PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• LARGE INTESTINAL CANCER •

Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15

Jie Li, Wei-Jian Guo, Qing-Yao Yang

Jie Li, Wei-Jian Guo, Department of Oncology, Cancer Center, Xin Hua Hospital, Shanghai Second Medical University, Shanghai 200092, China
Qing-Yao Yang, Department of Biology, Shanghai Teachers University, Shanghai 200234, China
Correspondence to: Dr. Jie Li, Department of Oncology, Cancer Center, Xin Hua Hospital, Shanghai Second Medical University, Shanghai 200092, China. ljee@citiz.net
Telephone: +86-21-65010796 Fax: +86-21-65010796
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Abstract

AIM: **Ursolic acid (UA) and oleanolic acid (OA) are triperpene acids having a similar chemical structure and are distributed wildly in plants all over the world. In recent years, it was found that they had marked anti-tumor effects. There is little literature currently available regarding their effects on colon carcinoma cells. The present study was designed to investigate their inhibitory effects on human colon carcinoma cell line HCT15.**

METHODS: **HCT15 cells were cultured with different drugs. The treated cells were stained with hematoxylin-eosin and their morphologic changes observed under a light microscope. The cytotoxicity of these drugs was evaluated by tetrazolium dye assay. Cell cycle analysis was performed by flow cytometry (FCM). Data were expressed as means±SEM and Analysis of variance and Student' *t*-test for individual comparisons.**

RESULTS: **Twenty-four to 72h after UA or OA 60μmol/L treatment, the numbers of dead cells and cell fragments were increased and most cells were dead at the 72nd hour. The cytotoxicity of UA was stronger than that of OA. Seventy-eight hours after 30μmol/L of UA or OA treatment, a number of cells were degenerated, but cell fragments were rarely seen. The IC_{50} values for UA and OA were 30 and 60μmol/L, respectively. Proliferation assay showed that proliferation of UA and OA-treated cells was slightly increased at 24h and significantly decreased at 48h and 60h, whereas untreated control cells maintained an exponential growth curve. Cell cycle analysis by FCM showed HCT15 cells treated with UA 30 and OA 60 for 36h and 72h gradually accumulated in G_0/G_1 phase (both drugs $P<0.05$ for 72h), with a concomitant decrease of cell populations in S phase (both drugs $P<0.01$ for 72h) and no detectable apoptotic fraction.**

CONCLUSION: **UA and OA have significant anti-tumor activity. The effect of UA is stronger than that of OA. The possible mechanism of action is that both drugs have an inhibitory effect on tumor cell proliferation through cell-cycle arrest.**

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INTRODUCTION

Ursolic acid (UA) and oleanolic acid (OA) are triperpene acids having a similar chemical structure and are distributed widely in plants all over the world[1-20]. They are of interest to scientists because of their biological activities. OA has antifungal[21,22], insecticidal[23], anti-HIV[24,25], diuretic[26], complement inhibitory[27], blood sugar depression[28] and gastrointestinal transit modulating[29] activities. UA and OA also possess liver-protection[30-33] and anti-inflammatory effects[34-37]. In recent years, it was found that they had marked anti-tumor effects and exhibited cytotoxic activity toward many cancer cell line in culture[38-44]. Concerning their effects on colon carcinoma cells, there is little available so far in the current literature. The present study was designed to investigate their inhibitory effects on the human colon carcinoma cell line HCT15.

MATERIALS AND METHODS

Drugs and reagents

UA and OA were gifts from Professor Qing-Yao Yang, Department of Biology, Shanghai Teachers University. UA was extracted from Catharantus roseus L. with purity 99%, and OA from Ziziphus jujuba Mill. with purity 98%. The drugs were dissolved in 100% ethanol and then diluted 10 times with RPMI-1640 as the working solution, the final concentration of ethanol being less than 2%. Me^a-2SO was purchased from the Sigma Company (USA).

Cell line and culture medium

HCT15 had been introduced from the NCI (USA) and was cultured and kept in this laboratory. The culture medium used was RPMI-1640 with 10% BSA (Huamei BG, Co Ltd, China).

Cell morphology observation

The morphology of the live cells was observed with an inverted microscope and the live and dead cells were identified after 1% Trypan blue staining[45]. Cell smear was stained with hematoxylin-eosin (HE).Cytotoxicity identification with tetrazolium dye assay (MTT)1.8×10^4 cells were inoculated to each of the 3 parallel wells on a 96-well plate and cultured over night. Different concentrations of UA and OA were added with a final volume of 0.2mL and cultured for 72 more hours. MTT 20μL (5g/L) was added to each well. 4h later samples were centrifuged and the supernatant was discarded. 180μL Me_2SO was added and the 570nm absorbance was read. The mean value of each concentration (3 well) was obtained. Experiments were repeated three times[46].

The inhibition rate (%) =(1-average rate of the treated/average of the control)×100

Flowcytometric detection of cell cycle

Cell cycle was determined by flowcytometric assays[47]. To 1ml of cell suspension with a concentration of 1×10^8/L, 30μmol/L UA and OA 60μmol/L were added. The cells were collected after being cultured for 36h and 72h, respectively. Collected cells were treated with absolute alcohol, then with 1% Triton X-100 (Sigma, USA) for 10min at room temperature. The samples were centrifuged, and the supernatant discarded. 0.01% Rnase was added and samples were shaken for 10min in a 37℃ water bath. Samples were stained with 0.05% propidium iodide for 10min in 4℃ in darkness. The cell cycle distribution was detected with flowcytometer (Model FACSCALIBAR, B.D., USA) and 10000 cells were analyzed with MODFIT software.

Data analysis

Data are displayed as percentage of control condition. Data were expressed as means±SEM and Analysis of variance and Student' *t*-test for individual comparisons. $P<0.05$ was considered statistically significant.

RESULTS

Changes in cell morphology

When HCT15 cells were treated with UA 60μmol/L for 24hours, a number of cells were found dead with a lot of cellular fragments. Among the intact cells, the dead ones accounted for more than 30%. Seventy-two hours later, the number of cells decreased significantly and the remaining cells became shrunken with disappearance of cellular refraction. On the smear stained with HE, a large amount of cellular fragments could be found. When treated with OA 60μmol/L for 24hours, a few cells were dead. There were relatively large amounts of cellular fragments and the dead cells accounted for about 15% at 72h. Within 72 hours of either UA or OA treatment at a concentration of 30μmol/L, a percentage of the cells became rugate at the periphery with blurred cellular borders. There was no obvious cell fragmentation. Relatively large amount of degenerated cells could be seen on HE stained smear, but degenerated cells with OA were markedly less than that with UA.

The cytotoxicity

Cell viability was significantly decreased by treatment of UA and OA for 72h in a dose-dependent manner. Their IC_{50} were 30μmol/L for UA and 60μmol/L for OA (Figure 1). Proliferation assay showed that proliferation of UA and OA-treated cells slightly increased at 24h and significantly decreased at 48h and 60h, whereas untreated control cells maintained exponential growth curves (Figure 2).

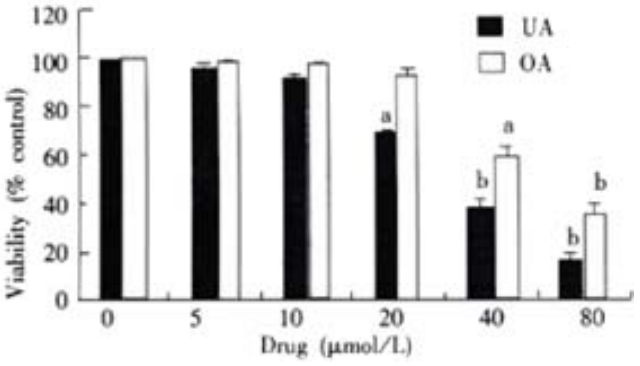

Figure 1 Effects of UA and OA on viability of colon carcinoma cell line. Relative cell viability was assessed by MTT assays. HCT15 cells were treated with various concentrations of UA and OA, respectively for 48h. Data points represent mean values of 3 replicates, with bar indicating SEM. $^aP<0.05$, $^bP<0.01$ *vs* control.

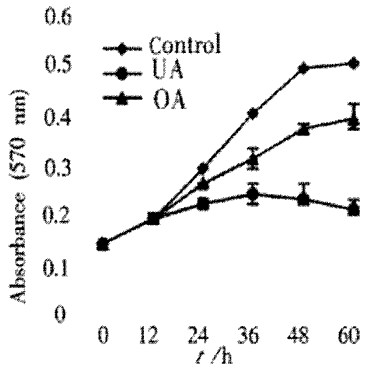

Figure 2 Effects of UA and OA on proliferation of HCT15 cells. HCT15 cells were treated with 30μmol/L UA and OA, respectively. The absorbance of 570 nm means the amount of living cells. Data points represent mean values of 3 replicates, with bar indicating SEM. $P<0.05$, $^bP<0.01$ *vs* control.

Change in cell cycle

When treated the HCT15 cells with different doses of UA and OA for different times, the cell cycle obtained by FCM were as shown in Table 1. HCT15 cells treated with UA 30μmol/L and OA 60μmol/L for 12h and 48h gradually accumulated in G_0/G_1 phase, with a concomitant decrease of cell population in S phase and no detectable apoptotic fraction.

Table 1 The cell cycle distribution of colon carcinoma cell line HCT15 treated with UA and OA ($n=3$ $\bar{x}\pm s$)

Cell cycle	Control		UA (30μmol/L)		OA (60μmol/L)	
	36h	72h	36h	72h	36h	72h
G_0+G_1	62±7	50±7	70±6	81±10[a]	68±9	76±6[a]
S	31±5	42±8	22±4[a]	9±3[b]	26±4	12±3[b]
G_2+M	7±2	8±2	8±3	10±2	6±1	12±2

$^aP<0.05$, $^bP<0.01$ *vs* control.

DISCUSSION

UA and OA both belong to pentacyclic triterpenoid acids. They have a similar molecular structure, but have different sites of the methyl group on the E loop: if the methyl group at C_{19} of UA is moved to C_{20}, it becomes OA. They are distributed widely in plants. In Korean traditional medicine, UA was used in anti-tumor therapy for a long time. Recently, it has been indicated in and outside China that UA and OA have a definitive antitumor activity by various routes[48-50].

This paper observed the anti-tumor activity of UA and OA on the HCT15 cells with some preliminary studies on their mechanism of action. With concentrations higher than their IC^{50}, there was obvious cell death and fragmentation. With the IC_{50} concentration, a few cell fragments were found, but cell death was also obvious. To investigate the effects of UA and OA on the viability of HCT15 cells, HCT15 viability was assessed by MTT assay. In addition, we performed a proliferation assay to identify the anti-proliferation effect of UA and OA. The results showed that cell viability was significantly decreased in a concentration-dependent manner and proliferation was markedly inhibited by both drugs. It was shown that both drugs possessed an inhibitory effect on HCT15 cells. The activity was significantly stronger with UA than with OA. According to changes in HCT15 cell morphology, UA and OA have a direct cytotoxic effect on HCT15 cells. Also it has been reported that UA exhibited both cytotoxic and cytostatic activity in A431 human epidermoid carcinoma cells[48] and OA has a cytotoxic activity against many cancer cell lines[43]. After incubation of the HCT15 cells with UA or OA for different times, the cell cycle was notably changed. When treated with IC_{50} concentration for 36 and 72 hours, the G_0/G_1 phase cells were gradually increased, with a concomitant decrease of cell population in S phase and no detectable apoptotic fraction. This result was in accordance with an inhibitory effect of UA and OA on HCT15 cells proliferation. These observations suggest that UA and OA may be involved in the action of the G_0/G_1 checkpoint and inhibition of DNA replication. Some studies have supported this inference. They have found that oleanane-type triterpenoids had inhibitory effects on DNA polymerase beta and DNA topoisomerases[51,52]. Many papers have demonstrated that UA can induce apoptosis[53,54]. But in the present study FCM assays showed that no apoptotic fraction in treated HCT15 cells. It is worth studying this further by using other methods of detecting apoptosis.

In conclusion, UA and OA have a definite anti-tumor activity on HCT15 cells. The effect of UA is stronger than that of OA. The possible mechanism of action is that both drugs have an inhibitory effect on tumor cell proliferation through cell-cycle arrest.

The toxicity of UA and OA is low and their distribution in plants is extensive. Besides their anti-tumor activity, they also possess immuno-regulatory and liver-protective effects. Therefore, they have

a bright future in clinical application. Further investigation to explore their potential in tumor treatment may prove to be worthwhile.

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