Ursolic Acid Enhances Apoptosis in K562 Cells

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Abstract **Objective** To investigate whether ursolic acid can inhibit proliferation of human leukemia cell line K562 and its cytotoxicity effect. **Methods** Inhibition of cell proliferation was determined by MTT assay and FCM. Cytotoxic effect was determined by "comet assay". Nuclear morphological fragmentation were observed by hoechst33258 fluorescent staining. Expression of Bcl-2 and Bax were assessed by immunochemical staining. Activation of Caspase-3 and expression of phosphotyrosine were detected by Western blotting. **Results** MTT assay showed that ursolic acid with the concentration more than 30 μmol/L could inhibit proliferation of K562 cells effectively in a dose-and-time-dependent manner. Apparent morphological changes, which showed index of apoptosis, were observed. Expression of Bcl-2 was down-regulated, but that of Bax was enhanced. Activation of Caspase-3 and down-regulation of phosphotyrosine was determined. **Conclusions** Ursolic acid can induce apoptosis in K562 cells in vitro. Up-regulation of Bax, activation of Caspase-3 and inhibition of BCR/ABL activity may contribute to its effects.

Key Words ursolic acid; apoptosis; K562 cell line; Bcl-2; Bax; Caspase-3; phosphotyrosine

Ursolic acid (UA) is a kind of triterpenoid compound which exists widely in natural plants and has multiple biological functions. It has been reported that UA indicates a wide variety of activities such as heamatoprotection, anti-inflammation, anti-atherosis, anti-tumor and immunomodulation. Recently, interest in its anti-tumor effect of UA is growing. As reported, Both oncogenesis and promotion of tumor are inhibited by UA. UA also has cytotoxic effect on leukemic cell line P-38 and L-1210, as well as human lung carcinoma cell A-59 [1]. But the mechanisms of these functions are still unknown. Here we investigated the effects of UA on human erythroleukemia K562 cells.

MATERIAL AND METHODS

Cell culture and preparation of UA

Human erythroleukemia K562 cells were subcultured every 2 days in RPMI-1640 (GIBCO) supplemented with 10% new-born bovine serum, 0.04% L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin at 37℃ in a 5% CO_2 humidified incubator. The cells in logarithmic growth phase were utilized. UA (Sigma) was dissolved in Me_2SO at concentration of 1 mmoL/L as stock solution and freezed at -20℃. Before used, the stock solution was diluted with RPMI-1640 at concentration ranging from 100 umoL/L to 500 umoL/L. Me_2SO volumes were kept equal in all groups.

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Cell viability

K562 cells were cultured at a density of 1.0×10^5/mL in 96-well plates. UA was triplicated into cell suspensions ensuring final concentration to be 0, 10, 20, 30, 40 and 50 umoL/L while Me_2SO capacity was kept at 0.05%(V/V). MTT was dissolved in phosphate-buffered saline (PBS) at 5mg/mL. After incubated with UA for 24 h, 48 h, 60 h and 72 h, cells received MTT (Final concentration: 1 mg/mL) and were incubated at 37℃ for further 4 h, then were centrifuged. After that, supernatants were discarded, Me_2SO was added into each well and samples were detected at the wavelength of 570nm. Cell growth inhibitory ratio = $[A_C$ (control)− A_{UA} (drug treated)] /A_C ×100%.

Comet assay

Briefly, cells treated with 40 μmol/L of UA were collected and rinsed once with PBS, cell density was adjusted to 1×10^6/mL. 1.1% agarose was laid on the slides, then covered with a coverslip on each layer. The coverslips were removed after 5 or 10 min. 130 μL of cell suspension and 20 μL of 1.1% low melting point agarose (agarose L) was mixed together. 75 μL of this mixture was added onto each slide. After 5 or 10 minitues´ so-

lidification at 4℃, the slides was dipped into lysis buffer at 4℃ for 1 h in the dark. The slides were then put into electrophoretic buffer for 30 min away from light. Then slides underwent electrophoresis followed by being put into 0.4 mol/L Tris-cl buffer for 15 min twice. The slides were stained with 50 μL of ethidium bromide (20 μg/mL). Results were observed on a fluorescence microscope at 510 ~ 560nm excitation wavelength. DNA injury rate=[(comet cell number in treated group-comet cell number in control group)/200] ×100%

Cell cycle analysis

Cells were collected and washed once with 5 mmol/L edetic acid-PBS, then were fixed with 70% ethanol. Before the cell cycle analysis, cells were gently centrifuged and resuspended in 500 μL of propidium iodide solution (100 ug/mL). Cell cycle was tested by a flow cytometer (Beckman).

Hoechst 33258 fluorescent staining

Centrifuged K562 cells were washed once and resuspended in PBS. Cell suspensions were added on slides and dried at room temperature followed by being fixed for 5 min. The slides were rinsed twice with PBS. Then cells were stained with hoechst 33258 (5 ug/mL, Sigma) for 10 min at 37℃ safe from light. Results were observed on a fluorescence microscopy at 450 nm.

Cell immunochemical staining

Cells were smeared on slides and dried. Then Bcl-2 and Bax were stained following instruction of S-P IHC kit (Zhongshan Biotechnology, Beijing). 3 typical domain fields on every slide were selected to be analysed.

Western blotting

4×10^6 treated and untreated cells were lysed with 2×SDS loading buffer, respectively. Then proteins were electrophoresed in 10% SDS-polyacrylamide gel for Caspase-3 and 8% SDS-polyacrylamide gel for phosphotyrosine and transferred to nitrocellulose membrane overnight. The membrane was blocked with 5% non-fat milk in TBS before co-incubated with anti-phosphotyrosine and anti-caspase-3 monoclonal antibodies in PBS for 8 h at 4℃. Then, after rinsed with PBS for 3 times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 3 h. The membrane was washed with PBS for 3 times and incubated with DAB solution for 15 min. Results were photographed by a digital scanner.

Statistical analysis

Student´s t-test was used to assess the significance of differences by SPSS software.

RESULTS

Cell viability

MTT assay showed that 10~50 umoL/L UA could inhibit K562 cell proliferation in a time-and-dose-dependent manner (Fig. 1).

Comet assay

After treated with 40 umoL/L of UA for 24 and 48 hours, the comet cells with vague DNA trails were observed. DNA injury rate(%) were 16.5 ±1.2; 20.8±1.4; 23.5±1.7, respectively. In control group cell form was normal, only a little comet cells were observed (Fig. 2).

Cell cycle analysis

Treatment with UA for 24 hours resulted in a G_2/M arrest and S-phase decline. After 48 hours, The G_2/M arrest was more apparent (Tab.1).

Hoechst 33258 fluorescent staining

After treatment with 40 umoL/L of UA for 48 hours, nuclei shrunk and were smaller, and the chromosomes were condensed and decomposed into pieces, which showed intensive fluorescence. In control group cells were stained uniformly and the fluorescence was weaker (Fig.3).

Immunochemical staining

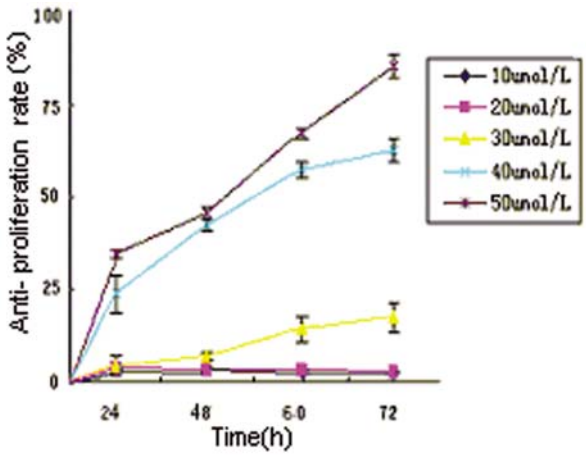


Fig. 1 Anti-proliferation effect of UA on K562 cells

Positive cells showed yellow or brown particles in cytosol. Compared with control group, density of Bcl-2 was reduced in treated groups, whereas that of Bax was enhanced obviously (Tab. 2).

Western blotting

After treated for 6, 12, 18, 24 hours with 50 umoL/L of UA, compared with control, inactive Caspase-3, which is a 32 KD protein, diminished in treated groups in a time-dependent manner. Meanwhile, cleavaged active fraction, which is 17KD, increased. What is more, phosphotyrosine designated at 210KD location in treated groups was down-regulated, and so were other phosphotyrosines (Fig.4).

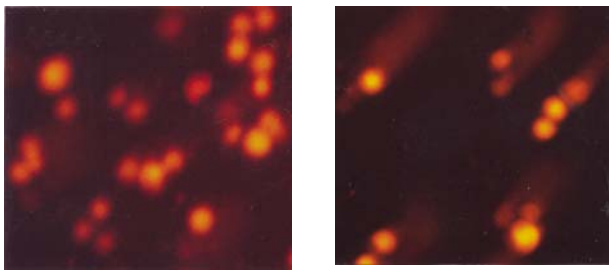
Table 1. Cell cycle arrest by UA

UA	Cell cycle distribution(%)		
	G0/G1	S	G2/M
Control	31.5	57.4	11.0
UA 24h	41.0	18.5	40.5
UA 48h	21.7	30.2	48.1

Table 2. Immunochemical staining on UA-treated K562 cells($\bar{x}$±S)

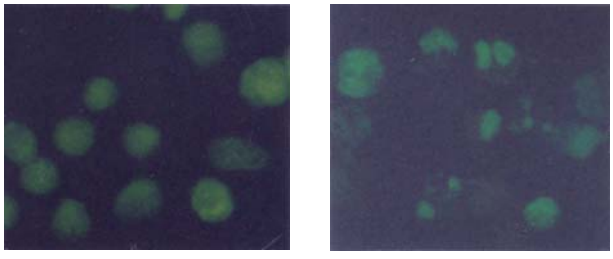
50 μmol/L UA	Bcl-2	Bax
Control	0.148±0.031	0.175±0.037
24 h	0.142±0.028	0.203±0.041*
48 h	0.134±0.019	0.264±0.032**
60 h	0.121±0.017*	0.322±0.036**

*p<0.05; **p<0.01 vs control



A B

Fig. 2 Comet assay for UA-treated K562 cells
A. Untreated cells as control
B. K562 cells treated with UA for 48 hours



A B

Fig. 3. Fluorescent staining on UA-treated K562 cells with Hoechst33258. (A) untreated cells; (B) K562 cells treated with 40μmol/L of UA for 48 hours.

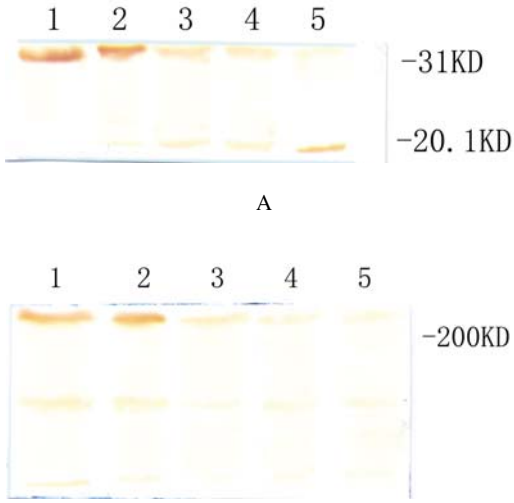


Fig. 4 Expression of Caspase-3(A) and phosphotyrosine(B) shown by Western blotting. The cells were untreated (1) or treated for 6 hours (2), 12 hours(3), 18 hours (4) and 24 hours (5).

DISCUSSION

Usolic acid (UA) is a kind of triterpenoid compound which exists widely in natural plants and has multiple biological functions. Its most remarkable function is anti-tumor effect. It has been identified that UA can not only regulate immune system including enhancing immune surveillance, but also directly kill some kinds of tumor cells non--specifically. Huang et al[2] found that UA was effective in inhibiting tumor generation induced by DMBA. Lien et al[3] reported UA shown cytotoxic effects on leukemia cell line HL-60 and CCRF-CEM. After injecting S-180 cells into mice whiling treating mice with UA, Hsu et al[4] observed that UA could inhibit the tumor generation and elongate survival interval. Cha et al [5] found that UA could down-regulate MMP-9 gene through inducing nuclear translocation of glucocorticoid receptor (GR) in HT1080 human fibrosarcoma cells. Rhew et al.[6] found that UA could suppress expressions of certain oncogenes, such as c-jun and c-fos. These study

partially revealed its anti-tumor effect, but the mechanism was poorly understood.

We firstly investigated its effect on proliferation of human erythroleukemia K562 cells. We found that UA could inhibit proliferation of K562 cells, which showed in a dose-dependent manner.

Comet assay, or termed single-cell gel electrophoresis assay, is a rapid and sensitive method for DNA damage evaluation at single-cell level. Our research demonstrated that UA caused DNA damage in K562 cells, which indicated DNA damage may be one way to inhibit K562 cell proliferation. We infer this damage as a result of apoptosis because the single-cell gel electrophoresis assay is usually for cells having integral membrane, but more information is needed. Cell cycle analysis showed UA treatment for 24 h led to G2/M arrest in K562 cells, and S-phase was down. When exposed longer, cells went into more significant cell cycle arrest, which showed UA may function towards K562 cells in G2/M phase. We also notice S-phase went up in this course, but this going-up may not represent cell regaining proliferation via self-repair. Papazisis et al.[7] used to prove that accumulation of apoptotic cells could enhance S-phase, and once apoptotic cells were removed, S-phase would get down again. It is well known that G2/M arrest is one of cell self-protective mechanisms, which provide enough time for repairing damaged DNA. When repair is successful, cells go on proliferating stage. If not, cell will undergo apoptosis or necrosis. Furthermore, we did observe apoptotic phenomena in UA-treated cells through fluorescent staining, suggesting apoptosis is one way to antagonize leukemia by UA.

The Bcl-2 family is regarded as the main regulator of apoptosis. This family consists of Bcl-2, Bcl-XL, Mcl-1, Al/ Bfl-1, Bax, Bcl-xs, Bak, Bik and Bad. These proteins form disomers and their ratio influences sensitivity and resisitance of tumor cells towards apoptotic inducers. We observed that UA caused down-regulation of Bcl-2 and up-regulation of Bax, resulting in weak resisitance for apoptosis and promoted sensitivity for apoptosis in K562 cells.

BCR-ABL fusion protein is a characteristic product of K562 cells and is also expressed in most chronic myeloid leukemia. It contains a TK domain and activates some signal transduction pathways such as RAS and STAT. The TK domain appears to be necessary for the transforming activity of the hybrid protein. It is known the protein kinase activity of the BCR-ABL protein results in its own phosphorylation. Here we investigated phosphotyrosine expression in K562 cells. Obviously, At the location where 210KD protein existed, UA inhibit tyrosine phosphorylation, which indicating that TK activity of BCR-ABL protein was probably inhibited significantly. Since we did not use anti-ABL antibody, this result was indirect, and we do not know whether BCR-ABL protein was cleaved or not. Further research is necessary. In other hand, Western blotting assay also showed that UA treatment caused cleavage of Caspase-3, which is regarded as a key in apoptotic pathway. The cleavage generates active form of Caspase-3, which is a 17KD protein. Activation of caspase-3 finally lead to nuclear DNA fragmentation as a characteristic of apoptosis.

Taken together, we investigated effects of UA on K562 cells, finding that UA can inhibit K562 cell proliferation and induce apoptosis. More experiments are needed to further our knowledge about its concrete mechanisms and its effects on tumor cells are to be studied.

REFERNCES

1. Lee KH, Lin YM, Wu TS, et al. The cytotoxic principles of Prunella vulgaris, Psychotria serpens and Hyptis capitata: Ursolic acid related derivatices. Planta Med, 1998, 54: 308–311.
2. Huang MT, Ho CT, Wang ZY, et al. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res, 1994, 54: 701–708.
3. Lien CC, Win C, Mei YC, et al. Antileukemic activity of selected natural products in Taiwan. Am J Chin Med 2003, 31: 37–46.
4. Hsu YH, Yang JJ, Lin CC. Effects of oleanolic acid and ursolic acid on inhibiting tumor growth and enhancing the recovery of hematopoietic system postirradiation in mice. Cancer Lett, 1997, 111:7–13.
5. Cha HJ, Park MT, Chung HY, et al. Ursolic acid-induced down-regulation of MMP-9 gene is mediated through the nuclear translocation of glucocorticoid receptor in HT1080 human fibrosarcoma cells. Oncongene, 1998, 16: 771–778.
6. Rhew TH, Park SM, Chung HY, et al. Effects of ursolic acid on oncogene expression detected by in situ hybridization in mice. Chemical Abs, 1993, 119:151741u.
7. Papazisis KT, Zambouli D, Kimoundri OT, et al. Protein tyrosine kinase inhibitor, genistein, enhance apoptosis and cell cycle arrest in K562 cells treated with γ-irradiation. Cancer Lett, 2000, 160: 107–113.