RESEARCH ARTICLE

Curcumin Induces Apoptosis in SGC-7901 Gastric Adenocarcinoma Cells via Regulation of Mitochondrial Signaling Pathways

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Abstract

Curcumin, a polyphenol compound derived from the rhizome of the plant *Curcuma longa* L. has been verified as an anticancer compound against several types of cancer. However, understanding of the molecular mechanisms by which it induces apoptosis is limited. In this study, the anticancer efficacy of curcumin was investigated in human gastric adenocarcinoma SGC-7901 cells. The results demonstrated that curcumin induced morphological changes and decreased cell viability. Apoptosis triggered by curcumin was visualized using Annexin V-FITC/7-AAD staining. Curcumin-induced apoptosis of SGC-7901 cells was associated with the dissipation of mitochondrial membrane potential (MMP) and the release of cytochrome c into the cytosol. Furthermore, the down-regulation of Bcl-2 and up-regulation of Bax that led to the cleavage of caspase-3 and increased cleaved PARP was observed in SGC-7901 cells treated with curcumin. Therefore, curcumin-induced apoptosis of SGC-7901 cells might be mediated through the mitochondria pathway, which gives the rationale for in vivo studies on the utilization of curcumin as a potential cancer therapeutic compound.

Keywords: Curcumin - human gastric adenocarcinoma - mitochondrial signaling pathway - apoptosis

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Introduction

Gastric carcinoma (GC) remains the second leading cause of cancer-related death worldwide (Huang et al., 2013). According to the statistics, the 5-year relative survival rate of gastric cancer patients is 24.3% (Shin et al., 2012). Only 20% of the patients are suitable for curative resection because the majority of diagnosed patients are locally advanced or metastatic (Ajani et al., 2013; Wang et al., 2013; Li et al., 2014). Chemotherapy is the main adjuvant treatment for postoperative and advanced gastric cancer therapy. Currently, platinum, 5-fuorouracil (5-FU), and taxanes are recommended as the first-line chemotherapy for the treatment of gastric cancer (Chen et al., 2013). However, the response rate of drug chemotherapy remains lower than 20% and the chemotherapeutics sometimes lead to severe toxicity at their therapeutic dose (Lorenzen et al., 2013). Therefore, to discover the effective and novel chemotherapy is requisite.

Curcumin (1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione), derived from the rhizome of the plant Curcuma longa L, has been used for thousands of years in Asia countries as a food additive, cosmetic, and as a traditional herbal medicine (Jiang et al., 2014; Shoji et al., 2014). Recently, amount of studies have shown that curcumin possessed the anticarcinogenic properties by modulating various mechanisms linked with the development and progression of cancer (Hasan et al., 2014). Apoptosis plays an important role in the treatment of cancer as it is a popular target of many treatment strategies (Wong, 2011; Li et al., 2013; Singh et al., 2013; Gopal et al., 2014; Li et al., 2014). The abundance of literature suggests that targeting apoptosis in cancer is feasible. However, the accurate pro-apoptotic role of curcumin and its underlying mechanism still have not been completely known. The present study attempts to determine the pro-apoptotic effect of curcumin and to elucidate the effect of curcumin on apoptosis involving in the collapse of mitochondrial function.

Materials and Methods

Drugs

Curcumin (Sigma-Aldrich, Inc., St. Louis, Mo, USA) was dissolved in DMSO at 20mM as a stock solution. The dilutions of all of the reagents were freshly prepared before each experiment.

Cell lines

The human gastric adenocarcinoma cell lines SGC-

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7901 which are poorly differentiated were purchased from Cell Bank, China Academy of Sciences (Shanghai, China). Cancer cells were maintained in RPMI-1640 (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), penicillin-streptomycin (100 IU/ml to 100 μg/ml), 2 mM glutamine, and 10 mM HEPES buffer at 37°C in a humidified atmosphere (5% CO_2-95% air). Cells were harvested by brief incubation in 0.02% (w/v) EDTA in PBS.

Growth and cell proliferation analysis

The proliferation of gastric adenocarcinoma cells was evaluated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. SGC-7901 cells (5×10^3 per well) seeded in 96-well plates were incubated with increasing concentrations of curcumin for 24, 48 and 72 h, respectively. The controls were treated with an equal volume of the drug's vehicle DMSO, but the applied concentration did not exhibit a modulating effect on cell growth. Thereafter, cell growth inhibition was evaluated by MTT assay as described elsewhere (Banjerdpongchai, 2013).

Staining of cells with Hoechst 33258

SGC-7901 cells seeded in 24-well plates (6×10^4 per well) were treated with increasing concentrations of curcumin for 24 h. Cancer cells were fixed and stained with Hoechst 33258 (Sigma, USA). The apoptotic cells were visualized with fluorescence microscope (Leica Microsystems Holdings GmbH, Germany). Cells were scored apoptotic if the nuclei presented chromatin condensation, marginalization or nuclear beading (Zhang et al., 2013).

Annexin V/FITC and 7-AAD staining analysis

SGC-7901 cells seeded in 6-well plates (1.5×10^5 per well) were treated with increasing concentrations of curcumin for 24 h. Cells were harvested and washed with cold PBS. The cell surface phosphatidylserine in apoptotic cells was quantitatively estimated by using Annexin V/FITC and 7-AAD apoptosis detection kit according to manufacturer's instructions (Roche, USA). Cell apoptosis was analyzed on a FACScan flow cytometry (Becton Dickinson, USA) (Xue et al., 2012). Triplicate experiments with triplicate samples were performed.

Mitochondria membrane permeability assay

The mitochondria membrane potential (MMP) was analyzed by using a JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3-tetraethylbenzimidazolocarbocyanine Iodide) fluorescence probe kit (Beyotime, China) as described previously (Hossein et al., 2013). The lipophilic and cationic fluorescent dye JC-1 is capable of selectively entering mitochondria, where it forms aggregates and emits red fluorescence when MMP is high. At low MMP, JC-1 cannot enter into mitochondria and forms monomers emitting green fluorescence. The ratio of green to red fluorescence provides an estimate of changes in MMP. Briefly, SGC-7901cells cultured in six-well plates exposed to 2.5, 5, and 10 μM curcumin for 24 h and then were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37°C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio.

Preparation of mitochondria and cytosol

Mitochondria/cytosol kit (Beyotime, China) was used to isolate mitochondria and cytosol according to the manufacture's protocol. After washing with cold PBS, cancer cells (5×10^7) were suspended in 500 μl of isolation buffer containing protease inhibitors and lysed on ice for 10 min. Cells were mechanically homogenized with Dunce grinder. The unbroken cells, debris and nuclei were discarded by centrifugation at 800 g for 10 min at 4°C. The supernatants were centrifuged at 12,000 g for 15 min at 4°C. The supernatant cytosol was collected and pellet fraction mitochondria were dissolved in 50 μl of lysis buffer.

Western blotting assay

Western blotting assay was performed to analyze the expressions of apoptotic and related mitochondrial molecules in SGC-7901 cells. Briefly, SGC-7901 cells (3×10^5) seeded in 6-well plates were exposed to various concentrations of curcumin for 72 h. The cells were harvested and lysates (50 μg of protein per lane) were fractionated by 10% SDS-PAGE as described below. The proteins were electro-transferred onto PVDF membranes, and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in TBS containing 5% nonfat milk. The bound antibodies were visualized by using an enhanced chemiluminescence reagent (Millipore, USA) followed by exposure to X-ray film. Data was expressed as the relative density of the protein normalized to β-actin. The percentages of increase or decrease of protein were estimated by comparison to vehicle control (100%) (Szaflarski et al., 2013). Triplicate experiments with triplicate samples were performed. The primary antibodies included anti-cytochrome c (Immunoway, YT1186), anti-caspase-3 (9662, Cell Signaling), anti-cleaved PARP (9541, Cell Signaling), anti-Bcl-2 (2772, Cell Signaling), anti-Bax (2872, Cell Signaling), and anti-β-actin (ab6276, Abcam).

Statistical analysis

Data was described as mean±S.D., and analyzed by one-way ANOVA. A p value less than 0.05 was considered statistically significant. Statistical analysis was done with SPSS/Win11.0 software (SPSS Inc., Chicago, IL.).

Results

Inhibition of human gastric adenocarcinoma cell proliferation

Human gastric adenocarcinoma were treated with curcumin for 24 h, 48 h, and 72 h, respectively and then subjected to the MTT assay. Curcumin effectively inhibited

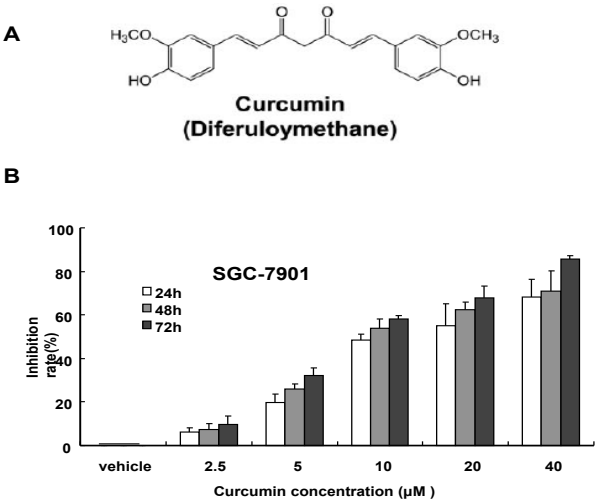

Figure 1. The Growth Inhibition Effect of SGC-7901Cells Induced by Curcumin. A) The chemical structure of curcumin. **B)** SGC-7901 cells were exposed to increasing concentrations of curcumin or an equal volume of the drug's vehicle DMSO for up to 72 h. Viable cells were evaluated by MTT assay and denoted as a percentage of untreated controls at the concurrent time point. The bars indicate mean±S.D. (n=3)

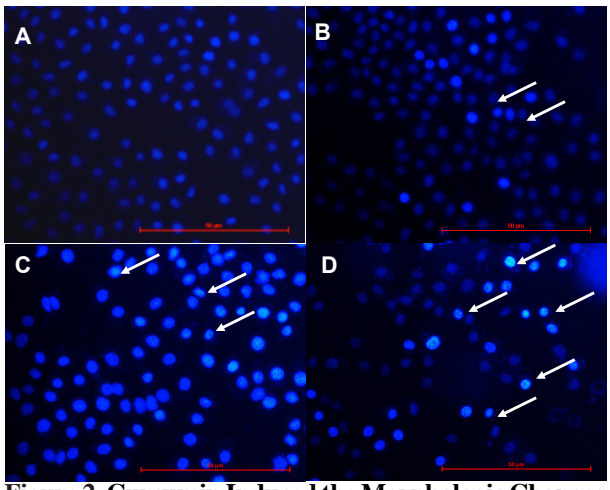

Figure 2. Curcumin Induced the Morphologic Changes of SGC-7901 Cells In Vitro. SGC-7901 cells treated with curcumin were subjected to Hoechst 33258 staining and viewed under microscope. **A)** Vehicle control; **B)** 5 μM; **C)** 10 μM; **D)** 20 μM. A scale bar, 50 μm. The white arrows indicated characteristics of apoptotic cells

the proliferation of human gastric adenocarcinoma SGC-7901 cells. As shown in Figure 1, the inhibition rate increased from 6.15% to 68.38% after treatment with curcumin for 24h, from 7.21% to 71.06% for 48 h, from 9.87% to 85.75% for 72h. The maximum inhibition rate of 85.75% was found with use of 40 μM for 72 h treatment. These results indicated that curcumin had a dose- and time-dependent antiproliferative effect on SGC-7901 cells in the range of 2.5-40 μM for up to 24 h, 48 h, and 72 h of exposure.

Induction of human gastric adenocarcinoma cell apoptosis

To evaluate the curcumin-induced cell apoptosis of SGC-7901 cells, we examined the morphologic changes by Hoechst 33258 staining (Figure 2). When SGC-7901 cells were cultured with 5μM, 10μM and 20 μM curcumin for 24 h, the apoptotic morphologic changes were

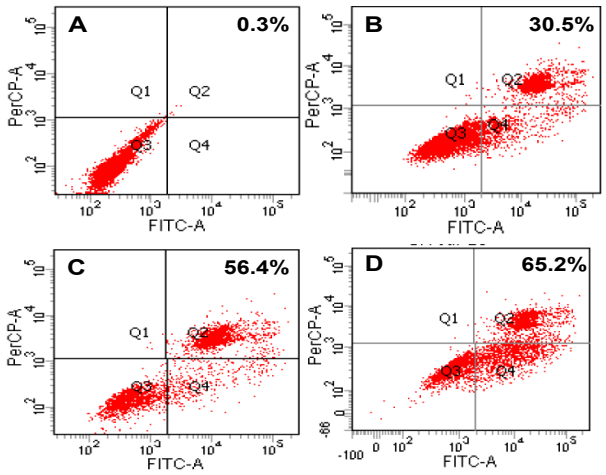

Figure 3. Detection of Apoptotic Cells After Annexin V/7-AAD Staining by Flow Cytometric Analysis. SGC-7901 cells were exposed to increasing concentrations of curcumin for 24 h. Cells were harvested and stained with AnnexinV/7-AAD. **A)** Vehicle control; **B)** 5 μM; **C)** 10 μM; **D)** 20 μM

observed as compared with the vehicle control. In vehicle control group, nuclei of SGC-7901cells were round and homogeneously stained (Figure 2A). However, curcumin-treated cells exhibited evident apoptosis characteristics including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation and chromatin compaction of late apoptotic appearance (Figure 2B-D).

SGC-7901cells were then stained with Annexin V/FITC and 7-AAD, and were analyzed by flow cytometry assay. The results showed the increase of apoptotic cells after exposure to curcumin for 24 h. In the concentrations of 5 μM, 10 μM and 20 μM of curcumin, the percentage of apoptotic cells was 30.5% to 56.4% and 65.2%, respectively, in SGC-7901 cells (Figure 3A-D).

Induction of MMP collapse

JC-1 fluorescence probe showed that MMP in SGC-7901 cells was significantly decreased after treatment with curcumin. As shown in Figure4A, the red fluorescence of JC-1 was gradually decreased and the green fluorescence was correspondingly increased after curcumin treatment. In the range of 5-20 μM, the ratios of green to red fluorescence were increased in a dose-dependent manner ($p<0.01$ *vs* vehicle control, Figure 4B). These results indicated the collapse of MMP in SGC-7901 cells after treatment with curcumin.

Release of cytochrome c from mitochondria to cytosol

The levels of cytochrome c were then examined by Western blotting assay. Cytochrome c in SGC-7901 cells was redistributed after curcumin treatment. In SGC-7901 cells, the level of cytochrome c in mitochondria was significantly decreased by 48.2%, 77.6%. Correspondingly, the levels of cytochrome c in cytosol were increased by 125.5%, 168.7%, respectively ($p<0.01$ *vs* vehicle control, Figure 5).

Increase of Bax/Bcl-2 ratio and apoptosis-related protein

Furthermore, we examined the expressions of Bax

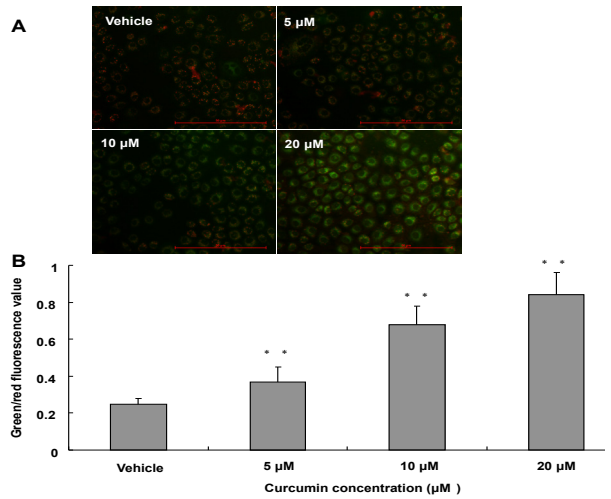

Figure 4. Curcumin Induced Mitochondrial Membrane Potentials Collapse in SGC-7901 Cells. SGC-7901 cells were stained with JC-1 probe and imaged by fluorescent microscope. The individual red and green average fluorescence intensities are expressed as the ratio of green to red fluorescence. **A)** A decrease of red fluorescence ratio indicates a shift correlating with a reduction in mitochondrial depolarization. Representative photographs of JC-1 staining in different groups. A scale bar, 50 μm. **B)** Quantitative analysis of the shift of mitochondrial red fluorescence to green fluorescence among groups. All values are denoted as means±S.D. from ten independent photographs shot in each group. Significant differences are indicated as: $p<0.01$ compared with vehicle control cells cultured in complete medium

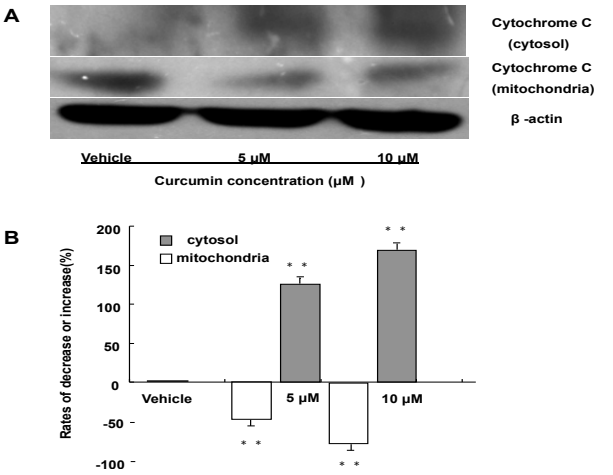

Figure 5. Curcumin Induced the Redistribution of Cytochrome c in SGC7901 Cells as Estimated by Western Blotting Assay. A) SGC7901 cells were exposed to various concentrations of curcumin and the levels of cytochrome c in mitochondria and cytosol were measured by Western blot analysis. **B)** Quantitative analysis of the expression of cytochrome c in mitochondria and cytosol. The bars indicated mean±S.D. (n=3). *$p<0.05$, **$p<0.01$ *vs* vehicle control. Triplicate experiments were performed with triplicate samples

and Bcl-2 and then analyzed the ratio of Bax/Bcl-2. As shown in Figure 6A-B, the level of Bax was significantly increased and Bcl-2 was obviously decreased in curcumin-treated cancer cells. Statistical analysis showed that curcumin in the range of 5μM increased the ratio of Bax/Bcl-2 by 50.2% ($p<0.05$ *vs* vehicle control), the ratio increased by 184% and 292.6% for 10μM-20μM ($p<0.01$ *vs* vehicle control), respectively.

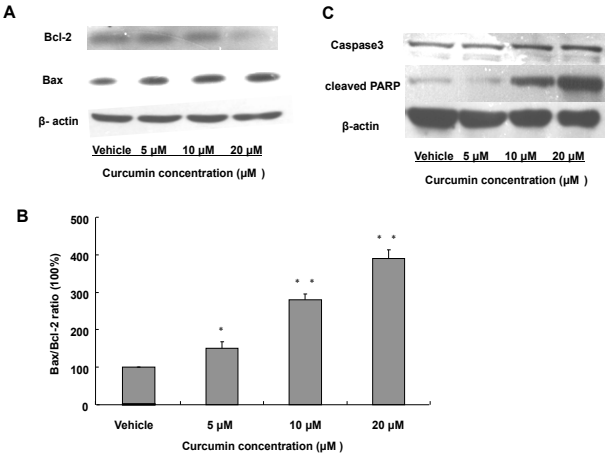

Figure 6. Curcumin increased the Bax/Bcl-2 ratio and apoptosis-related protein. A) SGC7901 cells were exposed to various concentrations of curcumin and the levels of Bax and Bcl-2 were measured by Western blot analysis. **B)** The ratio of Bax/Bcl-2 was calculated. The bars indicated mean±S.D. (n=3). *$p<0.05$, **$p<0.01$ *vs* vehicle control. Triplicate experiments were performed with triplicate samples. **C)** SGC7901 cells were exposed to various concentrations of curcumin and the levels of apoptosis-related protein Caspase3 and cleaved PARP were measured by Western blot analysis

Additionally, we measured the molecular alteration of apoptosis related proteins in curcumin-treated cells. Curcumin was found to activate the caspases cascade pathway as demonstrated by the increases of caspase-3 and cleaved PARP in SGC7901 cells. As shown in Figure6C, the levels of caspase-3 and cleaved PARP were significantly increased in SGC-7901 cells exposure to curcumin.

Discussion

A number of studies revealed that curcumin exerts its antitumor effects by apoptosis induction. However, the accurate pro-apoptotic role of curcumin and its underlying mechanism still have not been completely known. In the present study, the results showed that curcumin inhibited the proliferation of SGC-7901 cells in a concentration- and time-dependent manner. There was a significant growth inhibition from the concentration of 5 μM curcumin. Based on the results of Hoechst 33258 staining and Annexin V-FITC, we concluded that curcumin induced apoptosis in SGC-7901 cells.

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions (Zhang et al., 2013). However, the program of cell apoptosis in cancer cells is disrupted, thus results in the overgrowth of malignant cells (Wang et al., 2013). Induction of tumor cell apoptosis is the final goal of most anti-cancer drugs, as well as the potential pro-anticancer drugs (Rodriguez-Nieto et al., 2006). Two pathways are identified to be involved in apoptosis induction including death receptor-mediated extrinsic and mitochondria-mediated intrinsic pathways (Tomek et al., 2012). The intrinsic apoptotic pathway is characterized by permeability of the mitochondria and release of cytochrome c from mitochondria into the cytoplasm; and the extrinsic apoptotic pathway is activated by

death receptors on the plasma membrane such as tumor necrosis factor receptor 1 (TNFR1) and Fas/CD95 (Liu et al., 2013; Méndez et al., 2014; Sharoar et al., 2014; Tyagi et al., 2014). Both pathways ultimately lead to the activation of the executioner Caspases-3 via diverse proapoptotic signals and finally cell death (Ma et al., 2014). The cleavage of poly (-ADP-ribose) polymerase PARP which is a specific substrate for caspase-3, caused by hydrolyzation of caspase-3, can reflect the activity of caspase-3 (Gajek et al., 2014). In this study, 5 μM -20 μM curcumin remarkably increased the expression of caspase-3 and cleaved PARP. These results suggested that curcumin caused the apoptosis of SGC-7901 cells. However, it was worthy concerning which kind of pathway was involved the curcumin-induced apoptosis.

The intrinsic apoptotic pathway mediated by mitochondria was mainly triggered by the collapse of mitochondria membrane potential (Zhang et al., 2014). The collapse prompted the release of pro-apoptotic molecules cytochrome-c into the cytoplasm, which has been proposed as a 'point of no return' in mitochondrial pathway (Aporta et al., 2014). Besides, this pathway is closely regulated by a group of proteins belonging to the Bcl-2 family including the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) (Chan et al., 2004; Liu et al., 2005;Ko et al., 2011). The release of cytochrome c is associated with the increase of Bax and decrease of Bcl-2 followed by activating caspase-9, caspase-3 and cleavage of PARP (Lucena et al., 2013).Our data observed that cytochrome c in SGC-7901 cells was redistributed after curcumin treatment. The level of cytochrome c in mitochondria was significantly decreased and increased in cytosol correspondingly. Furthermore, the expression level of Bax protein was significantly up-regulated and the expression levels of Bcl-2 protein began to decrease simultaneously after curcumin treatment. The Bax/Bcl-2 ratio was up-regulated dramatically and ultimately induced the occurrence of mitochondria-mediated cell apoptosis. We thus suggested that the induction of apoptosis in SGC-7901 cells by curcumin might be due to the activation of mitochondria mediated intrinsic apoptosis pathway.

In conclusion, our results suggested that curcumin possessed the activity of anti-proliferation and apoptosis induction in human gastric adenocarcinoma SGC-7901 cells. Curcumin-induced apoptosis of SGC-7901 cells might be mediated through the mitochondria pathway. These results support the potential of curcumin to be developed as a promising agent for treatment of cancers.

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