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Cucurbitacin-I, a natural cell-permeable triterpenoid isolated from *Cucurbitaceae*, exerts potent anticancer effect in colon cancer

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ABSTRACT

Cucurbitacin-I is a triterpenoids found in medicinal plants and have diverse pharmacological and biological activities. In this study, the antitumor effects of cucurbitacin-I on colon cancer and possible roles in apoptosis and cell cycle arrest were investigated. Treatment of SW480 cells, a human colon cancer cells, with cucurbitacin-I decreased cell viability and cell proliferation in a concentration-dependent manner. Also, cucurbitacin-I induced G2/M phase cell cycle arrest in SW480 cells with a decreased expression of cell cycle proteins including cyclin B1, cyclin A, CDK1, and CDC25C. Moreover, cucurbitacin-I induced increased cleavage of caspase-3, -7, -8, -9, and poly ADP ribose polymerase. When we examined the inhibitory effect of cucurbitacin-I on tumor growth *in vivo*, cucurbitacin-I effectively inhibited the tumorigenicity and growth of CT-26 cells in syngenic BALB/c mice. In summary, the present study showed that cucurbitacin-I reduced colon cancer cell proliferation by enhancing apoptosis and causing cell cycle arrest at the G2/M phase.

1. Introduction

Natural compounds can modify cancer development [1]. Antitumor effects elicited by various natural compounds are due in part to the induction of cellular defense systems including the detoxifying and antioxidant enzymes system, as well as the inhibition of anti-inflammatory and anti-cell growth signaling pathways culminating in apoptosis and/or cell cycle arrest [2–5]. Among many natural compounds, phytochemicals, chemicals from plants that may affect health, but are not essential nutrients, are a good source of chemopreventive and therapeutic agents against cancer. Phytochemicals have fewer side effects, compared with chemically synthesized drugs, which has spurred efforts to identify new phytochemical antitumor agents.

Cucurbitacins are a group of highly oxidized tetracyclic triterpenoids initially identified in the *Cucurbitaceae* plant family, such as cucumber. In traditional medicine, cucurbitacin containing plants have been known for their antipyretic, analgesic, antiinflammatory, antimicrobial, and antitumor activities [6,7]. A recent study chronicled 12 main categories to group cucurbitacins and their derivatives according to their side-chain variations [7]. Cucurbitacins have potent pharmacological properties, such as antitumor, anti-inflammatory, and hepatoprotective effects [8,9]. Several molecular targets for cucurbitacins that have been discovered include fibrous-actin, signal transducer and activator of transcription 3 (STAT3), and cyclooxygenase-2 [10,11]. Like other cucurbitacins, cucurbitacin-I (Fig. 1A) inhibits cancer cell growth *in vitro* and *in vivo* tumor models by disrupting Janus kinase (JAK)/STAT3 signaling pathway [12,13]. Cucurbitacin-I potently inhibits cell growth in various human cancer cells including glioblastoma cells [14], hepatocellular carcinoma [15], B leukemic cells [13], nasopharyngeal carcinoma cells [16], and prostate cancer [17]. Cucurbitacin-I also suppresses cancer stem cell-like properties and enhances the response to chemoradiotherapy [18].

The anticancer effect of cucurbitacin-I and the underlying mechanism in colon cancer cells are unclear. This study explored the antitumor effect of cucurbitacin-I in colon cancer.

2. Methods

2.1. Reagents

Cucurbitacin-I was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI-1640 medium, EMEM medium, fetal

Abbreviations: HRP, horseradish peroxidase; JAK, Janus kinase; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; STAT, signal transducer and activator of transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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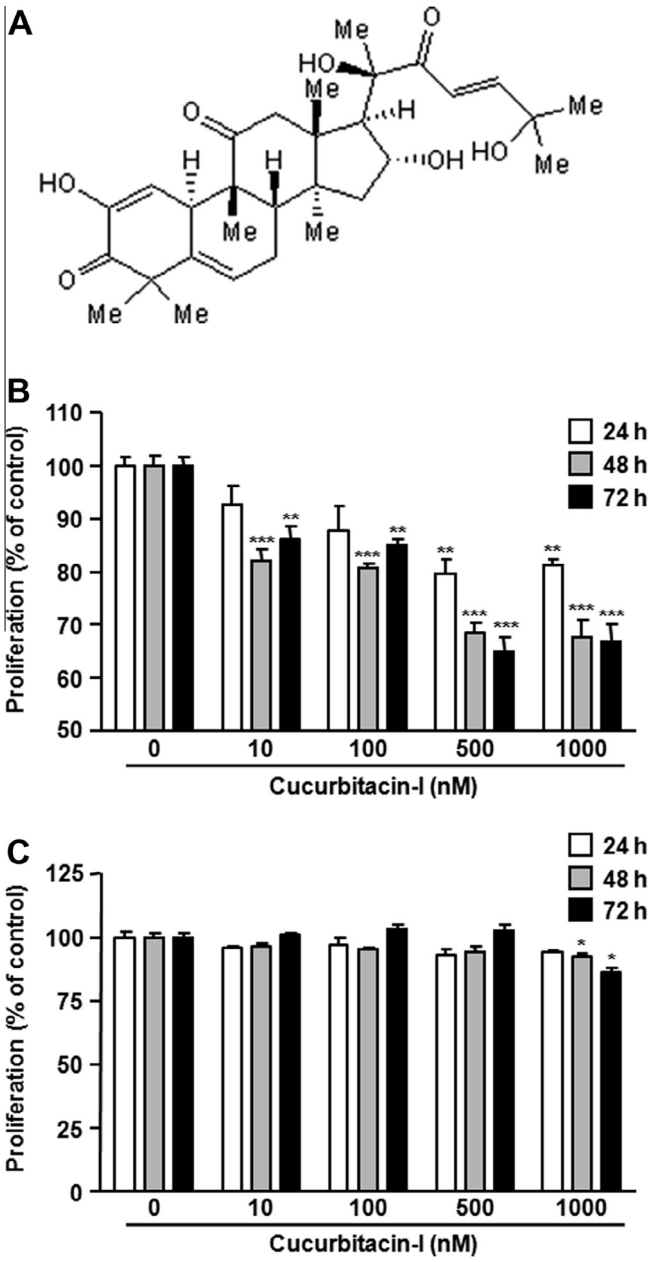

Fig. 1. Effects of cucurbitacin-I on colon cancer cell proliferation. (A) The structure of cucurbitacin-I. (B) SW480 cells and (C) CCD-18Co cells were treated with various dose of cucurbitacin-I for 24, 48 and 72 h, after which the proliferation was determined as described in Section 2. The results are reported as the mean ± S.E.M. of four independent experiments in triplicate. Statistical significance is based on the difference when compared with 0 nM-treated cells, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from Hyclone (Logan, UT, USA). Cell Titer 96 Aqueous One Solution was purchased from Promega (Madison, WI, USA). Fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection kit was obtained from BD Pharmingen (San Diego, CA). Dimethylsulfoxide (DMSO), RNase A, propidium iodide (PI) and anti-β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO, USA). Protein detection BCA kit was obtained from Thermo Scientific Pierce (Rockford, IL, USA). Primary and secondary antibodies, and horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti rabbit secondary anti-sera were purchased from Santa Cruz Biotechnology and Cell Signaling Technology (Danvers, MA, USA).

2.2. Cells and cell culture

Human colon cancer (SW480) and normal colon cell lines (CCD-18Co) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Murine colon carcinoma CT-26 cell line was obtained from the Korean Cell Bank (Seoul, Korea). SW480 and CT-26 cells were maintained in RPMI 1640 medium and CCD-18Co cells in EMEM medium at 37 °C with 5% CO_2 in a humidified atmosphere. All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells attained 70% confluence with a normal morphology and multiplication pattern, and were used for experiments between passages 3–12. Unless otherwise mentioned, control treatment refers to the cells treated with an equal amount of DMSO; the maximum amount of DMSO was maintained at <0.1% v/v.

2.3. Cell proliferation assay

SW480 and CCD-18Co cells (1×10^4 cells/well in 96-well plates) were treated for 72 h with different concentrations (0–1000 nM) of cucurbitacin-I. Cell viability was measured spectrophotometrically by the Cell Titer 96 Aqueous One Solution assay [19].

2.4. Cell cycle analysis

SW480 cells were plated in wells of a 48-well plate at 1.5×10^5 cells/well and incubated with cucurbitacin I at 0, 50, 100, or 500 nM for 48 h. Cells were harvested by centrifugation, washed twice with ice-cold phosphate buffered saline (PBS), and fixed in 70% ethanol at 4 °C overnight. Cells were then centrifuged at 200×*g* for 5 min, and the supernatant was discarded. The cells were washed with PBS and stained with 0.5 ml RNase A (10 μg/ml), and 0.5 ml of propidium iodide (PI; 10 μg/ml) for 45 min in the dark. The DNA content of the cells was measured on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA).

2.5. Annexin V staining

Apoptosis was measured using the FITC-Annexin V Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA). Briefly, SW480 cells were plated in wells of a 48-well plate at 1.5×10^5 cells/well and treated with various concentration (0, 50, 100, 500 nM) of cucurbitacin-I. After incubation at 37 °C for 48 h, the cells were washed twice with cold PBS and resuspended in 1× binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then, 1×10^5 cells in 100 μl binding buffer were transferred to 5 ml tubes and stained with 5 μl of FITC-Annexin V and 5 μl PI. The cells were gently vortexed and incubated at room temperature for 15 min. After washing the cells with 1× binding buffer to remove the excess FITC-Annexin V and PI, the cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). The data were analyzed using FlowJo software (TreeStar, Ashand, OR, USA).

2.6. Mitochondrial membrane potential analysis

The changes in relative mitochondrial membrane potential ($\Delta\Psi m$) were assessed using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzamid azolocarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA). SW480 cells were seeded in wells of a 24-well plate at 5×10^5 cells/well and treated with various concentrations (0, 50, 100, 500 nM) of cucurbitacin-I. After the 48 h treatment, the cells were incubated with 5 μM of JC-1 fluorescence dye for 15 min in a CO_2 incubator and washed slowly several times with PBS. Mitochondrial membrane