The mechanism underlying proliferation-inhibitory and apoptosis-inducing effects of curcumin on papillary thyroid cancer cells

Fei Song,1 Li Zhang, Hui-Xin Yu, Rong-Rong Lu, Jian-Dong Bao, Cheng Tan, Zhen Sun

Abstract

Recently, many studies on health benefits associated with curcumin have been reported. In this study, the effects of curcumin on apoptosis of papillary thyroid cancer cell line K1 and its potential mechanisms were investigated. Curcumin was found to significantly inhibit cell viability and promoted cell apoptosis in a dose-dependent manner. Moreover, curcumin-induced cell apoptosis was characterized with a rapid stimulation of reactive oxygen species (ROS) production. Furthermore, curcumin-induced ROS generation led to the loss of mitochondrial membrane potential (MMP) and the disturbance of intracellular Ca2+ concentration. A decrease in expression of Bcl-2 and the cleavage of poly ADP-ribose polymerase (PARP) were observed after exposure to curcumin. Results of this study may elucidate the curcumin-induced apoptosis effects on K1 cells. Thus, our results indicate a role of curcumin as health-promoting food ingredient, as well as a potential chemotherapeutic agent which is able to fight against papillary thyroid cancer.

1. Introduction

In recent years, extensive research has been carried out on the health promotion properties of different phytochemicals (Lantto, Colucci, Zvadov, Hiltunen, & Raasmaja, 2009). Among various phytochemicals as functional food constituents, polyphenols stand in the interest of many researchers due to their anti-oxidant, anti-inflammatory, anti-bacterial, anti-mutagenicity and anti-cancer properties (Parvathy, Negi, & Srinivas, 2009; Shishu & Kaur, 2008). Curcumin [bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione], derived from the plant turmeric (Curcuma longa), is among the best characterized polyphenols and primarily used as a food additive in several South Asian countries. It also has a long history to be used in treating inflammation and cancers in India and China (Miller, Chen, Woodiff, & Kansra, 2008). As reported, curcumin possessed the ability to inhibit the growth of a wide variety of tumour cells in multiple experimental model systems (Shi et al., 2006). It has been confirmed that the inhibitory effects of curcumin against proliferation of cultured cancer cells are attributed to its induction of cell cycle arrest and apoptosis.

Apoptosis, a morphologically distinct form of programmed cell death, is a closely regulated mechanism to control the removal of unwanted and/or harmful cells of an organism (Edinger & Thompson, 2004; Lockshin & Zakeri, 2004). It is also needed for the maintenance and function of homoeostasis, differentiation, embryonic development and the immune system (Kerr, Wyllie, & Currie, 1972). Impairments in apoptosis can be associated with several diseases, e.g., neurodegenerative diseases and cancer. Thus, induction of apoptosis in cancer cells is a potentially promising approach for cancer therapy. The mechanisms responsible for apoptosis induction by phytochemicals seem to be multiplex, including inhibition of NF-κB activation, down-regulation of the Bcl-2 and Bcl-Xi, levels, enhancement cytochrome c release, inducement of cell cycle arrest, up-regulation of growth arrest and DNA damage, and activation of p38 and caspase-3 (Sahu, Batra, & Srivastava, 2009; Shi et al., 2006; Shishodia, Sethi, & Aggarwal, 2005). However, the signalling pathways governing apoptosis are complex and the pro- and anti-apoptotic variations regulate cell survival change depending on the cell type. Thyroid cancer is the most common endocrine malignancy worldwide and constitutes 1% of all malignancies worldwide. Papillary thyroid cancer accounts for 80% of all thyroid cancers and its incidence has been rapidly rising in some European countries, the United States, China and other countries over the past three decades (Enewold et al., 2009; Kilfoy et al., 2009; Smailyte, Miseikyte-Kaubriene, & Kurtinaitis, 2006). Up to now, researchers have looked at the curcumin-induced apoptosis in papillary thyroid cancer cell line K1, however, this is an area which has not been fully elucidated and the cellular and molecular mechanisms have not really been investigated.

Recently, there are growing evidences that apoptosis is due to the production of reactive oxygen species (ROS). ROS have destructive actions on both DNA and proteins, and they have been suggested
as regulators of the process involved in the initiation of apoptotic signalling (Shih et al., 2004; Simon, Haj-Yehia, & Levi-Schaffer, 2000). Indeed, ROS have been demonstrated to perform certain functions in the early stages of apoptosis, and to induce the depolarization of mitochondrial membrane, which eventually results in an increase in the level of other pro-apoptotic molecules in the cytosol (Shih et al., 2004). However, the molecular mechanisms associated with the role of ROS have not to be clearly understood.

The aim of this study was to investigate the mechanism of curcumin-induced apoptosis in papillary thyroid cancer cells. The changes of intracellular ROS generation, mitochondrial membrane potential (MMP) and Ca\(^{2+}\) influx upon curcumin were evaluated. Additionally, the expression of apoptosis-related proteins Bcl-2 and poly ADP-ribose polymerase (PARP) were examined. Taken together, the induction of apoptosis by curcumin may provide a pivotal mechanism for its cancer chemopreventive action.

2. Materials and methods

2.1. Chemicals

Curcumin (Cur), N,N-dimethyl sulfoxide (DMSO), propidium iodide (PI), Hoechst 33342, and Rhodamine 123 were purchased from Sigma (St. Louis, MO, USA). Fluo-3/AM was bought from Dojin Laboratories (Kumamoto, Japan). Annexin V-fluorescein isothiocyanate (FITC) and PI apoptosis detection kit were bought from BD

![Fig. 1. Effects of curcumin on cell morphology and viability. (A) Effects of curcumin on morphology of K1 cells. K1 cells were exposed to different concentrations of curcumin (0–50 \(\mu\)M) for 24 h and cell morphological changes were assessed by microscopic examination. (B) PI and Hochest 33342 staining fluorescence images. After treatment with curcumin (0–50 \(\mu\)M) for 24 h, K1 cells were stained with PI (10 \(\mu\)g/ml) and Hochest 33342 (10 \(\mu\)g/ml) and then observed under a fluorescence microscope. (C) Effects of curcumin on cell death rate. The death rate is expressed as percentage of PI positive staining cells. All data represent as the means ± SEM of five independent experiments. SC, solvent control. *\(P < 0.05\) vs control, **\(P < 0.01\) vs control (Student’s two tailed t-test).]
Pharmingen (San Diego, CA, USA). Other drugs and reagents used in this study were as follows: 2’,7’-dichlorofluorescin diacetate (DCFH-DA, Cabiochem, USA), anti-β-actin, anti-poly ADP-ribose polymerase (PARP) and anti-Bcl-2 antibody (Beyotime, China). All other chemicals were of the highest analytical grade and purchased from common sources. Curcumin was dissolved in DMSO at 10 mg/ml, and stored at −20 °C until dilution before use.

2.2. Cell culture and drug treatment

Papillary thyroid cancer cell line K1 was obtained from the European Collection of Cell Cultures and maintained in complete medium [DMEM:F-12:MCDB105 = 2:1:1] containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in a humid atmosphere of 5% (v/v) CO2 and 95% (v/v) air at 37 °C. K1 cells in log phase were plated in cell culture plates (Corning, NY, USA) at a density of 8 × 10³ cells per well for 96-well plates and 4 × 10⁵ cells per well for 6-well plates. Then cells were treated with various concentrations (10, 20, 30, 40 and 50 μM) of curcumin for 24 h. Control cells were treated with the same medium without curcumin. As to the solvent control, it contains an equivalent amount of DMSO (0.18% DMSO) with the highest concentration as used in the curcumin-treatment group.

2.3. Cell viability assay

Cell viability was determined by PI and Hoechst 33342 staining (Zhao et al., 2010). After treatment with the indicated amount of curcumin (0–50 μM) for 24 h, cells were harvested and washed twice with phosphate-buffered saline (PBS, NaCl 140 mM, KCl 2.7 mM, Na₂HPO₄ 6.4 mM, H₂KO₄ 1.5 mM, pH 7.4). Then the cells were stained with PI (10 μg/ml) and Hoechst 33342 (10 μg/ml) at 37 °C for 10 min. After incubation, the plates were inspected with a fluorescence microscope (Olympus Optical, Japan). Cells were counted for five independent microscopic fields per well. Cell death rate (%) = (PI positive staining cells/total cell number) × 100%.

2.4. Cell apoptosis assay

Apoptosis characterized with translocation of phosphatidylserine to the cell surface was determined using an annexin V-FITC and PI apoptosis detection kit (Wang et al., 2011). Briefly, K1 cells were treated with the indicated amount of curcumin for 24 h, washed twice with PBS buffer, and stained with annexin V-FITC and PI, and analyzed by flow cytometry (FACS Cabilbur, Becton Dickinson, USA) according to the manufacturer’s instructions.

2.5. Measurement of ROS formation

Generation of ROS was measured by the oxidative-sensitive fluorescent probe DCFH-DA (Zhao, Zou, Lin, Shi, & Zhu, 2007). Intracellular ROS can oxidise DCFH-DA to the highly fluorescent compound dichlorofluorescein (DCF). At 0, 5, 30, or 60 min following the treatment with 40 μM curcumin, the medium was aspirated and replaced by DCFH-DA (10 μM) for a further 30 min at 37 °C. The cells were collected by pipetting, washed twice with PBS, and then analyzed by flow cytometry through FL1 channel.

2.6. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial inner membrane is negatively charged for being rich in negatively charged glycoprotein. A large accumulation of protons out of the inner membrane causes transmembrane potential, which can be assessed using the fluorescent rhodamine 123, a cell permeable cationic dye that preferentially enters mitochondria based on highly negative mitochondrial membrane potential (Laparra, Velez, Barbera, Farre, & Montoro, 2008). Depolarization of mitochondrial membrane potential during cell apoptosis results in the loss of rhodamine 123 from the mitochondria and a decrease
in intracellular fluorescence intensity. Briefly, at 24 h after the treatment with 0–40 μM curcumin, K1 cells were harvested, washed twice with PBS, incubated with 10 μM rhodamine 123 for 30 min at 37 °C in the dark and gently washed three times with PBS. The intracellular fluorescence intensity was analyzed by flow cytometry through FL1 channel.

2.7. Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ levels were determined with the Ca²⁺-sensitive fluorochrome Fluo-3/AM which can cross the cell membrane and be cut into Fluo-3 by intracellular esterase (Wang & Xu, 2005). The Fluo-3 can specifically combine with the Ca²⁺ and has a strong fluorescence. Cells at 6-well plate were collected after the treatment with 0–40 μM curcumin for 24 h, washed twice with PBS, then incubated with 3 μM Fluo-3/AM at 37 °C for 30 min in the dark and gently washed three times with PBS. The fluorescence was analyzed by flow cytometry through FL1 channel.

2.8. Western blot analysis

K1 cells with the indicated amount curcumin pretreatment were incubated for 24 h and then collected and washed with PBS. After centrifugation, cells were lysed in 20 μl lysis buffer [150 mM NaCl, 1% (w/v) NP-40, 0.02% (w/v) sodium azide, 10 μg/ml PMSF, 50 mM Tris–HCl (pH 8.0)] supplemented with additional protease inhibitor. The lysate was subjected to repeated freezing and thawing for three times and centrifuged at 12,000 × g for 5 min at 4 °C. The supernatant was collected and the protein concentration was determined using the Bradford assay (Bradford, 1976). After addition of sample loading buffer, protein samples were electrophoresed on a 15% SDS–PAGE and subsequently transferred onto a polyvinylidene fluoride membrane (Millipore, USA), were electrophoresed on a 15% SDS–PAGE and subsequently transferred onto a polyvinylidene fluoride membrane (Millipore, USA), after which the membrane was incubated in fresh blocking buffer (0.1% (v/v) Tween 20 in Tris-buffered saline, pH 7.4, containing 5% (w/v) skim milk) at room temperature for 1 h and then probed with the following antibodies: anti-β-actin (1:1000, v/v), anti-poly (ADP-ribose) polymerase (PARP) (1:1000, v/v), anti-Bcl-2 (1:500, v/v) in blocking buffer at 4 °C overnight. After three times washing each for 5 min with TBST (Tris-buffered saline with 0.1% (v/v) Tween 20) buffer at 4 °C overnight. After three times washing each for 5 min with TBST (Tris-buffered saline with 0.1% (v/v) Tween 20), the membrane was incubated with the appropriate HRP-conjugated secondary antibody (Goat anti-mouse IgG, 1:500 and Goat anti-rabbit IgG, 1:500, Santa cruz Biotechnology, California, USA) at room temperature for 1 h and then washed again three times in TBST buffer. The membrane was incubated with enhanced chemiluminescence substrate solution (Santa Cruz Biotechnology, California, USA) for 5 min according to the manufacturer’s instructions and visualised with autoradiography film.

2.9. Statistical analysis

Results were expressed as mean ± SEM. Two group comparisons were evaluated using the Student’s t-test. Differences were considered statistically significant when P < 0.05.

3. Results

3.1. Curcumin inhibits cell viability of K1 cells in a dose-dependent manner

Firstly, the effects of curcumin on K1 cell viability were investigated. The viability of K1 cells treated with curcumin at 0, 10, 20, 30, 40 or 50 μM for 24 h was determined by PI and Hoechst 33342 staining. Cell morphological changes were assessed by microscopic examination. During a 24 h period, curcumin treatment caused cell shrinkage, rounding and partial detachment, demonstrating the cytotoxic effects of curcumin on K1 cells as shown in Fig. 1A. Obviously, curcumin induced decrease of cell survival in a dose-dependent manner. As illustrated in Fig. 1B the amounts of the red-emitting fluorescent cells were increasing with the curcumin concentration mounting. The death ratio of K1 cells treated with curcumin at 10, 20, 30 and 40 μM was up to 1.14 ± 0.25%, 9.58 ± 3.29%, 23.29 ± 2.47% and 36.53 ± 2.10%, respectively (Fig. 1C). In the highest concentration group (50 μM), the dead cells increased to 45.04 ± 5.91% compared with controls (P < 0.01). There is no difference between the control and solvent control (P > 0.05).

3.2. Curcumin induces apoptosis in K1 cells

To determine whether the cell death was apoptotic, K1 cells were stained with Annexin V-FITC and PI followed by flow cytometry analysis. On the flow cytometry charts (Fig. 2), the Q3 represents the living cells, the Q4 refers to the early apoptosis cells and the Q2 means the late apoptosis. As shown in Fig. 2, upon incubation with 10–40 μM curcumin for 12 h, there were apparent shifts from Q3 to Q4 and Q2. The cells in Q2 steady increased with the increase of curcumin concentration, especially when the curcumin concentration exceeded 30 μM. At 30 and 40 μM, the apoptosis was dramatically up to 88.5% and 99.5%, respectively. Our results showed that curcumin promoted K1 cell apoptosis in a dose-dependent manner.

![Fig. 3. Effects of curcumin on ROS generation in K1 cells. (A) K1 cells were treated with 40 μM curcumin for 0, 5, 30 and 60 min, stained with DCFH-DA (10 μM) for 15 min and analyzed by flow cytometry. ROS in the cell converts the non-fluorescent dye into fluorescein, which emits green fluorescence. Fluorescence intensity is an indication of ROS levels in K1 cells. (B) Quantitative analysis of ROS accumulation in K1 cells. All data represent as the means ± SEM of three independent experiments. SC, solvent control. *P < 0.05 vs control (Student’s two tailed t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](https://example.com/fig3)
3.3. Curcumin induces intracellular ROS accumulation in K1 cells

ROS have been shown to be involved in cell proliferation and in apoptosis. In order to measure oxidation stress induced by curcumin, fluorescent dye DCFH-DA was used to examine ROS generation. When K1 cells were treated with curcumin at 40 μM, the fluorescent intensity increased to the maximum level at 5 min (from 17.97 ± 0.85 at 0 min to 51.58 ± 11.45 at 5 min), indicating a rapid increase in intracellular ROS production, after which the fluorescence intensity steadily reduced within 1 h as observed in Fig. 3A and B (57.5 ± 7.77 at 30 min; 51.58 ± 11.45 at 60 min).

3.4. Curcumin causes the loss of MMP in K1 cells

Loss of MMP has been shown to be an early event during apoptosis. We measured the MMP with rhodamine 123 to evaluate the function of mitochondria. At 24 h of curcumin treatment, the state of the MMP was examined by flow cytometry. As shown in Fig. 4A and B, the MMP rapidly declined in a dose-dependent manner. At 10–20 μM, MMP decreased slowly. However, compared with control cells, 30 μM curcumin-treatment group rapidly increased the proportion of rhodamine 123 negative cells from 3.26 ± 0.34% to 20.38 ± 8.17% (P < 0.05), and 40 μM curcumin further increased the proportion of rhodamine 123 negative cells to 71.64 ± 6.88% (P < 0.01), which was more than 2 times that of the 30 μM group. Our data indicate that exposure to curcumin of K1 cells resulted in MMP collapse.

3.5. Curcumin increases intracellular Ca²⁺ influx in K1 cells

An increase in intracellular Ca²⁺ concentration is recognised to be a factor for cell death and cell injury. Ca²⁺-sensitive fluorescence
probe Fluo-3/AM was employed to monitor alterations in intracellular Ca$^{2+}$ level by flow cytometry. As illustrated in Fig. 5A and B, in the control group, the level of intracellular free Ca$^{2+}$ was the lowest. When K1 cells were exposed to curcumin (10–40 µM) for 24 h, the Fluo-3 fluorescence moved to higher levels of intensity in a dose-dependent manner, indicating an increase in Ca$^{2+}$ influx. At 40 µM, intracellular Ca$^{2+}$ fluorescence increased dramatically to 30.86 ± 0.47%, more than 3 times that of the control group (12.46 ± 1.79%).

3.6. Curcumin effects on the protein expressions of PARP and Bcl-2

To determine whether caspase-dependent apoptotic activity could be modulated by curcumin, K1 cells were treated with different doses of curcumin for 24 h. The expression and cleavage of PARP was determined by western blot analysis. The results showed that PARP was rapidly cleaved from 116 to 89 kDa when the cells were exposed to 20 µM curcumin or more for 24 h (Fig. 6A and C).

Bcl-2 is a member of the Bcl-2 family of apoptosis regulator proteins. The expression of Bcl-2 in K1 cells were studied. Curcumin dramatically down-regulated the expression of Bcl-2 in K1 cells (Fig. 6B and D), and up to 30–50 µM curcumin treatment even no obvious Bcl-2 expression was observed.

4. Discussion

Curcumin is a nutraceutical, used worldwide for food as well as medicinal applications. Its beneficial connection with cancer...
prevention and treatment is without dispute. Curcumin as a plant-derived polyphenol occurs in remarkable amounts in the plant turmeric (C. longa). It also exhibits potent anti-oxidant and anti-tumour properties. Several studies have suggested that curcumin can induce apoptotic cell death in malignant cells. However, the mechanisms responsible for apoptosis induced by curcumin appear to be ill-defined.

The results in these studies showed that curcumin could inhibit proliferation and induce apoptosis in K1 cells in a dose-dependent manner. With the curcumin concentration increased, cell viability decreased significantly (Fig. 1) and the apoptosis increased steadily (Fig. 2). Furthermore, this study has highlighted how curcumin enhance cell apoptosis via a mechanism which is dependent on the generation of ROS. However, other reports have indicated that curcumin has the ability to inhibit ROS (Sandur et al., 2007), and consequently apoptosis, through its well known antioxidant properties. Oxidative stress is considered to be an important condition to promote cell death in response to a variety of signals and pathophysiological situations. In 1991, it has been already shown that hydrogen peroxide is able to induce apoptosis (Pierce, Parchment, & Lewellyn, 1991). Since then many researchers have demonstrated that ROS can induce apoptosis in many different cell systems (Simon et al., 2000; Xu et al., 2011). ROS, which is predominantly produced in the mitochondria, if excessive, may lead to the free radical attack of membrane phospholipids and loss of mitochondrial membrane potential, which releases apoptosis-inducing factors that activate caspase cascades and cause nuclear condensation (Thayyullathil, Chattoth, Hago, Patel, & Galadari, 2008). In this study, it was illustrated that curcumin induced a very rapid and significantly ROS generation in K1 cells, which could be readily detected only 5 min after drug treatment, leading to apoptotic signals (Fig. 3). The present findings corroborate the similar conclusion obtained by Hosseinzadeh et al., who recently reported that curcumin enhanced the apoptosis by doxorubicin via generation of ROS (Hosseinzadeh et al., 2011). Hence, these results supporting the hypothesis that curcumin leads to the rapid generation of ROS and this may play an important role in curcumin-induced apoptosis in K1 cells.

Moreover, ROS generation is also correlated with the decrease of MMP. Indeed, ROS have been demonstrated to induce the depolarization of the mitochondrial membrane, which in turn could result in the activation of mitochondrial apoptosis pathway and invoke several related mitochondrial pro-apoptotic factors (Wang, 2001). A rapid collapse of MMP is always found in some anticancer compounds-induced apoptosis in cancer cells (Chen et al., 2007). In this study, the MMP was investigated with fluorescent dye rhodamine 123. Our data clearly show that treatment with 10–40 μM curcumin could lead to a loss of mitochondrial transmembrane potential (Fig. 4), which is in agreement with the previous studies (Wang et al., 2011). This means that curcumin-induced apoptosis is related to the collapse of the MMP.

The collapse of the MMP implies the opening of mitochondrial permeability transition pore, which in turn could lead to the intracellular Ca2+ concentration increase (Wang et al., 2011). The intracellular Ca2+ level is another factor related to cell death with decrease of MMP, release of cytochrome c from mitochondria and the activation of apoptosis proteins caspase. Our findings demonstrated that as the curcumin concentration increased, intracellular Ca2+ congregated dramatically (Fig. 5). It was in accordance with the tendencies of cell apoptosis.

Caspases, represented by a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Among them, caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9. The activated caspase-3 could cleave the PARP, which is one protein related to a number of cellular processes involving mainly DNA repair and programmed cell death (Wang, 2001). In our study, the cleavage of PARP from its full-length form (116 kDa) to the cleaved form (89 kDa) was measured (Fig. 6A), which represents a biochemical hallmark of apoptosis. Thus, curcumin-induced cell death was accompanied by an increase in the activity of caspase, which then stimulated the molecular cascade of apoptosis.

On the other hand, members of the Bcl-2 family are critical for the regulation of apoptosis. Bcl-2 and its homologues could prevent disruption of mitochondrial membrane and release of cytochrome c and other pro-apoptotic factors (Tsujimoto, 2002). The expression
of Bcl-2 proteins might partly indicate the fate of the cells. A recent study has revealed a decrease in expression of Bcl-2 in a dose-dependent manner after being exposed to curcumin (Song et al., 2005). These results also suggest that curcumin induced apoptosis by down-regulating anti-apoptotic Bcl-2 (Fig. 6B).

In conclusion, our results have demonstrated that curcumin could significantly inhibit the proliferation and induce apoptosis in K1 cells. In the apoptotic process, curcumin induced intracellular formation of ROS followed by the collapse of MMP and the intracellular Ca\(^{2+}\) influx amount. Curcumin could also affect the expression of apoptosis-related proteins Bcl-2 and PARP. On the other hand, compared with some chemotherapy drugs, curcumin has low cytotoxic effects on normal cells (Syng-ai, Kumari, & Khar, 2004). Collectively, the results indicate a role of curcumin as health-promoting food constituents, as well as a potential chemotherapeutic agent against papillary thyroid cancer. The possible utilisation of plant-derived compounds as chemopreventive and health-promoting agents in the future has focused increasing attention on the understanding of their molecular mechanisms and targets of action.

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