Cell Killing and Radiosensitization by Caffeic Acid Phenethyl Ester (CAPE) in Lung Cancer Cells

Miao-Fen CHEN1,2, Chun-Te WU2,3, Yu-Jen CHEN4, Peter C. KENG5 and Wen-Cheng CHEN1*

CAPE and radiation/Lung cancer/Apoptosis/G2 arrest/Radiation sensitization.

Caffeic acid phenethyl ester (CAPE) is a biologically active ingredient of honeybee propolis. The cytotoxicity and radiation sensitization effects of CAPE were evaluated in human lung cancer A549 cells and normal lung fibroblast WI-38 cells. A549 cells treated with 6 µg/ml CAPE showed marked growth inhibition (60%) at 48 hr after treatments. During the same time, the number of viable cells decreased to 46% of the control value. In contrast, WI-38 cells showed 20% growth inhibition with no change in the number of viable cells under the same treatment conditions. At 72 hr after CAPE treatment (6 µg/ml), the percentage of apoptotic cells in A549 cultures increased significantly to 67% and an S/G2 arrest was also detected in the culture. Furthermore, there was a significant decrease in the level of intracellular glutathione and hydrogen peroxide contents within one hr after CAPE treatment, and the expression of cyclin B1 was reduced 6 hr after treatment. The radiation sensitization effect of CAPE on A549 cells was determined from the clonogenic survival curves, and the results showed a small but significant difference in radiation survival between cells treated with or without CAPE. Taken together, our results suggest that the effects of CAPE on differential cytotoxicity, apoptosis, and radiosensitization are associated with glutathione depletion that occurred shortly after treatments.

INTRODUCTION

Honeybee propolis has been widely used as a folk medicine. Caffeic acid phenethyl ester (CAPE) is a biologically active ingredient of honeybee propolis.1 Previous studies have shown that the compound elicits several interesting biological functions, including antiviral and anti-inflammatory effects, differential toxicity to cancer cells versus normal cells, and antioxidant effects to reduce intracellular free radicals in some instances.2-4 Seed et al. and Nair et al.5,6 have suggested that combination treatments with antioxidant would provide a new strategy to prevent radiation injury to normal tissues. Calikoglu et al. indicated that CAPE might be effective in protecting the injury of remote organs caused by oxidative stress and neutrophil accumulation that resulted from an ischemia-reperfusion injury.7 Based on these studied, CAPE might be a potential radioprotector for normal tissues. However, it is also a potent apoptosis-inducing agent as well as an antioxidant for tumors.8 CAPE-associated growth inhibition may be related to its effects on oxidative processes induced by mitogenic stimuli. The apoptosis of tumor cells can be induced via different pathways by various treatments, such as irradiation and chemotherapeutic agents.9,10 CAPE alters the redox state and induces apoptosis in transformed cells. Nagaoka et al. showed the high possibility of using CAPE and its analogues as a new class of chemo preventive agents for the treatment of colon cancer metastasis.11 Despite the multifunctional properties of CAPE, the cellular and molecular bases for the multiple activities have not yet been clearly understood. In this study, we chose malignant lung cancer as the study target because of the poor prognosis associated with treatment regimens, including radiotherapy and chemotherapy.12,13 Radiation resistance to dose levels typically used at clinics represents a major factor contributing to the high incidence of local failure. However, the therapeutic dose is limited by normal tissue tolerance to radiation damage.14,15 In the present study, we investigated the differential cytotoxic effects of CAPE on lung cancer cells and normal lung fibroblasts. A variety of factors that might be involved in apoptotic cell death were studied. Since free radicals play a key role in apoptosis, CAPE-induced changes in the anti-
dant system such as glutathione (GSH), which protects cells against oxidative stress\(^{16}\), were determined with a flow cytometric method. The radiation sensitization property of CAPE was evaluated for its potential use with radiation in cancer therapy.

**METHODS AND MATERIALS**

**Chemicals**

CAPE is a biologically active ingredient of honeybee propolis. It was purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell line and cell culture condition**

The human lung adenocarcinoma cell line, A549, was obtained from the American Type Culture Collection (ATCC). The immortal human normal lung fibroblasts, WI-38, were purchased from Bioreources Collection and Research Center (BCRC). The cells were maintained in DMEM supplemented with 10% fetal bovine serum in a 37°C incubator containing 5% CO\(_2\) and 95% air.

**Cell growth analysis**

Cells (A549 and WI-38) were seeded in the tissue culture flasks at a density of 10,000 cells/cm\(^2\). Various concentrations of CAPE (0, 2, 4, 6 µg/ml) were added to half the culture flasks, and the other half was used as untreated controls. On days 1 and 2 after treatment, the attached cells were trypsinized, and cell numbers were counted with a hemocytometer and trypan blue dye exclusion assay.

**Cell viability test**

To determine the toxic effects of CAPE, A549 and WI-38 cells were treated with various doses (0, 1, 2, 4, 6, 8, and 10 µg/ml) of CAPE for 1 hr, then resuspended in fresh medium and cultured for 2 days before MTT assay. For the assay, the cells were incubated with the MTT (tetrazolium compounds) for 4 hr, lysed with DMSO, and the color crystals were solubilized with an ELISA reader at a wavelength of 570 nm. We also used different cell numbers to perform the calibration of an MTT test to determine the linear regression relationship between the cell number and the color detected by ELISA.

**Measurements of Apoptosis by flow cytometry**

After incubation with 6 µg/ml CAPE for 24 and 72 hr, treated and untreated A549 cells were harvested, washed with PBS, and resuspended (1 × 10\(^6\)/ml) in Annexin-V-FLUOS labeling solution (Annexin-V-FLUOS staining kit, Roche) for 15 min in the dark at 37°C. The fluorescence was analyzed by a FACSCalibur flow cytometer (Epics Altra, Beckman Coulter, Taiwan). Green fluorescence was measured to indicate the proportion of cells undergoing apoptosis (FITC conjugated Annexin-V), and red fluorescence (propidium iodide) was measured to indicate the proportion of cells undergoing necrosis.

**Cell cycle analysis**

Flow cytometric analysis was performed to determine cell cycle changes after treatment with 6 µg/ml CAPE for various durations (6, 24, and 72 hr). The cells were washed and resuspended in PBS (1 × 10\(^6\)/ml) before being fixed in 75% ethanol. PI solution (50 µg/ml propidium iodide, 0.1% sodium citrate, 0.1% Nondiet P-40) was used to stain total cellular DNA at room temperature for 30 min before analysis by a FACSCalibur flow cytometer.

**Fluorocytometric analysis of intracellular GSH and \(\text{H}_2\text{O}_2\)**

Intracellular GSH and \(\text{H}_2\text{O}_2\) levels were assessed by staining the cells with 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probe) and 2′,7′-dichlororescin diacetate (DCFH-DA) (Molecular Probe), respectively. Briefly, PBS-washed A549 cells were incubated with 1 µM CMFDA and 10 µM DCFH-DA for 15 min at 37°C before treatment with CAPE. The cells were treated with 6 µg/ml CAPE, and the fluorocytometric analysis was performed every 20 min thereafter. The GSH and \(\text{H}_2\text{O}_2\) levels were measured from the FL-1 channel (green fluorescence) of the FACSCalibur.

**cDNA microarray analysis**

Total RNA was prepared from cells by an RNeasy mini kit (Qiagen). Total RNA was obtained from A549 cells treated with and without CAPE (6 µg/ml for 1 hr), respectively. The cDNA preparation, hybridization, and array data analysis (ImaGene 4.0 Biodiscovery, Inc.) were performed by the service at the U-vision Biotech. Inc., Taipei, Taiwan.

**Western blot analysis**

After treatment with 6 µg/ml CAPE for 6, 12, and 24 h, A549 cells were washed with PBS and lysed with extraction buffer (1% SDS, 10 mM sodium orthovanadate, 10 mM Tris, pH = 7.4). Cellular debris was cleared by centrifugation (1,300 rpm, 10 min, 4°C) and the protein concentration in the supernatant was determined by a protein assay kit (MicroBCA protein assay; Pierce, Rockford, IL). An equal amount of protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA). The membranes were probed with antihuman cyclin B1 mouse monoclonal antibody (BD Bioscience), COX-2 mouse monoclonal antibody (BD Bioscience), and housekeeping gene r-tubulin antibody (Santa Cruz Biotechnology, Inc.). The proteins were visualized by use of an HRP detection system (Pierce, Rockford, IL).

**Clonogenic assay**

Exponentially growing cells were counted, diluted, and seeded in triplicate at 1,000 cells per culture dish (35 mm\(^2\)). Thereafter the cells were treated with 0, 2, 4, or 6 µg/ml...
CAPE and Radiation Treatments in Cancer and Normal Lung Cells

CAPE for 1 h. After the CAPE was removed, the cells were seeded in petri dishes. Irradiation was performed with 6 MeV of electron beam by a linear accelerator at various doses (0, 2, 4, 6, and 8 Gy) at a dose rate of 300 cGy/min in a single fraction. After irradiation, the cells were allowed to grow in McCoy’s 5A medium containing 20% heat-inactivated FCS at 37°C in a humidified 5% CO₂, 95% air incubator. After 7 days, the culture dishes were stained with 0.4% crystal violet, and the colonies (≥50 cells) were counted. The surviving fraction was calculated as the mean number of colonies divided by the plating efficiency.

Statistics
Statistical analysis was performed by analysis of variance. All data were presented as mean ± standard deviation. Significant differences were considered at *p* < 0.05.

RESULTS

CAPE induces differential growth inhibition and cytotoxicity between A549 and WI-38 cells
The growth inhibitory effects of CAPE for both cell lines were evaluated at different time intervals after they were treated with a single dose of 0, 2, 4, and 6 µg/ml CAPE. The proliferation of A549 cells was significantly inhibited by CAPE in a dose- and time-dependent manner (Fig. 1). Treatments with 6 µg/ml CAPE caused marked growth inhibition (60% at 48 h). Contrary to A549 cells, WI-38 normal lung fibroblasts showed only a 20% growth inhibition after 6 µg/ml CAPE treatments for 48 h.

The cytotoxic effects of CAPE on human lung cancer cell
line and normal fibroblasts were determined by use of the MTT assay (Fig. 2). There was a liner relationship between the percentage of viable cells and the concentration of CAPE employed. In A549 cells, the percentage of viable cells decreased to 46%, from 70%, as the concentration of CAPE increased to 6 µg/ml, from 4 µg/ml. However, the toxicity was much less for normal lung fibroblasts, and there was no significant cytotoxicity detected at CAPE concentration below 6 µg/ml.

**Effect of CAPE on the induction of apoptosis**

The A549 cells were treated with 6 µg/ml CAPE for 24 and 72 h, and the percentage of apoptosis induced by CAPE was determined by the Annexin-V/PI staining and flow cytometric method. At 24 h after treatment, the percentage of apoptosis was 28%. At 72 h after treatment, the percentage of apoptosis increased significantly to 67% (Fig. 3).

**Cell cycle changes**

To determine the effects of CAPE on cell-cycle progression, we treated the A549 and WI-38 cells with 6 µg/ml for 1 h, and the cell-cycle distributions were determined at 24 and 72 hrs after treatments. The results are summarized in Fig. 4. For A 549 cells, the portion of the S and G2/M cells increased gradually at different times after CAPE treatments, suggesting that the cells were arrested in the G2/M phase of the cell cycle. Compared to the untreated control cells, the increase of the percentage of the S and G2/M cells was approximately 10% and 20% at 24 h and 72 h after treatment respectfully. Furthermore, there was a significant increase in the portion of cells with sub-G1 DNA content at 72 h after treatments, indicating the onset of apoptosis.

**Effect of CAPE on intracellular H2O2 level and GSH level**

As demonstrated in Fig. 6, the intracellular H2O2 level of CAPE-treated A549 cells decreased immediately after treatment and reached 32% of that untreated level at the end of 1 hr. After treatment with CAPE for an hour, the intracellular GSH level also decreased to 40% of the untreated A549 cells.

**Different gene expression profile after CAPE treatment**

Upon treatment with CAPE, the A549 cells underwent significant cell death via apoptosis and cell cycle arrest. Too identify genes involved in these effects, we compared gene expression profiles of the A549 cells with and without 6 µg/ml CAPE treatment, using cDNA microarray. The standard of
determination for differential expressed genes was that the absolute value of natural logarithm of the ratio of Cy5 to Cy3 was greater than 0.69; that is, the change of gene expression was more than 2 times. Of the total of 8,000 genes tested, 136 were overexpressed genes. These included heat shock protein, CD22 antigen, matrix metalloproteinase. Seventy-nine were under expressed genes including cyclin B1, cycloxygenase-2, survivin, and the RAS oncogene family.

Confirmation of the expression levels of cyclin B, COX-2 protein
Since cyclin B1 is necessary for cdc2 activity, which plays a crucial role in the G2/M transition, we conducted further experiments to examine the involvement of cyclin B1 repression in CAPE-induced G2/M arrest. By using the Western blot analysis, we found the expression level of cyclin B1 to be significantly depressed in the A549 cells treated with 6 µg/ml CAPE for more than 6 h (Fig. 5). Furthermore, CAPE treatment also decreased the COX-2 expression in A549 cells after 12–24 h of treatment.

Sensitization of A549 cells to radiation treatment by CAPE
To determine whether CAPE treatment influenced the radiosensitivity of A549 cells, we treated the cells with CAPE at various concentrations (0, 2, 4, and 6 µg/ml) for 1 h prior to radiation survival assays. Those treated with a dose of 6 µg/ml CAPE showed decreased radiation survival compared to those treated only with radiation. Significant differences were observed at higher radiation doses (Fig. 7). After normalizing the cytotoxicity of CAPE, we still found a significant difference in survival curves between treatments with radiation alone and radiation plus CAPE in the radiation dose range of 4–8 Gy. The ER (enhancement ratio) of CAPE at survival fraction 10% is 1.23. However, after the cells were treated with nontoxic doses of 2 or 4 µg/ml CAPE, no significant radiation sensitization effect was detected.

DISCUSSION
In our series, we used A549 cells as a lung cancer model to evaluate the possible therapeutic effect of CAPE and WI-38 cells to present the possible protection or complication effects in normal tissues. In this study, we found that CAPE had differential growth inhibition and cytotoxicity effects between...
the lung cancer cell line and normal lung fibroblast. The use-
ful therapeutic dose of CAPE for the cell line was 6 µg/ml,
and the tolerance dose of normal lung fibroblast for CAPE
appeared to be higher than the therapeutic dose. According
to the Chiao series, CAPE-associated growth inhibition for
tumor cells may be related to its effect on the oxidative stress,
and the growth inhibition may function through p53-indepen-
dent pathways. Nomura et al. reported that CAPE may induce
apoptosis through both p53-dependent and p53-independent
pathways, and its antitumor activity may have occurred
through the induction of apoptosis. Troll et al. showed that
tumor-promoting agents stimulated cell proliferation by
increasing the intracellular production of ROS. Frenkel also
pointed out that H2O2 was the intermediate precursor for OH
and singlet oxygen. The measurements of H2O2 formation
and DNA-base oxidation could provide a mean for determin-
ing the potential use of various agents such as tumor promot-
ers in cancer therapy. In our series and others, CAPE
was observed to inhibit oxidative processes. It could decrease
the generation of intracellular H2O2 measured by DCFH fluo-
rescence in A549 cells. Buttke et al. have reported that
alternative responses of cell proliferation and apoptosis were
dependent on the specific cellular redox balance at a given
time. Thomas et al. pointed out that an exposure to a low dose
(10–100 µM) of H2O2 induced apoptosis in a variety of cell
types, but that a high dose induced necrosis. The decrease in
H2O2 production has also been shown to be an early event
during dexamethasone-induced apoptosis in rat thymocytes.
As shown in our study, CAPE inhibited intracellular H2O2
production and the GSH level of A549 cells in a very rapid
and profound manner. Since GSH is a thiol antioxidant, the
depleting intracellular stores of GSH by CAPE can render
cells more susceptible to oxidative stress-induced apoptosis.
Furthermore, we found through cDNA microarray analysis
that CAPE could decrease survivin and cyclooxygenase-2
(COX-2) mRNA expressions. Survivin is a member of the
apoptosis protein (IAP) inhibitor family, which can suppress
(COX-2) mRNA expressions. Survivin is a member of the
apoptosis protein (IAP) inhibitor family, which can suppress
expression of COX-2 mRNA. We also found through the use of
RNA microarray analysis in our study, we identified several
up- and downregulated genes in human A549 cells after
CAPE treatment. Cyclin B1 reduction was observed at the
mRNA level. The decreased expression of cyclin B1 was fur-
ther proved by a Western blot analysis. The results suggest
that cyclin B1 repression is involved in the mechanism of G2
arrest induced by CAPE. However, the molecular mechanism
of how CAPE represses cyclin B1 expression remains
unknown.

Because lung cancer is relatively resistant to radiation treat-
ment, it is important to study how to increase the radiosensi-
tivity of lung cancer with different radiation sensitizing
agents. Loprevite et al. reported that the A549 cell line was
not radiosensitized by several different chemotherapeutic
agents such as gemcitabine, topocean. However, we dem-
onstrated that CAPE has a radiosensitization effect besides its
cytotoxic effect on lung tumor cells. CAPE inhibits cell-cycle
progression by arresting cells in the S/G2 phase, so the phe-
nomenon of radiosensitization cannot be adequately ex-
plained by the phase of cell-cycle arrest. The most sensitive
phase of cell cycle to radiation is the G2 and M phase, and the
S phase is relatively radioresistant. Moreover, we found that
the radiosensitization phenomenon caused by CAPE occurred
within a few hours after treatment, whereas the change of cell-
cycle distribution took place much later. The radiosensiti-
zation effect may be explained by the rapid decrease of GSH
induced by CAPE. The biological effects of radiation are pri-
mary from damaged DNA, which is the critical target for cell
survival. DNA lesions are caused by a large number of free
radicals produced by radiation. GSH can decrease radia-
tion-induced damage through its function as a free radical
scavenger. A high concentration of intracellular thiol is an
important way to resist cytotoxic and radiation damage in
cancer cells. In a series of studies by Chen et al. and
Chiao et al., they demonstrated that CAPE significantly
depleted intracellular GSH in HL-60 cells and other trans-
formed cells. We also demonstrated that CAPE decreased the
intracellular thiol levels, and this could explain the radiosensi-
tization effect in lung cancer cells. The regulation of intracel-
lar GSH requires a complex machinery involving the activ-
ation of stress kinases, redox-sensitive transcription factors
such as NF-κappa B, AP-1 and enzymes involved in GSH
synthesis. CAPE has been reported to be a potent inhibitor
of NF-κappa B and has been shown to induce the activities
of glutathione-S-transferase. All these possible mecha-
nisms by which CAPE depletes GSH levels and causes subse-
quent radiosensitization in tumor cells. Furthermore, several

series have reported that the COX-2 inhibitor can enhance tumor cell radiosensitivity.46,47 This is another possible mechanism for CAPE-induced radiosensitivity in tumors, since CAPE is capable of decreasing the expression of COX-2.

CONCLUSION

CAPE, a known antioxidant, induced differential cytotoxicity and apoptosis, GSH and H2O2 depletion, and cell cycle arrest (S/G2) in a human lung adenocarcinoma A549 cell line. CAPE also had a radiosensitization effect on lung cancer cell. Combined with the apparent benign effect on normal cells, CAPE may render it a useful adjuvant to radiotherapy for lung cancer. If CAPE can truly increase the local control of lung cancer by radiotherapy without normal lung toxicity in vivo, it will help us to overcome the problem of poor local control and the high incidence of complications in normal lung. In the future, we will begin a CAPE study in an animal model and further investigate the effects of combined CAPE and radiation on lung cancer control and normal lung protection.

REFERENCES