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Curcumin inhibits the proliferation of a human colorectal cancer cell line Caco-2 partially by both apoptosis and G2/M cell cycle arrest

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Abstract

The aim of this study was to assess the possible roles of the phytochemical compounds, curcumin, quercetin and resveratrol in the proliferation of human colorectal cancer cell line Caco-2. All three phytochemical compounds inhibited Caco-2 cell proliferation, with curcumin being more effective than quercetin and resveratrol. Investigations concerning DNA fragmentation in the nucleus, Bax and Bcl-2 mRNA expression levels, and caspase-3/7 activity indicated that curcumin induced apoptosis in Caco-2 cells through an increase in the Bax/Bcl-2 ratio and activation of caspase-3/7. Furthermore, the analysis of flow-cytometry showed that curcumin caused an arrest of G2/M phase in Caco-2 cells. These results suggest that curcumin suppresses Caco-2 proliferation partially via activation of the mitochondrial apoptotic pathway and cell cycle retardation.

Keywords: curcumin; colon cancer; proliferation; Caco-2; apoptosis; cell cycle arrest

1. Introduction

Colorectal cancer is one of the leading causes of death worldwide, being responsible for approximately 10% of total cancer-related mortality[1]. About 3–5% of colorectal cancers may be due to inherited genetic defects and up to 25% of patients may have some degree of familiarity for this disease, but the majority of colorectal cancers occur in a sporadic manner in the absence of a documented family history[2]. Among gastrointestinal cancers, colorectal cancer is a good candidate for chemoprevention due to the long precancerous stage that provides an opportunity to intervene before adenomas develop into cancers. Although standard clinical practice necessitates screening and surveillance for the early detection of colorectal cancers, preventive measures by dietary and lifestyle factors can be useful in decreasing the risk of colorectal cancer. Chemoprevention by use of natural compounds that have the potential to delay, prevent or reverse the development of colorectal cancer is a viable option.

Curcumin (diferuloylmethane) is an active ingredient of turmeric, a well known Indian spice that is derived from the dried roots of the plant *Curcuma Longa*. Its daily consumption as part of Indian cuisine not speaks for its safety, and it has been epidemiologically said to be responsible for a low incidence of colorectal cancer[3]. Previously, curcumin was reported to inhibit the proliferation of human colorectal cancer cell lines, HT-29 cells[4,5], Colo 205 cells[6], HCT-116 cells[5,7,10], LoVo cells[11,12], and SW-480 cells[4]. Of the other phytochemical compounds, quercetin[13-15] and resveratrol[16-18], which are both polyphenols and are found in many fruits and vegetables, have also been shown to possess an anti-proliferative effect on human colorectal cancer cells, HT-29 cells, Colo 201 cells, DLD-1 cells and/or SW-480 cells. However, little information is available concerning the simultaneous and comparative effect of all three phytochemical compounds on colorectal cancer cell lines.

Caco-2 cells are generally accepted as another well known human colorectal cancer cell line. Jiang *et al*[19] showed that HT-29 cells were more sensitive to curcumin than Caco-2 cells in inhibiting cell growth. Van Erk *et al*[20] also reported that expression of fewer genes was changed in Caco-2 cells than in HT-29 cells after exposure to curcumin. Furthermore, the previous observation that Caco-2 cells possess mutated p53[21] might lead researchers not to use Caco-2 cells for investigations of the curcumin effect on the colorectal cancer. However, in a mouse model with a mutation in the APC gene, curcumin reduced the number of colon tumors[22] or the multiplicity of colon adenomas[23]. It may therefore be valuable to investigate the effect of curcumin on Caco-2 proliferation in order to gain a more extensive understanding of its colon cancer-preventing potency.

In the present study, we showed that curcumin suppressed Caco-2 cells proliferation more potently than quercetin and resveratrol, and that the curcumin effect was partially due to an induction of apoptosis via Bax/Bcl-2 and caspase3/7, and partially due to G2/M cell cycle arrest.

2. Materials and Methods

2.1 Materials

Curcumin [≧98.5% (HPLC)], quercetin [≧95% (HPLC)], resveratrol [≧99% (GC)], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Curcumin, quercetin and resveratrol were dissolved in dimethyl sulfoxide (DMSO) and added to medium; the DMSO concentration (0.25%) used in the present study had no significant effect on Caco-2 proliferation.

2.2 Cell culture

Caco-2 human colon cancer cell lines were purchased from the European Collection of Cell Cultures (Salisbury, Wilts, UK) and cultured in Minimum Essential Medium (MEM; Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% FBS (Nichirei Biosciences Inc., Tokyo, Japan) and 1% non-essential amino acids (Life Technologies Corporation, Carlsbad, CA, USA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

2.3 Cell viability assay

Cell viability was measured by MTT assay, as we described previously[24,25]. Briefly, the cells were incubated with the test reagents at a density of 2.5×10^4 cells/2 mL/9.5 cm^2 well for 24 h. After incubation, the medium was removed and the cells were incubated with 1.1 mL of MTT solution (0.1 mL of 5 mg/mL MTT in 1 mL of medium) for 4 h. The product was eluted from cells by the addition of 20% SDS/0.01 M HCl, and absorbance at 595 nm was determined using an SH-1000Lab microplate reader (Corona Electric Co., Ltd, Ibaraki, Japan). Cell viability was calculated according to the following equation: cell viability (%) = (absorbance of experiment group/absorbance of control group) × 100.

2.4 Measurement of cyclooxygenase (COX)-2, Bax and Bcl-2 mRNA, and COX-2 protein expression levels

After curcumin treatment for 3, 6 and 12 h, the cells were collected by centrifugation, and then washed with ice-cold PBS.

2.4.1 Measurement of COX-2, Bax and Bcl-2 mRNA expression levels

Total cellular RNA was prepared using TRIzol reagent. One microgram of total RNA was reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis kit. The concentration and quality of the purified total RNA were determined spectrophotometrically at 260 nm and by the $OD_{260:280}$ ratio. mRNA expression was measured by a real time RT-PCR using a LightCycler FirstStart DNA Master^{plus} SYBR green reagent and LightCycler instrument (Roche). Results were expressed as the relative amount of the target mRNA to β-actin mRNA, and the values in the presence or absence of the drug were expressed as relative amounts to the control.

The primers for β-actin, COX-2, Bax and Bcl-2 were as follows: β-actin, sense, 5'-CCAACCGCGAGAAGATGA-3', antisense, 5'-CCAGAGGCGTACAGGGATAG-3'; COX-2, sense, 5'-GTCTGATGATGTATGCCACAATCTG-3', antisense, 5'-GATGCCAGTGATAGAGGGTGTTAAA-3'; Bcl-2, sense, 5'-TGC ACC TGA CGC CCT TCA C-3', antisense, 5'-AGA CAG CCA GGA GAA ATC AAA CAG-3'; Bax, sense, 5'-ACC AAG AAG CTG AGC GAG TGT C-3', antisense, 5'-ACA AAG ATG GTC ACG GTC TGC C-3'.

2.4.2 Measurement of COX-2 protein expression levels

Cell lysates were analyzed using a SDS-7.5% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes by electroblotting and the membranes were incubated overnight in TBS-T (0.14M NaCl, 20mM Tris and 0.1% Tween 20, pH 7.4) containing primary antibodies (rabbit polyclonal anti-human COX-2 antibody from Cayman Chemical Co., Ann Arbor, MI) and 3% nonfat dry milk. After incubation, the membranes were incubated with secondary goat HRP-linked anti-rabbit IgG antibody for 1 h, followed by ECL (Amersham-Pharmacia Corp., Buckingham, UK).

2.5 Detection of DNA fragmentation

DNA fragmentation in the nucleus was analyzed by an *in Situ* Cell Death Detection Kit, Fluorescein (Roche), as we described previously[26]. Caco-2 cells were seeded in 8-well Lab-Tech Chamber Slides (Greiner bio-one, Monroe, NC, USA) at 42,000 cells/1 mL/5 cm^2 well. One day after seeding, Caco-2 cells were incubated with curcumin for 6 h. TUNEL-positive nuclei were visualized by green coloring using a confocal laser scanning microscope (Carl Zeiss Co., Ltd., LSM-510). The intensity was recorded after excitation at 495 nm and emission at 530 nm, using appropriate software. Data were collected from at least 10 random sections per sample.

2.6 Measurement of caspase-3/7 activity

Changes in caspase-3/7 activity were assayed using a Apo-ONE® Homogenous Caspase-3/7 Assay kit (Promega) after incubation of Caco-2 cells with curcumin for 24 h.

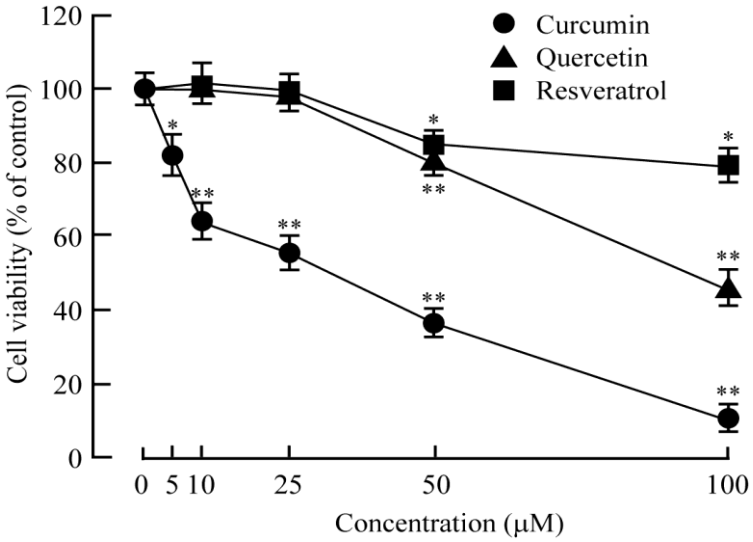
2.7 Cell cycle analysis

The cells were incubated with curcumin at a density of 1.0×10^6 cells/10 mL/55 cm^2 dish for 24 h, and then collected by centrifugation. The pellet was fixed with 70% ethanol cooled at -20°C on ice for 30 min. Following fixation, the cells were incubated with RNase (100 μg/mL) at room temperature for 30 min. The cells were treated with PI (50 μg/mL) in a dark place on ice for 30 min. The samples were filtrated through a nylon mesh (35 μm), and subjected to flow cytometry.

2.8 Statistics

Results are the means ± SEM. The significance of differences between two groups was assessed using the Student's *t*-test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA), followed by Scheffe's multiple range test. P-values less than 0.05 were considered significant.

Fig. 1. Effects of curcumin, quercetin and resveratrol on the proliferation of Caco-2 cells.



Caco-2 cells were treated with curcumin, quercetin and resveratrol for 24 h. Proliferation was assayed by an MTT. Data are expressed as the mean ± SEM (n=3-5). $^{*}P<0.05$, $^{**}P<0.01$; significantly different from the corresponding value in the absence of curcumin, quercetin or resveratrol.

3. Results

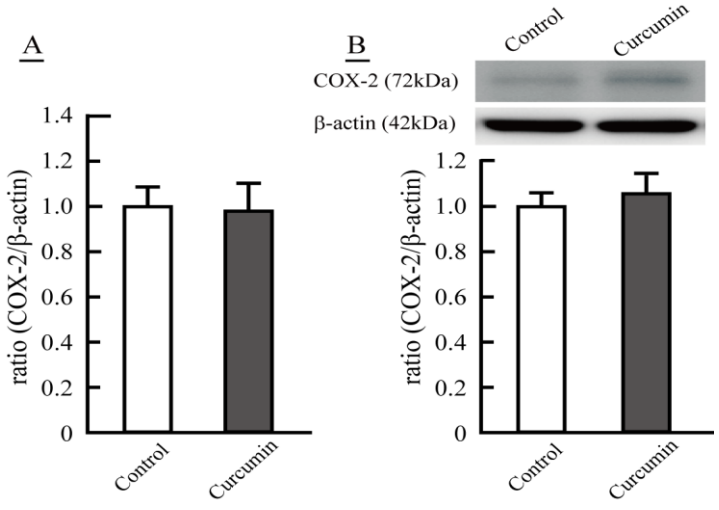
3.1 Curcumin suppressed the proliferation of Caco-2 cells more potently than quercetin and resveratrol

Fig. 1 illustrates the dose-dependent alterations in Caco-2 cell growth by curcumin, quercetin and resveratrol. When Caco-2 cells were incubated with curcumin at concentrations ranging from 5 to 100 μM at 24 h, a dose-dependent suppression in cell growth was obtained, and a significant inhibition appeared even at a 5 μM-curcumin. Quercetin and resveratrol also reduced the proliferation of Caco-2 cells, but the IC_{50} values for these drugs were estimated to be 100 μM or higher. Thus, it appears that curcumin inhibits Caco-2 proliferation more potently than quercetin and resveratrol.

3.2 Curcumin did not have any effect on the COX-2 expression

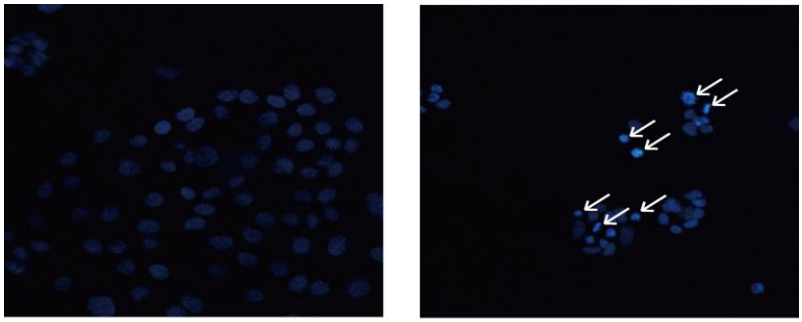
Wu *et al*[27] noted that COX-2 expression was dysregulated in many types of cancer including colorectal cancer, and COX-2-derived eicosanoids elicit multiple oncogenic signals to promote carcinogenesis. We therefore examined the effect of curcumin on the mRNA and protein expressions of COX-2 in Caco-2 cells using real-time RT-PCR and western blotting in a 3 h incubation (Fig. 2): 10 μM curcumin did not have any significant effect on either COX-2 mRNA or protein expression levels when compared to controls. Also, there were no significant alterations in the COX-2 mRNA and protein expression levels at 6 h and 12 h incubation (data not shown). Thus, it appears likely that curcumin has no effect on the COX-2 protein expression in Caco-2 cells.

Fig. 2. Quantification of mRNA (A) and protein (B) expressions of COX-2 in Caco-2 cells treated with curcumin.



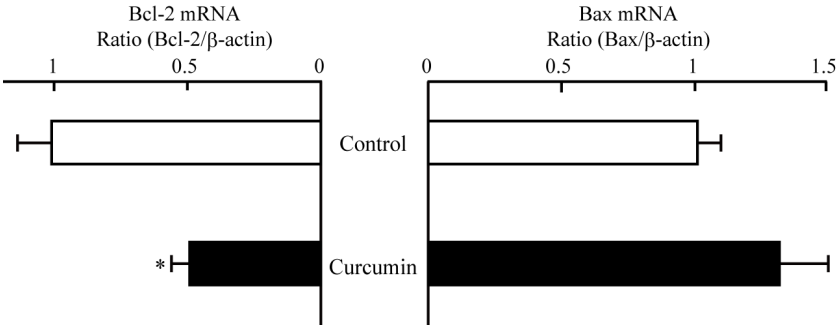
Caco-2 cells were treated with 10 μM curcumin for 3 h. mRNA and protein expressions of cyclooxygenase (COX)-2 were measured by use of a real-time RT-PCR and western blotting. Data are expressed as the mean ± SEM (n=3-5).

Fig. 3. Fluorescence image of DNA fragmentation in Caco-2 cells treated with curcumin.



Caco-2 cells were incubated with or without curcumin for 6 h, and then DNA fragmentation was monitored by an *in Situ* Cell Death Detection Kit, Fluorescein. Blue coloring indicates a cell nucleus stained by DAPI. TUNEL-positive nuclei were visualized by green coloring. Arrows indicate DNA fragmentation in the nucleus as clear light blue coloring (a mixture of blue coloring and green coloring). Data were collected from at least 10 random sections per sample. The data are representative of 4 experiments.

Fig. 4. Effect of curcumin on the mRNA expression levels of Bcl-2 and Bax in Caco-2 cells.

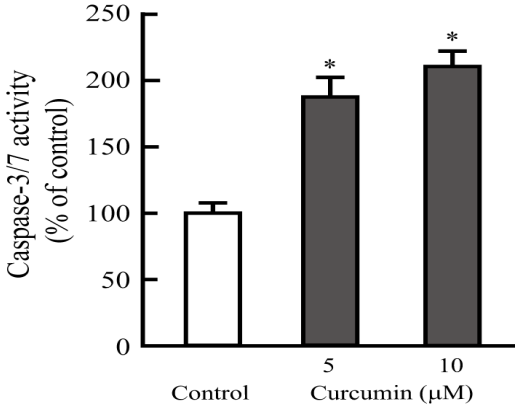


Caco-2 cells were treated with 10 μM curcumin for 3 h. mRNA and protein expressions of Bcl-2 and Bax were measured by use of a real-time RT-PCR. Data are expressed as the mean ± SEM (n=4-5). $^{*}P$<0.01; significantly different from control.

3.3 Curcumin caused apoptosis via an increase in the Bax/Bcl-2 ratio and activation of caspase3/7

As shown in Fig. 3, 10 μM curcumin-treatment for 6 h resulted in DNA fragmentation in the nucleus, measured by the TUNEL method; the control showed only blue coloring which indicates a cell nucleus stained by DAPI. DNA fragmentation was detected using the TUNEL method by green coloring (fluorescein). Many of the Caco-2 cells treated by 10 μM curcumin showed clear light blue coloring (a mixture of blue coloring and green coloring) implying DNA fragmentation in the nucleus. As indicated in Fig. 4, a three hour-treatment of curcumin (10μM) showed a tentative but not significant increase in Bax mRNA expression level, whereas it dramatically reduced the expression level of Bcl-2 (51% inhibition). Thus, the Bax/Bcl-2 ratio treated with curcumin was estimated to be 2.7-fold that of the control. Furthermore, the caspase-3/7 activity was measured after incubation of Caco-2 cells with curcumin for 24 h (Fig. 5). Curcumin significantly enhanced caspase 3/7 activity (5 and 10 μM curcumin; 1.9- and 2.1-fold, respectively). These findings show that curcumin induces apoptosis via an increase in the Bax/Bcl-2 ratio and activation of caspase-3/7.

Fig. 5. Effect of curcumin on the activity of caspase-3/7 in Caco-2 cells.

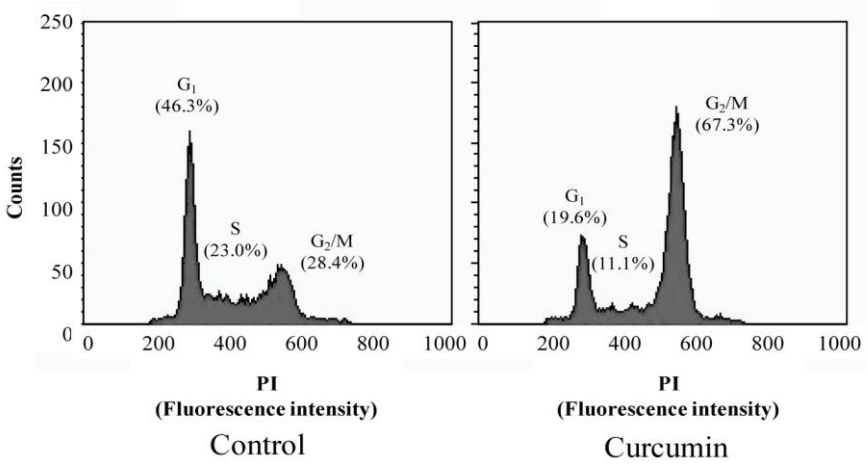


Caco-2 cells were treated with 5 and 10 μM curcumin for 24 h. Changes in caspase-3/7 activity were assayed using a Apo-ONE® Homogenous Caspase-3/7 Assay kit. Data are expressed as the mean ± SEM (n=3). $^{*}P$<0.01; significantly different from control.

3.4 Curcumin induced arrest of G2/M phase in Caco-2 cells

To determine whether curcumin-induced cell growth inhibition involves cell cycle changes, we examined the cell cycle phase distribution by flow cytometry (Fig. 6). A twenty-four-hour treatment of curcumin (10 μM) induced an accumulation of cells in the G2/M phase of the cell cycle. Concomitantly with this increase in the population of cells in the G2/M phase, a decrease in the population of cells in the G1 and S phases was observed. This result implies that curcumin induces G2/M phase arrest in Caco-2 cells.

Fig. 6. Effect of curcumin on cell cycle distribution of Caco-2 cells.



Caco-2 cells were treated with 10 μM curcumin for 24 h. Data are representative of three independent experiments.

4. Discussion

In rats, absorption of curcumin from the intestine was reported to be about 60%[28]. Curcumin and its metabolites formed in intestine and liver are mostly excreted in the feces[29,30], so the colon is a likely target for the anti-carcinogenic activity of curcumin. Moreover, the fact that humans were able to consume up to 8 grams of curcumin per day without toxic effects[31] makes curcumin a very interesting chemopreventive agent for colon cancer. Curcumin chemotherapy and chemoprevention of colon cancer presents many exciting possibilities. However, many aspects of the curcumin effect need to be further evaluated, investigated, and developed.

In the present study, we found that curcumin even at a concentration of 5 μM significantly decreased the cell viability of Caco-2 cells. Although quercetin and resveratrol did so, rather higher concentrations were needed for their inhibitory effects. The measurements of mRNA and protein expressions of COX-2 in Caco-2 cells using real-time RT-PCR and western blotting showed that the curcumin effect was independent of COX-2 protein expression. Investigations concerning DNA fragmentation in the nucleus, Bax and Bcl-2 mRNA expression levels, and the caspase-3/7 activity indicated that curcumin induced apoptosis in Caco-2 cells through an increase in the Bax/Bcl-2 ratio and activation of caspase-3/7. Furthermore, an analysis of flow-cytometry showed that curcumin caused an arrest of the G2/M phase in Caco-2 cells.

The concentrations of curcumin in normal and malignant colorectal tissue of twelve patients (5 females, 7 male, ages 47-72) receiving 3.6 grams of curcumin were reported to be 7.7-12.7 nmol/g[32]. It has also been estimated that the concentration of curcumin in the human intestinal lumen can be as high as 270 μM after consuming one gram of curcumin, with a meal and/or as supplements[29,30]. A similar concentration range for curcumin in the colon was reported by Wortelboer *et al*[33] Therefore, the present result that curcumin at 5 μM or higher significantly reduced Caco-2 proliferation may imply its potent preventive capacity against colon cancer under physiological conditions.

Lev-Ari *et al*[4] reported that curcumin inhibited COX-2 expression levels in HT-29 and SW-480 cells, and that these inhibitions were associated with growth retardation of the cells. Sandur *et al*[5] showed that curcumin potentiated radiation-induced cytotoxicity of HT-29, HCT-116 and SW-620 cells partially though a suppressive influence on COX-2 expression levels. In contrast, we found no significant alteration in COX-2 protein expression levels when Caco-2 cells were incubated with 10 μM curcumin. Thus, it seems likely that curcumin inhibits Caco-2 proliferation independently of COX-2 protein expression. This observation does not mean independence of COX-2 in the curcumin-induced inhibition of Caco-2 proliferation, because of possibility of its modulatory effect on the activity of COX-2. Further studies are needed to clarify the exact involvement of COX-2 in the curcumin-induced inhibition of Caco-2 proliferation.

Guo *et al*[12] showed that curcumin inhibited proliferation and induced apoptosis of LoVo cells by activating the mitochondrial apoptotic pathway, namely by increasing the Bax/Bcl-2 ratio and by activating caspase-3. The same results were reported by Shakibaei *et al*[9] and Chen *et al*[10], by use of HCT-116. These previous observations are in accordance with our present results that curcumin increased the Bax/Bcl-2 ratio and activated caspase-3/7 activity. Therefore, it appears that curcumin inhibits Caco-2 cell proliferation by activating the mitochondrial apoptotic pathway.

Chen *et al*[11] reported that LoVo cells treated with curcumin were largely accumulated in S and G2/M phases which

prevented cells from entering the next cell cycle. Using HCT-116, Jaiswal *et al*[7] and Chen *et al*[10] showed that curcumin treatment caused G2/M phase cell cycle arrest. The present result that curcumin induced G2/M cell cycle arrest in Caco-2 may mean that the curcumin effect on the cell cycle mainly emerges in the G2/M phase.

In conclusion, in the present study, we found that curcumin inhibited Caco-2 cell proliferation through both an activation of the mitochondrial apoptotic pathway, and G2/M cell cycle arrest. Our findings provide increased insight into the mechanism of action of curcumin in colon cancer cells, and may help researchers in this area to understand how this compound can protect against development of colon cancer.

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