Induction of apoptosis in human colon cancer HCT-116 cells by anthocyanins through suppression of Akt and activation of p38-MAPK

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Abstract. Anthocyanins belong to a class of flavonoids that exhibit important anti-oxidant and anti-inflammatory actions as well as chemotherapeutic effects. However, little is known concerning the molecular mechanisms by which these activities are exerted. In this study, we investigated the anthocyanins isolated from Vitis coignetiae Pulliat for their potential anti-proliferative and apoptotic effects on human colon cancer HCT-116 cells. These anthocyanins inhibited cell viability and induce apoptotic cell death of HCT-116 cells in a dose-dependent manner. The apoptotic cell death was caspase-dependent and the anthocyanins regulated anti-apoptotic proteins (IAPs). In addition, apoptosis was associated with activation of p38-MAPK and suppression of Akt. In conclusion, this study suggests that the anthocyanins isolated from Vitis coignetiae Pulliat induce apoptosis might at least in part through activating p38-MAPK and suppressing Akt in human colon cancer HCT-116 cells.

Introduction

The elderly population has been increasing due to developments in medical science. This elderly population has a high risk for cancer development, and the incidence of cancer and cancer-related mortality is expected to increase. These patients have a higher risk for serious side effects from conventional chemotherapeutic agents. Therefore, drug development is focusing on the less toxic drugs to improve the quality of life. The call for the development of a less toxic drug led us to be interested in the dietary agents because many dietary agents have recently been reported to have anti-cancer activities without notable side effects (1-3). In addition, with the growth of ecological movements, natural products have become more popular for the prevention or treatment of cancer. This has been paralleled by an increase in research focused on natural products.

Vitis coignetiae Pulliat (Meoru in Korea) belongs to the grape family. The fruit has traditionally been used in Korean folk medicine for the treatment of inflammatory disorders and cancer. The intense dark red hue reflects an abundance of anthocyanin pigments the anti-cancer activities of which have been reported in vitro and in vivo (4-6). Therefore, we postulated that the anthocyanins are major components for the anti-cancer activities of the Meoru. In the previous study (7), the composition of AIMs was falsely stated. The correct composition is: delphinidin-3,5-diglucoside; cyanidin-3,5-diglucoside; petunidin-3,5-diglucoside; delphinidin-3-glucoside; malvidin-3,5-diglucoside; peonidin-3,5-diglucoside; cyanidin-3-glucoside; petunidin-3-glucoside; peonidin-3-glucoside; malvidin-3-glucoside = 1.0:0.5:3.4:28.1:6.4:4.2:22.5:4.9:22.5, constituent ratio. Here we tested the anti-cancer activity of AIMs in human solid cancer with special focus on the underlying mechanisms of the anti-cancer activities.
Materials and methods

Cell culture and chemicals. Human colon cancer cells (HCT116) from the ATCC (Rockville, MD) were cultured in RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS (Gibco BRL, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator at 37°C in a humidiﬁed atmosphere of 95% air and 5% CO2. Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Antibodies against XIAP, cIAP-1, cIAP-2, Bcl-2, Bax, Bcl-XL, Bid, of poly (ADP-ribose) polymerase (PARP), caspase-3, -8 and -9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was from Sigma (Beverly, MA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin, and an enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL). All other chemicals not speciﬁcally cited here were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell viability assays. For the cell viability assay, the cells were seeded onto 24-well plates at a concentration of 5x10^4 cells/ml, grown to 70% confluence and then treated with the indicated concentration of AIMS for 48 h. Control cells were supplemented with complete media containing 0.1% DMSO (vehicle control). Following treatment, cell number and viability were determined by MTT assays.

Nuclear staining. After treatment with the indicated concentration of vitamin C, the cells were harvested, washed with phosphate-buffered saline (PBS) and ﬁxed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 2.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) solution for 10 min at room temperature. The cells were washed two times with PBS and observed under a ﬂuorescent microscope.

Flow cytometry assay. The cells were plated at a concentration of 2x10^5 cells/well in 6-well plates. Twenty-four hours later, the cells were subsequently treated with the indicated concentration of AIMS for 48 h. The cells were washed twice with cold PBS and then centrifuged. The pellet was ﬁxed in 75% (v/v) ethanol for 1 h at 4°C. The cells were washed once with PBS and resuspended in cold PI solution (50 μg/ml) containing RNase A (0.1 mg/ml) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry analyses were performed using FACScalibur (Becton-Dickinson, San Jose, CA). Forward light scatter characteristics were used to exclude the cell debris from the analysis. Approximately 10,000 cells were evaluated for each sample. Two independent experiments were performed.

Western blotting. The concentrations of cell lysate proteins were determined by means of the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine...
serum albumin as the standard. The protein was resolved by electrophoresis, electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then incubated with primary antibodies followed by secondary antibody conjugated to peroxidase. Blots were developed with an ECL detection system.

Determination of caspase activity. Caspase activities were determined by colorimetric assays using caspase-3, -8, and -9 activation kits according to the manufacturer’s instructions. The kits utilize synthetic tetrapeptides labeled with p-nitroanilide. Briefly, the cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37°C. The caspase activity was determined by measuring changes in absorbance at 405 nm using the microplate reader.

Statistical analyses. Each experiment was performed in triplicate. The results were expressed as mean ± SD. Significant differences were determined using the Student’s t-test. Statistical significance was defined as P<0.05.

Results

AIMs induce apoptosis in HCT-116 cells. To investigate the anti-tumor activity of AIMs, human colon cancer HCT116 cells were treated with various concentrations of AIMs for 48 h. We used a relatively low concentration range in which we previously demonstrated apoptotic activity in the hematologic malignant cells, U937 cells in vitro (7). The concentration range was easily attainable in vivo. The growth of HCT116 cells were inhibited by AIM treatment in a dose-dependent manner, and IC50 for the 48h-AIM treatment was <60 μg/ml in HCT-116 cells (Fig. 1A).

To assess how AIMs affected cell growth, we determined the effects of AIMs on the cell cycle of HCT-116 cells by flow cytometry. A significant accumulation of cells with sub-G1 DNA content was noted in a dose-dependent manner, but no other remarkably detectable cell cycle changes were observed in HCT-116 cells treated with the indicated concentration of AIMs for 48 h (Fig. 1B).

To investigate whether the viability decrease in HCT-116 cells was due to the induction of apoptosis, we assessed the changes in nuclear morphology of AIM-treated cells with DAPI staining. The DAPI staining revealed the condensed and fragmented nuclei at higher concentration. This is usually witnessed in apoptosis (Fig. 1C).

AIMs induce caspase-dependent apoptosis. Caspases are known as important mediators of apoptosis and contribute to leading cells undergoing apoptosis to irreversible cell death. Therefore, we measured the activation of caspase-3, -8 and -9 with both the Western blot and colorimetric activity assay. As shown in Fig. 2A, the treatment of AIMs decreased the
expression levels of pro-caspase-3, -8 and -9 in a concentration-dependent manner. To confirm this finding, we assayed proteolytic activity of caspases using colorimetric assay kits. We found that AIMs caused marked increases in the proteolytic activities of caspases (Fig. 2B). Western blot analysis also showed the progressive proteolytic cleavage of PARP protein, which is a downstream target of the activated caspase-3, in a concentration-dependent manner in HCT-116 cells after AIM treatment (Fig. 2C). These findings suggest that AIMs should induce apoptotic death through a caspase-dependent pathway.

AIMs modulate Bcl-2 and IAP family members. To investigate the effects of AIMs on the apoptosis-regulating proteins, we measured the levels of Bcl-2 and IAP family members, which play a crucial role in apoptosis conferring cancer cells drug resistance (8). Western blot analyses revealed that AIMs induced reduction in the expressions of anti-apoptotic proteins (XIAP, cIAP-1 and cIAP-2) whereas the expression of pro-apoptotic Bax remained unchanged at the concentration of ≤45 μg/ml (Fig. 2C). Our results suggested that suppression of the expression of anti-apoptotic proteins might be another mechanism through which AIMs enhance apoptosis in HCT-116 cells.

AIMs induce apoptosis at least in part through the activation of p38-MAPK. Mitogen-activated protein kinase (MAPK) is involved in cell proliferation, differentiation and apoptosis (9-11). To investigate the mechanism responsible for AIM-induced apoptosis, we assessed the changes in MAP kinase activation induced by AIMs. Western blot analysis revealed that 48-h treatment of AIMs activated phosphorylation of p38-MAPK and attenuated phosphorylation of JNK which are essential factors in apoptosis signaling (Fig. 3). By contrast, phosphorylation of ERK was stimulated by AIMs as early as 30 min and returned to basal status 24 h after AIM treatment. To confirm the involvement of the MAPK in AIM-induced apoptosis, we performed cell viability assay using p38 inhibitor (SB203580), JNK inhibitor (SP600125), and ERK inhibitor (PD98059). As shown in Fig. 4A and B, p38 inhibitor attenuated cell viability on MMT assay and DAPI staining. P38 inhibitor also attenuated activation of apoptosis-related enzyme (caspase-3) activated by AIMs (Fig. 4C). However, the JNK inhibitor and ERK inhibitor did not show a statistical significance in cell viability assay (data not shown). These findings suggest that AIM-induced apoptosis is mediated at least in part by the activation of p38-MAPK.

AIMs augment apoptosis in part by suppression of XIAP through suppression of phosphorylation of Akt. It has been reported that heat shock protein 90 (Hsp90), which should contribute to the stabilization of p-Akt, is over-expressed in colon cancer (12), and thus Akt/P3k pathway is activated in colon cancer cells. P-Akt is also an important factor regulating the expression of several pro-apoptotic or anti-apoptotic proteins (13-16). To investigate the possible involvement of PI3k/Akt pathway in AIM-induced apoptosis, we assessed...
the changes in phosphorylation of Akt induced by AIMs. Western blot analysis revealed that Akt was constitutively active in HCT-116 cells and the Akt activation was attenuated by AIMs treatment in time-dependent manner. To confirm the involvement of PI3k/Akt pathway in AIM-induced apoptosis, we performed cell viability assay using LY294002, a representative PI3k/Akt inhibitor. As shown in Fig. 5A and B, LY294002 accentuated cytotoxicity of AIMs on MMT assay and DAPI staining. To confirm this finding at the molecular level, we performed Western blot analysis for apoptosis-related enzyme and p-Akt. As shown in Fig. 5C, the suppression of Akt phosphorylation was related to activation of apoptosis-related enzyme (PARP and caspase-3). XIAP is an important anti-apoptotic protein which is known to be regulated by Akt pathway. We found AIMs inhibited the expression of XIAP in a dose-dependent manner in HCT-116 cells (Fig. 2) and 10 μM LY294002 had synergistic effects with 30 μg/ml or lower concentration of AIMs (Fig. 4). Akt phosphorylation was also positively related to expression of XIAP (Fig. 4C). Taken together, these findings suggest that AIM-induced apoptosis in HCT116 cells could be mediated in part by suppression of XIAP through inhibition p-Akt.

Discussion

This study is designed to determine whether AIMs have anti-cancer activities in human colon cancer cells and further to investigate the underlying mechanisms. In our ongoing efforts to determine the mechanism that mediate the effects of AIMs, we studied the involvement of MAPK and PI3K/AKT pathway. In this present study, we found that AIMs induced apoptosis in human colon cancer HCT-116 cells partly through activation of p38-MAPK and suppression of p-Akt. Our data provide evidence that AIMs may be a good potential anti-cancer agent, modulating p38-MAPK and p-Akt and inducing apoptosis because these are very important molecules in signaling for cancer cell survival and apoptosis. In addition, AIMs augmented the apoptosis by suppression of the anti-apoptotic proteins (XIAP, cIAP-1 and cIAP-2).

Recent evidence suggests that various anthocyanins should exert remarkable cytotoxic effects on malignant cells (3,4,6). These results indicated that apoptosis could be triggered by the activation of a set of caspases and their activation played important roles during apoptosis. In apoptotic processes, caspase-3 has been shown to play a pivotal role in the terminal and execution phase of apoptosis induced by diverse stimuli (17,18). We examined whether the caspase-3 protease is involved in AIM-induced cell death response. Furthermore, this study demonstrated that the activation of apoptosis-related enzyme (caspase-3) by AIMs might be related to the suppression of anti-apoptotic proteins. However, the mechanism of apoptosis triggered by anthocyanins was not fully understood whereas the apoptotic activity of anthocyanin is evident. MAP kinases are important factors in apoptosis signaling. A previous study demonstrated that anthocyanins induced apoptosis in hepatocellular carcinoma cells HepG2 through activation of JNK. In HepG2 cells, anthocyanin induced the production of ROS (4). ROS produced by chemical agents may induce JNK activation and subsequent apoptosis (19). However, AIMs did not induce
AIM-induced apoptosis in HCT-116 cells. Previous studies on the anti-tumor promotion of 60μg/ml has apoptotic activity in human colon cancer HCT-116 cells. Therefore, how these anti-apoptotic proteins are regulated by AIMs in HCT-116 warrants further investigation on NF-κB and related to drug resistance. These proteins can be regulated by p-AKT through IKK and subsequent activation of NF-κB (12). Therefore, how these anti-apoptotic proteins are regulated by AIMs in HCT-116 warrants further investigation on NF-κB.

Data presented here indicate that AIMs in the concentration of 60μg/ml has apoptotic activity in human colon cancer HCT-116 cells. Previous studies on the anti-tumor effect of anthocyanins demonstrated that the concentration of AIMs that induce apoptosis in vitro may be attainable in vivo in xenograft models without potential side effects (3,6).

In conclusion, AIMs induced apoptosis in human colon cancer HCT-116 cells. Akt and p38-MAPK are important protein kinases that regulate the apoptotic effect of AIMs on human colon cancer HCT-116 cells (Fig. 6). AIM-induced apoptosis in HCT116 cells was also mediated at least in part by suppression of XIAP through suppression of p-Akt which had a positive relationship with XIAP. This study provides evidence that AIMs might be a potential anti-cancer agent.

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References


