Caffeic Acid Phenethyl Ester Inhibits Cell Proliferation and Induces Apoptosis in Human Ovarian Cancer Cells

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Abstract − **The phenethyl ester of caffeic acid (CAPE), an active component of honeybee propolis extract, is shown to inhibit cancer growth previously. However, studies on human ovarian cancer are largely obscure. This study evaluated the effects of CAPE as a potential anti-proliferative and pro-apoptotic agent in the human ovarian cancer line, OVCAR-3. CAPE treated OVCAR-3 cells showed inhibition of cell viability and proliferation in a dose-dependent manner by WST-1 assay, LDH assay and bromodeoxyuridine (BrdU) incorporation assay. Furthermore, CAPE-mediated OVCAR-3 cell growth inhibition was associated with apoptotic changes as evident by cell cycle arrest and accumulation of cells in the apoptotic phase and DNA fragmentation. Taken together, CAPE inhibits cell proliferation via DNA synthesis reduction and induces apoptotic cell death via DNA damage, thus elucidating a novel, plausible mechanism of CAPE anti-tumorigenic property in OVCAR-3 cells.**

Key words : phenethyl ester of caffeic acid, DNA fragmentation, apoptosis, OVCAR-3

INTRODUCTION

Ovarian cancer is the fourth most common cancer in women and is the leading cause of death from all gynecological carcinomas (Zhang *et al*. 2004; Dundr 2010). Recently, pathogenesis of this disease remains to be elucidated and novel therapeutic agents are constantly being explored (Dundr 2010; Kandalaft *et al*. 2010). Moreover, the available experimental/human evidence on natural products intake and risk of ovarian cancer is limited. Thus, novel therapeutic approaches are direly needed. The phenethyl ester of caffeic acid (CAPE) is an active component of honeybee propolis extract and is used as a traditional medicine in the Far East (Grunberger *et al*. 1988), and has been shown to acts as an antiviral (Ho *et al*. 2005; Fruehauf and Meyskens 2007), anti-inflammatory (Orban *et al*. 2000; Lee *et al*. 2010), antiatherosclerotic agent (Hishikawa *et al*. 2005; Ho *et al*. 2009), and antitumoral (Nagaoka *et al*. 2003; Demestre *et al*. 2009; Szliszka *et al*. 2009) in diverse systems. Although these studies suggest that at least some of these effects of CAPE are due to its anticancer effects, it is not known whether CAPE can inhibits cell proliferation or induces apoptosis in human ovarian cancer cells. Therefore, we investigated the anticancer effect of CAPE in human ovarian cancer cell line, OVCAR3 and demonstrated for the first time that CAPE suppressed cell proliferation and induced apoptosis in ovarian cancer cells.

MATERIALS AND METHODS

1. Cell culture

The OVCAR3 cells, human ovarian cancer cell line, were obtained from the American Type Culture Collection (Rock-

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ville, MD, USA). Tissue culture media and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). The CAPE and all the other reagents, except where indicated, were purchased from sigma chemical company (St. Louis, Mo, USA).

CAPE was dissolved in DMSO and stored at −20°C. The OVCAR3 cell line was maintained in RPMI1640 medium supplemented with 10% FBS, penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}) and 1 mM glutamine. The cells were cultured at 37°C in a humidified atmosphere under 5% CO_2. For all the experiments, unless otherwise stated, 1×10^3 cells were seeded in 96-well plates and grown to confluence for 24 hrs, prior to being treated with CAPE or the carrier solvent DMSO. The final concentrations of CAPE used for all the experiments were prepared by diluting the stock with cell culture media. The cells were then treated with either the vehicle (DMSO) or CAPE at various concentrations for 48 hrs.

2. Cell viability

Cell viabilities were determined using WST-1 assays, and by measuring LDH release, which was quantified in supernatants using LDH kits. WST-1 and LDH assays were both performed according to the manufactures' instructions described previously (Lee *et al*. 2008a).

3. Cell proliferation

Cell proliferation was measured BrdU incorporation in place of thymidine during DNA synthesis before cell division by DNA precursor analog incorporation assay kit (Roche Diagnostics, Indianapolis, USA.) In brief, both the vehicle and CAPE-treated cells were labeled with BrdU (10 μM) for 6 h prior to incubation with anti-BrdU-peroxidase (10 μL $well^{-1}$) for 2 hrs. The immune complex was detected following the addition of trimethyl benzidine substrate and measured at 450 nm using an ELISA reader. Cell proliferation was expressed as the % BrdU incorporation.

4. Cell cycle analysis

Flow cytometry was used for cell cycle analysis. In brief, 3×10^5 cells were cultured in 6-well plates and treated with CAPE or vehicle for 48 hrs. Both the floating and adherent cells were collected and washed twice with DMSO prior to staining using propidium iodide. Modfit software (BD Biosciences, Mountain View, CA, USA) were used for data acquisition and analysis.

5. Caspase-3 activity and DNA fragmentation analysis

Caspase-3 assay and DNA Fragmentation were both performed according to the manufactures' instructions as described previously (Lee *et al*. 2008b). Briefly, cell lysates were incubated in reaction buffer in the presence of 50 μM fluorogenic substrates (Biomol, Germany), preferentially cleaved by caspase-3 (AC-DEVD-AMC). Cleavage of the fluorogenic substrates for 60 min at 37°C was measured using a fluorescence reader (Varioskan, Thermo Electon Co.) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. DNA samples for DNA laddering were extracted from lysates using a total DNA separator kit (Promega, USA) and electrophoretically separated in a 0.9% agarose gel, which were treated ethidium bromide and visualized and photographed under ultraviolet light.

6. Statistical analysis

All data were expressed as the mean ± S.D. from three independent experiments, performed in triplicate. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the student t test, using $p < 0.05$ as the level of significance.

RESULTS

1. Effect of CAPE on cell cytotoxicity and proliferation

The cytotoxicity effects of CAPE in OVCAR3 cells were quantified using LDH and WST-1 assays. CAPE fully induced cell cytotoxicity in a dose-dependent manner based on WST-1 assay results (Fig. 1A). In particular, CAPE also induced the leakage of LDH from OVCAR3 cells (Fig. 1B). Moreover, CAPE inhibited DNA synthesis in a dose-dependent manner as evident by the observed decrease in the incorporation of BrdU into DNA in the CAPE-treated cells compared to the controls (Fig. 2A).

2. Effects of CAPE on cell cycle

To explore CAPE on cell cycle arrest in ovarian cancer

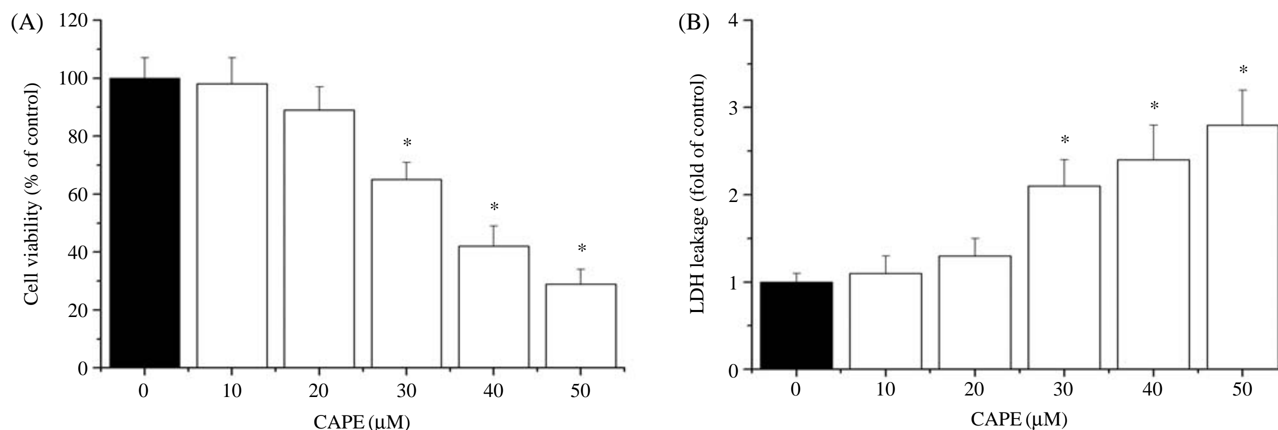

Fig. 1. Effect of CAPE on cytotoxicity of OVCAR-3 cells. The cells were seeded at an initial density of 1×10^3 cells $well^{-1}$. Cells were treated with various concentrations of CAPE were treated for 48 hrs. Cell viability were estimated by the WST-1 assay (A) and LDH leakage assay (B), as described in Materials and Methods. The cytotoxicity was determined by relative absorbance normalized to the control cells. Each bar represents the mean ± S.D. calculated from three independent experiments. *$p < 0.05$ Compared to control.

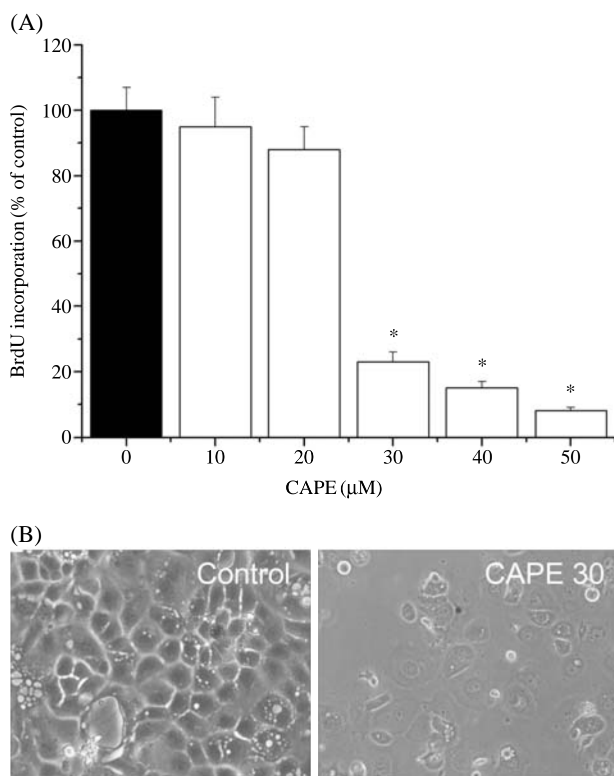

Fig. 2. Effect of CAPE on proliferation of OVCAR-3 cells. Cells were treated with various concentrations of CAPE were treated for 48 hrs. (A) DNA synthesis was measured by BrdU incorporation assay as described in Materials and Methods. (B) Cellular morphology of OVCAR-3 cells. Each bar represents the mean ± S.D. calculated from three independent experiments. *$p < 0.05$ Compared to control.

cells, we further analyzed FACS analysis. As the results, CAPE increased the percentage of OCVAR-3 cells in the G_1-phase (Fig. 3A) with an associated decrease in the percentage of cells in S-phase (DNA synthesis) (Fig. 3B) and G_2-phase (Fig. 3C) of the cell cycle when compared to the untreated controls. Furthermore, the percentage of cells in the apoptosis phase (G0 arrest) was increased when compared to the controls (Fig. 3D).

3. Effects of CAPE on apoptosis

The CAPE-mediated OVCAR-3 inhibition of cell proliferation/survival was associated with apoptotic changes. At CAPE concentrations where inhibition of cell proliferation/survival was observed (10 ~ 50 μM), the cells were shrunken, rounded and detached from the cell culture substratum (Fig. 2B). Furthermore, DNA fragmentation/laddering (characteristic of apoptosis) was observed when the cells were treated with CAPE, indicating cell death by apoptosis in these cells while no DNA laddering was observed in the untreated cells (controls) (Fig. 4). The apoptotic cells were identified by the caspase-3 assay kit. As the result, CAPE-treated cells increased in a does dependent manner, when compared to control cells (Fig. 5).

DISCUSSION

The flavonoids and phenolic components found in propolis are known to affect the apoptosis of various cancer cells and may play an important role in cancer chemoprevention (Shukla and Gupta 2006; Syed *et al.* 2008; Demestre *et al.*

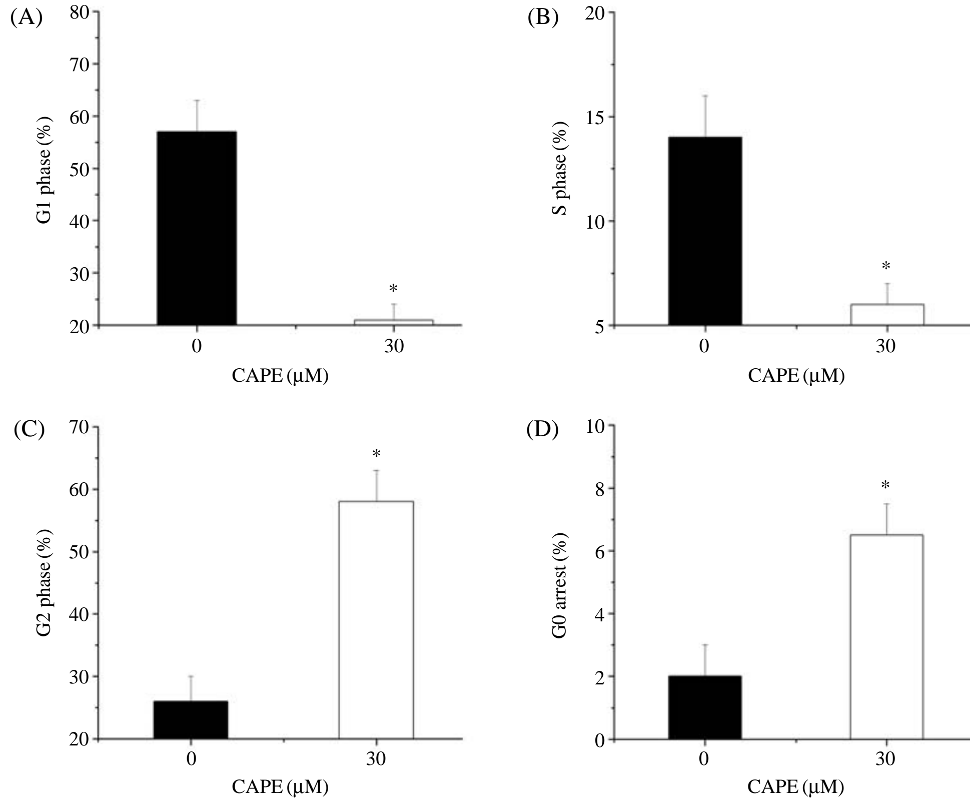

Fig. 3. Effect of CAPE on cell cycle progression. After 48 hrs of CAPE- treatment, the cell cycle phase distribution was determined by FACS analysis as described in Materials and Methods. Each bar represents the mean ± S.D. calculated from three independent experiments. *$p < 0.05$ Compared to control.

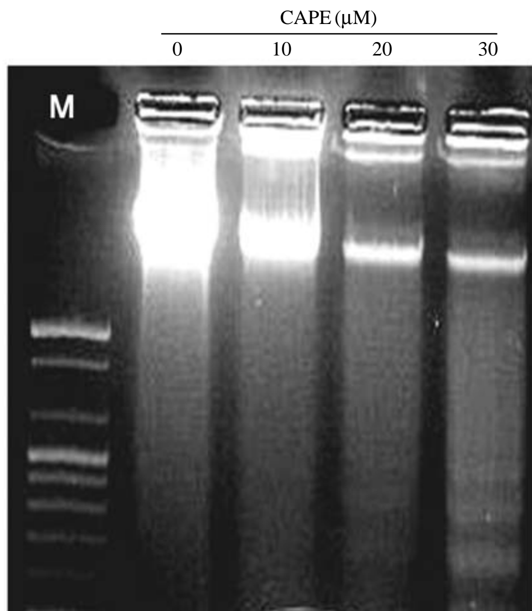

Fig. 4. DNA fragmentation analysis of OVCAR-3 cells treated with CAPE for 48 hrs. Lane 1: Protein marker (M); lane 2: vehicle-treated cells; lane 3; CAPE (10 μM)-treated cells; lane 4: CAPE (20 μM)-treated cells; lane 5: CAPE (30 μM)-treated cells.

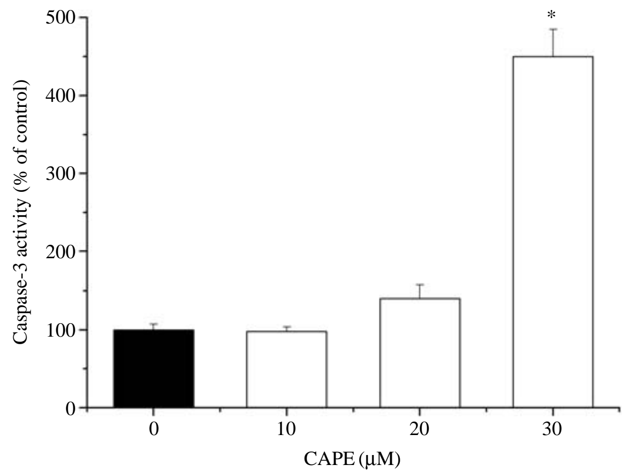

Fig. 5. Effect of CAPE on caspase-3 activation of OVCAR-3 cells. After 48 hrs of CAPE-treatment, the caspase-3 activation was determined as described in Materials and Methods. Each bar represents the mean ± S.D. calculated from three independent experiments. *$p < 0.05$ Compared to control.

2009; Szliszka *et al.* 2009). The present study showed that CAPE inhibited the viability of OVCAR-3 cells. This study

had the advantage that the MTT and LDH assay was used, which measures the mitochondrial activity and cell membrane damage of cells. CAPE-treated cells lost the ability to provide and maintain energy for metabolic activity and survival indicating that CAPE caused cellular damage. DNA replication occurs before cell division/doubling, thus the measurement of DNA synthesis, is an attractive marker for cell proliferation. A decrease in BrdU-labeled DNA was noted in the CAPE treated cells, suggesting that CAPE is an inhibitor of DNA synthesis and cell proliferation in OVCAR-3 cells. Moreover, the percentage of OVCAR-3-treated cells in the apoptotic phase was significantly increased as compared to the untreated cells, suggesting a block in cell cycle progression leading to programmed cell death. This was in agreement with previous studies which established that apoptosis is the likely mechanism of action of honeybee propolis extract for inhibiting cell growth (Kamatou *et al*. 2008). Thus, the activation of apoptosis is believed to play a critical role in both the chemo-prevention and treatment of human carcinomas.

The biochemical hallmark of apoptosis is genomic DNA fragmentation and caspase-3 activity increasing that commits the cell to die. The nuclear endonuclease enzyme cleaves DNA resulting in DNA fragments through some kind of caspase activites. In the previous study, CAPE has the potent prooxidant potential in DNA cleavage, and *ortho*-dihydroxyl functionality of CAPE may be a major determination of its prooxidant activity (Wang *et al*. 2008). In present study, CAPE confirmed by the labeling the DNA fragments and Caspase-3 assay, and indicating that CAPE induced apoptosis in the OVCAR-3 cells by causing DNA damage. In recent study, ethanolic extract of propolis augments TRAIL-induced apoptotic death in prostate cancer cells (Szliszka *et al*. 2009). Because its phenolic components may be one of the mechanisms responsible for their cancer preventive effects, more studies are needed to further explore this mecanisms.

Taken together, the anti-proliferative and proapoptotic properties of CAPE in the human ovarian cancer cell line, OVCAR-3, were supported by the observed reduction in cell survival and DNA synthesis, as well as cell cycle arrest and the accumulation of cells in the apoptotic phase caused by DNA damage.

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