RESEARCH ARTICLE

Anti Cancer Effects of *Cnidium officinale* Makino Extract Mediated through Apoptosis and Cell Cycle Arrest in the HT-29 Human Colorectal Cancer Cell Line

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Abstract

The anti cancer properties and underlying cell death mechanisms induced by an extract of the roots of *Cnidium officinale* Makino (COM) were investigated. An ethanolic extract of COM inhibited proliferation of human colon cancer cells (HT-29) with both dose- and time-dependence. Analysis of the cell cycle after treatment of HT-29 cells with various concentrations of COM showed reduction of cellular proliferation via G1 phase arrest. Apoptotic effects of COM on HT-29 cells were confirmed with the annexin V-propidium iodide apoptosis test. RT-PCR and Western blotting both revealed that COM extract dose-dependently increased the expression of p53, p21, Bax and caspase-3. Anti-apoptotic factor Bcl-2 expression was down regulated as well as those of cyclin D1 and CDK4. These data suggest that COM has anti cancer properties by inducing apoptosis and cell cycle arrest in HT-29 cells and could have possible therapeutic potential against human colon adenocarcinoma.

Keywords: *Cnidium officinale* Makino - HT-29 cells - apoptosis - cell cycle - P53 - P21 - Caspase-3

Asian Pac J Cancer Prev, **15 (13)**, 5117-5121

Introduction

Colon cancer is a serious health concern throughout the world. Currently, colon cancer is the third most common cancer for both males and females and accounts for about 9% of cancer deaths per year (Shang et al., 2012). Although some progress has been made in the prevention and management of this disease, the outcome is essentially incurable when it reaches the advanced stage. Emerging evidence has demonstrated that many natural products extracted from plant sources possess some anti cancer properties. As a result, plant derived drug research has made a significant progress in anti cancer therapies. Since cancer is a result of unregulated proliferation of cell and continuous cell survival, effective strategies for preventing tumor growth includes inhibition of cell proliferation and apoptosis (Abdullah et al., 2010).

The root of *Cnidium officinale* Makino (COM) has been used as a medicinal plant for a long time in Asia. It is a perennial plant of the family Umbelliferae and is one of the important traditional herbal medicines used for the treatment of diverse diseases. This plant contains many volatile phthalide derivatives which have been shown to have diverse pharmacological activities which include being a sedative, immune modulator, anti-anemia, anti-fungal, smooth muscle relaxant, and anti-complementary properties (Bae et al., 2011; Dela Cruz et al., 2013). In addition to this, several scientists have also reported about the antitumor, anti-metastatic and anti-angiogenic activities of COM (Haranaka et al., 1998; Onishi et al., 1998; Kwak et al., 2002). However, limited information about the molecular mechanisms by which these antitumor properties has been known.

The aims of this study were to evaluate the anti cancer properties of an extract prepared from the roots of COM and to determine the mechanism of cell death elicited by the extract in HT-29 cells.

Materials and Methods

Plant material and extract preparation

Dried roots of *Cnidium officinale* Makino was freeze dried and pulverized to powder form. Dried powder root of COM (300g) was then soaked in 80% ethanol for 24 hours. The extracts were collected and the same process was repeated three times. The total extract was collected, filtered and evaporated in a rotary evaporator at 50°C under reduced pressure. The end product was freeze dried and the powdered extract was kept in deep freezer (-70°C).

Cell lines and cell culture

HT-29 human colon cancer cells were obtained from the Korean Cell Line Bank (KCLB). The cells were maintained in Dulbecco's Modified Eagle's medium

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(DMEM), supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100mg/ml), and 3.7mg/ml of $NaHCO_3$. Cells were cultured in a humidified atmosphere and incubated at 37°C in 5% CO_2.

Cell viability assay

Cell counting kit-8 (CCK-8, Dojindo, Japan) was used to determine cell viability, according to the manufacturer's instructions. HT-29 cells were seeded in a 96-well plate ($1x10^5$ cells/ml), incubated in DMEM at 37°C in 5% CO_2 for 24 hours. The cells were pretreated with 50, 100, 200 and 400µg/ml of COM for 24 and 48 hours. After treatment, medium containing COM was removed and was replaced with fresh media containing 10µL of CCK-8 solution and incubated at 37°C for two hours. Absorbance at 450nm was measured with an ELISA plate reader. For the treated cells, viability is expressed as the percentage of control cells.

Cell cycle analysis

Flow cytometry analysis was used to measure the proportion of HT-29 cancer cells in the different stages of the cell cycle. HT-29 cells were seeded in 6-well plates at $1x10^6$ cells/ml for 24 hours. The cells were then treated with increasing concentrations of COM (0-400µg/ml) for 48 hours. After treatment, the cells were harvested and washed twice with phosphate buffered saline (PBS). Each sample was fixed in 1mL of 70% ethanol for 2 hours at -20°C. After fixing, the samples were centrifuged, ethanol was removed, and cells were resuspended in PBS containing 50µg/ml propidium iodide (PI) and 100µg/ml RNAse A and incubated in the dark for 30 minutes at room temperature (RT). Cell cycle was analyzed using the BD FACSCalibur Flow Cytometer. The data was analyzed using the BD CellQuest Pro software.

Apoptosis detection by annexin V staining

Apoptosis was examined using BD Biosciences FITC Annexin V Apoptosis Detection Kit. All adhering and floating cells were harvested after incubation for 24 hours with 0, 50, 100, 200 and 400 µg/ml COM. Cells were washed with ice cold PBS, centrifuged and the cell pellet was resuspended in Annexin binding buffer at a concentration of $1x10^6$ cells/ml. Then, 100µl of this cell suspension was transferred to a culture tube, to which 5µl of FITC Annexin V and 5µl of PI were added. The samples were gently vortexed and incubated in the dark for 15 minutes at RT. Addition of 400µl Annexin binding buffer was then performed before analysis by flow cytometry.

RNA isolation and polymerase chain reaction analysis

Total RNA was isolated from COM treated cells using Trizol (Invitrogen, USA) according to the manufacturer's protocol. RNA samples were reverse-transcribed with M-MuLV reverse transcriptase (Fermentas, Lithuania) and specific primers were used to amplify P53, Bax, Bcl-2, Caspase-3, P21, Cyclin D1 and CDK 4. The optimum number of cycles for each primer was determined experimentally. The "housekeeping gene" β-actin was used to verify that equal amounts of RNA were added in the PCR reaction. All gene expression values were normalized against the β-actin expression.

Western blot analysis

The cells treated with 0-400µg/ml COM for 24 hours were lysed using a protein extraction solution (Intron Biotechnology). Total protein was determined by the Bio-Rad protein assay. Next, 30µg of protein was diluted and heated at 95°C for 10 minutes prior to SDS-PAGE gel analysis (10% and 12%). The proteins were then transferred to nitrocellulose membranes and blocked overnight with 5% skim milk in TBST (20mM Tris-HCl, pH 7.6, 140mM NaCl, 0.1% Tween 20). The membranes were then rinsed four times with TBST and incubated for 2 hours with 2% skim milk containing 1:1000 dilution of primary antibody: p53, p21, Bax, Bcl-2, Caspase 3, cyclin D1 and CDK4. After four washes with TBST buffer, the membranes were incubated for 2 hours in horse radish peroxidase-conjugated secondary antibody diluted at 1:2000. The membranes were washed again and developed using enhanced chemiluminescence (ECL Western Blot Analysis System Kit, Amersham Biosciences).

Statistical analysis

All experiments were performed in triplicate and the results were expressed as means±S.D. Differences between means were evaluated using ANOVA test (one-way) followed by Duncan Multiple Range Test and $p<0.05$ was taken as statistically significant.

Results

COM inhibits the proliferation of HT-29 cells

Before the investigation of the anti-cancer activity of COM extract, the cytotoxicity of COM on normal cells was evaluated using intestinal epithelial (IEC-6) cell line. COM stimulated the proliferation of IEC-6 cells and no cytotoxicity was observed when the concentration was not higher than 400µg/ml (Figure 1). Human colorectal cancer cell line (HT-29) was used to examine the anti-proliferative activity of COM extract by CCK-8 assay. As shown in Figure 2, COM showed a potent cytotoxic activity in HT-29 cells. There was a significant, dose-dependent decrease in cell viability for cells incubated for both 24 and 48 hours. The data suggests that COM extract induced a concentration dependent inhibition in HT-29 cells with IC_{50} value of 305.0246µg/ml after 48 hours of incubation but showed no cytotoxic effect in normal IEC-6 cells.

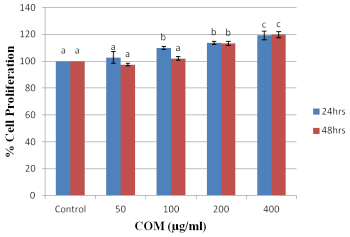

Figure 1. Effects of Ethanol Extract of COM on the Proliferation of IEC-6 Cells Incubated for 24 and 48 Hours. Data are means±SE (n=3). Means with different superscript are significantly different at $p<0.05$

COM extract induces a G1 phase cell cycle arrest in HT-29 cells

Flow cytometry analysis was performed to investigate whether COM induces cell cycle regulation in HT-29 cells (Figure 3). DNA histogram analysis revealed that COM induced a dose-dependent increase in the number of cells within the G0-G1 phase from 64% in the control group to 75.05% in 400μg/ml COM treated cells. However, concomitant to this increase, a dose dependent decrease in cell population in the S (from 16.03% to 7.81%) and G2-M phase (16.7% to 9.85%) was also observed in the HT-29 treated cells, meaning that the progression of cell cycle from the G1 phase was inhibited by COM treatment. The data in Figure 3 clearly shows that COM extract dose dependently inhibited the cellular proliferation of HT-29 cells via G0/G1 phase arrest of the cell cycle.

COM extract induces apoptosis in human colorectal cancer (HT-29) cells

Figure 4 shows the apoptotic effect of COM in HT-29 cells using double staining of FITC-conjugated Annexin V and PI. Data shows that treatment of COM extract significantly increased the number of early and late apoptotic cells (6.52% in untreated cells versus 42.34%, 58.85%, 67.65%, and 71.09% in 50, 100, 200 and 400μg/ml, respectively). COM extract at increasing concentration induced a higher rate of apoptosis compared to control.

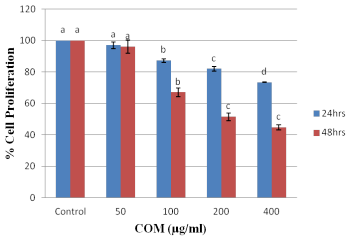

Figure 2. Anti-proliferative Effect of Ethanol Extract of COM on HT-29 Cells Incubated for 24 and 48 Hours. Data are means±SE (n=3). Means with different superscript are significantly different at p<0.05

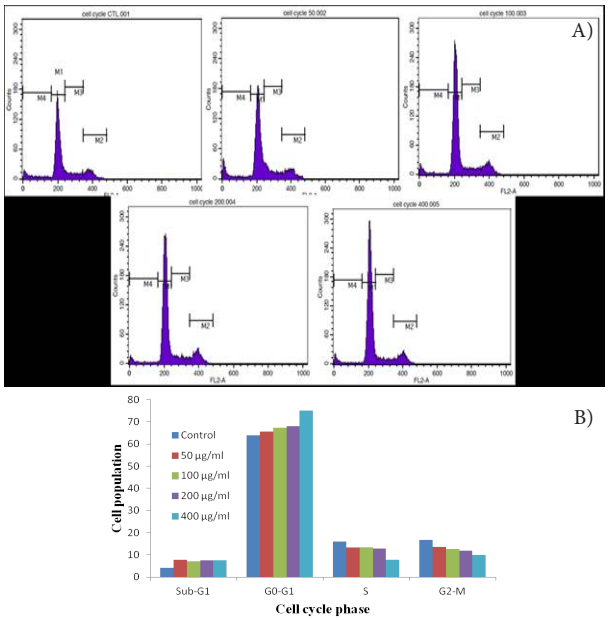

Figure 3. The Effect of *Cnidium officinale* Makino Extract on HT-29 Cell Cycle. A) Cells treated with different concentrations of COM for 48hrs and analyzed by flow cytometry after staining with PI; B) Histogram showing the number of cells in each phase of the cell cycle

Gene expression changes induced by COM

To further understand the mechanism of COM induced cell cycle arrest and apoptosis in HT-29 cells, the expressions of cell cycle and apoptosis related genes were examined with RT-PCR (Figure 5). Compared to untreated cells, the expressions of p53, p21, Bax and Caspase-3 dose-dependently increased after treatment with COM for 24 hours. The level of anti-apoptotic factor Bcl-2 expression was down regulated as well as cell cycle regulators cyclin D1 and CDK4.

Effects of COM on the expression of cell cycle and apoptosis-related proteins

To investigate the possible molecular mechanism by which COM extract triggered cell cycle regulation and apoptosis in HT-29 cells, the protein expressions of several cell cycle and apoptosis related proteins in HT-29 treated with COM was evaluated by Western blot analysis. Western blot data showed up regulation of tumor-suppressor protein p53, which controls cell growth

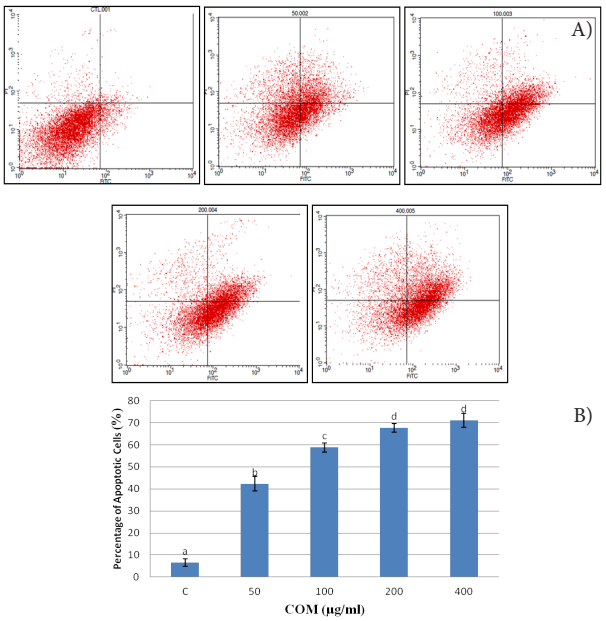

Figure 4. Flow Cytometric Analysis of Apoptosis Induction by COM in HT-29 Cells. A) Representative cytograms of apoptosis in HT-29 cells incubated for 48 hours; B) Percentage of apoptotic cells after 48 hours incubation with varying COM extract concentration. Data are means±SE (n=3). Means with different superscript are significantly different at p<0.05

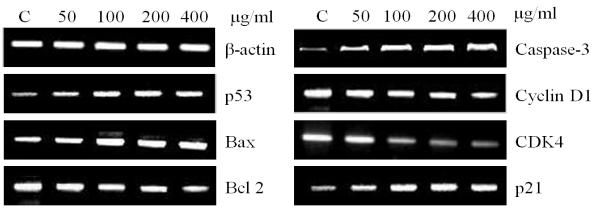

Figure 5. Effect of COM on mRNA Expression of Cell Cycle and Apoptosis Related Genes in HT-29 Cells

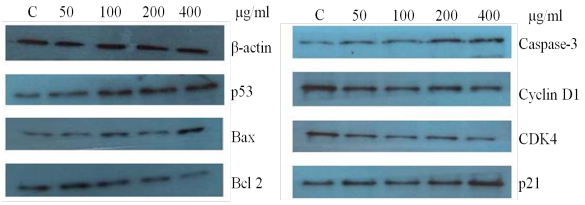

Figure 6. Expression of Cell Cycle and Apoptosis Related Proteins in HT-29 Cells Treated with Increasing Concentrations of COM Extract

through cell cycle arrest (p21 activation) and apoptosis (Bax activation and Bcl-2 inhibition).

Discussion

In our present investigation, we examined the anti-cancer effects of COM extract on colorectal cancer cell line HT-29. One characteristic of a good anti-cancer agent is the ability to selectively induce cell death in cancer cells with very minimal cytotoxicity to normal cells. The results of the cell proliferation assay showed that COM exerts a potent cytotoxic effect on cancer cells but no detrimental effect on normal cells.

The inhibition of proliferation in HT-29 cells was a result of cell cycle arrest and apoptosis induction, as evidenced by the FACS analyses of COM treated HT-29 cells showing a G0/G1 phase cell cycle arrest (Figure 3) and high percentage of Annexin-positive cells in contrast to the negligible percentage in control cells (Figure 4).

The p53 protein lies at the center of stress response pathways that prevent the proliferation and survival of potentially malignant cells. Many kinds of stress activate p53, including DNA damage, telomere attrition, oncogene activation, hypoxia and loss of normal growth and survival signals (Ryan et al., 2001; Jo et al., 2012). The activation of p53 can trigger several responses in the cell including differentiation, senescence, DNA repair and the inhibition of angiogenesis, but most understood is the ability of p53 to induce cell cycle arrest and apoptotic cell death (Bates, 1999; Jin and Levine, 2001; Li et al., 2011). Our results show that COM extract activates p53 and in the process inhibits the growth and proliferation of stressed HT-29 cells by cell cycle arrest and apoptosis.

Upon activation of p53, it promotes the expression of a number of genes that are involved in apoptosis including Bax, a proapoptotic member of the Bcl-2 family. In most cases, this p53-induced apoptosis proceeds through mitochondrial release of cytochrome c, which leads to caspase activation (Oda et al., 2000; Cory and Adams, 2002; Kuwana et al., 2002). Caspases are the most important protein that modulates the apoptotic response. Caspase-3 is the key executioner and hallmark of apoptosis which causes cleavage of many cellular substrates that eventually leads to cell death (Earnshaw et al., 1999; Kim et al., 2002). RT-PCR and Western blot data shows that COM up regulated the expression of proapoptotic factor Bax and down regulated the activation of pro-survival Bcl-2 gene. Our data suggests that the activation of p53 in COM treated HT-29 cells triggers the activation of Bax and inactivation of Bcl-2, causing a cascade of events leading to the activation of caspase-3 and finally cell death.

Another factor that has been shown to be up-regulated by the p53 tumor suppressor gene *in vitro* in response to DNA-damaging agents and is thought to be an integral part of the p53-mediated growth-arrest pathway is p21 (Haupt et al., 2003; Mcleod et al., 2013). P21, a potent cyclin-dependent kinase inhibitor regulates the progression of cells in the G0-G1 phase of the cell cycle and induction of these proteins causes a blockade of the G1 to S transition, thereby resulting in a G0-G1 phase arrest of the cell cycle (Pavletich, 2009). Cyclin D1 is a member of the G1 cyclins which plays an important role in the progression of G1 to S phase of the cell cycle in normal cells through the activation of cyclin-dependent kinase 4. It is evident from the results that COM induced cell cycle arrest in HT-29 cells at the G0/G1 phase through the suppression of CyclinD1/CDK4 complex, via the up regulation of p53 and p21.

The findings obtained in this study have shown that COM possesses cytolytic and cytostatic properties by inducing apoptosis and cell cycle arrest against HT-29 cells. The results show that *Cnidium officinale* Makino may be developed as a promising agent against colon cancer and deserves to be investigated further.

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