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Curcumin induces apoptosis through the mitochondria-mediated apoptotic pathway in HT-29 cells*

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Abstract: Objective: To investigate the effects of curcumin on release of cytochrome *c* and expressions of Bcl-2, Bax, Bad, Bcl-xL, caspase-3, poly ADP-ribose polymerase (PARP), and survivin of HT-29 cells. Methods: HT-29 cells were treated with curcumin (0~80 μmol/L) for 24 h. The release of cytochrome *c* from the mitochondria and the apoptosis-related proteins Bax, Bcl-2, Bcl-xL, Bad, caspase-3, PARP, and survivin were determined by Western blot analysis and their mRNA expressions by reverse transcriptase-polymerase chain reaction (RT-PCR). Results: Curcumin significantly induced the growth inhibition and apoptosis of HT-29 cells. A decrease in expressions of Bcl-2, Bcl-xL and survivin was observed after exposure to 10~80 μmol/L curcumin, while the levels of Bax and Bad increased in the curcumin-treated cells. Curcumin also induced the release of cytochrome *c*, the activation of caspase-3, and the cleavage of PARP in a dose-dependent manner. Conclusion: These data suggest that curcumin induced the HT-29 cell apoptosis possibly via the mitochondria-mediated pathway.

Key words: Curcumin, Apoptosis, Mitochondrial pathway, HT-29 cells
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INTRODUCTION

Colorectal cancer is one of the leading causes of death and is a major public health problem in western countries. The incidence of colorectal cancer is also increasing among Chinese populations. Chemoprevention is a promising strategy because other therapies are not effective in controlling gastrointestinal cancers. Recently, much attention has been focused on identifying naturally occurring chemopreventive compounds that can inhibit, retard, or reverse the process of multistage carcinogenesis with minimal toxicity. Between 1981 and 2002, almost 74% of all drugs approved for cancer were either natural products or based on natural products (Newman *et al*., 2003).

Curcumin is an important polyphenol extracted from the rhizomes of *Curcuma longa* L. Several studies have shown that curcumin exerts antioxidant, anti-inflammatory, anti-carcinogenic and chemopreventive activities on many tumor cells (Shishodia *et al*., 2005b). Curcumin has been used for a long time as a naturally occurring medicine in treatment of many diseases (Sharma *et al*., 2005). Several studies have shown that curcumin possesses anti-proliferative and anti-carcinogenic properties in a wide variety of cell lines and animals (Gao *et al*., 2004; Aggarwal *et al*., 2006). Curcumin is described to efficiently induce apoptosis in various cell lines, including NIH 3T3, HL-60, K562, H520, and HeLa (Bhaumik *et al*., 2000; Sen *et al*., 2005). Apoptosis is a regulated process involving activation of various molecules and initiation of cell death. Induction of apoptosis in tumor cells is a potentially promising approach for cancer therapy (Green, 2000). The signaling pathway of apoptosis can proceed via the activation of caspases and the recruitment of proteins in Bcl-2 family (Cory and Adams, 2002). Some evidence indicates that curcumin can cause proteolytic cleavage of

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poly-ADP-ribose polymerase, activate caspase-3, reduce expression of Bcl-xL, and up-regulate pro-apoptotic members, Bax and Bad, in non-small cell lung cancer (NSCLC) cells (Sen *et al*., 2005; Shishodia *et al*., 2005a). Curcumin also suppresses activation of nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), and signal transducer and activator of transcription 3 (STAT3) (Aggarwal *et al*., 2006). However, because the anti- and pro-apoptotic signaling pathways are variable in different types of cells, the exact mechanism of curcumin inducing apoptosis of HT-29 cells is still unknown.

In the present study, we investigated the mechanisms underlying the cytotoxic effect of curcumin on colorectal cancer cell line (HT-29), assessing its influence on the balance between pro-apoptotic pathways, such as apoptotic cascade through caspases (caspases-3, PARP) and some Bcl-2 family members (Bad and Bax), and anti-apoptotic pathways, namely, Bcl-2, Bcl-xL, and survivin in HT-29 cells.

MATERIALS AND METHODS

Reagents

RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were purchased from GIBCO (Gaithersburg, USA). Curcumin, sodium dodecylsulfate (SDS), ponceaus, dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF), bovine serum albumin (BSA), Hoechst H33258, and JC-1 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-Bcl-2, anti-Bax, anti-Bad, anti-cytochrome *c*, anti-Bcl-xL, anti-pro-caspase-3, anti-PARP (poly ADP-ribose polymerase) antibodies, and horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Epitomics (Burlingame, USA). Anti-survivin was obtained from Cell Signaling Technology (Boston, USA). Nitrocellulose membrane and the enhanced chemiluminescence (ECL) detection system were purchased from Amersham (Pittsburgh, USA). Caspase-3 assay kits and cell lysis buffer for Western blot analysis were purchased from Beyotime Biotech, China. Reverse transcriptase and first strand buffer were from Takara, Japan. Other reagents used were of analytical grade and were procured locally.

Cell culture and treatment

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Upon reaching 70%~80% confluence, the cells were exposed to 0~80 μmol/L curcumin for 24 h.

Cell viability assay

Viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazoliumbromide) assay. HT-29 cells were plated at a density of 6000 cells per well in 96-well plates. After 24 h, the cells were treated with different concentrations of curcumin (0, 10, 20, 40, 60, and 80 μmol/L). After 8 and 24 h treatment, media containing curcumin were carefully removed and cells were washed twice with phosphate buffered saline (PBS). 25 μl MTT solution dissolved in the culture medium at the final concentration of 0.5 mmol/L was added to each well, and the plates were wrapped with aluminium foil and incubated for another 4 h at 37 °C. Then the medium was totally removed and 200 μl dimethyl sulfoxide was added to solubilize MTT tetrazolium crystal. Finally, the plates were shaken and the optical density was determined at 570 nm (OD_{570}) using a Benchmark Plus microplate reader (Bio-Rad, USA). At least three independent experiments were performed.

Hoechst 33258 staining

Chromatin condensation was detected by nuclear staining with Hoechst 33258 (Porn-Ares *et al*., 1997). After treatment with 0~80 μmol/L curcumin for 16 h, cells were harvested and washed with PBS thrice. Then the cells were stained with 1 μl of Hoechst 333258 (5 mg/ml; Sigma) in 1 ml basal medium and incubated at room temperature in the dark for 15 min. Stained cells were imaged under a fluorescent microscope by using 350 nm stimulation and 460 nm emission.

Measurement of caspase-3 activities

Caspase-3 activities were measured as previously described (Piwocka *et al*., 1999). Briefly, cells were lysed in a buffer containing 5 mmol/L Tris (pH=8), 20 mmol/L ethylenediamine tetraacetate

(EDTA), and 0.5% (v/v) Triton-X100. Reaction mixture contained 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7), 10% (v/v) glycerol, 2 mmol/L dithiothreitol, 50 μg protein per condition, and 200 μmol/L *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-*p*NA) as substrate. After incubation for 24 h at 37 °C, each absorbance in each well was measured at 405 nm with a microplate enzyme-linked immunosorbent assay (ELISA) reader.

Mitochondrial membrane potential assay

Mitochondrial potential was assessed using the fluorescent potentiometric dye JC-1 as described previously (Desai *et al*., 2002; Shang *et al*., 2005). Briefly, at 12 or 24 h after the treatment with 0~80 μmol/L curcumin, HT-29 cells were harvested, washed twice with PBS, and centrifuged for 8 min at 450×*g* at room temperature. Then the cells were suspended with JC-1 (5 μg/ml) in serum-free RPMI-1640 and incubated for 15 min at 37 °C. After staining, the cells were collected at room temperature and washed thrice with pre-warmed PBS. The cell pellet was then resuspended in 1 ml of PBS. JC-1 fluorescence was quantitated using a fluorescence plate reader (Molecular Devices, MDS analytical Technologies, USA) at 37 °C. The fluorescence of the JC-1 monomer was measured at 485 nm of excitation wavelength/530 nm of emission wavelength. The fluorescence of the JC-1 aggregate was measured at 535 nm of excitation wavelength/590 nm of emission wavelength. For each experiment, the ratios of JC-1 aggregate to JC-1 monomer were normalized to untreated controls; values reported, therefore, represent a percentage of mitochondrial function in untreated cells.

RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted with Trizol isolation reagent (Invitrogen, USA). Sample RNA content was quantified by measuring absorbance at 260 nm. Reverse-transcription (RT) was performed with PrimeScript One Step RT-PCR Kit (Takara, Japan) according to the manufacturer's instructions. Table 1 shows the synthetic oligonucleotide primer sequences used for RT-PCR and the product sizes. β-actin served as a control. A total 20 μl of reaction mixture contained 4 μl RT product, 2.5 U Taq DNA polymerase, 20 μmol/L dNTP, 0.1 μmol/L primer, and 1× Taq DNA polymerase buffer (Takara, Japan). The reaction mixture was incubated in a thermocycler (Perkin-Elmer, CA, USA) programmed to predenature at 94 °C for 10 min, denature at 94 °C for 30 s, anneal at 55 °C for 30 s, and extend at 72 °C for 1 min. The last cycle was followed by incubation at 72 °C for 10 min and cooling to 4 °C. For each combination of primers, the PCR amplification kinetics was determined, the number of cycles corresponding to the plateau was counted, and PCR was conducted within an exponential range. The PCR products were analyzed in 1.5% (v/v) agarose gels. Densitometry was performed using Quantity One software. The data were recorded as the ratio of sample to internal standard.

Western blot analysis

Western blot analysis was done as previously described with minor modifications to detect the expressions of caspases-3, PARP, Bcl-2, Bcl-xL, Bad, Bax, and survivin proteins (Chen *et al*., 2008). After treatment for 24 h, the cells were washed thrice with ice-cold PBS. The total proteins were solubilized and extracted with 100 μl lysis buffer (20 mmol/L Tris

Table 1 The synthetic primers used for RT-PCR

Apoptosis modulator	Sense primer (5′−3′)	Anti-sense primer (5′−3′)	PCR conditions*	Size (bp)
Bcl-2	CAGCTGCACCTGACGCCCTT	GCCTCCGTTATCCTGGATCC	62 (28)	231
Bcl-xL	GGAGCTGGTGGTTGACTTTCT	CCGGAAGAGTTCATTCACTAC	56 (29)	379
Bax	TGCTTCAGGGTTTCATCCAG	GGCGGCAATCATCCTCTG	57 (27)	169
Survivin	GGACCACCGCATCTCTACAT	GCACTTTCTTCGCAGTTTCC	57 (30)	338
Caspase-3	CTCGGTCTGGTACAGATGTCGATG	GGTTAACCCGGGTAAGAATGTGCA	63 (28)	533
β-actin	CTACAATGAGCTGCGTGTGG	TAGCTCTTCTCCAGGGAGGA	57 (24)	450

*Annealing temperature in °C (number of cycles)

(pH 7.5), 150 mmol/L NaCl, 1% (v/v) Triton X-100, 1 mmol/L sodium orthovanadate, 100 μmol/L β-glycerophosphate, 5 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 10 μg/ml leupeptin, and 2 mmol/L phenylmethanesulfonyl fluoride (PMSF)). The lysates were used to estimate their protein content with bicinchoninic acid (BCA) protein assay. Equal amounts of protein (50 μg) from each sample were subjected to electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, using 10%, 12%, or 15% (v/v) acrylamide gels. After electrophoresis, proteins were electroblotted to a Hybond-C Extra nitrocellulose membrane (Amersham, USA). The membrane was blocked at room temperature with 5% (w/v) nonfat dry milk in Tris buffered saline (TBS) containing 0.3% (v/v) Tween (TBS-T). Then the membrane was washed thrice with TBS-T and incubated overnight at 4 °C with the primary antibody, rabbit monoclonal anti-caspases-3 (1:5000, v/v), anti-PARP (1:1000, v/v), anti-Bax (1:1000, v/v), anti-Bcl-2 (1:1000, v/v), anti-Bad (1:1000, v/v), anti-Bcl-xL (1:1000, v/v), or mouse monoclonal antibodies anti-survivin (1:1000, v/v), followed by 1-h incubation with a 1:5000 (v/v) dilution of the appropriate horseradish peroxidase-conjugated secondary antibody. After incubation, the membrane was washed with TBS-T for three times, and the antigen-antibody complexes were visualized by enhanced chemiluminescence and exposure to X-ray film (Kodak, Eastman Kodak, USA) for 0.5 to 30 min.

Analysis of cytochrome *c* release

Cytochrome *c* release from mitochondria was evaluated by Western blot analysis of cytosolic protein samples (Bae *et al*., 2003; Grishko *et al*., 2005). HT-29 cells were harvested after treatment with curcumin for 24 h and mixed with 100 μl cold lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L NaF, 150 mmol/L NaCl, 1 mmol/L ethylene glycol tetraacetic acid (EGTA), 1 mmol/L PMSF, 1% (v/v) nonidet P 40 (NP-40), and 10 μg/ml leupeptin), followed by centrifugation at 10000×*g* for 30 min at 4 °C. The supernatant was centrifuged two more times under the same conditions to remove nuclear debris. The cytosolic extracts were used for Western blot analysis to detect the cytochrome *c* release. The subsequent Western blot analysis was performed as described above.

Statistical analysis

Results are presented as mean±*SEM* (standard error of the mean). Comparisons between multiple groups were performed using the one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered to be significant at $P<0.05$.

RESULTS

Effects of curcumin on cell viability

Different concentrations of curcumin (10~80 μmol/L) at different time intervals (8 and 24 h) had cytotoxicity effects on HT-29 cells in a dose-dependent manner, as determined by the MTT assay (Table 2). Treatment with 40~80 μmol/L curcumin for 8 h significantly reduced the cell viability (11.64% dead cells at the highest concentration 80 μmol/L) compared with controls ($P<0.05$). Curcumin significantly decreased cell proliferation after 24 h of exposure in a dose-dependent manner (89.13% dead cells at the highest concentration 80 μmol/L) compared with controls ($P<0.01$), with an estimated 50% of inhibitory concentration (IC_{50}) value of 40.7 μmol/L (Table 2).

Table 2 Effect of curcumin on viability of HT-29 cells*

Curcumin (μmol/L)	Dead cells (%)	
	8 h	24 h
0	2.37±0.57^{a}	2.98±0.82^{a}
10	3.28±0.62^{a}	9.76±1.25^{b}
20	4.31±0.85^{ab}	17.48±2.94^{c}
40	6.22±1.41^{bc}	38.52±7.89^{d}
60	8.99±2.01^{cd}	54.03±6.27^{e}
80	11.64±3.63^{d}	89.13±8.12^{f}

*The viability is expressed as percentage of dead cells (mean±*SEM*; n=8~10); Time×concentration interaction was significant; Means in a column with different superscript letters were significantly different, $P<0.05$

Effects of curcumin on cell nuclear morphology

The Hoechst 33258 staining method was used to determine the apoptosis rates in HT-29 cells. The results show that when treated with curcumin for 16 h, the ratio of apoptotic cells significantly increased in a dose-dependent manner (Fig.1), which suggests that when exposed to curcumin, HT-29 cells underwent the typical morphologic changes of apoptosis.

Effects of curcumin on caspase-3 activities

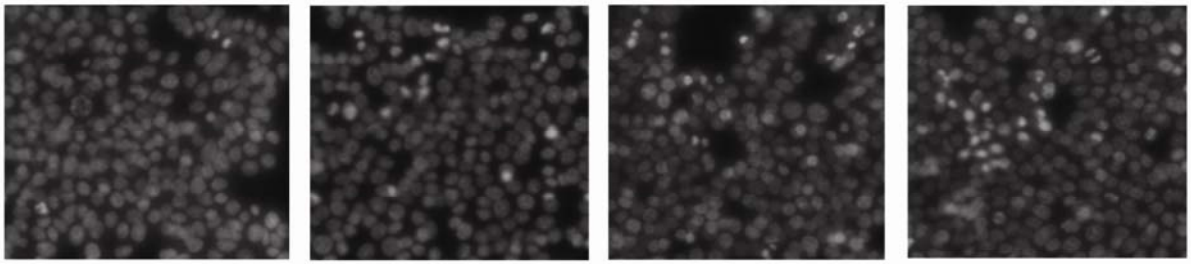
To investigate whether curcumin-induced apoptosis is dependent upon caspase activation, we examined the activities of caspase-3 in HT-29 cells treated with curcumin for 12 or 24 h. In cells treated with 10, 20, 40, 60, or 80 μmol/L curcumin for 12 h, the activities of caspase-3 were enhanced by 2.3-, 2.7-, 3.2-, 4.2-, and 4.5-fold, respectively, compared with control group. In cells treated with 10~80 μmol/L curcumin for 24 h, the activities of caspase-3 also significantly increased (Fig.2).

Effects of curcumin on loss of mitochondrial membrane potential ($\Delta\Psi_m$) of HT-29 cells

Loss of mitochondrial membrane potential ($\Delta\Psi_m$) has been shown to be an early event during apoptosis. We measured the $\Delta\Psi_m$ with JC-1 to appraise the function of mitochondria. At 12 or 24 h after curcumin treatment, we examined the state of the $\Delta\Psi_m$ by measuring the ratio of the red- to green-emitted fluorescence in control and curcumin-incubated HT-29 cells. As shown in Fig.3, the $\Delta\Psi_m$ significantly declined at 12 or 24 h in a dose-dependent manner. Our data indicate that exposure to curcumin resulted in a depolarization and $\Delta\Psi_m$ collapse.

Effect of curcumin on the mRNA expressions of Bax, Bcl-2, Bcl-xL, caspase-3, and survivin

In order to study the effects of curcumin on Bax, Bcl-2, Bcl-XL, caspase-3, and survivin productions on the transcriptional level, cells were treated with 10~80 μmol/L curcumin for 24 h, then the total RNA of cells were isolated, and RT-PCR was performed as described in the previous section. PCR products visualized by ultraviolet (UV) transillumination showed increase in the expression of Bax and significant decrease of the expression of Bcl-2 (Figs.4a and 4b). This suggests that curcumin (40~80 μmol/L) reduced ratio of Bcl-2/Bax at transcriptional level. As showed in Figs.4a and 4b, the anti-apoptotic proteins Bcl-xL and survivin were down-regulated at transcriptional level in HT-29 cells incubated with curcumin for 24 h. However, the caspase-3 gene transcription had not been significantly affected by curcumin treatment (Figs.4a and 4b).



(a) (b) (c) (d)

Fig.1 Effects of curcumin on the morphology of HT-29 cells were assayed by Hoechst 33342 staining

After treatment with curcumin for 16 h, HT-29 cells were stained with Hoechst 33342 and then were observed under a fluorescence microscope. (a) Control; (b) 20 μmol/L curcumin; (c) 40 μmol/L curcumin; (d) 60 μmol/L curcumin

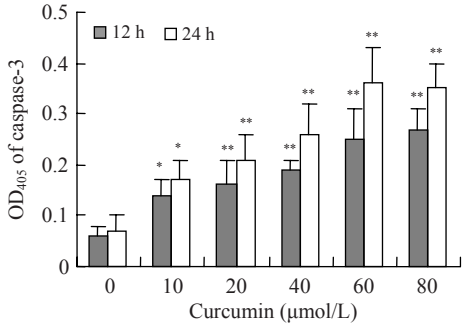


Fig.2 Effect of curcumin on caspase-3 activity in HT-29 cells

After 12 or 24 h treatment with or without curcumin, caspase-3 activities were determined by incubation of 50 μg total protein with DEVD-*p*NA substrates (200 μmol/L). $^{*}P<0.05$ and $^{**}P<0.01$ vs control

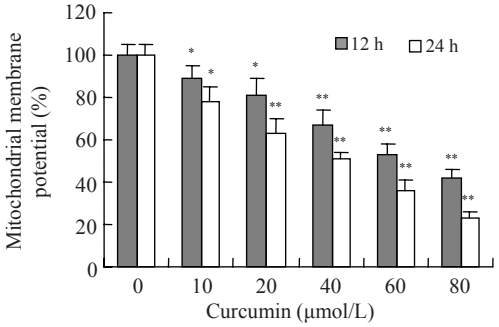


Fig.3 Effects of curcumin on mitochondrial membrane potential

HT-29 cells were treated with or without curcumin for 12 or 24 h, and then incubated with JC-1. Mitochondrial membrane potential of control is defined as 100%; $^{*}P<0.05$ and $^{**}P<0.01$ vs control

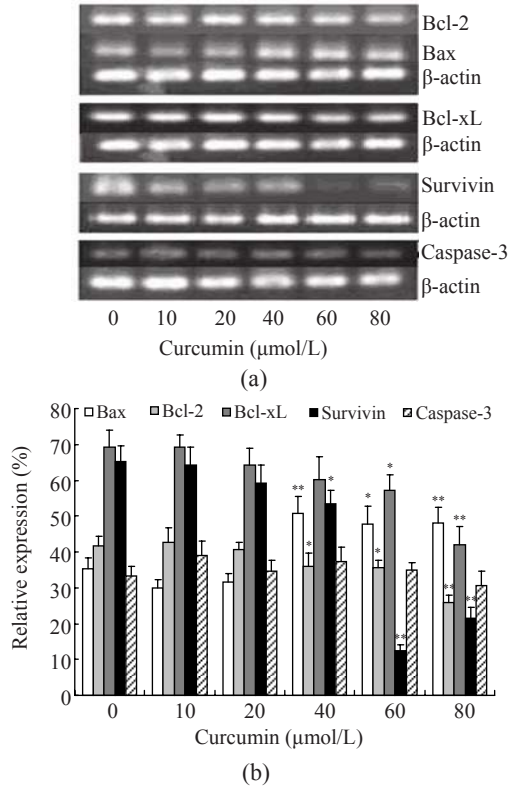


Fig.4 Effect of curcumin treatment on mRNA expression of Bax, Bcl-2 , Bcl-xL, survivin and caspase-3 in HT-29 cells

(a) The mRNA levels of Bax, Bcl-2, Bcl-xL, survivin, and caspase-3 in curcumin-treated and untreated HT-29 cells were analyzed by RT-PCR. β-actin served as internal marker; (b) The mRNA levels of Bax, Bcl-2, Bcl-xL, survivin, and caspase-3 quantified by densitometric analysis of the three autoradiographs. $^{*}P<0.05$ and $^{**}P<0.01$ vs control

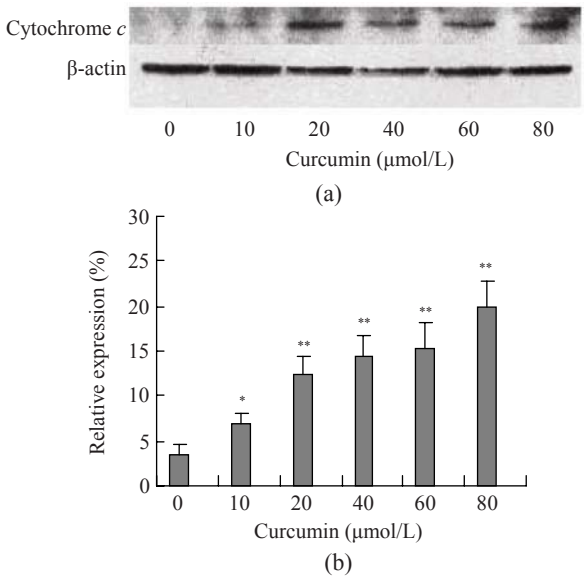


Fig.5 Effect of curcumin on the release of cytochrome *c*

(a) HT-29 cells were treated with the indicated concentrations of curcumin for 24 h. The cytochrome *c* in cytosolic fraction was assayed by Western blot; (b) Data obtained from Western blot represented in (a) were analyzed using Bandscan software. Each column represents the mean± *SEM* of four independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ vs control

Effect of curcumin on cytochrome *c* release

Cytochrome *c* release is known to be a key event during mitochondria-dependent apoptosis. Therefore, we attempted to examine whether treatment with curcumin for 24 h could enhance the release of cytochrome *c* from the mitochondria into the cytosol. The level of cytochrome *c* was determined by Western blot analysis. The release of cytochrome *c* was significantly increased in HT-29 cells incubated with 20~80 μmol/L curcumin (Fig.5).

Effect of curcumin on the protein expressions of Bad, Bax, caspase-3, Bcl-2, Bcl-xL, survivin, and PARP

In mitochondrial pathway of apoptosis, a number of signals can cause changes in mitochondrial membrane potential and mitochondrial permeability transition resulting in the release of cytochrome *c*. The release of cytochrome *c* from the mitochondria is facilitated by Bax and blocked by Bcl-2 (Kluck *et al*., 1997). Therefore, we studied the role of curcumin on the expression of proteins involved in the mitochondrial pathway. The results show that curcumin treatment resulted in down-regulation of the level of Bcl-2 and up-regulation of the level of Bax (Fig.6a). Therefore, the ratio of Bcl-2/Bax was significantly decreased. Bad is another pro-apoptotic member of the Bcl-2 family, which promotes cell death by binding to Bcl-xL. The results show that curcumin significantly down-regulated the level of Bcl-xL and up-regulated the level of Bad (Fig.6b).

Cytochrome *c* release is known to be a key event during mitochondria-dependent apoptosis, which, in turn, induces mitochondrial downstream caspase-3 activation. Pro-caspase-3 is processed into two subunits of 20 and 12 kDa. The 20 kDa subunit is autoprocessed into an active subunit of 17 kDa. The results of Western blot show that curcumin treatment dose-dependently induced the cleavage of pro-caspase-3 in HT-29 cells (Fig.6c). Furthermore, curcumin treatment also induced more cleavage of

caspase-3 substrate, PARP, compared with the control (Fig.6c).

Survivin, a member of the inhibitors of an apoptosis protein family, is a recently discovered protein that plays a key role in regulation of apoptosis and cell division. Survivin expression is significantly increased in colorectal cancer (Tan *et al*., 2005). Curcumin treatment dramatically reduced the level of survivin in HT-29 cells (Fig.6d).

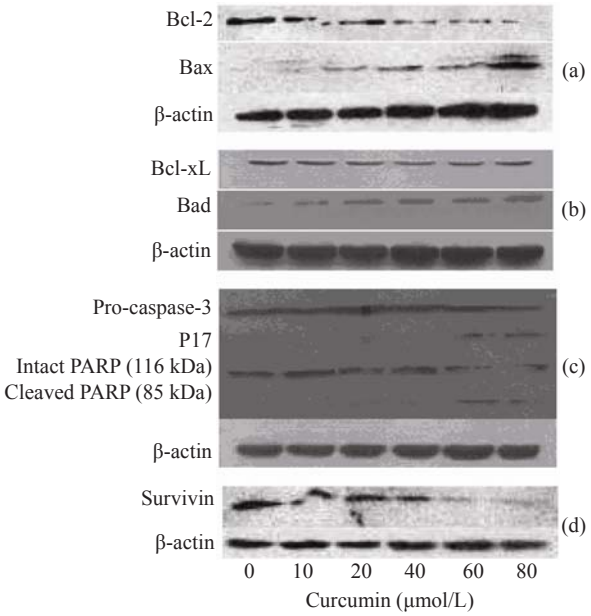


Fig.6 Effect of curcumin treatment on protein expressions of Bax (a), Bcl-2 (a), Bad (b), Bcl-xL (b), caspase-3 (c), PARP (c), and survivin (d)

HT-29 cells were treated with different concentrations of curcumin for 24 h. Bax, Bcl-2, Bad, Bcl-xL, caspase-3, PARP, and survivin were assayed by Western blot. Equal amounts of total cellular protein (50 μg) were resolved on 10%~15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and β-actin was used as an internal control

DISCUSSION

Curcumin, a polyphenol derived from the plant *Curcuma longa*, has been shown to inhibit the growth of a wide variety of tumor cells in multiple experimental model systems. As reported, the mechanisms responsible for apoptosis induction by curcumin seem to be varied, including inhibition of Akt dephosphorylation and NF-κB activation, down-regulation of the levels of Bcl-2 and Bcl-XL, increase of cytochrome *c* release, up-regulation of growth arrest and DNA damage gene (GADD153), and activation of p38 and caspase-3 (Pan *et al*., 2001; Woo *et al*., 2003; Scott and Loo, 2004; Shishodia *et al*., 2005a). However, the signaling pathways governing apoptosis in mammalian cells are complex and the pro- and anti-apoptotic variations regulate cell survival change according to cell type (Cory and Adams, 2002). The cellular and molecular mechanisms underlying curcumin-induced apoptosis in HT-29 cells have not been well defined.

Mitochondrial and death receptor pathways are two major apoptosis signaling pathways. Many studies have shown that the collapse of the mitochondrial membrane potential and the redistribution of cytochrome *c* are early steps in the apoptotic cascade (Kluck *et al*., 1997; Wang, 2001). A rapid collapse of $\Delta\Psi_m$ is always found in some anticancer compounds-induced apoptosis in cancer cells (Chen *et al*., 2007). In this study, we investigated the effects of curcumin on the $\Delta\Psi_m$ with fluorescent dye JC-1. JC-1 was chosen because it has been described as a reliable probe for analyzing $\Delta\Psi_m$ changes in intact cells, whereas other probes show a lower sensitivity (e.g., rhodamine 123) or noncoherent behavior due to a high sensitivity to changes in the plasma membrane potential (e.g., $DiOC_6(3)$) (Salvioli *et al*., 1997). Changes in the plasma membrane potential do not seem to affect the JC-1 status (Salvioli *et al*., 1997). The previous studies suggested that the $\Delta\Psi_m$ could be reduced by curcumin at the concentration of 10 μmol/L or above (Rashmi *et al*., 2003; Cao *et al*., 2007). Our data clearly show that treatment with 10~80 μmol/L curcumin could lead to a collapse of mitochondrial transmembrane potential, which is in agreement with the previous studies. This means that curcumin-induced apoptosis is related to the collapse of the mitochondrial membrane potential.

Cytochrome *c* is a critical factor in apoptotic process, which releases from the mitochondria into the cytosol during cell apoptosis (Kluck *et al*., 1997). The released cytochrome *c* forms an "apoptosome" of Apaf-1, cytochrome *c*, and caspase-9, which subsequently cleaves the effector caspase-3 (Li *et al*., 1997). Many studies have shown that curcumin can induce the release of cytochrome *c* from the mitochondria in different cell lines (Rashmi *et al*., 2003; Sen *et al*., 2005; Cao *et al*., 2007). In the present

study, we also observed that curcumin markedly increased the release of cytochrome *c* from the mitochondria to the cytosol in HT-29 cells. All these studies indicate that the levels of cytochrome *c* in the cytosolic fraction increased dramatically when the dosage of curcumin was 10 μmol/L or above. This provides a direct link between the mitochondria and the curcumin-induced apoptosis.

Bcl-2 family and survivin function through different pathways in the regulation of cell apoptosis. Bcl-2 family mainly affects the mitochondrial pathways (Reed, 1998). However, survivin directly blocks the processing and activating of effectors caspase-3 and caspase-7, which act on a common downstream of both apoptosis signaling pathways (Suzuki *et al*., 2000). Therefore, to explore molecular mechanisms of curcumin, we examined the expression levels of both Bcl-2 family and survivin.

Bcl-2 and its homologues could prevent mitochondrial membrane disruption and the release of cytochrome *c* and other pro-apoptotic factors, while Bax promotes these events. The total expression rate of these two contrary proteins might partly indicate the fate of cells. The ratio of Bcl-2/Bax is usually regarded as a criterion in programmed cell death. An increase in expression of Bax and a decrease in expression of Bcl-2 were observed in a time-dependent manner after being exposed to 50 μmol/L curcumin, while the expression of the Bcl-xL was unchanged (Song *et al*., 2005). Our results show that curcumin induced apoptosis by down-regulating anti-apoptotic Bcl-2 and Bcl-xL, and up-regulating pro-apoptotic Bax and Bad, thereby decreasing the Bcl-2/Bax and Bcl-xL/Bad ratios. Our result about Bcl-2/Bax ratio is in agreement with previous studies (Song *et al*., 2005; Shi *et al*., 2006). However, we found that the Bcl-xL level was also reduced by curcumin, which is inconsistent with the results of Song *et al*.(2005). The discrepant results may be attributed to the different concentrations of curcumin applied. In Song *et al*.(2005)'s study, HT-29 cells were treated with 50 μmol/L curcumin for 0~24 h, whereas we incubated the cells with 0~80 μmol/L curcumin for 24 h and the level of Bcl-xL was significantly decreased at the concentration of 60 and 80 μmol/L.

Caspases, represented by a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9. The activated caspase-3 could cleave the PARP, which is one of the hallmarks of apoptosis (Wang, 2001). Song *et al*.(2005) has shown that curcumin could decrease the level of pro-caspase-3 in HT-29 cells. In this study, we found that curcumin could decrease the level of pro-caspase-3, leading to activation of caspase-3 and increase of cleavage of PARP protein in HT-29 cells. Thus, curcumin-induced HT-29 cell death was accompanied by an increase in the activity of caspase-3, which then stimulated the molecular cascade of apoptosis.

Survivin, a recently discovered protein and a member of the inhibitors of apoptosis protein family, plays a key role in regulation of apoptosis and cell division (Li and Brattain, 2006). Survivin is abundantly expressed in fetal tissues for survival, but not in normal adult tissues. This would make survivin an advantageous target for treatment of cancer in adults. Some studies have shown that survivin is highly expressed in colorectal cancer and the high level of survivin expression is a potential biomarker for colorectal cancer and a potential indicator for good candidate for gene targeting therapy to cancer (Tan *et al*., 2005; Khor *et al*., 2006; Watson, 2006; Lam *et al*., 2008). Curcumin has been shown to markedly reduce the level of survivin to inhibit the growth and differentiation of KU7 bladder cancer cells (Chadalapaka *et al*., 2008). However, curcumin did not change the expression of survivin in DA-MB-231 cells (Moiseeva *et al*., 2007). In the present study, we observed that curcumin markedly down-regulated the expression of survivin in HT-29 cells, which, to our knowledge, is the first demonstration that curcumin could down-regulate the expression of the anti-apoptotic protein survivin in HT-29 cells.

In conclusion, our results show that curcumin could significantly inhibit the proliferation and induce apoptosis in HT-29 cells via the mitochondrial cell death pathway. In the apoptotic process, curcumin-induced decreases of Bcl-2/Bax and Bcl-xL/Bad ratios followed by the activation of caspase-3, the release of cytochrome *c*, and the collapse of $\Delta\Psi_m$. Curcumin could also clearly down-regulate the level of another important anti-apoptotic protein, survivin. Results of this study may provide an explanation for effects of curcumin on the apoptosis of HT-29 cells. The role of curcumin in inducing apoptosis of HT-29

cells indicates that curcumin may be a potential chemotherapeutic agent.

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