Original Article
Curcumin induces apoptosis in breast cancer cells and inhibits tumor growth *in vitro* and *in vivo*

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Abstract: Curcumin has shown therapeutic and/or adjuvant therapeutic effects on the treatment of some patients with breast cancer. However, its mechanisms of action are largely unknown. This study was designed to investigate its antitumor effect and underlying mechanisms in human breast cancer MDA-MB-231 and MCF-7 cells. The MTT assay was used to evaluate cell viability, and flow cytometry, acridine orange staining and transmission electron microscopy were used to detect apoptosis for cultured cells. The protein expression in cells was evaluated by western blot analysis. Breast tumors were established by subcutaneous injection of MDA-MB-231 cells in nude BALB/c mice, and curcumin was administered to the mice. The size of tumors was monitored and the weight of tumors was examined. The exposure of breast cancer cells to curcumin resulted in growth inhibition and the induction of apoptosis in a dose-dependent manner. We also found that the expression of Bcl-2 protein decreased and the expression of Bax protein increased which lead to an increase of the Bax/Bcl-2 ratio. In mice bearing MDA-MB-231 xenograft tumors, administration of curcumin showed a significant decrease of tumor volumes and tumor weight compared with the control. Our results showed that curcumin exhibited antitumor effects in breast cancer cells with an induction of apoptosis.

Keywords: Curcumin, breast carcinoma, apoptosis, nude mice

Introduction

Breast cancer is the second leading cause of cancer-related deaths among females in the United States. Its rate in China and other Asian countries is also increasing rapidly [1]. To find novel natural compounds with low toxicity and high selectivity for killing cancer cells is an important area in cancer research [2]. To date, chemotherapy has been the most frequently used treatment for breast cancer and other cancers. However, some normal cells are destroyed as well by this method of treatment. Due to their wide range of biological activities and low toxicity in animal models, some natural products have been used as alternative treatments for cancers including breast cancer [3, 4].

Curcumin has been widely studied for its anti-inflammatory, anti-angiogenic, antioxidant, wound healing and anti-cancer effects because of its medicinal properties in Indian and Chinese systems of medicine [5, 6]. Moreover, extensive research has shown that curcumin possesses anti-proliferative and anti-carcinogenic properties in a wide variety of cell lines and animals [7]. In addition, recent studies have shown that curcumin, either alone or in combination with other anticancer agents, can efficiently induce apoptosis. This is evidenced by its inhibitory effects on the growth of a number of tumor cell lines both in vitro and in vivo, including melanoma, mantle cell lymphoma, hepatic, prostatic, ovarian and pancreatic carcinomas [8]. Apoptosis is a tightly regulated process of programmed cell death, including the activation of various molecules for initiating cell death. Specific activation of apoptosis in tumor cells offers a promising approach for cancer therapy. However, the specific mechanisms of curcumin induced cytotoxicity remain controversial due to the variable anti- and pro-apoptotic signaling pathways in different cell types. Furthermore,
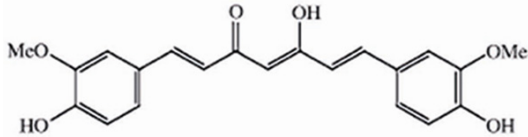
Figure 1. Chemical structures of curcumin.

the exact mechanism by which curcumin exerts its apoptotic effects in breast cancer cells still remains unclear.

The present study examined the anti-proliferative activity of curcumin and its effect on the apoptosis of breast cancer cells. Then, the levels of several important proteins that are strongly associated with the signal-transduction pathway of apoptosis were measured to establish the anticancer mechanism of curcumin. Furthermore, we used a nude mouse model to confirm the antitumor effect of curcumin in vivo.

Materials and methods

Reagents

Curcumin was purchased from Sigma-Aldrich Corporation and was prepared with dimethyl sulfoxide (DMSO) at the concentration of 10 mM, stored as small aliquots at -20°C, and thawed and diluted as needed in cell culture medium. Its chemical structures are shown in **Figure 1**. DMSO, propidium iodide (PI) and trypsin were purchased from Sigma (St. Louis, MO, USA). RPMI-1640, penicillin, streptomycin and other cell culture supplies were from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA). Monoclonal Bcl-2, Bax and GAPDH primary antibodies, as well as second antibody goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl trazolium bromide] and acridine orange (AO) were obtained from Fluka (Ronkonkoma, NY, USA).

Cell line and culture

Two human breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the Cancer Research Institute of Beijing, China. These cells were cultivated in T75 tissue culture flasks in RPMI-1640 supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml strepto-

mycin, 2 mM L-glutamine, and 20 mM hydroxyethyl piperazine ethanesulfonic acid, and incubated in a humidified incubator containing 5% CO_2 at 37°C.

MTT assay

Cell viability was assessed using MTT assay. Breast cancer (5×10^3) cells were seeded in 200 µl of RPMI-1640 medium into 96-well plates, and cultured overnight. Then the medium was replaced with fresh RPMI-1640 or the same media containing different concentrations of curcumin. After a further incubation for 24 or 48 h, 50 µl of MTT (2 mg/ml) was added to each well followed by 4 h incubation. The medium was discarded and 150 µl of dimethyl sulfoxide was added into each well, and incubated for 20 min. The OD490 nm was measured. The cell viability index was calculated according to the formula: (experimental OD value/control OD value) × 100%.

Flow cytometric analysis

Breast cancer cells were exposed to curcumin. The attached and floating cells were mixed and washed with PBS. The cells were centrifuged, and the pellets were resuspended with 5 ml of cold 70% ethanol and fixed overnight at 4°C. The fixed cells were washed twice in PBS. Then 50 µl of RNase (10 µg/ml) and 25 µl of propidium iodide (1 mg/ml) were added to the cells for 30 min at room temperature in the dark. Flow cytometric analysis was performed with a FACS Caliber. For each analysis, 10,000 events were collected and analyzed with Cell Quest software.

Transmission electron microscopy

The breast cancer cells were trypsinized and then fixed in ice-cold 2.5% electron microscopy grade glutaraldehyde in PBS (pH 7.3). The specimens were rinsed with PBS, postfixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30-90%), and embedded in Epon. Semithin (300 nm) sections were cut using a Reichart Ultracut, stained with 0.5% toluidine blue, and examined under a light microscope. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate, and examined on a transmission electron microscope at × 5,000 magnification.
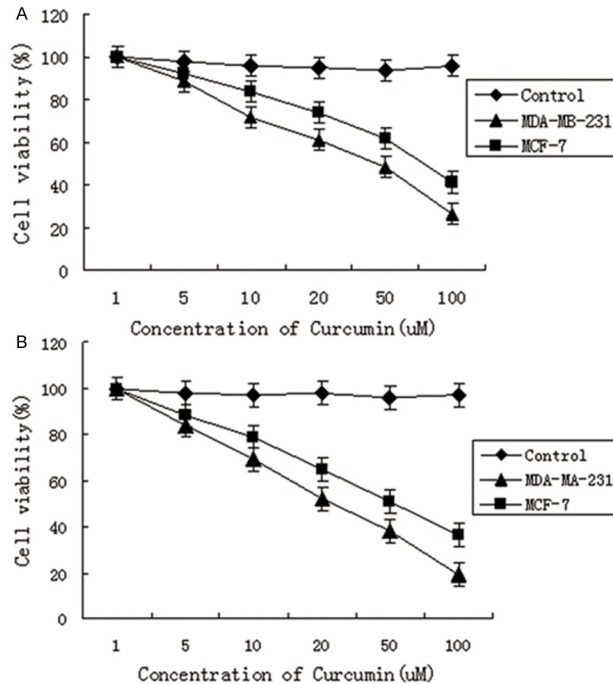
Figure 2. Effect of curcumin on the proliferation of MDA-MB-231 and MCF-7. Cells were treated with different concentrations of curcumin for 24 h (A) and 48 h (B). The results are expressed as the mean ± SD of three independent experiments.

In situ detection of apoptosis

Breast cancer cells were cultured to subconfluence in a 24-chamber slide with 10% FCS containing RPMI-1640. The medium then was changed to test solutions. After incubation for 24 h or 48 h, apoptosis was quantified by fluorescent staining methods: the detection of AO/EB condensed nuclei by fluorescent microscopy. AO/EB staining identifies alive, early apoptotic, late apoptotic, and necrotic cells. Subconfluent cells (70-80% confluent) in 24-well uncoated plates were exposed to apoptotic stimuli for 48 h. Breast cancer cells in 24 wells were gently washed with PBS and immediately treated with acridine orange (100 µg/mL) for 5 min and ethidium bromide (100 µg/mL) for 5 min. Each well was then examined immediately under an epifluorescence microscope. For evaluation by fluorescence microscopy, an excitation wavelength of 455 nm is used. Apoptosis is defined by morphological criteria. Cells containing normal nuclear chromatin will exhibit green nuclear staining. Cells containing fragmented nuclear chromatin will exhibit orange to red nuclear staining.

Western blotting

Total cellular protein was extracted using analysis buffer and quantified using protein quantification reagents from Bio-Rad. Next, 60 µg of the protein were suspended in 5 × reducing sample buffer, boiled for 5 min, electrophoresed on 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was blocked in 1% BSA/0.05% Tween/PBS solution overnight at 4°C, followed by incubation with the primary antibody for 24 h. A horseradish peroxidase-labeled goat anti-mouse IgG was used as the secondary antibody. The blots were then developed by incubation in a chemiluminescence substrate and exposed to X-ray film.

Animal experiment

Female BALB/c nude mice, 35-40 days old and weighing 20-22 g, were supplied by Shanghai Slac Laboratory Animal Limited Company. The mice were kept under sterile conditions and fed a sterilized mouse diet and water. Animals were anaesthetized via inhalation of isoflurance and a tumor cell suspension of 2×10^6 MDA-MB-231 cells in 0.1 ml RPMI-1640 was injected subcutaneously into the dorsa of each mouse. When tumors reached around 60 mm^3 at about 2 weeks, the mice were randomly assigned to three groups (n = 8/group). The mice were treated with curcumin 50 ug/kg, 200 ug/kg or saline (control) by intraperitoneal injections every other day for 4 weeks. Tumor size was measured once every 4 days in two perpendicular dimensions with vernier calipers and converted to tumor volume using the formula: $(ab^2)/2$, where a and b refer to the longer and shorter dimensions, respectively. The body weight of the animals was measured once every 4 days at the same time as the tumor dimension measurement and the mortality was monitored daily. After the treatments, all mice were sacrificed and tumor was segregated and weighed. All experiments were carried out according to the Guidelines for Animal Experimentation of the Qingdao University. The experimental protocol was approved by the Ethics Committee for Animal Experimentation, and was performed according to the Guidelines for Animal Experimentation
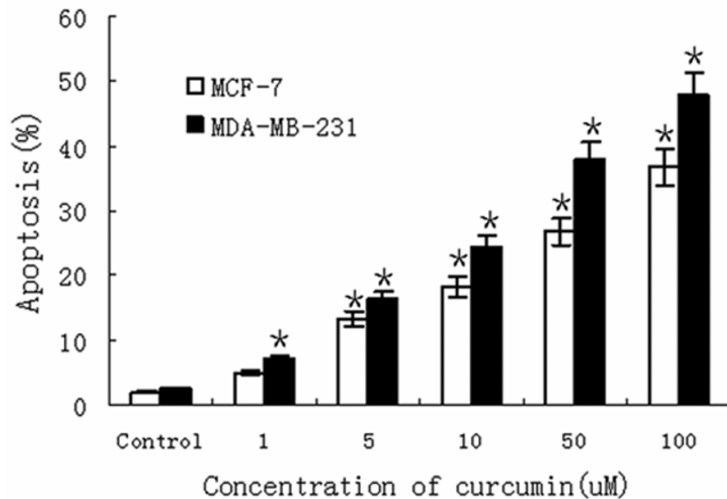
Figure 3. Percentages of MDA-MB-231 and MCF-7 cells in the sub-G1 group (apoptosis) after treatment with Curcumin for 48 h. *$P < 0.05$.
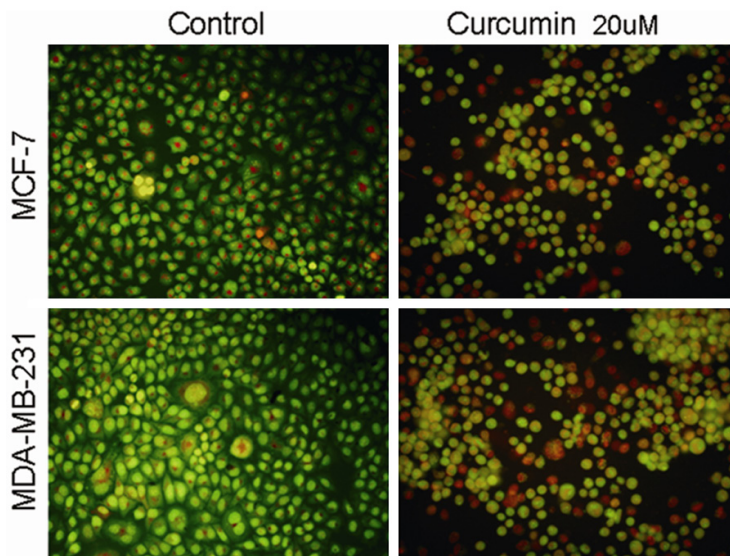
Figure 4. Cell apoptosis observed by acridine orange/ethidium bromide (AO/EB) staining at 48 h. Cells containing normal nuclear chromatin exhibit green nuclear staining. Cells containing fragmented nuclear chromatin exhibit orange to red nuclear staining. (× 100).

of the Qingdao University and to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis

All data were summarized as mean ± SE, where appropriate. The student's t-test was performed for the comparison of control and curcumin treatment groups. Differences were considered statistically significant when the *P*-value was ≤ 0.05.

Results

Cell growth inhibition

To determine the growth inhibitory activity of curcumin, breast cancer cells MDA-MB-231 and MCF-7 were treated with curcumin for 24 h or 48 h and viable cells were measured by MTT assay. MDA-MB-231 and MCF-7 cells exposed to curcumin resulted in a significant decrease in viable cells in a time- and dose-dependent manner (**Figure 2**). Effective concentrations were selected for all further mechanistic studies.
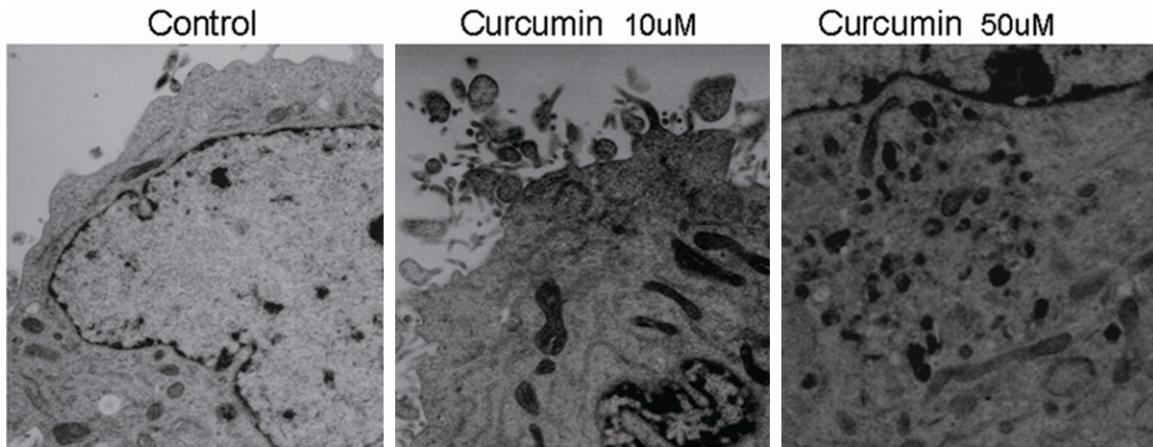
Induction of apoptosis by curcumin

In order to determine whether the growth inhibition by curcumin was associated with apoptosis, breast cancer cells treated with curcumin were analyzed for the amount of sub-G1 DNA by flow cytometry of fixed nuclei to quantify the degree of apoptosis. As shown in **Figure 3**, treatment of breast cancer cells with different concentrations of curcumin resulted in an increase of the number of apoptotic cells in a dose-dependent manner. We further examined the morphological changes of breast cancer cells treated with curcumin by fluorescence microscopy. The control cells displayed an intact nuclear structure, while cells treated with curcumin had chromosomal condensation and formation of apoptotic bodies (**Figure 4**). The mainultra-microstructural changes seen in all treated groups were chromatin aggregation, mitochondrial denaturation and apoptotic body formation, as well as cytoplasmic compartments, swelling and disappearance of mitochondrial cristate (**Figure 5**).
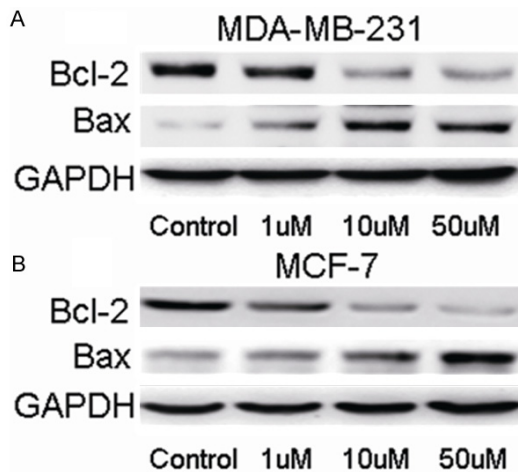
Effect of curcumin on the expression of Bcl-2 and Bax

To further investigate the molecular basis for curcumin-induced apoptosis in breast cancer

Figure 5. Morphology change of apoptosis in human breast cancer cells induced by curcumin under transmission electron microscope at 48 h. (× 5,000).

Figure 6. Effects of curcumin on the expression of Bcl-2 and Bax in MDA-MB-231 (A) and MCF-7 cells (B). Cells were treated with various concentrations of curcumin for 48 h. Western blots for each protein were done at least twice using independently prepared lysates.

cells, the constitutive expression of typical apoptosis-related proteins Bcl-2 and Bax were analyzed by Western blot. As shown in **Figure 6**, the pro-apoptotic protein Bax was induced in curcumin-treated cells, whereas the expression of antiapoptotic protein Bcl-2 was inhibited after treatment with curcumin for 48 h. This suggested an increase of Bax/Bcl-2 ratios might be involved in apoptosis induced by curcumin.

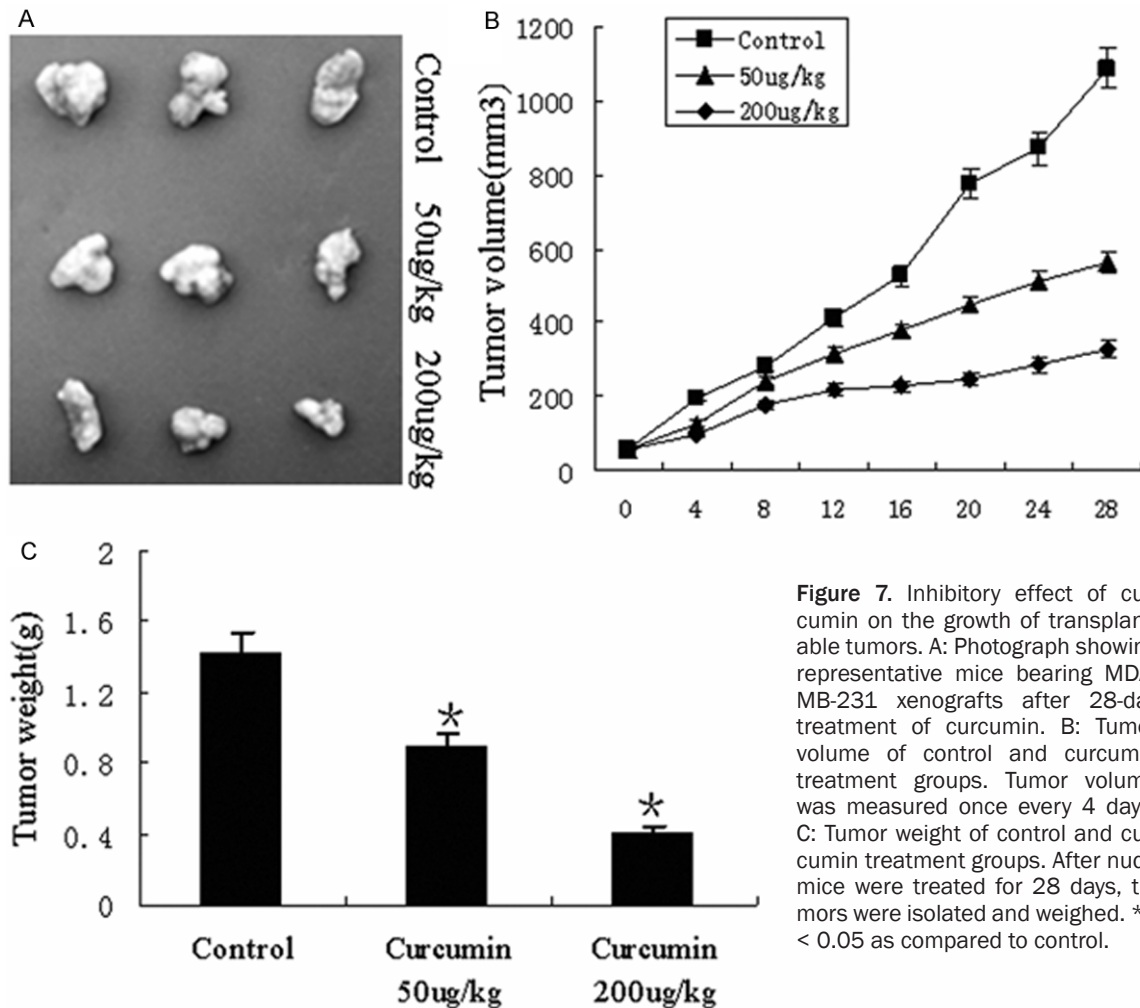*Curcumin inhibits the growth of transplantable tumors*

Tumor xenografts transplanted by human breast cancer MDA-MB-231 cells were used to evaluate the antitumor effect of curcumin in vivo. Tumors in curcumin treated mice were visibly smaller than in saline treated mice (**Figure 7A**). The tumor volume in curcumin treated mice was less than in negative control mice at the same measurement day (**Figure 7B**), indicating that curcumin significantly inhibited tumor growth during the 4-week treatment. As shown in **Figure 7C**, the weight of tumors was also reduced significantly with curcumin treatment. None of the mice died during the treatment.

Discussion

For centuries, curcumin has been consumed in the diet and used as an herbal medicine in several Far Eastern countries. Curcumin has potent anti-tumor effects on a variety of tumor cell lines in vitro and chemopreventive effects in murine carcinoma models [9]. It is also important to emphasize that to date, no curcumin-related toxicity was observed in either experimental animals or humans, even at very high doses [10]. However, the signaling pathways regulating apoptosis are complex in mammalian cells since their mechanisms vary according to cell type. Although curcumin induces apoptosis by affecting the ubiquitin-proteasome pathway, the exact mechanism remains unclear. Currently, many studies have been focused on identifying natural products promoting apoptosis in malignant cells. In addition, the cellular morphological changes and molecular mechanisms underlying curcumin-induced apoptosis in breast cells have not been well documented.

Figure 7. Inhibitory effect of curcumin on the growth of transplantable tumors. A: Photograph showing representative mice bearing MDA-MB-231 xenografts after 28-day treatment of curcumin. B: Tumor volume of control and curcumin treatment groups. Tumor volume was measured once every 4 days. C: Tumor weight of control and curcumin treatment groups. After nude mice were treated for 28 days, tumors were isolated and weighed. *$P < 0.05$ as compared to control.

Our present study demonstrated that plasmic atrophy, nuclear shrinkage, and formed extracellular and/or intracellular apoptotic bodies were observed under a transmission electron microscope. Furthermore, apoptosis was quantified by two methods: the detection of acridine orange/ethidium bromide-stained condensed nuclei by fluorescent microscopy and the detection of surface expression of phosphatidylserine by flow cytometry. The 48 h apoptotic indexes of MDA-MB-231 cells which were tested by flow cytometry with PI staining were 47.8 ± 5.21% in curcumin group. Compared with the control group, statistically significant differences were found. In the control group, spontaneous apoptosis occurred in breast cancer cells, but the apoptotic index was low. Therefore, we thought that curcumin could induce apoptosis of breast cancer cells, cause exfoliation, and eventually inhibit tumor growth. To test the physiological relevance of in vitro curcumin-induced antitumor effects in vivo, the antitumor effects of curcumin were evaluated in MDA-MB-231 xenografts. Curcumin significantly inhibited tumor growth of the MDA-MB-231 xenografts without causing mortality or other noticeable major side effects. These observations are in agreement with our in vitro studies showing that treatment of breast cancer cells with curcumin results in a dose- and time-dependent induction of apoptosis.

In general, the signaling pathway of apoptosis is complex, but can be divided largely into a Fas/Fas ligand and a mitochondrial pathway [11]. The tumor suppressor p53 is a transcriptional factor which is involved in apoptosis partly by inducing Bax expression [12, 13]. Bcl-2, an inhibitor of the mitochondrial apoptosis pathway, exerts its action by blocking proapoptotic counterparts, which in turn prevents the release of cytochrome c and the activation of

caspases [14, 15]. In our study, there was a dose-dependent decrease of Bcl-2 in curcumin treated breast cancer cells, while the levels of Bax were increased, resulting in an increase in ratio of Bax/Bcl-2. These observations show that curcumin-induced apoptosis in human breast cancer cells was triggered by the down-regulation of Bcl-2 and the up-regulation of Bax.

In summary, our present findings indicate that curcumin significantly inhibited growth of human breast cancer cells MDA-MB-231 and MCF-7 by inducing apoptosis in a dose- and time-dependent manner, accompanied by an increase in Bax/Bcl-2 ratios. Moreover, we demonstrated the tumor growth inhibition effect of curcumin in a nude mouse model. Given the potential and safety of curcumin, it is a promising candidate for therapy of breast cancer, although additional studies are needed.

Acknowledgements

Disclosure of conflict of interest

None.

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