RESEARCH ARTICLE

Curcumin Inhibits Human Non-small Cell Lung Cancer A549 Cell Proliferation Through Regulation of Bcl-2/Bax and Cytochrome C

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Abstract

   We intended to study the mechanism of the inhibitory action of curcumin on human non-small cell lung cancer A549 cell. The cell growth was determined by CCK-8 assay, and the results indicated that curcumin inhibited the cell proliferation in a concentration dependent manner. And to further confirm the relative anti-cancer mechanism of curcumin, RT-PCR was carried out to analysis the expression of relative apoptotic proteins Bax, Bcl-2. We found that curcumin could up-regulate the expression of Bax but down-regulate the expression of Bcl-2 in A549 cells. In addition, curcumin affect the mitochondrial apoptosis pathway. These results suggested that curcumin inhibited cancer cell growth through the regulation of Bcl-2/Bax and affect the mitochondrial apoptosis pathway.

Keywords: Curcumin - Bcl-2 - Bax - cytochrome C - apoptosis - NSCLC cells

Introduction

   Lung cancer has the highest incidence and mortality rate of all malignancies. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, the majority of which are at an advanced stage and unresectable when diagnosed (Parkin et al., 2005). Surgery is the most common treatment given with curative intent. Other treatments including radiotherapy, combined chemoradiotherapy and adjuvant chemotherapy are commonly used as well (NCCC, 2011). However, the side effects of the radiotherapy or chemotherapy promote much work on the new effective medicine in treating NSCLC. Traditional Chinese Medicine (TCM) is widely used for cancer treatment in China. Many support its use in treatment for cancer, yet scientific evidence for the effect of TCM needs to be established.

   Curcumin, the principal polyphenolic cucuminoid, extracted from the turmeric rhizome Curcuma longa Linn, has been vastly reported for its biological activities, including anti-inflammatory (Schaffer et al., 2011), anti-oxidant (Speciale et al., 2011), anti-infection (Na et al., 2011) and anti-cancer (Bansal et al., 2011). Moreover, curcumin can cross the blood-brain barrier and is neuroprotective in neurological disorders (Song et al., 2012). Recently, interest in studying the anti-tumor mechanism of curcumin seems to be mounting for it is effective and safe. Curcumin may be able to modulate multiple cellular pathways involved in carcinogenesis and thus behaves as a multi-targeted drug. The mechanisms involved including cell proliferation, cell cycle, migration, invasion and angiogenesis though suppression of the Janus kinase-STAT3 (Yang et al., 2012), the upregulaton of α-antitrypsin (Xu et al., 2012) and HIF-1α mechanisms (Wang et al., 2013).

   In this paper, we try to verify the anti-cancer mechanism of curcumin in the A549 cells of NSCLC. Therefore, we carried out RT-PCR and Western blot assay to evaluate the expression of Bcl-2, Bax and cytochrome C, which are important factors involved in cancer cell.

Materials and Methods

Materials

   In this study, we prepared the following materials: curcumin (Sigma-Aldrich, St. Louis, MO), β-actin antibody (Sigma), cytochrome C monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), real-time polymerase chain reaction (RT-PCR) kit (GIBCO, Grand Island, NY, USA); Amplification of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) upstream and downstream primers were synthesized by Shanghai Shenggong Biological Engineering Technology Service Company (Shanghai, China).

Cell culture and treatment

   Human NSCLC A549 cells were purchased from Shanghai cell bank of the Chinese Academy of Sciences

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Table 1. The Primers Used in the Experiment for RT-PCR

	Forward Prime	Reverse Primer	Amplified Fragment Length
Bcl-2	5'-CTT TTG CTG TGG GGT TTT GT-3'	5'-GTC ATT CTG GCC TCT CTT GC-3'	490bp
Bax	5'-GGA GCT GCA GAG GAT GAT TG-3'	5'-CCT CCC AGA AAA ATG CCA TA-3'	515bp
GAPDH	5'-GAA GGT GAA GGT CGG AGT-3'	5'-GAA GAT GGT GAT GGG ATT TC-3'	226bp

(Shanghai, China). Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-stretomycin at 37 °C in a humidified incubator with 5% CO_2.

Curcumin was dissolved in DMSO in 10 mol/ml stock solution and kept at 4 °C. Curcumin were freshly diluted with DMSO of 0, 5, 10, 20 and 40 μmol/ml, respectively. The cells were treated with different concentrations of curcumin for 24 h and then evaluated as described later.

Cell viability assay after cells were co-treated with curcumin

Cell viability was determined with a Cell-Counting Kit-8 (CCK-8, Dojindo Laboratories, Tokyo, Japan). A549 cells were plated into 96-well plates at the density of 5×10^5 cells/well and cultured for 24 h. And then curcumin was added to the wells with the final concentration of 0, 5, 10, 20 and 40 μmol/l, while only the wells with DMSO was set as the control group. After the cells were co-incubated for 24 h, 10 μl of the CCK-8 solution was added to each well, followed by incubation for 2 h at 37 °C. The resulting color was assayed at 450 nm using a microplate reader.

RT-PCR for detection of Bcl-2 and Bax levels in A549 cells

RT-PCR was used to evaluate the Bcl-2 and Bax expression in A549 cells. The primers used in the experiment showed in Chart 1 and GAPDH was used as an internal standard to normalize protein levels.

Cells in logarithmic phase were harvested and seeded into 12-well plates at the density of 5×10^5 cells/well and cultured for 24 h. And then the medium was replaced by DMSO and curcumin at 0-40μmol/l 2ml and cultured for 24 h.

The total RNA was extracted with Trizol according to the kit's instruction. The purity of the total RNA was determined by UV in OD260/OD280 (>1.8) and the integrity was evaluated by 1% agarose gel electrophoresis (AGE) (28S/18S is about 2). The RT-PCR was performed according to the manufacturer's instruction: the RT-PCR reaction consisted of denaturing for 1 min at 94 °C, followed by 32, 32 and 34 cycles of denaturing for 30 s at 94 °C, annealing for 1min at 56 °C, 60°C and 56°C for GAPDH, Bcl-2 and Bax, and extension for 2 min at 72 °C.

Electrophoresis of the PCR products were analyzed by the Syngene image analysis system. The threshold cycle (Ct) values of Bcl-2/GAPDH and Bax/GAPDH were used for calculation of the relative expression ratios. All quantitative PCR reactions were performed in triplicate.

Detection of changes in mitochondrial membrane potential and release of mitochondrial cytochrome C

Cells were treated with 5, 10, 20, 40 μmol/L curcumin (reference group with 0.5% DMSO) for 48 h, then washed with PBS twice, supplemented with 10 μmol/L rhodamine 123 dye and incubated in culture box at 37 °C under 5% CO_2 for 10 min. The cells were centrifuged and washed two times with cell medium. After heavy suspension in the medium and incubation at 37 °C for 60 min under 5% CO_2, wavelength 480 nm and 530 nm stimulation were applied on the cells. Changes of mitochondrial membrane potential were assessed using flow cytometry. Treatment with quercetin was prior to incubation with the precooling cell lysates (20 mmol/L HEPES, pH7.5, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 250 mmol/L sucrose and protease inhibitor mixture) for 1 h at 4 °C, followed by centrifugation at 12,000 g at 4 °C for 5 min, then supernatant was collected and the cells were further centrifuged at 100,000 g for 30 min to harvest cytoplasmic protein components.

Western blot was performed to analyze cytochrome C levels

Cells at a density of 2×10^6 /well were seeded into 6-well plates and incubated at 37 °C for 24 h. Curcumin of 0, 5, 10, 20 and 40μmol/l was added to the wells and co-incubated for 24 h. And then the medium was removed and total protein was extracted from A549 cells with RIPA lysis buffer and protein concentration was determined by the BCA assay. The equal amount of proteins was subjected to SDS-PAGE (12%) and electro-transferred to PVDF membrane. After blocking with 5% non-fat dry milk for 60 min, the membrane was incubated with cytochrome C and β-actin antibodies overnight at 4 °C with a gentle agitation, respectively. The blots were washed with PBS for 3 times followed by secondary antibody reactions with AP-labeled anti-mouse IgG for 60 min at room temperature. Finally, the slices were washed with PBS for 3 times, 5 min for each time; signals were detected with an enhanced chemiluminescence system (Minipore, Bedford, MA).

Statistical analysis

Data are expressed as mean ± SEM. The statistical significance of differences between means was determined by one-way analysis of variance (one-way ANOVA) followed by Dunnett's test or Newman-Keuls post hoc test (SPSS version 13.0 software). A value of $P<0.05$ was considered to be statistically significant.

Results

Effects of curcumin on A549 cell proliferation

To evaluate the anti-proliferation effect of curcumin on A549 cells in vitro, CCK-8 was used as described above. The results showed that curcumin could inhibit cells proliferation in a dose independent manner. We found that in the same time interval, the rates mounted with the concentration of curcumin increasing, and were statistical different form the control group significantly ($P<0.05$).
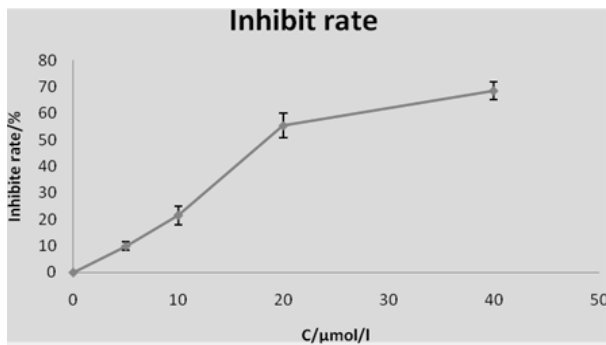
Figure 1. The Inhibited Effect of Curcumin on A549 Cells. Cells were exposed to 0, 5, 10, 20 and 40 μmol/ml of curcumin for 24 h. The resulting color was assayed at 450 nm using a microplate reader. Analysis of variance showed that the growth of A549 cells was inhibited by curcumin in a concentration-dependent manner ($P<0.05$)
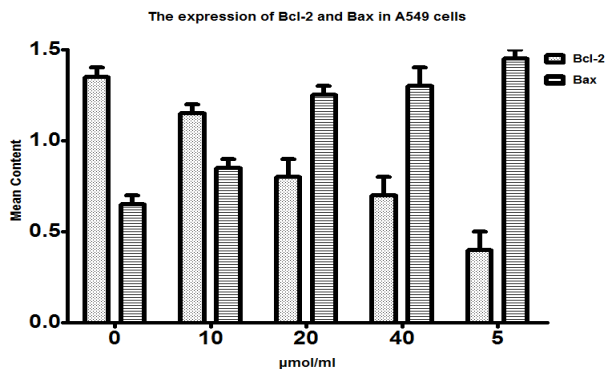

Figure 3. RT-PCR to Determine the Effect of Curcumin on the Expression of Bcl-2 and Bax in A549 Cells after Treated with 0-40 μmol/ml of Curcumin for 24 h. Changes in A549 cells were expressed as the ration compared to GAPDH. The ratio of Bcl-2/Bax was decreased with the dose increased

Figure 2. RT-PCR to determine the effect of curcumin on the expression of Bcl-2 and Bax in A549 cells after treated with 0-40 μmol/ml of curcumin for 24 h. Changes in A549 cells were expressed as the ration compared to GAPDH. Curcumin down regulated the level of Bcl-2 but up-regulated the level of Bax in A549 cells

Figure 4. Curcumin Can Significantly Lower Mitochondrial Membrane Potential ($P < 0.05$)
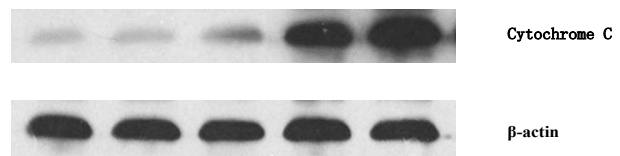
Figure 5. Western Blot was Used to Detect Cytochrome C Release

Dose response curves in A549 cells showed that 5 μmol/l could inhibit cell viability, and the concentration of 5-40 μmol/l, 40 μmol/l of curcumin showed the strongest suppressed effect on A549 cells (Figure 1).

Effect of curcumin on Bcl-2 and Bax expression in A549 cells

We carried out quantitative RT-PCR assay to determine the expression of Bcl-2 and Bax in A549 cells. Cells that treated with 0-40 nmol/ml curcumin were cultured for 24 h. Statistical analysis revealed that curcumin promote the expression of Bax in the treatment group, and was significantly different with the control group ($P<0.05$); while curcumin inhibited the expression of Bcl-2 in the treatment group, and that there was significant difference between the treatment group and the control group. We also observed the significant differences of Bax/Bcl-2 between the treatment group and the control group (Figure 2, 3).

Changes of mitochondrial membrane potential and release of cytochrome C

In order to investigate the role of mitochondrial pathway in curcumin-induced A549 cells apoptosis, curcumin of different concentrations were applied on
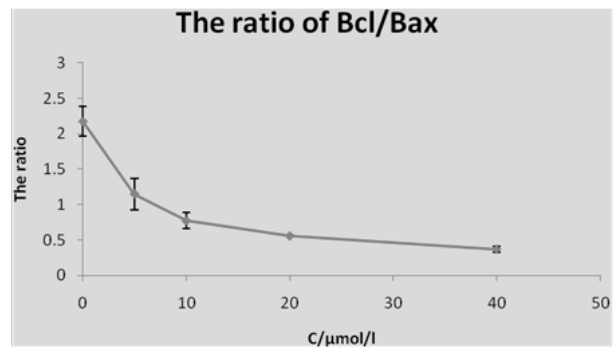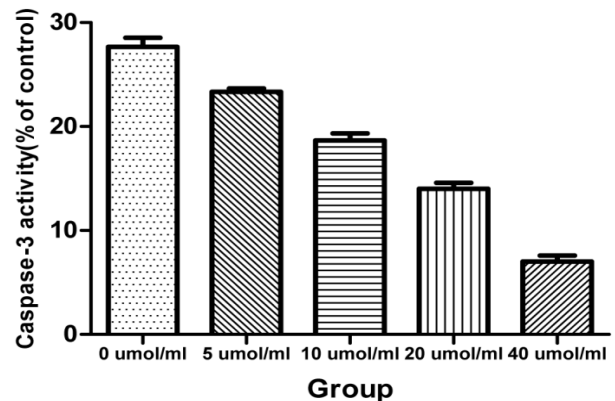
cells for 48 h before mitochondrial membrane potential detection and cytosolic cytochrome C levels measurement by Western blot. The results showed the use of curcumin on cells after 40 h can significantly decrease the mitochondrial membrane potential ($P < 0.05$) in a dose-dependent manner (Figure 4).

Western blot analysis showed that cytochrome C was released from mitochondria to cytoplasm (Figure 5). These data indicate that the mitochondrial apoptotic pathway may be involved in lung cancer A549 cell apoptosis induced by curcumin.

Discussion

Curcumin, the bioactive component extracted from turmeric rhizome Curcuma longa Linn, has been widely studied with respect to its potential anti-cancer activity in vivo and in vitro. The mechanisms involved are complicated. Curcumin can promote inhibition or arrest cell cycle at all stages through increasing p53 and p21 expression (Jaiswal et al., 2002). In addition, curcumin

inhibits cancer cell growth by stimulating the activation of casepase-3, casepase-7 and caspase-8 (Notarbartolo et al., 2005; Howells et al., 2007). Furthermore, curcumin affects cancer cell proliferation by suppressing TNF-induced NF-ᴋB-dependent gene products (COX-2, cyclin-D, c-myc) (Aggarwal et al., 2006).

Bcl-2 family proteins, which have either pro- or anti-apoptotic activities, have been studied intensively for the past decade owing to their importance in the regulation of apoptosis, tumorigenesis and cellular responses to anti-cancer therapy (Youle et al., 2008). In the Bcl-2 protein family, proapoptotic member Bax and antiapoptotic member Bcl-2 are the active effectors and regulators, and the ratio between Bcl-2 and Bax affects apoptosis induction (Pettersson et al., 2002). According to Sato et al, there are two non-mutually exclusive possibilities: (i) Bcl-2 could induce a pathway that actively maintains cell survival, with Bax serving as a negative regulator of Bcl-2, or (ii) Bax could directly or indirectly generate death signals, with Bcl-2 serving in this case as a dominant inhibitor of Bax (Sato et al., 1994).

In the present study, RT-PCR were adopted to analyze the levels of Bcl-2 and Bax in the A549 cells. Statistical analysis revealed that curcumin stimulated the expression of Bax but inhibited the expression of Bcl-2 in the treatment group. We also observed the significant differences of Bax/Bcl-2 between the treatment group and the control group. These data are consistent with curcumin-inhibited cancer cell growth associated with the balance of Bcl-2/Bax.

There are two main ways of cell apoptosis. One way is to activate apoptotic enzyme caspase within the cell through extracellular signals, the other way is the mitochondrial pathway. Mitochondria play an important role in the process of apoptosis. Mitochondrial membrane potential stimulates the mitochondrial membrane to open, resulting in the release of cytochrome C into cytoplasm, activation of the caspase pathway, and degradation of important intracellular proteins, consequently inducing apoptosis.

The results of this study showed that using curcumin in cells for 40 h can significantly reduce the mitochondrial membrane potential in a dose-dependent manner. In addition, Western blot confirmed the release of cytochrome C from mitochondria to the cytoplasm, suggesting that the mitochondrial apoptosis pathway is important in curcumin-induced lung cancer cell line apoptosis.

In conclusion, this paper indicates that curcumin is a potential growth inhibitor of human non-small cell lung cancer A549 cells. And we have demonstrated the possible anti-cancer mechanism may be the modulation of Bcl-2/Bax and affect the mitochondrial apoptosis pathway by curcumin. These findings are consistent with many studies and may provide a molecular basis for the development of novel natural anticancer agents for NSCLC.

Acknowledgements

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