Anticancer Drug

Anti-tumor Effect and Its Mechanisms of Ursolic Acid on Human Esophageal Carcinoma Cell Eca-109 *in Vivo*

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CLC number: R735.1 Document code: A Article ID: 1000-9604(2008)03-0205-06

10.1007/s11670-008-0205-y

ABSTRACT

Objective: To investigate the anti-tumor effect and possible mechanisms of ursolic acid on human esophageal carcinoma *in vivo*. **Methods:** A transplanted tumor model by injecting Eca-109 cells into subcutaneous tissue of BALB/c nude mice was established. 40 nude mice bearing tumors were randomly divided into 4 groups and 0.2 ml saline or 0.2 ml ursolic acid (25–100 $mg \cdot kg^{-1} \cdot d^{-1}$) was injected into abdominal cavity respectively once everyday and lasted for fourteen days. The changes of tumor volume were measured continuously and tumor inhibition rate was calculated. The morphological changes of apoptosis were observed by electron microscope. The expressions of COX-2, bcl-2 and Bax protein in transplanted tumors were detected by immunohistochemistry. At last the PGE2 level of transplanted tumors was detected by radioimmunoassay. **Results:** Treatment of nude mice with 25, 50, or 100 $mg \cdot kg^{-1} \cdot d^{-1}$ of ursolic acid significantly inhibited the growth of the human esophageal carcinoma tumor in nude mice and induced Eca-109 cells apoptosis as demonstrated by electron microscopy analyses. The expressions of COX-2 and bcl-2 in the transplanted tumors were decreased in ursolic acid groups, while the Bax increased. The PGE2 level of transplanted tumors was decreased in ursolic acid groups with a dose-related manner. **Conclusion:** Ursolic acid has anti-tumor effects against human esophageal carcinoma cells *in vivo*, which are likely mediated via induction of tumor cell apoptosis and inhibition of COX-2 and PGE2.

Key words: Ursolic acid; Esophageal carcinoma; Apoptosis; COX-2; PGE2

Ursolic acid (UA), a pentacyclic triterpene acid, is widely distributed in medical herbs and edible plants, such as ligustrum lucidum, hedyotis diffusa, sambucus chinensis, forsythia, hawthorn, apples and other fruits. UA has been shown to possess hypoglycemic[1], anti-inflammatory[2], anti-viral[3], anti-oxidation[4] and immunomodulatory activity[5]. Now, attention has been focused on its antitumor activity. Some studies have shown that UA exhibits growth inhibition properties against many human cancer cell lines *in vitro*, including HepG2 Hepatoma cells[6], MCF-7 breast cancer cells, Caco-2 colon cancer cells[7] and SNG-II endometrial cancer cells[8]. And these could be explained through inhibition of DNA replication, induction of Ca2C release, inhibition of protein tyrosine kinases, and activation of caspases to induce cancer cell apoptosis. Another UA-induced apoptotic mechanism involves the down-regulation of the cellular inhibitor of apoptosis gene and inhibition of NF-kB activity. However, the effect of UA on cancer cells *in vivo* has rarely been reported. In this study, we investigated the effects of UA on the inhibition of proliferation, induction of apoptosis and expression of apoptosis-related proteins in transplanted human esophageal carcinoma in nude mice at the cell and molecular level, and further clarified its anti-tumor mechanisms.

Received: May 9, 2008; **Accepted:** Jul. 11, 2008

This work was supported by the Natural Science Foundation Project of Chongqing Sci & Tech Committee (CSCT, 2006BB5297).

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MATERIALS AND METHODS

Materials

Human esophageal carcinoma cell line ECa-109

was supplied by the department of pathophysiology of Chongqing University of Medical Sciences. Ursolic acid was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 culture medium were obtained from GIBCO BRL (USA). The mouse monoclonal antibodies against human Cox-2, Bax, and bcl were purchased from Santa Cruz Biotechnology, Inc (USA). Sp-9000 Immunohistochemistry kits and horseradish peroxidase (HPR)-rabbit anti-mouse antibody (the second Ab) were purchased from Beijing Zhongshan Biotechnology Co (China). PGE2 Radioimmunoassay kits was obtained from Institute of Hematology of Suzhou University (China).

Cell Culture and Preparation of UA

Eca-109 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37℃ in a humidified atmosphere containing 5% CO_2 . UA was dissolved in saline.

Animal Experiment

A total of 50 mature male normal BALB/c nude mice, aged 6–8 w and weighing 15–20 g, were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Sciences. Animals were housed in micro-isolator cages in a barrier facility under pathogen-free conditions at a monitored ambient temperature of 22℃. To establish tumors in the BALB/c nude mice, Eca-109 cells were subcutaneously injected into the forelimb region of 40 nude mice at a cell density of 1×10^7 cells suspended in 100 μl of PBS. Tumors were allowed to grow until the tumor volume was increased to approximately 0.2 cm^3. They were then randomly divided into four groups (10/group), and given daily peritoneal injections (200 μl) of following treatments for fourteen days: the first group was kept as control and treated with saline, the other groups were treated with UA (25, 50, 100 $mg\cdot kg^{-1}\cdot d^{-1}$), respectively. The nude mice were killed 12 h after last injection and tumor inhibition rate of UA was calculated. Tumor inhibition rate (IR)=(average weight of control group−average weight of treated-group)/average weight of control group×100%. IRM<30 was considered ineffective, while IR≥30 was considered effective[9].

Electron Microscopy

Tumor tissues (1 mm^3) were pre-fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer at 4℃ for 2 h, and then rinsed thoroughly in phosphate buffer and postfixed in 1% OsO_4 at 4℃ for 30 min. After being fixed, tissues were dehydrated through a graded ethanol series, and embedded in Epon (Epoxy resin, Spi supplies, West chester, USA). The ultrastructure of cells was analyzed in ultrathin sections (70 nm) in a TEM (Hitachi H-800, Tokyo, Japan) after the sections were stained with uranyl acetate and lead citrate.

Immunohistochemistry

The tumor tissues were fixed with 4% paraformaldehyde at room temperature for 24 h. The paraffin-embedded specimens were cut into sections with a thickness of 5 μm. Immunohistochemical staining for COX-2, bcl-2 and Bax was performed according to the standard streptavidin-peroxidase method described in the procedure program of streptavidin-peroxidase reagents kit. A previously known positive tumor tissue was used as a positive control. The immunohistochemical results were quantitatively analyzed by a biological image analysis system which consists of Nikon800u biology microscopy, Spot2 digital color camera and Metomorph biological image analysis software.

Radioimmunoassay

The frozen tumor tissues (about 50 mg) were homogenized in 1 ml 0.9% sodium chloride solution and incubated in a shaking bath (37℃) for 15 min, then centrifuged at 7500 g for 15 min. Subsequently, the level of PGE2 in tissues' supernatant was detected using PGE2 (I125) RIA kit.

Statistical Analysis

All data were expressed as $\bar{x}\pm s$. Statistical analysis were carried out by using the statistic software SAS 9.0 and t-test. $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of UA on Tumor Growth in the Esophageal Cancer Model in Vivo

The body weight of nude mice (control group) was lower but there was no significant difference