Cytotoxic Activity from *Curcuma zedoaria* Through Mitochondrial Activation on Ovarian Cancer Cells

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α-Curcumene is one of the physiologically active components of *Curcuma zedoaria*, which is believed to perform anti-tumor activities, the mechanisms of which are poorly understood. In the present study, we investigated the mechanism of the apoptotic effect of α-curcumene on the growth of human ovarian cancer, SiHa cells. Upon treatment with α-curcumene, cell viability of SiHa cells was inhibited > 73% for 48 h incubation. α-Curcumene treatment showed a characteristic nucleosomal DNA fragmentation pattern and the percentage of sub-diploid cells was increased in a concentration-dependent manner, hallmark features of apoptosis. Mitochondrial cytochrome c activation and an *in vitro* caspase-3 activity assay demonstrated that the activation of caspases accompanies the apoptotic effect of α-curcumene, which mediates cell death. These results suggest that the apoptotic effect of α-curcumene on SiHa cells may converge caspase-3 activation through the release of mitochondrial cytochrome c.

**Key words:** Cytotoxic activity, α-Curcumene, Mitochondrial activation, ovarian cancer cells

**INTRODUCTION**

Anti-cancer effect and biological properties of *Curcuma zedoaria* rhizomes have been extensively reported by Lobo et al. (1). In terms of anticancer properties, previous studies showed that polysaccharides and protein-bound polysaccharides of *C. zedoaria* could inhibit the growth of sarcoma-180 (2,3). A few papers showed that the essential oil from *C. zedoaria* had an antiproliferative effect on MCF-7, HL-60 and OVCAR-3 cells (4-7). However, there are very few reports on the active components responsible for the cytotoxic effects, even less on the underlying mechanism of cell death elicited by the active components (8,9). It is highly desirable to have compounds that can cause cancer cell death via apoptosis. Apoptosis eliminates malignant or cancer cells without damaging normal cells and surrounding tissues (10). Apoptosis is characterised by cell morphological changes, chromatin condensation, DNA cleavage, and nuclear fragmentation. There are two main apoptotic pathways—the intrinsic or mitochondrial pathway and the extrinsic pathway which involves ligand binding to a death receptor, where both pathways subsequently cause activation of the caspase cascade which then trigger an ordered series of biochemical events that lead to cell changes (morphology) and death (11-13).

In our previous study, we suggested that the putative component of hexane fraction of *C. zedoaria* showing cytotoxic activity in SiHa cells might be a α-curcumene (14). In our continuing search for anticancer agent, we herein report the apoptotic effect of α-curcumene on ovarian cancer cell, SiHa cells and suggest its mitochondrial cytochrome c activation as its pharmacological mechanism.

**MATERIALS AND METHODS**

*Isolation and identification of α-curcumene.* Powdered Curcuma zedoaria (200 g) was extracted with methanol. The methanol extract (57 g) was then suspended in distilled water and partitioned with hexane. The hexane fraction (25 g) was loaded on a silica gel column and eluted with a hexane-acetone gradient (30 : 1 to 1 : 1) to afford 27 fractions. Fraction S5 (6.3 g) was further separated using a silica gel column chromatography with an elution of a hexane-acetone gradient (50 : 1 to 1 : 1), and 16 fractions were obtained. Fraction S5-5 (1.0 g) was further fractionated with silica gel
column chromatography, α-curcumene (190 mg) was identified by UV, NMR and MS data (15,16).

**MTT assay.** This is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan by mitochondrial enzymes as previously described (17). SiHa cells were seeded at a density of $5 \times 10^4$ cells per well in 24-well plates and incubated for 24 hr. α-Curcumene was dissolved in PBS and added to the culture media at concentrations of 0–400 µM range, and the cells were incubated for 24 hr and 48 hr. 120 µl of stock MTT solution was added into each well under the dark condition, and plates were incubated at 37°C for 4 hr. After centrifugation, 1 ml of the diluted DMSO with ethylalcohol (1 : 1) was added, which was performed to dissolve formazan. After shaking for 10 min at room temperature, 100 µl of each solution was transferred to 96-well plates, and the absorbance value of each well was read at 540 nm using ELISA reader (Model 550 Microplate Reader, Bio-Rad, USA).

**DNA isolation and electrophoresis.** After being treated with or without α-curcumene for 24 hr, the cells were washed twice with ice-cold PBS and lysed with lysis buffer (10 mM Tris-HCl pH 7.4, 2 mM EDTA and 0.5% Triton X-100) at 4°C for 30 min (18). DNA was isolated with phenol-chloroform extraction, and treated with 100 ng/ml RNase A (Sigma). Electrophoresis of the DNA was performed on a 1.5% agarose gel in a TAE buffer, and photographed under UV light after staining the gel with ethidium bromide.

**Quantitative analysis of fragment DNA.** SiHa cells were incubated in growth medium for 4 hr with 1 µCi/ml [³H]-thymidine (Amersham Pharmacia Biotech., UK). Then the cells were washed twice with PBS and lysed with lysis buffer (10 mM Tris-Cl, pH 7.4, 2 mM EDTA and 0.5% Triton X-100) at 4°C for 30 min (18). DNA was isolated with phenol-chloroform extraction, and treated with 100 ng/ml RNase A (Sigma). Electrophoresis of the DNA was performed on a 1.5% agarose gel in a TAE buffer, and photographed under UV light after staining the gel with ethidium bromide.

**Preparation of cytosolic extracts and immunoblotting.** After treatment of α-curcumene for 24 hr, the cells were collected and resuspended in 500 µl of extraction buffer (50 mM Pipes-KOH, 220 mM mannitol, 68 mM sucrose, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors). After 30 min incubation on ice, cells were homogenized using a glass dounce and a tight pestle (50 strokes). Cell homogenates were centrifuged and 10 µl of protein was loaded on 15% SDS-polyacrylamide gels (20). Mitochondrial cytochrome c was detected with anti-cytochrome c monoclonal antibody (PharMingen).

**Caspase-3 assay.** After treatment of α-curcumene for 24 hr, SiHa cells were harvested, washed twice with ice-cold PBS, and resuspended in lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml aprotonin, 20 µg/ml leupeptin). The rest of the protocol followed the manufacturer’s instruction (Bio-Fad Lab., Hercules, CA, USA). The fluorescence was measured in a microplate reader (BIO-TEK Instruments, Winooski, VT, USA) using 360 nm excitation and 530 nm emission. Data were expressed fold-induction of caspase-3 activity compared to that of control cells.

**Immunoblot analysis.** SiHa cells were treated with α-curcumene for 24 hr and lysed with lysis buffer (40 mM Tris-HCl 7.4, 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors). Fifty µg of total protein were electrophoresed using 15% SDS-polyacrylamide gels and used for immunoblot analysis using anti-Bcl-2, anti-actin, anti-caspase-3 polyclonal antibodies and anti-p21 monoclonal antibody (Santa Cruz Inc., Santa Cruz, CA, USA). Monoclonal anti p53 and polyclonal anti-Bax antibodies were purchased from Calbiochem (Cambridge, MA, USA).

**Flowcytometry analysis.** After treatment with α-curcumene for 24 hr, the cells were washed with cold PBS and resuspended in PBS. DNA contents of cells were measured using a DNA staining kit (CyleTest Plus DNA Reagent Kit, Becton Dickinson, Heidelberg, Germany). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit protocol. Data were acquired using CellQuest Software with a FACS calibur (Becton Dickison) flow cytometry system using 20,000 cells per analysis. Cell cycle distributions were calculated using ModFit LT 2.0 software (Verity Software House, Topsham, ME, USA).

**RESULTS**

**Inhibition of cell viability by α-curcumene.** α-Curcumene showed a dose dependent inhibitory effect on SiHa cell proliferation. The cell viability was inhibited $> 73\%$ in SiHa cells exposed to 400 µM of α-curcumene, viability of SiHa cells was inhibited in a concentration dependent manner (Fig. 1). Inhibition percentage of 48 hr incubation is a little bit larger than that of 24 hr incubation at 300 µM of α-curcumene, and those were almost same at 400 µM of α-curcumene.

**Effect of α-curcumene on DNA fragmentation.** The apoptotic response, as judged by the appearance of a DNA ladder, was examined by gel electrophoresis in order to
assess the apoptotic effect of α-curcumene (21). A characteristic nucleosomal DNA fragmentation pattern, which is the biochemical hallmark of apoptosis, was detected 24 hr after exposure to 300 and 400 µM of α-curcumene (Fig. 2A). The percentage of DNA fragmentation showed the same results in the quantitative analysis of fragmented DNA using [3H]-thymidine incorporation test (Fig. 2B). Fragmented DNA was increased significantly by 300 or 400 µM α-curcumene treatment.

**Effect of α-curcumene on sub-diploid cell population.**

α-curcumene-mediated apoptosis induction in SiHa cells was confirmed by the quantitation of apoptotic sub-diploid cells. As shown in Fig. 3, the percentage of sub-diploid cells was increased to 36.2%, and 43.6% by α-curcumene treatment at concentrations of 300, and 400 µM, respectively, in SiHa cells. These results suggest that α-curcumene induces clear apoptosis in SiHa cells in the range of 200–400 µM.

**Cytochrome c release and caspase-3 activation.**

Activation of caspases is regulated by the release of cyto-
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The present study showed that cytochrome c release was markedly induced by treatment with \( \alpha \)-curcumene for 24 hr (Fig. 4A). We confirmed these results using a caspase-3 activity assay. As shown in Fig. 4B, caspase-3 activities were increased 2.9 fold, 3.4 fold, by treatment with \( \alpha \)-curcumene 300, 400 \( \mu \)M, respectively. These results suggest that \( \alpha \)-curcumene induces apoptosis through the release of mitochondrial cytochrome c; a complex form with Apaf-1 subsequently activates caspase-3.

**DISCUSSION**

The present results clearly demonstrate that \( \alpha \)-curcumene induces apoptosis in human ovarian cancer cell, SiHa cells, which demonstrates the previous suggestion that active fraction, H2-3-1 of *C. zedoaria* that showed antiproliferative activity on SiHa cells involved \( \alpha \)-curcumene (14). Our results also suggest that the induction of apoptotic cell death by \( \alpha \)-curcumene occurred via the mitochondrial pathway that activates caspase-3.

Apoptosis is a systematically regulated process that involves the expression of many gene products. Of the major genes that regulate apoptosis, the anti-apoptotic Bcl-2 gene and the pro-apoptotic Bax gene are of particular interest. Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope, and may register damage to these compartments and affect their behavior, perhaps by modifying the flux of small molecules or proteins (24). However, pro-apoptotic Bax protein translocates to mitochondria upon exposure to stimuli of apoptosis (25), and induces the release of cytochrome c and activation of caspase *in vitro* (26). Thus, the pro-survival Bcl-2 subfamily and pro-apoptotic Bax subfamily can oppositely regulate apoptosis through the control of cytochrome c release from mitochondria, resulting in caspase activation (26). Caspases were implicated in apoptosis with the discovery that CED-3, the product of a gene required for cell death in the nematode *C. elegans*, is related to mammalian interleukin 1B-converting enzyme (27,28). Our studies showed that \( \alpha \)-curcumene treatment to SiHa cells caused a concentration-dependent activation of caspase-3, one of the main executers of the apoptotic process (29,30). Our data also showed that activation of caspase-3 is regulated by the release of cytochrome c from mitochondria to the cytosol (Fig. 4). From these results, it is also suggested that released cytochrome c enters the cytosol and might activate caspase-9 after forming a complex with Apaf-1 and a pro-form of caspase-9 (12). Therefore, our data indicate that the pathway for apoptosis by \( \alpha \)-curcumene exists, in part, due to cytochrome c release and caspase activation, resulting in apoptosis.

Taken together, these findings suggest that \( \alpha \)-curcumene exhibits apoptotic effects through a mitochondrial pathway that activates caspase-3. Since the safety of this plant for human consumption has been known for many years, this might be a new type of dietary cancer-chemopreventive plant.

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