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Cyanidin 3-Glucoside and Peonidin 3-Glucoside Inhibit Tumor Cell Growth and Induce Apoptosis In Vitro and Suppress Tumor Growth In Vivo

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Abstract: *Dietary polyphenols, including anthocyanins, are suggested to be involved in the protective effects of fruits and vegetables against cancer. However, anticancer effects of peonidin 3-glucoside have not been clearly demonstrated, with only limited studies being available concerning the inhibitory effect of cyanidin 3-glucoside for tumor cell growth. Therefore, in this study, we have isolated and identified the two bioactive compounds, peonidin 3-glucoside and cyanidin 3-glucoside, from* Oryza sativa *L. indica, to treat various cancer cells. The results showed that, among analyzed cell lines, HS578T was the most sensitive to peonidin 3-glucoside and cyanidin 3-glucoside. Treatment with peonidin 3-glucoside or cyanidin 3-glucoside resulted in a strong inhibitory effect on cell growth via G2/M arrest. Regarding cell cycle–related proteins, peonidin 3-glucoside treatment resulted in down-regulation of protein levels of cyclin-dependent kinase (CDK)-1, CDK-2, cyclin B1, and cyclin E, whereas cyanidin 3-glucoside could decrease the protein levels of CDK-1, CDK-2, cyclin B1, and cyclin D1. In addition, cyanidin 3-glucoside or peonidin 3-glucoside also induced caspase-3 activation, chromatin condensation, and cell death. Furthermore, anthocyanins from* O. sativa *L. indica were evidenced by their inhibition on the growth of Lewis lung carcinoma cells in vivo.*

Introduction

Antitumor effects can be attributed to altered biochemical mechanisms, including inhibitions of proliferation, growth arrest at one or more checkpoints of the cell cycle, enhanced apoptosis, and modulation of signal transduction pathways, which are related to altered expressions of key enzymes (1). Cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs), and cyclins are important regulators of cell cycle progression, and a higher expression of these molecules accompanied by increased CDK activity has been observed in most cancer cells, which could be causally involved in deregulated and unchecked cell proliferation (1–3). CDKIs are tumor-suppressor proteins that down-regulate cell cycle progression by binding with active CDK–cyclin complexes and thereby inhibiting their kinase activities (2,3). In addition to antiproliferation and cell cycle checkpoint regulation, apoptosis is one of the major mechanisms of cancer suppression. Apoptotic cells showed characteristics such as cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, the formation of apoptotic bodies, and caspase activation (4). In particular, casapse-3 has been shown to be a key component of the apoptotic machinery to be activated in apoptotic cells and in turn cleaves downstream cellular proteins including poly ADP-ribose polymerase (PARP) protein, the cleavage of which is a hallmark of apoptosis (5).

Natural products have been used for therapeutic purposes in traditional and folk medicine; for example, several anticancer drugs originated from natural sources, including the well-known taxol, flavonoid, and colchicines (6–8), have been used in cancer chemotherapy. Because antiproliferative and cytotoxic drugs have had great success and will likely continue to play a major role in cancer treatment, we are interested in finding new active compounds from natural sources and studying their biological effects. According to these studies, black rice is a major rice cereal crop in the developing world and an important staple food in many Asian countries. It has been well documented that consumption of rice bran, which contains tocopherols, tocotrienols, and γ-oryzanol, can produce a hypocholesterolemic effect and antioxidant activity (9,10). Other reports in hypercholesterolemic rabbits have indicated that feeding black rice in place of white rice could decrease

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atherosclerotic plaque formation and increase antioxidant status by increasing serum high-density lipoprotein cholesterol and apolipoprotein (apo) A-1 concentrations (11). A recent study also showed that supplementation of black rice extracts in atherogenic diets could dramatically reduce inflammation and oxidative stress in addition to the modulation of atherosclerotic lesions in apo-E–deficient mice (12). The major anthocyanin extracts from black rice (*Oryza sativa* L. indica), including cyanidin 3-glucoside and peonidin 3-glucoside, have notable antioxidant and anti-inflammatory properties for potential use in nutraceuticals (13). Moreover, a recent report showed that cyanidin and malvidin isolated from black rice (*O. sativa* cvs. Heuginjubyeo) have exerted cytotoxicity through arrest of the G2/M phase of the cell cycle and induction of apoptosis on U937, a human leukemia cell (14). However, peonidin 3-glucoside has not been studied for its anticancer effects, whereas only little is known about the potential of cyanidin 3-glucoside in retarding tumor growth, and thus the underlying molecular mechanisms are not well defined yet. Therefore, two bioactive compounds, cyanidin 3-glucoside and peonidin 3-glucoside, were isolated and identified from *O. sativa* L. indica. The purpose of the present study was to characterize the growth-inhibitory and apoptotic effects of these compounds on human carcinoma cells, whereas the changes of cell cycle progression and the inhibitory effect of anthocyanins on tumor cell growth in vivo were also investigated. The improvement effect against cancer by combining peonidin 3-glucoside or cyanidin 3-glucoside with doxorubicin was also studied.

Materials and Methods

Chemicals

Black rice (*O. sativa* L. indica) was obtained from the Taichung area, Taiwan. Cyanidin 3-glucoside and peonidin 3-glucoside standards were obtained from Extrasynthese AA Co. (Genay Cedex, France). Bio-Gel P-2 resin was obtained from Bio-Rad Laboratories (Hercules, CA). Dulbecco's modified Eagle's medium was obtained from Life Technologies (Grand Island, NY), and fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 4′-6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St. Louis, MO). A monoclonal antibody against α-tubulin was purchased from Sigma. Rabbit polyclonal antibodies against CDK-1, CDK-2, cyclin B1, cyclin D1, and cyclin E were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a rabbit polyclonal antibody against cleaved caspase-3 and a monoclonal antibody against cleaved PARP were purchased from BD Transduction Laboratories (San Diego, CA). An ECL Plus detection kit was obtained from Amersham Life Sciences, Inc. (Piscataway, NJ).

Isolation and Identification of Peonidin 3-Glucoside and Cyanidin 3-Glucoside From Black Rice

The aleurone layer of black rice was recovered by an abrasive dehuller (Satake) at a yield of 10% and then used for the extraction of anthocyanins. The pigmented fractions (1 kg) were extracted by incubation with methanol containing 1% HCl overnight at room temperature followed by filtration through Whatman filter paper No 4. Methanol was removed by rotary evaporation under 40°C, and the pigmented fraction extracts were stored for further study. The obtained pigments were then dissolved in deionized water, and this concentrated solution was chromatographed and eluted via a 40 × 2.5 cm Diaion HP-20 column with methanol. After evaporation of the organic solvents, the resultant extract was dissolved in deionized water and then lyophilized to become powders (6.4 g) of anthocyanin fractions (*O. sativa* L. anthocyanins, OAs), yielding a recovery ratio of 0.64% (13,15).

OAs were further purified by a 40 × 2.5 cm Bio-Gel P-2 column (Bio-Rad Laboratories) with aqueous acetic acid, pH 2.5, and monitored by spectrophotometer at 520 nm with a Hitachi L-4000 UV-vis detector. The anthocyanin-rich fractions were collected and purified by high-performance liquid chromatography (HPLC) L-6220 Intelligent pump using an L-4500 diode array detector (Hitachi Instruments, Inc., Tokyo). Reversed-phase chromatographic separation was carried out on a 50 × 2.1 mm, 2.5 μm Xterra MS C18 column (Waters Corp., Milford, MA), and the Colbox column oven (Hipoint Scientific Co., Kaohsiung, Taiwan) was set at 40°C with a linear gradient mobile phase containing solvent A (water), solvent B (methanol), and solvent C (5% formic acid in water, vol/vol) at a flow rate of 0.3 ml/min. Mobile phase composition increased from A:B:C 70/10/20 to 40/40/20 within 5 min before returning to initial conditions for 3 min for the next sample. Injection volume was 5 μl. Purified anthocyanins were determined by mass spectrometry (MS) [VG Platform II liquid chromatography (LC)-MS, Micromass Co., Cheshire, UK] operated in the ESI-positive ion mode. Positive ion mode was applied with conditions as described in our previous study (15). Anthocyanins were identified by both retention time and mass profile in comparison with authentic standards.

The purity of the collected compounds after separation by Bio-Gel P-2 column was further separated by HPLC and was estimated by LC-MS to be 98%. By LC-MS, the compounds were identified to be peonidin 3-glucoside ($[M+H]^+$, *m/z* 463) and cyanidin 3-glucoside ($[M+H]^+$, *m/z* 449). These were also confirmed by spiking with authentic cyanidin 3-glucoside and peonidin 3-glucoside standards.

Cell Culture

Cell lines, including HS578T from human breast carcinoma, AGS from human gastric adenocarcinoma, SKHep-1 and Huh-7 from human hepatocellular carcinoma, HeLa

from human cervical carcinoma, and Lewis lung carcinoma (LLC) from mouse lung carcinoma, were obtained from the American Type Cell Culture (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml streptomycin sulfate. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cell Growth Assay

HS578T cells were plated in medium containing 10% FBS at a density of 1×10^4 cells/ml in 24-well plates and allowed to adhere for 24 h. The medium was then changed to fresh medium containing the test samples (cyanidin 3-glucoside or peonidin 3-glucoside), and the cells were further cultured for an additional 24, 48, or 72 h. To see if the effect of a conventional anticancer drug could be improved, cells were cultured and treated with either doxorubicin (0, 0.05, 0.1, 0.5, and 1 μM) alone or in combination with peonidin 3-glucoside or cyanidin 3-glucoside (2 μM) for 5 days. Afterward, cells were harvested, and viable cell number was counted in a hemocytometer by trypan blue exclusion (16).

Determination of Cell Viability (MTT Assay)

To evaluate the cell viability of cyanidin 3-glucoside and peonidin 3-glucoside, an MTT colorimetric assay was performed (17). Cells were seeded onto 24-well plates at a density of 1×10^4 cells/well. In concentration-dependent assays, cells were treated with OAs (0, 25, 50, 100, 200, and 500 μg/ml), cyanidin 3-glucoside (0, 30, 50, and 100 μM), or peonidin 3-glucoside (0, 30, 50, and 100 μM) for 48 h. In time-dependent assays, cells were treated with 10 μM cyanidin 3-glucoside or peonidin 3-glucoside for 24, 48, or 72 h. After the exposure period, media were removed, and cells were washed with phosphate-buffered saline (PBS) followed by incubation with 0.5 mg/ml MTT in culture medium for an additional 4 h. Afterward, 1 ml of lysis buffer (isopropyl alcohol containing 10% Triton X-100 and 0.1 N HCl) was added to dissolve the formazan formed and then measured spectrophotometrically at 570 nm.

Western Blot Analysis

After indicated cyanidin 3-glucoside or peonidin 3-glucoside treatments, the medium was removed and cells were rinsed with PBS twice. After an addition of 0.1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 mg/ml leupeptin, and 1 mg/ml aprotinin), cells were scraped followed by a vortex at 0°C for 10 min, and then cell lysate was subjected to a centrifugation of 10,000 g for 10 min at 4°C. Resultant protein samples were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane as previously described (18). The blot was subsequently incubated with 5% nonfat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h to block nonspecific binding followed by incubation with a monoclonal antibody against α-tubulin, CDK-1, CDK-2, cyclin B1, cyclin D1, cyclin E, cleaved caspase-3, or cleaved PARP for 2 h and then with a peroxidase-conjugated secondary antibody for 1 h. All incubations were carried out at 37°C, and intensive TBS-T washing was performed between incubations. After the final TBS-T washing, protein expression was detected by chemiluminescence using an ECL Plus detection kit, and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corp., San Leandro, CA).

Cell Cycle Analysis

HS578T cells were grown at a similar confluence as in cell growth assay and treated with cyanidin 3-glucoside (0, 5, and 10 μM) or peonidin 3-glucoside (0, 10, and 30 μM) in a complete medium for 48 h. At the end of treatment, cells were collected after a brief incubation with trypsin-EDTA and subjected to cell cycle analysis (19). Briefly, 1×10^5 cells were fixed in 1 ml of 70% ethanol for 30 min and then suspended in 1 ml of propidium iodide (PI) solution [25 μg/ml PI (wt/vol), 0.1 mM EDTA and 10 μg/ml RNase (wt/vol) in PBS] for 30 min in the dark. Cell cycle distribution was then analyzed by flow cytometric analysis using Cell-Quest software (Becton Dickinson, San Jose, CA).

DAPI Staining

Single-cell suspensions of treated cells were washed with PBS, fixed with 70% ethanol for 20 min at room temperature, and washed again with PBS. Cells were then treated with DAPI (0.6 μg/ml PBS), incubated for 5 min, and washed again with PBS for 5 min, and chromatin fluorescence was observed under a UV-light microscope. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation (20).

Measurement of Tumor Growth in Lewis Lung Carcinoma–Bearing Mice

C57BL/6 male mice 5–6 wk old (National Taiwan University Animal Center, Taiwan) were housed with a regular 12-h light/12-h dark cycle and ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO). Mice were injected subcutaneously with 2×10^6 LLC cells (0.1 ml/mouse) into the right front axilla. The day of cell implantation was designated Day 0, and tumor growth and tumor appearance were assessed daily after cell injection. On the next day (Day 1), mice were randomly divided into two groups ($n = 5$ for each group) to be fed by oral gavage with saline (control) and OAs (0.5%, wt/wt, daily) suspended in saline.

In tumor-bearing animals, the growth of tumors was measured daily during the study using vernier calipers. The tumor volumes were calculated using the following formula: tumor volume = 0.5 × long diameter × short diameter × short diameter. Thirty days following LLC cell injection, animals were euthanized with a high dosage of anesthetic, and the primary tumors were separated from the surrounding muscles and dermis, weighed, and fixed in formalin (21).

Statistics

Statistical significances of difference throughout this study were calculated by Student's *t*-test (SigmaStat 2.0, Jandel Scientific, San Rafael, CA).

Results

Cell Growth Inhibitory Effect of OAs on HS578T and LLC Cells

The effects of a treatment with OAs, a crude mixture, at 100 µg/ml for 48 h at 37°C, on the cell growth of six cell lines (HS578T, AGS, SKHep-1, Huh-7, HeLa, and LLC) were examined by MTT assay (Fig. 1A). The results showed that cell growth inhibitory effects of OAs were various between these cell lines with the greatest effect, in a dose-dependent way, on HS578T (Fig. 1B) and LLC cells (Fig. 1C), whereas no significant inhibitory effects were seen on AGS, SKHep-1, Huh-7, or HeLa.

Cell Growth Inhibitory Effects of Purified Anthocyanins Cyanidin 3-Glucoside and Peonidin 3-Glucoside on HS578T Cells

Cell growth inhibitory effects of cyanidin 3-glucoside or peonidin 3-glucoside on HS578T growth were determined. In MTT assay, cyanidin 3-glucoside and peonidin 3-glucoside showed a dose-dependent inhibitory effect (Fig. 2A). From this result, doses of 10 µM were chosen for the subsequent time-course experiments (Fig. 2B). A treatment with 10 µM of cyanidin 3-glucoside or peonidin 3-glucoside was conducted for 24, 48, or 72 h, and at the end of each treatment viable cells were counted. The results showed that cyanidin 3-glucoside and peonidin 3-glucoside decreased viable cell significantly in time-dependent manner (Fig. 2C). Results from the same procedures performed on MCF10A (a normal breast epithelial cell line), MRC-5 (a normal lung cell line), and Chang liver (a normal liver cell line) revealed that these compounds did not have any significant cytotoxicity on these cell lines (data not shown).

Next, the impact of cyanidin 3-glucoside or peonidin 3-glucoside on cell cycle progression was examined. After a 48-h treatment with cyanidin 3-glucoside (0, 5, and 10 µM) or peonidin 3-glucoside (0, 10, and 30 µM), cells were stained with PI and then subjected to flow cytometric analysis. Cell cycle distribution analysis showed that treatment

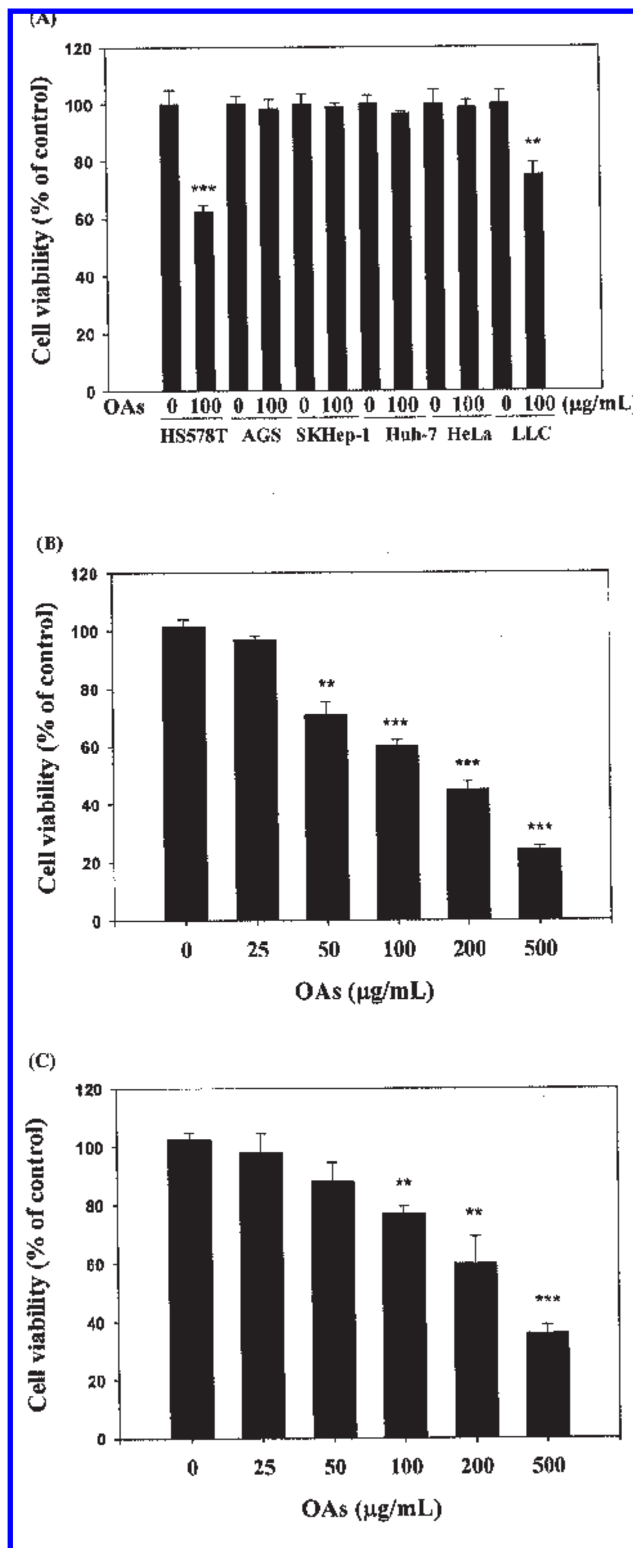

Figure 1. Effects of *Oryza sativa* L. anthocyanins (OAs) on the growth of various tumor cells. Cell growth inhibitory effects were determined by 3-[4, 5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide assay after (A) HS578T, AGS, SKHep-1, Huh-7, HeLa, and LLC cells were treated with OAs at 100 <ml for 48 h or (B) HS578T and (C) LLC cells were treated with 0, 25, 50, 100, 200, and 500 µg/ml of OAs for 48 h. The data shown were mean ± SD of three independent experiments (**$P < 0.01$; ***$P < 0.001$).

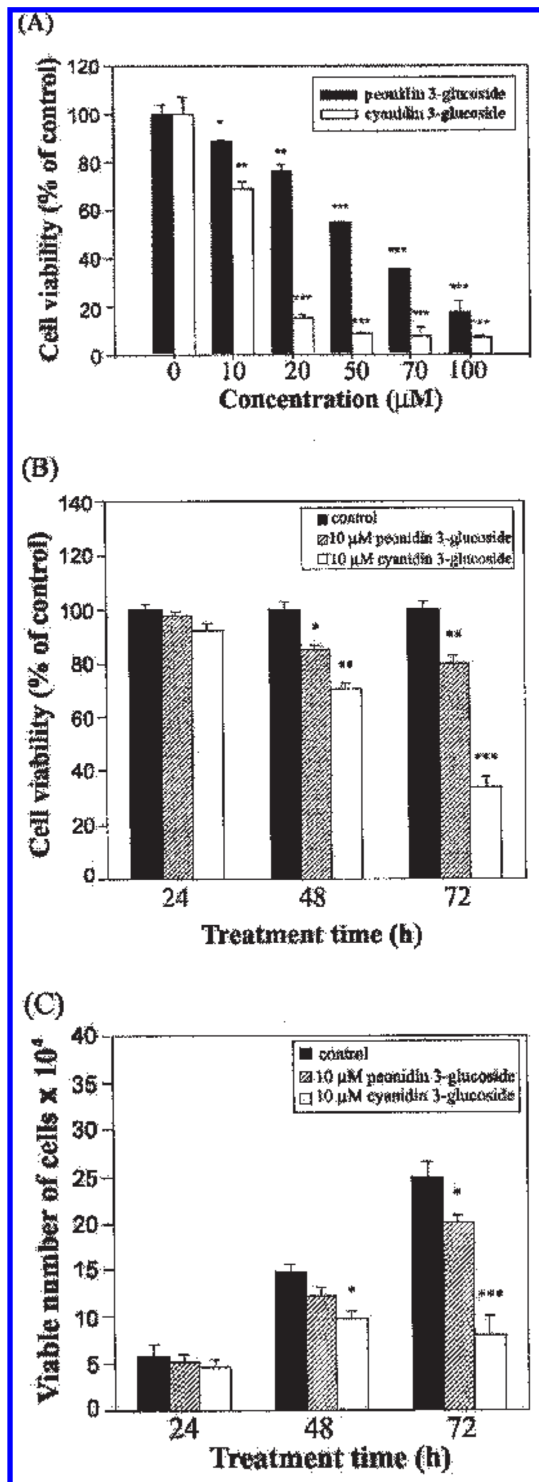

Figure 2. Inhibitory effects of cyanidin 3-glucoside and peonidin 3-glucoside on cell growth of HS578T cells. (A) In concentration-dependent assays, cells were treated with cyanidin 3-glucoside or peonidin 3-glucoside at concentrations of 0, 10, 30, 50, 70, and 100 µM for 48 h. (B) In time-dependent assays, cells were treated with cyanidin 3-glucoside or peonidin 3-glucoside at 10 µM for 24, 48, and 72 h, and then cell viability was detected using the 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide test. (C) Cells were treated with 10 µM cyanidin 3-glucoside or peonidin 3-glucoside for 24, 48, and 72 h, and then viable cells were collected and counted using a hemocytometer. The data shown were mean ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

with cyanidin 3-glucoside and peonidin 3-glucoside resulted in an increase in the G2/M population together with a decrease in the S-phase population after a 48-h treatment (Fig. 3).

Down-regulation of the Expressions of CDKs and Cyclins by Cyanidin 3-Glucoside and Peonidin 3-Glucoside

The ability of cyanidin 3-glucoside or peonidin 3-glucoside to modulate the expression of CDKs and cyclins in HS578T cells was determined. Western blot analysis showed that peonidin 3-glucoside treatment at 0, 5, 10, and 30 µM resulted in a significant decrease in the expression of CDK-1, cyclin B1, and cyclin E, and a slight decrease in the expression of CDK-2 (Fig. 4A), and a cyanidin 3-glucoside treatment at 0, 2.5, 5, and 10 µM resulted in a significant reduction in the expression of CDK-1, CDK-2, cyclin B1, and cyclin D1 (Fig. 4B) in a dose-dependent manner. However, peonidin 3-glucoside did not show any noticeable effect on the protein levels of cyclin D1, and cyanidin 3-glucoside also has no effect on the protein levels of cyclin E.

Apoptotic Effect of Cyanidin 3-Glucoside and Peonidin 3-Glucoside at High Dose

The capability of cyanidin 3-glucoside or peonidin 3-glucoside to induce apoptotic death of HS578T cells was investigated using PI staining followed by flow cytometric analysis and DAPI staining. A treatment with peonidin 3-glucoside at 30 and 50 µM or cyanidin 3-glucoside at 10 and 30 µM for 48 h resulted in a dose-dependent increase in apoptotic cell population by as determined by flow cytometric analysis (Fig. 5A). After 48-h incubation with 30 and 50 µM peonidin 3-glucoside or 10 and 30 µM cyanidin 3-glucoside, apoptotic cell bodies and chromatin condensation were observed in HS578T cells by DAPI staining (Fig. 5B). Furthermore, the ability of cyanidin 3-glucoside or peonidin 3-glucoside to modulate the expression of caspase-3 and PARP in HS578T cells was determined. Western blot analysis showed that cyanidin 3-glucoside or peonidin 3-glucoside treatment resulted in a significant increase in the active subunit of caspase-3 (Fig. 5C) and PARP (Fig. 5D).

Anticancer Effects of OAs In Vivo

With black rice is a derivable dietary supplement mainly containing peonidin 3-glucoside and cyanidin 3-glucoside, and the in vivo anticancer effects of the crude extract, OA, were further demonstrated by engrafting LLC cells to C57BL/6 mice treated with saline or OA. The injected animals were examined daily for tumor growth. Small solid tumors were observed at 8 days following cell inoculation, and a 1.5-fold reduction of tumor volume by OA feeding was seen on Day 20 and a 2.1-fold reduction on Day 29, com-

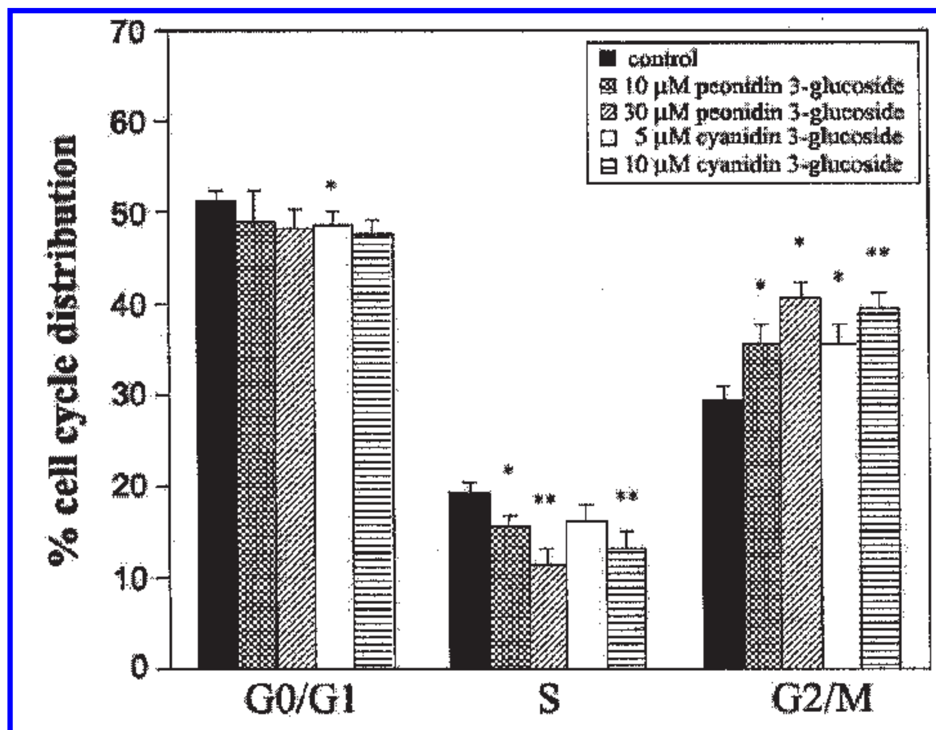

Figure 3. Induction of cell arrest by cyanidin 3-glucoside and peonidin 3-glucoside on HS578T cells. For the measurement of cell cycle distribution, cells were harvested after a 48-h treatment of cyanidin 3-glucoside or peonidin 3-glucoside and then treated with propidium iodide for 30 min followed by flow cytometric analysis for cell cycle distribution. The data shown were mean ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$).

pared with control animals (Fig. 6A). Moreover, by Day 30, OA feeding induced a 2.5-fold reduction in tumor weight (Fig. 6B) without any apparent signs of toxicity of OAs by diet consumption and body weight monitoring throughout the experiment. The purified anthocyanins cyanidin 3-glucoside and peonidin 3-glucoside also have the inhibitory effect on the tumor growth in mice (data not shown).

Peonidin 3-Glucoside and Cyanidin 3-Glucoside Improve the Activity of Doxorubicin

To assess the effect of an addition of peonidin 3-glucoside and cyanidin 3-glucoside for the anticancer effect of doxorubicin, HS578T cells were treated with either different doses of doxorubicin (0, 0.05, 0.1, 0.5, and 1 μM) alone or a combination with a low concentration of peonidin 3-glucoside or cyanidin 3-glucoside (2 μM) for 5 days. As shown in Fig. 7, the TC_{50}, producing 50% toxicity by extrapolation from the two closest tested doses, of doxorubicin alone was approximately 0.36 μM. However, a supplement of 2 μM of peonidin 3-glucoside and cyanidin 3-glucosine could cause the TC_{50} of doxorubicin to reduce to 0.19 and 0.08 μM, respectively. The analysis of cell growth showed that these agents could improve the inhibitory effect of doxorubicin on cell growth. According to the present data, it was further shown that the beneficial effect of cyanidin 3-glucoside on the action of doxorubicin was greater than that of peonidin 3-glucoside.

Discussion

The finding of the present study showed that cyanidin 3-glucoside and peonidin 3-glucoside have strong inhibitory effects on cell growth of highly metastatic breast cancer HS578T cells and that such effects involved significant alterations in cell cycle regulators to result in G2/M arrests.

In recent years, naturally occurring plant products have been getting increased attention for the prevention and/or intervention of the early stage of carcinogenesis and neoplastic progression before the occurrence of invasive malignant diseases. On the basis of this idea, certain foods, including many grains, fruits, and vegetables, as well as phytochemicals of diversified pharmacological efficacies have been shown to offer significant protection against various cancers (22). Furthermore, there is an increased focus on providing a scientific basis to use these agents in the prevention strategy for people with a high risk for cancer. In this regard, extensive investigations with anthocyanins have shown promising results against different cancers, including human leukemia (HL60 and Molt 4B), liver cancer (HepG2), and colon cancer (HCT116) in vitro (23–26). In the present study, we first provided evidence to prove the potential of cyanidin 3-glucoside

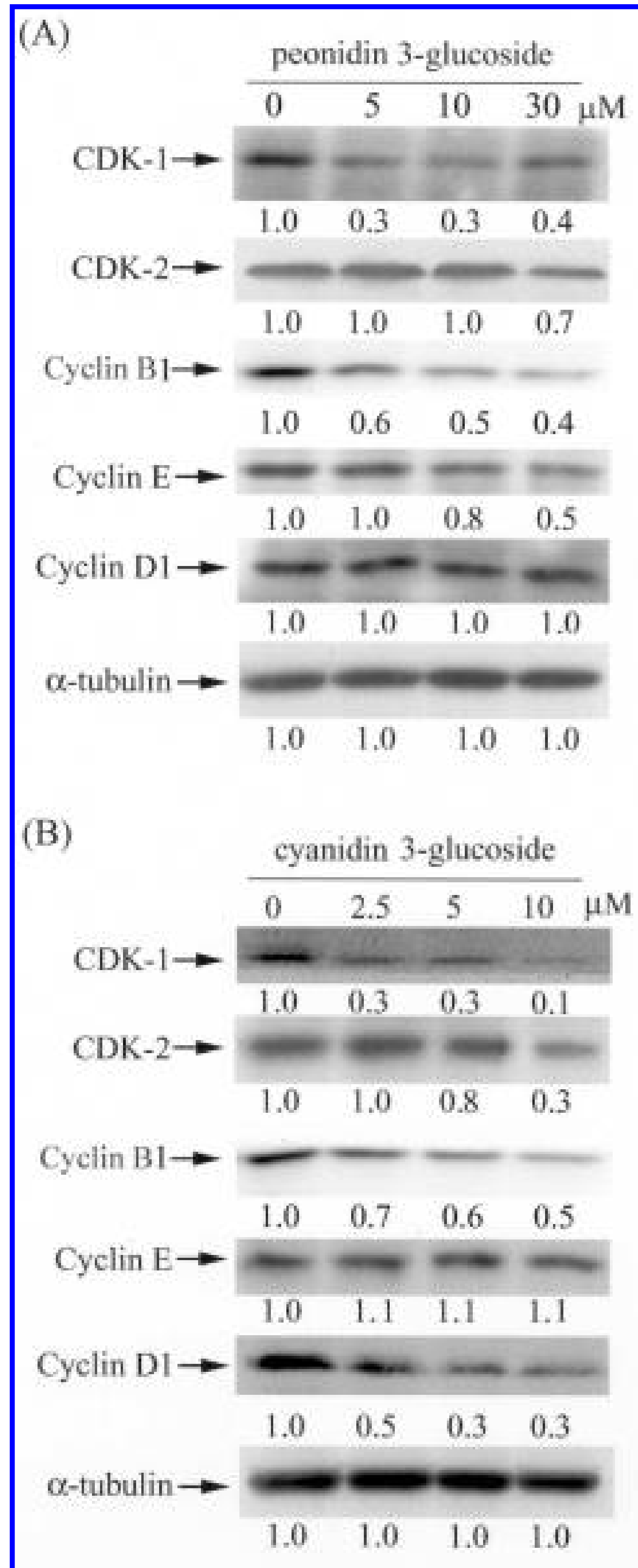

Figure 4. Effect of cyanidin 3-glucoside or peonidin 3-glucoside on cell cycle regulators. Cells were cultured in the presence of (A) peonidin 3-glucoside (0, 5, 10, and 30 μM) or (B) cyanidin 3-glucoside (0, 2.5, 5, and 10 μM) for 48 h. Cell lysates were subjected to dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting. The membranes were probed with antibodies against CDK-1, CDK-2, cyclin B1, cyclin E, or cyclin D1 together with α-tubulin as an internal control. Signals of proteins were visualized with an ECL detection system. Results from three repeated and separated experiments were similar.

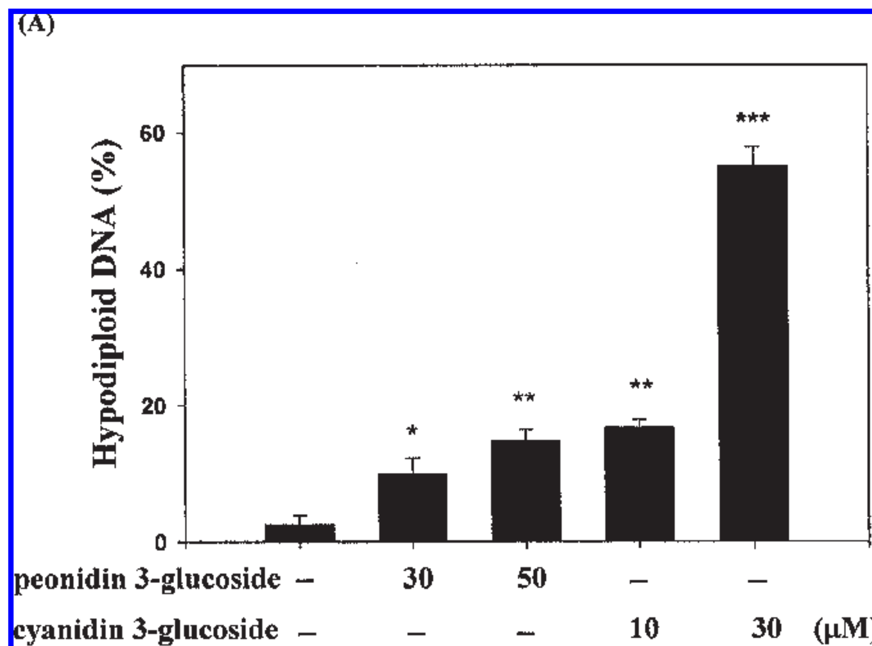

Figure 5. Apoptotic patterns of HS578T cells treated with cyanidin 3-glucoside and peonidin 3-glucoside. Cells cultured with peonidin 3-glucoside (0, 30, and 50 μM) or cyanidin 3-glucoside (0, 10, and 30 μM) were examined for apoptosis. (A) For determining sub–G1 hypodiploid cells, cyanidin 3-glucoside– or peonidin 3-glucoside–treated cells were harvested at the indicated times and treated with propidium iodide for 30 min and then analyzed by flow cytometry. (B) For nuclear morphology of HS578T cells, cells were then stained with 4′-6-diamidino-2-phenylindole and observed under a UV-light microscope. Arrows showed areas of intense fluorescence staining with condensed nuclei (at a magnification of 200×). In the Western blot assay, cell lysates were subjected to dodecyl sulfate-polyacrylamide gel electrophoresis, and the membranes were probed with antibodies against cleaved caspase-3 (C) and cleaved poly ADP-ribose polymerase (D), with α-tubulin as an internal control. Signals of proteins were visualized with an ECL detection system. The data shown were mean ± SD from three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

and peonidin 3-glucoside against cell growth and induced apoptosis of human breast cancer.

Mammalian cell growth and proliferation are mediated via cell cycle progression, and defects in cell cycle are one of the most common features of cancer cells because cancer cells divide under conditions in which their normal counterparts do not (27,28). G2/M transition is regulated by a sequential activation and deactivation of CDK-regulatory proteins and cyclin complexes. Protein tyrosine phosphatase cdc25C functions as a mitotic activator by dephosphorylating CDK-1, which forms a complex with cyclin B1 and drives the cell from the G2 to the M phase. A group of proteins called CDKIs modulates the activity of the CDK–cyclin complexes resulting in a hypophosphorylation of pRb and cell cycle arrest (1–3). Due to various genetic and epigenetic alterations, CDK-1 and cyclin B1 were often overexpressed in various cancers (29). Consistent with these facts, cyanidin 3-glucoside and peonidin 3-glucoside could down-regulate CDK-1 and cyclin B1, and these molecular alternations by cyanidin 3-glucoside and peonidin 3-glucoside could have led to a G2/M arrest, which was the effective target of many chemopreventive or chemotherapeutic agents (14,30). We also found that treatment with peonidin 3-glucoside resulted in a significantly decreased expression of cyclin E together with a lightly decreased expression of CDK-2, and a cyanidin 3-glucoside treatment resulted in a significant reduction in the expression of CDK-2 and cyclin D1 in a dose-dependent manner. The inhibitory effects on protein levels of CDK-2, cyclin D1, and cyclin E of peonidin 3-glucoside or cyanidin 3-glucoside may imply that other pathways related to cell cycle progression may be involved.

Apoptosis and associated cellular events have profound effects on the progression of benign to malignant phenotype and could be an ideal target for the therapy of various cancer cells (31). Our results revealed an induction of caspase-3 activation, chromatin condensation, and cell death of HS578T cells by cyanidin 3-glucoside or peonidin 3-glucoside, revealing another (together with growth inhibitory) anticancer effect of cyanidin 3-glucoside or peonidin 3-glucoside on breast cancer cells. Recently, several studies have shown that the ortho-dihydroxyphenyl structure on the B-ring of anthocyanins appears to be essential for the inhibitory action on the 12-*O*-tetradecanoylphorbol-13-acetate–induced cell transformation and activator protein-1 transactivation (32). According to the present data, it was further shown that the anticancer effects of cyanidin 3-glucoside were greater than those of peonidin 3-glucoside. Such variation may be risen from the different chemical structures of the R1 group, –OH and $–OCH_3$, respectively, of cyanidin 3-glucoside and peonidin 3-glucoside (32).

The major drawbacks of many effective cancer chemotherapeutic agents are systemic toxicity and drug re-

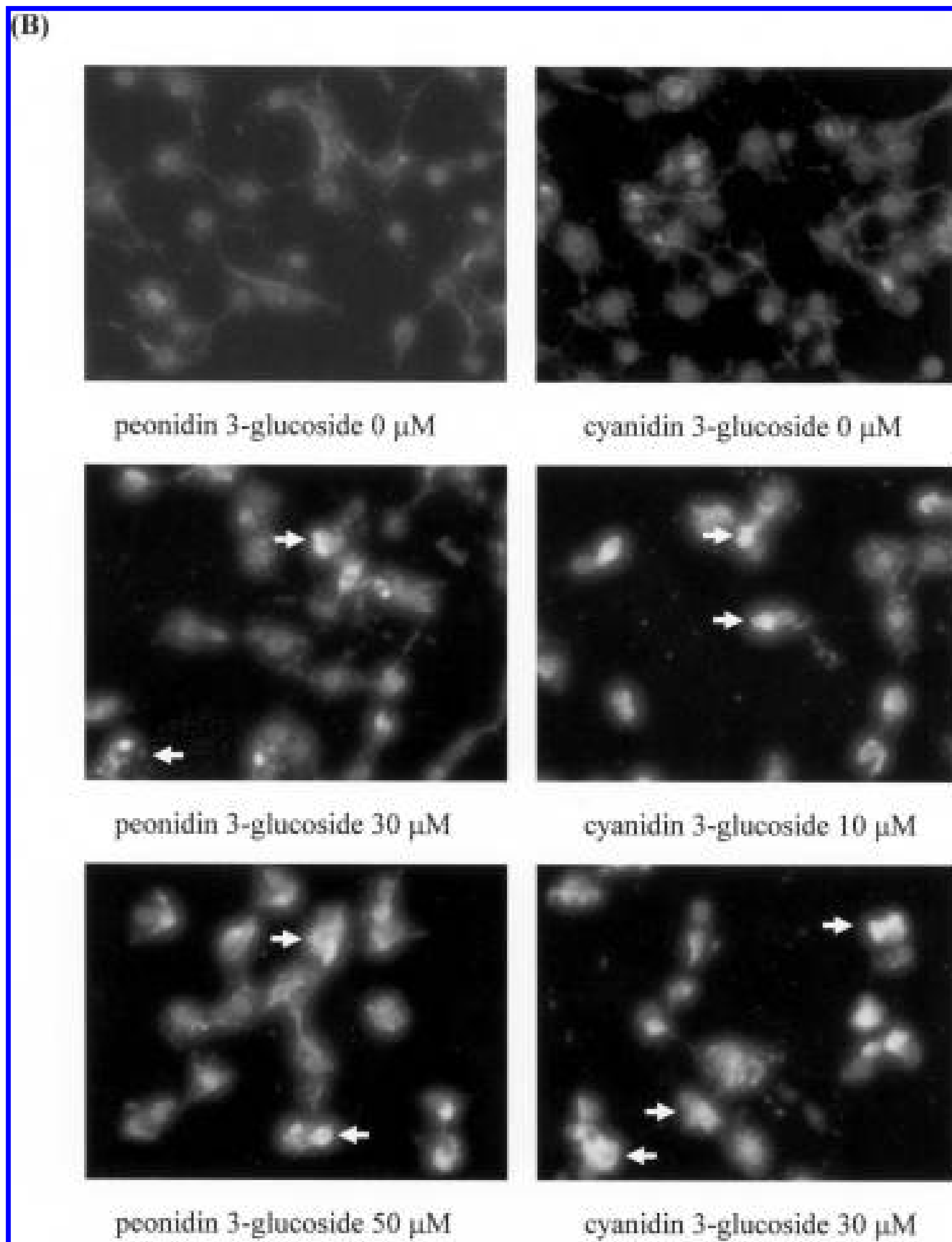

Figure 5. (Continued)

sistance. To overcome such problems, studies of combination chemotherapy have been focused to find compounds with a known action mechanism that could increase the therapeutic index of clinical anticancer drugs (33,34). Doxorubicin is one of the clinical chemotherapy agents with a broad therapeutic activity against various cancers including breast cancer (34). However, the success of doxorubicin chemotherapy has been limited due to its significant toxicity (34). Therefore, the reduction in doxorubicin-caused systemic toxicity has been a major challenge in maximizing the beneficial outcome of this therapy for cancer. In this regard, dietary supplement as well as phytotherapeutic agents with high anticancer activity and less toxicity to normal tissues have been suggested as possible candidates for their capability to improve the efficacy of anticancer drugs (22,35). Accumulating evidence indicated that treatment with a low concentration (2 μM) of peonidin 3-glucoside or cyanidin 3-glucoside does not affect the viability of HS578T cells but improves the efficacy of doxorubicin. Plasma concentration of anthocyanins responding to an oral administration has been well estab-

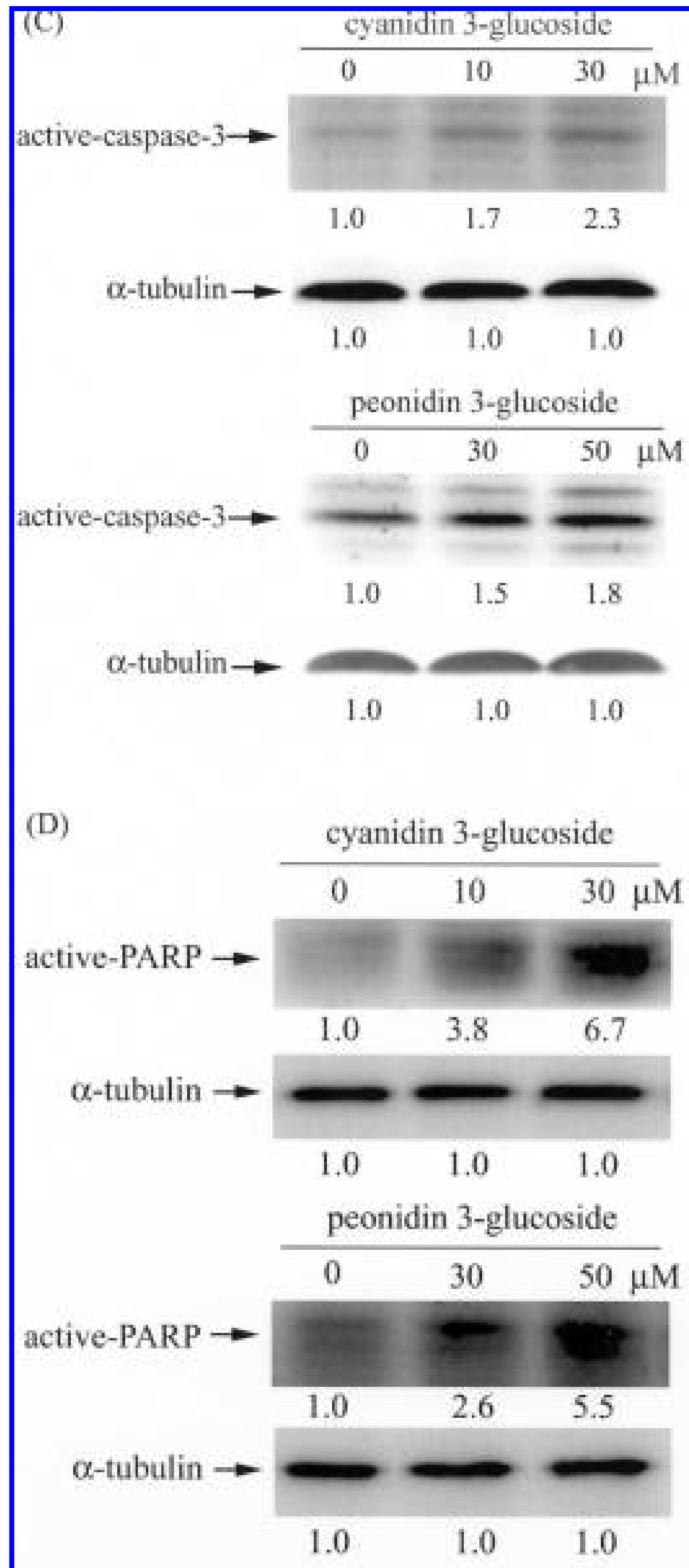

Figure 5. (Continued)

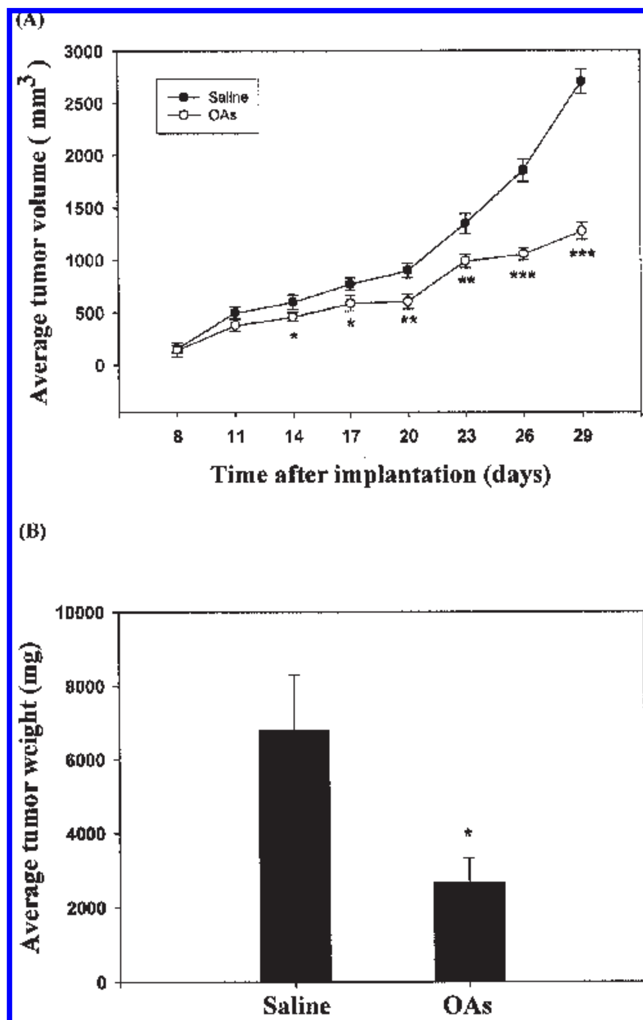

Figure 6. In vivo anticancer effects of *Oryza sativa* L. anthocyanins (OAs). After subcutaneous implantation of LLC cells, C57BL/6 mice were treated with saline or OAs as described in **Materials and Methods** and then analyzed for the growth of tumor (A) and the weight of primary tumor (B). Values represented the mean ± SD (*P < 0.05; **P < 0.01; ***P < 0.001; OAs compared with saline).

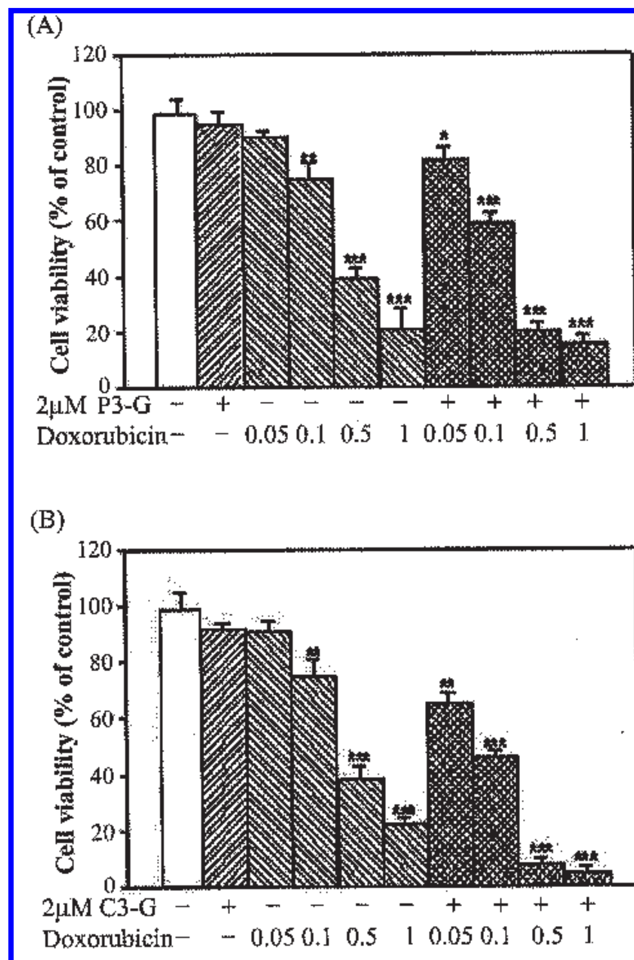

Figure 7. Improvement effect of peonidin 3-glucoside and cyanidin 3-glucoside with doxorubicin on HS578T cells. Cells were cultured and treated with either different doses of doxorubicin (0, 0.05, 0.1, 0.5, and 1 µM) alone or in combination with peonidin 3-glucoside (P3-G; 2 µM) or cyanidin 3-glucoside (C3-G; 2 µM) or cyanidin 3-glucoside (C3-G; 2 µM), and cell numbers were determined at the end of a 5-day treatment. In each case, the data shown were mean ± SD of three independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001).

lished. It was reported that human plasma concentration of anthocyanins reached 0.115 µM at 1.25–1.75 h after an oral ingestion of 3.58 mg/kg of black current concentrate (36). In a study of rats, following an oral administration of purified cyanidin 3-glucoside (800 µmol/kg of body weight), anthocyanins were detected in the plasma, and the C_{max} values were 0.840 ± 0.190 µM at 0.5–2.0 h after administration (36). Therefore, we prove a deduction that such efficient requirements could easily be fulfilled in serum of cancer patients through consumption as a dietary supplement, contributing to anticancer therapy for breast cancer.

From these results, we believe that cyanidin 3-glucoside and peonidin 3-glucoside may exert cell growth inhibition through an arrest of the G2/M phase of the cell cycle, inhibition of cell proliferation, and induction of apoptosis in vitro, whereas OAs, cyanidin 3-glucoside, and peonidin 3-glucoside could inhibit tumor growth in vivo. Moreover, peonidin 3-glucoside and cyanidin 3-glucoside exerted an improvement on the inhibitory effect of doxorubicin on cell growth of HS578T cells. Therefore, bioactive anthocyanins isolated from *O. sativa* L. indica may act as a potential candidate for cancer chemoprevention.

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References

1. Grana X and Reddy EP: Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* **11,** 211–219, 1995.
2. Swanton C: Cell-cycle targeted therapies. *Lancet Oncol* **5,** 27–36, 2004.
3. MacLachlan TK, Sang N, and Giordano A: Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. *Crit Rev Eukaryot Gene Expr* **5,** 127–156, 1995.
4. Steller H: Mechanisms and genes of cellular suicide. *Science* **267,** 1445–1449, 1995.
5. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, and Earnshaw WC: Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371,** 346–347, 1994.
6. Pezzuto JM: Plant-derived anticancer agents. *Biochem Pharmacol* **53,** 121–133, 1997.
7. Fakih M, Yagoda A, Replogle T, Lehr JE, and Pienta KJ: Inhibition of prostate cancer growth by estramustine and colchicine. *Prostate* **26,** 310–315, 1995.
8. Brown DM, Kelly GE, and Husband AJ: Flavonoid compounds in maintenance of prostate health and prevention and treatment of cancer. *Mol Biotechnol* **30,** 253–270, 2005.
9. Xu Z, Hua N, and Godber JS: Antioxidant activity of tocopherols, tocotrienols, and gamma-oryzanol components from rice bran against cholesterol oxidation accelerated by 2,2′-azobis(2-methyl-propionamidine) dihydrochloride. *J Agric Food Chem* **49,** 2077–2081, 2001.
10. Sugano M and Tsuji E: Rice bran oil and cholesterol metabolism. *J Nutr* **127,** S521–S524, 1997.
11. Ling WH, Cheng QX, Ma J, and Wang T: Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. *J Nutr* **131,** 1421–1426, 2001.
12. Xia M, Ling WH, Ma J, Kitts DD, and Zawistowski J: Supplementation of diets with the black rice pigment fraction attenuates atherosclerotic plaque formation in apolipoprotein e deficient mice. *J Nutr* **133,** 744–751, 2003.
13. Hu C, Zawistowski J, Ling W, and Kitts DD: Black rice (Oryza sativa L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. *J Agric Food Chem* **51,** 5271–5277, 2003.
14. Hyun JW and Chung HS: Cyanidin and malvidin from Oryza sativa cv. Heugjinjubyeo mediate cytotoxicity against human monocytic leukemia cells by arrest of G(2)/M phase and induction of apoptosis. *J Agric Food Chem* **52,** 2213–2217, 2004.
15. Chen PN, Chu SC, Chiou HL, Kuo WH, Chiang CL, et al.: Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. *Cancer Lett* 2006 (in press).
16. Singh RP, Tyagi AK, Zhao J, and Agarwal R: Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. *Carcinogenesis* **23,** 499–510, 2002.
17. Lai Y, Tse CM, and Unadkat JD: Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs. *J Biol Chem* **279,** 4490–4497, 2004.
18. Chiang WL, Chu SC, Yang SS, Li MC, Lai JC, et al.: The aberrant expression of cytosolic carbonic anhydrase and its clinical significance in human non-small cell lung cancer. *Cancer Lett* **188,** 199–205, 2002.
19. Zi X, Grasso AW, Kung HJ, and Agarwal R: A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. *Cancer Res* **58,** 1920–1929, 1998.
20. Cazillis M, Bringuier AF, Delautier D, Buisine M, Bernuau D, et al.: Disruption of MKK4 signaling reveals its tumor-suppressor role in embryonic stem cells. *Oncogene* **23,** 4735–4744, 2004.
21. Sigounas G, Sallah S, and Sigounas VY: Erythropoietin modulates the anticancer activity of chemotherapeutic drugs in a murine lung cancer model. *Cancer Lett* **214,** 171–179, 2004.
22. Kelloff GJ: Perspectives on cancer chemoprevention research and drug development. *Adv Cancer Res* **78,** 199–334, 2000.
23. Katsuzaki H, Hibasami H, Ohwaki S, Ishikawa K, Imai K, et al.: Cyanidin 3-O-beta-D-glucoside isolated from skin of black Glycine max and other anthocyanins isolated from skin of red grape induce apoptosis in human lymphoid leukemia Molt 4B cells. *Oncol Rep* **10,** 297–300, 2003.
24. Katsube N, Iwashita K, Tsushida T, Yamaki K, and Kobori M: Induction of apoptosis in cancer cells by Bilberry (Vaccinium myrtillus) and the anthocyanins. *J Agric Food Chem* **51,** 68–75, 2003.
25. Bagchi D, Sen CK, Bagchi M, and Atalay M: Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochemistry (Moscow)* **69,** 75–80, 2004.
26. Liu M, Li XQ, Weber C, Lee CY, Brown J, et al.: Antioxidant and antiproliferative activities of raspberries. *J Agric Food Chem* **50,** 2926–2930, 2002.
27. Elledge SJ and Harper JW: Cdk inhibitors: on the threshold of checkpoints and development. *Curr Opin Cell Biol* **6,** 847–852, 1994.
28. Sherr CJ: G1 phase progression: cycling on cue. *Cell* **79,** 551–555, 1994.
29. Abal M, Bras-Goncalves R, Judde JG, Fsihi H, De Cremoux P, et al.: Enhanced sensitivity to irinotecan by Cdk1 inhibition in the p53-deficient HT29 human colon cancer cell line. *Oncogene* **23,** 1737–1744, 2004.
30. Singh SV, Herman-Antosiewicz A, Singh AV, Lew KL, Srivastava SK, et al.: Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. *J Biol Chem* **279,** 25813–25822, 2004.
31. Stumm S, Meyer A, Lindner M, Bastert G, Wallwiener D, et al.: Paclitaxel treatment of breast cancer cell lines modulates Fas/Fas ligand expression and induces apoptosis which can be inhibited through the CD40 receptor. *Oncology* **66,** 101–111, 2004.
32. Hou DX, Kai K, Li JJ, Lin S, Terahara N, et al.: Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure-activity relationship and molecular mechanisms. *Carcinogenesis* **25**, 29–36, 2004.
33. Raghavan D, Koczwara B, and Javle M: Evolving strategies of cytotoxic chemotherapy for advanced prostate cancer. *Eur J Cancer* **33,** 566–574, 1997.
34. Von Hoff DD, Layard MW, Basa P, Davis HL Jr, Von Hoff AL, et al.: Risk factors for doxorubicin-induced congestive heart failure. *Ann Intern Med* **91,** 710–717, 1979.
35. Tyagi AK, Agarwal C, Chan DC, and Agarwal R: Synergistic anti-cancer effects of silibinin with conventional cytotoxic agents doxorubicin, cisplatin and carboplatin against human breast carcinoma MCF-7 and MDA-MB468 cells. *Oncol Rep* **11,** 493–499, 2004.
36. Matsumoto H, Inaba H, Kishi M, Tominaga S, Hirayama M, et al.: Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J Agric Food Chem* **49**, 1546–1551, 2001.