Caffeic Acid Induces Apoptosis in Human Cervical Cancer Cells Through the Mitochondrial Pathway

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SUMMARY

Objective: The anti-proliferation effect of caffeic acid (3,4-dihydroxycinnamic acid), isolated from *Ocimum gratissimum* Linn, on human cervical cancer cells (HeLa cells) was examined to elucidate the associated mechanism and death mode.

Materials and Methods: Flow cytometry showed that caffeic acid treatment results in dramatically increased apoptosis of HeLa cells. Western blot analysis revealed that caffeic acid activates various processed caspases.

Results: Caffeic acid significantly reduced proliferation of HeLa cells in a concentration-dependent manner. Morphological evidence of apoptosis, including nuclei fragmentation was clearly observed 24 and 48 hours after exposure to caffeic acid (1 mM and 10 mM) by flow cytometry. Time-dependent inhibition was also observed. Caffeic acid decreased levels of uncleaved caspase-3 and Bcl-2, and induced cleaved caspase-3 and p53.

Conclusion: Caffeic acid induces apoptosis by inhibiting Bcl-2 activity, leading to release of cytochrome c and subsequent activation of caspase-3, indicating that caffeic acid induces apoptosis via the mitochondrial apoptotic pathway. This also suggests that caffeic acid has a strong anti-tumor effect and may be a promising chemopreventive or chemotherapeutic agent. [*Taiwan J Obstet Gynecol* 2010;49(4):419–424]

Key Words: caffeic acid, cervical cancer cell line, cytochrome c, flow-cytometry

Introduction

Cervical cancer is characterized by the rapid and uncontrolled growth of abnormal cells on the cervix. Although the worldwide incidence has decreased, primarily because of the widespread use of cervical screening programs, it is still the second most common gynecological malignancy. Cervical cancer is highly curable when diagnosed early and treated promptly. Primary treatment for cervical cancer depends on the stage at which it is diagnosed and includes surgery, combined radiation therapy and chemotherapy, or both [1].

In normal cell function and tissue homeostasis, proliferation and apoptosis are balanced. Cancer might reflect abnormal *in vivo* proliferation that is not balanced by compensatory apoptosis [2]. The growth speed of embryo cells and malignant cells are significantly faster than that of normal cells; therefore, cell death and cell growth mechanisms in cancer are already beyond control [3].

The mechanism of apoptosis is a controlled form of cell death [4]. Some researchers have pointed out the relationship between apoptosis and chloroquine. This compound can inhibit proliferation of colon cancer and induce apoptosis [5]. Therefore, the mechanism of apoptosis is an important factor in developing cancer treatments.

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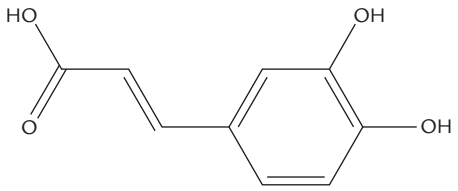

Figure 1. Chemical structure of caffeic acid.

Flavonoids and other polyphenolic compounds derived from fruits and berries have been shown to induce apoptotic pathways and to suppress proliferation of various types of cancer cells such as leukemic cells and liver cancer cells [6–12]. Caffeic acid is a simple phenolic compound (Figure 1) found primarily in coffee. Studies have shown that it has anti-oxidative [13], anti-inflammatory [14], and anti-allergic [15] activities.

In this study, the mechanism by which caffeic acid inhibits cell proliferation and the pathway of its pro-apoptotic effects are examined in human cervical cancer cells (HeLa).

Materials and Methods

Cell lines

Human cervical cancer cells (HeLa cells) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% FBS, 1% penicillin, and 100 μg/mL streptomycin (Life Technologies Inc., Grand Island, NY, USA) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Drugs and reagents

3-(4,5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Life Technologies Inc.; antibodies to caspase-3, p53, Bcl-2, and cytochrome c were from Cell Signaling Technology (Beverly, MA, USA); all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

MTT assay

Cells were seeded in a 96-well plate at 1×10^3 cells per well and cultured for 24 hours. Cells were then incubated with different concentrations of caffeic acid (0.5, 1, 2.5, 5 or 10 mM) for 24 hours.

After incubation, MTT was dissolved in PBS at 5 mg/mL and then added to culture medium at a final concentration of 0.5%. After incubation at 37°C for 4 hours, the medium was gently aspirated and 150 μL DMSO was added to each well to dissolve any formazan crystals. The plate was shaken for 10 minutes to allow complete solubilization. Cell viability was determined spectrophotometrically by measuring the absorbance at 570 nm using a 96-well plate reader.

Flow cytometric detection of apoptosis by PI staining

DNA content was analyzed by PI staining of permeabilized cells; apoptosis was identified by the presence of fragmented DNA in cells of the sub-G_1 phase. After exposure of HeLa cells (2×10^6 cells) to various concentrations of caffeic acid for 24 and 48 hours, the cells were washed twice with PBS, fixed with ice-cold 80% methanol, and stored at −20°C for 24 hours. Subsequently, the cells were pelleted, suspended in PBS, and incubated with 0.2 mg/mL RNaseA and 2% Triton X-100 for 1 hour at room temperature. Propidium iodide was added to a final concentration of 10 μg/mL and incubation was continued for at least 18 hours in the dark before flow cytometry. Flow cytometry was performed using a Becton Dickinson FACSCalibur cytometer with an argon ion laser (488 nm) as the excitation source and Cell Quest (version 3.3) software to analyze DNA content.

Gel electrophoresis and western blot analysis

Cells were incubated for 24 hours in the presence of indicated concentrations of caffeic acid. After the incubation, the cells were harvested and lysed. Mitochondrial protein and cytosolic protein were isolated using a mitochondrial isolation kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Proteins were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For western blot analysis, proteins were electrophoresed on a 10–12% linear gradient SDS polyacrylamide gel, followed by immunoblot on nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk in PBS with 0.1% Tween-20 for 1 hour at room temperature. Finally, proteins were visualized using an enhanced chemiluminescence detection system (Cell Signaling Technology, Beverly, MA, USA) after incubation with antibodies to caspase-3, cytochrome c, p53, or actin at 4°C overnight. Proteins were then incubated with peroxidase-labeled anti-rabbit antibody or peroxidase-labeled anti-mouse antibody for 1 hour at room temperature. Peroxidase activity was visualized on X-ray film in a darkroom.

Statistical analysis

Results are representative of independent experiments performed in triplicate; data are expressed as mean ± standard deviation, and were evaluated by one-way

analysis of variance followed by the Dunnett's test. A p value <0.05 indicates statistical significance ($*p<0.01$).

Results

Caffeic acid inhibited proliferation of HeLa cells in a concentration-dependent manner

To examine the effects of caffeic acid on HeLa cells, we treated cells with various concentrations of caffeic acid. As shown in Figure 2, caffeic acid significantly reduced the proliferation of HeLa cells in a dose-dependent manner.

Caffeic acid induced apoptosis in a concentration- and time-dependent manner

Cells were treated with caffeic acid for 24 hours or 48 hours. Flow cytometry revealed a hypodiploid DNA peak following treatment with 1 mM caffeic acid (Figure 3A-2). The proportion of cells in the sub-G_1 phase increased from 8.9% after exposure to 1 mM caffeic acid to 53.37% following exposure to 5 mM of caffeic acid (Figures 3A-1 and 3A-3).

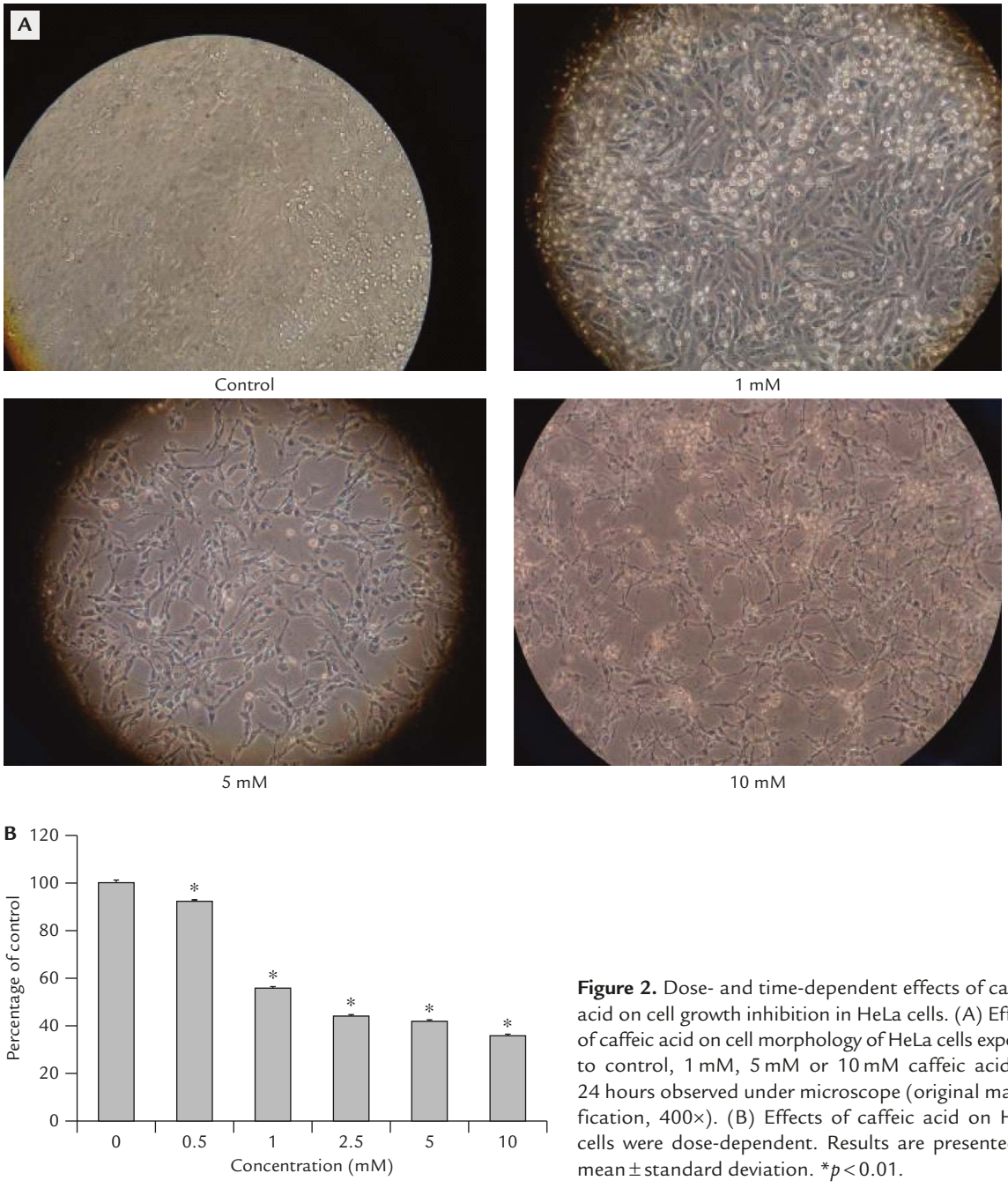

Figure 2. Dose- and time-dependent effects of caffeic acid on cell growth inhibition in HeLa cells. (A) Effects of caffeic acid on cell morphology of HeLa cells exposed to control, 1 mM, 5 mM or 10 mM caffeic acid for 24 hours observed under microscope (original magnification, 400×). (B) Effects of caffeic acid on HeLa cells were dose-dependent. Results are presented as mean ± standard deviation. $*p<0.01$.

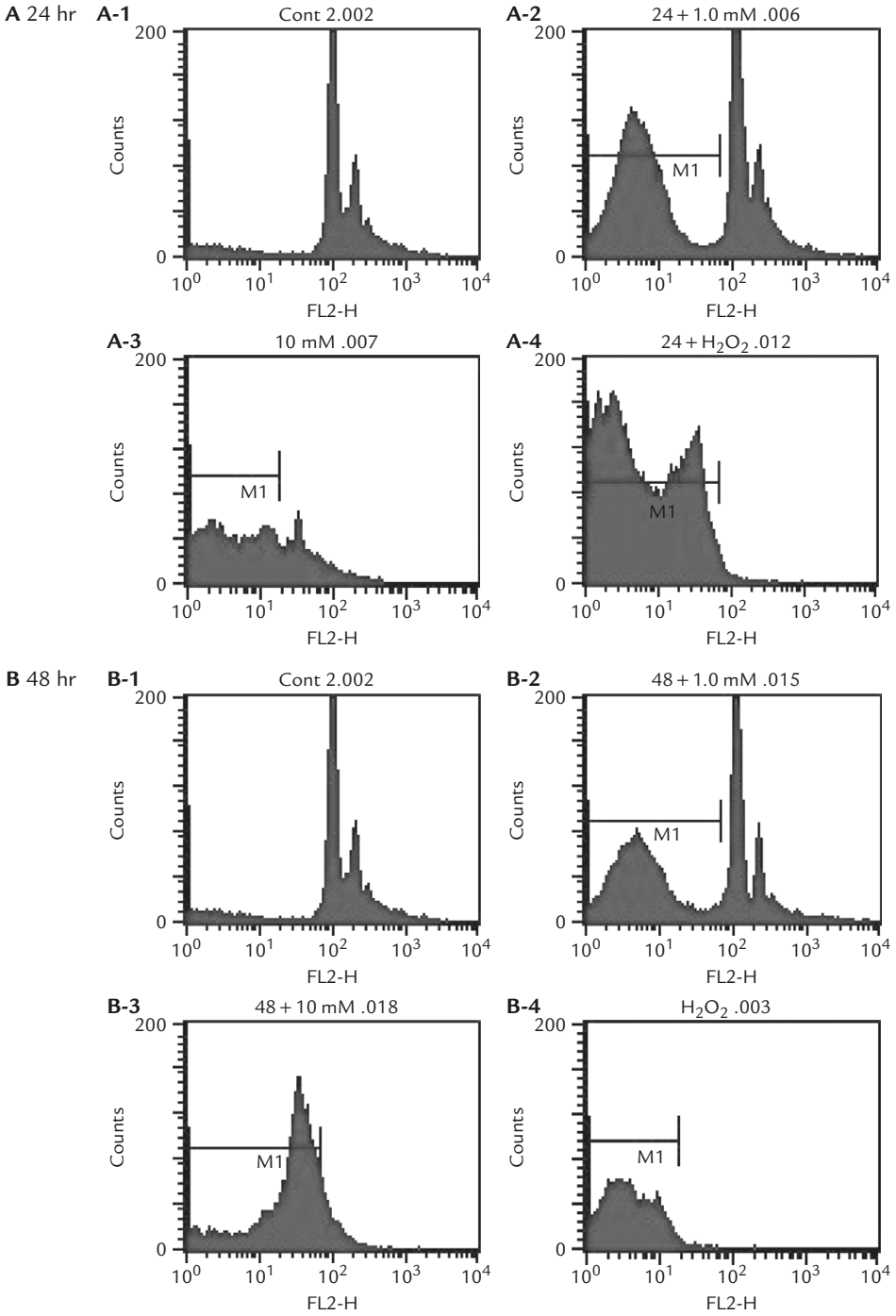

Figure 3. Effects of caffeic acid on HeLa cell DNA fragmentation. HeLa cells were exposed to solvent, 1 mM or 10 mM caffeic acid for 24 or 48 hours, then fixed and stained with PI. Cell percentage within the hypodiploid DNA region was determined by flow cytometry. (A) Cells in the sub-G_1 phase increased from 8.9% (A-1) to 62.42% (A-3) following exposure to 1 mM and 10 mM of caffeic acid for 24 hours, respectively. (B) Cells in the sub-G_1 phase increased from 8.9% (B-1) to 83.94% (B-3) following exposure to 1 mM and 10 mM of caffeic acid for 48 hours, respectively. Cells in the sub-G_1 phase were 50.42% (A-2) after 24-hour incubation of HeLa cells with 1 mM caffeic acid and 53.37% (B-2) after 48-hour incubation; H_2O_2 was used as an internal control. Over 90% of cells were in the sub-G_1 phase (A-4, B-4).

HeLa cells were pre-treated for 48 hours with 10 mM caffeic acid. The proportion of cells in the sub-G_1 phase increased from 8.9% after exposure to 1 mM caffeic acid to 83.94% following exposure to 10 mM caffeic acid (Figures 3B-1 and 3B-3).

Gel electrophoresis and western blot analysis

Caffeic acid induced apoptosis in HeLa Cells via a caspase-3-dependent pathway

To elucidate the role of caspases in caffeic acid-induced apoptosis, whole cell lysates were prepared from HeLa

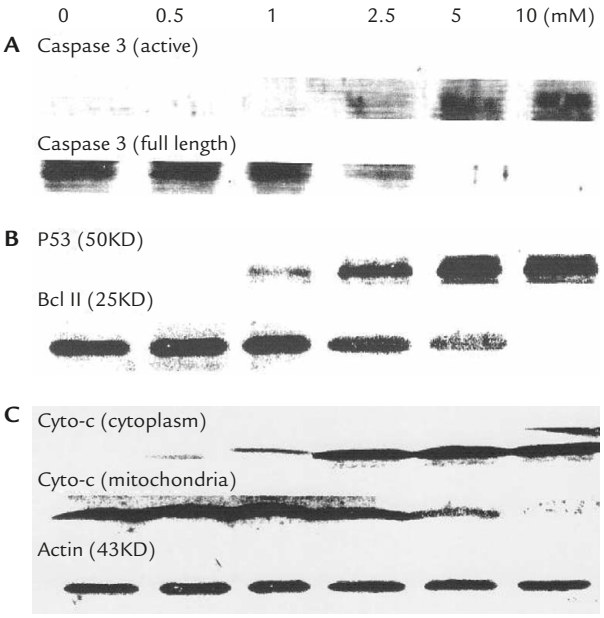

Figure 4. HeLa cells treated with various concentrations (0–10 mM) of caffeic acid for 12 hours; actin was used as an internal loading control. (A) Cleaved and uncleaved caspase-3 levels determined by western blot. (B) Effects of caffeic acid on Bcl-2 and p53 levels. (C) Western blot of antibodies specific for cytochrome c in mitochondrial and cytosolic lysates.

cells that had been stimulated by various concentrations of caffeic acid; levels of cleaved caspase-3 and full caspase-3 were examined. As shown in Figure 4A, western blot analysis revealed that levels of cleaved caspase-3 protein increased and levels of uncleaved caspase-3 protein decreased, in a dose-dependent manner.

Caffeic acid induces the release of cytochrome c, downregulates Bcl-2, and upregulates p53 protein expression

As caspase-3 was activated in caffeic acid-treated cultures, the role of mitochondria in caffeic acid-induced apoptosis was further studied by examining the effects of caffeic acid on the levels of Bcl-2 and cytochrome c. Western blot analysis indicated that the anti-apoptotic protein Bcl-2 was down-regulated, and that the levels of the apoptosis promoter p53 increased in HeLa cells after treatment with 10 mM caffeic acid (Figure 4B). The release of cytochrome c was detected following exposure to 1 mM caffeic acid; cytochrome c levels in the mitochondria decreased in a concentration-dependent manner (Figure 4C). These results suggest that cytochrome c plays a direct role in caffeic acid-induced apoptosis.

Discussion

Clinically, interstitial cells in the body cavity can be confused with malignant cells and are likely to cause diagnostic errors. To avoid this confusion, flow cytometry must be used [16]. In this study, it was used to detect cell apoptosis.

Many anticancer medicines are extracted from plants. Earlier research points out that plant phytochemicals inhibit proliferation in breast cancer [17] and ovary cancer cell lines [18]. In this study, we have investigated the mechanisms by which caffeic acid induces apoptosis in human cervical cancer cells [19].

After incubation with various concentrations of caffeic acid for 24 hours, cell viability was markedly reduced (Figure 2A). Caspase-3 activity was stronger after incubation with 10 mM caffeic acid than after incubation with concentrations below 10 mM. In addition, less obvious morphological changes were noted in cells with stronger caspase-3 activity (Figure 4A). The MTT assay showed cell viability reduced from 58% after incubation with 1 mM caffeic acid to 37% after treatment with 10 mM caffeic acid (Figure 2B), while DNA fragmentation was seen in 83.94% of cells after exposure to 10 mM caffeic acid for 48 hours (Figure 3B-3).

Apoptosis is initiated by two distinct signaling pathways. The intrinsic apoptotic pathway is dominated by the Bcl-2 family, which determines the release of cytochrome c from mitochondria. Exposure of HeLa cells to caffeic acid leads to the disappearance of the anti-apoptotic Bcl-2 protein on the mitochondria and the release of cytochrome c into the cytosol (Figure 4C). During the apoptotic process, the tumor suppressor protein p53 stimulates a wide network of biochemical signals in response to DNA damage. Here, we examined the effects of caffeic acid on p53 protein expression and found it expressed in a concentration-dependent manner (Figure 3B). These findings indicate that caffeic acid induces apoptosis via the mitochondrial apoptotic pathway.

Cervical cancer is the most common gynecologic malignancy. The disease is curable if diagnosed early and treated promptly. In early stages, radiation therapy or postoperative total pelvic irradiation can improve survival rates [20]. Neo-adjuvant chemotherapy dramatically reduces the rate of local recurrence and distant metastases in high-risk patients [21,22]. Radiation therapy combined with chemotherapy has also been shown to be an effective treatment for cervical cancer [23]. Our findings suggest that caffeic acid produces a strong anti-tumor effect and, therefore, may be a promising chemopreventive or chemotherapeutic agent.

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