CENTELLA ASIATICA EXTRACT INDUCES CELL CYCLE ARREST IN CACO-2 HUMAN COLON CANCER CELLS

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Abstract Centella asiatica is traditionally a medicinal plant frequently employed in the practice of Thai folk medicine. This study examined the anti-tumor activity of the crude water extract of C. asiatica using human colon adenocarcinoma-derived Caco-2 cells. C. asiatica extract reduced the proliferation rate of Caco-2 cells significantly in a concentration- and time-dependent manner. The mechanism of cancer cell growth inhibition was shown to occur via cell cycle arrest. The extract induced S and G2-M arrest in Caco-2 cells accompanied apoptosis induction. The extract also increased the accumulation of cyclin B1 protein in the cells. These findings indicate that C. asiatica extract inhibited cell proliferation of Caco-2 cells through modification of the cell cycle events and this cell cycle arrest is associated, at least in part, with increased accumulation of cyclin B1 protein. Chiang Mai Med Bull 2005;44(1):21-28.

Keywords: centella asiatica, colon adenocarcinoma cell lines, cell proliferation, cell cycle arrest, cyclin B1

The research for bioactive natural products and their analogues as chemotherapy agents continues in preclinical development.1 Various phytochemicals originating from medicinal plants have been shown to have anti-tumor activities, and some of them are currently used in clinical fields.2 Our laboratory has shown that some medicinal plants exhibit inhibitory effects on the formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF).3-6 Centella asiatica Linn. is a medicinal plant traditionally used in Asia. This plant possesses many kinds of biological activities such as the elevation of antioxidant level in

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wound healing\textsuperscript{(7)} and anti-herpes simplex virus activity.\textsuperscript{(8)} Crude extracts of this plant have been shown to inhibit the proliferation of transformed cell lines and ascites tumor cells and retard the development of solid and ascites tumors.\textsuperscript{(9)} Moreover, our previous report found that \textit{C. asiatica} extract inhibited the formation of AOM-induced ACF in rat colons and had a chemopreventive effect on colon tumorigenesis.\textsuperscript{(10)} Principle active components of \textit{C. asiatica} are pentacyclic triterpenes.\textsuperscript{(11)}

Cellular homeostasis critically depends on a balance of cell proliferation, differentiation and death.\textsuperscript{(12)} Since uncontrolled cell proliferation has essential roles in carcinogenesis, regulation of cell proliferation is important for cancer prevention.\textsuperscript{(13)} Some phytochemicals have been reported to show anti-tumor activity by apoptosis induction and/or cell cycle arrest.\textsuperscript{(14-15)} Cell cycle is regulated by two processes: oscillating changes in the activity of the cell cycle machinery and specific proteolysis of the cell cycle regulators.\textsuperscript{(16)} Cyclin-dependent kinase (CDKs), stage-specific cyclins and inhibitors of cyclin-dependent kinases (CKIs) act as regulators of the cell cycle.\textsuperscript{(17)} Progression from S to G\textsubscript{2}-M phase is regulated by the accumulation of cyclin B1, which binds to the CDK1 catalytic subunit to form an active complex.\textsuperscript{(18)}

In this study, we examined the effects of \textit{C. asiatica} extract on the growth of Caco-2 cells including cell proliferation, cell cycle, and cyclin B1 protein level to elucidate the mechanism(s) of antitumorigenesis of this plant.

**Materials and methods**

**Preparation of plant extract**

\textit{C. asiatica} was purchased from local markets in Chiang Mai, Thailand. The fresh plant (100 g) was washed with water, cut into small pieces and extracted with 200 mL of cold distilled water for 4 hr. The extract was centrifuged at 4,000x\textsuperscript{g} for 15 min before the supernatant was filtered through paper (Whatman No.1). The filtrate was evaporated in a rotatory evaporator under reduced pressure at 45-50 °C prior to lyophilization. The yield of water extract from the fresh plant was 2.5%.

**Cell line and culture condition**

Caco-2 cells (RCB0988, Riken Cell Bank, Tsukuba Science City, Japan) were cultured in MEM (ICN Biomedical, Ohio, USA) containing 20% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY), 100 units/mL penicillin, 100 µg/mL streptomycin sulfate and 2 mM glutamine. Cells were grown at 37 °C in a fully humidified atmosphere containing 5% CO\textsubscript{2}. The number of viable cells was determined using a hemocytometer based on the exclusion of trypan blue dye. Twenty four hours after seeding, the cells were treated with \textit{C. asiatica} extract at various concentrations.

**Cell proliferation assay**

Cell proliferation was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] cell proliferation kit (Promega, Corp., Madison, WI, USA), which colorimetri-
Centella asiatica extract induces cell cycle arrest. Cells were plated at $10^5$ cells/well in 96-well culture plates and treated with 1-16 mg/mL of Centella asiatica extract for 24, 48, 72 and 96 hr. Then, 20 µL of MTS solution was added to each well according to the manufacturer’s instructions. The mixtures were incubated for 1 hr at 37 °C and the formazan produced was measured using a microplate reader spectrophotometer (Corona Electric, Tokyo, Japan) at 490 and 630 nm. The results were expressed by the number of cells.

Flow cytometric analysis of the cell cycle
To examine the effect of Centella asiatica extract on cell cycle phase distribution, the cells were treated with the extract at concentrations of 2-8 mg/mL for various times and then resuspended in 500 µL of phosphate-buffered saline (PBS) with 35% ethanol at 37 °C for 30 min. The cells were washed 2 times with PBS and resuspended in 500 µL of PBS containing 2 µL of RNaseA (1 mg/mL, Wako, Osaka, Japan) and 2 µL of RNaseT1 (3340 units/mL, Wako), and incubated at 37 °C for 1 hr. They were stained with 50 µg/mL of propidium iodide (PI) and fluorescence was detected with a flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Tokyo, Japan). The Phoenix Flow System was used to make DNA content frequency histograms and analyze the data.

Western blot analysis
Whole cell lysates were prepared as described previously. Briefly, cells were harvested by centrifugation at 1,000 x g for 5 min at 4 °C. The cell pellets (3x10^6 cells) were washed once with ice-cold PBS and resuspended with 100 µL of the chilled lysis buffer containing 20 µg/mL leupeptin, 20 µg/mL aprotinin and 0.2 mM phenylmethylsulfonylfluoride (PMSF). The cells were disrupted by passing them through a G27 needle 10 times. After centrifugation in a microcentrifuge at 750xg for 5 min at 4 °C, the supernatants were centrifuged again at 15,000xg for 15 min at 4 °C. The supernatants were divided into aliquots and stored at -20 °C. Protein concentration was determined using a Coomassie protein assay kit (Pierce, Rockford, USA) according to the manufacturer’s instructions. Samples were subjected to 12% SDS (sodium dodecyl sulfate)-polyacrylamide gel (Wako) electrophoresis with 200 V for 35 min and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) with 20 V for 1 h. The membranes were blocked in Tween 20-Tris-buffered saline (TTBS) containing 2% bovine serum albumin (BSA) for 1 hr and probed overnight with a primary antibody mouse anti-cyclin B1 (1:2000; Upstate Biotechnology, Lake Placid, NY) at 4 °C. After washing with TTBS, the membranes were incubated further with primary antibody mouse anti-β-actin (1:5000; Sigma) for 1 hr at 37 °C. Primary antibody binding was detected with a goat anti-mouse IgG conjugated with alkaline phosphatase (1:2000; Sigma) and visualized by an enhanced chemiluminescence.
method using disodium-3(4-methoxyspiro{1,2-dioxetane-3-2’-(5’-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) (CSPD) (Boehringer, Manheim Germany).

Results

*C. asiatica* extract reduces the proliferation of Caco-2 cells

*C. asiatica* extract significantly reduced the proliferation rate of Caco-2 cells in concentration- and time-dependent manners (Fig. 1). Caco-2 cells treated with the extract grew very slowly, and the number of cells treated with 1 mg/mL of *C. asiatica* for 96 hr was approximately one-fifth of the medium control.

Effect of *C. asiatica* extract on the cell cycle

Caco-2 cells were treated with *C. asiatica* extract at concentrations of 2, 4 and 8 mg/mL for 48, 72 and 96 h, respectively and then subjected to flow cytometric analysis after staining DNA with PI. Histograms of the flow cytometric data are shown in Fig. 2. In the control culture, most of the cells were distributed in the G0/G1 phase (Table 1). In contrast, 89% of the cells were arrested in the S phase after 96 hr of treatment with 8 mg/mL of the extract. The number of Caco-2 cells distributed in the S phase increased in a time-dependent manner at 2, 4 and 8 mg/mL of the extract, at 48, 72 and 96 hr. The induction of S phase arrest was clearly dose-dependent at 96 h. Sub-G1 peak, which means the appearance of apoptotic cells, was observed slightly at concentrations of 4 and 8 mg/mL after 72 and 96 hr exposure respectively.

![Figure 1](image)

Figure 1. Effect of *C. asiatica* extract on the growth of Caco-2. Cells of 1x10^4 were cultured with or without *C. asiatica* extract (open circles) at concentrations of 1 (closed circles), 2 (open triangles), 4 (closed triangles), 8 (open squares), 16 (closed squares) mg/mL for 48, 72 and 96 hr. Cell numbers were determined by the MTS assay using various concentrations of the cells as standard. Values are means±SD (n=3). * Significant difference compared with all test concentrations of *C. asiatica* extract at the same time, p<0.0001 (ANOVA).
These results indicate that *C. asiatica* extract induced S and G2-M phase arrests in Caco-2 cells and led to apoptosis induction in consequence.

**C. asiatica extract induces cyclin B1 accumulation**

Fig. 3 shows the level of cyclin B1 protein in Caco-2 cells treated with solvent or 2, 4 and 8 mg/mL of the extract for 96 h. Cyclin B1 accumulated distinctively at a concentration of 8 mg/mL in Caco-2 cells.

**Discussion**

*C. asiatica* is a plant used for traditional medicine. Crude extract of this plant has been shown to inhibit the proliferation of ascites tumor cells *in vitro* and retard the development of transplanted tumors. Several different classes of food constituents and some phytochemicals have been reported to enable modulation of both cellular proliferation and programmed cell death. In this study, we investigated the inhibitory effect of the crude water extract of this plant on cell proliferation and the cell cycle distribution of the colon adenocarcinoma cell line. *C. asiatica* extract inhibited the proliferation of Caco-2 cells in dose- and time-dependent manners. Cell cycle analysis showed that Caco-2 cells were preferentially arrested at the S phase. Apoptosis was observed at concentrations of 4 and 8 mg/mL after 72 and 96 hr respectively exposures, indicating that the extract reduced the proliferation of both cells via cell cycle arrest, and led to apoptosis. Irreversible arrest of the cell cycle leads to apoptosis, and it may be a possible mechanism underlying the inhibition of cancer cell growth.

Although the majority of cells in Caco-2 still accumulated in the S phase.
Table 1. Cell cycle analysis of Caco-2 cells. Percentage distribution of cells treated with C. asiatica extract at doses of 2, 4 and 8 mg/mL.

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<tr>
<th>Concentration (mg/mL)</th>
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Figure 3. Effect of C. asiatica extract on the levels of cyclin B1 in Caco-2 cells. The cells were treated with and without C. asiatica extract at 2, 4 and 8 mg/mL for 96 hr. Forty µg protein from each sample were electrophoresed and Western blot was carried out using antibody against cyclin B1, and β-actin was detected as a loading control.

at 96 hr, some were arrested at the G2-M phase. This difference in response may be due to the possible effect of several differently-active components in the crude extract of C. asiatica. Cell cycle is regulated by oscillating changes in the activity of the cell cycle machinery, and specific proteolysis of the cell cycle regulators. Progression from the S to G2-M phase is regulated by the accumulation of cyclin B1, which binds the to cdk1 catalytic subunit to form an active complex. During mitosis, a specific ubiquitin-ligase complex, known as an anaphase-promoting complex (APC), is activated to initiate proteolysis of various mitosis regulators. Cyclin B1, cyclin A and a number of other regulatory proteins are degraded by APC-mediated specific proteolysis during and after mitosis. Proteolysis of cyclin B1 is universally required as an exit from mitosis. In our study, cyclin B1 protein levels in Caco-2 cells were distinctly higher after treatment with the extract. Therefore, C. asiatica extract affects the cell cycle through accumulation of cyclin B1, probably by modification of APC activity or its regulation. Although lower concentrations of C. asiatica extract, at less than 1 mg/mL, did not exhibit any significant cytotoxicity on Caco-2 cells (data not shown), this might be improved with a long period of incubation. The water extract used in this study probably contained various kinds of constituents, partially purification of the extract, which might may reduce the concentration used in the cell culture.

These results indicated that C. asiatica extract inhibits the proliferation of colon cancer cells through modification of cell cycle progression. C. asiatica is usually taken as a traditional medicine in Thailand. Therefore, the detailed mechanisms of C.asiatica cell cycle arrest and its other possible modes of action should be clarified before intro-
Centella asiatica extract induces cell cycle arrest

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References

สารสกัดบัวบกเหนี่ยวนำการหยุดวัฏจักรของเซลล์มะเร็งล่าสุดให้ใหญ่ของคนชนิด 
Caco-2

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บทคัดย่อ: นักวิจัยจัดเป็นพืชสมุนไพรซึ่งนิยมนำมาใช้กันมากในทางการแพทย์แผนโบราณของไทย การศึกษาครั้งนี้เพื่อตรวจหาฤทธิ์ต้านมะเร็งของสารสกัดบัวบกบ_handles โดยใช้เซลล์มะเร็งล่าสุดให้ใหญ่ชนิด Caco-2 ผลการศึกษาพบว่าสารสกัดบัวบกเหนี่ยวนำการรับของเซลล์มะเร็งล่าสุดให้ใหญ่ชนิด Caco-2 ทำให้การเจริญเติบโตของเซลล์เร็วขึ้น แต่ไม่ทำให้เซลล์ตาย ในการศึกษานี้สรุปได้ว่าสารสกัดบัวบกเหนี่ยวนำการหยุดวัฏจักรเซลล์มะเร็ง Caco-2 ที่ระยะ S และ G2-M และพบว่ามีการตายของเซลล์เกิดขึ้นร่วมกัน สารสกัดบัวบกเหนี่ยวนำการหยุดวัฏจักรเซลล์มะเร็ง Caco-2 แต่ไม่ทำให้เซลล์ตาย ผลการศึกษานี้มีความเกี่ยวข้องกับการสะสมของโปรตีน cyclin B1 ที่เหมาะสม สารสกัดบัวบกเหนี่ยวนำการหยุดวัฏจักรเซลล์มะเร็ง Caco-2 ทำให้เซลล์ตาย ผลการศึกษานี้มีการเกี่ยวข้องกับการสะสมของโปรตีน cyclin B1 ที่เหมาะสม

คำสำคัญ: บัวบก เซลล์มะเร็งล่าสุดให้ใหญ่ การแบ่งตัวของเซลล์ การหยุดวัฏจักรเซลล์ cyclin B1