

## Curcumin-induced Apoptosis of Human Colon Cancer Colo 205 Cells through the Production of ROS, Ca<sup>2+</sup> and the Activation of Caspase-3

CHIN-CHENG SU<sup>1,6</sup>, JAUNG-GENG LIN<sup>1</sup>, TE-MAO LI<sup>1</sup>, JING-GUNG CHUNG<sup>2,3</sup>,  
JAI-SING YANG<sup>4</sup>, SIU-WAN IP<sup>5</sup>, WEN-CHUAN LIN<sup>4</sup> and GUANG-WEI CHEN<sup>1</sup>

<sup>1</sup>School of Chinese Medicine, Departments of <sup>2</sup>Microbiology, <sup>4</sup>Pharmacology and <sup>5</sup>Nutrition, <sup>3</sup>School of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung City 404, Taiwan;

<sup>6</sup>Division of General Surgery, Department of Surgery, Buddhist Tzu Chi General Hospital, No 707, Sec. 3, Chung Yang Road, Hualien, 970, Taiwan, R.O.C.

**Abstract.** Curcumin (diferuloylmethane), the yellow pigment in turmeric (*Curcuma longa*), is known to inhibit proliferation of cancer cells by arresting them at various phases of the cell cycle and to induce apoptosis in tumor cells. Curcumin-induced apoptosis mainly involves the activation of caspase-3 and mitochondria-mediated pathway in various cancer cells of different tissue origin. In the present study, the induction of apoptosis and cytotoxicity by curcumin in colon cancer colo 205 cells was investigated by using flow cytometry. The results demonstrated that curcumin induced cytotoxicity and apoptosis dose- and time-dependently. Curcumin induced the production of reactive oxygen species (ROS) and Ca<sup>2+</sup>, decreased the levels of mitochondria membrane potential and induced caspase-3 activity. Curcumin also promoted the expression of Bax, cytochrome C, p53 and p21 but inhibited the expression of Bcl-2. These observations suggest that curcumin may have a possible therapeutic potential in colon cancer patients.

Cytostatic and cytotoxic-based therapies rely on the induction of apoptosis in target cells (1). The modulation of the apoptotic response in human prostatic cancer cells has provided new hope for therapeutic strategies for this disease (2, 3).

Colon cancer is a major cause of death in the human population. Based on reports from the "People's Health Bureau of Taiwan", it was demonstrated that about 16

people per 100,000 per year die of colorectal cancer. Currently, therapeutic approaches for human colorectal cancer in Taiwan include radiotherapy, chemotherapy and surgery. However, conventional strategies for the treatment of this cancer are not yet satisfactory. In the past 40 years, 5-fluorouracil has been routinely included in treatment regimens for colorectal cancer (4). So far, new agents acting on novel cancer target are under development.

Many compounds purified from plants, such as taxol (5) and camptothecin (6), have revealed anticancer activity and induce cancer cells to differentiate and undergo apoptosis. Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), the active portion of turmeric (*Curcuma longa* L.), has been shown to have significant antioxidant activity both *in vitro* and *in vivo* (7-9). Curcumin presents anti-inflammatory, anticarcinogenic, antiviral, hypolipidemic and anti-infectious activities (8-11). Many studies have shown that curcumin efficiently induced apoptosis in various cell lines, including HL-60, K562, MCF-7, HeLa (12) and HT29 (13). Curcumin also leads to apoptosis in scleroderma lung fibroblasts (SLF) without affecting normal lung fibroblasts (NLF) (14). Curcumin exhibited anti-oxidant properties and inhibited both JNK activation and mitochondrial release of cytochrome *c* in a concentration-dependent manner in several cancer cell lines (15). Our previous studies have shown that curcumin inhibited N-acetyltransferase activity and gene expression in human colon cancer colo 205 cells (16).

In the present study, the effect of curcumin on the molecular mechanism of growth and apoptosis in colo 205 cells was investigated.

### Materials and Methods

**Chemicals and reagents.** Curcumin, trypan blue, tris-HCl, Triton X-100, propidium iodide (PI), leupeptin and ribonuclease-A were

*Correspondence to:* Guang-Wei Chen, Ph.D., School of Chinese Medicine, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +886 4 2205 1613, Fax: +886 4 2205 2295, e-mail: ckwei@mail.cmu.edu.tw

**Key Words:** Curcumin, apoptosis, ROS, Ca<sup>2+</sup>, caspase-3, colon cancer cells.

obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA, potassium phosphates and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was purchased from Boehringer Mannheim (Mannheim, Germany).

*Human colon cancer cell line (colo 205).* The human colon cancer cell line (colo 205: human colon adenocarcinoma) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75-cm<sup>3</sup> tissue culture flasks and grown at 37°C in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and 1% glutamine in a humidified 5% CO<sub>2</sub> and 95% air atmosphere. All data presented in this report are from at least 3 independent experiments.

*Effects of curcumin on the viability of colo 205 cells as determined by flow cytometry.* The colo 205 cells were cultured in 12-well plates at a density of 5x10<sup>3</sup> cells/well for 24 h. Various concentrations of curcumin were added at final concentrations of 0, 5, 10, 20 and 50 µM, while only DMSO (solvent) were added for the control regimen. The cells were then grown for different periods of time. To determine cell viability, the flow cytometric protocol was used, as described previously (17, 18).

*Comet assay for DNA damage in colo 205 cells after treatment with curcumin.* The Comet assay was used as described by Wang *et al.* (19) with some modifications. Cells were treated with curcumin 50 µM for 12 and 24 h. Conventional microscope slides were covered with an 85 µl solution of 0.5% normal melting point agarose (NMP) and 0.5% low melting point agarose (LMP) in PBS (pH 7.4), and allowed to dry at room temperature. Approximately 10 µl (2.5x10<sup>5</sup> cells/ml) were gently mixed with 75 µl of 0.5% (w/v) of LMP in PBS (pH 7.4) and 75 µl of this suspension were rapidly layered onto the slides which were precoated with a mixture of 0.5% NMP and 0.5% LMP, and covered with a coverslip. The coverslip was removed and cells were immersed in a freshly made lysis solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris and 1% (v/v) Triton X-100 at pH 10) at 4°C for 1 h. The slides were then placed in a double row in a 260 mm-wide horizontal electrophoresis tank containing 0.3 M of NaOH and 1 mM of Na<sub>2</sub>EDTA for 10 min. After electrophoresis (30 V, 300 mA) for 15 min at 4°C, the slides were soaked in a cold neutralizing buffer (400 mM Tris buffer, pH 7.0) at 4°C for 5 min. Slides were dried in methanol for 5 min, and stored in a low humidity environment before staining with 40 µl propidium iodide (2.5 µg/ml) and examined by fluorescence microscopy (19).

*Flow cytometry analysis of apoptosis in colo 205 cells treated with various concentrations of curcumin.* The percentage of cells in G0/G1-, S- and G2/M-phases and apoptosis were determined by flow cytometry, as described previously (17). Briefly, 5x10<sup>5</sup> colo 205 cells/well in 12-well plates were incubated with curcumin at 0, 5, 10, 20 and 50 µM for various time periods and the cells were then harvested by centrifugation. The cells were subsequently washed with PBS and were fixed gently (drop by drop) in 70% ethanol (in PBS), were placed on ice overnight and were then resuspended in PBS containing 40 µg/mL PI, 0.1 mg/mL RNase (Sigma) and 0/1% Triton X-100. After 30 min at 37°C in the dark, the cells were

analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. The cell cycle and apoptosis were determined and analyzed (17).

*Poly (ADP ribose) monoclonal antibody assay.* The colo 205 cells were cultured in 12-well plates at a density of 5x10<sup>5</sup> cells/well for 24 h. Various concentrations of curcumin were added, while only DMSO (solvent) was added for the control regimen. The cells were grown at 37°C in a humidified 5% CO<sub>2</sub> for 12 h for the poly (ADP ribose) monoclonal antibody assay (Alexis, San Diego, CA, USA), and were washed twice with PBS. The supernatant was removed and added to 50 µl 1% FITC-conjugated goat anti-mouse IgG antibody (secondary antibody) for 30 min in the dark, was then washed with PBS and examined under a fluorescence microscope (20).

*Examination of apoptosis by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.* The cells (5x10<sup>4</sup> cells/ml) were treated with or without various concentrations of curcumin at 0, 5, 10, 20 and 50 µM for 48 h and were then isolated for DAPI staining. The cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 µg/ml DAPI for 5 min at 37°C. The cells were then washed with PBS and examined by fluorescence microscopy (Nikon, 200X).

*Flow cytometry analysis of caspase-3 activity in colo 205 cells treated with various concentrations of curcumin.* The caspase-3 activity of the colo 205 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using PhiphiLux green (10 µM) (OncoImmunin, Inc., MD, USA). Approximately 5x10<sup>5</sup> cells/ml were treated with or without various concentrations of curcumin at 0, 5, 10, 20 and 50 µM for 24 h to detect the caspase-3 activity. The cells were harvested and washed twice, re-suspended in 25 µl of PhiphiLux green, incubated at 37°C for 60 min and analyzed by flow cytometry (21).

*Inhibition of curcumin-induced apoptosis by the caspase-3 inhibitor z-DEVD-fmk in colo 205 cells.* In order to examine whether or not caspase-3 activation was involved in the apoptosis triggered by curcumin, the colo 205 cells were pretreated with the caspase-3 inhibitor z-DEVD-fmk 3 h prior to treatment with 20 µM curcumin. Apoptosis and caspase-3 activity were then determined by flow cytometry, as described above.

*Flow cytometry detection of reactive oxygen species (ROS) in colo 205 cells after treatment with curcumin.* The levels of ROS from colo 205 cells were examined and determined by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) for staining. The cells were treated with or without various concentrations (0, 5, 10, 20 and 50 µM) of curcumin for 6 h to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 µl of 2,7-dichlorodihydrofluorescein diacetate solution (10 µM), incubated at 37°C for 30 min and analyzed by flow cytometry (22).

*Flow cytometry detection of Ca<sup>2+</sup> concentrations in colo 205 cells after treatment with curcumin.* The Ca<sup>2+</sup> levels in colo 205 cells were determined by flow cytometry (Becton Dickinson FACS Calibur) using Indo 1/AM (Calbiochem, La Jolla, CA, USA) for staining. The cells were pre-treated with BAPTA (Ca<sup>2+</sup> chelator) and with or without various concentrations (0, 5, 10, 20 and 50 µM) of curcumin for 24 h, harvested and washed twice. One portion was

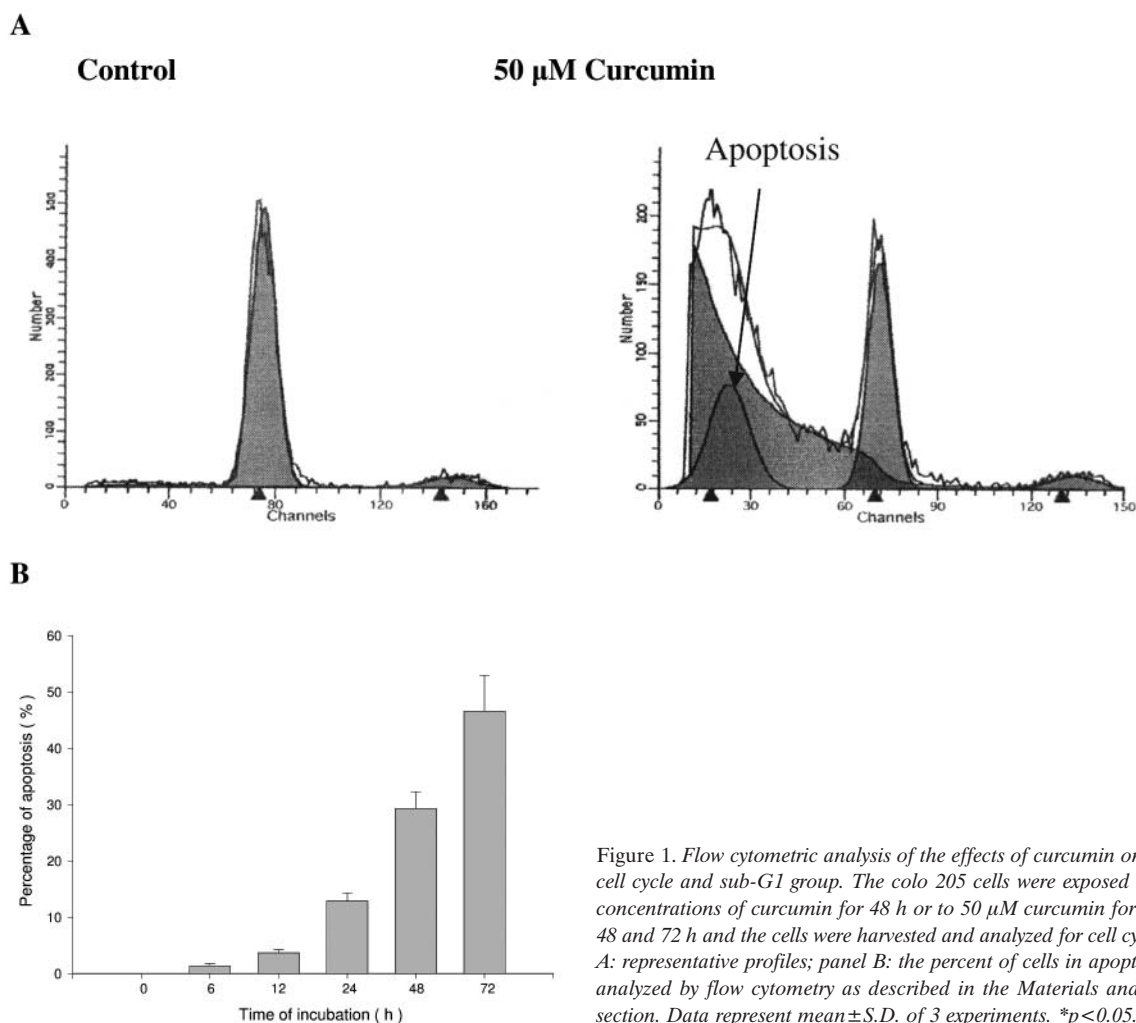


Figure 1. Flow cytometric analysis of the effects of curcumin on colo 205 cell cycle and sub-G1 group. The colo 205 cells were exposed to various concentrations of curcumin for 48 h or to 50  $\mu$ M curcumin for 6, 12, 24, 48 and 72 h and the cells were harvested and analyzed for cell cycle (panel A: representative profiles; panel B: the percent of cells in apoptosis) were analyzed by flow cytometry as described in the Materials and Methods section. Data represent mean  $\pm$  S.D. of 3 experiments. \* $p < 0.05$ .

kept for apoptosis analysis and the other was re-suspended in Indo 1/AM (3  $\mu$ g/ml), incubated at 37°C for 30 min, and the changes of  $\text{Ca}^{2+}$  concentrations were analyzed by flow cytometry (23).

*Flow cytometry detection of mitochondrial membrane potential in colo 205 cells after treatment with curcumin.* The mitochondrial membrane potential levels in colo 205 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using DiOC<sub>6</sub> (4 mol/L) for staining. The cells were treated with or without various concentrations (0, 5, 10, 20 and 50  $\mu$ M) of curcumin for 24 h, harvested and washed twice, re-suspended in 500  $\mu$ l of DiOC<sub>6</sub> (4 mol/L) and incubated at 37°C for 30 min. The changes of mitochondrial membrane potential were analyzed by flow cytometry (24).

*Western blotting for the examination of the effect of curcumin on p53, Bcl-2, Bax, Chk2 and cytochrome c levels in colo 205 cells.* Total proteins were collected from colo 205 cells after treatment with or without 20  $\mu$ M of curcumin for 6, 12 and 24 h before the p53, p21, Bcl-2, Bax, Chk2 and cytochrome c levels were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (22, 24)

*Statistical analysis.* The Student's *t*-test was used to analyze the statistical analysis between the curcumin-treated and control groups.

## Results

*Induction of apoptosis by curcumin.* Cell cycle and apoptosis were detected by PI-staining and the Annexin V method after various time periods of continuous exposure to curcumin before flow cytometry analysis. As shown in Figure 1A and B, curcumin induced apoptosis (sub-G1 group) in a time-dependent manner.

*Induction of DNA damage by curcumin as examined by the Comet assay.* The occurrence of DNA damage in colo 205 cells was detected by the Comet assay after 24 h of continuous exposure to curcumin. As shown in Figure 2, curcumin induced DNA damage in a concentration-dependent manner.

*Induction of apoptosis by curcumin in colo 205 cells as determined by poly (ADP-ribose) fluorescence staining.* Apoptosis and DNA damage in cells may produce poly (ADP-ribose) polymerase (PARP) for repair purposes. PARP-positive fluorescence staining at 5-50  $\mu\text{M}$  of curcumin is shown in Figure 3.

*Induction of apoptosis by curcumin as examined by DAPI staining.* Apoptosis was detected by the DAPI staining method after 48 h of continuous exposure to curcumin followed by fluorescence microscopy. As shown in Figure 4, curcumin induced apoptosis in a concentration-dependent manner.

*Inhibition of curcumin-induced caspase-3 activity and apoptosis by the caspase inhibitor z-VAD-fmk.* The purpose of these studies was to examine whether or not caspase-3 activation is involved in the apoptosis of cells triggered by curcumin. The results indicated that curcumin increased caspase-3 activity and apoptosis (Figure 5A and B). The cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (z-VAD-fmk), 3 h prior to the treatment with curcumin. z-VAD-fmk decreased caspase-3 activity and apoptosis. After treatment with curcumin and z-VAD-fmk, inhibition of curcumin-mediated caspase-3 activation was accompanied by the marked attenuation of curcumin-induced apoptotic cell death (Figure 5B).

*Effects of curcumin on the production of reactive oxygen species (ROS).* The levels of ROS were significantly different between the curcumin-treated group and the control group. Increasing the time of incubation and dose of curcumin led to an increase in the levels of ROS in the examined colo 205 cells (Figure 6A and B). The effects of curcumin on the levels of ROS were dose- and time-dependent.

*Effects of curcumin on the production of  $\text{Ca}^{2+}$ .* The  $\text{Ca}^{2+}$  concentrations were significantly different between the curcumin-treated group and the control group. Increasing the time of incubation and dose of curcumin led to an increase in the concentrations of  $\text{Ca}^{2+}$  in the examined colo 205 cells (Figure 7A and B). The effects of curcumin on the levels of  $\text{Ca}^{2+}$  were dose- and time-dependent.

*Effects of curcumin on the mitochondrial membrane potential (MMP).* The levels of mitochondria membrane potential (MMP) were significantly different between the curcumin-treated and the control group. Increasing the time of incubation and dose of curcumin led to a decrease in the MMP in the examined colo 205 cells (Figure 8A and B). The effects of curcumin on the levels of MMP were dose- and time-dependent.

*Inhibition of curcumin-induced  $\text{Ca}^{2+}$  concentrations and apoptosis by the  $\text{Ca}^{2+}$  chelator (BAPTA).* The aim of these experiments was to examine whether or not  $\text{Ca}^{2+}$  was involved in curcumin-triggered apoptosis of colo 205 cells. The chelator BAPTA was used to capture  $\text{Ca}^{2+}$  and then apoptosis was examined. The data indicated that BAPTA abolished  $\text{Ca}^{2+}$  thereby decreasing the percentage of apoptosis in these cells (Figure 9A and B).

*Western blotting investigation of the effect of curcumin on p53, p21, Bcl-2, Chk2, Bax and cytochrome c levels.* The results indicated that curcumin decreased the expressions of p53 (Figure 10A), p21 (Figure 10B) and Bcl-2 (Figure 10F), but increased the expressions of Chk2 (Figure 10C), Bax (Figure 10D) and the release of cytochrome c (Figure 10E).

## Discussion

Many experiments have demonstrated that curcumin can induce apoptosis in cancer cells and that these pathways depend on mitochondria or caspase-3 activation. Those effects also involve ROS production in some cases. The action of curcumin may, thus, depend on the cell type. In the present study, the effects of curcumin on the colon cancer colo 205 cells were examined for a better understanding of the molecular mechanism of apoptosis induced by curcumin. The curcumin induced apoptosis was shown by the results of flow cytometric analysis, Comet assay, poly (ADP-ribose) fluorescence staining and DAPI staining. Our data also indicated that curcumin induced apoptosis in human colo 205 cells through the mitochondrial pathway (Chk2 and Bax up-regulations, p53 and p21 down-expression, Bcl-2 down regulation), decreased the mitochondrial membrane potential, increased cytochrome c release and caspase-3 activation, as determined by Western blotting.

In addition, curcumin was found to induce ROS production earlier than 15 min after curcumin treatment for up to 2 h, when the ROS production started to decline. After 6 h of curcumin treatment, the ROS levels were back to normal, indicating the anti-oxidant function of curcumin. These observations are in agreement with the report that curcumin-treated cells led to earlier ROS production (25). Other investigators have demonstrated that curcumin induced ROS production shortly after treatment and the reduction in membrane mobility induced by curcumin was attributed to ROS production (26). However, other studies also showed that curcumin inhibited ROS production *in vitro*, demonstrating that curcumin significantly attenuates methylglyoxal-induced ROS formation and suggesting that ROS triggers cytochrome c release, caspase activation and subsequent apoptotic biochemical changes (27). Although we added anti-oxidant (hSOD) that led to the decrease in the levels of curcumin cytotoxicity, the amount of ROS was still



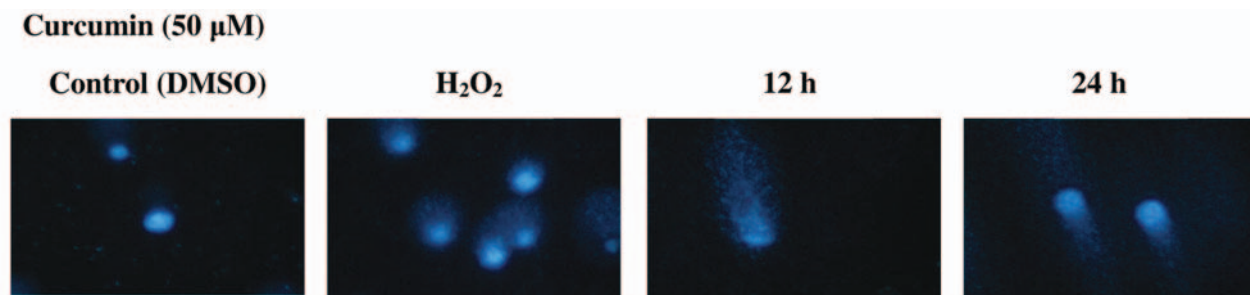


Figure 2. DNA damage determination after curcumin treatment. The colo 205 cells were incubated with 50  $\mu\text{M}$  curcumin for 12 and 24 h and DNA damage was determined by the Comet Assay as described in the Materials and Methods section.

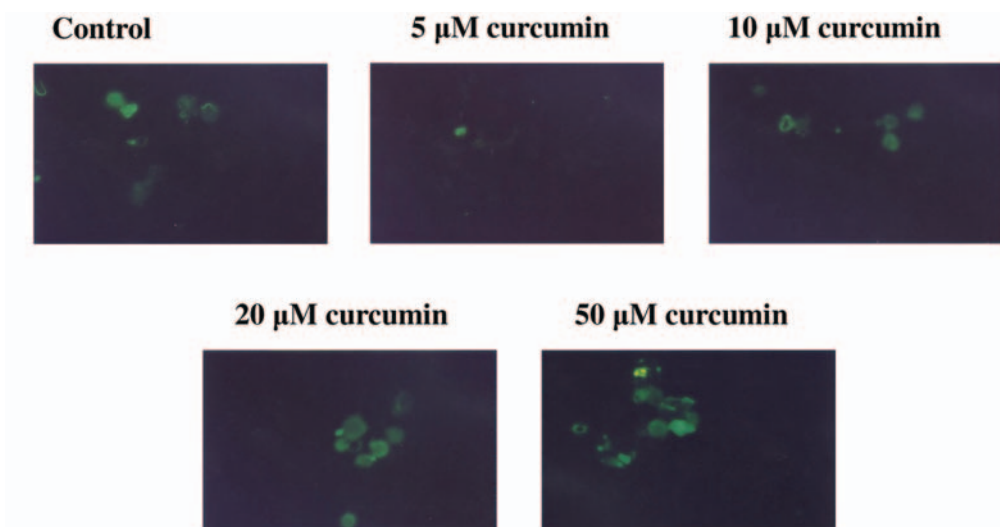


Figure 3. Curcumin induced DNA damage in colo 205 cells, which might produce poly (ADP-ribose) polymerase (PARP) for repair. The colo 205 cells were cultured in 12-well plates at a density of  $1 \times 10^5$  cells/well with or without curcumin treatment for 24 h. The cells were then fixed, examined with PARP monoclonal antibody assay and were viewed by fluorescence microscopy. The fluorescence is shown in the treatment for 5-50  $\mu\text{M}$  of curcumin.

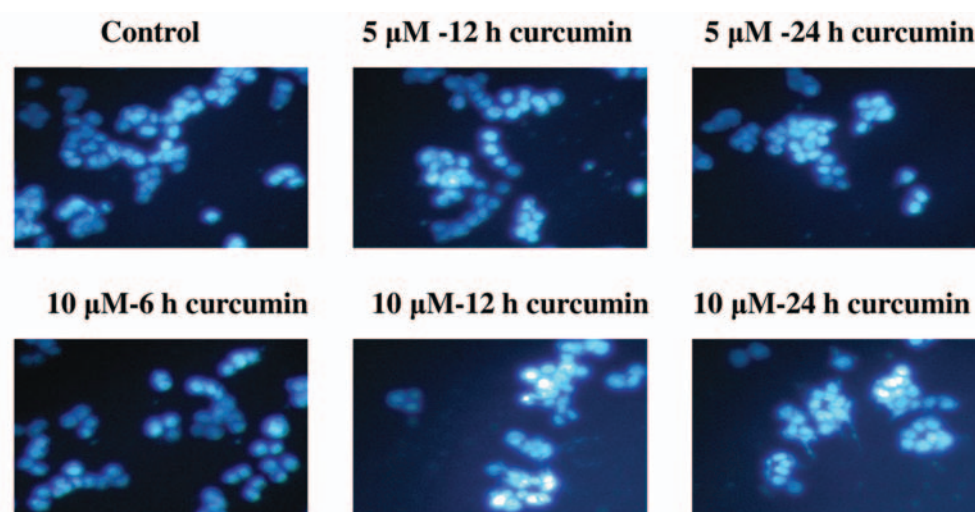


Figure 4. DAPI staining analysis for the effects of curcumin on apoptosis. The colo 205 cells were incubated with various concentrations of curcumin for 6, 12, 24 and 48 h and apoptosis was determined by DAPI staining as described in the Materials and Methods section.

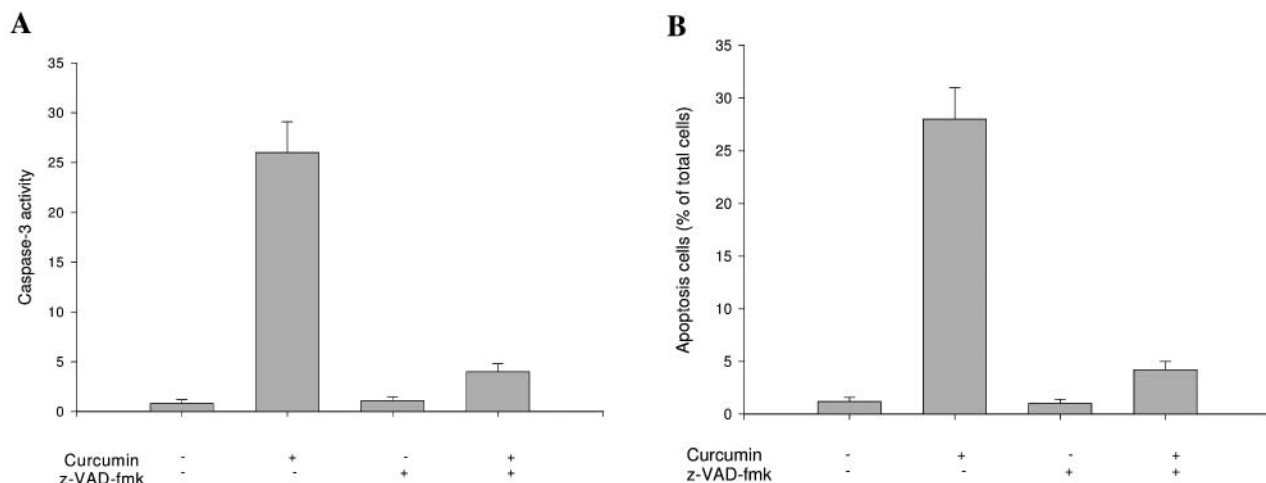


Figure 5. Flow cytometric analysis of the effects of curcumin on caspase-3 activity and apoptosis. The colo 205 cells were incubated with 20  $\mu$ M curcumin and with or without z-VAD-fmk for caspase-3 activity (panel A) and apoptosis determination (panel B), as described in the Materials and Methods section. Data represents mean  $\pm$  S.D. of 3 experiments. \* $p < 0.05$ .

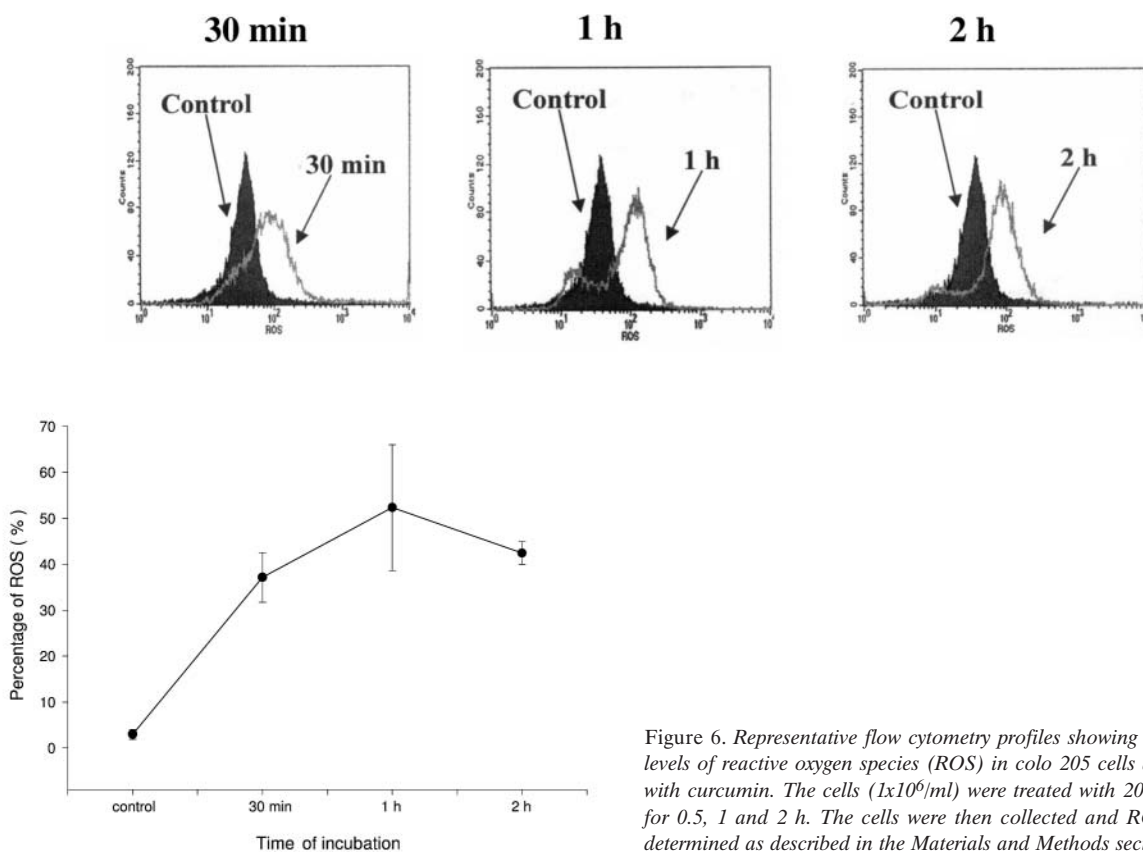


Figure 6. Representative flow cytometry profiles showing changes in the levels of reactive oxygen species (ROS) in colo 205 cells after treatment with curcumin. The cells ( $1 \times 10^6$ /ml) were treated with 20  $\mu$ M curcumin for 0.5, 1 and 2 h. The cells were then collected and ROS levels were determined as described in the Materials and Methods section.

slightly higher than in the control (data not shown). The hSOD did not completely block ROS production. Therefore, other pathways or modes of action may be involved the cascade of events leading to apoptosis, including histone

acetylation,  $Ca^{2+}$  production and a plausible widespread thiol oxidizing action. However, the data from the present study suggested the involvement of ROS in the induction of cell death of colo 205 cells after curcumin treatment.

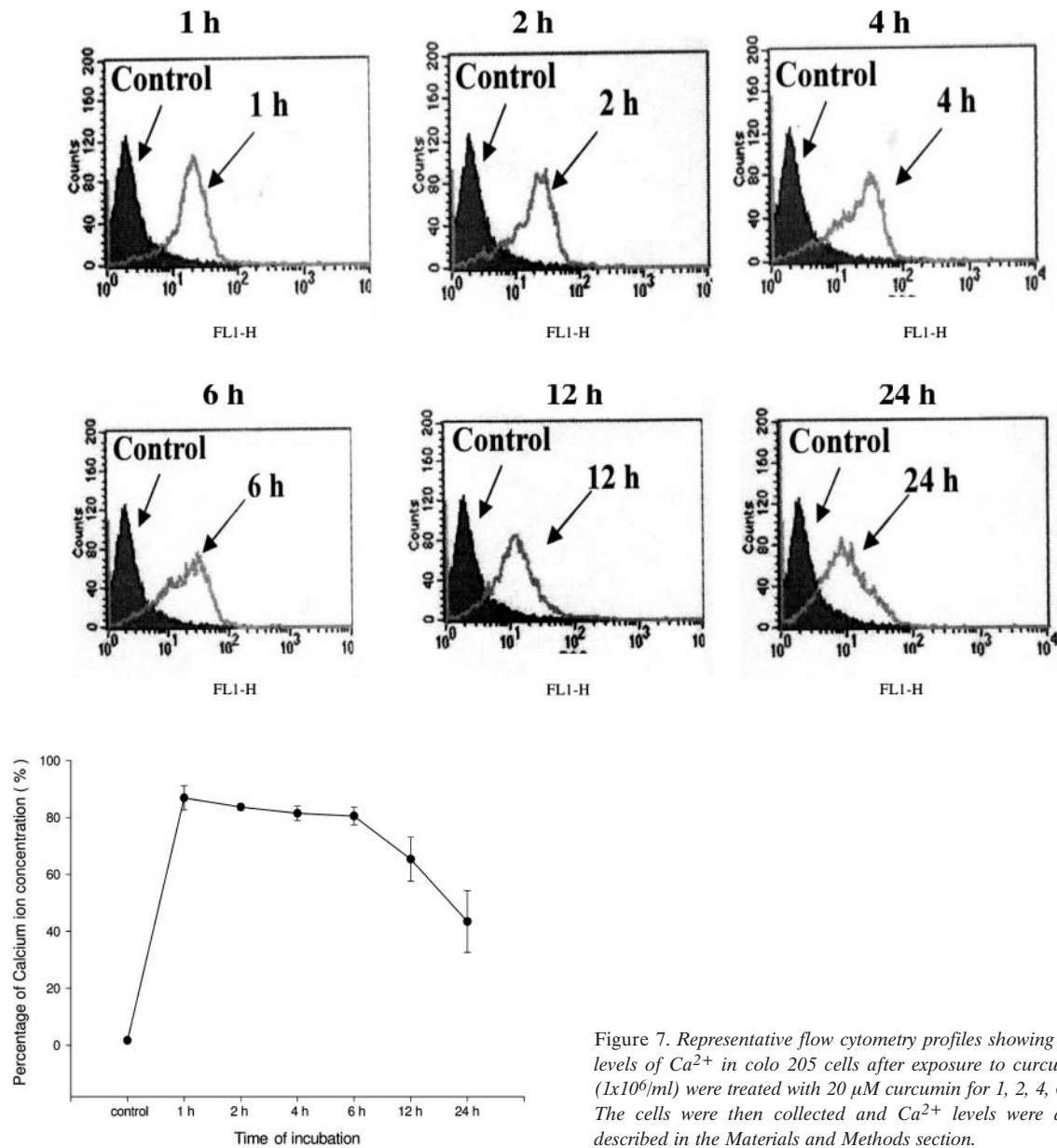


Figure 7. Representative flow cytometry profiles showing changes in the levels of  $\text{Ca}^{2+}$  in colo 205 cells after exposure to curcumin. The cells ( $1 \times 10^6/\text{ml}$ ) were treated with  $20 \mu\text{M}$  curcumin for 1, 2, 4, 6, 12 and 24 h. The cells were then collected and  $\text{Ca}^{2+}$  levels were determined, as described in the Materials and Methods section.

We also investigated Chk2 expression by Western blotting. Our results demonstrated that curcumin increased the levels of Chk2, Bax, and cytochrome *c* release, but inhibited the expressions of p53, p21 and Bcl-2 in a dose-dependent manner. Therefore, the present results demonstrated that curcumin induced ROS production, leading to an increase in Chk phosphorylation. Our data also indicated that curcumin promoted the levels of  $\text{Ca}^{2+}$  in the examined cells and that these effects were dose- and time-dependent. Moreover, curcumin induced  $\text{Ca}^{2+}$  production and the capture of  $\text{Ca}^{2+}$  by the chelator

(BAPTA) leading to a decrease in the levels of  $\text{Ca}^{2+}$  production and MMP in the examined cells. Apparently,  $\text{Ca}^{2+}$  plays an important role in curcumin-induced apoptosis. Disruption of the cellular  $\text{Ca}^{2+}$  homeostasis is a critical event in apoptosis (28, 29).

Our Western blot data indicated that curcumin decreased the expression of Bcl-2 in colo 205 cells. This is in agreement with other reports which have demonstrated that the overexpression of Bcl-2 results in reduced levels of  $\text{Ca}^{2+}$  production on endoplasmic reticulum (ER),  $[\text{Ca}^{2+}]_{\text{ER}}$ , whereas the down-regulation of Bcl-2 led to the increase of

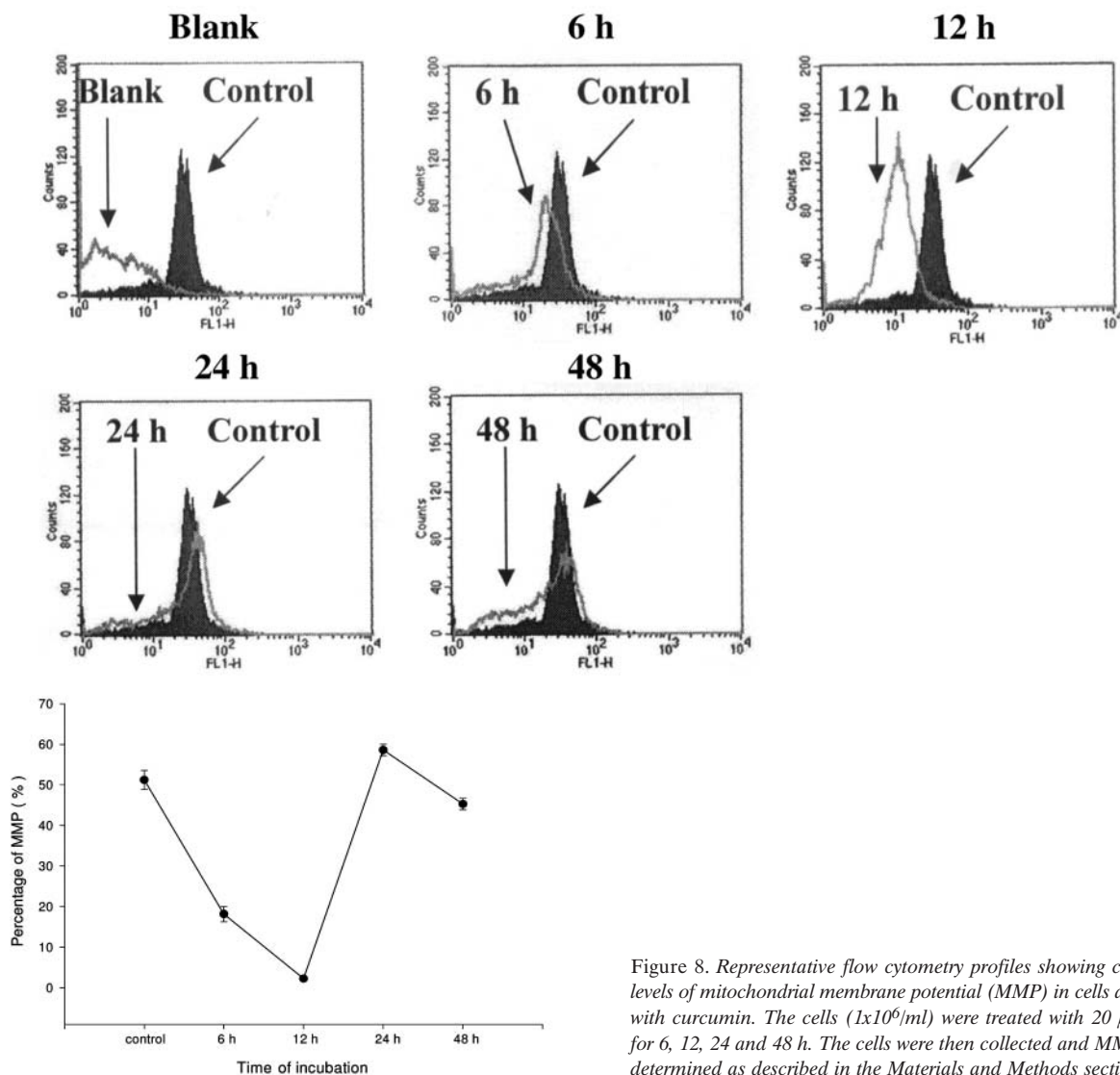


Figure 8. Representative flow cytometry profiles showing changes in the levels of mitochondrial membrane potential (MMP) in cells after treatment with curcumin. The cells ( $1 \times 10^6/ml$ ) were treated with  $20 \mu M$  curcumin for 6, 12, 24 and 48 h. The cells were then collected and MMP levels were determined as described in the Materials and Methods section.

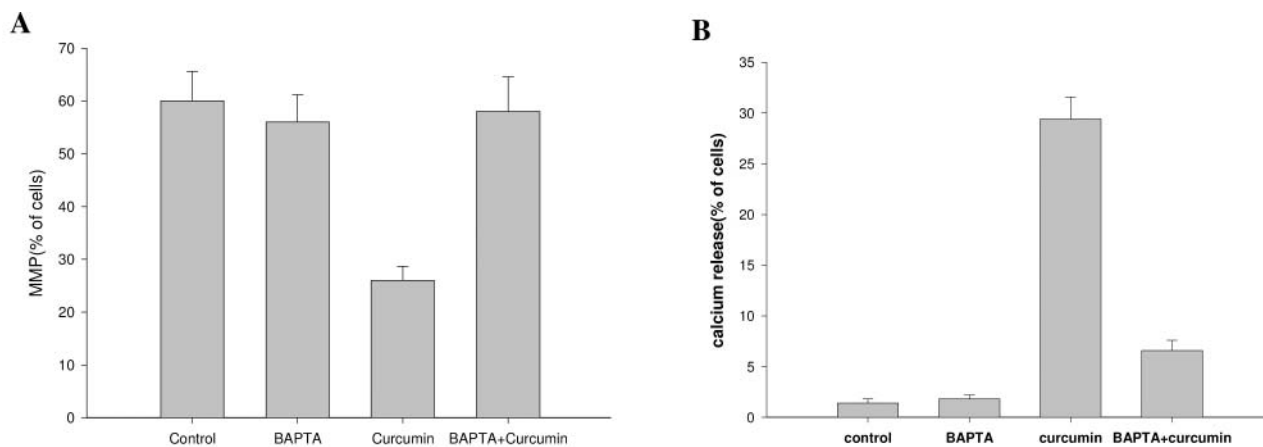


Figure 9. Flow cytometric analysis of the effects of curcumin on  $Ca^{2+}$  concentration and apoptosis. The cells were incubated with  $20 \mu M$  curcumin and/or with or without BAPTA treatment for  $Ca^{2+}$  concentration (panel A) and apoptosis determination (panel B) as described in the Materials and Methods section. Data represent mean  $\pm$  S.D. of 3 experiments. \* $p < 0.05$ .



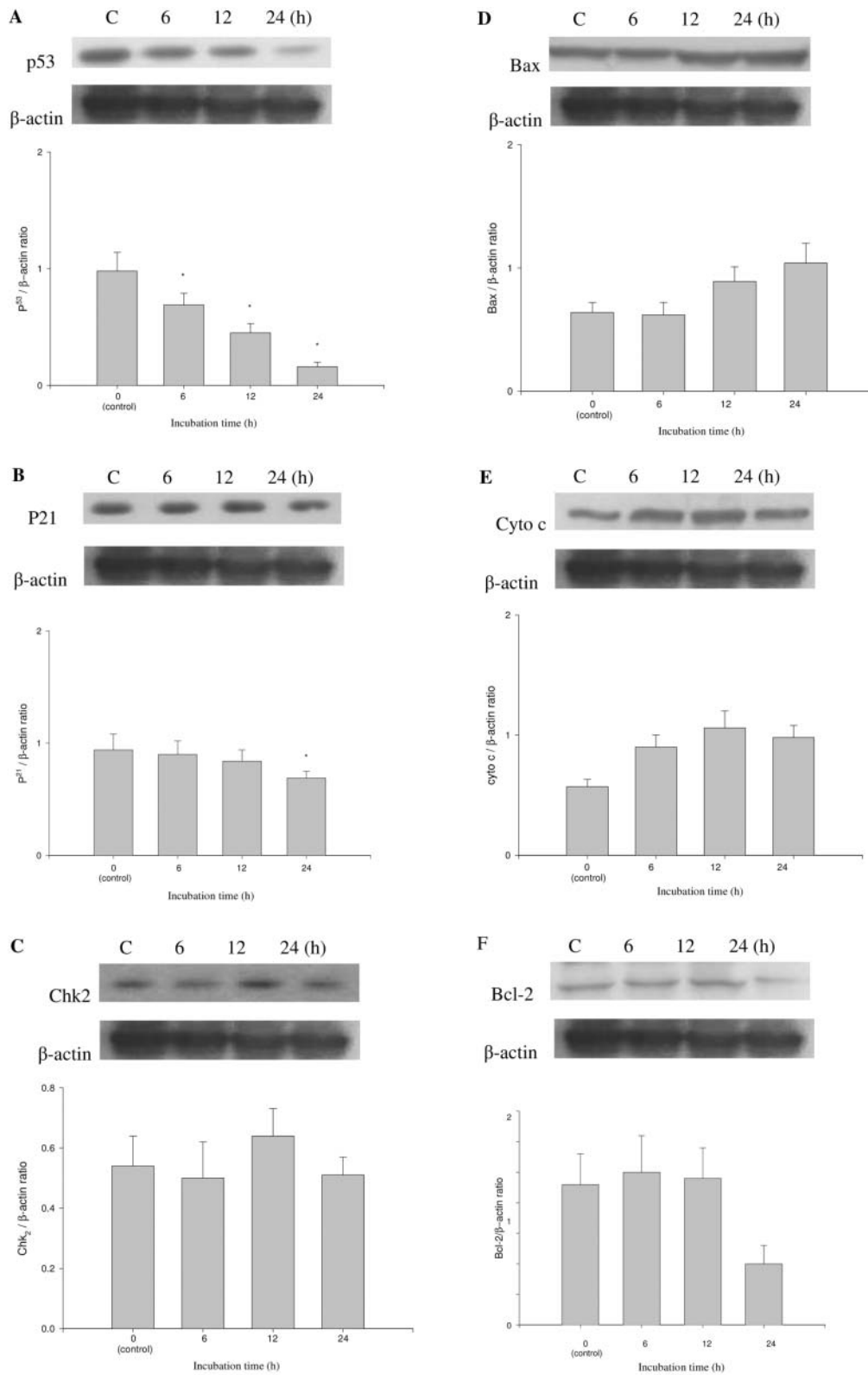


Figure 10. Representative Western blot showing changes in the levels of p53, p21, Chk2, Bax, cytochrome c and Bcl-2 in colo 205 cells after treatment with curcumin. The colo 205 cells ( $5 \times 10^6/ml$ ) were treated with section  $20 \mu M$  curcumin for 6, 12 and 24 h. Then cytosolic fraction and total protein were prepared and determined as described in the Materials and Methods. The evaluation of the levels of p53 (panel A), p21 (panel B), Chk2 (panel C), Bax (panel D), cytochrome c (panel E) and Bcl-2 (panel F) expressions were estimated by Western blotting as described in the Materials and Methods section.

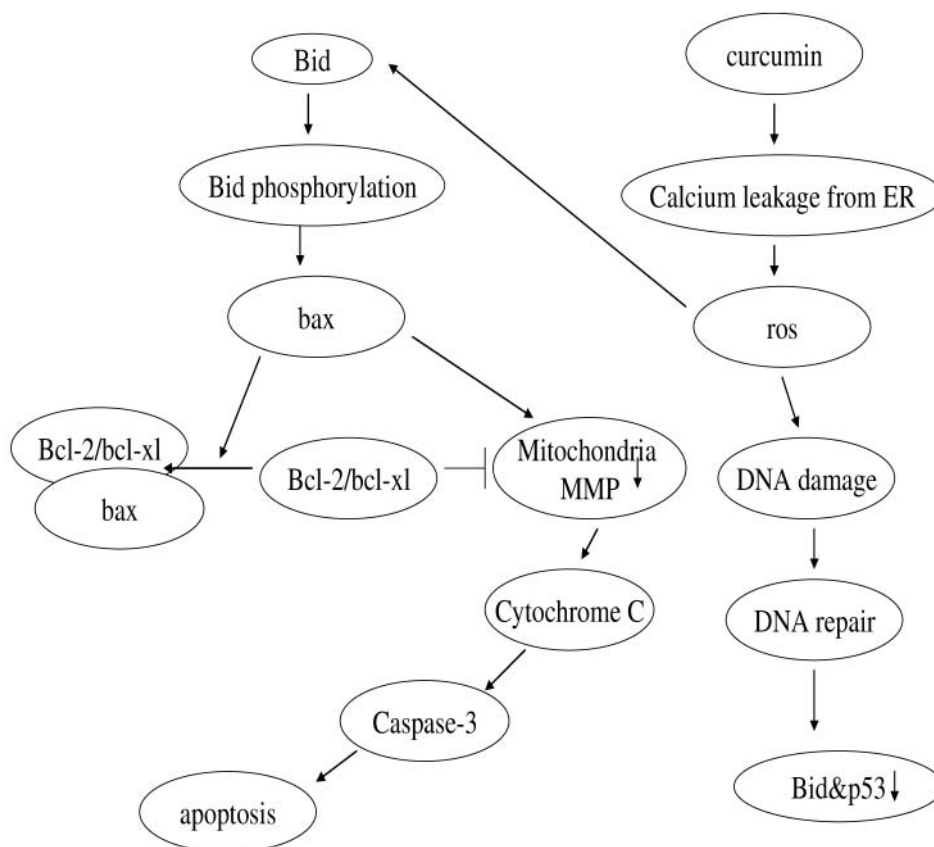


Figure 11. The proposed signal pathways of curcumin-induced apoptosis in human colon cancer colo 205 cells.

[Ca<sup>2+</sup>]<sub>ER</sub> (29-31). It has been well documented that massive depletion of the ER Ca<sup>2+</sup> store consists an ER stress condition that initiates apoptosis (32). Our data is in agreement with this concept since BAPTA (Ca<sup>2+</sup> chelator) depleted the concentration of Ca<sup>2+</sup>, leading to a blockage of the caspase-3-dependent apoptosis pathway in the curcumin-treated cells. Thus, depletion of ER calcium *per se* promotes activation of an apoptotic cascade. We also used catalase and N-acetylcysteine treatment to prevent the accumulation of ROS, but these radical scavengers did not block the elevation of intracellular Ca<sup>2+</sup> (data not shown). Therefore, the pathways for curcumin-induced apoptosis are mitochondria-dependent since curcumin decreased the MMP, led to cytochrome *c* release and caused the activation of caspase-3 (Figure 11).

In conclusion, the increase of [Ca<sup>2+</sup>] is a key mediator in curcumin-induced apoptosis in colo 205 cells, because: (i) after curcumin exposure of colo 205 cells, there were rapid and sustained increases in [Ca<sup>2+</sup>]; (ii) the increase of [Ca<sup>2+</sup>] precedes ROS production and caspase-3 activation; (iii) an intracellular Ca<sup>2+</sup> chelator (BAPTA), abolished curcumin-

induced [Ca<sup>2+</sup>] elevation and increased the MMP levels leading to the decrease of cytochrome *c* release, which further prevented caspase-3 activation and apoptosis.

### Acknowledgements

This study was supported by grant DMR-94-025 from the Research Section of China Medical University Hospital, Taichung City, Taiwan, R.O.C.

### References

- 1 Lotan R: Retinoids and apoptosis: implications for cancer chemoprevention and therapy. *JNCI* 87: 1655-1657, 1995.
- 2 Martikainen P, Kyprianou N, Tucker RW and Isaacs JT: Programmed death of nonproliferating and androgen-independent prostatic cancer cells. *Cancer Res* 51: 4693-4700, 1991.
- 3 Kyprianou N, Bains A and Jacobs S: Induction of apoptosis in androgen-independent human prostatic cancer cells undergoing thymineless death. *Prostate* 25: 66-75, 1994.
- 4 Christopoulou A: Chemotherapy in metastatic colorectal cancer. *Tech Coloproctol* 8(Suppl 1): 43-46, 2004.

- 5 Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME and Huang Y: Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia* 7: 563-568, 1993.
- 6 Kaufman SH: Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res* 49: 5870-5878, 1989.
- 7 Sharma OP: Antioxidant activity of curcumin and related compounds. *Biochem Pharmacol* 25: 1811-1812, 1976.
- 8 Joe B, Vijaykumar M and Lokesh BR: Biological properties of curcumin-cellular and molecular mechanisms of action. *Crit Rev Food Sci Nutr* 44: 97-111, 2004.
- 9 Krishnaswamy K, Goud VK, Sesikera B, Mukundan MA and Krishna TP: Retardation of experimental tumorigenesis and reduction in DNA adducts by turmeric and curcumin. *Nutr Cancer* 30: 163-166, 1998.
- 10 Aggarwal BB, Kumar A and Bharti AC: Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* 23: 363-398, 2003.
- 11 Araujo CC and Leon LL: Biological activities of *Curcuma longa* L. *Mem Inst Oswaldo Cruz* 96: 723-728, 2001.
- 12 Roy M, Chakraborty S, Siddiqi M and Bhattacharya RK: Induction of apoptosis in tumor cells by natural phenolic compounds. *Asian Pac J Cancer Prev* 3: 61-67, 2002.
- 13 Goel A, Boland CR and Chauhan DP: Specific inhibition of cyclooxygenase-2 (COX-2) expression by dietary curcumin in HT-29 human colon cancer cells. *Cancer Lett* 172: 111-118, 2001.
- 14 Tourkina E, Gooz P, Oates JC, Ludwicka-Bradley A, Silver RM and Hoffman S: Curcumin-induced apoptosis in scleroderma lung fibroblasts: role of protein kinase cepsilon. *Am J Respir Cell Mol Biol* 31: 28-35, 2004.
- 15 Duvoix A, Blasius R, Delhalle S, Schneckeburger M, Morceau F, Henry E, Dicato M and Diederich M: Chemopreventive and therapeutic effects of curcumin. *Cancer Lett* 232: 181-190, 2005.
- 16 Chen JC, Hwang JM, Chen GW, Tsou MF, Hsia TC and Chung JG: Curcumin decreases the DNA adduct formation, arylamines N-acetyltransferase activity and gene expression in human colon tumor cells (colo 205). *In Vivo* 17: 301-309, 2003.
- 17 Lu H F, Sue CC, Yu CS, Chen SC, Chen GW and Chung JG: Diallyl disulfide (DADS) induced apoptosis undergo caspase-3 activity in human bladder cancer T24 cells. *Food Chem Toxicol* 42: 1543-1552, 2004.
- 18 Chung JG, Yeh KT, Wu SL, Hsu NY, Chen GW, Yeh YW and Ho HC: Novel transmembrane GTPase of non-small cell lung cancer identified by mRNA differential display. *Cancer Res* 61: 8873-8879, 2001.
- 19 Wang SC, Chung JG, Chen CH and Chen SC: 2- and 4-Aminobiphenyls induce oxidative DNA damage in human hepatoma (Hep G2) cells *via* different mechanisms. *Mutat Res* 593: 9-21, 2006.
- 20 Li YC, Tyan YS, Kuo HM, Chang WC, Hsia TC and Chung JG: Baicalein induced *in vitro* apoptosis undergo caspases activity in human promyelocytic leukemia HL-60 cells. *Food Chem Toxicol* 42: 37-43, 2004.
- 21 Loop T, Dovi-Akue D, Frick M, Roesslein M, Egger L, Humar M, Hoetzel A, Schmidt R, Borner C, Pahl HL, Geiger K and Pannen BH: Volatile anesthetics induce caspase-dependent, mitochondria-mediated apoptosis in human T lymphocytes *in vitro*. *Anesthesiology* 102: 1147-1157, 2005.
- 22 Lu HF, Sue CC, Yu CS, Chen SC, Chen GW and Chung JG: Diallyl disulfide (DADS) induced apoptosis undergo caspase-3 activity in human bladder cancer T24 cells. *Food Chem Toxicol* 42: 1543-1552, 2004.
- 23 Park EK, Kwon KB, Park KI, Park BH and Jhee EC: Role of Ca<sup>2+</sup> in diallyl disulfide-induced apoptotic cell death of HCT-15 cells. *Experim Mol Medicine* 34: 250-257, 2002.
- 24 Lin JG, Chen GW, Li TM, Chou ST, Tan TW and Chung JG: Aloe-emodin induce apoptosis in human bladder cancer cells (T24) through p53-dependent apoptotic pathway. *J Urology* 175: 343-347, 2005.
- 25 Chattopadhyay I, Bandyopadhyay U, Biswas K, Maity P and Banerjee RK: Indomethacin inactivates gastric peroxidase to induce reactive-oxygen-mediated gastric mucosal injury and curcumin protects it by preventing peroxidase inactivation and scavenging reactive oxygen. *Free Radic Biol Med* 40: 1397-1408, 2006.
- 26 Atsumi T, Fujisawa S and Tonosaki K: Relationship between intracellular ROS production and membrane mobility in curcumin- and tetrahydrocurcumin-treated human gingival fibroblasts and human submandibular gland carcinoma cells. *Oral Dis* 11: 236-242, 2005.
- 27 Chan WH, Wu HJ and Hsuw YD: Curcumin inhibits ROS formation and apoptosis in methylglyoxal-treated human hepatoma G2 cells. *Ann NY Acad Sci* 1042: 372-378, 2005.
- 28 McConkey DJ and Orrenius S: The role of calcium in the regulation of apoptosis. *J Leukoc Biol* 59: 775-783, 1996.
- 29 Pinton P, Ferrari D, Rapizzi E, Di Virgilio FD, Pozzan T and Rizzuto R: The Ca<sup>2+</sup> concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. *EMBO J* 20: 2690-2701, 2001.
- 30 Vanden Abeele F, Skryma R, Shuba Y, Van Coppenolle F, Slomianny C, Roudbaraki M, Mauroy B, Wuytack F and Prevarskaya N: Bcl-2-dependent modulation of Ca<sup>2+</sup> homeostasis and store-operated channels in prostate cancer cells. *Cancer Cell* 1: 169-179, 2000.
- 31 Takadera T and Ohyashiki T: Apoptotic cell death and caspase 3 (CPP32) activation induced by calcium ionophore at low concentrations and their prevention by nerve growth factor in PC12 cells. *Eur J Biochem* 249: 8-12, 1997.
- 32 Shen HM, Dong SY and Ong CN: Critical role of calcium overloading in cadmium-induced apoptosis in mouse thymocytes. *Toxicol Appl Pharmacol* 171: 12-19, 2001.

Received December 27, 2005

Revised July 31, 2006

Accepted September 4, 2006