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Anticancer Activities of Plant Triterpenoids, Ursolic Acid and Oleanoic Acid

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In order to determine whether triterpenoids have anticancer activities, we initially studied the tumor-cell differentiating activity by using F9 teratocarcinoma stem cells as a model system. The triterpene acids, ursolic acid (UA) and oleanolic acid (OA), brought about a morphological alteration of F9 stem cells into the differentiated state. This strikingly resembles the differentiation of parieral endoderm-like cells by retinoic acid. These results present the possibility that F9 stem cells were induced to differentiate by these agents. Moreover, UA and OA inhibited embryogenic angiogenesis on the chick embryo CAMs. In addition, UA has an inhibitory effect on the invasive activity of the HT1080 cells in a concentration-dependent manner. Taken together, we suggest that triterpenoids including UA and OA have various anticancer activities-such as the induction of tumor cell differentiation, antiangiogenic activity, and anti-invasive activity.

Key Words: Anti-invasive activity, Triterpenoid

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INTRODUCTION

Cancer is a disease involving loss of cellular growth control and disruption of cell differentiation. Recent approaches to therapy for various types of cancer have focused on drugs that induce the differentiation of maturation-resistant cells causing the disease[1].

In addition, angiogenesis is important for the progressive growth of solid tumors and also permits the shedding of metastatic tumors from the primary site. In tumor-associated angiogenesis, angiogenic factors from tumors stimulate endothelial cells within a venule to degrade the vascular basement membrane (BM) and to migrate into surrounding tissues toward the tumor mass and to promote the proliferation of endothelial cells in a capillary sprout. There is much evidence that angiogenesis is important for the progressive growth of solid tumors[2,3]. Consequently, inhibition of angiogenesis might lead to control of tumor growth and metastasis. Thus, treatment of angiogenesis inhibitors might be a novel strategy for tumor growth inhibition.

Moreover, invasion into surrounding tissues is a characteristic feature of malignant tumors, and such invasiveness is also required for tumor cells to form metastatic colonies. An essential pattern of this process includes degradation of the extracellular matrix (ECM) and BM. Many proteolytic enzymes produced by tumor cells have been reported to degrade components of the ECM and BM.

In this paper, we firstly studied the cell differentiation activity of plant triterpenoids, ursolic acid (UA), oleanolic acid (OA) (Fig. 1), utilizing F9 teratocarcinoma stem cells as a model system[4,5], because F9 stem cells show very low spontaneous differentiation under normal condition and can be induced to differentiate into two stages of mammalian cell development, a primitive endoderm-like phenotype in response to retinoic acid, and a parietal endoderm-like phenotype in response to retinoic acid in combination with dibutyryl cyclic AMP (Bt2cAMP)[6,7]. Secandly, these triterpenoids were examined for anti-angiogenic activities by using the chick embryo chorioallantoic membrane (CAM) assay. Then we finally examined the anti-invasive activity of UA on the highly metastatic HT1080 human fibrosarcoma cell line with an *in vitro* invasion assay

MATERIALS AND METHODS

1) Cell culture

The F9 mouse teratocarcinoma stem cell line was obtained from the American Type Culture Collection (ATCC CRL 1720, USA). The cells were cultured on gelatinized tissue-culture flasks in a manner similar to that described by Strickland and Madhavi (1978)[8]. F9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovin serum (FBS) and penicillin (100 μg/ml) (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. For Southwestern blot analysis and gel-shift assay, F9 cells were cultured in Iscove's modified Dulbecco's medium (Gibco).

2) Differentiation of F9 teratocarcinoma stem cells

For induction of differentiation, F9 stem cells were trypsinized and seeded onto a gelatinized T75 culture flask containing DMEM supplemented with 2.5% FBS, and incubated for 24h prior to the addition of drugs. To induce differentiation by retinoic acid, 1μM all-*trans*-retinoic acid, 0.5 mM Bt_2cAMP and 0.25 mM theophyline(RACT) were added to F9 stem cells. When F9 cells were differentiated by dexamethasone, 25 μM dexamethasone, 0.5 mM Bt_2cAMP and 0.25 mM theophylline(DEXCT) were added and incubated for 7 days. In the case of UA-induced differentiation, F9 stem cells treated with 7.5 μM UA (Sigma), 0.5 mM Bt_2cAMP and 0.25 mM theophylline (UACT) were incubated for 7 days. In the case of OA-induced differentiation, 37.5 μM OA (Sigma), 0.5 mM Bt_2cAMP and 0.25 mM theophylline (OACT) were used and cells were harvested 7 days later. These agents were added every 2 days with one medium change. The morphology of the cells was visualized under a phase-contrast microscope.

3) CAM assay for angiogenic inhibitor

The fertilized chicken eggs used in this study were kept in a humidified egg incubator at 37°C. After 3 days incubation, about 2 ml of albumin was aspirated from the eggs with an 18-gauge hypodermic needle through a small hole drilled at the narrow of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. On day 4, the shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away[9]. Embryos with chorioallantois of 3~5 mm in diameter were employed for the assay of antiangiogenic activity. Five microliters of an aqueous, salt-free solution of each sample were applied to sterile Thermanox 15-mm disks and allowed dry under laminar flow conditions[10]. The loaded-disks were inverted and applied to the CAM surface of 4, 5-day-old embryos through the windows. The air sac ends of the embryo with shells were covered

with scotch tape. Two days later, an appropriate volume of a 10% fat emulsion was injected using a 33-gauge needle into the 6.5 -day embryo chorioallantois so that the vascular network of CAM stood out against the white background of lipid. The anti-angiogenic response was assessed by measuring an avascular zone of the CAM beneath the disk. When the CAM showed an avascular zone of 3 mm or larger in diameter, the response was scored as a positive according to the method of Crum et al.[11]. Only the frequency was monitored, therefore it was not indicated whether a higher dose also yielded larger avascular zones. At least 20 eggs were used for each dose of agent. Finally, the chorioallantois were photographed. Data on the incidence of anti-angiogenic activity were analyzed by means of the *t*-test, with $P < 0.05$ as the level of significance.

4) Cell culture and treatment with UA

HT1080 human fibrosarcoma cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin and were incubated at 37°C in a humidified atmosphere containing 5% CO^2 in air. UA was obtained from Sigma Chemical Co. (St. Louis, MO), and the cells were treated with 5, 7.5, or 10 μM of UA. After a 3- or 6-day incubation.

5) In vitro invasion assay

In vitro invasion assay was carried out by the method of Saiki *et al.*[12]. Invasion was measured by use of 24-well transwell units with 8 μm porosity polycarbonate filters. The lower side of the filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μl of 0.5 mg/ml reconstituted basement membrane substance (Matrigel; Collaborative Research. Lexington, Ky). The coated filters were air- dried for 1h prior to the addition of the cells. The lower compartment contained 600 μl of DMEM containing 0.1 mg/ml BSA, and 5×10^4 cells were resuspended in 100 μl DMEM and placed in the upper part of a transwell plate. The same concentration of UA as that being cultured was treated in the upper and lower parts of the transwell plate, and cells were incubated for 16 h in a humidified atmosphere of 5% CO_2 at 37°C. Cells were fixed with methanol and stained with H&E. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter with optical microscopy at ×400. Thirteen fields were counted for each assay. Each sample was assayed in triplicate, and each assay was repeated twice.

RESULTS

1) Differentiation-inducing activity of triterpenoids

We have found that UA and OA brought about a morphological alteration of F9 stem cells to the differentiated state. F9 teratocarcinoma stem cells grew in culture as closely packed colonies, and it was difficult to distinguish cell-cell boundaries (Fig. 2S). However, F9 cells, cultured in the presence of UACT or OACT for 7 days became round, as did the differentiate F9 cells treated with RACT, and stopped growing (Fig. 2R, U, O). These results suggest that these agents are able to change the morphology of F9 teratocarcinoma stem cells into the differentiated form of endoderm cells. The morphology of F9 cells treated with Bt2cAMP and theophylline (CT) alone were not changed (data not shown). Accordingly, these results suggest that UA and OA has a morphology changing effect on the F9 stem cells like that of RA.

2) Anti-angiogenic activity of triterpenoids

Dose-response relationships for the inhibition of embryonic angiogenesis are shown in Fig. 3. Retinoic acid, which is known to have anti-angiogenic activity, was used as a control[13]. UA and OA caused avascular zones reflecting anti-angiogenic activity on the treated CAMs, while treatment with an empty coverslip (control) did not produce an effective response. UA and OA produced a slightly weaker anti-angiogenic effect when compared with that of retinoic acid.

UA was a more potent angiogenic inhibitor than OA. The minimum doses required for producing an avascular zone in the CAM were 2 μg and 5μg per egg for UA and OA, respectively. The doses required for half-maximal inhibitor (ID_{50}) were 5 μg and 40 μg per CAM for UA and OA, respectively. The highest dose of UA (100 μg per egg) completely inhibited embryonic angiogenesis and the area of avascular zone was roughly proportional to the frequency of an avascular zone (data not shown). No signs of thrombosis or hemorrhage were detected over the dose range used.

3) Anti-invasive activity of UA on HT1080 cells.

We examined the effect of UA on HT1080 cell invasion through a reconstituted basement membrane (Matrigel) in a transwell chamber. The invasion of HT1080 cells through Matrigel to the collagen-coated lower surface of the filters was inhibited by UA in a concentration-dependent manner (Fig. 4). Treatment with 10 μM of UA for 6 days inhibited the invasion of HT1080 cells into

Matrigel by about 80% compared with a control.

DISCUSSION

Tumor progression required disruption of cell differentiation, angiogenesis, and invasion into surrounding tissue. In order to determine whether triterpene acids act as prevention of tumor, we have screened using F9 teratocarcinoma stem cells as a model system in the work. The triterpene acids, UA and OA, brought about a morphological alteration of F9 stem cells into the differentiated state (Fig. 2). This strikingly resembles the differentiation of parietal endoderm-like cells by RACT. It suggest the possibility that F9 stem cells were induced to differentiate by these agents.

The next study was designed to determine whether UA and OA act as inhibitors of angiogenesis on the CAM assay[14]. There is much evidence that angiogenesis is important for the progressive growth of solid tumors[15]. Angiogenesis also permits the shedding of metastatic tumors from the primary site[3]. Consequently, inhibition of angiogenesis may lead to control of tumor growth and metastasis[16,17]. Therefore, inhibitors of angiogenesis could be inhibitors of tumor growth. Several inhibitors of angiogenesis have been identified from normal tissues or cells, such as angiostatin[18], endostatin[19], TNP-470, protamine, platelet factor-4, tumor necrosis factor, angiostatic steroids, angiostatic antibiotics, and angiostatic vitamins[20]. During searching of novel inhibitor of angiogenesis, we found that UA and OA showed strong inhibitory effects on embryonic angiogenesis in chick embryo chorioallantoic membranes. UA was effective at doses as low as 2 μg/egg, with an ID_{50} value of 5 μg/egg, and OA was effective at doses as low as 5 μg/egg with an ID_{50} value of 40 μg/egg. Therefore, UA had more potent anti-angiogenic activity than did OA.

Metastasis is a multistep process that involves invasion in a number of sequential steps: invasion through BM, intravasation, extravasation, metastatic invasion into the tissues of distant organs, and again intravasation to start another metastatic cascade[21]. In this cascade of metastasis, the invasion of BM by tumor cells is thought to be one of the critical steps. Indeed, the ability of tumor cells to degrade components of BM has been reported to be correlated with the metastatic potential of cells[22]. The present study shows that UA has an inhibitory effect on the invasive activity of the HT1080 cells in a concentration-dependent manner, suggesting the possibility that UA has a capability to inhibit tumor cell invasion. From these results, we suggest that UA and OA should be a subject for further *in vivo* animal studies in order to determine the possibility of clinical trials.

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Fig. 1. Structures of ursolic acid and oleanolic acid.

Fig. 2. S, R, U, O Phase-contrast photomicrographs of live cultures of F9 cells before and after exposure to triterpene acids. F9 cells growing exponentially in the undifferentiated state (S), cultured in the presence 1 μM retinoic acid, 0.5 mM Bt_2cAMP and 0.25 mM theophylline (RACT) (R), 7.5 μM ursolic acid (UA), 0.5 mM Bt_2cAMP and 0.25 mM theophylline (UACT) (U), 37.5 μM oleanolic acid (OA), 0.5 mM Bt_2cAMP and 0.25 mM theophylline (OACT) (O).

Fig. 3. Inhibitory effects of retinoic acid, ursolic acid, and oleanolic acid on embryonic angiogenesis. Coverslips loaded with indicated dose of drugs were implanted on the 4.5-day CAM, and their anti-angiogenic effects were by measuring and avascular zone 2 days after implantation of the sample. Retinoic acid was included as a positive control. The response relationships for the appearance of an avascular zone were dose-dependent. $*P<0.05$ compared to the control; $***P<0.001$ compared to the control.

Fig. 4. In vitro invasion assay of HT1080 cells after treatment with UA. A, light microscopic examination of UA-untreated HT1080 cells (left) and 10 μM HT1080 cells after 6 days of treatment (right) (×200). B, anti-invasive activity of UA. After treatment with 5, 7.5, or 10 μM UA for 3 or 6 days, the cultured HT1080 cells were incubated in a transwell chamber for 16 h. The number of invaded cells was conunted, and mean values were determined under ×400 light microscopy.