

Curcumin induces human HT-29 colon adenocarcinoma cell apoptosis by activating p53 and regulating apoptosis-related protein expression

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Abstract

Curcumin, a major yellow pigment and active component of turmeric, has multiple anti-cancer properties. However, its molecular targets and mechanisms of action on human colon adenocarcinoma cells are unknown. In the present study, we examined the effects of curcumin on the proliferation of human colon adenocarcinoma HT-29 cells by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method and confirmed the curcumin-induced apoptosis by morphology and DNA ladder formation. At the same time, p53, phospho-p53 (Ser15), and other apoptosis-related proteins such as Bax, Bcl-2, Bcl-x_L, pro-caspase-3, and pro-caspase-9 were determined by Western blot analysis. The colon adenocarcinoma cells were treated with curcumin (0-75 μ M) for 0-24 h. We observed that p53 was highly expressed in HT-29 cells and curcumin could up-regulate the serine phosphorylation of p53 in a time- and concentration-dependent manner. An increase in expression of the pro-apoptotic factor Bax and a decrease in expression of the anti-apoptotic factor Bcl-2 were also observed in a time-dependent manner after exposure of 50 μ M curcumin, while the expression of the anti-apoptotic factor Bcl-x_L was unchanged. Curcumin could also down-regulate the expression of pro-caspase-3 and pro-caspase-9 in a time-dependent manner. These data suggest a possible underlying molecular mechanism whereby curcumin could induce the apoptosis signaling pathway in human HT-29 colon adenocarcinoma cells by p53 activation and by the regulation of apoptosis-related proteins. This property of curcumin suggests that it could have a possible therapeutic potential in colon adenocarcinoma patients.

Key words

- Curcumin
- Apoptosis
- HT-29 cells
- p53
- Phosphorylation
- DNA ladder

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Introduction

Curcumin is the major yellow pigment in turmeric which is derived from the herb *Curcuma longa* Linn (1). Consumption of turmeric or curcumin has been associated with many beneficial effects on human health and

is currently receiving attention from cancer investigators for its chemopreventive properties against human malignancies. Recent studies have demonstrated that curcumin can be used for cancer prevention for its anti-inflammatory, anti-oxidant, anti-cancer, anti-proliferative, and anti-atherogenic properties

(2,3). *In vivo*, curcumin suppresses carcinogenesis of the skin (4), the forestomach (5), the colon (6), the breast (7), and the liver (8) in mice, and *in vitro*, it has been shown to inhibit the growth of a wide variety of tumor cell lines (9). Although the mechanisms of the anti-cancer action of curcumin are not fully understood, in recent years, curcumin-induced apoptosis by targeting mitochondria, affecting p53-related signaling or blocking NF- κ B activation, has emerged as the major anti-cancer mechanism (10).

The p53 protein has emerged as a key tumor suppressor protein by playing a central role in cellular stress response pathways. Through these pathways, one of its roles is to survey cellular stress and to induce apoptosis. p53 can promote apoptosis by several mechanisms (11). The Bcl-2 family has been shown to be a p53 target. Bax, the pro-apoptotic member, is up-regulated in a number of systems during p53-mediated apoptosis (12). On the other hand, down-regulation of Bcl-2, the anti-apoptotic member, has also been demonstrated during apoptosis (12). Recent studies have proposed that the alteration of the Bcl-x_L (a pro-proliferative member of the Bcl-2 family) and Bax ratio is one of the important factors which decide the fate of a cell (13). However, the mechanism of curcumin-induced apoptosis involved in the regulation of the balance between these pro- and anti-apoptotic proteins is not fully clear.

Colon cancer is a serious health problem in most developed countries and is one of the leading causes of cancer mortality throughout the world (14). To date, chemoprevention is a major strategy since other therapies have not been effective in controlling either the high incidence or low survival rate of colon cancer (15). The anti-cancer properties of curcumin on colon cancer have been demonstrated *in vivo* (6,16) and, *in vitro*, curcumin has been shown to inhibit the growth of human colon cancer cells independent of cyclooxygenase-2 (COX-2) expression (17,18). In order to extend the observations on the potential inhibi-

tion of human colon adenocarcinoma cells by curcumin, in the present study we evaluated the effect of curcumin on HT-29 cells *in vitro*, and investigated the possible underlying molecular mechanism. The study showed that curcumin can induce the apoptosis signaling pathway in human HT-29 colon adenocarcinoma cells through p53 activation and the regulation of apoptosis-related proteins.

Material and Methods

Antibodies

Mouse anti-p53 monoclonal antibody and rabbit anti-phospho-p53 (Ser15) polyclonal antibodies were purchased from Calbiochem (Darmstadt, Germany). Rabbit anti-Bax, Bcl-x_L, Bcl-2, pro-caspase-3, and pro-caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-actin monoclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology.

Cell culture and curcumin treatment

The human HT-29 colon adenocarcinoma cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Curcumin (Sigma) was dissolved in DMSO at a concentration of 5 mM and was stored in a dark-colored bottle at -20°C as a stock solution. The stock was diluted to the required concentration with serum-free medium immediately before use. Before treatment with curcumin, the medium was removed when cells were about

80% confluent, the cells were starved overnight in serum-free medium and then exposed to curcumin at different concentrations (0-75 μ M) and for different periods of time (0-72 h). Cells grown in medium containing an equivalent amount of DMSO without curcumin served as control.

Cell viability assay

Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) method. Briefly, cells were plated onto a 96-well plate at a density of 5×10^3 cells/well in 200 μ L of medium. After 24 h they were treated with a series of curcumin concentrations (0-75 μ M) for 12, 24, 36, 48, and 72 h. After treatment, medium containing curcumin was carefully removed, and 100 μ L MTT solution (0.25 mg/mL in PBS) was added to each well. After 4 h of incubation at 37°C, MTT was discarded and 200 μ L of extraction buffer (90% DMSO, 10% 0.1 mol/L glycine-NaOH, pH 10.0) was added to each well, followed by shaking for 30 min. Absorbance (A) at 570 nm was measured with an ELISA plate reader, with the extraction buffer used as a blank. Percent cell viability was calculated as follows: A of the experimental group/A of the control group \times 100%.

Cell morphology studies

After treatment with 50 μ M curcumin for 24 h, the cells, cultured on glass coverslips, were washed three times with PBS, fixed with 3% paraformaldehyde for 10 min at room temperature, and then washed in PBS again, and the morphological changes were observed. Some cells were incubated with 10 μ g/mL Hoechst 33258 (Calbiochem, San Diego, CA, USA) for 10 min. In addition, when the cells had been treated for the indicated time, both adherent cells and floating cells were centrifuged and the pellet was resuspended in 2 μ L acridine orange and ethidium bromide (AO/EB, AO 100 μ g/mL and EB 100 μ g/mL in

PBS). Cell morphology was then observed by fluorescence microscopy (Leica DM IRB, Wetzlar, Germany).

DNA fragmentation assay

Briefly, cells (2×10^6) were harvested and lysed in cold buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.5% NP-40 on ice for 40 min. Lysates were centrifuged and the pellets were resuspended in cold buffer containing 10 mM Tris-HCl, pH 8.0, 350 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT on ice for 20 min. Lysates were then extracted once with a phenol:chloroform:isoamyl alcohol mixture (25:24:1) and DNA was precipitated with 10 mM $MgCl_2$ and 2.5 volumes 100% ethanol overnight at -20°C. DNA was collected by centrifugation at 14,000 g for 20 min, resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) plus 0.1 mg/mL RNase A, and incubated at 37°C for 1 h. Proteinase K (1 mg/mL) was added and the mixture was incubated at 37°C for an additional hour. Fragmented DNA was then electrophoresed in 1.5% agarose gels containing 0.5 μ g/mL EB.

Western blot analysis

After harvesting, the experimental cells were washed twice with PBS and lysed in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 20 mM KCl, 1.5 mM $MgCl_2$, 50 mM β -GPA, 10 mM NaF, 0.5% NP-40, plus proteinase and phosphatase inhibitors) for 10 min and centrifuged at 12,000 g for 10 min at 4°C. Total protein, as determined by the Bio-Rad protein assay, was mixed with 4X loading buffer, and pre-heated at 95°C for 10 min. The samples were then loaded on SDS-polyacrylamide gel and the proteins were transferred to a PVDF membrane for 1.5 h using a semi-dry transfer system (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 0.25% gelatin for 1.5 h at room temperature and then incubated

for 1.5 h with primary antibody. After hybridization with the primary antibody, the membrane was washed with Tris-buffered saline containing Tween-20 three times, then incubated with HRP-labeled secondary antibody for 1 h at room temperature and washed with Tris-buffered saline containing Tween-20 three times. Final detection was performed with ECL enhanced chemiluminescence Western blotting reagents (Amersham, Pharmacia Biotech, Piscataway, NJ, USA). The blot was then stripped in buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 200 mM 2-mercaptoethanol) at 50°C for 30 min. After extensive washing, the same blot was used to probe for the next protein, beginning from the blocking step.

Results

Effect of curcumin on the growth of HT-29 cells

We examined the effects of different concentration of curcumin on the viability of

Figure 1. Effect of curcumin on HT-29 cell viability. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Data are reported as the means \pm SD of three separate experiments.

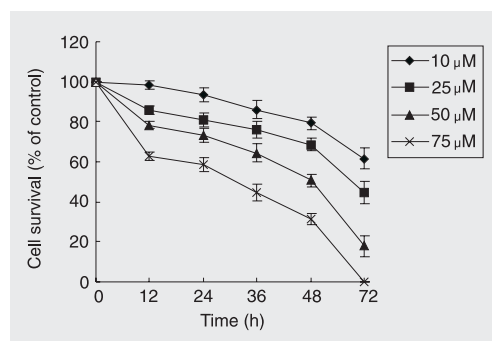
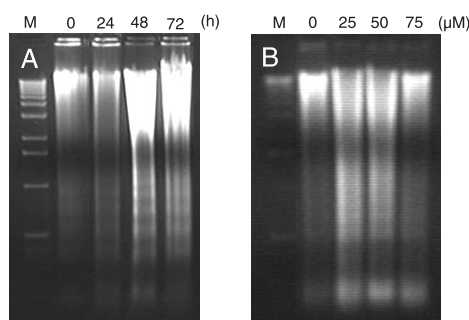


Figure 2. Curcumin induces DNA fragmentation in HT-29 cells. *A*, Cells were treated with 50 μ M curcumin and the control was treated with medium containing an equivalent amount of DMSO without curcumin. At the indicated times, the cells were harvested and lysed. DNA fragmentation was examined by agarose gel electrophoresis. *B*, Cells were incubated in medium containing curcumin at the indicated concentrations for 48 h. The cells were harvested and lysed. DNA fragmentation was examined by agarose gel electrophoresis. (M: 1 kb DNA marker).



HT-29 cells for 12, 24, 36, 48, and 72 h by the MTT method (Figure 1). Curcumin inhibited growth in a concentration- and time-dependent manner. At the concentration used here (50 and 75 μ M), a significant loss of viability was detectable during the 48 h of treatment. The cell growth-inhibiting rate was about 80% at 50 μ M curcumin concentration after 72 h of treatment. Furthermore, DNA fragmentation, a hallmark event in cell apoptosis, was also detected in a concentration- and time-dependent manner in HT-29 cells (Figure 2A,B). The results showed a typical ladder pattern of internucleosomal fragmentation in HT-29 cells treated with 25 and 50 μ M curcumin for 48 and 72 h.

Effect of curcumin on cell morphology of HT-29 cells

To determine curcumin-induced apoptosis of HT-29 cells, we first examined the changes in cell morphology after exposure to curcumin. Twenty-four hours after exposure to 50 μ M curcumin, HT-29 cells began to show cell shrinkage, rounding and fragmentation, thus taking on the typical appearance of apoptotic cells when compared to untreated cells (Figure 3A). We also analyzed changes in cell morphology by Hoechst 33258 (Figure 3B) and AO/EB (Figure 3C) staining. The curcumin-treated cells also exhibited morphological changes indicative of apoptosis, including chromatin condensation and nuclear fragmentation.

Effect of curcumin on the serine phosphorylation of p53.

p53 has been shown to be involved in apoptosis induced by a broad range of agents. In order to understand the exact mechanism of curcumin-induced apoptosis in HT-29 cells, we first examined the activation of p53 in the presence of 50 μ M curcumin for different periods of time or at different curcumin concentrations for 6 h. Results showed

very little or no change of total p53 expression for different exposure times or at different concentrations for 6 h (Figure 4A, B). However, a notable change was observed in p53 phosphorylation. Upon treatment with 50 μ M curcumin, the serine phosphorylation level of p53 started to increase as early as after 1 h, and reached a high level at 6 h (Figure 4A). The serine phosphorylation level of p53 also started to increase in a concentration-dependent manner. Upon treatment with 75 μ M curcumin, the serine phosphorylation level of p53 reached a maximum level after

6 h as compared to other curcumin concentrations (Figure 4B).

Effect of curcumin on the expression of apoptosis-related proteins

To further understand the mechanism of curcumin-induced apoptosis in HT-29 cells, we also examined the expression of apoptosis-related proteins. Bcl-x_L levels were moderately high in HT-29 cells and remained almost unchanged after curcumin treatment, but Bcl-2 levels decreased after 4 h. On the

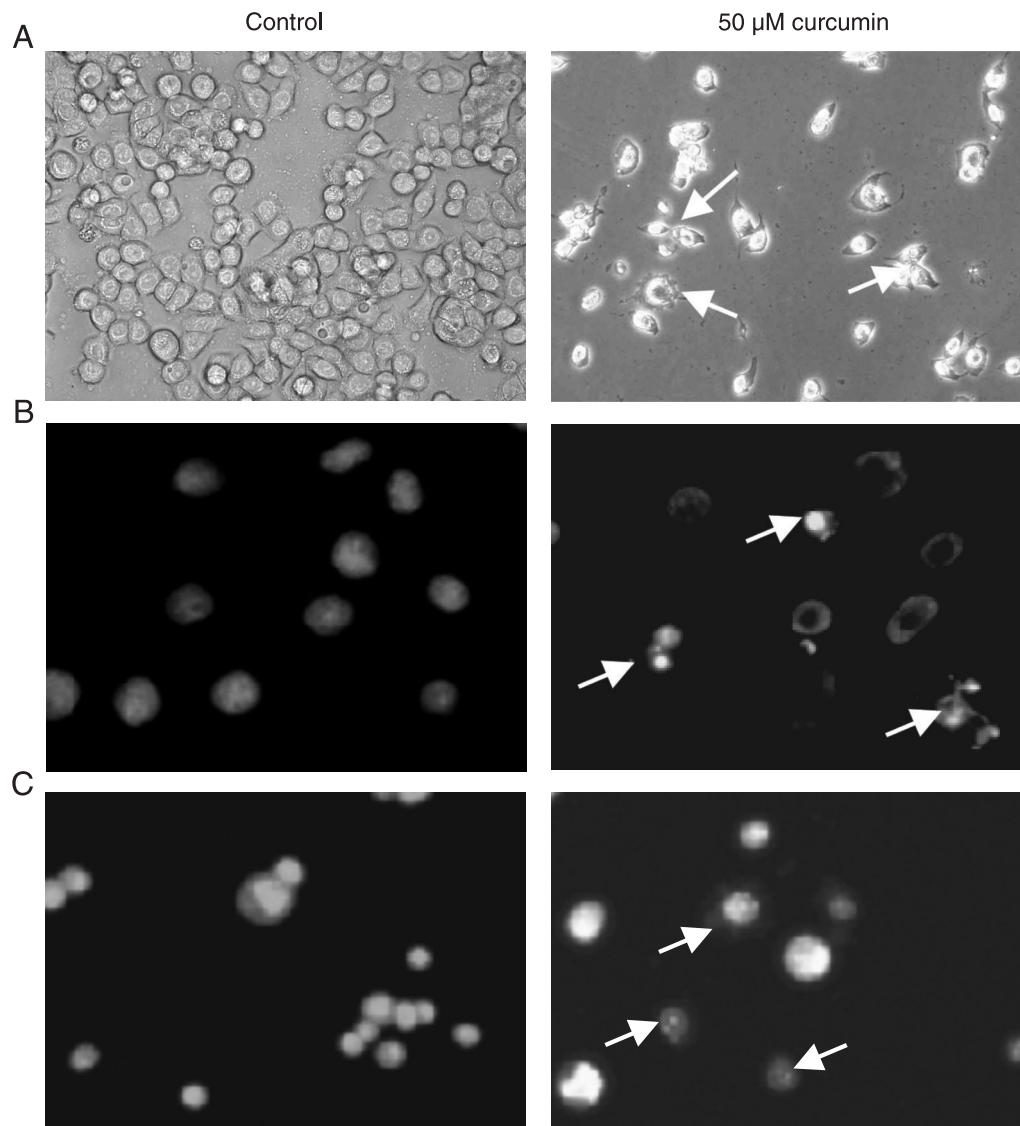


Figure 3. Morphological changes of HT-29 cells after exposure to curcumin. HT-29 cells were treated with DMSO (control) or 50 μ M curcumin for 24 h. A, The images were captured by phase contrast microscopy (Leica DM IRB). Original magnification, 200X. B, Cells were fixed and stained with Hoechst 33258, and the images were captured by fluorescence microscopy (Leica DM IRB). Original magnification, 400X. C, Cells were harvested and stained with AO/EB, and the images were captured by fluorescence microscopy (Leica DM IRB). Original magnification, 400X. The arrows indicate cells undergoing apoptosis.

other hand, the Bax levels increased significantly and remained higher after 4 h (Figure 5A). The levels of pro-caspase-3 and pro-caspase-9 zymogen in HT-29 cells treated with 50 μ M curcumin were low and decreased in a time-dependent manner. Simultaneously, we observed a clear reduction of pro-caspase-9 zymogen expression after 6 h of curcumin treatment (Figure 5B).

Discussion

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a hydrophobic molecule that passes easily through the plasma membrane into the cytosol (19). This phenolic substance inhibits tumor initiation induced by various carcinogens and has also been shown to inhibit the

growth of many human cancer cell lines *in vitro* (9). A number of mechanisms of cell proliferation inhibition and apoptosis induction by curcumin have been demonstrated. The present results and other literature reports (17,18) show that curcumin strongly reduces the viability of HT-29 colon adenocarcinoma cells by the induction of apoptosis. However, the signaling pathways governing apoptosis in mammalian cells are complex and the pro- and anti-apoptotic variations regulating cell survival change according to cell type (20). The cellular and molecular mechanisms underlying curcumin-induced apoptosis in HT-29 cells have not been well defined. Therefore, it is interesting to study the mechanism of action of curcumin on HT-29 cells.

In agreement with a previous study (18),

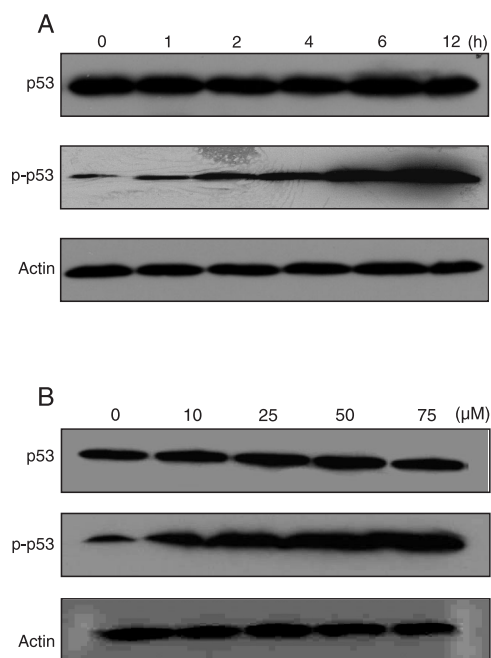


Figure 4. Enhanced serine phosphorylation of p53 in HT-29 cells by treatment with curcumin. **A**, Cells were incubated in serum-free medium overnight and then further incubated for 0, 1, 2, 4, 6, and 12 h in the presence of 50 μ M curcumin. **B**, Cells were incubated in serum-free medium overnight and then further incubated for 6 h in the presence of 0, 10, 25, 50, and 75 μ M curcumin, respectively. Equal amounts of cell lysates were resolved by SDS-PAGE and analyzed by Western blot using specific antibodies. The blots were re-probed with anti-actin antibody to confirm equal protein loading.

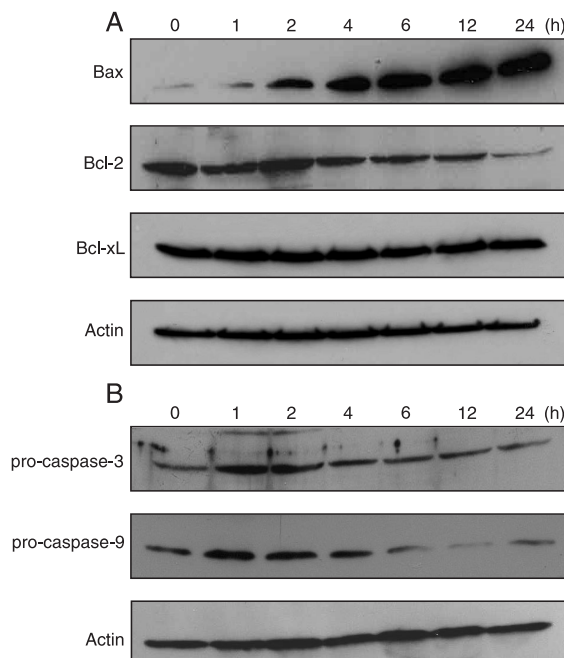


Figure 5. Expression of the apoptosis-related proteins Bax, Bcl-2, Bcl-x_L, pro-caspase-3, and pro-caspase-9 in HT-29 cells by treatment with curcumin. **A**, Cells were incubated in serum-free medium overnight and further incubated for 0, 1, 2, 4, 6, 12, and 24 h in the presence of 50 μ M curcumin. **B**, Cells were incubated in serum-free medium overnight and then further incubated for 6 h in the presence of 0, 10, 25, 50, and 75 μ M curcumin, respectively. Equal amounts of cell lysates were resolved by SDS-PAGE and analyzed by Western blot using specific antibodies. The blots were re-probed with anti-actin antibody to confirm equal protein loading.

the present investigation showed that curcumin inhibited the growth of HT-29 colon adenocarcinoma cells in a concentration- and time-dependent manner. In our study, however, we observed that a cell growth inhibitory rate of about 80% occurred at the curcumin concentration of 50 μM after 72 h of treatment. In contrast to our results, in a previous report the same cell growth inhibitory effect of curcumin was observed at about 75 μM after 72 h of treatment (18). We also observed a typical ladder pattern of internucleosomal fragmentation through DNA fragmentation in HT-29 cells treated with 50 μM curcumin for 48 h. Similar effects of curcumin in inhibiting cell growth have been previously reported for other colon cancer lines (21,22).

In a study of the mechanism of action of curcumin on HT-29 cells, Hanif et al. (17) reported that curcumin inhibits the growth of human colon cancer cells independent of COX-2 expression. Goel et al. (18) also reported that curcumin markedly inhibited the mRNA and protein expression of COX-2, but not of COX-1. In the present study, we propose another possible underlying molecular mechanism of curcumin-induced HT-29 cell apoptosis via the activation of p53 and the regulation of apoptosis-related proteins. Despite its central role in cell apoptosis, the mechanism of p53-mediated apoptosis after cellular stress remains unclear. Current evidence indicates that the mode of action of p53-mediated apoptosis involves transactivation of target genes and direct signaling events that are transcription independent (23). It has been proposed that p53 may induce two sets of genes upon stress signals. One set, such as p21/*waf-1* and GADD45, mainly functions in cell growth control, and the other, such as Bax and Bcl-2, acts on apoptosis (24).

In the present study, we observed that p53 was highly expressed in HT-29 cells, but the total p53 protein was almost unchanged. This was in agreement with previ-

ous reports (25,26). However, we found that the serine phosphorylation level of p53 was enhanced prominently when HT-29 cells were treated by curcumin. A recent study implicated that curcumin could impair p53 function required for serine phosphorylation of p53 in colon cancer cells (27). The activation of p53 by curcumin would affect the expression of its downstream effectors, such as the Bcl-2 family proteins. This implies a possible underlying molecular mechanism of curcumin action on HT-29 cells.

It is well recognized that the Bcl-2 family proteins are central regulators of apoptosis and the Bcl-2 family members act like checkpoints through which survival and death signals pass before they determine the fate of the cell (20). In our study, the high serine phosphorylation level of p53 was shown to be capable of both down-regulating the anti-apoptotic factor, Bcl-2 and up-regulating the pro-apoptotic factor Bax, thereby decreasing the Bcl-2/Bax ratio and disposing to apoptosis. Interestingly, curcumin induced apoptosis with an increased serine phosphorylation level of p53 which transactivates Bax expression. But in these cells, Bcl-x_L levels remained almost unchanged, thereby shifting the Bcl-x_L/Bax ratio towards apoptosis. The activation of the p53-mediated apoptotic signaling pathway may play an important role in apoptosis by modulating the Bcl-2/Bax or Bcl-x_L/Bax ratio, as also reported for breast cancer cells (28).

p53-mediated transcriptionally activated pro-apoptotic proteins are usually classified into two groups including the death-receptor pathway and the mitochondrial apoptotic pathway. Caspases are the key proteins that modulate the apoptotic response and are represented by a family of cysteine proteases (29). Caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9. These activated caspases cleave many cellular substrates, ultimately leading to cell death (29). We found that curcumin could down-regulate

pro-caspase-9 and pro-caspase-3 expression in a time-dependent manner on the HT-29 cells. Our results agree with data reported for other cancer cell lines (30,31).

Taken together, our results show that curcumin induces apoptosis in HT-29 colon adenocarcinoma cells by up-regulating the serine phosphorylation level of p53 and the

level of Bax, while down-regulating the levels of Bcl-2, pro-caspase-3, and pro-caspase-9. These findings suggest a mechanism of curcumin action on HT-29 cells and should further establish its use as a valid chemopreventive and chemotherapeutic agent in colon cancer.

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