Chin J Integr Med 2006 Jun; 12(2) : 126－131

EXPERIMENTAL WORK AND RESEARCH

Growth Inhibition and Apoptosis Inducing Mechanisms of Curcumin on Human Ovarian Cancer Cell Line A2780*

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ABSTRACT **Objective:** To explore the growth inhibition effects and apoptosis inducing mechanisms of curcumin on human ovarian cancer cell line A2780. **Methods:** After treatment with 10－50 μmol/L curcumin for 6－24 h, the growth activity of A2780 cancer cells were studied by [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetry. Cellular apoptosis was inspected by flow cytometery and acridine orange-ethidium bromide fluorescent staining methods. The fragmentation of cellular chromosome DNA was detected by DNA ladder, the ultrastructural change was observed under a transmission electron microscope, and the protein levels of nuclear factor-κappa B (NF-κB, P65) and cysteinyl aspartate specific protease-3 (Caspase-3) in ovarian cancer cells were measured by immunohistochemistry. **Results:** After treatment with various concentrations of curcumin, the growth inhibition rates of cancer cells reached 62.05%－89.24%, with sub-G_1 peaks appearing on histogram. Part of the cancer cells showed characteristic morphological changes of apoptosis under fluorescence and electron microscopes, and the rate of apoptosis was 21.5%－33.5%. The protein expression of NF-κB was decreased, while that of Caspase-3 was increased in a time-dependent manner. **Conclusion:** Curcumin could significantly inhibit the growth of human ovarian cancer cells; inducing apoptosis through up-regulating Caspase-3 and down-regulating gene expression of NF-κB is probably one of its molecular mechanisms.

KEY WORDS curcumin, ovarian cancer, NF-κB gene, Caspase-3 gene, apoptosis

Curcumin, a phenolic pigment extracted from curcuma, is the major effective component of that traditional Chinese medicine and used as a dietary coloring agent and a natural condiment. Recent research reports have shown that curcumin could selectively inhibit the proliferation and induce the apoptosis of many tumor cells, but the involved mechanisms still unclear[1,2]. The phenomenon that curcumin induces apoptosis of tumor cells could be seen in numerous cancers, however, the effects of curcumin on human ovarian cancer cells have not been reported yet. In this research, the apoptosis inducing effect of curcumin on ovarian cancer cell line A2780 and its influence on gene expression of NF-κB and Caspase-3 were studied.

METHODS

Material and Reagents

Pure dried curcumin powder was purchased from Sigma Company and prepared into 1 mmol/L stock solution with dimethyl sulphoxide (DMSO). Acridine orange, ethidium bromide, propidium iodide and [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were also purchased from Sigma Company. Proteinase K and DMSO were purchased from Roche Company. Monoclonal mouse anti-human NF-κB and Caspase-3 antibodies were purchased from Beijing Zhongshan Biotechnology Company.

The human ovarian cell line A2780 obtained from Cancer Institute of Chinese Academy of Medical Sciences was cultured in RPMI 1640 with 10% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin under standard conditions (37℃, 5% CO_2).

The human ovarian cancer cell line A2780 cells were inoculated at 1.2×10^8/L density into 96-well chamber plate with 100 μl per well. Every group had triplicate wells. When the cells were anchored to the plates, the stock solution of curcumin was diluted into various concentrations (10 μmol/L, 20 μmol/L, 30 μmol/L, 40 μmol/L, and 50 μmol/L)

* Supported by National Natural Science Foundation of China (No. 30200284)

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with RPMI 1640 and added into the wells, 100 μl in each well, then the cells were incubated at 37℃, 5% CO_2, ready for different detection.

Besides, a control group of cell culture was set up, which was operated in the same way as described above but with no curcumin added, that is, the concentration of curcumin solution added was equal to zero.

Detection of Cell Growth Activity *in vitro*

After 6 h, 12 h, 18 h and 24 h of culture, each well was added with 20 μl of 0.5% MTT and cultured for another 4 h. Then the supernate was discarded and 100 μl of DMSO was added to each well and agitated to dissolve the crystal. The absorbance (A) values of the samples were read on enzyme-labeled Mini-reader Ⅱ at the wave length of 570 nm.

The cellular proliferation inhibition rate (CPIR) was calculated by the following formula. CPIR = (1− average A value of experimental group/average A value of control group) × 100%.

After being treated with curcumin for 12 h, 2×10^6 A2780 cells were collected, washed with phosphate-buffer saline (PBS, pH 7.2) twice, and fixed in 5 ml of 70% ethanol overnight at 4℃. Then the cells were washed again with PBS, incubated with buffer including RNase 200 μl (1 mg/ml) for 30 min at 37℃ by water bath, stained with 400 μl of propidium iodide (50 μg/ml) for 30 min at room temperature with no light. The apoptotic "sub-G_1" peaks were detected with flow cytometry (Becton Dickson Company) using CELLQEST software.

DNA Ladder Detection

Referring to literature[3], 1×10^6 cells were collected and re-suspended in 20 μl of PBS and reacted with 400 μl of cell splitting solution for 10 min. After centrifugation, the supernate was collected and sodium dodecyl sulfate added to make the concentration to 1%. Then the sample was incubated with RNase (terminal concentration in 50 μg/ml) at 56℃ for 1 h, managed with proteinase K in terminal concentration of 2.5 μg/μl overnight at 37℃, and the DNA was deposited with ethanol. The electrophoresis with 1% agarose gel was performed for DNA observation and photo taking done under ultraviolet light.

Detection of Apoptosis Rate (AR)

Referring to literature[4], the number of vital cells (VN), early apoptotic cells with intact cell membrane (VA), late stage apoptotic cells with incompetent cell membrane (NVA), and non-apoptotic dead cells (NVN) in the control group and the tested groups, after being treated with various concentrations of curcumin for 12 h, were observed and counted under a fluorescence microscope respectively and analyzed with high resolution pathological image analysis system type 1000 (HPIAS1000) purchased from Tongji Medical Collage of Huazhong University of Science and Technology, and the AR of cells was calculated by the following formula: AR = (VA+NVA)/(VN+NVN+VA+NVA)×100%.

Observation of Ultrastructure of Apoptotic Cells

After being treated with 20 μmol/L curcumin for 12 h, cancer cells in the sample were digested with 0.125% trypsinase and 0.01% ethylene diamine tetra-acetic acid (EDTA), rinsed by PBS, fixed with 2.5% glutaraldehyde for 30 min, washed again by PBS, and suspended. After routine embedding and sectioning, the cellular ultrastructure in the sample was observed under a transmission electron microscope.

Gene Expression Assay of NF-κB and Caspase-3

The percentage of NF-κB and Caspase-3 protein expression was detected using the strept-peroxidase (SP) immunohistochemical method, according to the instruction in the test kit. The cell creeping slices made by the cancer cells gotten from the control group and the tested groups were, after being treated with different concentrations of curcumin for various time points, fixed with acetone. After the non-specific binding sites were blocked with 10% normal goat serum for 30 min, the slices were incubated with mouse monoclonal anti-human antibody of NF-κB and Caspase-3 respectively, and followed by incubating with goat anti-mouse IgG as the second antibody. After treated with streptavidin-peroxidase, the slices were stained with 3, 3'-Diaminobenzidine (DAB) and counter-stained with haematoxyin. The sample for negative control was treated by PBS instead of the first antibody.

Both the positive rate and intensity were adopted to estimate the results. The positive cells expressed brown granules in cytoplasm. To score the positive rate, 500 cells were counted under a light microscope using HPIAS-1000 and calculated by the following formula: positive cells rate = (the number of positive cells in 500 cells)/500×100%.

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Statistical Analysis

Statistical analysis was performed using the Student's *t* test and ANOVA (analysis of variance).

RESULTS

Growth Activity of Cancer Cells

See Figure 1. The *in vitro* growth of A2780 cells in the control group was active, while in the tested groups, it decreased to a certain extent after treated with 10－50 μmol/L curcumin for 6－24 h in a time- and dose-dependent manner, the growth inhibition rates being 62.05%－89.24%. There was significant difference among the effects of curcumin of the same concentration acting at various time points ($P<0.01$), and significant difference among those of different concentrations acting at the same time point ($P<0.05$). The 12 h half inhibitory concentration (IC_{50}) of curcumin was 41.6 μmol/L.

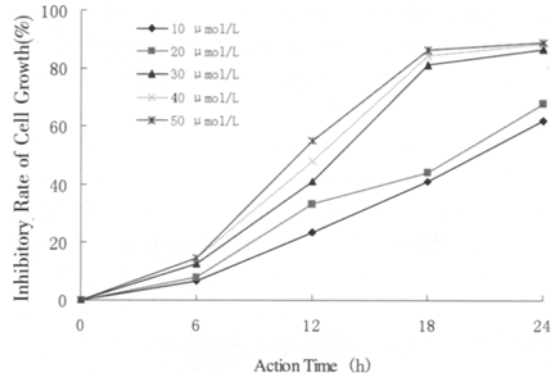

Figure 1. Growth Inhibition Effects on A2780 Cells by Various Concentrations of Curcumin

Dynamic Apoptosis Inducing Effects

See Figure 2. After being treated with 10－50 μmol/L curcumin, significant sub - G_1 peaks

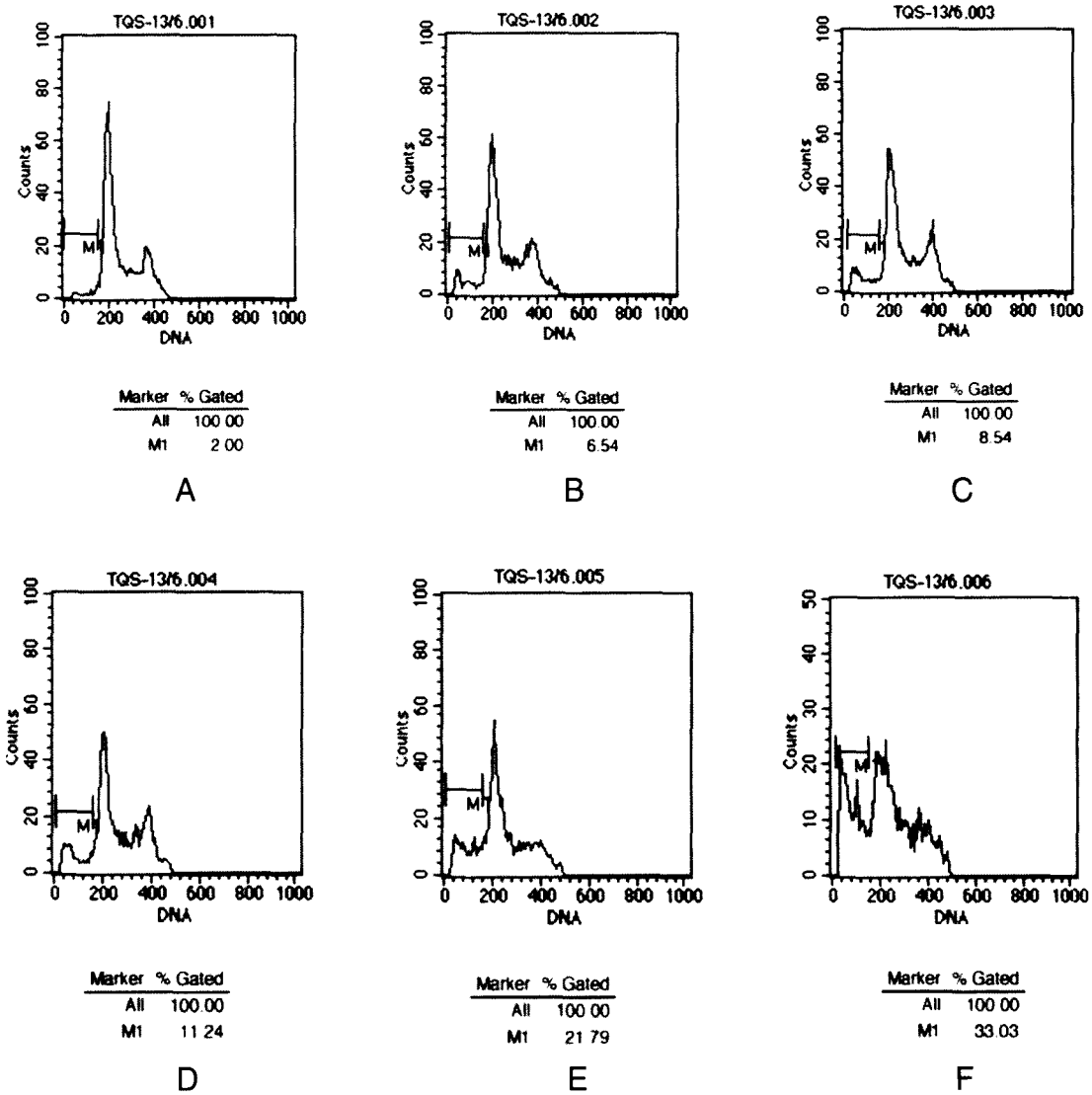

Figure 2. Apoptosis Inducing Effects of Various Concentrations of Curcumin on A2780 Cells Detected by Flow Cytometry

Notes: A: Control group; B: 10 μmol/L curcumin; C: 20 μmol/L curcumin; D: 30 μmol/L curcumin; E: 40 μmol/L curcumin; F: 50 μmol/L curcumin

showed on FCM with the amplitude increased along with the concentration of curcumin.

Biochemical Detection of Cellular Apoptosis

See Figure 3. The 1% agar-gel electrophoresis image of the sample of the control group showed regular striae of genom, while in the tested groups, the segment of DNA 180－200 bp in size, i.e. typical DNA ladder, were shown after the samples were treated with various concentrations of curcumin for 12 h.

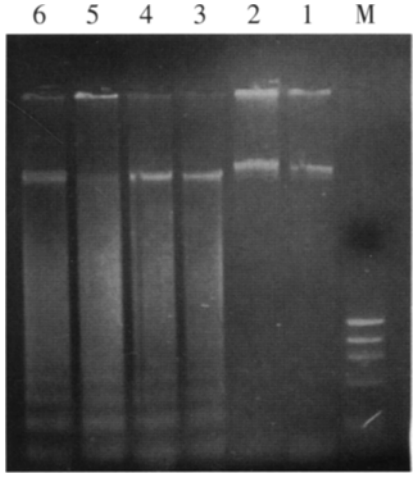

Figure 3. Apoptosis of Ovary Cancer Cells Induced by Various Concentrations of Curcumin for 12 h Assayed by DNA Ladder Notes: M: Φ174/Hinf marker; 1－2: control group; 3: 10 μmol/L curcumin; 4: 20 μmol/L curcumin; 5: 30 μmol/Lcurcumin; 6: 40 μmol/L curcumin

Ultrastructure of Apoptotic Cells

See Figure 4. After being treated with 20 μmol/L curcumin for 12 h, part of the A2780 cells presented typical ultrastructure changes of a-poptosis under electron microscope, such as cell shrinkage with pyknotic nucleus and disappearance of nucleolus, concentrated chromatin congregating close to the nuclear membrane, crenate and tortuous cell, but the cell membrane was still intact.

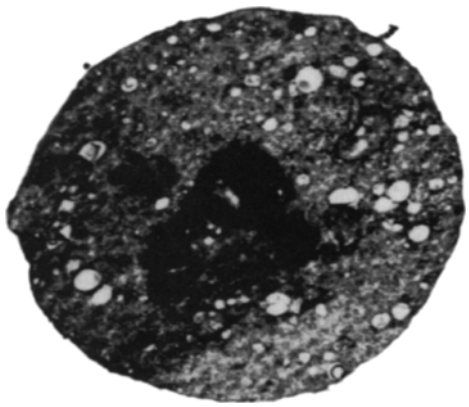

Figure 4. Electron Microscope Observation of Apoptotic Cells Treated with 20 μmol/L Curcumin for 12 h (8000×)

Relation between Apoptosis Rate and Concentration of Curcumin

Nucleus of early apoptotic cells (VA) showed shrunken fluorescence under fluorescence microscope, and nucleus of late apoptotic cells (NVA) showed shrunken orange. Although the nucleus of active cells (VN) and non-apoptotic dead cells (NVN) also showed green and orange fluorescence respectively, they had normal cellular structure. The apoptosis rates, calculated on the basis of above criteria, in various groups after the cells being acted by curcumin for 12 h were listed in detail in Table 1, showing significant statistical difference in comparison of apoptotic rate in the tested groups of various curcumin concentrations respectively with that in the control ($P<0.05$).

Table 1. Apoptosis of A2780 Cells Detected by Acridine Orange-ethidium Bromide Fluorescent Staining after Treatment with Various Concentrations of Curcumin for 12 h

Group	Cell Number				Summation	Apoptosis Rate (%)
	VA	NVA	NV	NVN		
Control	8	5	179	8	200	6.5
10 μmol/L Curcumin	12	31	140	17	200	21.5*
20 μmol/L Curcumin	17	34	138	11	200	25.5*
30 μmol/L Curcumin	19	38	129	14	200	28.5*
40 μmol/L Curcumin	21	41	126	12	200	31.0*
50 μmol/L Curcumin	24	43	122	11	200	33.5*

Notes: * $P<0.05$ compared with the control group

Effects of Curcumin on NF-κB and Caspase-3 Expression

See Table 2. After being treated with curcumin for 12 h, the NF-κB (P65) positive rates of A2780 cells decreased, while those of Caspase-3 increased in time- and dose-dependent manner, the respective difference between the control group and the tested groups of various concentrations was significant ($P<0.05$).

Table 2. Effects of Various Concentrations of Curcumin on NF-κB and Caspase-3 Expression ($\bar{x}\pm s$)

Group	Times of Tests	NF-κB	Caspase-3
Control	5	75.0±2.45	5.9±0.79
10 μmol/L Curcumin	3	55.0±5.57*	15.6±1.78*
20 μmol/L Curcumin	3	51.3±3.53*	18.8±3.75*
30 μmol/L Curcumin	3	46.0±3.00*	21.5±3.97*
40 μmol/L Curcumin	3	44.1±1.01*	31.3±3.21*
50 μmol/L Curcumin	3	31.3±1.53*	37.5±2.18*

Notes: * $P<0.05$ compared with the control group

DISCUSSION

Curcuma is widely used as a spice and coloring

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agent in foods, also as cosmetics and drugs. At the same time, it is one of the traditional Chinese medicines used to treat different kinds of inflammation (e. g. hepatitis). Curcumin (diferulomethane, $C_{12}H_{36}O_5$, FW 364. 5) is the major component of the Curcuma species, which has shown to have potent antioxidant, anti-inflammatory activities, and could inhibit the carcinogen-DNA adduct and tumorigenesis in several animal models.

Recently, curcumin has been considered by oncologists as a potential third generation cancer chemopreventive agent, though its involved molecular mechanism has not been well elucidated. It is proposed[5] by *in vitro* studies that curcumin may suppress tumor cell proliferation and induce apoptosis by blocking multiple pathways and regulating multiple tumor surface markers. Its action targets might vary from genome (DNA) level to the messenger RNA level and the enzyme (protein) level.

In this study, we have explored whether some apoptotic signal transduction genes, such as NF-κB and Caspase-3, play any role in the apoptosis inducing effects of curcumin. It was found that curcumin could induce apoptosis of human ovarian cancer cell line A2780 in this research: (1) the results of MTT assay and acridine orange-ethidium bromide fluorescent staining indicated that curcumin could both inhibit the growth and induce the apoptosis of A2780 cells *in vitro*. After treated by curcumin, the apoptotic cells increased in a time-dependent manner. The apoptosis inducing effect of curcumin was further confirmed from ultrastructural morphological viewpoint through transmission electron microscopy. (2) After treated with 10－50 μmol/L curcumin for 12 h, A2780 cells showed significant peak of sub-G_1 on DNA histogram.

After A2780 cells were treated with curcumin of various concentrations on creep slides for 12 h, it was found that the NF-κB (P65) expression was decreased, while that of Caspase-3 increased in a dose-dependent manner. Based on current references, these results gave us the following clues.

1. Caspase family is the key effector molecule on apoptotic signal transduction pathway triggered by Fas/Apo-1[6], the incision effects on aspartate residues make caspase family members activate with each other, in the manner of zymogen activation, and thus result in cascade magnification. At the same time, Caspases-3 is the most important effector caspase within the downstream of cascade, manipulating the incision of enzyme, which is one of the caspase family members closest involved in apoptosis. Caspases-3 could irreversibly destroy the key substance of cell survival, finally leading cells to apoptosis through such mechanisms as activating DNase, segmenting poly (ADP-ribose) polymerase, and degrading cellular framework proteins and certain cancer proteins[7]. Our results indicated that curcumin could activate the apoptotic effector Caspase-3 and had significant function in inducing apoptosis of human ovary cancer cells. There has been no other reports about the effects of curcumin on Caspase-3 expression heretofore.

2. NF-κB takes part in transcription regulation of many genes, such as cytokines, adhesive factors and growth factors, etc. It was also closely related to the immunological reaction, inflammation and tumor formation. The expression of many NF-κB related factors, such as tumor necrosis factor-α (TNF-α), granulocyte macrophage colony stimulating factor, intercellular adhesion molecule-1 and platelet-derived growth factor, increase in numerous diseases. It was considered that one of the molecular mechanisms involved in inducing apoptosis on ovary cancer cells might be the inhibitory effect of curcumin on NF-κB expression, which could result in reducing the transcription of some key genes responsible for cell proliferation.

3. It was demonstrated that NF-κB regulates the activity of Caspase-8 and Caspase-3 in apoptotic signal transduction pathway[8,9]: NF-κB acts in the earliest period of signal transduction, and inhibits at first the activity of Caspase-8 to block the cascade response activated by a series downstream caspases. Results of the study showed that curcumin could inhibit the expression of NF-κB (P65), which probably plays a negative role in regulating the early pathway of caspases on apoptosis.

4. It has been reported by some researchers that blocking activity of NF-κB could enhance the sensitivity of cancer cells to radiation and chemotherapeutic drugs, and increase the cell apoptotic effects induced by TNF-α[10]. Thus, we suppose that curcumin might cooperate with TNF-α in anti-tumor effects, and thus further research is being done by us.

Along with the advance of research, we have enough reasons to believe that curcumin, as a kind of condiment, dietary natural coloring agent and tradition Chinese Medicine, shall exhibit its expansive prospect of application as an anti-cancer medicine as well.

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(Received May 30, 2005)