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Original Article

The role of parthenolide in the inhibition of proliferation and regulation of apoptosis of the human cervical carcinoma HeLa cells

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Abstract: Parthenolide has good antitumor effects, and in this study, we examined the effect of parthenolide on different kinds of cancer cells. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the effect of parthenolide on the inhibition of proliferation of the human cervical carcinoma HeLa cells. We determined the rate of apoptosis using flow cytometry. The expression levels of caspase-9, caspase-8, and caspase-3 were detected using substance P (SP) immunocytochemistry. The results of the MTT assay showed that parthenolide significantly inhibited the growth of different kinds of cancer cells, and we observed the sub-G1 peak using flow cytometry. The fragment characteristic to cytochrome C was detected using a monoclonal antibody of cytochrome C to determine the effect of treatment with parthenolide on the cytochrome C levels in HeLa cells. The level of cytochrome c increased 24 h after treatment with parthenolide. The level of cytochrome C at 24 h was significantly higher than that at 0 h; subsequently, the cytochrome c levels decreased. Results of immunocytochemical staining showed that parthenolide affected the expression levels of caspase-9, caspase-8, and caspase-3 in the HeLa cells. The positive rate of protein expression calculated using the formula showed a statistically significant difference in the expression levels of these proteins ($P < 0.05$). The results showed that parthenolide could alter the expression of caspase-9, caspase-8, and caspase-3.

Keywords: Prthenolid, HeLa cells, apoptotic, caspase-9, caspase-8, caspase-3, cytochrome C

Introduction

Over the last few decades, tumors have been routinely diagnosed in clinical diseases. A majority of the tumors are malignant and thus incurable, which remain to be a big challenge for the doctors. The conventional methods for managing tumors include surgery, radiotherapy, and chemotherapy. However, because of the cytotoxic effects of the chemotherapeutic agents currently used clinically for treatment, which cause extensive damage to the patients' body, many researchers have started focusing on developing novel antitumor drugs using the traditional Chinese medicines, which have few side effects, to improve patient survival. Parthenolide [1-3] belongs to the half terpene lactones, and it is an active ingredient of medicinal plants in Mexico and India. Parthenolide has been commonly used as an herbal active ingredient mainly for the treatment of migraine, inflammation, and tumors. With an increase in the studies on Chinese medicines with antitumor potential, several researchers have started focusing on the effects of parthenolide. In this study, we examined the antitumor effects of parthenolide on different kinds of tumor cells [4], and we determined the effects of parthenolide on the expression of caspase-9, caspase-8, and caspase-3 in HeLa cells by using immunohistochemistry (IHC). We used propidium iodide (PI) staining and flow cytometry [5-7] to determine apoptosis and cell cycle changes caused by parthenolide in human cervical cancer HeLa cells; the levels of cytochrome c [8-10] were detected using western blotting. We aimed to

Table 1. Proliferation inhibition of parthenolide on various tumor cells ($\bar{x}$±s, n = 3)

Cell line	Dose (μg/ml)				
	5	10	20	40	80
A-549	20.35±0.21	31.74±0.74	48.92±0.57	73.41±0.12	82.36±0.91
HepG2	18.27±1.54	27.30±0.52	46.51±0.73	69.82±0.85	79.26±0.46
Hep3B	17.33±1.24	29.25±0.78	45.78±0.46	70.43±0.51	78.77±0.34
B-16	23.54±0.85	36.15±0.49	51.98±0.97	79.64±0.67	85.71±0.73
HeLa	37.41±0.57	48.90±1.08	71.35±1.29	89.59±0.95	93.05±0.89

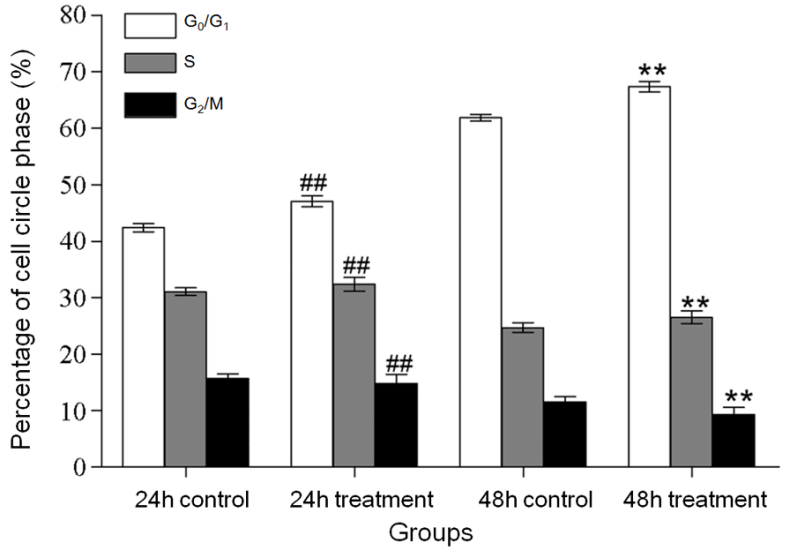

Figure 1. Influences of Prthenolid on the distribution of HeLa cycle.

elucidate the mechanism underlying the antitumor effects of parthenolide on HeLa cells.

Materials and methods

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

Tumor cells in logarithmic growth phase were selected and a cell suspension containing 10 × 10^6 cells/mL was prepared by mechanical blowing. Then, the cells were subcultured in 96-well plates containing 20 μL suspension/well and then placed in an incubator at 37°C and 5% CO_2. After 4 h, different concentrations of parthenolide were added to the cells in the test group. Four wells for every concentration of parthenolide were used as the control group. We added 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) to each well 20 h after treatment, and then 150 μL dimethylsulfoxide (DMSO) was added after culturing for another 4 h and the supernatant was separated. The absorbance (A) of the supernatant was measured at 492 nm by using an enzyme-linked immunosorbent assay (ELISA) reader after slightly vortexing the liquid for 10 min. The rate of inhibition of proliferation of tumor cells (%) = [1-A value in the treatment wells/A value in control wells] × 100%.

An optimal concentration of parthenolide determined using the MTT assay was selected for the next experiments.

Flow cytometry

HeLa cells in logarithmic growth phase were selected and a cell suspension containing 10 × 10^6 cells/mL was prepared by cultivating the cells in a 100-mL culture flask. Parthenolide was added into the suspension to achieve a final concentration of 10 μg/mL. Meanwhile, control group was set. Cells were collected 24 and 48 h after cultivation. After rinsing the cells with phosphate buffered saline (PBS) solution twice, PBS was added to create a cell suspension. The cells were stained with Cycle TESTTM PLUS DNA Kit, and red fluorescence at an excitation wavelength of 488 nm was recorded using a flow cytometer to test the rate of apoptosis and cell cycle distribution in HeLa cells. The co-efficient of variance (CV) value was adjusted within 5% by taking concentrated red blood cells (CRBCs) as the standard sample before detecting 20,000 cells for each sample in both treatment and control groups three times. Cell cycle distribution was analyzed using the Mod Fit 3.0 software, and single parameter analysis of DNA content was conducted for the sample to obtain the percentage of viable and apoptotic cells. According to the distribution of cells in each phase of the cell

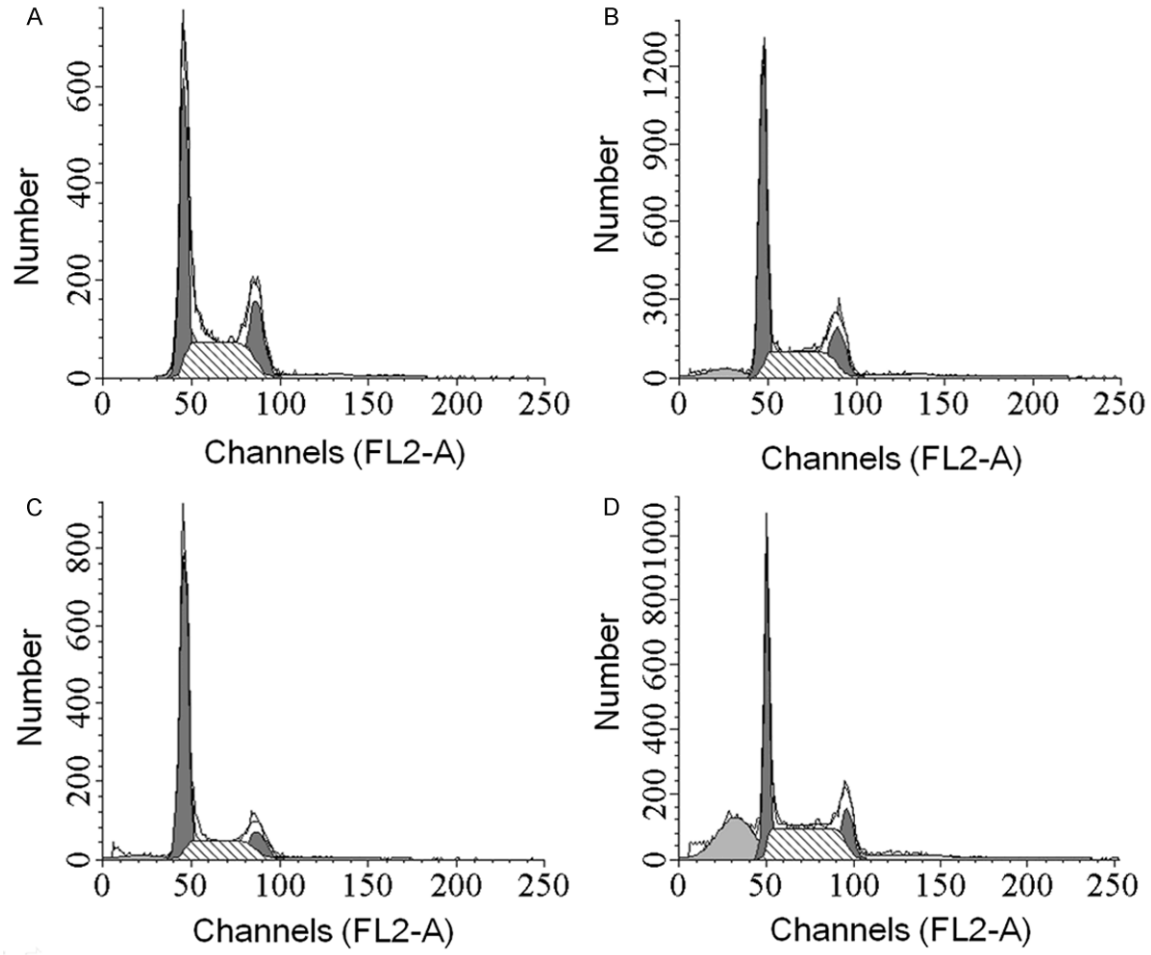

Figure 2. HeLa cell cycle detected by flow cytometer. A. Control group at the 24th hour; B. Prthenolid at the 24th hour; C. Control group at the 48th hour; D. Prthenolid at the 48th hour.

cycle, the proliferation index was calculated for comparison of cell proliferation activity. PI = (S + G2/M)/(G0/G1 + S + G2/M) × 100%.

Western blotting

Cells in each phase of the cell cycle were collected after treatment with parthenolide, and the plasma membrane was lysed selectively by using a digitonin solution (consisting of 220 mmol/L KCl, 5 mmol/L KH_2PO_4, 10 mmol/L HEPES, 2 mmol/L ethylenediaminetetraacetic acid disodium salt [EGTA], 0.15 mg/ml digitonin, and a protease inhibitor, pH 7.4) without damaging the mitochondrial membrane. After incubating for 10 min at 4°C, the cells were centrifuged at 12,000 rpm/min for 5 min. The supernatant was used as the protein in the cytolymph, and then, the levels of cytochrome c in the cells were detected using western blotting.

Immunocytochemistry

The control group was prepared, and 20 μg/mL parthenolide was added to the treatment group and the cells were cultured for 24 h. The immunocytochemical staining was performed according to the protocol specified in the S-P kit. The results showed that cells with clear brown particles inside were positive. Caspase-9, caspase-8, and caspase-3 were predominantly expressed in the cytoplasm or in both the cytoplasm and the nucleus. Cells not stained or slightly stained were negative. Every index was observed on three smears with five views at 400× higher magnification for each. The number of positively stained cells and total cells were counted in every view to calculate the positive expression rate (positive expression rate [%] = number of positive cells/total cell number × 100%). The tissue section with known

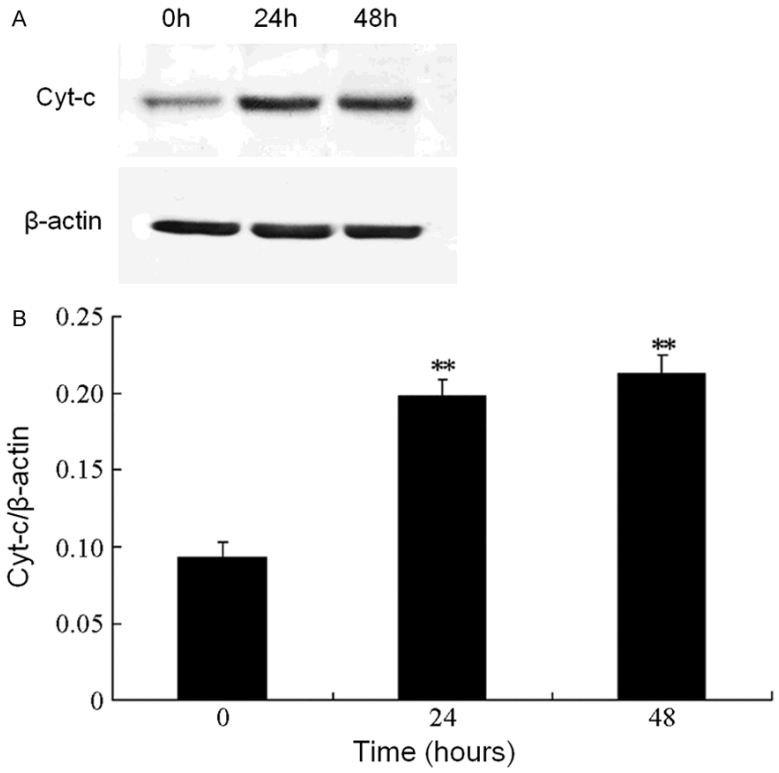

Figure 3. Influences of Prthenolid on the Expression Cyt-c. **Comparison with 0 h: $P < 0.01$.

positive matter was the positive control, and PBS was the negative control instead of primary antibody in every group.

Statistical analysis

The SPSS 13.0 software was used for statistical analysis; $\bar{x} \pm s$ was used to express data; *t* test was used to compare the rate of inhibition detected using the MTT assay; correlation analysis and curve regression were used to calculate the half maximal inhibition concentration (IC_{50}). $P < 0.05$ indicated a statistically significant difference.

Results

MTT assay

Parthenolide showed a dose-dependent increase in the inhibition of proliferation of A-549 human lung cancer cells, HepG2 and Hep3B human liver cancer cells, HeLa human cervical cancer cells, and B-16 murine melanoma cells over a period of 24 h (**Table 1**). The IC_{50} of parthenolide after 24 h was 10 μg/mL.

Cell cycle

The results of flow cytometry were as follows: at 24 h, the cells in the control group in the G0/G1 phase were 42.39±0.74, in the S stage were 31.05±0.68, and in the G2/M phase were 15.68±0.81; the cells in the treatment group in the G0/G1 phase were 61.85±0.59, in the S stage were 24.67±0.88, and in the G2/M phase were 11.52±0.94. At 48 h, the cells in the control group in the G0/G1 phase were 47.06±0.98, in the S stage were 32.38±1.25, and in the G2/M were 14.81±1.61; the cells in the treatment group in the G0/G1 phase were 67.35±0.96, in the S stage were 26.51±1.14, and in the G2/M phase were 9.28±1.31. The proliferation index at 24 h of cells in the control and the treatment groups was 52.43±1.55 and 36.91±1.82, respectively, and that at 48 h was 50.07±1.67 and 34.70±0.94, respectively. The data show a decrease in the number of HeLa cells in the G0/G1 phase after treatment with 10 μg/mL parthenolide for 24 and 48 h. Then, the cells are transitioned from the G1 phase to S phase. With an increase in the treatment time, the number of cells in the G0/G1 phase increases, while the number of cells in the S phase decreases. Compared to the control group, all treatment groups showed a statistically significant difference ($P < 0.01$ or $P < 0.05$) in the number of cells in the G0/G1 phase at different time points (**Figures 1**, **2**).

Cytochrome c levels

Our results show that the fragment characteristic to cytochrome c can be detected by using a monoclonal antibody of cytochrome C. While the expression of cytochrome c was low at 0 h, the cytochrome c levels in the cytoplasm increased significantly after treatment with parthenolide for 24 and 48 h ($P < 0.05$). However, no statistically significant difference was

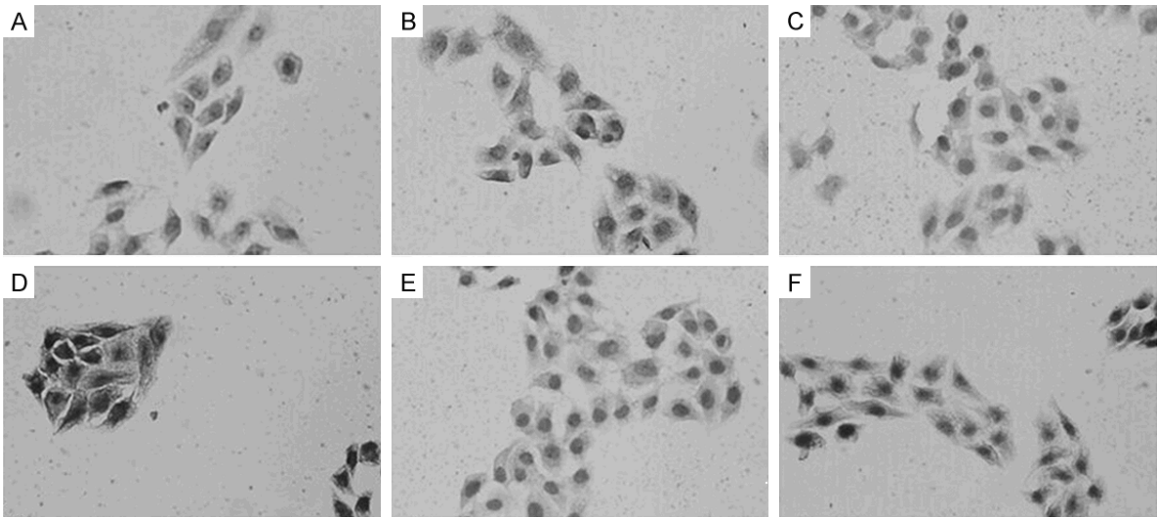

Figure 4. Influences of Prthenolid on the Caspase-9, Caspase-8, Caspase-3 of HeLa. A. Change of Caspase-9 in HeLa Cell of Control Group in 24 hours; B. Change of Caspase-9 in HeLa Cell under Effect of Prthenolid in 24 hours; C. Change of Caspase-8 in HeLa Cell of Control Group in 24 hours; D. Change of Caspase-8 in HeLa Cell under Effect of Prthenolid in 24 hours; E. Change of Caspase-3 in HeLa Cell of Control Group in 24 hours; F. Change of Caspase-3 in HeLa Cell under Effect of Prthenolid in 24 hours.

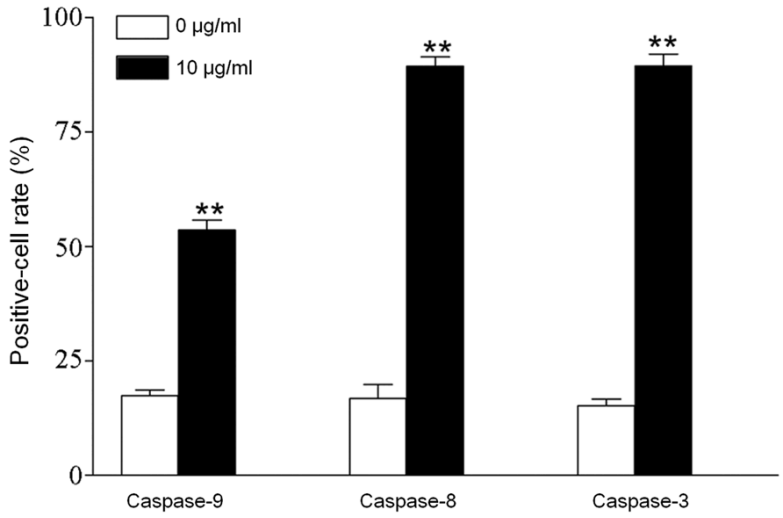

Figure 5. Influences of prthenolid on the expression of caspase-9, caspase-8, and caspase-3.

observed in the cytochrome c levels between 24 and 48 h ($P > 0.05$). Thus, our results indicate that parthenolide can promote the efflux of cytochrome c from mitochondria leading to apoptosis (**Figure 3**).

Immunocytochemistry

Apoptosis occurred in the treatment group after treatment of HeLa cells with 10 μg/mL of parthenolide for 24 h. The expression levels of caspase-9, caspase-8, and caspase-3 decreased after treatment with parthenolide, and the results of microscopic examination in each group are similar to those of expression of caspases. The levels of caspase-9, caspase-8, and caspase-3 in the control group were 17.37±1.27, 16.82±3.06, and 15.21±1.46, respectively, and those in the treatment group were 53.59±2.15, 89.39±1.98, and 89.42±2.57, respectively. The dark color shades of cells in the view show that the expression degree quantity increase. Statistical analysis using the *t* test indicated a statistically significant difference in the expression of caspases between the control group and the group treated with 10 μg/mL parthenolide ($P < 0.01$) (**Figures 4**, **5**).

Discussion

The MTT colorimetric method can be used to determine the action of succinate dehydrogenase in the mitochondria by measuring the absorbance of the royal purple formazan crys-

tals that are formed in the cells. However, the formazan crystals are not formed in dead cells [11]. DMSO can dissolve the formazan in the cells. Measurement of the absorbance using the ELISA Reader at 490 nm can indirectly reflect the amount of viable cells. The amount of crystallization induced by MTT is proportional to the number of cells.

The MTT assay showed that parthenolide had inhibitory effects on various tumor cell lines. Treatment with 80 μg/mL of parthenolide showed an inhibition rate of approximately 80% in various cell lines. Therefore, parthenolide has good anti-proliferative effects on tumor cells.

A key step in apoptosis is the release of cytochrome c from the mitochondrion. Cytochrome c released into the cytoplasm binds with apoptosis protease activating factor 1 (Apaf-1) and is stored as a complex with dATP [12] to form a polymer and drive caspase to form an apoptosome. Caspases plays a vital role in apoptosis [13]. Apoptosis is an irreversible process of cascade reaction for limited hydrolyzed substrate of caspase. To date, more than 10 caspases have been discovered. With high homology and similar structure, caspase modules are all caspase [14]. Caspases can be divided into two categories according to their function: one participates in cell processing and the other in apoptosis [15]. Caspases are present in the form of proenzymes; the caspase family generally has a locus for cysteine activation and substance pyrolysis. Cytochrome c can form a polymer that combines with Apaf-1 under the presence of dATP, and this polymer can form an apoptosome with caspase-9. Thus, caspase-9 is activated, and it further activates other caspases such as caspase-3, thus inducing caspase-mediated apoptosis [16, 17].

Our results indicate that parthenolide can affect the activation of caspase-9 and caspase-3. The expression levels of caspase-9 and caspase-3 are increased significantly after 24 and 48 h, which indicates that the apoptosis of HeLa cells is mediated by the release of cytochrome c and activation of caspases.

The results of flow cytometry indicate that compared to the control group, the treatment group showed a decrease in the number of cells in the G2/M and S phases. This indicates that cells in the DNA synthesis phase showed a marked decrease, and that parthenolide exerts antitumor effects by interfering with the cell cycle.

Disclosure of conflict of interest

None.

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