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Original article

Novel effects of glycyrrhetinic acid on the central nervous system tumorigenic progenitor cells: Induction of actin disruption and tumor cell-selective toxicity

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ABSTRACT

Licorice extracts are used worldwide in foods and medicines, and glycyrrhetinic acid (GA) is a licorice component that has been reported to induce various important biological activities. In the present study, we show that GA induces actin disruption and has tumor cell-selective toxic properties, and that its selectivity is superior to those of all the clinically available antitumor agents tested. The cytotoxic activity of GA and the tested antitumor agents showed better correlation with the partition coefficient (log *P*) values rather than the polar surface area (PSA) values. For selective toxicity against tumor cells, GA was most effective at 10 μM that was the same concentration as the previously reported maximum plasma GA level reached in humans ingesting licorice. These results suggest that GA could be utilized as a promising chemopreventive and therapeutic antitumor agent. The underlying mechanisms involved in the selective toxicity to tumor cells by GA are also preliminarily discussed.

1. Introduction

Licorice extracts and the principal licorice component glycyrrhizin (GL) are used worldwide in foods and medicines. The sweet taste of licorice roots arises from GL, which is reputed to be at least 50 times sweeter than refined sugar. Owing to this sweetness, GL is extensively used as a natural sweetener and flavoring additive [1]. GL has been approved for use in foods by most national and supranational regulatory agencies, and the World Health Organization suggested that consumption of GL at 100 mg/day would be unlikely to cause adverse effects [2]. However, excessive intake of GL may cause serious adverse effects, such as disruption of glucocorticoid metabolism. GL is a saponin compound comprising a triterpenoid aglycone, glycyrrhetinic acid (GA; Fig. 1), conjugated to a disaccharide of glucuronic acid. When orally administered, GL is hydrolyzed to the pharmacologically active GA [3]. GA influences glucocorticoid metabolism by inhibiting 11β-hydroxysteroid dehydrogenase type 2, which prevents conversion of active cortisol to inactive cortisone. In turn, this results in unrestricted activation of mineral ocorticoid receptors by cortisol, leading to increased sodium retention, exaggerated renal potassium loss, low aldosterone levels and hypertension [4]. Excessive licorice intake has also been associated with higher blood pressure [5]. Therefore, considering the growing use of licorice and its potential adverse clinical effects, physicians are encouraged to obtain detailed dietary histories when patients present with hypoaldosteronism and high blood pressure [6].

Although excessive intake of any foods may cause certain adverse effects, their adequate consumption can be helpful for advancement of health and prevention of disease. For example,

Abbreviations: CNS, central nervous system; EGF, epidermal growth factor; GA, glycyrrhetinic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, glycyrrhizin; *r*/*m* HM-SFME-1, human c-Ha-*ras* and mouse c-*myc* cotransfected highly metastatic serum-free mouse embryo-1; SFME, serum-free mouse embryo; UA, ursolic acid.

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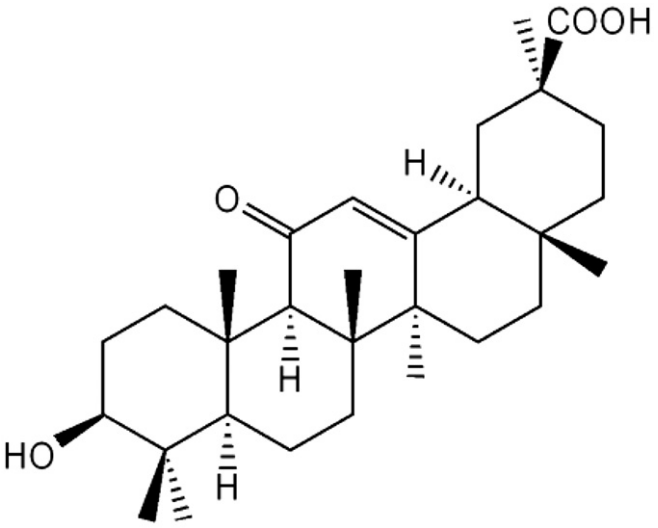

Fig. 1. The structure of GA.

recent epidemiologic studies have found a correlation between populations with higher consumption of selenium, vitamin E, fruits and tomatoes and a lower overall risk of cancer in humans [7,8]. For chemoprevention of human cancers, since the development of more effective and safer agents has been highly expected in recent years, natural products from plants and their synthetic derivatives have been anticipated to play important roles in creating new and better chemopreventive and therapeutic agents [9,10]. Consistent with this notion, several single natural compounds from plants, such as the green tea polyphenol epigallocatechin gallate [11] and apple ursolic acid (UA) [12], are being studied as anticancer agents. GA is also used as an antitoxic and immunological regulatory agent for the prevention or treatment of viral infection, inflammation and anaphylaxis [13,14]. However, despite its wide use in the market and great effects on some physiological aspects, to the best of our knowledge, no studies have investigated the antitumor effects of GA by comparing normal cells with tumor cells. It is of the utmost importance that an antitumor agent should only affect tumor cells and have no adverse effects on normal cells. Therefore, to examine the efficacy of an antitumor agent from a practical point of view, it is essential to carry out comparisons of its effects on normal and tumor cells.

In the present study, normal serum-free mouse embryo (SFME) cells and human c-Ha-*ras* and mouse c-*myc* cotransfected highly metastatic SFME-1 (*r*/*m* HM-SFME-1) cells were treated with GA and its efficacy as an antitumor agent was investigated. Furthermore, a new antitumor agent should be more potent or at least better in a particular aspect than clinically available agents. Hence, the potency of GA was compared with those of some clinically available antitumor agents. Finally, the possible mechanisms underlying the inhibition of the tumor cell growth following exposure to this licorice compound are discussed.

2. Materials and methods

2.1. Reagents

GA was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Adriamycin and fluorouracil were from Kyowa Hakko Industry Co. Ltd. (Tokyo, Japan). Cytarabine and etoposide were from Nippon Shinyaku Co. Ltd. (Kyoto, Japan) and Nippon Kayaku Co. Ltd. (Tokyo, Japan), respectively.

2.2. Cell lines and cell culture

SFME cells were a gift from Dr. S. Shirahata (Kyushu University, Fukuoka, Japan) and *r*/*m* HM-SFME-1 cells were taken from our cell stocks [15]. The basal nutrient medium was a 1:1 mixture of DME and nutrient mixture F-12 Ham (DME/F-12) [16,17], supplemented with sodium bicarbonate, sodium selenite and gentamicin sulfate. Cells were maintained in DME/F-12 supplemented with insulin, transferrin and epidermal growth factor (EGF) in 60-mm diameter dishes precoated with bovine fibronectin (Biomedical Technologies, Cambridge, MA) in a humidified atmosphere containing 20% O_2 and 5% CO_2 at 37 °C.

2.3. Measurement of the antiproliferative activity

Cells plated at 1×10^4 cells/well in 96-well microplates were treated with the test compounds at half confluency. After culture for another 24 h, the cell numbers were determined by the MTT assay [18].

2.4. Western blotting analysis

Proteins were extracted with PBS containing 1 mM PMSF, 1 mM EDTA, 2 mM 2-mercaptoethanol and 1% Triton X-100 at 4 °C for 3.5 h. For western blotting analysis, aliquots of proteins were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and probed with a primary antibody followed by a secondary antibody. The primary antibodies used were mouse monoclonal anti-β-actin (Sigma, St. Louis, MO) and mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody used was AP-conjugated anti-mouse IgG_1 (Chemicon International, Temecula, CA). Images of the positive western blotting bands were obtained by scanning and the densities were determined using an LAS-3000 image analyzer (Fuji Film, Tokyo, Japan).

2.5. Fluorescence staining and confocal laser scanning microscopic observation

Cells were fixed with 3.7% formaldehyde in PBS and permeabilized in PBS containing 0.1% Triton X-100. For F-actin labeling, cells were incubated with rhodamine–phalloidin. After washing and mounting with ProLong Gold anti-fade reagent (Molecular Probes, Eugene, OR), the cells were observed by confocal microscopy using an LSM510 META confocal laser scanning microscope equipped with Ar and He–Ne lasers (Carl Zeiss Japan, Tokyo, Japan) or a BIOREVO BZ-9000 fluorescence microscope system (Keyence, Osaka, Japan). Images were captured using 40×, 63× and 100× oil immersion objective lenses, analyzed and processed with the software Image Browser (Carl Zeiss Japan).

2.6. Calculation of partition coefficients between 1-octanol and aqueous phases (log P) and polar surface area (PSA)

Spartan '06 program (Wavefunction Inc., Irvine, CA) was adopted for calculation of log *P* from Crippen model and PSA.

2.7. Statistical analysis

Experiments were performed in triplicate and repeated at least three times. The values quoted are given as means ± SD. A two-tailed Student's *t*-test was used to evaluate differences between two experimental groups. Ordinary or repeated-measures analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to evaluate the statistical significance of differences

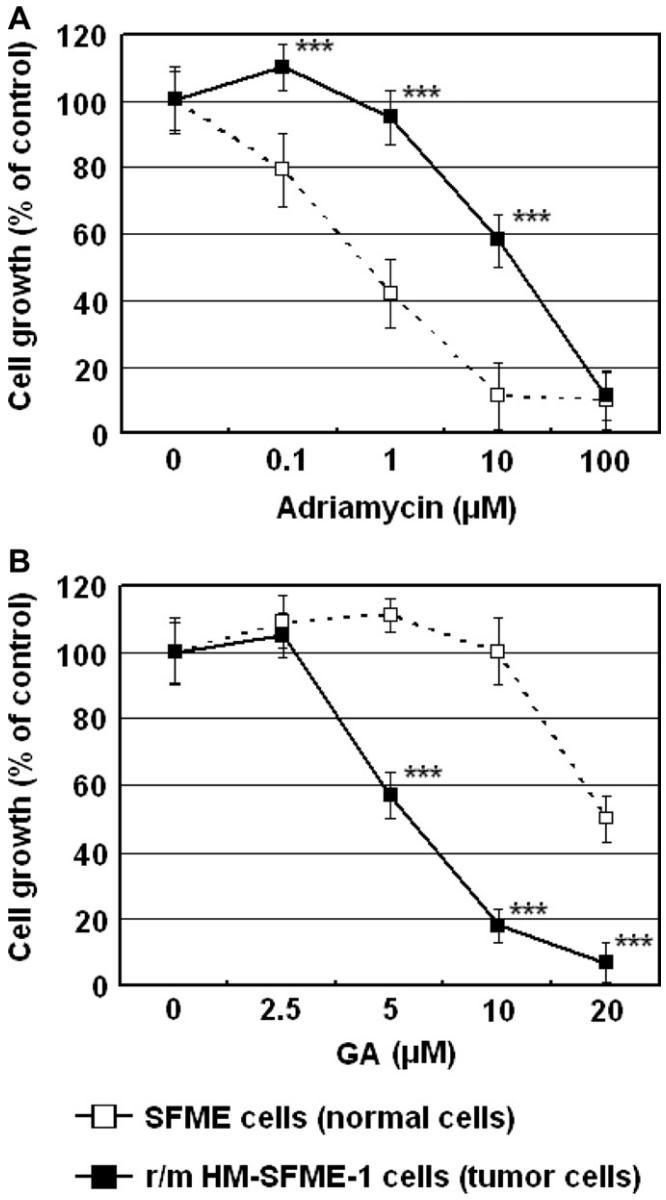

Fig. 2. Antiproliferative effects of adriamycin and GA on normal SFME and tumorigenic *r*/*m* HM-SFME-1 cells. Normal SFME and tumorigenic *r*/*m* HM-SFME-1 cells were treated with various concentrations of adriamycin (A) or GA (B) for 24 h. Adriamycin inhibits the cell growth of the normal cells more strongly than that of the tumor cells. However, GA is capable of selectively affecting the tumor cells. Each point is the mean ± SD of at least 3 experiments. ***$P < 0.001$ by Dunnett's test.

Table 1
Mean IC_{50} values of the cells treated with clinically available antitumor agents and glycyrrhetinic acid (GA) for 24 h.[a]

Compounds	SFME cells (μM)	*r*/*m* HM-SFME-1 cells (μM)	logP	PSA (A^2)
Adriamycin	0.8 ± 0.2[b]	64.0 ± 31.9***[c]	−0.75	142.14
Cytarabine	0.8 ± 0.1	33.3 ± 8.5***	−2.32	95.83
Etoposide	0.5 ± 0.1	9.0 ± 2.5***	1.67	130.10
Fluorouracil	3.2 ± 1.2	48.5 ± 36.2***	−1.31	51.31
GA	18.0 ± 4.0	7.3 ± 3.7***	7.11	64.02

[a] The partition coefficient (log *P*) and polar surface area (PSA) values of each compound are also shown.
[b] Values indicate the mean ± SD of at least 3 experiments.
[c] ***$P < 0.001$ by Student's *t*-test compared with the tumorigenic *r*/*m* HM-SFME-1 cells and normal SFME cells. GA shows lower IC_{50} values for the tumor cells than for the normal cells.

between multiple groups. IC_{50} values were obtained using Prism 4.0 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. GA is selectively toxic against tumor cells without impeding normal cell growth

We previously reported that UA from apples scarcely affected the viability of normal cells (SFME cells), but markedly suppressed the growth of tumor cells (*r*/*m* HM-SFME-1 cells) at 10 μM [12]. This ability of UA prompted us to investigate whether there were other components in plants that can inhibit tumor cell growth without hindering normal cell growth. To this end, normal and tumor cells were treated with GA, a licorice compound, at 2.5–20 μM for 24 h and their effects on cell growth were examined. An antitumor agent, adriamycin was also used as a reference compound. As shown in Fig. 2A, adriamycin was always more toxic to the normal cells than to the tumor cells at any concentration tested, while GA was more toxic to the tumor cells (Fig. 2B). The selective toxicity of GA was most effective at 10 μM, inhibiting more than 80% of the tumor cell growth.

3.2. GA is more potent than some clinically available antitumor agents in inhibiting tumor cell growth without severely affecting normal cells

Even if a compound is capable of inhibiting tumor cell growth without severely affecting normal cells, it is not useful unless it is more potent than clinically available antitumor agents. Therefore, the mean IC_{50} values of the cells treated with adriamycin, cytarabine, etoposide, fluorouracil (clinically available antitumor agents) and GA were determined. Among the antitumor agents tested, no

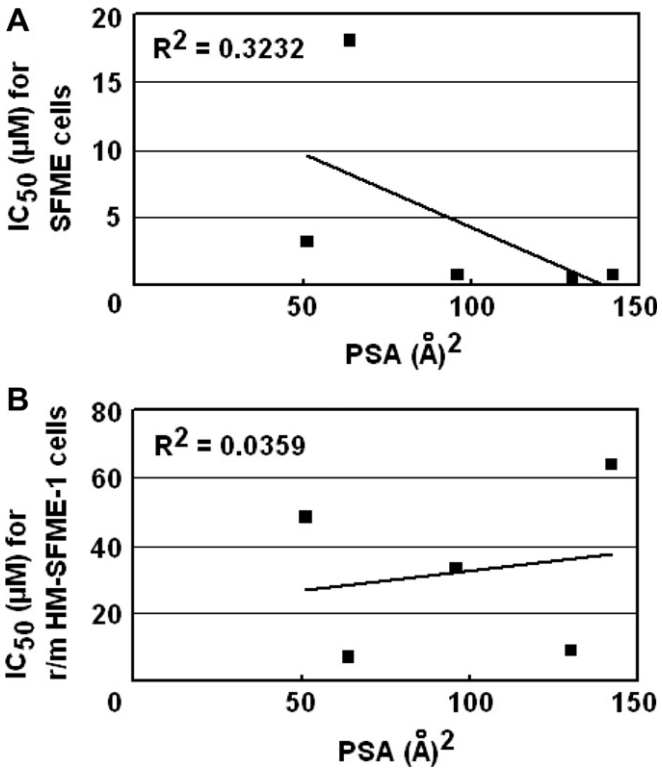

Fig. 3. Linear regression curves for the interaction of PSA with IC_{50} of cell growth for SFME (A) and *r*/*m* HM-SFME-1 (B) cells. No correlation is found between PSA and IC_{50} values of the tested compounds. Each value is described in Table 1.

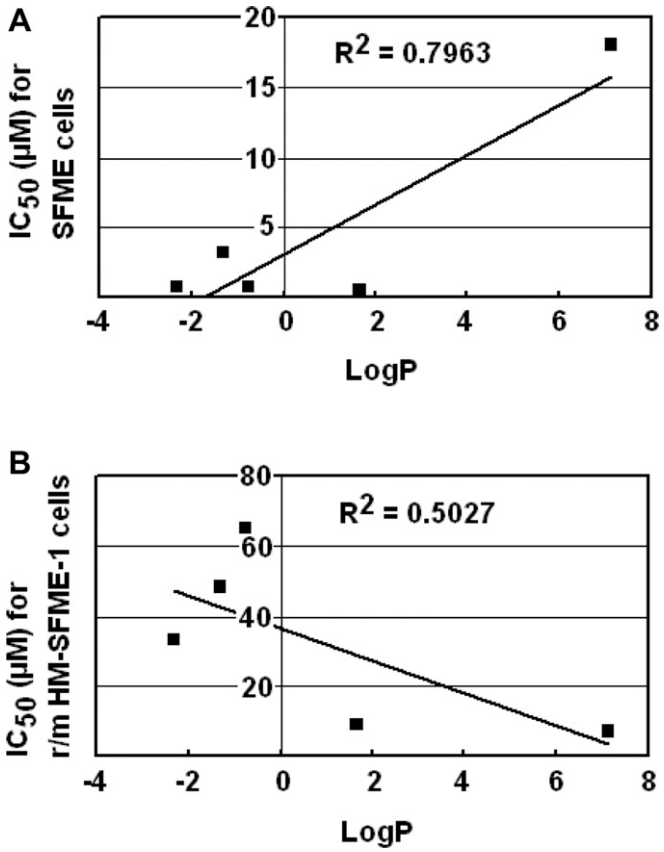

Fig. 4. Linear regression curves for the interaction of log *P* with IC_{50} of cell growth for SFME (A) and *r*/*m* HM-SFME-1 (B) cells. A significant ($P < 0.05$ by null hypothesis) direct or an inverse correlation between log *P* and IC_{50} values of each compound is noted for the normal or the tumor cells, respectively. Each value is described in Table 1.

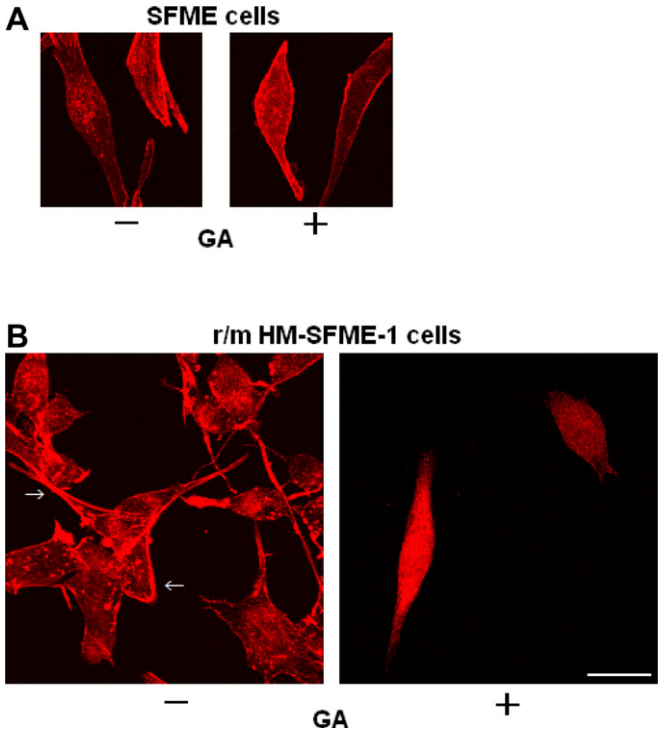

Fig. 5. GA disrupts F-actin. SFME and *r*/*m* HM-SFME-1 cells were treated with GA at IC_{50} for 8 h and its effects on F-actin extensions were analyzed. (A) Fluorescence staining of F-actin protein and confocal laser scanning microscopic observation of SFME cells. GA treatment does not induce any changes in F-actin in the normal cells, (B) Fluorescence staining of F-actin protein and confocal laser scanning microscopic observation of *r*/*m* HM-SFME-1 cells. In the right + GA panel, the GA-treated tumor cells exhibit disappearance of the F-actin extensions, and instead the staining is rather granulated and condensed. Loss of the F-actin extensions at the periphery of the cell membrane is particularly obvious and the tumor cells are no longer capable of maintaining the characteristic broad lamellipodia. Arrows, F-actin extensions at the periphery of the cell membrane. Bar 20 μm.

agent inhibited 50% of the tumor cells at a lower concentration than that for the normal cells (Table 1). In contrast, GA showed lower IC_{50} values for the tumor cells than for the normal cells.

3.3. Cytotoxic activity of GA and the tested antitumor agents shows better correlation with log P values than PSA values

Noshita et al. [19] reported a significant correlation between PSA and biological activities of an antitumor compound from wasabi (*Wasabia japonica*) and its synthetic derivatives. However, no correlation was found between PSA and IC_{50} values of the tested compounds in our present study (Fig. 3A and B). In analyzing lipophilicity and toxic effects of some compounds from plants, Jiao et al. [20] reported that the log *P* values measured for some antitumor compounds are consistent with those predicted on the basis of their chemical structures and that log *P* values for measurement of the relative lipophilicity could be applied. A good direct or an inverse correlation between log *P* and IC_{50} values of each compound was noted for the normal or the tumor cells, respectively (Fig. 4A and B).

3.4. GA induces actin disruption

It has been reported that interference with actin functions associated with the integrity of the cytoskeleton could be utilized as a strategy for developing novel antitumor treatments [21]. We analyzed the effects of an 8-h GA treatment at the IC_{50} value on the F-actin cytoskeletons in the normal and tumor cells by fluorescence microscopy. As shown in Fig. 5, the normal cells were rather elongated (Fig. 5A, left panel) while the tumor cells were relatively rounder and showed broad lamellipodia (Fig. 5B, left panel), and both cells displayed broad F-actin extensions. Although GA treatment did not induce any changes in F-actin in the normal cells (Fig. 5A, right panel), the GA-treated tumor cells exhibited disappearance of the F-actin extensions, and instead the staining was rather granulated and condensed (Fig. 5B, right panel). The loss of F-actin extensions at the periphery of the cell membrane was particularly obvious and the tumor cells were no longer capable of maintaining the characteristic broad lamellipodia. In the GA-treated tumor cells, β-actin protein was slightly downregulated (Fig. 6A and B), although the normal cells were unaffected.

4. Discussion

SFME cells, which were established by Loo et al. [22], were originally derived from a 16-day-old whole Balb/c mouse embryo, and are maintained in a serum-free culture medium. These cells 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 the central nervous system (CNS) progenitor cells [23,24]. SFME cells were cotransfected with the human c-Ha-*ras* and mouse c-*myc* genes, and the resulting cells were designated *ras*/*myc* SFME cells [25]. While SFME cells are non-tumorigenic *in vivo* and require EGF for their

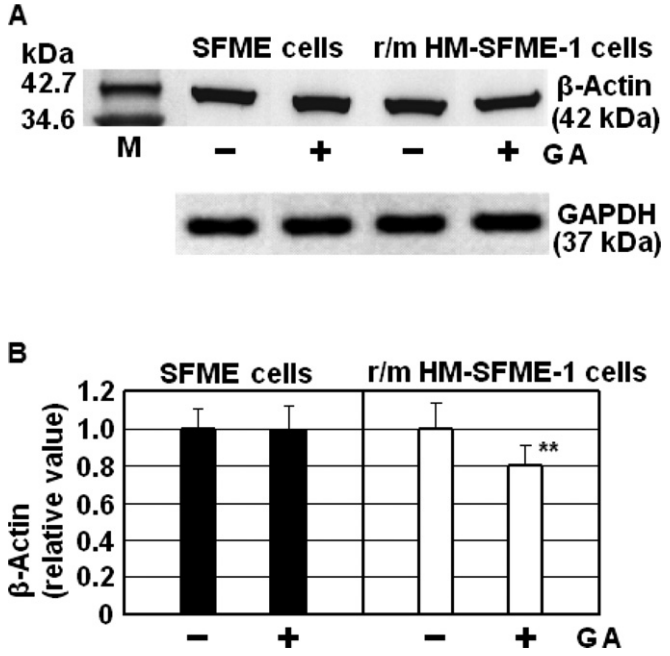

Fig. 6. GA downregulates β-actin protein. SFME and *r*/*m* HM-SFME-1 cells were treated with GA at IC_{50} for 8 h and its effects on β-actin protein were analyzed. (A) A typical image obtained by the western blotting analysis. GAPDH was used as a loading control. M: Size markers. (B) Images of the western blotting analysis were captured, analyzed and processed with the software Image Browser (Carl Zeiss Japan). In the GA-treated tumor cells, β-actin protein is slightly downregulated. Each point is the mean ± SD of at least 3 experiments. $^{**}P < 0.01$ by Student's *t*-test.

survival, growth and proliferation [23,24], *ras*/*myc* SFME cells are tumorigenic and do not require any growth factors, such as EGF [25]. Another line of SFME-derived tumorigenic cells are *r*/*m* HM-SFME-1 cells, which were established by selecting *ras*/*myc* SFME cells that only metastasize to the lungs of Balb/c mice [15]. Analyzing the characteristics and behaviors of the normal and tumorigenic SFME cells could be of great importance in the field of medicinal plant studies for cancer prevention and therapy, because simple comparisons of these cells may contribute to our understanding of the behavioral differences between normal and tumor cells in the CNS in their responses to antitumor agents.

In the present study, we analyzed normal and tumor cells from the same lineage in the CNS and found the selective toxicity of GA, a licorice compound, against the tumor cells. The selectivity of GA was such that, at 10 μM, it inhibited more than 80% of the tumor cell growth within 24 h without affecting the normal cells. Its efficacy was also revealed to be more potent than those of some clinically available antitumor agents. After we realized these unique properties of GA against the tumor cells, our interest shifted toward elucidating the mechanisms for the selective cytotoxicity. Log *P* and PSA are very important parameters to estimate and optimize drug molecules to cross membranes, particularly the blood–brain barrier [26]. We found a significant direct or an inverse correlation between log *P* and IC_{50} values for the normal or the tumor cells, respectively, which suggests that a certain degree of the lower log *P* value is necessary for the toxic effects against the tumor cells. We also found that GA disrupted F-actin extensions and downregulated β-actin protein in the tumor cells. These results indicate that GA induces qualitative and quantitative disruption in the actin cytoskeleton, which could lead to loss of functionality for cell growth or proliferation. It has been reported that some agents bind directly to cytoskeletal proteins, disrupt the integrity of tumor cells and inhibit cell growth and proliferation [27,28]. Although further studies are required to elucidate whether the GA-mediated interference with the cytoskeletal protein was a direct action or an indirect effect through some signaling pathways, our present study clearly reveals that disruption of the actin cytoskeleton was one of the important factors that led to the induction of the selective tumor cell death.

Selectivity is an important issue for cancer prevention and therapy. An ideal antitumor agent should be toxic toward malignant cells with minimum toxicity toward normal cells. However, only a few studies, such as those on β-phenylethyl isothiocyanate [29] and lupeol [30], have reported selective toxicity against tumor cells utilizing natural compounds. In the present study, we have revealed that GA, a licorice compound, has a tumor cell-selective toxic property possibly through disruption of the actin cytoskeleton, and that its selectivity was superior to those of the clinically available antitumor agents tested. To the best of our knowledge, there are no other detailed studies that have described these characteristics of GA. Furthermore, plasma GA levels have been reported to reach 10 μM in humans ingesting licorice [31], and this is exactly the same concentration that showed the selective tumor cell toxicity most effectively in the present study. Although validation studies supporting the utilization of GA in clinical practice are warranted, and further investigations are required to elucidate the mechanisms underlying the selective tumor cell toxicity, our present study clearly suggests that GA could be a promising chemopreventive and therapeutic antitumor agent.

Acknowledgements

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