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Codonopsis lanceolata extract induces G0/G1 arrest and apoptosis in human colon tumor HT-29 cells – Involvement of ROS generation and polyamine depletion

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ABSTRACT

Codonopsis lanceolata (Campanulasea) is widely distributed and grown in Asia and has been in use as traditional medicine for long time. The *n*-butanol fraction (BF) of *C. lanceolata* significantly inhibited human colon cancer HT-29 cell growth in a dose- and time-dependent manner by inducing G0/G1 phase arrest and apoptosis. The inhibition was associated with intracellular ROS generation and polyamine depletion as evidenced by HPLC quantitatively. Additionally, semi-quantitative RT-PCR revealed enhanced expression of caspase-3, p53, and the Bax/Bcl-2 ratio and reduced expression of survivin in HT-29 cells treated with BF. Furthermore, western blot analysis of p53, JNK, and caspase-3 showed that ROS generation was accompanied by JNK activation. Increase of the Bax/Bcl-2 ratio and activation of caspase-3 might be due to intracellular polyamine depletion. Conclusively, the findings of this study imply a critical role of ROS and polyamine depletion in the anticancer effects of *C. lanceolata* root extract.

1. Introduction

Codonopsis lanceolata (Bench et Hook) (Campanulacea), widely distributed in East Asia, contains various active compounds such as tannins, saponins, polyphenolics, alkaloids, essential oils, steroids etc. It has been extensively used as herbal medicine for bronchitis and coughs (Lee et al., 2002) and has also been used as an antidote in traditional folk medicine (Wang et al., 1995). Saponins from *C. lanceolata* has been reported to produce anti-inflammatory effects in a xylene-induced model of ear edema in mouse (Xu et al., 2008). Recently, lancemaside A, which is a main constituent of *C. lanceolata* was reported to potently inhibit LPS-stimulated, TLR-4-linked NF-κB activation of 293-hTLR4-hemagglutinin (HA) cells (Joh et al., 2010).

Although there have been several ethnopharmacological studies on the anti-cytotoxic effects of the plant (Pulla et al., 2008; Purev et al., 2008) the underlying issues regarding its mode of action remain largely unexplained and requires further investigation.

It is well established that reactive oxygen species (ROS) are mediators of intracellular signaling cascades. Excessive ROS generation can induce redox-signaling pathways, including those involved in oxidative stress, loss of cell function, cell cycle arrest, and apoptosis (Sauer et al., 2001). ROS signaling also induces mitochondrial-dependent apoptosis through the activation of apoptosis signal-regulating kinase 1 (ASK1)/mitogen-activated protein kinase (MAPK) pathways and up-regulates the proapoptotic Bcl-2 proteins Bax or Bak, consequently affecting mitochondrial membrane permeability and cell death (Ling et al., 2003; Zhang and Chen, 2004; Kim et al., 2005; Moungjaroen et al., 2006; Kuo et al., 2007).

Polyamines have been proposed in the past to have possible connections linking cell death to cellular proliferation (Stefanelli et al., 2001). Available literatures have also showed complex interplay of polyamines with apoptotic cell death (Nikolaus and Francis, 2005). Polyamines have been shown to exert both negative and positive regulatory effects on apoptosis depending on the cell type and environmental signals (Thomas and Thomas, 2001). Both up-regulation and down-regulation of polyamine levels were reported during apoptosis (Schiller et al., 2005). The interaction of polyamines with cell cycle events and apoptosis are closely connected (Igarashi and Kashiwagi, 2010).

Various chemical agents with strong apoptosis-inducing activity but minimal toxicity have potential as anticancer drugs. As a herb, *C. lanceolata* is widely used in food preparation, but its medicinal application has not been explored yet in South Korea. Thus, as part of our program on screening for natural compounds from plants with potential chemo-preventive effects, we investigated

Abbreviations: 5-Fu, fluorourcine; BF, *n*-butanol fraction; Glu, glutathione; HPLC, high pressure liquid chromatography; JNK, c-Jun N-terminal kinase; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction.

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the effects of *C. lanceolata* extract on cancer cells and the molecular mechanism by which it induces apoptosis in human HT-29 colon cancer cells.

2. Materials and methods

2.1. Preparation of roots extracts

Air-dried *C. lanceolata* roots obtained from Hengcheng, South Korea, was pulverized into fine flour using a milling machine. Root flour (100 g) was extracted with 2 L boiling distilled water three times for 4 h. The combined extract (6 L) was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The dried extracts were then suspended in 1 L water and further fractionated by *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol in a stepwise manner, repeated three times with each solvent. Removal of the solvents afforded 103.2 mg, 89.3 mg, 127.3 mg, 10.3 g, and 11.4 g of the hexane, dichloromethane, ethyl acetate, *n*-butanol and water fractions, respectively. In this study the *n*-butanol fraction (BF) was used for the bioactivity assays.

2.2. Cancer cell culture and cytotoxicity MTT assay

The human colon tumor cell line HT-29 was obtained from the American Type Cell Culture (Rockville, MD, USA) and cultured in RPMI-1640 medium (Gibco, MD, USA) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The growth-inhibitory effect of BF was measured using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay. HT-29 cells (2×10^5 cells/mL) were incubated with BF (50, 100, 150, or 200 μg/mL) in 96-well plates for 48 h. After incubation, the culture medium was removed and 50 μl MTT reagents (2 mg/mL) were added to each well. The plates were incubated at 37 °C for 4 h. DMSO (100%, 150 μL) was added to each well. The metabolized MTT product was dissolved in DMSO and quantified by measuring the optical density at 550 nm on a microplate reader (Dynex Technologies, USA). IC_{50} is the concentration of BF required to inhibit growth by 50% and was calculated from the equation of the logarithmic line of best fit (Microsoft Excel). The cytotoxicity of BF in HEK293 cells were measured using the same MTT assay described above.

2.3. Cell cycle analysis

Cells were exposed to 0, 50, 100, 150, or 200 μg/mL BF for 24 h and 48 h. Afterwards, cells were harvested, washed with PBS, fixed in 70% ethanol for 1 h at −20 °C, and then stained with propidium iodide (PI) solution (20 μg/mL PI, 0.1 mM EDTA, 10 μg/mL RNase, and 1% Triton X-100 in PBS) for 30 min in the dark. DNA content was measured using a FACS flow cytometer analysis system, and at least 10,000 cells were analyzed for each experimental treatment. PI fluorescence at 585 nm was detected in the FL-2 channel. Samples were initially examined by gating on a FL-2 area versus FL-2 width dot plot to exclude debris and subsequently examined on a DNA content histogram. Data analysis was performed using WinMDI version 2.9 cell cycle analysis software (Scripps Research Institute).

2.4. Measurement of intracellular ROS

A flow cytometer (FACScan, BD Biosciences, San Jose, CA, USA) was used to analyze intracellular reactive oxygen species with the fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, Invitrogen), which passively diffuses into the cell and is cleaved and oxidized to 2′,7′-dichlorofluorescein. Intracellular H_2O_2 or –OH in the presence of peroxidase changes DCFH to the highly fluorescent compound DCF. Thus, the fluorescent intensity is proportional to the amount of peroxides which are produced by the cells. Following exposure to the drug, the cells were trypsinized and washed with ice-cold PBS. Then added 1 ml of PBS containing 20 μM DCFDA, and incubated the cells for 30 min at 37 °C. The fluorescence emission from DCF was analyzed via FACScan flow cytometry (Becton–Dickinson, San Jose, CA). Three independent samples of 10,000 cells were analyzed for each experimental condition.

2.5. HPLC analysis of intracellular polyamine levels

HT-29 (2×10^5) cells were incubated with BF (0, 10, 50, 100, 150, 200 μg/mL) or 5-FU, (25 μg/mL), fluorouracil, Sigma), for 24 h. Cells were collected and counted using a Neubauer haemocytometer, washed twice with PBS and extracted for polyamine analysis as described by Kabra et al. (1986). Polyamine extract (10 μL) was injected onto the HPLC and analyzed using a Phenomenex Luna 5 μm, 25 cm × 4.6 mm, C-18 HPLC column. The mobile phase was acetonitrile (A) and water (B). For a better separation a 0.4 mL/min flow rate and a gradient elution mode were used: time = 0 min, A:B (95:5); time = 25 min, A:B (37:63); time = 30 - min, A:B (0:100); time = 40 min, A:B (90:5). The quantitative analysis was carried out by the external standard (putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride) method.

2.6. RT-PCR analysis of BF-treated cells

Total RNA was extracted from treated cells with or without (control) BF using Trizol reagent. For cDNA synthesis, 2 μg RNA was reverse transcribed with moloney murine leukemia virus reverse transcriptase (RT). Semi-quantitative PCR was performed with oligonucleotides as previously described (Yin et al., 2009) using primers specific for Bcl-2, Bax, p53, caspase-3, survivin and β-actin as control. The products of the RT-PCR were separated by electrophoresis using 1.2% agarose gel and stained with ethidium bromide. PCR products were visualized by UV transillumination.

2.7. Western blot analysis of BF-treated cells

The HT-29 cells were washed twice with PBS and lysed in lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 20 mM KCl, 1.5 mM $MgCl_2$, 50 mM β-GPA, 10 mM NaF, 0.5% NP-40, plus proteinase and phosphatase inhibitors) for 10 min and centrifuged at 12,000*g* for 10 min at 4 °C. Total protein, as determined by the Bio-Rad protein assay, was mixed with 4× loading buffer, and pre-heated at 95 °C for 5 min. The samples were separated on SDS denaturing 10% polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF). The membranes were blocked with 5% skim milk, washed, and probed with primary antibodies against p53, Caspase-3, JNK (Santa Cruz Biotechnology) and GAPDH (Sigma–Aldrich), respectively. After washing, the membranes were incubated with corresponding secondary antibodies and visualized by enhanced chemiluminescence (ECL) in accordance with the manufacturer's recommendations (Amersham).

2.8. Statistical analysis

Results were expressed as the mean ± SD of at least three experiments performed using different *in vitro* cell preparations. Statistically significant differences were determined using a one-way ANOVA by SPSS 11.5 software. Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Effects of BF on cell cytotoxicity

HT-29 and HEK293 cells were used to examine the anti-proliferation activity and cytotoxicity of the BF extract using the MTT-based assay. In HEK293 cells, BF extract showed non-cytotoxic activity (data not shown). In HT-29 cells, extract of BF inhibited cell growth in a dose- and time-dependent manner and showed inhibition of growth rate on 24 h basis by 3.10 ± 0.3%, 6.12 ± 1.1%, 10.56 ± 1.7%, 17.27 ± 0.5%, and 32.14 ± 0.7% at 10, 50, 100, 150 and 200 μg/mL, respectively. Moreover, the percent inhibition of the HT-29 cell growth rate 48 h after exposure to the same BF extracts were 4.80 ± 0.87%, 16.02 ± 1.03%, 18.33 ± 2.64%, 30.78 ± 1.93% and 66.12 ± 1.52%, respectively (Fig. 1).

3.2. BF extract induces sub-G1 phase accumulation and G0/G1 arrest

To further evaluate the effects of the BF extract on HT-29 cell proliferation, we determined the cell cycle distribution by flow cytometry. After BF treatment for 24 h and 48 h, cellular DNA was stained with PI, and flow cytometric analysis was performed. The

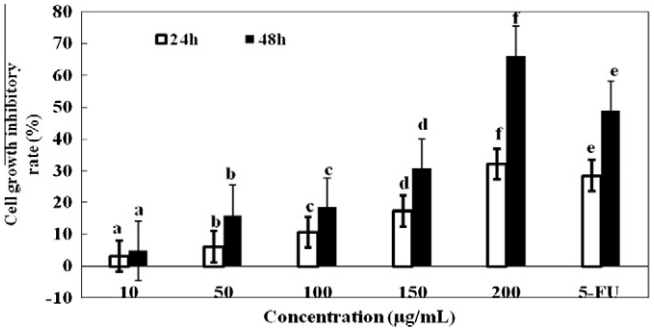

Fig. 1. *In vitro* inhibition rate of HT-29 cells by BF. As reference 5-Fu (50 μg/mL) is included. Data are the mean ± SD of three independent experiments. Lowercase letters indicate significant differences according to ANOVA test ($P < 0.05$).