

Original Research Article

Curcumin induces apoptosis in lung cancer cells via mitochondria-dependent signal pathways

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Sent for review: 9 June 2018

Revised accepted: 25 September 2018

Abstract

Purpose: To investigate the effect of curcumin on the growth and viability of lung cancer cells.

Methods: The viability of curcumin-treated A-427 and A-549 cells, and changes in their morphologies were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and phase-contrast microscopy, respectively. Flow cytometry and western blot assays were employed for the determination of apoptosis and changes in protein expressions, respectively.

Results: Curcumin treatment altered the morphologies of A-427 and A-549 lung cancer cells. The viability of A-427 cells was reduced to 89, 71, 49, 26 and 25 %, respectively, on treatment with 5, 10, 15, 20 and 25 μ M curcumin, while the corresponding decreases in viability of A-549 cells treated with the same doses of curcumin were 91, 74, 53, 31 and 29 %, respectively. Treatment with 20 μ M curcumin resulted in 62.87 % apoptosis in A-549 lung cancer cells. Curcumin increased the proportion of cells in G0/G1 phase in a concentration-dependent manner, and increased the expressions of p21 and p-p53 proteins in A-427 cells, while the expressions of cyclin E and Cdc25c were decreased. Curcumin treatment also enhanced the expressions of Bax, cleavage-caspase-8 and PARP in A-549 cells.

Conclusion: Curcumin inhibits lung cancer growth by inducing apoptosis and cell cycle arrest. Thus, curcumin has a promising potential for the treatment of lung cancer.

Keywords: Curcumin, Lung cancer, Apoptosis, Viability, Intrinsic, Reactive oxygen species

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INTRODUCTION

Lung cancer is one of the leading causes of carcinoma-related deaths, with over 225,000 new cases yearly in USA alone [1]. The prognosis of lung cancer is very poor despite adjuvant chemotherapy and radical resection [2-4]. The poor success associated with available treatment strategies and recurrence of lung cancer require

research aimed at discovering novel compounds capable of effectively inhibiting cancer growth.

The removal of unwanted cells from organisms occurs in a well programmed manner known as apoptosis. Apoptosis involves extrinsic and intrinsic pathways [5,6]. In the extrinsic pathway, cellular surface markers like tumor necrosis factor (TNF) and CD95/Fas induce apoptosis by interacting with specific ligands [6,7]. On the

other hand, the intrinsic pathway of apoptosis is induced by DNA damage and production of ROS within the cell [6]. Activation of caspases regulate expressions of various members of Bcl-2 families involved in induction of cell apoptosis [8,9]. Most of the anti-cancer chemotherapeutic agents exhibit their effects through induction of cell apoptosis.

Curcumin is one of the most active compounds present in turmeric, and it is used widely for the treatment for inflammation [10]. In addition, curcumin is used for the treatment of chronic diseases like pancreatitis and arthritis [11]. There are reports that curcumin is useful in the prevention of various bone degenerative disorders [12-15]. In the present study, the effect of curcumin on cell cycle arrest and apoptosis induction in lung cancer cells was investigated.

EXPERIMENTAL

Cell culture

A-427 and A-549 lung cancer cell lines were supplied by the Shanghai Institute of Biochemistry and Cellular Biology, Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin in a humidified atmosphere with 5 % CO₂.

Assessment of cell morphology

A-427 and A-549 cells were plated, each at a concentration of 2 x 10⁵ cells per well into 12-well plates and cultured for 24 h. The cells were then incubated with curcumin (0, 5, 10, 15, 20 and 25 μM) for 48 h at 37 °C. After incubation, the cells were examined for morphological changes using a phase-contrast microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Proliferation in A-427 and A-549 lung cancer cell lines was determined using MTT assay. The density of the cells was adjusted to 2 x 10⁵ cells per mL in a volume of 190 μL/well in 96-well plates. After 24 h of incubation in an incubator at 37 °C under 5 % CO₂ atmosphere, the cells were treated with curcumin at concentrations of 5, 10, 15, 20 and 25 μM, followed by incubation for 48 h. After incubation, 20 μL of MTT solution (5 mg/mL) was put into each well of the plate and incubation was continued for additional 4 h. Thereafter, the supernatant was discarded and

150 μL DMSO was added to each well to dissolve the resultant formazan crystals. The absorbance of each well was read in triplicate at a wavelength of 490 nm.

Flow cytometric analysis

The A-427 and A-549 cells were incubated separately for 48 h with 5, 10, 15, 20 and 25 μM curcumin, harvested by trypsinization, and subsequently washed three times using phosphate-buffered saline. The cells were then re-suspended in binding buffer at a density of 2 x 10⁵ cells per ml. The cell suspension was treated with annexin-V-fluorescein isothiocyanate (5 μL; FITC) and propidium iodide (5 μL; PI), followed by incubation for 15 min in the dark at room temperature. Apoptosis in the cell suspension was assessed using FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Cell cycle analysis

Each cell line (A-427 and A-549 cells) was incubated for 48 h with 5, 10, 15, 20 and 25 μM curcumin and then harvested by trypsinization and subsequently washed three times using phosphate-buffered saline. The cells were then isolated and fixed overnight in 70 % ethyl alcohol at 4 °C. The cells were kept in the dark at 37 °C and re-suspended in PBS containing PI (40 μg/mL), RNase (0.1 mg/mL) and Triton X-100 (0.1 %) for 40 min. Thereafter, they were subjected to cell cycle analysis at a wavelength of 488 nm using flow cytometer equipped with an argon ion laser.

Determination of ROS generation and Ca²⁺ ion concentration

In the determination of ROS production, A-427 cells at a density of 2 x 10⁵ cells per mL were incubated with 25 μM curcumin for 48 h. Then, the cells were harvested and re-suspended in 550 μL of 10 μM solution of 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA). Cellular Ca²⁺ ion concentration was determined by treating the cells with 3 μg/mL solution of 1-[2-amino-5-(6-carboxyindol-2-yl) phenoxy]-2-(2'-amino-5' methylphenoxy) ethane-N, N, N', N'-tetra acetic acid pentaacetoxymethyl ester. The cells were then incubated for 45 min and subsequently subjected to flow cytometry.

Western blot assay

A-427 cells were harvested after 48 h of treatment with various concentrations of curcumin (5, 10, 15, 20 and 25 μM). The cells

were lysed with lysis buffer [40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, and 0.1% nonidet P-40], and 30 µg of protein was used for Western blot analysis. The protein separation was performed using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred onto nitrocellulose membranes by electroblotting, and the membranes were incubated overnight with primary antibodies against cyclin E, Cdc25c, p21, p-p53 (Santa Cruz Biotechnology, CA), Bcl-2, Bax, caspase-9, AIF, Endo G, caspase-3, PARP and caspase-8 (R&D Systems, Minneapolis, USA). Thereafter, the membranes were washed with PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Wuhan Boster Biological Technology, Ltd; 1:10,000) for 1 h at room temperature. β-actin was used as a loading control.

Statistical analysis

The results are presented as mean ± SD (n = 3). Statistical analysis of data was performed with Statistical Package for Social Sciences (SPSS for Windows, version 17.0; SPSS, Inc., Chicago, IL, USA). The data analysis was carried out using monofactorial analysis of variance. $P < 0.05$ was taken as statistically significant.

RESULTS

Curcumin altered the morphology and inhibited viability of lung cancer cells

Phase-contrast microscopy showed that curcumin changed the morphology of A-427 and A-549 lung cancer cells. The changes in cell morphology increased with increase in dose of curcumin from 10 to 20 µM (Figure 1 A). The curcumin treatment inhibited the viability of A-427 and A-549 lung carcinoma cells in concentration- and time-dependent manners. The viability of A-427 cells was reduced to 89, 71, 49, 26 and 25 %, respectively on treatment with 5, 10, 15, 20 and 25 µM curcumin for 48 h. Similarly, in A-549 cells, viability was decreased to 91, 74, 53, 31 and 29 %, respectively on treatment with 5, 10, 15, 20 and 25 µM curcumin for 48 h (Figure 1 B).

Curcumin induced apoptosis in lung carcinoma cells

Curcumin treatment induced apoptosis in A-549 lung cancer cells after 48 h (Figure 2). The percentage of apoptosis in A-549 lung carcinoma cells was 62.87 % on treatment with 20 µM curcumin. Incubation of A-549 cells with curcumin at doses of 5, 10 and 15 µM led to

18.24, 36.78 and 57.89 % apoptosis, respectively.

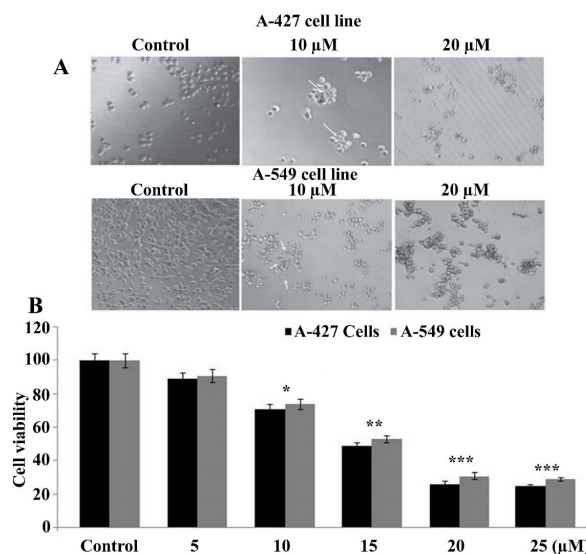


Figure 1: Changes in morphology and viability of A-427 and A-549 lung cancer cells by curcumin. (A) Changes in morphology due to incubation of the cells with 10 and 20 µM curcumin for 48 h. (B) Changes in viability after incubating the cells with 5, 10, 15, 20 and 25 µM curcumin for 48 h. The experiments were carried out in triplicates and results presented are the mean of three measurements; * $p < 0.05$, ** $p < 0.02$ and *** $p < 0.01$, compared to cells in the control group

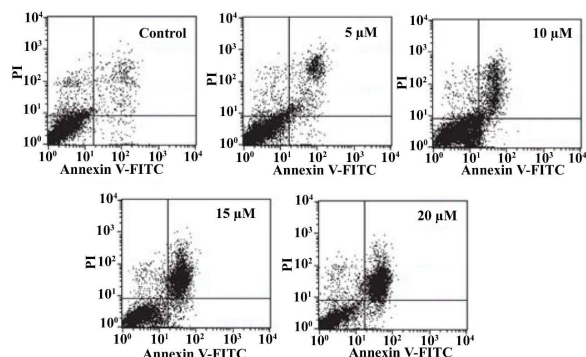


Figure 2: Induction of apoptosis in A-549 lung carcinoma cells by curcumin

Curcumin arrested cell cycle in G0/G1 phase in A-549 lung carcinoma cells

Curcumin treatment enhanced the proportion of cells in G0/G1 phase in a dose-dependent manner, and decrease cells in the S phase. The proportion of A-549 cells in G0/G1 phase was 39.23, 46.49, 52.67 and 62.71 %, respectively on incubation with 0, 10, 15 and 20 µM curcumin (Figure 3). On treatment with 0, 10, 15 and 20 µM curcumin, the percentages of cells in the S phase were 23.81, 16.34, 9.21 and 7.45, respectively (Figure 3).

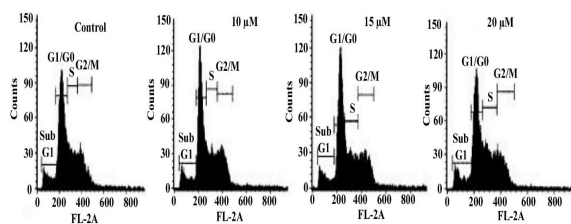


Figure 3: Curcumin treatment increased percentage of A-549 cells in G0/G1 phase

Curcumin enhanced ROS generation and Ca^{2+} ion concentration in A-549 cells

Results from flow cytometry showed that treatment of A-549 cells with 5, 10, 15 and 20 μ M curcumin for 48 h caused increases in ROS generation and Ca^{2+} ion concentration (Figure 4).

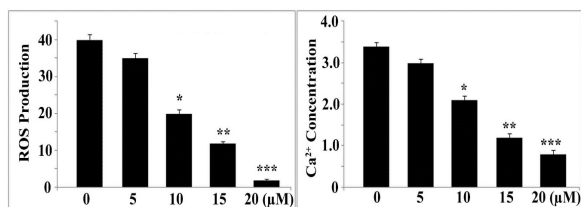


Figure 4: Curcumin treatment increased generation of ROS and Ca^{2+} ions in A-549 cells; * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$, compared to cells in the control group

Curcumin altered expressions of proteins involved in apoptosis

Curcumin treatment for 48 h increased the expressions of p21 and p-p53 proteins in A-427 cells, but decreased the expressions of cyclin E and Cdc25c in A-549 cells (Figure 5). It was also found that expressions of Bax and cleaved-caspase-8, -9, -3 were increased by curcumin in lung carcinoma cells. Moreover, the levels of AIF, Endo G and PARP were enhanced in A-549 cells on incubation with curcumin for 48 h (Figure 5). However, curcumin reduced the expression of cytosolic Bcl-2.

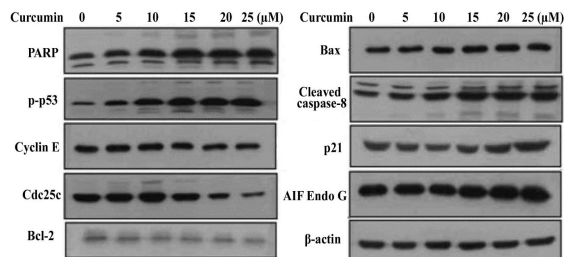


Figure 5: Changes in the expressions of cyclins E, Cdc25c, cleaved-caspase, AIF Endo G and PARP in A-549 cells treated with curcumin

DISCUSSION

Plant-derived natural products such as taxanes and vinca alkaloids have been found to inhibit growth and progression different cancers [16]. In the present study, the effects of curcumin (active ingredient of turmeric) on viability, apoptosis induction and progression of cell cycle were investigated in lung carcinoma cells. The study showed that curcumin reduced viability, induced apoptosis and arrested cell cycle progression in the lung carcinoma cells.

Incubation of A-427 and A-549 cells with curcumin led to induction of apoptosis. The progression of cell cycle is maintained by various complexes of protein kinases such as cyclins [17]. After activation, D/cdk2, 4, 5 and 6 cyclins regulate progression to G1 phase whereas the activation of E/cdk2 cyclin maintains progression to G1/S phase [18,19]. Progression to S phase is regulated by A/cdk2 activation, while that of G2/M is regulated by activation of A and B/cdk1 cyclins [18,19]. The current study showed that curcumin treatment caused cell cycle arrest of the lung cancer cells in G0/G1 phase. Examination of the cell cycle protein expression showed that incubation with curcumin promoted expressions of p53 and p21. Curcumin treatment down-regulated Cdc25c and cyclin E expressions in the lung carcinoma cells.

These findings suggest that curcumin treatment results in arrest of cell cycle in G0/G1 phase by suppressing the expressions of Cdc25c and cyclin E. Curcumin treatment of lung cancer cells also caused release of AIF and Endo G from the mitochondria, resulting in decreased membrane potential. This suggests that curcumin-mediated onset of apoptosis in lung carcinoma cells proceeds through the mitochondrial pathway. Apoptosis induction also involves reduction in the expression of Bcl-2 [20,21]. It has been reported that members of Bcl-2 family change the mitochondrial membrane permeability, leading to caspase activation and consequently cell apoptosis [8,9,22]. The present study showed that curcumin treatment increased Bcl-2, and activated caspases and PARP in the lung cancer cells.

Studies have shown that apoptosis and cell cycle arrest are also induced in the lung carcinoma cells by increased production of ROS [21,23]. In the present study, treatment of lung cancer cells with curcumin increased production of ROS. There are reports that higher expressions of p21, Bak, Bad and TNF- α result from the activation of JNK [24-27]. The results obtained in the present study showed higher expressions of p21, Bak,

Bad and TNF- α in A-549 lung carcinoma cells on incubation with curcumin, when compared with cells in the control group. These findings suggest that curcumin exerts its effect through activation of JNK pathway.

CONCLUSION

Curcumin inhibits lung cancer growth by inducing apoptosis and cell cycle arrest. The suppression of lung cancer cell growth by curcumin involves mitochondrial-dependent signal pathways. Thus, curcumin can be potentially developed as a therapeutic agent for lung cancer.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hui Wang designed the study and compiled the data. Wei Qiao carried out the experiments and performed the literature study. Both authors wrote and approved the article for publication.

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