Ochnaflavone, a Natural Biflavonoid, Induces Cell Cycle Arrest and Apoptosis in HCT-15 Human Colon Cancer Cells

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(Received June 1, 2009; Revised June 12, 2009; Accepted June 17, 2009)

Abstract - Ochnaflavone is a natural biflavonoid and mainly found in the caulis of Lonicera japonica (Caprifoliaceae). Biological activities such as anti-inflammatory and anti-atherogenic effects have been previously reported. The anticancer activity of ochnaflavone, however, has been poorly elucidated yet. In the present study, we investigated the effect of ochnaflavone on the growth inhibitory activity in cultured human colon cancer cell line HCT-15. Ochnaflavone inhibited the proliferation of the cancer cells with an IC50 value of 4.1 μM. Flow cytometric analysis showed that ochnaflavone arrested cell cycle progression in the G2/M phase, and induced the increase of sub-G1 peak in a concentration-dependent manner. Induction of cell cycle arrest was correlated with the modulation of the expression of cell cycle regulating proteins including cdc2 (Tyr15), cyclin A, cyclin B1 and cyclin E. The increase of sub-G1 peak by the higher concentrations of ochnaflavone (over 20 μM) was closely related to the induction of apoptosis, which was evidenced by the induction of DNA fragmentation, activation of caspase-3, -8 and -9, and cleavage of poly-(ADP-ribose) polymerase. These findings suggest that the cell cycle arrest and induction of apoptosis might be one possible mechanism of actions for the anti-proliferative activity of ochnaflavone in human colon cancer cells.

Keywords: Ochnaflavone, Cell cycle arrest, Apoptosis, HCT15 human colon cancer cells

INTRODUCTION

Flavonoids which are a typical polyphenol have been reported to have a variety of biological activities including anti-oxidant, metal chelating, immunostimulatory, liver protection, anti-inflammatory, and anti-carcinogenesis (Butenko et al., 1993; Grange et al., 1999; Ielpo et al., 2000; Sharon and Nora, 2000; Chowdhury et al., 2002; Molina et al., 2003).

Biflavonoid, a dimer of flavonoid, is generally found in the plants of gymnosperms, and the limited pharmacological activities including inhibition of phosphodiesterase, aldose reductase of lens, and histamine release by mast cell were reported (Beretz et al., 1979; Amella et al., 2000; Sharon and Nora, 2000; Chowdhury et al., 2002; Molina et al., 2003).

Ochnaflavone, a naturally occurring biflavonoid, is mainly found in the medicinal plant Lonicera japonica (Caprifoliaceae). Several biological activities mediated by ochnaflavone include the inhibition of phospholipase A2 in rat platelet, lymphocyte proliferation, and arachidonate release from rat peritoneal macrophage (Chang et al., 1994; Lee et al., 1995; Lee et al., 1997). The anti-atherogenic activity of ochnaflavone was also revealed with the correlation of inhibition of human vascular smooth muscle cell proliferation induced by TNF-α via regulation of cell cycle related proteins, ERK and MMP-9 (Suh et al., 2006b). Recent study also exhibited the inhibitory activity of CCl4-induced PE degradation in rat liver microsome (Moon et al., 2006) and anti-inflammatory effects with the down-regulation of inducible nitric oxide, cyclooxygenase-2 and 5-lipoxygenase through NF-κB and ERK pathway by ochnaflavone (Son et al., 2006; Suh et al., 2006a). However, the anti-proliferative effect of ochnaflavone against human cancer cells has been poorly elucidated yet. We herein report for the first time that ochnaflavone exhibits the anti-proliferation of human colon cancer cells with the induction of cell cycle arrest and apoptosis.

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MATERIALS AND METHODS

Chemicals
Trichloroacetic acid (TCA), sulforhodamine B, ribonuclease A (RNase A), propidium iodide (PI), bisbenzimide H 33258 (Hoechst 33258), and mouse monoclonal anti-β-actin primary antibody were purchased from Sigma (St. Louis, MO, USA). Rosewell park memorial institute (RPMI) medium 1640, fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100×), trypsin-ethylenediaminetetraacetic acid (EDTA) solution (1×) and antibiotic-antimycotics solution (PSF) were from Invitrogen Co. (Grand Island, NY, USA). Rabbit anti-cyclin A, cyclin B1, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-caspase-3, anti-caspase-9, anti-phospho-cdc2 (Tyr15), anti-phospho-cyclin B1, and mouse anti-caspase-8 primary antibody were obtained from Cell Signaling (Denver, MA, USA). Mouse monoclonal anti-PARP and anti-cyclin E were from BD Biosciences (San Diego, CA, USA). Ochnaflavone (Fig. 1A) isolated from the caulis of Lonicera japonica was provided by Dr. Sam Sik Kang, a coauthor, College of Pharmacy, Seoul National University, Seoul, Korea.

Cell culture
Human colon cancer HCT-15 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea), and the cells were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The cells were maintained at 37°C in humidified atmosphere with 5% CO2.

Evaluation of growth inhibitory potential
HCT-15 cells (5×10⁴ cells/ml) were treated with various concentrations of ochnaflavone for 3 days. After incubation, cells were fixed with 10% TCA solution, and the cell viability was determined with sulforhodamine B (SRB) protein staining method (Lee et al., 1998). The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC₅₀ values were calculated using non-linear regression analysis (percent survival versus concentration).

DNA fragmentation assay
HCT-15 cells were plated in 100 mm culture dish at a density of 1×10⁶ cells/dish. Twenty-four hours later, fresh media containing test sample were added to cultured dishes. After treatment for 24 h, the cells were washed with PBS and lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 1.0% NP-40. After centrifugation, 1% SDS and RNase A (5 μg/μl) were added to the supernatants, and then incubated at 56°C for 2 h. Subsequently, proteinase K (2.5 μg/μl) was added and then incubated at 37°C for 2 h. DNA was precipitated with 0.5 volume of 10 M ammonium acetate and 2.5 volumes of cold ethanol at −20°C overnight. Precipitated DNA was dissolved in 50 μl of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA. DNA samples (4 μg) were resolved by electrophoresis in 2% agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, Oregon, USA), and visualized (Lee et al., 2002).

Analysis of cell cycle dynamics by flow cytometry
Cell cycle analysis by flow cytometry was performed as previously described (Lee et al., 2002). Briefly, HCT-15 cells were plated at a density of 1×10⁶ cells per 100-mm culture dish and incubated for 24 h. Fresh media containing test samples were added to culture dishes. After 24 h,
the floating and adherent cells were collected and washed with PBS twice. The cells were fixed with 70% ethanol, and incubated with a staining solution containing 0.2% NP-40, RNase A (30 μg/ml), and propidium iodide (50 μg/ml) in phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer (FACScalibur, BD, Biosciences). At least 20,000 cells were used for each analysis, and results were displayed as histograms. Cell cycle distribution was analysed using the ModFit LT 2.0 program.

Evaluation on the expression of cell cycle regulating proteins and apoptosis-related proteins

Cells were treated with various concentrations of ochnaflavone for 24 h. After incubation, cells were lysed and protein concentrations were determined by BCA method. Each protein (30-50 μg) was subjected to 10% SDS-PAGE. Proteins were transferred onto PVDF membranes by electrophoretic transfer, and membranes were treated for 1 h with blocking buffer [5% non-fat dry milk in phosphate-buffered saline-0.1% Tween 20 (PBST)]. Membranes were then incubated with indicated antibodies overnight at 4°C, washed three times for 5 min with PBST. After washing, membranes were incubated with HRP-conjugated anti-mouse IgG diluted 1:1,500 in PBST for 3 h at room temperature, washed three times for 5 min with PBST, and detected using ECL reagent (Lab Frontier, Seoul) (Lee et al., 2002).

Statistical analysis

Data were presented as the mean ± SE for the indicated number of independently performed experiments. Figure data are shown as one representative of at least three independent experiments.

RESULTS

Growth inhibitory effects of ochnaflavone on HCT-15 human colon cancer cells

The growth inhibitory potential of ochnaflavone was determined in cultured HCT-15 human colon cancer cells by a colorimetric SRB protein dye staining method. As a result, ochnaflavone exhibited a remarkable growth inhibitory effect against HCT-15 human colon cancer cells with the IC50 value of approximately 4.1 μM (Fig. 1B). The concentrations of the compound up to 10 μM exhibited mainly cell cycle arrest, but over 20 μM of the compound caused a cytotoxic effect with the observation of floating dead cells.

Changes of cell cycle distribution by ochnaflavone

HCT-15 cells were treated with ochnaflavone (0-40 μM) for 24 h, and the distribution of cells in various compartments of the cell cycle was analyzed by flow cytometry. The cell cycle distribution by ochnaflavone seemed to be highly dependent on the concentration of the compound. When treated with ochnaflavone of 5 μM cells were significantly accumulated in the G2/M phase, but 10 μM of the compound evoked the accumulation of S phase. In addition, the higher concentrations of ochnaflavone with 20 and 40 μM increased in the sub-G1 phase, indicative of apoptotic peaks, during incubation time (Fig. 2).
Induction of DNA fragmentation by ochnaflavone

To further investigate whether the cytotoxic effect of ochnaflavone was associated with apoptosis, DNA was extracted from the cells exposed to the compound for 24 h and subjected to electrophoresis in agarose gel. DNA fragmentation characteristic for apoptosis was clearly detected by exposure of ochnaflavone in a concentration-dependent manner as illustrated in Fig. 3.

Effects of ochnaflavone on the expression of cell cycle regulatory proteins and apoptosis related proteins

Based on the cell cycle distribution data evoked by ochnaflavone, in order to investigate whether ochnaflavone affects the expression of cell cycle regulatory proteins, the cells were treated with various concentrations of the compound for 24 h, and then the protein expressions were determined by western blot analysis. As depicted in Fig. 4A, the lower concentration of ochnaflavone (5 μM) increased the expression of phosphorylated cdc2 (Tyr-15) which is the inactive form of cdc2 (p-cdc2, Tyr-15) and this event seemed to be related with the interruption of the exit of G2/M phase of cell cycle. This might result in the accumulation of G2/M phase of cell distribution. In addition, apoptotic phenomena induced by the treatment of the higher concentrations of ochnaflavone (over 20 μM) were correlated with the activation of caspases. The expressions of procaspase-9, -8, and -3 were downregulated and thus the active forms of cleaved corresponding caspases were formed by the treatment of ochnaflavone (Fig. 4B). PARP cleavage which is the target of caspases was also induced, and these events subsequently affect to the induction of apoptosis.

DISCUSSION

Cancer is the main cause of death in the world. Especially, colon cancer is the forth abundant causes of cancer mortality in Korea and the second in Western countries. One of the promising strategies in controlling colorectal cancer progression is considered cancer chemoprevention by taking dietary factors. Various compounds derived from natural products have been reported...
to modulate the growth of colorectal cancer cells and thus are considered to be cancer chemopreventive agents (Bhanot and Möller, 2009). Ochnaflavone is a natural biflavonoid and has been demonstrated to have anti-inflammatory and anti-arthrogenic activities (Lee et al., 1997; Moon et al., 2006; Son et al., 2006; Suh et al., 2006a; Suh et al., 2006b; Kim et al., 2008). Since chronic inflammation is highly correlated with colorectal carcinogenesis (Wada, 2009; Bhanot and Möller, 2009), anti-inflammatory effect of ochnaflavone might suggest its possible role in colon cancer chemoprevention. However, the direct effects of ochnaflavone against cancer cells have been poorly examined yet. The present study evaluated the effect of ochnaflavone on the proliferation of cultured human colorectal cancer HCT-15 cells and further investigated the growth inhibitory mechanisms of action. Primarily, the anti-proliferative activity of ochnaflavone in human colon cancer HCT-15 cells was examined. Ochnaflavone concentration-dependently inhibited the cell proliferation and the IC50 value for 3 days incubation was 4.1 μM (Fig. 1B). The IC50 value of ellipticine, a positive control, was 1.0 μM in the same culture condition. Based on the potential anti-proliferative activity of ochnaflavone we further investigated the mechanisms of action in terms of cell cycle analysis and expressions of cyclin-dependent regulatory proteins in HCT-15 cells. Flow cytometric analysis indicated that ochnaflavone significantly induced the accumulation of cell population in the G2/M phase at 5 μM (control; 28.3%, treatment; 59.35%), and the accumulation of S phase of cells was pronounced at 10 μM (control; 39.68%, treatment; G2/M 83.95%) during the incubation of the compound for 24 h. These results suggested that ochnaflavone differentially induced cell cycle arrest in a concentration-dependent manner. In addition, the higher concentrations of ochnaflavone (20 or 40 μM) dramatically increased the sub-G1 peaks (34.83 and 49.19%, respectively), indicative of apoptotic phase. Therefore, the cell cycle arrest and induction of apoptosis might be one possible mechanisms of action in the growth inhibitory effect of ochnaflavone in human colon cancer cells. To further confirm the cell cycle arrest and apoptosis induced by ochnaflavone we examined the cell cycle regulatory proteins in HCT-15 cells. Generally, cell cycle progression is regulated by cyclin/cyclin dependent kinase (CDK) complex. Although CDK expression is constant during the cell cycle, expression of cyclin is changed according to the each phase of cell cycle. First, cyclin D is normally increased during mid/late G1 phase, and subsequent increase of cyclin E is needed for entry to S phase. Up-regulation of cyclin A and cyclin B leads up to S to G2/M phase entry. In addition, cyclins/CDKs are regulated by several factors, such as kinase (WEE1, PLK) and phosphatase (CDC25C) (Russell and Nurse, 1987; Lundgren et al., 1991; Gu et al., 1992; Porter and Donoghue, 2003). When the times of G2 phase pass to M phase PLK transfers phosphate to the site of ser-147 of cyclin B, and then cyclin B moves into nucleus and combines with cdc2 (Toyoshima-Morimoto et al., 2001). Moreover, CDC25C removes the phosphate group at the site of cdc2 (Tyr-15), resulting in M phase progression. In this view, the induction of cell cycle arrest by ochnaflavone was highly correlated with the formation of inactive form of cdc2 (Tyr-15) (p-cdc2, phosphorylated cdc2 (Tyr-15)) (Fig. 4A). The increased inactive form of cdc2 interfered the cell cycle exit from G2/M phase and thus arrested in this phase of cell cycle. Increase of cyclin B at 5 μM of ochnaflavone was also thought to be in part in the process of inactivation of cdc-2. Although the accumulation of S phase was evoked by the treatment of 10 μM of ochnaflavone the cell cycle regulatory proteins were not dramatically changed. However, the cdc2 inactivation and the increase of cyclin A, B1 and E1 might be still in part related to the arrest. The more detailed examination needs to be remained. In addition, the growth inhibition by ochnaflavone was also related with the induction of apoptotic cell death with the treatment of over 10 μM. As depicted in Fig. 2, over 20 μM of ochnaflavone drastically increased the cell cycle distribution of the sub-G1 phase, indicative of apoptotic peaks. The induction of apoptosis was also confirmed by the observation of DNA fragmentation (Fig. 3). One of well-known phenomena of apoptosis is the activation of caspases (Ahn et al., 2009). Ochnaflavone-mediated apoptosis seems to be explained the consequential activation of caspase-8, -9 and caspase-3 and thus induces the cleavage of PARP, a target protein of caspases.

In summary, this study suggests that the growth inhibitory effects of ochnaflavone against human colon cancer cells are related to the cell cycle arrest and induction of apoptosis. The cell cycle arrest is closely correlated with cdc 2 inactivation and apoptosis is associated with the sequential activation of caspases. Along with the potential cancer chemopreventive potential of ochnaflavone in vitro this study further suggests one plausible mechanism of actions in the growth inhibitory effect of ochnaflavone in human colon cancer cells.

ACKNOWLEDGMENTS

This study was supported by a grant Studies on the Identification of the Efficacy of Biologically Active Components from Oriental Herbal Medicines from the Korea Food
and Drug Administration (2007).

REFERENCES


