Isolation of Ursolic Acid from Apple Peels and Its Specific Efficacy as a Potent Antitumor Agent

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Dried apple peels were extracted with *n*-hexane, chloroform, and methanol successively. The portion of the chloroform extract that showed the strongest cytotoxic activity was purified by silica gel chromatography to isolate ursolic acid (UA). The amount of the isolated UA was 0.71% of the dried peels. Normal mouse embryo cells [serum-free mouse embryo (SFME) cells] and tumorigenic human c-Ha-*ras*- and mouse c-*myc*-transformed SFME cells [*r*/*m* highly metastatic (HM)-SFME-1 cells] were treated with various concentrations of UA (2.5–20 μM) to investigate its effects on cell growth. UA at 10 μM appeared very effective at suppressing the tumor cell growth, affecting more than 82% of *r*/*m* HM-SFME-1 cells, while it inhibited cell growth in only about 7% of SFME cells. Tumorigenic *r*/*m* HM-SFME-1 cells were also treated with various concentrations (2.5–10 μM) of epidermal growth factor (EGF) or aminoguanidine (AG) in the presence of UA (2.5–10 μM). Neither EGF nor AG seemed to have any effect on UA-inhibited cell growth. In the present study, it is revealed that UA could be a very effective and promising agent for antitumor treatments, as it specifically affects tumorigenic cells yet appears to cause very little harm to normal cells.

Key words —— apple peels, ursolic acid, serum-free mouse embryo (SFME), tumorigenic cells, cancer

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INTRODUCTION

Consumption of fruits and vegetables has been associated with a low incidence of cancers and other degenerative diseases. Apples are among the most consumed fruits worldwide and previous studies have suggested that apple intake could play an important role in the prevention of carcinogenesis and in the inhibition of tumor progression.[1–5] Apple peel has been documented to exhibit more potent antitumor activity than apple flesh,[2,6,7] which suggests that apple peels provide the major portion of bioactive phytochemicals and that they could be beneficial for health as a source of natural bioactive components.[6,7] Identification of the bioactive components of apple peels that may be responsible for antitumor activity has not been explored until recently, but some of the components have been identified by bioactivity-guided fractionation of apple peels; one of these was characterized as ursolic acid (UA: 3β-hydroxy-urs-12-en-28-oic acid).[5]

Triterpenoids exist widely in nature and are used for medicinal purposes in Asia. Although they are utilized in many Asian countries, this class of molecule, which resembles steroids in chemical structure, biogenesis and pleiotropic actions, has not impacted on the practice of Western medicine.[8] Triterpenoids, like steroids, are formed in nature by the cyclization of squalene, with the retention of all 30 carbon atoms in molecules, such as UA. UA, a pentacyclic triterpene acid, is found in berries, leaves, flowers, fruits, and medicinal plants, such as *Calluna vulgaris*, *Eriobotrya japonica*, *Eugenia jumbolana, Glechoma hederaceae, Ocimum sanctum*, and *Rosemarinus officinalis* in the form of free acid or as an aglycone of triterpenoid saponins,[9–13] and is known to possess antiinflammatory, hepatoprotective, antiulcer, antiatherosclerotic, hypolipidemic, and antitumor effects.[11,14–17] It has attracted a great deal of attention because of its effects on cancer cells in particular, including inhibition of cell growth and induction of apoptosis. It was shown to induce calcium-dependent apoptosis of human Daudi cells and human leukemic

cells.[18,19] It also suppressed phorbol 12-myristate 13-acetate-induced inflammation and tumor promotion in mouse skin,[11] and these effects were attributed, in part, to inhibition of prostaglandin synthesis,[11,16] although the underlying mechanisms are not fully understood. Furthermore, its antitumor effects include induction of tumor cell differentiation,[20] antitumor promotion,[9,10] antiangiogenic effects,[21] and antiinvasive activity.[22]

The mouse embryo cells, established by Loo *et al.*,[23] were originally derived from a 16-day-old whole Balb/c mouse embryo, and are maintained in a serum-free culture medium. These cells were termed serum-free mouse embryo (SFME) cells. They do not undergo growth crisis, maintain their diploid karyotype for extended passages, and are non-tumorigenic *in vivo*. Consequently, they are non-transformed, behave as primary cultures, have a finite lifespan, and display the characteristics of central nervous system (CNS) progenitor cells.[24,25] SFME cells were co-transfected with human c-Ha-*ras* and mouse c-*myc* genes, and these cells were designated as *ras/myc* SFME cells.[26] Whereas SFME cells are non-tumorigenic *in vivo* and require epidermal growth factor (EGF) for their survival, growth and proliferation,[24,25] *ras/myc* SFME cells are tumorigenic without requiring any growth factors such as EGF.[26] Another line of SFME-derived tumorigenic cells is the highly metastatic (HM) *ras/myc* SFME (*r/m* HM-SFME-1) cells, which was established by selecting *ras/myc* SFME cells that only metastasize to the lungs of Balb/c mice.[27] Analyzing the characteristics and behavior of the tumorigenic SFME cells could be of great importance to the field of medicinal plant studies, because a simple comparison of normal SFME and tumorigenic SFME cells may contribute to our understanding of the behavioral differences between normal and cancer cells in the CNS in response to antitumor medicines.

Although UA has been isolated from several plants and some physiological aspects of UA have been studied, to the best of our knowledge, there have not been any reports that identified UA from apple peels and investigated the antitumor effects of UA by comparing normal and tumorigenic cells. It is of utmost importance that an antitumor agent should affect only tumor cells, and have no adverse effects on normal cells. That is, to examine the efficacy of an antitumor agent from a practical point of view, it is essential that a comparison be made of its effects on normal and tumorigenic cells. Therefore, in the present study, UA was isolated from apple peels, and normal SFME cells and tumorigenic *r/m* HM-SFME-1 cells were treated with UA to investigate the efficacy of UA as an antitumor agent. In addition, *r/m* HM-SFME-1 cells were treated with EGF or aminoguanidine (AG) in the presence of UA to elucidate the possible mechanisms underlying the suppression of tumor cell growth by UA.

MATERIALS AND METHODS

Plant Material —— Apples (*Malus pumila*) of the Fuji variety were purchased from a commercial orchard (Aomori, Japan). The peels were collected and dried at room temperature for 10 days prior to extraction. The voucher specimen is deposited in the herbarium of the Department of Clinical Pharmacy, Aomori University (Aomori, Japan).

Chromatographic Materials —— Silica gel (Wakogel C-200) for column chromatography and precoated silica gel 60 TLC plates (DC-Fertigplatten Kieselgel 60 F_{254} and DC-Alufolien Kieselgel 60 F_{254}) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Merck KGaA (Darmstadt, Germany), respectively.

Instrumentation —— ^{1}H-Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL JNM-AL300 NMR spectrometer (JEOL Ltd., Tokyo, Japan) and chemical shifts were recorded in δ units. Infrared (IR) spectra were recorded in KBr on a JASCO FT/IR-5300 spectrometer (JASCO Corporation, Tokyo, Japan).

Extraction, Isolation and Purification Procedures of UA from Apple Peels —— The dried apple peels (280 g) were extracted three times (each 24 hr) with 1000 ml of *n*-hexane, chloroform, and methanol (all three substances from Wako Pure Chemical Industries, Ltd.), successively, at room temperature. After filtration, the solvent was evaporated under reduced pressure to obtain *n*-hexane (5.9 g), chloroform (11.8 g), and methanol extracts (186 g), respectively. A part (11.3 g) of the chloroform extract was purified by silica gel chromatography, eluted isocratically with chloroform/methanol (20:1, v/v), and 11 fractions were collected. One (2.9 g) of the collected fractions showed cytotoxic activity against mouse leukemia P388 cells. Mouse leukemia P388 and its vincristine (VCR) selected multidrug resistant cells were kindly pro-

vided by Dr. M. Inaba (Cancer Chemotherapy Center, Tokyo, Japan). The cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum and 60 mg/ml of kanamycin. To analyze the cytotoxic activity, P388 cells (1×10^4 cells/well) were plated in 96-well microplates and incubated for 24 hr in a humidified 20% O_2 and 5% CO_2 atmosphere at 37C. The cells were treated in triplicate with graded concentrations of VCR in the absence or presence of the collected fractions, and were then re-incubated for 72 hr. The cytotoxic activity was shown as the IC_{50} value. A part (1.5 g) of the fraction that showed the cytotoxic activity was further purified by silica gel chromatography and again eluted isocratically with chloroform/methanol (20:1, v/v) to obtain UA (1.0 g).

Measurement of the Antitumor Activity of UA —— SFME cells were a gift from Dr. S. Shirahata (Kyushu University, Fukuoka, Japan) and *r/m* HM-SFME-1 cells were from our cell stocks.[27] The basal nutrient medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DME/F-12; Sigma, St. Louis, MO, U.S.A.),[28,29] supplemented with sodium bicarbonate (1.2 mg/ml; Sigma), sodium selenite (10 nM), and gentamicin sulfate (10 μg/ml; both from Wako Pure Chemical Industries, Ltd.). Cells were maintained in DME/F-12 supplemented with insulin (10 μg/ml) and transferrin (25 μg/ml; both from Sigma), with (for SFME cells) or without (for *r/m* HM-SFME-1 cells) EGF (50 ng/ml; Sigma), in 60 mm-diameter dishes pre-coated with bovine fibronectin (10 μg/ml; Biomedical Technologies Inc., Stoughton, MA, U.S.A.) in a humidified atmosphere containing 20% O_2 and 5% CO_2 at 37°C. Cells cultured continuously in the serum-free medium were detached from stock dishes by trypsinization, and then diluted, centrifuged, re-suspended, plated at 1×10^4 cells/well in 96-well microplates, and cultured in culture medium with (2.5–20 μM) or without UA (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), or with a combination of UA (2.5–10 μM) and EGF (2.5–10 μM) or AG (2.5–10 μM; Wako Pure Chemical Industries, Ltd.). Cells were cultured for 72 hr and cell numbers were determined by colorimetric assays utilizing the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Wako Pure Chemical Industries, Ltd.).[30]

RESULTS AND DISCUSSION

Identification of the Purified UA from Apple Peels

Dried peels (280 g) of apples (*Malus pumila*) of the Fuji variety, cultivated in Aomori, Japan, were extracted with *n*-hexane, chloroform and methanol, successively, to furnish fractions of 5.9 g, 11.8 g, and 186 g, respectively. These extracts showed cytotoxic activities against mouse leukemia P388 cells with IC_{50} values of > 25, 13, and > 25 μg/ml, respectively. Interestingly enough, it was also found that the chloroform extract showed stronger cytotoxic activity against VCR-resistant P388 leukemia cells than normal P388 leukemia cells with IC_{50} values of 5.5 μg/ml for VCR-resistant and 6.9 μg/ml for normal cells, respectively. The portion of the chloroform extract (11.3 g) that showed the strongest cytotoxic activity was purified by silica gel chromatography while monitoring the cytotoxic activity to isolate ursolic acid together with a trace amount of oleanolic acid. The identification of these components was performed by making direct comparisons of them with commercially available authentic standard samples. Figure 1 shows representative ^{1}H-NMR spectra of the UA isolated from apple peels

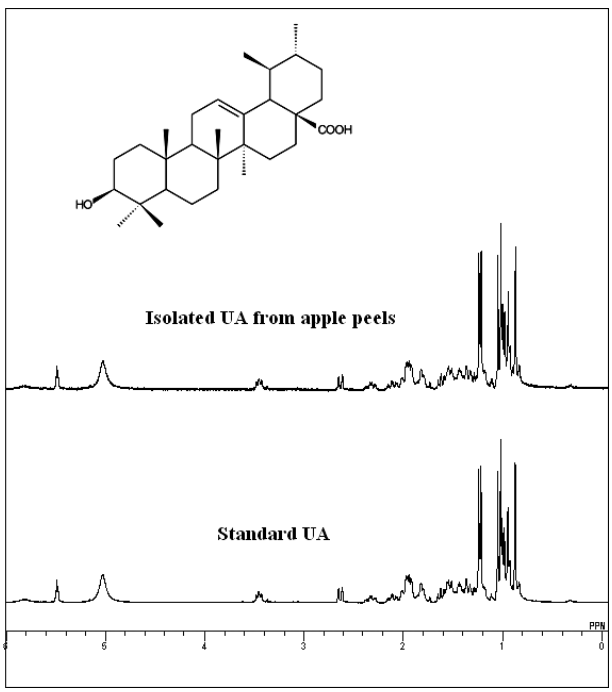


Fig. 1. Representative ^{1}H-NMR (Pyridine-d5, 300 MHz) Spectra of the UA Isolated from Apple Peels and the Standard UA

The structure of UA is also shown.

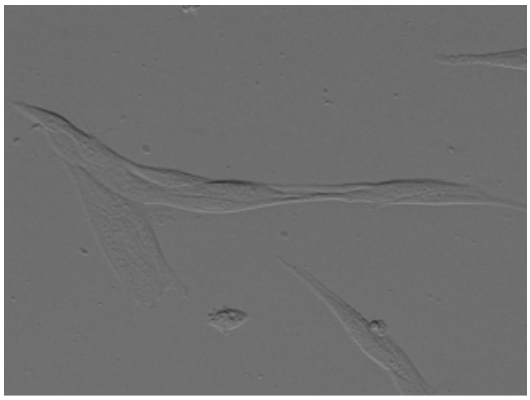
and the standard UA. The structure of UA is also shown. The spectra of the isolated UA were in good agreement with the standard UA. Recently, it was reported that UA was isolated from apple peels of the Red Delicious variety, and that the obtained UA was about 0.15% of the apple peels.[5] In the present study, the amount of the isolated UA from 280 g of apple peels of the Fuji variety was 2.0 g (0.71%). ^{13}C-NMR, IR and TLC (R_f = 0.50, $CHCl_3$/MeOH, 30:1, v/v) data on the UA isolated from apple peels also agreed with those on the standard UA.

Profiles of SFME and *r/m* HM-SFME-1 Cells

In the present study, SFME cells, a CNS normal cell line, and *r/m* HM-SFME-1 cells, an SFME-derived tumorigenic cell line, were used. The *r/m* HM-SFME-1 cells were highly metastatic to the lungs of Balb/c mice.[27] To show the morphological differences between SFME and *r/m* HM-SFME-1 cells, phase-contrast images of representative cells are presented in Fig. 2. SFME cells were rather elongated, while the tumorigenic *r/m* HM-SFME-1 cells were rounder and smaller. From a practical point of view, it is of great importance that an antitumor agent should affect only tumor cells and not inflict any abnormalities upon normal cells. When investigating the efficacy of an antitumor agent from a plant source, analysis of the characteristics and behavior of *r/m* HM-SFME-1 cells could be of great use, because a simple comparison of an agent's effects on normal SFME and tumorigenic SFME cells may provide new insights into understanding the behavioral differences between normal and cancer cells in the CNS in response to antitumor medicines.

Efficacy of UA as a Potent Antitumor Agent

Normal SFME cells and tumorigenic *r/m* HM-SFME-1 cells were treated with various concentrations of UA (2.5–20 μM) to investigate its effects on cell growth (Fig. 3). UA from apple peels has been reported to show antiproliferative activities on human HepG2 liver cancer, MCF-7 breast cancer, and Caco-2 colon cancer cells, with EC_{50} values of 87.4, 14.4, and 34.4 μM, respectively.[5] In the present study, UA scarcely affected the viability of SFME cells at 2.5–10 μM; however, it markedly suppressed the growth of *r/m* HM-SFME-1 cells at 5 μM. From a practical point of view, UA at 10 μM appeared to be very effective, affecting growth in more than 82% of *r/m* HM-SFME-1 cells, yet inhibiting growth in only about 7% of SFME cells. These results suggest that UA could possibly be a very effective and promising agent for antitumor treatments, as it affects tumorigenic cells and appears to cause very little harm to normal cells. At 20 μM, the highest concentration tested in the present study, UA suppressed more than 90% of *r/m* HM-SFME-1 cells, whereas it affected about 60% of SFME cells.



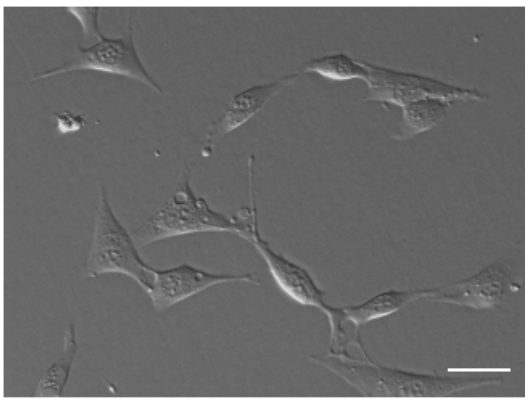


Fig. 2. Phase-contrast Images of Representative SFME and *r/m* HM-SFME-1 Cells

Mouse embryo cells cultured multipassage in basal nutrient medium supplemented with serum eventually undergo growth crisis or senescence. Replacing serum in the culture medium by growth factors and other supplements allows extended culture of some cell types that cannot be maintained on a long-term basis in conventional serum-containing media. SFME cells can be initiated and maintained on a multipassage basis in a rich nutrient medium supplemented with insulin, transferrin, and EGF. The cells derived in this manner display several unique properties. They do not exhibit growth crisis or gross chromosomal aberration and are nontumorigenic in syngeneic or athymic mice. SFME cells were co-transfected with human c-Ha-*ras* and mouse c-*myc* genes, and the cells that are tumorigenic and metastasize to the lungs of Balb/c mice were designated as *r/m* HM-SFME-1 cells. Analyzing the characteristics and behavior of the normal and tumorigenic SFME cells could be of great importance to the field of medicinal plant studies, because a simple comparison of these cells may contribute to our understanding of the behavioral differences between normal and cancer cells in response to antitumor medicines. Scale bar, 20 μm.

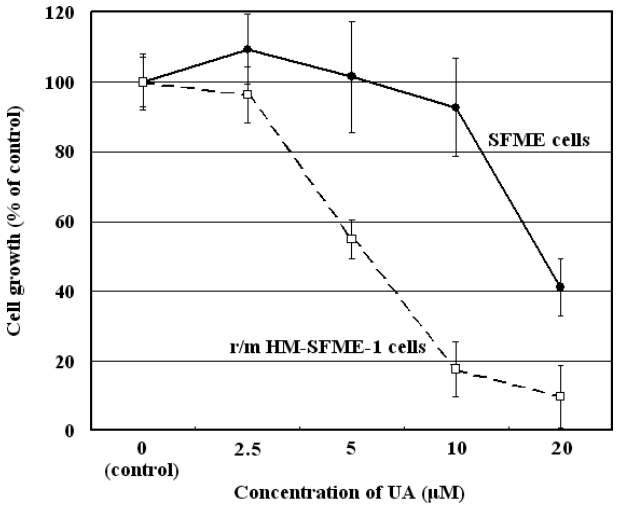


Fig. 3. Suppression of the Growth of Tumorigenic *r*/*m* HM-SFME-1 Cells by UA

Normal SFME cells and tumorigenic *r*/*m* HM-SFME-1 cells were treated with various concentrations of UA (2.5–20 μM) to investigate its effects on cell growth. Each point is the mean ± standard deviation of at least 6 experiments.

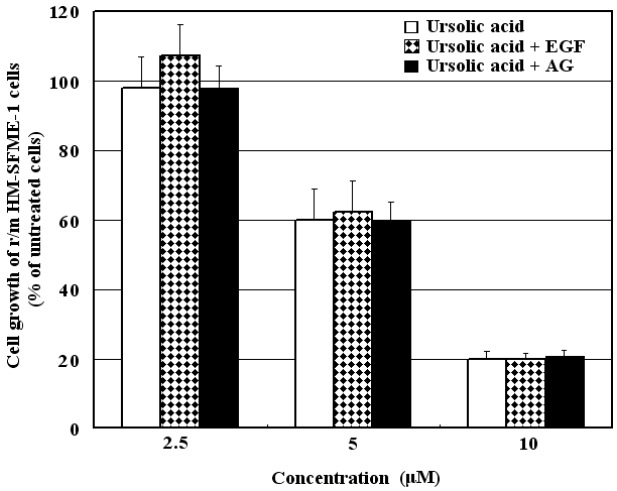


Fig. 4. Cell Growth of Tumorigenic *r*/*m* HM-SFME-1 Cells Treated with Various Concentrations (2.5–10 μM) of EGF or AG in the Presence of UA (2.5–10 μM)

Each point is the mean ± standard deviation of at least 6 experiments.

Tumorigenic *r*/*m* HM-SFME-1 cells were also treated with various concentrations (2.5–10 μM) of EGF or AG in the presence of UA (2.5–10 μM), and their combined effects on cell growth were analyzed (Fig. 4) to investigate the possible mechanisms underlying the suppression of tumor cell growth induced by UA. Growth factors, such as tumor necrosis factor (TNF) and EGF, are essential for cells to survive. TNF has been documented as a growth factor for some cell types,[31,32] and EGF is required for the survival, growth and proliferation of normal SFME cells.[24,25] However, tumorigenic *r*/*m* HM-SFME-1 cells are independent of EGF because they are capable of producing growth factors.[26] We hypothesized that the antiproliferative effect of UA on *r*/*m* HM-SFME-1 cells (Fig. 3) was possibly due to the suppression of growth factor production, and we treated the cells with EGF in the presence of UA (Fig. 4) to determine whether or not tumor cell growth would recover. However, the inhibitory effect of UA was not nullified, suggesting that UA affects growth factors other than EGF, or that its inhibitory effect is based on mechanisms that do not involve any growth factors. Furthermore, UA has also been reported to have effects on nitric oxide (NO) production. UA induced NO in resting macrophages,[33] while it attenuated the expression of inducible NO synthase (iNOS) in macrophages.[8] Therefore, we presumed that the inhibition of *r*/*m* HM-SFME-1 cell growth induced by UA (Fig. 3) might be attributable to NO production. We treated the cells with AG, an iNOS inhibitor (Fig. 4), even though studies of the roles of NO in tumor biology have yielded mixed results. For example, high levels of NO derived from tumor and/or host cells caused cell death and resulted in the suppression of tumor growth and metastasis.[34–37] On the other hand, NO production in various tumors has been positively correlated with the degree of malignancy, including tumor growth, invasiveness, metastasis,[38,39] angiogenesis,[40] and inhibition of apoptosis.[41] In the present study, AG did not seem to have any effect on UA-inhibited cell growth (Fig. 4), indicating that growth suppression by UA may involve factors other than NO. Meanwhile, pharmacokinetic studies of UA have been reported and it was suggested that UA could rapidly be absorbed and may have high binding activity to organs.[42] Further studies will be expected to elucidate the mechanisms underlying the specific growth suppression of tumorigenic cells induced by UA and its efficacy as a potent antitumor agent.

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