INTRODUCTION

Colon cancer is the third most common type of cancer in the world (Stewart and Wild, 2014). Currently available treatment for colon cancer is a surgical resection combined with chemotherapy or radiation therapy, but these are limited by their side effects. Although many chemotherapeutic agents have been developed, there is still a need to explore potential therapeutic agents with advanced efficacy against colorectal cancer cells.

Columbianadin (1-(8S)-8,9-dihydro-2-oxo-2H-furo[2,3-h]-1-benzopyran-8-yl]-1-methylethyl-[(2Z)-2-methylebutenoic acid] ester, CBN), a coumarin-type compound, is mainly found in the root of Angelica decursiva Fr. Et Sav (Umbelliferae) (Lim et al., 2014). Although various biological activities of CBN have been reported, including calcium-channel blocking activity (Tammela et al., 2004), cytotoxic activity against various cancer cell lines (Yang et al., 2008), analgesic activities (Chen et al., 1995), intestinal absorption and transportation (Yang et al., 2008), and anti-inflammatory activity (Zhang et al., 2012), the anti-cancer activity of CBN and its mechanism of action against human colon cancer cells remain to be elucidated.

Apoptosis and necrosis are well-known as mechanisms of cancer cell death. Apoptosis is a programmed cell death and characterized by the activation of caspases, pseudopod retraction, pyknosis, chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies (Kerr et al., 1972). Apoptosis is triggered by either intrinsic or extrinsic pathways in response to various stimuli of cell death, and it is associated with a specific caspase cascade (Galluzzi L, 2008). Necrosis is morphologically characterized by the swelling of organelles, increase in cell volume, disruption of the plasma membrane, and loss of intracellular contents (Wu et al., 2012). In addition, necroptosis, also known as a regulated necrosis, is found to be a new mechanism of programmed cell death that is quite different from apoptosis. Therefore, necroptosis is considered as a new target for cancer cell death (Christofferson and Yuan, 2010). Necroptosis has been shown to be generally dependent on caspase-8 and receptor interacting protein kinase 3 (RIP-3). RIP-3 is an essential factor for necroptosis because RIP-3 is a component of necrosome, which is one of the initiators of necroptosis. Necrosome is also linked to the production of reactive oxygen species (ROS) by RIP-3, which increases mitochondrial ROS production through the activation of metabolism-related enzymes (Zhang et al., 2009). ROS is able to induce cell death through both...
apoptosis and necroptosis.

ROS is termed as an oxidative stress inducer and an important second messenger in cellular metabolism. Therefore, imbalance of redox status is associated with human pathogenesis including cancers. ROS has a variety of roles in cancer cells such as cell survival, proliferation, angiogenesis, and metastasis (D’Autréaux and Toledano, 2007; Clerkin et al., 2004; Hyoudou et al., 2008). ROS is also considered as a tumor suppressor (Ramsey and Sharpless, 2006). Overproduction of ROS can induce cancer cell death (Renschler, 2004; Toler et al., 2006). Since ROS has two opposite functions in cancer therapy both pro- or anti-oxidant therapies have been recommended for treatment of cancers (Pellicano et al., 2004; Hyoudou et al., 2006; Ozben, 2007; Hyoudou et al., 2008).

ROS mainly consists of a superoxide anion radical (O$_2^•$), a singlet oxygen, hydrogen peroxide (H$_2$O$_2$), and a highly reactive hydroxyl radical. In ROS generation, superoxide dismutase (SOD), glutathione peroxidase 1 (GPx), catalase, thioredoxin (Trx), and peroxiredoxin (Prx) play central roles (Ames, 1983; Cerutti, 1988). There are two types of intracellular SOD in mammalian cells. Cu, Zn-SOD (SOD-1) is found in the cytoplasm and nucleus, and MnSOD (SOD-2) is located in the mitochondrial matrix (Mülliken et al., 1991; Church et al., 1992). SOD is responsible for transformation of superoxide anion into hydrogen peroxide.

The glutathione (GSH) system is comprised of nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase (GR) and GSH supported by glutaredoxin. The GSH system is regulated by GSH-related enzymes such as GPx, GR and glutathione transferase (GST). Hydrogen peroxide can be converted into oxygen and water by catalase or reduced into water by GPx (Cerutti et al., 1988; Birben et al., 2012). GPx is found mainly in the cytosol and mitochondrial matrix, and catalase is largely contained in peroxisomes.

Prx and Trx are composed in the thioredoxin system. Antioxidant enzymes play crucial roles in protecting cells from oxidative stress (Khan et al., 2010). Therefore, targeting antioxidant enzymatic activities could serve as an important strategy for developing therapeutic agents for cancer treatment. In addition, the enhanced understanding of the mechanism in drug actions can be achieved by studying the effects of anticancer drugs on the activity and expression level of antioxidant enzymes.

Based on this information, we investigated the cellular mechanism of a natural product CBN-mediated cell death in terms of ROS in colon cancer cells.

**MATERIALS AND METHODS**

**Materials**

Bicinchoninic acid (BCA), trichloroacetic acid (TCA), sulfoboradine B (SRB), propidium iodide, RNase A, dimethyl sulfoxide (DMSO) and catalase were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco Modified Eagle Medium (DMEM)/high glucose, Minimum Essential Media (MEM), fetal bovine serum (FBS), trypsin-EDTA solution (1X), antibiotic-antimycotic solution (100X), and PBS (1X) were purchased from HyClone Laboratories, Inc. (South Logan, UT, USA). Anti-caspase-9, anti-cleaved caspase-9, anti-caspase-3, anti-BID, anti-p-p53 (Ser15), anti-Prlx1, anti-caspase-8, and anti-cleaved caspase-8 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-cleaved caspase-3, anti-Bax, anti-Bcl-2, anti-p53, anti-catalase, anti-SOD-1, anti-SOD-2, anti-GPx-1, anti-Trx, anti-PrlxII, anti-β-actin, and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-BIM, anti-PARP, anti-cleaved PARP, and fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit I were purchased from BD Biosciences (CA, USA). Anti-RIP-3 was purchased from Abcam (Cambridge, MA, USA). 2’3’-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) was purchased from Invitrogen (Carlsbad, CA, USA).

**Test compound**

Columbianadine (CBN, Fig. 1) was isolated from the root of Angelica decursiva as described (Lim et al., 2014) and provided by Dr. Jae-Soo Choi, a co-author, in Pukyung National University, Korea. CBN was dissolved in 100% DMSO, and stored as stock solution (20 μM) at -20°C until use.

**Cell cultures**

HCT116 (human colorectal cancer cells), A549 (human lung cancer cells), SK-HEP-1 (human liver cancer cells), K562 (human erythroleukemic cells), SNU638 (human gastric cancer cells), MDA-MB-231 (human breast cancer cells), HEK293 (human embryonic kidney cells), and MRC5 (human normal lung epithelial cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in the medium (RPMI 1640 for HCT116, A549, K562, and SNU638 cells; DMEM for SK-HEP-1, MDA-MB-231, and HEK293 cells; MEM for MRC-5 cells) supplemented with 10% FBS and antibiotics-antimycotics (PSA; 100 units/mL penicillin G sodium, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B) in a 37°C humidified incubator with 5% CO$_2$.

**Cell proliferation assay**

The effect of CBN on the cell proliferation was evaluated by SRB cellular protein-staining method (Skehan et al., 1990). The cells were seeded in 96-well plates with various concentrations of CBN and incubated at 37°C in a humidified incubator with 5% CO$_2$ for 48 and 72 h. The cells were fixed with 10% TCA solution for 30 min at 4°C, washed 5 times with tap water, and dried in the air. The cells were stained with 0.4% SRB in 1% acetic acid solution for 1 h at room temperature. After washing out the unbound dye and drying, the stained cells were dissolved in 10 mM Tris buffer (pH 10.0), and the absorbance was measured at 515 nm. Cell viability was calculated by comparing to the absorbance of the vehicle-treated control group. The IC$_{50}$ values were calculated by non-linear regression analysis using the Table Curve 2D v5.01 software (Systat Software Inc., Richmond, CA, USA).
Cell cycle analysis

Analysis on the effects of CBN on cell cycle distribution was performed by the method previously described (Pozarowski and Darzynkiewicz, 2004). The cells were treated with or without CBN for 24 h in 10% FBS-supplemented medium. The cells harvested, washed twice, and fixed with 80% cold ethanol overnight at -20°C. The fixed cells were pelleted, washed with ice-cold PBS, and resuspended in staining solution containing 50 μg/mL PI and 100 μg/mL RNase. After 1 h of incubation at room temperature in the dark, the fluorescence-activated cells were sorted and cellular DNA content was analyzed with a FACSCalibur® flow cytometer (BD Biosciences, CA, USA), and data were evaluated using CellQuest 3.0.1 software (BD Biosciences, CA, USA). The DNA content of 20,000 cells in each group was analyzed and the results were demonstrated as histograms of DNA content. The distribution of cells in each phase of cell cycle was calculated using ModFit LT 2.0 program.

Western blot analysis

Human colon cancer cells exposed with various concentrations of CBN for 48 h were lysed and protein concentrations were determined by BCA method. The total proteins (40 μg) in each cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with blocking buffer (5% bovine serum albumin (BSA) in TBST) for 1 h at room temperature and then further incubated with specific antibodies diluted in 2.5% BSA in TBST overnight at 4°C. After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature and then visualized by HRP-chemiluminescent detection kit (Lab Frontier, Seoul, Korea) using LAS-4000 Imager (Fuji Film Corp., Tokyo, Japan).

Annexin V/PI staining

Cellular death was evaluated through FITC Annexin V apoptosis detection kit (BD Biosciences, CA, USA) as described by the manufacture’s instruction. Briefly, HCT116 cells were collected 48 h after the CBN treatment, and washed twice in cold PBS and then resuspended in 1X binding buffer. The cells (100 μL) were transferred to a 5 mL culture tube containing 5 μL of Annexin V-FITC and 5 μL of propidium iodide, and then were incubated for 15 min at room temperature in the dark. After 1X binding buffer was added into each tube, the stained cells were analyzed by FACSCalibur flow cytometer. The indicated cell concentrations calculated using CellQuest software. Data acquisition was conducted by collecting 20,000 cells per tube and the numbers of viable and dead cells were determined for each experimental condition.

Measurement of ROS production

Human colon cancer cells were plated in 6-well plates and incubated overnight. The cells were then treated with various concentrations of CBN or 100 U/mL catalase for 48 h. The cellular ROS contents were measured by incubating the cells with 5 μM DCFH-DA for 30 min, followed by a flow cytometry using a FACSCalibur flow cytometer at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Fig. 2. Growth inhibitory effects of CBN in HCT116 human colon cancer cells. (A) Anti-proliferative effects of CBN in HCT116 cells. The cells were treated with the indicated concentrations of CBN for 48 and 72 h. Cell proliferation was measured by SRB assay and plotted as a percentage. Data represent the mean ± SD in a triplicate test. (B) Morphological changes mediated by the treatment of CBN in HCT116 cells. The cells were treated with the indicated concentrations of CBN for 48 h. The morphology was observed under a phase-contrast microscope (40X).

Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.). Statistical analyses were carried out using the student’s t test. Differences were considered statistically significant at *p<0.05, **p<0.01.

RESULTS

Growth inhibitory effects of CBN in HCT116 human colon cancer cells

In order to investigate the effects of colombianadin (CBN) on the growth of human cancer cells, a panel of human solid cancer cell lines (A549 lung cancer cells, SK-HEP-1 liver cancer cells, K562 erythroleukemic cells, SNU638 gastric cancer cells, MDA-MB-231 breast cancer cells, and HCT116 colon cancer cells) and normal cell lines (HEK293 embryonic kidney cells and MRC5 normal lung epithelial cells) were treated with various concentrations of CBN for 3 days, and the cell viabilities were measured by SRB assay. Among the tested cell lines, HCT116 colon cancer cell line was found to be the
most sensitive cell line (IC50; 32.4 μM) compared to the other cancer cell lines including a normal cell lines (all cell lines, IC50; >300 μM) (data not shown). CBN showed the most effective growth inhibitory activity against human colorectal cancer cells. Accordingly, further study was performed using HCT116 cells to give the detailed growth-inhibitory mechanism of action mediated by CBN. As shown in Fig. 2A, the cells treated with various concentrations of CBN (0-100 μM) exhibited a dose- and time-dependent growth inhibition with an IC50 value of 47.2 and 32.4 μM after 48 and 72 h incubation, respectively. Morphological changes were examined using a phase-contrast microscope. Treatment of various concentrations (12.5, 25, and 50 μM) of CBN for 48 h in HCT116 cells decreased the number of cells and increased the floating cells. Apparent morphological changes with round-shape and dying cells were also observed at 25 and 50 μM CBN-treated cells (Fig. 2B).

Cell cycle distribution and expressions of apoptosis-related proteins by CBN

To identify cell death mechanism by CBN, the cell cycle distribution was analyzed by a flow cytometer. Although any specific cell cycle arrest was not manifested, treating various concentrations of CBN (0-50 μM) for 48 h increased the sub-G1 phase of cells in a concentration-dependent manner. In particular, 50 μM CBN greatly elevated the level of the sub-G1 peak from 3.0 to 20.3% (Fig. 3A). The sub-G1 peak is generally occurred when cell death is induced by apoptosis or necroptosis. These results suggest that the growth-inhibitory activity of CBN might be in part related to the induction of cell death that is either apoptosis or necroptosis.

To further confirm whether the induction of the sub-G1 peak mediated by CBN was related to the expressions of apoptosis-associated regulatory proteins, Western blot analysis was performed in HCT-116 cells. As shown in Fig. 3B, the expressions of cleaved caspase-9 and cleaved caspase-3, Bcl-2-associated X protein (Bax), and p53 were elevated, but the expressions of B cell lymphoma 2 (Bcl-2), Bim, and BH3 interacting death domain (Bid) were down-regulated after the treatment of 25 μM CBN, which supports the induction of apoptosis by 25 μM CBN in HCT-116 cells. Despite the fact that cell death was increased after the treatment of 50 μM CBN, the most anti-apoptotic markers were interestingly down-regulated in comparison with 25 μM CBN treated cells. These results indicated that cell death induced by 50 μM CBN is not dependent on apoptotic cell death.

Induction of cell death by CBN with apoptosis and necroptosis and expressions of necroptosis associated protein RIP-3

To further confirm the cell death mechanism mediated by CBN in HCT116 colon cancer cells, the analysis of Annexin V/PI staining was performed. The cells treated with 25 μM CBN showed increase in population stained with Annexin V+/PI+, a biomarker of apoptosis. However, the cells stained with
CBN is associated with the up-regulation of RIP-3 and down-regulation of cleaved caspase-8 in human colon cancer cells.

Fig. 4. Cell death induction and expressions of necroptosis-related proteins by CBN. (A) Flow cytometric analysis of the mode of cell death mediated by CBN. The cells were treated with the indicated concentrations of CBN for 48 h and analyzed by Annexin V/PI staining as described in Materials and Methods. Annexin V+/PI− and annexin V+/PI+ staining, respectively. (B) Effects of CBN on the expressions of necroptosis related proteins in HCT116 cells. The cells were treated with indicated concentrations of CBN for 48 h and the protein expressions were measured by Western blot analysis.

Accumulation of ROS in CBN-induced HCT116 cancer cell death

Evidences suggest that the elevation of reactive oxygen species (ROS) production is associated with apoptotic and necroptotic cancer cell death by various anticancer agents (Morgan et al., 2007). To further investigate whether CBN-induced cell death is mediated with the accumulation of ROS in colon cancer cells, the intracellular ROS level was measured by a flow cytometry after the treatment of CBN for 48 h. As shown in Fig. 5A, CBN increased the accumulation of ROS in a concentration-dependent manner. In particular, the ROS level was significantly increased from 8.7% in control group to 62.3% in 50 μM CBN-treated group. The elevated ROS level was effectively recovered by the co-treatment of catalase (100 U/ml), an inhibitor of ROS. Therefore, these results indicate that the necroptotic cell death induced by CBN might in part be associated with oxidative stress in colon cancer cells.

The level of ROS production is finely regulated by intracellular antioxidant enzyme systems, and these antioxidant enzymes either up-regulate or down-regulate to protect cells from the produced ROS. Therefore, we investigated the expressions of major antioxidant enzymes in the cells after the treatment of CBN by Western blot analysis. The expressions of catalase and superoxide dismutase-1 (SOD-1) were suppressed by the treatment of 50 μM CBN, but the expressions of catalase and superoxide dismutase-1 (SOD-1) were suppressed by the treatment of 50 μM CBN, but the expressions...
of superoxide dismutase-2 (SOD-2) and glutathione peroxidase-1 (GPx-1) which are usually located in mitochondrial matrix were activated by CBN treatment as depicted in Fig. 5B. In contrast, the expressions of thioredoxin (Trx), peroxiredoxin (Prx-1), and peroxiredoxin I/II (Prx I/II) which are components of thioredoxin system were not changed by CBN. These data demonstrate that the regulation of antioxidant enzymes by CBN is independent of thioredoxin system, but is associated with SOD-1, SOD-2, GPx, and catalase in HCT116 cells.

**DISCUSSION**

Natural products have played important roles in drug development and discovery. Also, many anti-cancer agents such as camptothecins, etoposide, vinca alkaloids, and taxoids practiced in clinics are originated from natural products (Mann, 2002; Szychowski et al., 2014). In this study, we described the potential of columbianadin (CBN) as an anti-cancer agent and elucidated the underlying mechanism of anti-proliferative activity in human colorectal cancer cells. CBN is one of the main bioactive compounds isolated from the roots of *Angelica decursiva*, which has been used in Oriental medicine for the treatment of asthma, pain, upper respiratory tract infection, and cough remedy. Previous studies also revealed that CBN has the potential to inhibit the proliferation of HL-60 human leukemia cells, KB human nasopharyngeal carcinoma cells, and E-J human bladder carcinoma cells *in vitro* (Yang et al., 2007). However, an anti-tumor activity of CBN against human colon cancer cells has not been reported, and its underlying mechanisms of action for the growth-inhibitory activity of cancer cells need to be identified. We report for the first time that CBN induced cancer cell death with apoptosis and necroptosis in human colorectal cancer cells.

Primarily, we found that CBN-induced cell death of colorectal cancer cells was a dose-dependent dual mode of action of, apoptosis and necroptosis. Double staining with Annexin V and PI, which is a useful tool for distinguishing between necroptosis and apoptosis, exhibited the induction of apoptosis (+/-) at the low concentration of 25 μM CBN and necroptosis (+/+ ) at the high concentration of 50 μM CBN. Loss of plasma membrane integrity and constriction of cell morphology by the treatment of CBN were also correlated with the induction of necroptosis (Fig. 3A).

Recent findings suggest that necroptosis is a new form of programmed cell death (regulated necrosis) and thus considered as a novel target to control the cancer cell growth. Indeed, several compounds including cyclosporine A and staurosporine induced necrotic cell death in cancer cells (Dunai et al., 2012; Ouyang et al., 2012). A natural compound, shikonin was also reported as a necroptosis inducing agent in glioma cells (Huang et al., 2013). One plausible mechanism of necroptosis is associated with the modulation of RIP-1 and RIP-3. RIP-3 is important for necroptosis because RIP-3 is modulated by the caspase-8-FLIP complex (Oberst et al., 2011). Recent report also suggests necroptosis can be modulated by RIP-3 in RIP-1 independent manner (Upton et al., 2012). In this study, we found that the induction of necroptosis...
sis by CBN was more correlated with the activation of RIP-3 compared to that of RIP-1, and down-regulation of caspase-8 cleavage (Fig. 4C).

Accumulation of ROS was found to be dose-dependent in CBN-treated cells, which can trigger cell death in cancer cells. Generally, the ROS level is higher in cancer cells than in normal cells. However, an irreversible oxidative stress caused by the ROS overproduction can effectively kill cancer cells (Kong and Lillehei, 1998). The induction of apoptosis through both receptor and mitochondria is highly associated with ROS (Ozben, 2007). When apoptosis occurs through a receptor, Fas ligand (FasL) triggers ROS formation that is primarily derived from NADPH oxidase. FasL-mediated ROS induces the ubiquitination and degradation by proteasome of FLICE-like inhibitory protein (FLIP) for activating Fas which recruit the Fas-associated death domain (FADD) and caspase-8 and subsequently induce apoptosis (Denning et al., 2002; Uchikura et al., 2004; Medan et al., 2005; Reinherz et al., 2005; Wang et al., 2007). Mitochondria-mediated apoptosis is commonly provides an opening of permeability transition (PT) pore complex that leads to cytochrome c release, apoptosisome formation, and caspases activation.

Necrotic cell death is associated with ROS from both mitochondria and NADPH oxidase-derived ROS. The accumulation of ROS is induced by RIP, TNF receptor associated factor 2 (TRAF2) and Fas-associated protein with death domain (FADD) in tumor necrosis factors (TNF)-induced necrotic cell death (Jacob et al., 2005; Kim et al., 2007). Recent report suggests RIP-3/ mixed lineage kinase domain-like (MLKL)-dependent pathway is a mechanism of regulated necrosis, and FADD, RIP-1, RIP-3, inactive caspase-8 and TNF receptor-associated death domain (TRADD) complex is named necroosome (Vandenabeele et al., 2010). The formation of necroosome initiated by RIP-3 causes accumulation of ROS level (Fiers et al., 1999). In this study, we found that the induction of oxidative stress by CBN might be also correlated with the induction of apoptosis and necroptosis in HCT-116 colon cancer cells.

In addition, cellular ROS levels are in part regulated by cellular antioxidant enzymes. Therefore, the levels of antioxidant enzymes were determined after the treatment of CBN in human colon cancer cells. CBN down-regulates the expressions of catalase and SOD-1 and up-regulates the expression of SOD-2 and GPx-1 after the treatment of 50 μM CBN. However, the Trx and Prx systems were unaffected by CBN. Although the expression of GPx and SOD-2 were increased, the overall imbalance of antioxidant enzymes caused by CBN may leads to ROS accumulation and subsequently induces cancer cell death. Additional study about the upstream of necrotic signaling pathway mediated by CBN will be needed to further develop our findings.

In conclusion, our data demonstrated that CBN causes cell death in HCT116 human colon cancer cells by inducing apoptosis at the low concentrations and necroptosis at the high concentration. Moreover, oxidative stress mediated by CBN in part activated apoptosis and necroptosis. Accordingly, CBN can potentially be a natural source-derived drug to treat colon cancer that modulates ROS through the imbalance of cellular antioxidant enzymes.

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REFERENCES


Upton, J. W., Kaiser, W. J. and Mocarski, E. S. (2012) DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIFRA. Cell Host Microbe. 11, 290-297.


