EFFECTS OF CURCUMIN ON PROLIFERATION AND APOPTOSIS OF HUMAN CERVICAL CARCINOMA HeLa CELLS *IN VITRO*

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ABSTRACT

Objective: To investigate the regulatory effect of curcumin on proliferation and apoptosis in human cervical carcinoma cell line HeLa *in vitro*. Methods: Human cervical carcinoma cell line Hela was cultured *in vitro*. HeLa cells were treated with 10~50 μmol/L curcumin for 24~72 h and the growth inhibition rates of HeLa cells were measured by MTT method. Cell apoptosis was inspected by electron microscopy. In addition, the expression of bcl-2, bcl-xl and caspase-3 protein in HeLa cell were observed by SP immunohistochemistry. Results: Curcumin inhibited the proliferation of HeLa cells on a dose-depending manner. Peak of subG1 appeared on DNA histogram in FCM. A portion of the cells presented the characteristic morphological changes of apoptosis under the electron microscope. The bcl-2, bcl-xl expression was decreased while Caspase-3 expression was increased. Conclusion: Curcumin could significantly inhibit the growth of HeLa cells; inducing apoptosis through up-regulating Caspase-3 and down-regulating expression of bcl-2 and bcl-xl was probably one of its molecular mechanisms.

Key words: Curcumin; Cervical carcinoma; Apoptosis; Immunohistochemistry; Flow cytometry

Curcumin, a deferuloymethane, is a major active component of the food flavor turmetric (Curcumal Longa). Because of its stable colour and luster and low toxicity, curcumin has been widely used as food additive and stain. Recently, it has been reported to posses antiinflammatory, antioxidation and antiviral activities. Now, attention has been focused on its antitumor activity[1]. *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[2]. However, few reports has been found about the effect of curcumin on human cervical carcinoma. In our study, we investigated the effect of curcumin on proliferation and apoptosis of human cervical carcinoma HeLa cells *in vitro*.

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MATERIALS AND METHODS

Materials

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

Cells Culture

HeLa cells were maintained in RPMI-1640 containing 10% FBS and antibiotic-antimycotic. The cells were cultured in a humidified incubator containing 5% CO_2 at 37°C.

MTT Assay

Cell growth was measured by a modified MTT assay. About 1×10^5 cells/well were plated in 96-well microtiter plates and incubated overnight. Cells were then treated with 10, 25, 50 μmol/L curcumin for 24, 48, 72 h. The 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 producted. 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 absorbance of treated cells relative to those of the control wells were calculated and expressed as percentage of growth inhibition.

Flow Cytometry

For DNA content analysis, cells were treated with 10, 25, 50 μmol/L curcumin for 48 h. 1×10^6 cells were harvested, pelleted, washed with phosphate-buffed salin (PBS), and resuspended in PBS containing propidium iodide (PI) (ClonTECH) 20 mg/L and ribonuclease A 1 g/L. Fixed cells were examined by flow cytometry, and percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub-G1). Cell cycle analysis was performed using the same experimental conditions and distributions using the cell Fix Program. All measurements were carried out under the same instrumental settings.

Electron Microscopy

Cells were planted in 50 ml plastic culture flask. In the absence or presence of curcumin 25 μmol/L, incubation was carried out 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 1.5 ml for 5 min. The section were prepared and photographed under Hitach 600 Electron microscopy (Japan).

Immunohistochemistry

HeLa cells treated with 25 μmol/L curcumin for 48 h were fixed by stock acetone and stained with DAB. The detection procedure was done as described in Kit protocol. PBS instead of the first antibodies was used in the negative control. The bcl-2, bcl-xl 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%.

Statistics Analysis.

The data were presented as 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. $P<0.05$ is considered to be statistically significant.

RESULTS

Effects of Curcumin of Cell Proliferation

The effects of curcumin on growth of HeLa cells 24, 48, 72 h after treatment are shown in Figure 1. It was showed that cells of control group growth actively. Compared with control group, the growth of cells treated with the different concentration curcumin was inhibited significantly in a concentration and time-dependent manner. After 72 h, inhibition rate is in the range of 11%~45.8%. Statistical analysis showed that the deference between the different time was very significant ($P<0.01$). Compared with the control group, the difference of the group of 10 μmol/L was significant ($P<0.05$) and between the groups of 25 μmol/L and 50 μmol/L there was also significant difference ($P<0.01$).

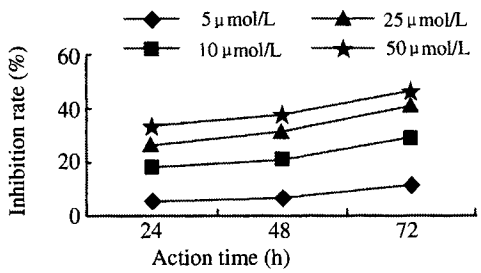

Fig. 1. The inhibitory effects of different concentration curcumin on the growth of HeLa cells.

Kenetic Changes of Apoptosis of Hela Cells Induced by Curcumin

After HeLa cells were treated with 10, 25, 50 μmol/L curcumin for 48 h, the cell cycle distribution and rate of apoptosis of were shown in Table 1. In the control group, low subdiploid peak appeared before diploid peak. With increase of concentration of curcumin, the subdiploid peak became higher and reached 11.96%, 12.06%, 22.4% respectively in 10, 25, 50 μmol/L group. Meanwhile, a significantly increase in the percentages of S phase cells was observed along with increase of concentration.

Effects of Curcumin on HeLa Cells Ultrastructure

By electron microscopy, we observed that in the control group, no apoptotic cell was found, however, in 25 μmol/L curcumin group, a lot of apoptotic cells could be found which were smaller in size; the cytoplasm was dense and chromatin aggregated peripherally under the nuclear memebrane, into well-delimited dense masses of various shapes and sizes (Figure 2).

Effects of Curcumin on Expression of bcl-2, bcl-xl, Caspase-3

After treatment with 25 μmol/L curcumin for 48 h, the expression of bcl-2, bcl-xl decreased, while the expression of Caspase-3 increased. The difference of

expression rate between control group and curcumin group was significant ($P<0.05$) (Figure 3 and Table 2).

Table 1. The effects of different concentration curcumin on the cell cycle distribution and the apoptotic rate of HeLa cells

Groups	The cell cycle distribution (%)			The apoptotic rate (%) (the sub-G_1 peak)
	G_0/G_1	S	G_2/M	
Control	58.55	31.64	9.81	5.54
Curcumin (μmol/L)				
10	58.09	33.99	7.92	11.96
25	48.21	41.19	10.67	12.06
50	49.94	42.47	7.58	22.40

Table 2. The effects of curcumin on the rate of expression of bcl-2, bcl-xl, Caspase-3 ($\bar{x}\pm s$, %) by SP

Groups	bcl-2	bcl-xl	Caspase-3
Control	0.74±1.1	0.75±1.5	0.06±2.1
Curcumin (25μmol/L)	0.31±1.2	0.38±1.3	0.27±2.2

Compared with the control group, $P<0.05$

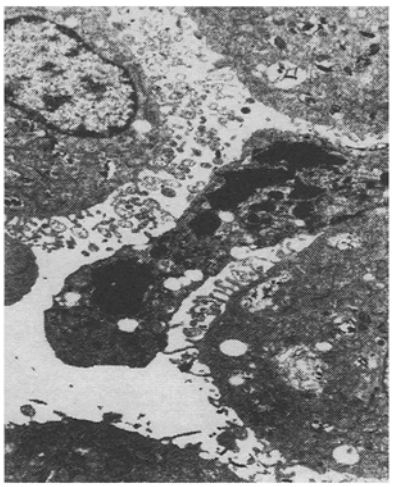

Fig. 2. The morphological changes of HeLa cells treated with 25 μmol/L curcumin under microscopy (× 12000).

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. But the mechanism is not clear yet. It has been reported that curcumin can inhibit the proliferation of tumor cells and induce apoptosis through the multiple pathways and tumor-related factors. In the present report, we selected the HeLa cell line to analyse the effect of curcumin on proliferation and induction of apoptosis, and investigate its probable mechanisms.

Our results showed that curcumin could inhibit the growth of HeLa cells significantly. Furthermore we found its inhibitory effect is in a time and concentration-dependent manner by the MTT method.

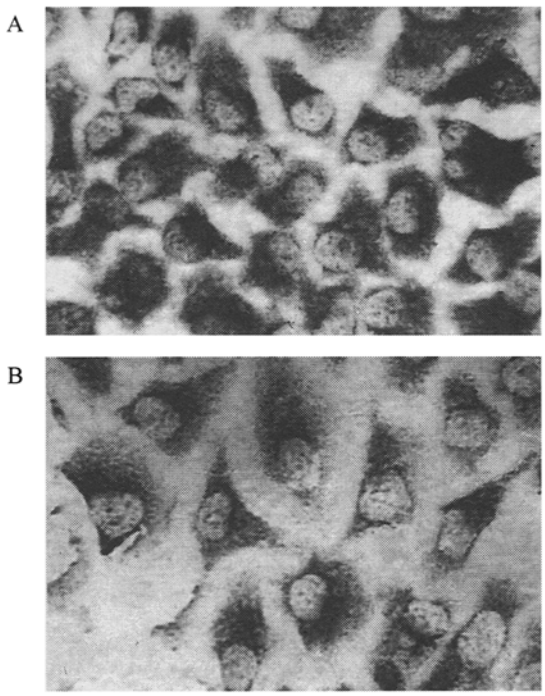

Fig. 3 (A). bcl-2 expression of HeLa cells inspected by SP (control group) (×400). (B). bcl-2 expression of HeLa cells inspected by SP (25 μmol/L curcumin) (×400).

In many studies, curcumin was proved to induce apoptosis of multiple cell lines and this feature might be an important mechanism of its antitumor effect. In our study, we investigated the effect of curcumin on inducing apoptosis by FCM, electron microscopy and immunohistochemistry. Our results showed curcumin could induce apoptosis of HeLa cells in a concentration

dependent manner. Many apoptotic bodies were found under electron microscopy; furthermore, curcumin changed the distribution of cell cycle and increased the percentage of S phase cells.

Cell apoptosis can be regulated by multiple apoptosis-related proteins. The most important are two families. (a) bcl-2 family. In this family, these related proteins can either promote apoptosis (e.g., Bax, Bad) or inhibit the process (e.g., bcl-xl)[3]. Bcl-2 and bcl-xl are considered the most important antiapoptotic proteins. Anto et al.[4] reported that the growth of the human T-cell line Jurkat which expresses no bcl-xl was apparently inhibited by curcumin, whereas Hut-78, another T-cell line, which overexpresses bcl-xl, was not. So they considered that bcl-xl was a superior inhibitor of curcumin-induced apoptosis. In most other studies however, no significant difference was found in the activity between bcl-2 and bcl-xl for suppression of curcumin-induced apoptosis. Our results showed curcumin could down-regulate the expression of bcl-2 and bcl-xl which indicated it may be one of the mechanism of curcumin-induced apoptosis. However, we did not find significant difference in effects of curcumin on both proteins. (b) Caspase family. This proteolytic cascade, now including more than ten members, can be divided functionally into two basic groups-initiator and execution. In particular, Caspase-3 is considered as direct executor of apoptosis[5]. Our results showed that curcumin could up-regulate the expression of Caspase-3 and promote apoptosis of HeLa cells. In addition, recent study showed that cleavage of Caspse-3 could make bcl-2 and bcl-xl inactive and a membrane-combined Caspase-3, which was found recently, could be inhibited by bcl-2[6]. So the effects between bcl-2 and different type Capase-3 is very important in cells apoptosis. Our study demonstrate that curcumin can induce apoptosis of HeLa cells in vitro, suggesting that down-regulating bcl-2 and bcl-xl and up-regulating the expression of Caspase-3 may be one of its mechanisms.

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