Curcumin inhibits intracellular fatty acid synthase and induces apoptosis in human breast cancer MDA-MB-231 cells

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Abstract. High levels of fatty acid synthase (FAS) expression have been found in many tumors, including prostate, breast, and ovarian cancers, and inhibition of FAS has been reported to obstruct tumor growth *in vitro* and *in vivo*. Curcumin is one of the major active ingredients of *Curcuma longa*, which has been proven to inhibit the growth of cancer cells. In the present study, we investigated the potential activity of curcumin as a FAS inhibitor for chemoprevention of breast cancer. As a result, curcumin induced human breast cancer MDA-MB-231 cell apoptosis with the half-inhibitory concentration value of 3.63±0.26 μg/ml, and blocked FAS activity, expression and mRNA level in a dose-dependent manner. Curcumin also regulated B-cell lymphoma 2 (Bcl-2), Bax and p-Akt protein expression in MDA-MB-231 cells. Moreover, FAS knockdown showed similar effect as curcumin. All these results suggested that curcumin may induce cell apoptosis via inhibiting FAS.

Introduction

Fatty acid synthase (EC 2.3.1.85, ab. FAS) is a key metabolic enzyme which catalyzing the *de novo* synthesis of long chain saturated fatty acids from acetyl-CoA (Ac-CoA) and malonyl-CoA (Mal-CoA) in the presence of the reducing substrate nicotinamide adenine dinucleotide phosphate (NADPH) (1). Most human tissues, except liver and adipose tissue, exhibit low expression of FAS. However, the expression of FAS is especially high in a variety of common human cancers (2-5). It has been reported that many FAS inhibitors, such as cerulenin, C75, orlistat and epigallocatechin gallate (EGCG), have joint weight-loss and antitumor effects (6-9). Therefore, FAS may be a dual therapeutic target for treating both obesity and cancer.

Curcumin (Fig. 1), a hydrophobic polyphenol derived from the rhizome of *Curcuma longa*, possesses wide pharmacological activities including respiratory conditions, inflammation, breast disorders, diabetic wounds, and certain tumors (10). Curcumin induces cell death in some cancers, such as gastric and colon cancers (11), human melanoma (12), and lung cancer (13) without major cytotoxic effects on healthy cells (14-16). However, the mechanism involved is not fully understood.

In our previous study, we found curcumin to show both fast-binding and slow-binding inhibition to FAS with a half-inhibitory concentration (IC_{50}) value of 10.5 μg/ml (17). Compared with EGCG and cerulenin, two classical FAS inhibitors, curcumin showed stronger inhibitory activity. In the present study, we investigated the effect of curcumin on FAS overexpressed human breast cancer cells. We demonstrated that curcumin inhibited FAS activity and expression in MDA-MB-231 cells. Curcumin also regulated pro-apoptotic and anti-apoptotic proteins such as Bax, B-cell lymphoma 2 (Bcl-2), Akt, phosphorylate-Akt (p-Akt) expression in a dose-dependent manner.

Materials and methods

Reagents. Ac-CoA, Mal-CoA, NADPH, DMSO, Hoechst 33258 and curcumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Beijing, China). FAS antibody for immunoblotting was obtained from BD Biosciences Pharmingen

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Abbreviations: Ac-CoA, acetyl CoA; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGCG, epigallocatechin gallate; FAS, fatty acid synthase; FBS, fetal bovine serum; IC_{50}, half-inhibitory concentration; Mal-CoA, malonyl-CoA; MAT, malonyl/acetyltransferase; MTT, 3-4,5-dimethylthiazol-2-yl-2,3-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline

Key words: curcumin, fatty acid synthase, inhibitor, breast cancer, MDA-MB-231 cells, apoptosis

(Shanghai, China). GAPDH antibody was purchased from Cell Signaling Technology, Inc. (Shanghai, China). 3-4,5-dimethylthiazol-2-yl-2,3-diphenyltetrazolium bromide (MTT), phosphate-buffered saline (PBS) and the TRIzol reagent were purchased from Invitrogen (Beijing, China). Annexin V-FITC/PI apoptosis detection kit was purchased from Mbchem (Shanghai, China). The PCR primers for human β-actin and FAS were synthesized by SBS Genetech Co., Ltd. (Beijing, China). M-MLV, Rnasin and Oligo (dT) were purchased from Promega (Beijing, China). ECL and PVDF membrane were obtained from Millipore (Beijing, China).

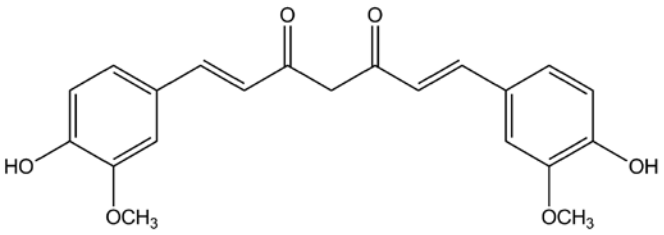

Figure 1. Chemical structure of curcumin.

Cell viability assay. Tests were performed in 96-well plates. MDA-MB-231 cells were cultured in the plates until confluence, cells were incubated with either DMSO (1:1,000) or increasing concentrations of curcumin for 24 h (37˚C, 5% CO_2). The medium was then changed to a fresh one with 0.5 mg/ml MTT. After 4 h of incubation at 37˚C, the plates were again decanted, and 150 μl of DMSO was added to solubilize the formazan crystals present in viable cells. The plate was analyzed by spectrometry at the wavelength of 492 nm by a microplate spectrophotometer (Multiskan, MK3). Data were obtained from the average of five wells, and the assay was repeated three times.

Immunoblot analysis. Cells were washed three times with ice-cold PBS and harvested in RIPA lysis buffer with 1 mM PMSF, and then lysed on ice for 5 min. The homogenate was centrifuged at 12,000 rpm for 30 min at 4˚C and supernatant was collected for FAS analysis. Equal protein extracts were separated by SDS-PAGE, then electrophoretically transferred to PVDF membranes. Incubation with primary and secondary antibodies was performed in Tris-buffered saline containing 5% non-fat dry milk for 2 h or more. After incubation, membranes were washed in Tris-buffered saline containing 0.1% Tween-20. ECL was used for detection. Blots were reprobed with an antibody against GAPDH as a loading control of protein loading and transfer.

RNA isolation and RT-PCR analysis. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions and reverse-transcribed by RiboClone M-MLV(H^-) cDNA technology. The synthesized single-stranded cDNA was used for amplification of a specific target. The β-actin gene was amplified as a loading control. The PCR conditions were denatured at 95˚C for 5 min and followed by 30 cycles (95˚C, 15 sec, 55˚C, 15 sec, 72˚C, 30 sec). The primer sequences were as follows: FAS forward, 5'-AGATCCTGGAACGAGAACACGAT-3' and reverse, 5'-GAGACGTGTCACTCCTGGACTTG-3'; and β-actin forward, 5'-GTGGGCCGCTCTAGGCACCAA-3' and reverse, 5'-CTCTTTGATGTCACGACGATTTC-3'.

The amplified products were visualized on 1% agarose gels. Quantification was performed in duplicate and the experiments were repeated independently three times. We used ImageJ software to analyze the results of gels.

Cell FAS activity assay. FAS activity in cells was assessed in a routine manner with some modifications. In brief, after cells were harvested, pelleted by centrifugation, resuspended in cold assay buffer (100 mM potassium phosphate buffer, 1 mM EDTA, 1 mM PMSF and 1 mM dithiolthreitol, pH 7.0), ultrasonically disrupted and centrifuged at 12,000 rpm for 30 min at 4˚C, the supernatant was collected for the overall reaction assay. Fifty microliters of supernatant was added to the reaction mix contained 25 mM KH_2PO_4-K_2HPO_4 buffer, 0.25 mM EDTA, 0.25 mM dithiothreitol, 30 μM Ac-CoA, 100 μM Mal-CoA, 350 μM NADPH (pH 7.0) in a total volume of 200 μl. Protein content in the supernatant was determined using a bicinchoninic acid (BCA) assay (Pierce) and results were expressed as the specific activity of FAS (U/mg).

Hoechst 33258 staining. MDA-MB-231 cells were seeded on 24-well culture dishes and cultured in the plates until confluence. Cells were treated with indicated dose of curcumin. After 24 h incubated in 37˚C, 5% CO_2 incubator, the medium was changed to a fresh one with 0.5 μg/ml Hoechst 33258 and the dish was incubated in an incubator for 30 min, then the cells were washed three times with PBS. Nuclear staining was examined under the fluorescence microscope and images were captured using ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Annexin V/propidium iodide (PI) dual staining. MDA-MB-231 cells were treated with indicated concentrations of curcumin. Then cells were harvested, and the percentage of cells undergoing apoptosis was measured by fluorescence microscopy after staining with fluorescein-conjugated Annexin V and PI staining 5 min in the dark.

RNA interference. MDA-MB-231 cells were transiently transfected with a small interfering RNA (siRNA) that silences expression of FAS. The FAS-targeted siRNAs (sense, CCCUGAGAUCCCAGCGCUGdTdT and antisense, CAGCGCUGGGAUCUCAGGGdTdT) were synthesized by Invitrogen. For transient expression, cell lines were transfected by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After incubating the cells for 6 h, the lipid and siRNA complex was removed, and fresh growth medium was added. Cells were lysed 24 h after transfection, and specific protein levels were determined by western blot analysis with specific antibodies against the targeted proteins and GAPDH as control.

Results

Effect of curcumin on the viability of MDA-MB-231 cells. The anti-viability effect of curcumin on MDA-MB-231 cells was

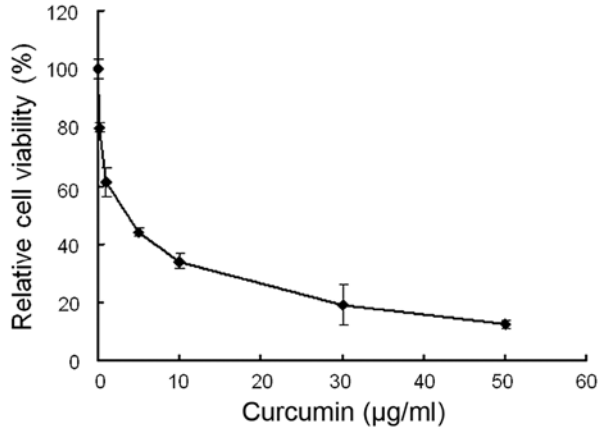

Figure 2. Dose-dependent inhibitory effects of curcumin on cell viability of MDA-MB-231 cells. Cell viability was determined by MTT assay. MDA-MB-231 cells were incubated with curcumin for 24 h at the concentrations 0-50 μg/ml. IC_{50}=3.63±0.26 μg/ml. Bars represent means ± SD.

examined by MTT assay. Following treatment with 0-50 μg/ml curcumin, cell viability was significantly decreased. As shown in Fig. 2, curcumin reduced cell viability in a dose-dependent manner with an IC_{50} value of 3.63±0.26 μg/ml.

Curcumin induces MDA-MB-231 cells apoptosis. According to the results of MTT assay, the apoptotic effect of curcumin (0, 5, 10 and 15 μg/ml) on MDA-MB-231 cells was examined by using Hoechst 33258 staining and the Annexin V/PI dual staining. After exposure to four concentrations of curcumin for 24 h, apoptotic MDA-MB-231 cells were demonstrated by the result of Hoechst 33258 staining, which revealed a cell membrane permeability increase and nuclear condensation (Fig. 3), and by Annexin V/PI dual staining, where cells were observed to be Annexin V-FITC- and PI-positive, indicating that they were in end-stage apoptosis or already dead (Fig. 4).

Curcumin downregulated FAS expression and inhibited FAS activity in MDA-MB-231 cells. Curcumin has been reported to be a highly active FAS inhibitor, however, no previous studies focused on the activity of curcumin on intracellular FAS activity in breast cancer cells. Therefore, we measured the effects of curcumin on the expression and activity of FAS in MDA-MB-231 cells. As shown in Fig. 5, compared with control, the cells treated with curcumin showed much lower expression level of FAS. Compared with control, curcumin showed a dose-dependent inhibition on FAS activity. As shown in Fig. 6, the relative activities of FAS were reduced by 6.8% at 5 μg/ml, 41.1% at 10 μg/ml and 59.7% at 15 μg/ml. These results indicated that curcumin potently inhibits FAS activity in this breast cancer cell line.

Effects of curcumin on FAS mRNA levels in MDA-MB-231 cells. To further explore the mechanism underlying the suppression of cancer cells by curcumin, the mRNA level of FAS was also examined. As shown in Fig. 7, compared with control, curcumin treated cells showed significantly lower mRNA levels of FAS.

Effects of curcumin on Bax and Bcl-2 proteins. Bax is a pro-apoptotic protein and plays a key role in mitochondrial stress-induced cell apoptosis. Increased levels of Bax could promote cells apoptosis. Bcl-2 is an anti-apoptotic protein that can inhibit apoptosis induced by cytochrome *c* release. Decreased Bcl-2 protein levels may lead to cell apoptosis. As shown in Fig. 5, after curcumin administration, the expression level of Bax showed a dose-dependent increase. In contrast, curcumin decreased Bcl-2 expression in a dose-dependent manner.

Curcumin upregulated Akt phosphorylation in MDA-MB-231 cells. The activity of curcumin on Akt was also determined. After treated with 0, 5, 10 and 15 μg/ml curcumin for 24 h, MDA-MB-231 cells were collected and cell lysates were subjected to western blot analysis for phospho-Akt. The result in Fig. 5 showed that curcumin increased the phosphorylation of Akt at Ser473 in a dose-dependent manner, without affecting total Akt expression.

Effect of siRNA mediated FAS silencing on MDA-MB-231 cells. Because overexpression of FAS protected immortalized cancer cells from apoptosis, we wanted to assess the effect of short interfering RNA to FAS on apoptosis in MDA-MB-231 cells. To accomplish this, MDA-MB-231 cells were transfected with FAS siRNA (0, 200, 300 and 500 nM), then the expression levels of FAS, Akt, Bcl-2 were measured 48 h after transfection by western blotting. As shown in Fig. 8, compared

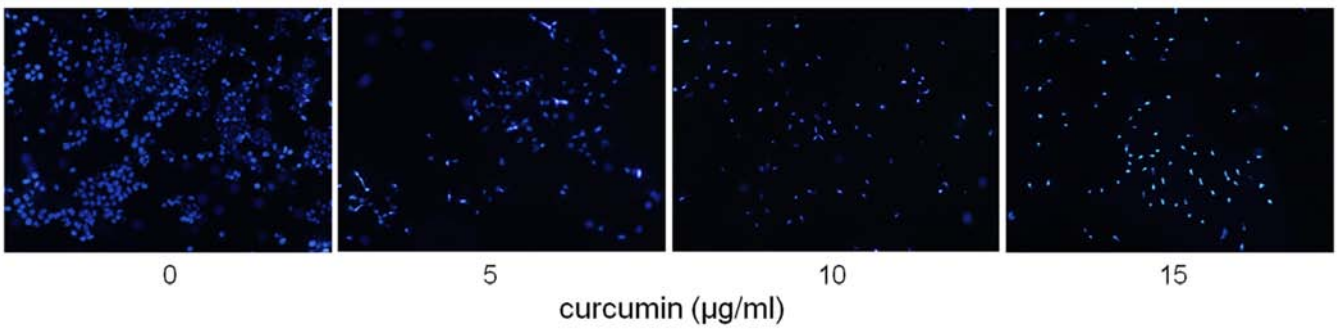

Figure 3. Hoechst 33258 staining in curcumin treated MDA-MB-231 cells. Cell culture was performed as described in Materials and methods. Images of MDA-MB-231 cells were taken after Hoechst 33258 staining, the concentrations of curcumin were: 0, 5, 10 and 15 μg/ml. Original magnification, x200.

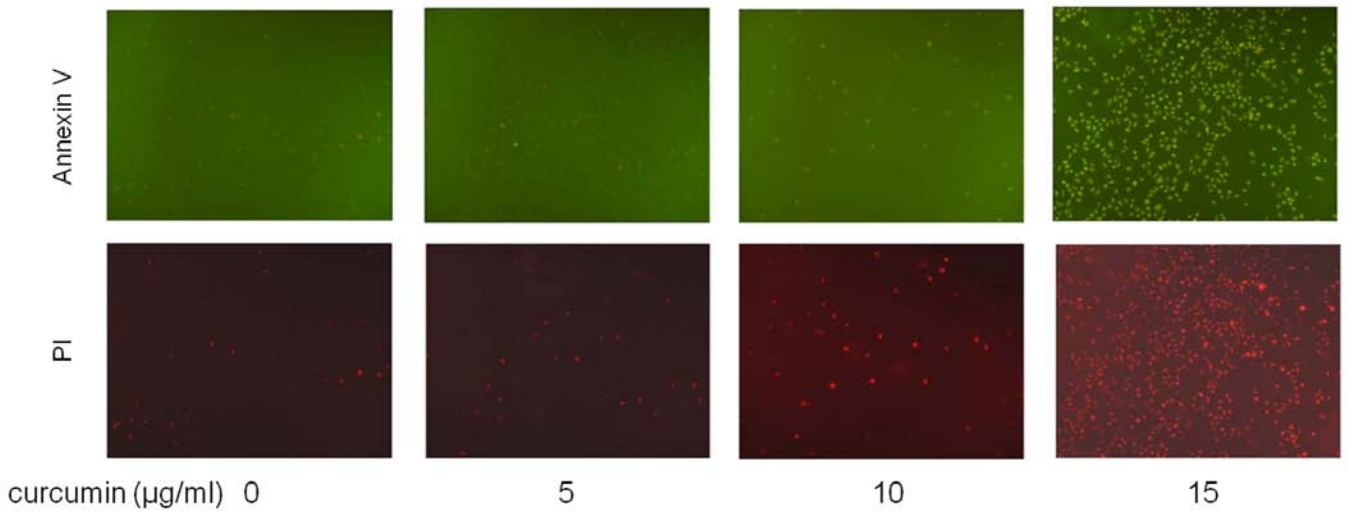

Figure 4. Annexin V/PI dual staining in curcumin treated MDA-MB-231 cells. Cell culture was performed as described in Materials and methods. Images of MDA-MB-231 cells were taken after Annexin V and PI dual staining, the concentrations of curcumin were: 0, 5, 10 and 15 μg/ml. Original magnification, x200.

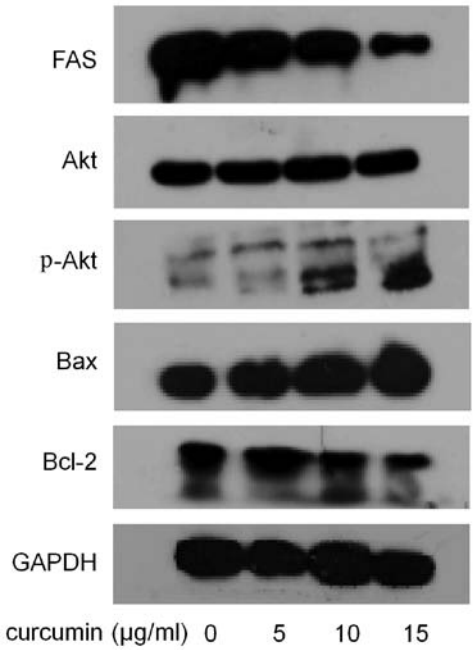

Figure 5. Regulation of curcumin on the expression levels of FAS, Akt, p-Akt, Bax, and Bcl-2 in MDA-MB-231 cells. Representative western blots showing a dose-dependent inhibition of FAS, Bcl-2 expression levels but upregulating the expression levels of p-Akt, Bax in MDA-MB-231 cells after treating with curcumin at 24 h.

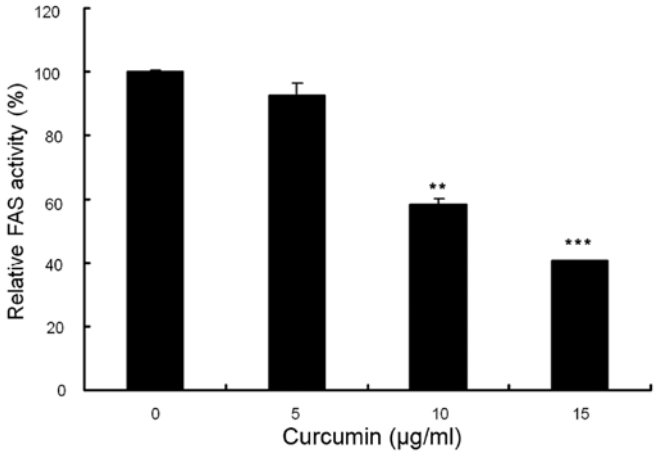

Figure 6. Inhibitory effects of curcumin on FAS activities in MDA-MB-231 cells. The dose-dependent inhibition of FAS by curcumin at the concentrations of 0, 5, 10 and 15 μg/ml was measured. Values represent the mean ± SD of triplicate determinations. **P<0.01 compared to control; ***P<0.001 compared to control.

with the control, the cells treated with siRNA showed much lower level of FAS. Reduction of Bcl-2 expression showed a dose-dependent manner with increase of siRNA, but total Akt expression had no change.

Discussion

FAS is a key enzyme catalyzing the synthesis of long-chain fatty acids from small molecule carbon unit *in vivo*. According to the biochemical and pharmacological studies in recent years, FAS is believed to be a dual target of treating both obesity and cancer (18,19). Fatty acid biosynthesis is dependent on FAS to satisfy the needs of cell division and proliferation. FAS expression level in cancer cells is much higher than that of normal tissue cells (20). There is a correlation between expression and prognosis in malignant tumors (21). RNAi knockdown experiments have shown that multiple cancer cell lines depend on FAS for proliferation and survival (22). FAS appears to play a key role in tumor initiation and propagation for many malignancies and represents an attractive target for cancer treatment. Inhibition of FAS has emerged as a promising therapeutic target in cancer, and numerous inhibitors have been investigated (19,21). However, severe pharmacological limitations have challenged their clinical testing.

Curcumin is a phenolic pigment extracted from *Curcuma longa*, which is an effective anti-mutagenic and anticancer agent. In Chinese medicine, *Curcuma longa* has long been used as a medicinal plant (23). Numerous studies have suggested

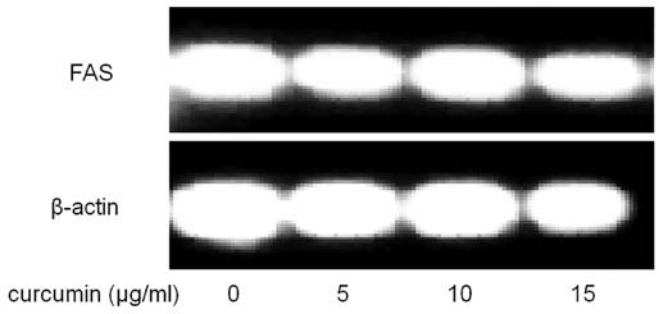

Figure 7. Effect of curcumin on FAS mRNA. MDA-MB-231 cells were treated with 0, 5, 10 and 15 μg/ml curcumin. After 24 h, mRNA was extracted and quantified via RT-PCR, and normalized to β-actin mRNA. Data were normalized to control cells without curcumin (0 μg/ml).

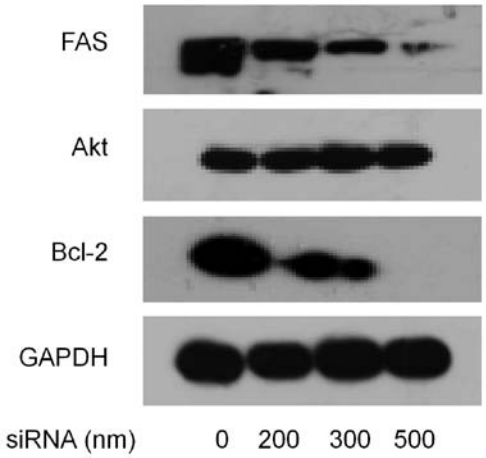

Figure 8. Regulation of siRNA on the expression levels of FAS, Akt, and Bcl-2 in MDA-MB-231 cells. MDA-MB-231 cells transfected with siRNA-targeting FAS or with the control were subjected to immunoblotting analyses with antibodies against FAS, Akt, Bcl-2, and GAPDH.

that curcumin can inhibit the proliferation of cancer cells and promote cancer cells apoptosis (24,25). Yet, little is known about its mechanism that inhibits the growth of cancer cells.

Since curcumin is a highly active FAS inhibitor, this study is focus on the relationship between the apoptotic effect and FAS inhibitory effect of curcumin. We found that treatment with curcumin produced a dose-dependent decrease in the viability of MDA-MB-231 cells. Hoechst 33258 staining, and the Annexin V/PI dual staining results indicated that curcumin induced apoptosis dose-dependently (Figs. 3 and 4). After treating with curcumin, the mRNA, expression level, as well as activity of FAS were also decreased in a dose-dependent manner (Figs. 5-7). These results indicated that curcumin potently blocked FAS in MDA-MB-231 cells.

Cell death signaling frequently converges on mitochondria, which is controlled by the activities of pro- and anti-apoptotic Bcl-2 family proteins (26-31). In the present study, we found that curcumin treatment decreased anti-apoptotic Bcl-2 and increased pro-apoptotic Bax proteins, thereby causing a significant increase in the Bax/Bcl-2 ratio in MDA-MB-231 cells. In addition, FAS siRNA also downregulated the expression level of Bcl-2. These results indicated that bcl-2 family was involved in curcumin-induced apoptosis.

The activation of Akt is one of the most frequent alterations observed in human cancer cells (32-34). Inhibition of FAS activity preferentially inhibits cell growth and induces apoptosis in a number of cancel cells (35). Considerable evidence demonstrates that phosphatidylinositol 3-kinase (PI3K)/Akt signaling plays an important role in cancer progression and has been linked with FAS expression in cancer cells (36,37). In the present study, to assess whether the activity of Akt was affected by curcumin, we explored the phosphorylation of Akt in MDA-MB-231 cells after treatment with curcumin and FAS siRNA. The results showed that p-Akt was noticeably increased when treated with curcumin in MDA-MB-231 cells. However, expression levels of Akt phosphorylation were not detected in MDA-MB-231 cells exposed to increasing concentrations of FAS siRNA. Moreover, the total levels of the corresponding Akt were not altered in either curcumin or siRNA treated breast cancer cells. These results demonstrated that PI3K/AKT signaling pathway may be involved in curcumin-induced apoptosis, but the detailed mechanisms involved are not fully understood.

In conclusion, curcumin was able to induce MDA-MB-231 cell apoptosis via inhibiting intracellular FAS activity and downregulating FAS expression and mRNA level. FAS inhibition plays a great role in curcumin regulated signal proteins including Bax, Bcl-2, Akt and p-Akt. FAS knockdown by siRNA also illustrated that FAS inhibition closely related to cell apoptosis. These results support the important role of FAS in MDA-MB-231 cells and suggest that FAS is the target that curcumin act on. Our findings combined with its well-documented pharmacological safety profile make curcumin a promising drug candidate for the treatment of breast cancer.

Acknowledgements

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