Anti-Tumor Effect of Curcumin on Human Cervical Carcinoma HeLa Cells *In Vitro* and *In Vivo*

ZHAO Jing (赵敬)[1]**, ZHAO Yong (赵涌)[2]*, ZHANG Yan (张燕)[3], CHEN Wei (陈伟)[2]

[1]*Department of Pathophysiology,* [2]*Department of Pathology, Congqing Medical University, Chongqing 400016;* [3]*Department of Pathophysiology, Chuanbei Medical College, Nanchong*

CLC number: R737.33 Document code: A Article ID: 1000-9604(2007)01-0032-05
10.1007/s11670-007-0032-6

ABSTRACT

Objective: To investigate the anti-tumor effect of curcumin on human cervical carcinoma HeLa cells *in vitro* and *in vivo*. **Methods:** (1) Human cervical carcinoma cell line HeLa was cultured in *vitro*. HeLa cells were treated with 5-50 μmol/L curcumin for 24. 48, 72 h and the growth inhibition rates of HeLa cells were measured by MTT method. Cell apoptosis was inspected by electron microscopy and flow cytometry (FCM). (2) A transplanted tumor model by injecting HeLa cells into subcutaneous tissue of BABL/C mice was established and its growth curve was measured. 30 BABL/C mice with tumors were divided into 2 groups at random and 0.2 ml saline or 0.2 ml 250 μmol/L curcumin was injected into abdominal cavity respectively once everyday and lasted for ten days. The changes of tumor volume were measured continuously and tumor inhibition rate was calculated. At last the expressions of caspase-3 and bax protein in transplanted tumors were detected by immunohistochemistry. **Results**: (1) Curcumin inhibited the proliferation of Lela cells on a dose-depending manner. Apoptosis of cells could be observed by FCM. Partial cells presented the characteristic morphological changes of apoptosis under electron microseope. (2) When 1×10^7 HeLa cells were inoculated for each mouse, 100% of the mice developed growing tumors after seven days. An inhibition effect was observed in treatment group, and the inhibition rate of curcumin was 74.33%. The expressions of caspase-3 and bax in the transplanted tumors were increased in curcumin group. **Conclusion**: Curcumin is effective as an anti-cancer drug not only in *vitro* but also *in vivo*.

Key words: **Curcumin; Cervix neoplasm; Apoptosis; Immunohistochemistry; Flow cytometry**

Curcumin, a deferuloymethane, is a major active component of the food flavor turmeric (Curcuma Longa). Because of its stable colour and luster and low toxicity, curcumin has been widely used as food additive and coloring agent. Recently, it has been reported to possess anti-inflammatory, antioxidation and antiviral activities. Now, attention has been focused on its antitumor activity[1, 2]. *In vitro* curcumin was found to induce apoptosis of a wide variety of tumor cells including mice sarcoma S180 cells, human colon carcinoma HT-29 cells, human renal carcinoma 293 cells, human liver carcinoma HepG2 cells etc[3]. In our previous study, we have some primary results in the effect of curcumin on human cervical carcinoma[4]. In this study, we further investigated the anti-tumor effect of curcumin on HeLa cells, both *in vitro* and *in vivo*.

Received: Dec. 23, 2006; **Accepted :** Jan. 26, 2007
***Author** to whom correspondence should be addressed.
*E-mail: blys01@163.com
**E-mail: yangyang741022@sina.com

MATERIALS AND METHODS

Materials

HeLa cell line was supplied by the department of pathophysiology of Chongqing Medical University. Curcumin and 3-(4, 5-demethy-2-thiazolyl)-2, 5-dephenyl-2h-tetrazolium-bromid (MTT) were obtained from Sigma (St Louis, Mo, USA). RPMI-1640 and fetal bovine serum (FBS) were from Hyclone. The mouse monoclonal anti-Bax and anti-Caspase-3 antibodies were purchased from Fuzhou Maixin Biotechnology Co. (China).

MTT Assay

Cell growth was measured by a modified MTT assay. About 1×10^5 cells/well were plated in 96-well

microplates and incubated overnight. Cells were then treated with 5, 10, 25 and 50 μmol/L curcumin for 24, 48 and 72 h. Then 20 μl stock MTT was added to each well and the cells were further incubated at 37°C for 4 h. The supernatant was removed and 200 μl DMSO in isopropanol was added to each well to solubilize the formazan product. The absorbance at wavelength of 570 nm was measured by a micro ELISA reader (Sigma). The negative control well contained medium only. The ratios of the absorbances of treated cells relative to those of the control wells were calculated and expressed as percentage of growth inhibition.

Flow Cytometry

The induction of apoptosis was analyzed by using the Annexin V-PE Apoptosis Detection Kit I according to the manufacturer's instructions[5]. Briefly, cells were inoculated at a cell density of 1.0×10^5 cells/ml, treated with 5, 10, 25 and 50 μmol/L curcumin and cultured for 48 h. After cultivation, these cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then resuspended in a binding buffer [10 mmol/l HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$] at a cell density of 1.0×10^6 cells/ml. The cell suspension (100 μl) was transferred to a 5-ml culture tube to which 5 μl of annexin V-PE and 5 μl of 7-AAD were added as fluorescent dyes. After incubation for 15 min at 25°C in the dark, 400 μl of binding buffer was added to the solution. Flow cytometry analysis was performed within 1 h according to the manufacturer's instructions.

Electron Microscopy

Cells were planted in 50 ml plastic culture flask and incubated in the absence or presence of 25 μmol/L curcumin at 37°C in 5% CO_2 for 48 h. Cells were collected into 1.5 ml Ep tubes and fixed with 2.5% paraformaldehyde for 5 min. Sections were prepared and photographed under Hitach 600 electron microscopy (Japan).

Animal Experiment

Five-week-old female normal BALB/c mice, purchased from our university, were used in this study and maintained under extremely clean conditions. To establish tumors in the BALB/c mice, HeLa cells were subcutaneously injected into the mid-dorsal region of the mice at a cell density of 1×10^7 cells suspended in 100 μl of PBS. Tumors were allowed to grow until the tumor volume was increased to approximately 0.2 cm^3. After tumor growth, the mice were injected with 250 μmol/L of curcumin in 200 μl PBS or the same volume of PBS via abdominal cavity once everyday and lasted for ten days. The changes of tumor volume were measured continuously and tumor inhibition rate of curcumin was calculated. Tumor inhibition rate (IR)=(average weight of control group-average weight of curcumin group)/average weight of control group×100%. IR<30 was considered ineffective, while IR≥30 was considered effective[6, 7].

Immunohistochemistry

The tumor tissues were fixed with 10% paraformaldehyde at room temperature for 24 h. The paraffin-embedded specimens were cut into sections with a thickness of 5 μm. The detection procedure was done as described in Kit protocol[8]. PBS instead of the first antibodies was used in the negative control. The Bax and Caspase-3 positive cells were defined when there was an aggregation of brown particles in the cytoplasm of the tumor cells. And the rate of the positive expressions = (the positive cells/500 tumor cells) × 100%.

Statistical Analysis

The data were mean values of at least three different experiments and expressed as $\bar{x}\pm s$. The student's *t*-test was used to compare data. $P<0.058$ was considered to be statistically significant.

RESULTS

Effects of Curcumin on Cell Proliferation

The effects of curcumin on growth of HeLa cells are shown in Fig. 1. It was showed that cells of control group growth actively. Compared with control group, the growth of cells treated with different concentrations of curcumin was inhibited significantly in a concentration and time-dependent manner. After 72 h, inhibition rate was in the range of 11%-45.8%. Statistical analysis showed that the differences between samples with different treatment times were very significant ($P<0.01$). Compared with the control group, the differences of the groups of 5 and 10 μmol/L were significant ($P<0.05$) and between the groups of 25 μmol/L and 50 μmol/L were very significant ($P<0.01$).

Apoptosis of HeLa Cells Induced by Curcumin

Flow cytometry analysis of the apoptosis induced by curcumin was carried out by performing annexin

V-PE and 7-AAD staining. As shown in Fig. 2, the annexin V-PE+/7-AAD cell population (lower right quadrant) was markedly increased from 2.82% to 12.37% by curcumin treatment. The lower right quadrant represents the early stage of apoptosis. This result suggests that curcumin induces apoptosis.

Morphologic Changes of HeLa Cells Induced by Curcumin

By electron microscopy, we observed that in the control group, no apoptotic cell was found. However, in 25 μmol/L curcumin group, many apoptotic cells could be found which showed smaller size, dense cytoplasm and aggregated chromatin which distributed peripherally under the nuclear membrane with well-delimited dense masses of various shapes and sizes (Fig. 3).

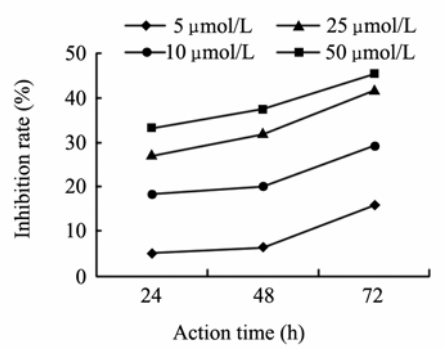


Fig. 1. The inhibitory effects of curcumin at different concentrations on HeLa cells growth

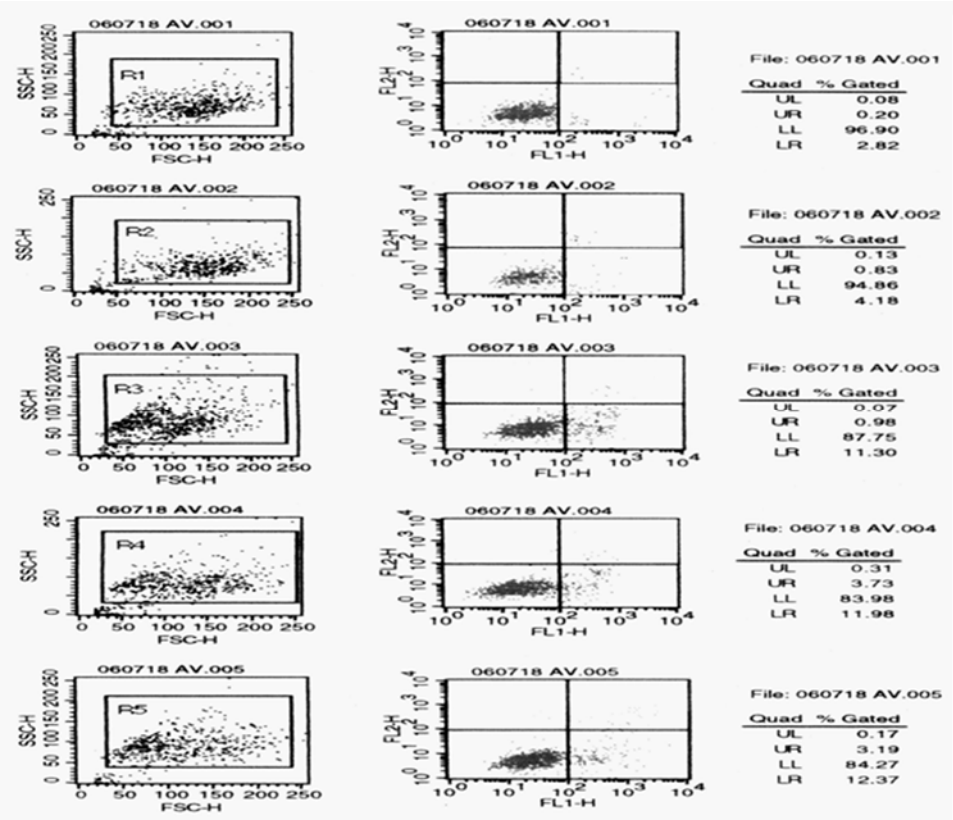


Fig. 2. Apoptosis rate of curcumin-treated HeLa cells by FCM

Anti-tumor Effect of Curcumin *In Vivo*

We examined the effects of curcumin on the growth of subcutaneous HeLa tumors. As indicated in Tab. 1 and 2, the growth of the tumors in BALB/c mice injected with curcumin via abdominal cavity was significantly delayed when compared with those of the control mice injected with PBS. No death of mice caused by curcumin injection was observed.

Tab. 1. Body weight of BALB/C mice before and after treatment

Group	n	Control ($\bar{x}\pm s$)	Curcumin ($\bar{x}\pm s$)
Weight before treatment (g)	15	21.55±1.037	21.85±1.306
Weight after treatment (g)	15	23.07±1.056*	21.18±1.042**

Compared with the weight before treatment, *P<0.05, **P>0.05

Effects of Curcumin on the Expressions of Bax and Caspase-3

Treated with 25 μmol/L curcumin for 48 h, the expressions of Bax and Caspase-3 increased. The difference of expression rates between control group and curcumin group was significant (P<0.05) (Fig. 4, 5).

DISCUSSION

The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest in phytochemicals. Curcumin is one such compound, which has been shown to inhibit the growth of a wide variety of tumor cells[9-12]. But the mechanism is not clear yet. It has been reported that curcumin can inhibit the proliferation of tumor cells and induce apoptosis through multiple pathways and tumor-related factors[13]. In our previous report, we have analyzed the effects of curcumin on proliferation and apoptosis *in vitro*.

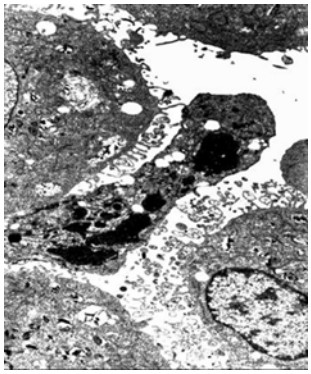
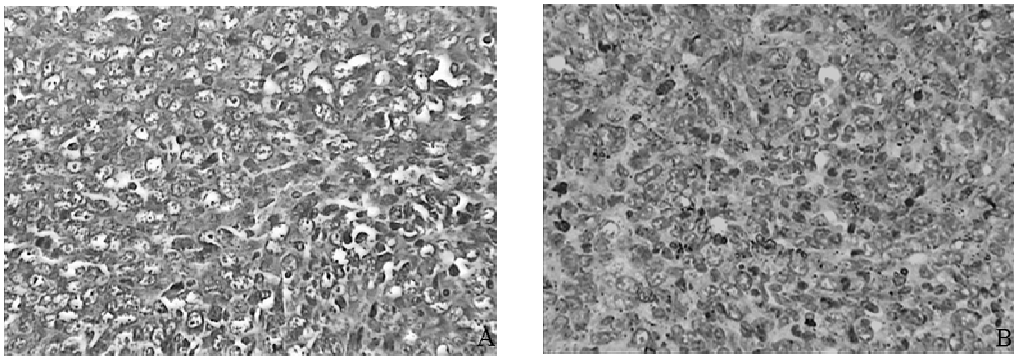
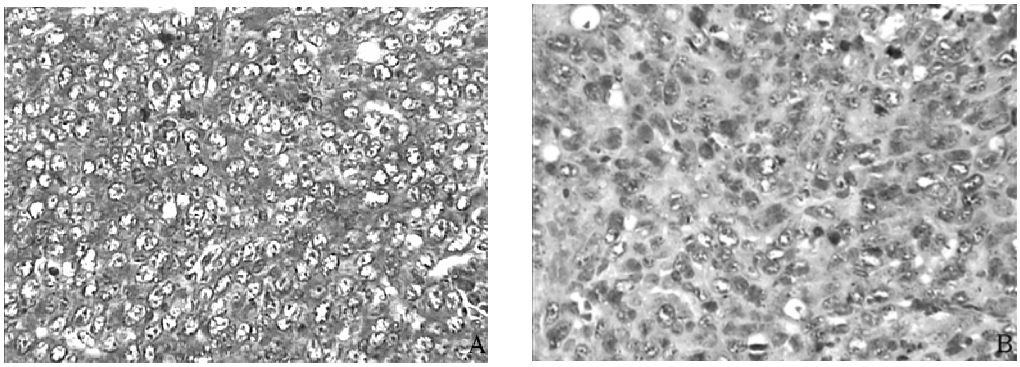


Fig. 3. Morphologic changes of curcumin-treated HeLa cells by EM (× 12000)



A: Control group B: Curcumin-treated group

Fig. 4. The expression of Caspase-3 in transplanted tumor DAB × 400



A: Curcumin-treated group B: Control group

Fig. 5. The expression of Bax in transplanted tumor DAB × 400

Tab. 2. Inhibition rates of transplantation tumor after injection of curcumin

Group	n	Tumor volume (mm^3, $\bar{x}\pm s$)	Average weight of tumor (g, $\bar{x}\pm s$)	IR
Control	15	350±34.95	1.50±0.23	---
Curcumin	15	53±8.52	0.39±0.06	74.33

Compared with the control: P<0.05

The major purpose of this study was the evaluation of the anti-cancer effect of curcumin *in vivo*. Before the *in vivo* investigation, we began the study by examining the *in vitro* cytotoxic effect of curcumin on HeLa cells. Curcumin was observed to induce cell death in HeLa cells in a dose-dependent manner. The morphological changes of apoptosis were observed under electron microscope. Obvious apoptosis of cells could be observed by FCM. These findings suggested that the model of the cell death induced by curcumin is via the apoptosis pathway.

Curcumin also showed the anti-tumor effects on HeLa tumors established in BALB/c mice, demonstrating that the effect of curcumin is manifest not only *in vitro* but also *in vivo*. The cell death induced by curcumin in the HeLa tumors *in vivo* was also due to apoptosis, as demonstrated by the expression of apoptosis related proteins such as Bax and Caspase-3.

In addition, the administration of curcumin daily did not cause death in the mice. This means that the side-effects of curcumin such as anaphylactic shock may not be so serious. These desirable properties suggest that curcumin has the potential to be a novel drug for cancer therapy.

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