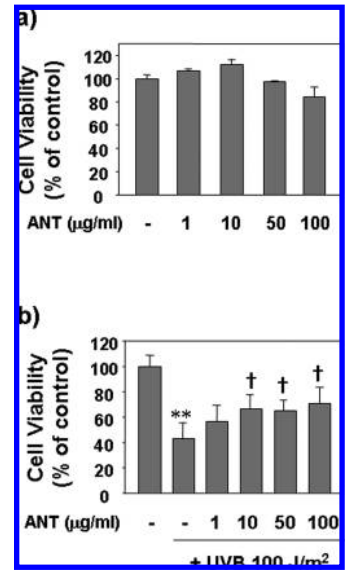

Figure 2. Effects of anthocyanins on UVB-mediated cytotoxicity in HaCaT. (**A**) Cells were incubated with various concentrations of anthocyanins (1, 10, 50, and 100 μg/mL) for 24 h, and the cell viability was measured by trypan blue analysis. (**B**) Cells were treated with anthocyanins as described in Materials and Methods. After incubation, cells were subjected to trypan blue analysis. Data represent means $\pm$ SEMs of three independent experiments ($^{**}P < 0.01$, significance as compared with the control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, significance as compared with UVB irradiation) ($n = 3$).

cells were pelleted at 1500 rpm and washed with 10 mL of ice-cold phosphate-buffered saline (PBS). The pellets were resuspended in 70% ethanol and then stored on ice for 60 min. Cells were then permeabilized in a reagent consisting of 0.5% Triton X-100, 230 μg/mL RNaseA, and propidium iodide to 50 μg/mL in PBS. The samples were kept at 37 °C for 30 min followed by flow cytometry analysis (Becton Dickinson FACScan), and the apoptotic populations (sub-G1 phase) were analyzed using the CellQuest program. The cells with DNA content less than the G0/G1 amount present in untreated cells were considered to be apoptotic cells.

Data Analysis. Scanning densitometry was performed using an Image Master VDS (Pharmacia Biotech Inc., San Francisco, CA). Treatment groups were compared using one-way analysis of variance (ANOVA), and the Newman−Keuls test was used to locate any significant differences identified in the ANOVA. The data are presented as the mean $\pm$ standard error of the mean (SEM). $P < 0.05$ or $p < 0.01$ was accepted as significant.

RESULTS

Reduction of UVB-Induced ROS Production by Anthocyanins in the Human Keratinocyte Cell Line, HaCaT. We determined the effect of anthocyanins on UVB-induced ROS production. UVB (100 J/m^2) significantly induced ROS production as compared to untreated cells (UVB 37.4% vs control 1.1%), whereas pretreatment with anthocyanins prevented UVB-induced ROS generation at doses of 10 or 100 μg/mL (37.4 to 11.1 or 13.1%, respectively) (**Figure 1**).

Effects of Anthocyanins on UVB-Mediated Cytotoxicity in HaCaT. When we examined the cell viability of keratinocytes in response to anthocyanins, anthocyanins did not influence cell viability in cultured HaCaT cells by trypan blue assay even at a high concentration ($\sim$100 μg/mL) (**Figure 2A**). Instead, anthocyanins protected HaCaT cells from UVB-induced cytotoxicity. Cell viability was decreased by UVB 100 J/m^2 (about 57%), which was significantly increased by pretreatment with anthocyanins in a concentration-dependent manner (1, 10, 50, and 100 μg/mL) (**Figure 2B**).

Protective Effect of Anthocyanins on UVB-Induced Apoptotic Cell Death in HaCaT. To determine if anthocyanins could exert a protective effect on UVB-induced apoptotic cell death in keratinocytes, TUNEL assay and FACS analysis were performed in HaCaT. UVB irradiation increased TUNEL-positive cells about 25% at 100 J/m^2, which was reduced to 6% by anthocyanins at 100 μg/mL (**Figure 3**). In addition, 100 J/m^2 UVB increased the sub-G1 % age to 18.7%, which was reduced by anthocyanins in a dose-dependent manner (1, 10, 50, and 100 μg/mL) (**Figure 4**). In particular, 100 μg/mL anthocyanins showed a prominent protective effect upon UVB (100 J/m^2)-induced cytotoxicity (decrease of apoptosis by UVB from 18.7 to 4.1%).

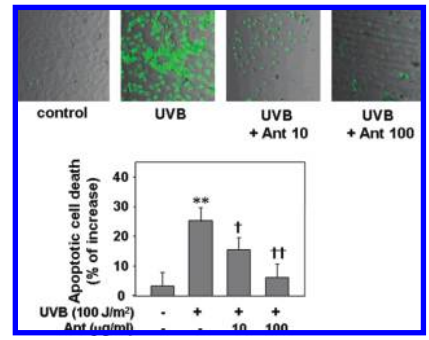

Figure 3. Protective effect of anthocyanins on UVB-induced apoptotic cell death in HaCaT by TUNEL assay. Cells were treated the same as described in the Materials and Methods, and then, the TUNEL assay was performed according to the manufacturer's protocol. The experiments were performed three times; data represent means $\pm$ SEMs of three independent experiments as a bar graph, and representative figures are presented ($^{**}P < 0.01$, significance as compared with the control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, significance as compared with UVB irradiation).

Molecular Mechanisms by Which Anthocyanins Block the Apoptotic Pathway Activated by UVB. It was previously demonstrated that caspases are involved in apoptosis induced by different stimuli and that UVB induces apoptosis via the

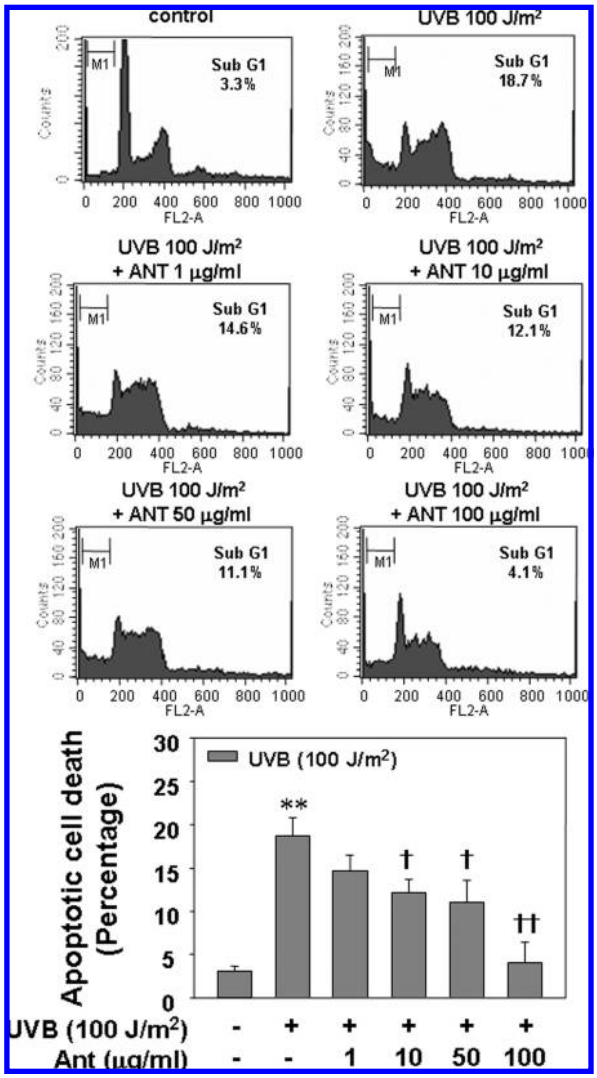

Figure 4. Protective effect of anthocyanins on UVB-induced apoptotic cell death in HaCaT by FACS analysis. Cells were treated with anthocyanins, and then, FACS analysis was performed as described in the Materials and Methods. Data represent means ± SEMs of three independent experiments (**$P < 0.01$, significance as compared with the control; †$P < 0.05$ and ††$P < 0.01$, significance as compared with UVB irradiation).

caspase-dependent pathway. Thus, the level of cleaved caspase-3 as a marker of apoptosis was determined. Cleaved caspase-3 was clearly detected in the UVB (100 J/m^2)-irradiated cells, and this level was decreased by anthocyanins (**Figure 5**). In particular, 50 or 100 μg/mL anthocyanins completely blocked the level of cleaved caspase-3. When the production of pro-apoptotic Bax and antiapoptotic Bcl-2 was measured by Western blot analysis, UVB irradiation induced pro-apoptotic Bax, which was effectively inhibited by anthocyanins, whereas the anti-apoptotic Bcl-2 level was decreased by UVB irradiation, which was recovered by anthocyanins. These results suggest that UVB irradiation induces apoptosis via caspase pathway activation and induction of proapoptotic Bax protein, which are blocked by anthocyanins.

Effect of Anthocyanins on UVB-Induced Apoptosis in Hairless Mice. To examine the effect of anthocyanins on UVB-induced apoptosis in vivo, the TUNEL assay was performed in UVB-irradiated hairless mice with or without anthocyanins. UVB increased TUNEL-positive cells in the epidermis, which was inhibited by anthocyanins (50 and 100 mg/kg) (**Figure 6**).

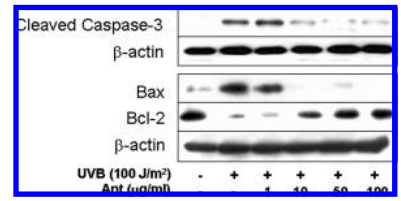

Figure 5. Immunodetections of cleaved caspase-3, Bcl-2, or Bax in the UVB-irradiated cells with/without anthocyanins. Cells were treated as described in **Figures 3** and **4**. After treatment, total cell lysates were extracted, and 30 μg of the total protein for caspase-3, Bcl-2, or Bax was electrophoresed on 12% polyacrylamide gels. Western blot analysis was performed using the anticleaved caspase-3 (cpp20), anti-Bcl-2, or anti-Bax antibodies. The results were confirmed by three independent experiments.

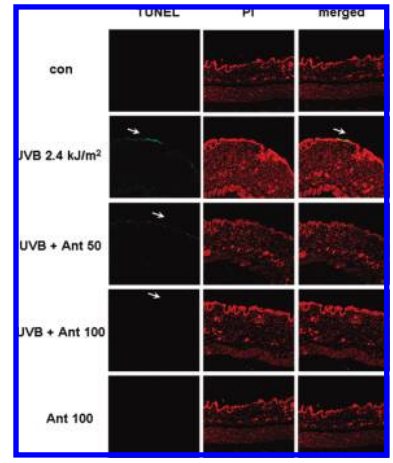

Figure 6. Effect of anthocyanins on UVB-induced apoptotic cell death in hairless mice. Immediately after UVB irradiation, anthocyanins were applied to the hairless mice, and 24 h later, the dorsal skin (ca. 1 cm × 2 cm) was excised from each mouse as described in the Materials and Methods. The TUNEL assay was performed as described in the Materials and Methods. Results were confirmed by three independent experiments ($n = 9$). All photographs were taken at a magnification of 100× (scale bar = 50 μm).

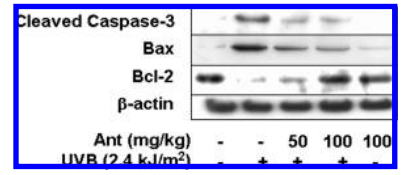

Figure 7. Effect of anthocyanins on UVB-activated apoptotic-related pathways. Western blot analysis was of hairless mice exposed as described in **Figure 6**, and Western blot analysis was performed using the dorsal skin (ca. 1 cm × 2 cm) excised from each mouse. Results were confirmed by three independent experiments ($n = 9$).

In addition, to determine if the TUNEL-positive cells actually undergo apoptosis, total protein was extracted from the skin of hairless mice, and cleaved caspase-3 and apoptosis-related proteins, Bcl-2 and Bax, were measured. As expected, cleaved caspase-3 and Bax proapoptotic protein levels were increased in UVB-treated mice skin, which were decreased by treatment of anthocyanins. UVB-mediated decrease of Bcl-2 antiapoptotic protein level was recovered by anthocyanins (**Figure 7**).

DISCUSSION

The present study showed that novel anthocyanins from black soybean [*G. max* (L.) Merr] protected keratinocytes from UVB-

induced cytotoxicity and apoptosis through the prevention of caspase-3 pathway activation and reduction of proapoptotic Bax protein level. Furthermore, topical application of anthocyanins effectively protected apoptotic cell death in mouse skin as determined by the TUNEL assay and Western blot analysis. The beneficial effects of anthocyanins may begin with regulation of UVB-mediated ROS production. Anthocyanins inhibited ROS production at early time points after UVB exposure in our study. UVB-generated ROS play a key role in mediating most resultant biological responses, including components of apoptosis pathways such as caspases activation. Thus, the protective effect of anthocyanins on UVB-induced apoptosis may be due to inhibition of caspases pathway by blocking ROS production. ROS activate a number of signaling pathways as well as cause lipid peroxidation in cellular membranes and induce oxidative damage to DNA and cellular proteins. UV light can also damage nuclear DNA due to direct absorption. Caspase activation in response to UV radiation occurs mainly via the intrinsic, mitochondria-driven pathway, downstream of cytochrome *c* release. Caspase-3 is activated by caspase-8 activated via the death receptor-mediated pathway (*14, 15*) and by caspase-9 activated via the mitochondrial activation-mediated pathway (*16*). In the mitochondrial activation-mediated pathway, the pro-apoptotic Bcl-2 members, such as Bax, are redistributed from cytosol to mitochondria. This event is followed by a reduction in the mitochondrial transmembrane potential and the release of mitochondrial cytochrome *c* to the cytosol. The released cytochrome *c* participates in the process leading to caspase-9 activation.

ROS have also been suggested to play a role as second-messenger molecules in the signaling and regulation of gene expressions related to the biological effects elicited by UVB. All of the activation mechanisms that lead to nuclear factor-κB (NF-κB) translocation have been suggested to involve ROS (*17, 18*). NF-κB is upregulated by UVB in human skin in vivo (*19*) and HaCaT human keratinocytes in culture (*20, 21*). UV-generated ROS can also activate diverse downstream signaling molecules mitogen-activated protein kinase (MAPK) and PI3 kinase. Recently, we also reported that UVB activated MAPK, NF-κB, and PI3 kinase/Akt pathway, but anthocyanins regulated the NF-κB pathway and PI3 kinase/Akt pathway activated by UVB but not MAPK (submitted data). Actually, the PI3 kinase/Akt pathway is known as a survival pathway. Ionizing or UV radiation-induced cellular survival signaling pathways protect cells from apoptosis; however, at the same time, it may induce the development of cancer (*22*). Thus, it is important to maintain a balance between apoptosis and survival.

Taken together, this study shows that anthocyanins from black soybean have antioxidant effects along with down-regulation of ROS generation and apoptosis by UV radiation, suggesting that they could be useful compounds to prevent UV-induced damage. Furthermore, this study proposes a molecular mechanism by which anthocyanins prevent UV radiation-mediated apoptosis. Thus, this study may help to prevent and treat UVB-induced photoaging and help to develop a therapeutic strategy to target these pathways.

ABBREVIATIONS USED

ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemoluminescence; ERK, extracellular regulated kinase; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κ B; ROS, reactive oxygen species; TBS-T, Tris-buffered saline/Tween 20; UV, ultraviolet; PI3K, phosphatidylinositol 3-kinase; TUNEL, TdT-mediated dUTP nick end labeling.

LITERATURE CITED

(1) Elder, D. E. Human melanocytic neoplasms and their etiologic relationship with sunlight. *J. Invest. Dermatol.* **1989**, *92*, 297S–303S.

(2) Nomura, T. H.; Nakajima, T.; Hongyo, E.; Taniguchi, K.; Fukuda, L.; Li, Y.; Kurooka, M.; Sutoh, K.; Hande, P. M.; Kawaguchi, T.; Ueda, M.; Takatera, H. Induction of cancer, actinic keratosis, and specific p53 mutations by UVB light in human skin maintained in severe combined immunodeficient mice. *Cancer Res.* **1997**, *57*, 2081–2084.

(3) Wong, G.; Gupta, R.; Dixon, K. M.; Deo, S. S.; Choong, S. M.; Halliday, G. M.; Bishop, G. E.; Ishizuka, S.; Norma, A. W.; Posner, G. H.; Mason, R. S. 1,25-Dihydroxivitamin D and three low-calcemic analogs decrease UV-induced DNA damage via rapid response pathway. *J. Steroid Biochem. Mol. Biol.* **2004**, *89–90*, 567–470.

(4) Morley, N.; Curnow, A.; Salter, L.; Campbell, S.; Gould, D. N-Acetyl-L-cysteine prevents DNA damage induced by UVA, UVB and visible radiation in human fibroblasts. *J. Photochem. Photobiol. B* **2003**, *72*, 55–60.

(5) (a) Kuroda, C.; Wada, M. The colouring matter of kuromame. *Proc. Imp. Acad. (Tokyo)* **1988**, *9*, 17. (b) Manabe, T.; Kubo, S.; Kodama, M., III. Some properties of an anthocyanin pigment isolated from black soybean peels. *Nippon Shokuhin Kogyo Gakkaish* **1965**, *12*, 472.

(6) Yoshikura, K.; Hamaguchi, Y. Anthocyanins of the black soybean. *Eiyo To Shokuryo* **1969**, *22*, 367.

(7) Taylor, B. H. Environmental and chemical evaluation of variations in hilum and seed coat colors in soybean. M.S. Thesis, University of Arkansas, Fayetteville, AR, 1976.

(8) Yoshida, K.; Sato, Y.; Okuno, R.; Kameda, K.; Isobe, M.; Kondo, T. Structural analysis and measurement of anthocyanin from colored seed coats of *Vigna*, *Phaseolus*, and *Glycine* lugumes. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 589–593.

(9) Choung, M. G.; Baek, I. Y.; Kang, S. T.; Han, W. Y.; Shin, D. C.; Moon, H. P.; Kang, K. H. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). *J. Agric. Food Chem.* **2001**, *49*, 5848–5851.

(10) Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. Suppression of tumor cell growth by anthocyanins in vitro. *Cancer Invest.* **1995**, *13*, 590–594.

(11) Kim, H. J.; Tsoy, I.; Park, J. M.; Chung, J. I.; Shin, S. C.; Chang, K. C. Anthocyanins from soybean seed coat inhibit the expression of TNF-alpha-induced genes associated with ischemia/reperfusion in endothelial cell by NF-kappaB-dependent pathway and reduce rat myocardial damages incurred by ischemia and reperfusion in vivo. *FEBS Lett.* **2006**, *580*, 1391–1397.

(12) Kim, H. J.; Kang, S. Y.; Kim, S. J.; Kim, S. H.; Kim, T. Y. Potentiation of UVB-induced apoptosis by novel phytosphingosine derivative, tetraacetyl phytosphingosine in HaCaT cell and mouse skin. *Apoptosis* **2004**, *9*, 449–456.

(13) Telford, W. G.; King, L. E.; Fraker, P. J. Rapid quantitation of apoptosis in pure and heterogeneous cell populations using flow cytometry. *J. Immunol. Methods* **1994**, *172*, 1–16.

(14) Deveraux, Q. L.; Roy, N.; Stennicke, H. R.; Van Arsdale, T.; Zhou, Q.; Srinivasula, S. M.; Alnemri, E. S.; Salvesen, G. S.; Reed, J. C. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J.* **1998**, *17*, 2215–2223.

(15) Kluck, R. M.; Bossy-Wetzel, E.; Green, D. R.; Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **1997**, *275*, 1132–1136.

(16) Gross, A.; Jockel, J.; Wei, M. C.; Korsmeyer, S. J. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J.* **1998**, *17*, 3878–3885.

(17) Schreck, R.; Rieber, P.; Baeuerle, P. A. Reactive oxygen intermediates as apparently widely used messengers in the

activation of the NF-κB transcription factor and HIV-1. *EMBO J.* **1991**, *10*, 2247–2258.

(18) Schreck, R.; Meier, B.; Mannel, D. N.; Droge, W.; Baeuerle, P. A. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J. Exp. Med.* **1992**, *175*, 1181–1194.

(19) Fisher, G. J.; Datta, S. C.; Talwar, H. S.; Wang, Z. Q.; Varani, J.; Kang, S.; Voorhees, J. J. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* **1996**, *379*, 335–339.

(20) Saliou, C.; Rimbach, G.; Moini, H.; McLaughlin, L.; Hosseini, S.; Lee, J.; Watson, R. R.; Packer, L. Solar ultraviolet-induced erythema in human skin and nuclear factor-kappa-B-dependent gene expression in keratinocytes are modulated by a French maritime pine bark extract. *Free Radical Biol. Med.* **2001**, *30*, 154–160.

(21) Kitazawa, M.; Nakano, T.; Chuujou, H.; Shiojiri, E.; Iwasaki, K.; Sakamoto, K. Intracellular redox regulation by a cystine derivative suppresses UV-induced NF-kappa B activation. *FEBS Lett.* **2002**, *526*, 106–110.

(22) Zhan, M.; Han, Z. C. Phosphatidylinositide 3-kinase/AKT in radiation responses. *Histol. Histopathol.* **2004**, *19*, 915–923.

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