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Effects of Ursolic Acid on the Proliferation and Apoptosis of Human Ovarian Cancer Cells

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Summary：This study examined the effects of ursolic acid (UA) on the proliferation and apoptosis of a human ovarian cancer cell line, CAOV3. The CAOV3 cells were cultured in the RPMI 1640 media and treated with different concentrations of UA (0, 10, 20, 40 μmol/L). The proliferation rate of the CAOV3 cells was determined by MTT assay. The apoptosis rate was measured by flow cytometry. ERK activity was detected by immunoprecipitation and the expressions of p-ERK1/2, MKP-1, Bax and Bcl-2 by Western blotting. The results showed that the proliferation rate was significantly decreased in the cells treated with UA as compared with that in the non-treated cells (P<0.05). The intracellular ERK activity and p-ERK1/2 expression were also reduced in the UA-treated cells, while the MKP-1 expression was elevated. Moreover, the apoptosis was found in the CAOV3 cells exposed to UA; the Bax expression was increased and the Bcl-2 expression decreased. The apoptosis rate in the UA-treated cells was much higher than that in the non-treated cells (P<0.05). It is concluded that UA can inhibit the proliferation of CAOV3 cells by suppressing the ERK activity and the expression of p-ERK1/2. And it can also induce the apoptosis of the CAOV3 cells by up-regulating the Bax expression and down-regulating the Bcl-2 expression.
Key words：ursolic acid; ovarian cancer; proliferation; apoptosis

Epithelial ovarian carcinoma is an aggressive malignancy associated with high morbidity and mortality in gynecological practice. Currently no effective treatment has been available. Ursolic acid (UA), a pentacyclic triterpene compound, found widely in natural plants, had been reported to possess anti-inflammatory, liver-protecting, lipid-lowering activities[1, 2], and has recently been found to be capable of inhibiting various types of cancer cells and inducing their apoptosis[3–5]. Yet, no studies on the inhibitory effects of UA on the ovarian cancer cells have been available. In this study, a human ovarian cancer cell line, CAOV3 cells, was treated with UA of different concentrations. The proliferation and apoptosis of the CAOV3 cells were examined and the possible mechanism explored.

1 MATERIALS AND METHODS

1.1 Chemicals and Instruments

The human ovarian cancer cell line (CAOV3) used in this study was provided by China Center for Type Culture Collection (CCTCC), Wuhan University, China. UA was a product of Sigma Co., USA. RPMI 1640 media, fetal calf serum and trypsin were purchased from Hyclone Co., USA. Extracellular signal-regulated kinase (ERK) assay kit was from Chemicon Co., USA and the standard protein from New England Biolab Co., USA. Other materials including mouse anti-ERK antibody, mouse anti-MKP-1 antibody, mouse anti-Bax antibody, mouse anti-Bcl-2 antibody, alkaline phosphatase goat anti-mouse antibody and alkaline phosphatase color development kit were obtained from Beijing Zhongshan Biotechnology Co., Ltd., China. Flow cytometer was from Beckman Co., USA.

1.2 Cell Culture

The CAOV3 cells were maintained in the RPMI 1640 media supplemented with 10% fetal calf serum, penicillin (1×10^5 U/L) and streptomycin (1×10^5 U/L). They were cultured and passaged in a humidified incubator in an atmosphere of 5% CO_2/95% O_2 at 37°C. The cells at passage 3–6 were used in the following experiments.

1.3 Grouping

The CAOV3 cells were randomly divided into four groups: control group, in which cells were not exposed to UA, and 3 UA Groups, in which the cells were treated with UA of different concentrations (10, 20, 40 μmol/L).

1.4 Intervention Factors

The cells at the logarithmic phase were collected and seeded into the culture bottles at a density of 1×10^5/mL. The culture media were replaced 24 h later and the unattached cells were removed. When the cells reached subconfluence, they were cultured in the serum-free media for another 12 h. Thereafter, different concentrations of UA were added to the media to a final volume of 2 mL. The cells were collected after 12-h cul-

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ture and were measured as follows[6].

1.5 MTT Assay

When the cells were grown to mid-exponential phase, they were seeded into a 96-well plate at an initial density of 1×10^5cells/well and incubated in a humidified incubator in 5% CO_2 at 37°C. After subconfluence was reached, 200-μL serum-free media were added. Twelve h after the plating, the cells were treated with different concentrations of UA. After a 48-h incubation, the media were replaced with MTT (5 mg/mL). Then, the cells were cultured for a further 4 h, which was followed by the addition of 150-μL DMSO to each well and oscillation for 10 min. The absorbance (*A*) value, which was used to reflect cell viability, was measured at a wavelength of 620 nm (the reference wavelength was set at 490 nm) by using an ELISA reader. The cell growth curve was drawn and the proliferation rate (PR) of the cells was calculated by following formula: PR= B/A×100%, where A and B respectively represent the *A* value of the control group and the UA groups.

1.6 Measurement of ERK Activity

The cell protein (0.5 mL) were sampled, and treated with anti-p-ERK antibody (10 μL) at 4°C for 12 h and then with 20-μL agarose in a ratio of 50% (v/v) at 4°C for 2 h. After the centrifugation (2 000 g, 4°C, 1 min), lysis buffer (1 mL) was added to the mixture and incubated at 4°C for 10 min. Afterwards, another centrifugation was conducted. The above procedure was repeated twice. The supernatants were subsequently removed and the sediments were measured for the ERK activity. The *A* values were obtained by using a microplate reader at a wavelength of 450 nm and the ERK activities were calculated from a standard curve, which was drawn on the basis of the *A* values of the standard protein.

1.7 Detection of Apoptosis Rate by Flow Cytometry

The cells in each group were collected and measured by using the annexin Ⅴ-FITC apoptosis detection kit in accordance with the manufacturer's instructions. Briefly, binding buffer was diluted with the double-distilled water at the proportion of 1:4. The cells were washed with PBS, and then re-suspended in the binding buffer, with the cell density adjusted to 2×10^5/mL. The cell suspension (195 μL) was obtained, and annexin Ⅴ-FITC (5 μL) and PI (10 μL) were added respectively. The mixture was kept at room temperature for 30 min and then measured for apoptosis by flow cytometry.

1.8 Detection of p-ERK1/2, MKP-1, Bax and Bcl-2 by Western Blotting

After the cells were washed briefly with PBS, they were lysed with cell lysis buffer, five times that of cell solution in volume, for 20 min. Total cell lysate (40 μg) was cleared by centrifugation and boiled with the same volume of 3×SDS sample buffer for 5 min, and the purified protein extracts were then resolved by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to PVDF membranes, which were blocked and then incubated overnight at 4°C with mouse anti-ERK, anti-MKP-1, anti-Bax and anti-Bcl-2 antibodies, diluted to 1:1000 in tris-buffer saline containing 0.1% tween-20 (TBST). The membranes were then washed and incubated at room temperature for 2 h with alkaline phosphatase goat anti-mouse antibody (dilution 1:1000) in TBST. Resolved proteins were electroblotted onto Hybond nitrocellulose ECL membranes (Amersham Bioscience Co, USA) and then visualized by using enhanced chemiluminescence. The protein signals were quantified by scanning densitometry using bio-image analysis software (Quantity-One 4.1.0, Bio-Rad, USA). The results from each group were expressed as integrated intensity that was compared for statistical difference.

1.9 Statistical Analysis

The data were expressed as $\bar{x}\pm s$. Statistical differences were determined by using one-way ANOVA and were considered to be significant at a P value <0.05.

2 RESULTS

2.1 Inhibition of CAOV3 Cell Proliferation by UA

The MTT assay showed that the proliferation rate was significantly decreased in the UA-treated cells when compared with the non-treated cells (P<0.05 or P<0.01) (table 1). And UA inhibited the proliferation of the cells in a concentration-dependent fashion.

Table 1 Effects of UA on the proliferation of the CAOV3 cells (*n*=6)

Groups	*A* value	Proliferation rate (% of control)
Control	1.712±0.168	100
UA (μmol/L)		
10	1.539±0.226*	89.89±10.21*
20	1.229±0.126**	71.79±9.22**
40	1.119±0.106**	65.36±9.38**

*P<0.05, **P<0.01as compared with the control group

2.2 Suppression of the ERK Activity of CAOV3 Cells

The ERK activity was 100% in the control group, and (88.99±9.37)%, (76.39±9.21)%, (65.32±8.27)% in the groups of different UA concentrations (10, 20, 40 μmol/L). The ERK activity was concentration-dependently reduced in the UA-treated cells.

2.3 Down-regulation of p-ERK1/2 Expression and Up-regulation of MKP-1 Expression by UA in CAOV3 Cells

Western blotting revealed that UA inhibited the expression of p-ERK1/2 in a concentration-dependent manner, and at the same time increased the expression of MKP-1. Statistical differences were found in the p-ERK1/2 expression and the MKP-1 expression between the control group and the UA groups (P<0.05 for all) (table 2, fig. 1).

Table 2 Effects of UA on the expressions of p-ERK1/2 and MKP-1 in the CAOV3 cells (*n*=6)

Groups	p-ERK1/2	MKP-1
Control	0.94±0.13	0.49±0.06
UA (μmol/L)		
10	0.88±0.12*	0.58±0.10*
20	0.85±0.12**	0.71±0.09**
40	0.71±0.11**	0.79±0.11**

*P<0.05, **P<0.01 as compared with the control group

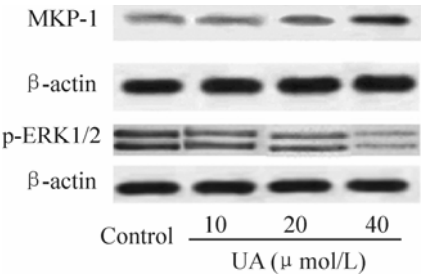

Fig. 1 Western blot analysis of p-ERK1/2 and MKP-1

2.4 Promotion of CAOV3 Cell Apoptosis by UA

Flow cytometry showed that UA could induce the apoptosis of the CAOV3 cells. And the apoptosis rate increased with the concentration of UA. There were significant differences in the apoptosis rate between the control group and different concentrations of US groups (P<0.05 for all) (fig. 2).

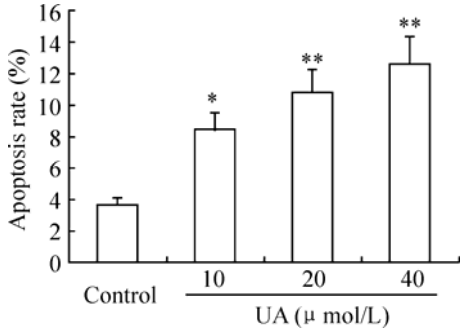

Fig. 2 Effects of UA on the apoptosis of CAOV3 cells
$^{*}P$<0.05, $^{**}P$<0.01 as compared with the control group (n=6)

2.5 Down-regulation of Bcl-2 Expression and Up-regulation of Bax Expression by UA

Western blotting revealed that the Bax expression was elevated and the Bcl-2 expression reduced in the UA-treated cells as compared with those in the non-treated cells, and the Bax/Bcl-2 ratio increased with the concentration of UA (fig. 3, table 3).

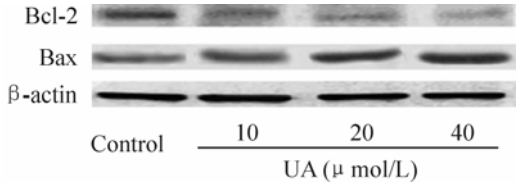

Fig. 3 Western blot analysis of Bcl-2 and Bax

Table 3 Effects of UA on the expressions of Bcl-2 and Bax in the CAOV3 Cells (n=6)

Groups	Bcl-2	Bax	Bax/Bcl-2
Control UA (μmol/L)	0.83±0.13	0.56±0.09	0.67±0.10
10	0.62±0.10*	0.63±0.10*	1.02±0.12*
20	0.56±0.09**	0.72±0.09**	1.28±0.19**
40	0.42±0.05**	0.87±0.12**	2.07±0.23**

$^{*}P$<0.05, $^{**}P$<0.01 as compared with the control group

3 DISCUSSION

ERK, a MAPK family member, can be activated by a good many growth factors and cell factors[7]. The activated ERK was found to cause the malignant transformation of cells by activating the oncogenes through transcription regulation and play an important role in cell growth, development, division, death and malignant transformation[8–10]. UA has recently drawn a great deal of attention for its effects on cancer cells, including inhibition of tumor cell growth and induction of apoptosis[11–16]. In this study, we found that UA could inhibit the proliferation of the CAOV3 cells in a concentration-dependent manner and the ERK activity was decreased at the same time in the cells treated with different concentrations of UA. The p-ERK1/2 expression was also reduced and the expression of MKP-1, a key enzyme that inactivates and regulates the ERK, was increased. On the basis of the findings we speculated that UA may inhibit the CAOV3 cell growth by inhibiting the ERK activity and the p-ERK1/2 expression, and increasing the MKP-1 expression.

The apoptosis of tumor cells is a type of cell death taking place actively under the circumstances where many kinds of genes are involved[17, 18]. The genes such as p53, c-myc, Fas, Bcl-2/Bax were found to participate in the apoptosis of cancer cells[19–22]. P53, c-jun, c-fos, caspase family could induce the apoptosis and Bcl-2 family could suppress the development of apoptosis[23, 24]. In this study, we found that UA induced CAOV3 cell apoptosis, and meanwhile, increased the Bax expression, decreased the Bcl-2 expression, thereby increasing the Bax/Bcl-2 ratio. The inhibitory role of UA in the CAOV3 cell growth *in vitro* may be related to the cell apoptosis, which may explain the anti-tumor effect of UA.

Identification of an anti-tumor agent with low toxicity has always been a hot research topic in oncological field. Our study suggested that UA may be used against ovarian cancer in the future clinical practice. In this study, UA was found to be able to inhibit the proliferation of CAOV3, a human ovarian cancer cell line, and the mechanism is speculated to involve the inhibition of the ERK activity and the p-ERK1/2 expression, development of cell apoptosis, as evidenced by an increase in Bax expression and a decrease in Bcl-2 expression. However, these results do not rule out the possibility of other signaling pathways through which UA exerts its inhibitory effects on tumor cells. More investigations *in vivo* and *in vitro* about UA on many aspects are still warranted for further clarification.

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