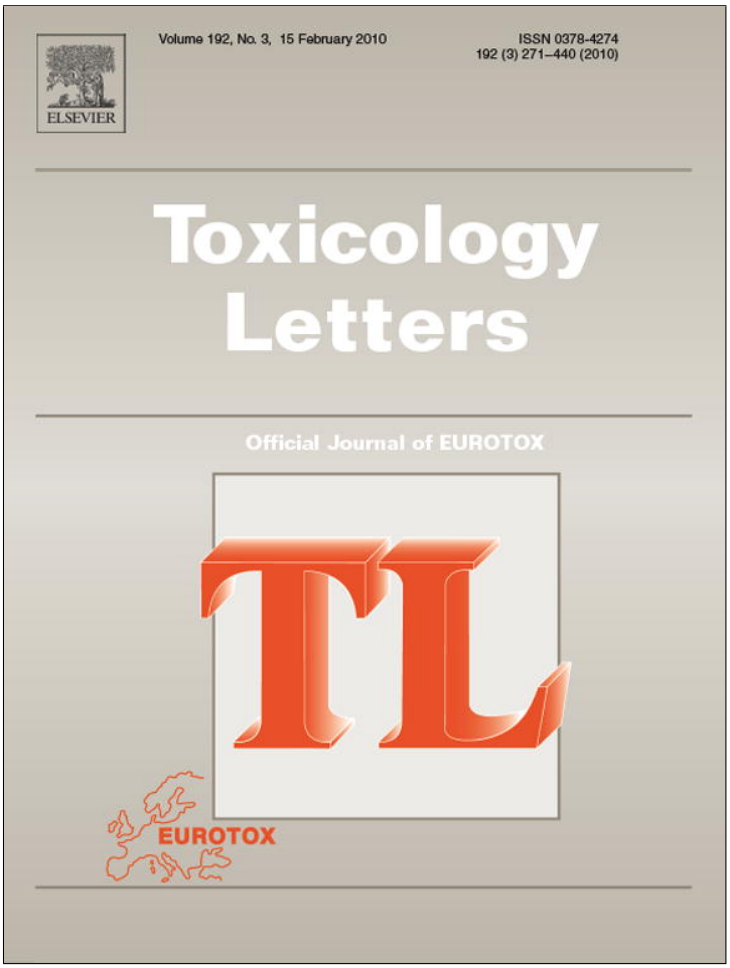

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Selective cytotoxicity of glycyrrhetinic acid against tumorigenic r/m HM-SFME-1 cells: Potential involvement of H-Ras downregulation

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ABSTRACT

With the intensive need for the development of more effective and safer agents for chemoprevention and therapy of human cancer, natural products from plants have been expected to play significant roles in creating new and better chemopreventive and therapeutic agents. Selectivity is also an important issue in cancer prevention and therapy. In the present study, normal serum-free mouse embryo (SFME) and tumorigenic human c-Ha-ras and mouse c-myc cotransfected highly metastatic serum-free mouse embryo-1 (r/m HM-SFME-1) cells were treated with various concentrations of clinically available antitumor agents or glycyrrhetinic acid (GA), and the antiproliferative effects of these compounds were determined by the MTT assay. Western blotting analysis, RT-PCR, fluorescence staining and confocal laser scanning microscopic observation were adopted to analyze H-Ras regulation. GA exhibited the tumor cell-selective toxicity through H-Ras downregulation, and its selectivity was superior to those of all the clinically available antitumor agents examined. For the selective toxicity of tumor cells, GA was most effective at 10 μM. Interestingly, this concentration was the same as the previously reported maximum plasma GA level reached in humans ingesting licorice. These results in the present study suggest that GA with its cytotoxic effects could be utilized as a promising chemopreventive and therapeutic antitumor agent.

1. Introduction

Licorice extracts and the principal licorice component glycyrrhizin (GL; Fig. 1A) are used worldwide in foods, tobacco 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 (Isbrucker and Burdock, 2006). GL is a saponin compound comprising a triterpenoid aglycone, glycyrrhetinic acid (GA; Fig. 1B), conjugated to a disaccharide of glucuronic acid, and the pathway for the biosynthesis of GL has recently been proposed (Seki et al., 2008). When orally administered, GL is hydrolyzed to the pharmacologically active GA (Akao, 1998). In recent years, the development of more effective and safer agents has been intensively required for chemoprevention of human cancers, and natural products from plants and their synthetic derivatives have been expected to play important roles in creating new and better chemopreventive and therapeutic agents (Martin et al., 2007). Consistent with this notion, several single natural compounds from plants, such as the green tea polyphenol epigallocatechin gallate (Nihal et al., 2005) and apple ursolic acid (UA; Yamaguchi et al., 2008), 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 (Shibata, 2000; Armanini et al., 2002). 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 cytotoxic effects of GA by comparing normal cells with tumor cells. It is of the utmost

Abbreviations: CNS, central nervous system; EGF, epidermal growth factor; GA, glycyrrhetinic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, glycyrrhizin; KLF-4, Kruppel-like factor-4; MA, manumycin A; M/O, membrane/organelle; 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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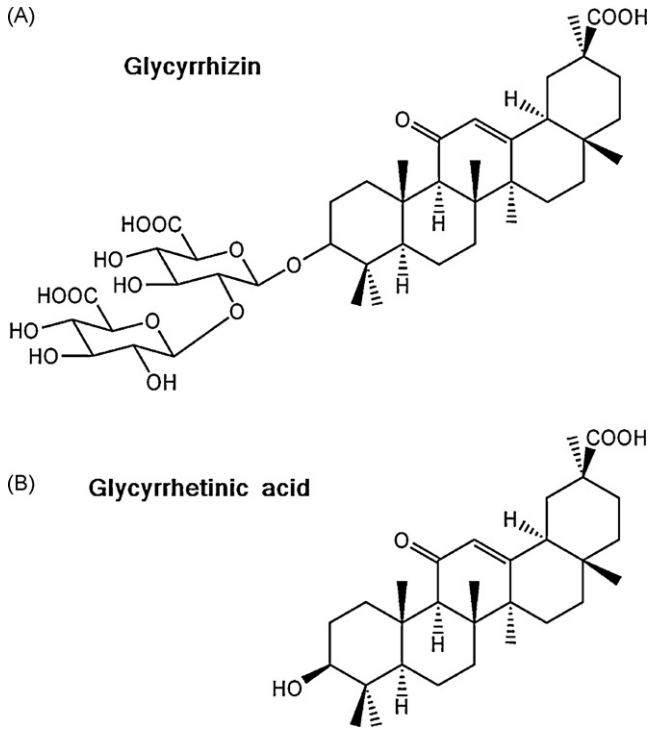

Fig. 1. The structures of GL (A) and GA (B).

importance that an antitumor agent should only affect tumor cells and have no adverse effects on normal cells.

Serum-free mouse embryo (SFME) cells, which were established by Loo et al. (1987), 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 central nervous system (CNS) progenitor cells (Loo et al., 1989; Rawson et al., 1990). 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 (Rawson et al., 1991). While SFME cells are non-tumorigenic in vivo and require epidermal growth factor (EGF) for their survival, growth and proliferation (Loo et al., 1989; Rawson et al., 1990), ras/myc SFME cells are tumorigenic and do not require any growth factors, such as EGF (Rawson et al., 1991). Another SFME-derived tumorigenic cell line is the human c-Ha-ras and mouse c-myc cotransfected highly metastatic SFME-1 (r/m HM-SFME-1) cell line, which was established by selecting ras/myc SFME cells that only metastasize to the lungs of Balb/c mice (Nomura et al., 1993). Analyzing the characteristics and behaviors of normal and tumorigenic SFME cells could be of great importance in the field of toxicological 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, normal SFME cells and tumorigenic r/m HM-SFME-1 cells were treated with GA to investigate its efficacy as a cytotoxic agent against tumor cells. Furthermore, a new antitumor agent should be more potent or at least better in a particular aspect than clinically available agents. Therefore, the potency of GA was compared with those of some clinically available antitumor agents. Finally, the possible mechanism underlying the selective cytotoxic effects of GA through H-Ras downregulation is discussed.

2. Materials and methods

2.1. Materials

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

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 (Nomura et al., 1993). The basal nutrient medium was a 1:1 mixture of DME and nutrient mixture F-12 Ham (DME/F-12) (Ham and McKeehan, 1979; Mather and Sato, 1979), supplemented with sodium bicarbonate, sodium selenite and gentamicin sulfate. Cells were maintained in DME/F-12 supplemented with insulin, transferrin and 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 (Carmichael et al., 1987).

2.4. RT-PCR

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) and chloroform. Aliquots (1 μg) of the extracted RNA were reverse-transcribed to cDNA using an RNA PCR kit (TaKaRa Biochemicals, Tokyo, Japan) according to the manufacturer's instructions. Aliquots of the cDNA samples were subjected to PCR using AmpliTaq Gold with Gene Amp 10× PCR Gold Buffer (Applied Biosystems Japan, Tokyo, Japan). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified with upstream (sense) and downstream (antisense) primers of 5′-GTGGCAAAGTGGAGATTGTTGC-3′ and 5′-TTTCTCGTGGTTCACACCCATC-3′, respectively (Grassi et al., 2004). The expected PCR product was 344 bp. The H-Ras gene was amplified with upstream (sense) and downstream (antisense) primers of 5′-ATGACGGAATATAAGCTGGT-3′ and 5′-CGCCAGGCTCACCTCTATA-3′, respectively (Matano et al., 1995). The expected PCR product was 123 bp. The PCR products were electrophoresed in a 1.5% agarose gel and transferred to a positively charged nylon membrane. The transferred PCR products were hybridized with probes that had been labeled with a DIG Oligonucleotide Tailing Kit (Roche Diagnostics K.K., Tokyo, Japan). The membrane was washed, exposed and analyzed with a lumino-image analyzer (LAS-3000; Fuji Film, Tokyo, Japan).

2.5. 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 subcellular extraction of proteins, a Calbiochem® ProteoExtract® Subcellular Proteome Extraction Kit (MERCK Japan, Tokyo, Japan) was used according to the manufacturer's instructions. 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-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse monoclonal anti-H-Ras (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody used was AP-conjugated anti-mouse IgG_1 (Chemicon International, Temecula, CA). Visualization of the antigen–antibody complexes was performed with 33 μl of 5-bromo-4-chloro-3-indolyl phosphate, 66 μl of nitroblue tetrazolium and 40 μl of 1 M $MgCl_2$ in 10 ml of 0.1 M Tris–HCl buffer (pH 9.5). Images of the positive bands were obtained by scanning and the densities were determined using an LAS-3000 image analyzer (Fuji Film, Tokyo, Japan).

2.6. 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 H-Ras labeling, cells were blocked in Tris-buffered saline containing 0.05% Tween-20 and 3% non-fat dried milk. Next, the cells were incubated with a mouse monoclonal anti-H-Ras antibody (Santa Cruz Biotechnology, Santa Cruz, CA), washed with PBS and incubated with HiLyte Fluor™ 488-conjugated goat anti-mouse IgG (AnaSpec, San Diego, CA). 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× objective lenses, analyzed and processed with the software Image Browser (Carl Zeiss Japan, Tokyo, Japan).

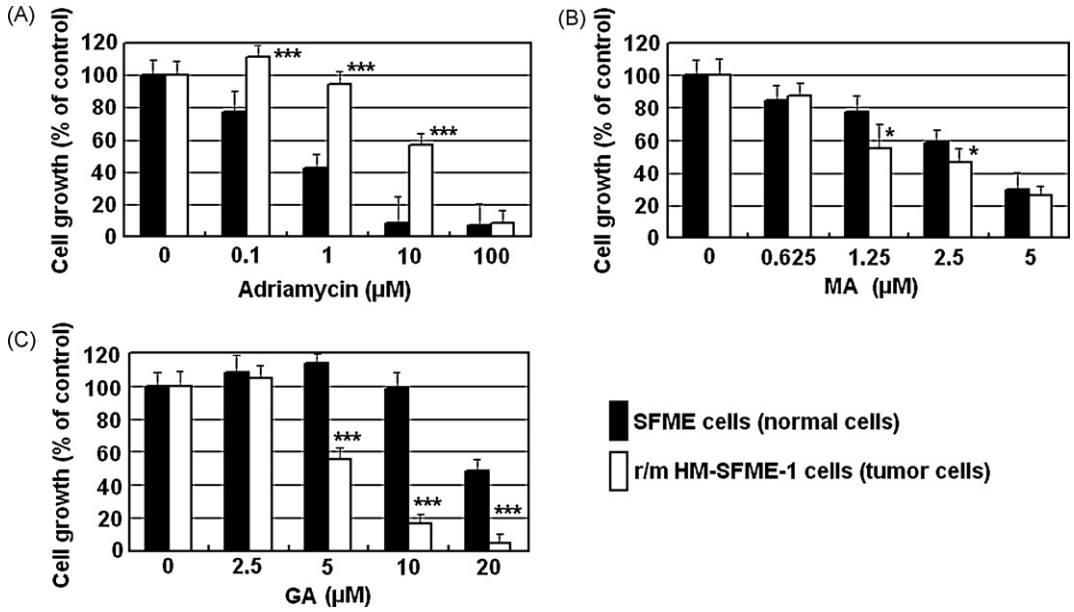

Fig. 2. Antiproliferative effects of adriamycin, MA and GA on normal SFME and tumorigenic r/m HM-SFME-1 cells. (A–C) Normal SFME and tumorigenic r/m HM-SFME-1 cells were treated with various concentrations of adriamycin (A), MA (B) or GA (C) for 24 h. Adriamycin inhibits the cell growth of the normal cells more strongly than that of the tumor cells. However, MA is capable of selectively affecting the tumor cells, and GA is more potent than MA in its selectivity. Each point is the mean ± SD of at least six experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ by Dunnett's test.

2.7. Statistical analysis

Experiments were performed in triplicate and repeated at least six 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 or a two-tailed multiple *t*-test with the Bonferroni correction was used to evaluate the statistical significance of differences between multiple groups. IC_{50} values were obtained using Prism 4.0 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Antiproliferative effects of adriamycin, MA and GA on normal SFME and tumorigenic r/m HM-SFME-1 cells

The antiproliferative effects of 24-h treatments with adriamycin, MA (both clinically available antitumor agents) and GA were determined by a colorimetric assay (MTT assay). As shown in Fig. 2A, adriamycin inhibited the growth of the normal cells more strongly than that of the tumor cells. However, MA was capable of selectively inhibiting the growth of the tumor cells (Fig. 2B), and GA was more potent than MA in its selectivity (Fig. 2C). GA in the range from 2.5 to 10 μM was not toxic to the normal cells at all. In contrast, more than 80% of the proliferative capability of the tumor cells was inhibited by GA at 10 μM.

3.2. GA is more potent than some clinically available antitumor agents in selective toxicity against tumor cells

The mean 24-h IC_{50} values of the cells treated with adriamycin, cisplatin, cytarabine, etoposide, fluorouracil, MA (clinically available antitumor agents) and GA were determined (Table 1). Among the antitumor agents tested, MA was the only agent that inhibited 50% of the tumor cells at a lower concentration than that for the normal cells. GA also showed lower IC_{50} values for the tumor cells than for the normal cells. The difference in IC_{50} values for GA between the normal and the tumor cells was greater than that for MA.

3.3. Selective tumor cell toxicity by GA can be detected as early as 1 h

For any antitumor chemotherapy, the time for treatment has to be as short as possible for patients' quality of life. Short-term dose-intensive chemotherapy exhibited excellent efficacy for disease-free survival rates and overall survival rates for lymphoma (Lee et al., 2001; Di Nicola et al., 2004), which indicates the importance of high-dose and less time-consuming treatments for anticancer chemotherapy. As shown in Fig. 2 and Table 1, MA and GA exhibited selective toxicity against the tumor cells over 24 h of treatment. Those results prompted us to investigate whether the selective toxicity against the tumor cells could be induced immediately after the antitumor agent treatment, possibly within 1 h. To this end, we treated the normal and tumor cells with MA or GA at 90% growth-inhibitory concentrations (7.2 and 5.5 μM for the normal and tumor cells with MA, respectively; 27 and 18 μM for the normal and tumor cells with GA, respectively), and observed the cells microscopically to determine whether the selective toxicity against the tumor cells could be detected as early as 1 h after the

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

Compounds	SFME cells (μM)	r/m HM-SFME-1 cells (μM)
Adriamycin	0.8 ± 0.2	64.0 ± 31.9***
Cisplatin	14.5 ± 1.5	27.1 ± 5.8***
Cytarabine	0.8 ± 0.1	33.3 ± 8.5***
Etoposide	0.5 ± 0.1	9.0 ± 2.5***
Fluorouracil	3.2 ± 1.2	48.5 ± 36.2***
Manumycin A (MA)	4.5 ± 2.9	2.4 ± 1.6**
GA	18.0 ± 4.0	7.3 ± 3.7***

Among the clinically available antitumor agents tested, MA is the only agent that inhibits 50% of the tumor cells at a lower concentration than that for the normal cells. GA shows lower IC_{50} values for the tumor cells than for the normal cells. Each point is the mean ± SD of at least six experiments.

** $P < 0.01$ by Student's *t*-test compared with the tumorigenic r/m HM-SFME-1 cells and normal SFME cells.

*** $P < 0.001$ by Student's *t*-test compared with the tumorigenic r/m HM-SFME-1 cells and normal SFME cells.

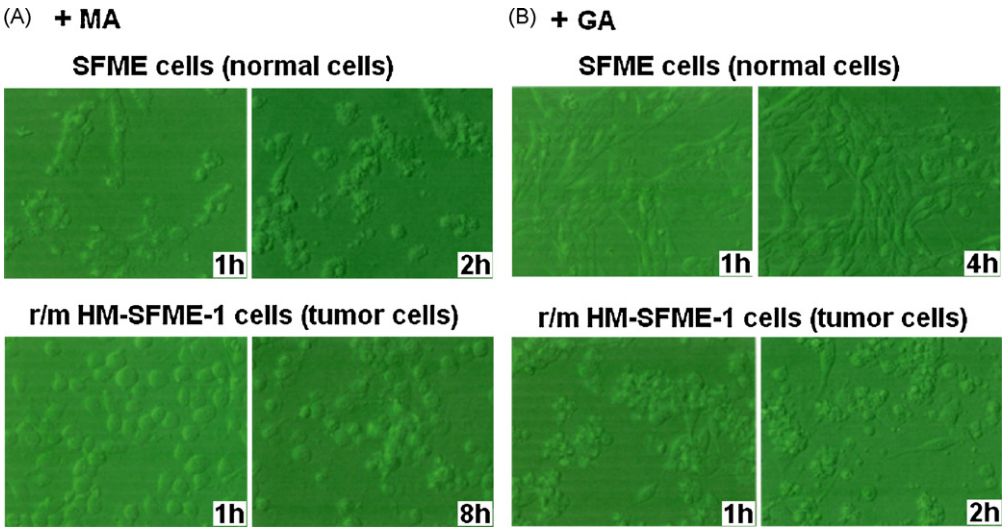

Fig. 3. Selective tumor cell toxicity by GA can be detected as early as 1 h. Normal SFME and tumorigenic r/m HM-SFME-1 cells were treated with MA or GA at IC_{90} values, and the cells were observed microscopically to determine whether the selective toxicity against the tumor cells could be detected as early as 1 h after the antitumor agent treatment. (A) Obvious toxicity by MA is observed at an earlier time point in the normal cells (2 h) than in the tumor cells (8 h). (B) In contrast, GA only affects the normal cells after 4 h, while toxicity against the tumor cells is even observed at 1 h.

antitumor agent treatment. As shown in Fig. 3A, obvious toxicity by MA was observed at an earlier time point in the normal cells (2 h) than in the tumor cells (8 h). In contrast, GA only affected the normal cells after 4 h, while cytotoxic effects on the tumor cells were even observed at 1 h (Fig. 3B). These results suggest that GA at the IC_{90} value is effective as early as 1 h after the treatment and that GA is more potent than MA in the spontaneous selective toxicity against the tumor cells.

3.4. GA downregulates H-Ras in tumor cells

r/m HM-SFME-1 cells were established by cotransfecting SFME cells with the human c-Ha-ras (to immortalize the cells) and mouse c-myc (to strengthen the ras effects) genes and selecting cells that only metastasized to the lungs of Balb/c mice (Nomura et al., 1993). The only difference between the normal and tumor cells in the present study was that the tumor cells were designed to strongly show the characteristics of the ras gene. MA, which showed the selective toxicity (Fig. 2 and Table 1), is a farnesyltransferase inhibitor that was originally identified as an effective tumoricidal agent against several cancers, especially those harboring constitutively active Ras (Kainuma et al., 1997; Frassanito et al., 2002). Therefore, we surmised that Ras regulation would be the key to elucidating the mechanism underlying the selectivity of GA toward the tumor cells (Figs. 2 and 3 and Table 1). Fig. 4 shows the expression levels of H-Ras mRNA and protein in the normal and tumor cells after 8 h of treatment with MA or GA at IC_{50} values. With MA treatment, H-Ras mRNA was slightly upregulated in the normal cells but downregulated in the tumor cells, while H-Ras protein was slightly downregulated in both cell types. GA had no effects on H-Ras mRNA in both the normal and tumor cells, but significantly downregulated H-Ras protein in the tumor cells. These results indicate that both MA and GA act toward downregulation of H-Ras protein in the tumor cells, and that the regulation of H-Ras protein is caused by a post-transcriptional event, rather than a transcriptional event, because there does not seem to be a correlation between the expression levels of H-Ras mRNA and protein.

As shown in Fig. 4, even the comparatively short-term 8-h exposure to MA or GA downregulated H-Ras protein. These findings prompted us to investigate the timing and part of the cell involved in the downregulation of H-Ras protein in the tumor cells. r/m HM-SFME-1 cells were treated with MA for 6 h or 8 h at IC_{50} values, and the expression levels of H-Ras protein in the cytosolic and membrane/organelle (M/O) fractions were analyzed. Exposure to MA decreased H-Ras protein in both fractions at 8 h, but not at 6 h (Fig. 5A), indicating that the downregulation of H-Ras protein is

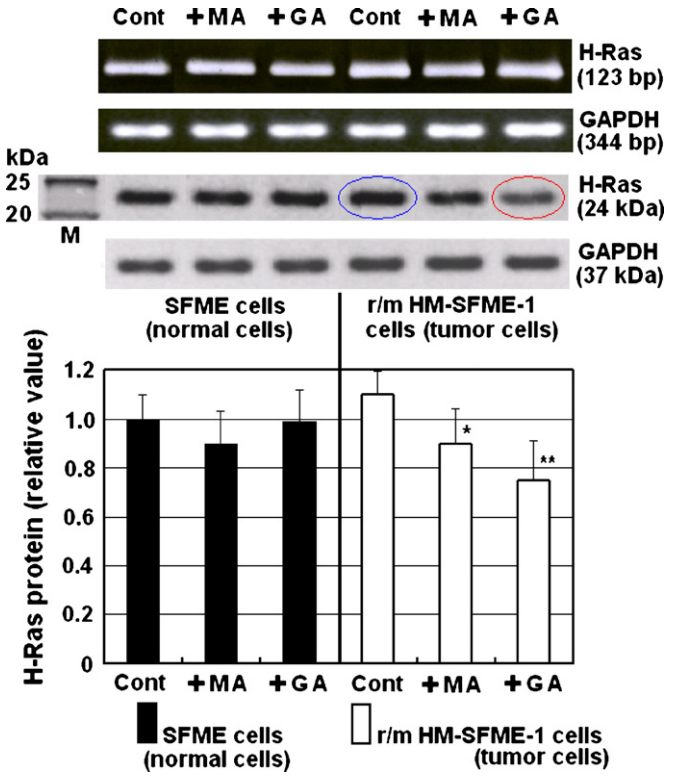

Fig. 4. GA downregulates H-Ras in tumor cells. Normal SFME and tumorigenic r/m HM-SFME-1 cells were treated with MA or GA at IC_{50} values for 8 h. MA and GA both downregulate H-Ras protein in the tumor cells. GAPDH was used as a loading control. Each point is the mean ± SD of at least six experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ by a two-tailed multiple *t*-test with the Bonferroni correction. M, size markers; Cont, control; blue circle, control tumor cells; red circle, GA-treated tumor cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

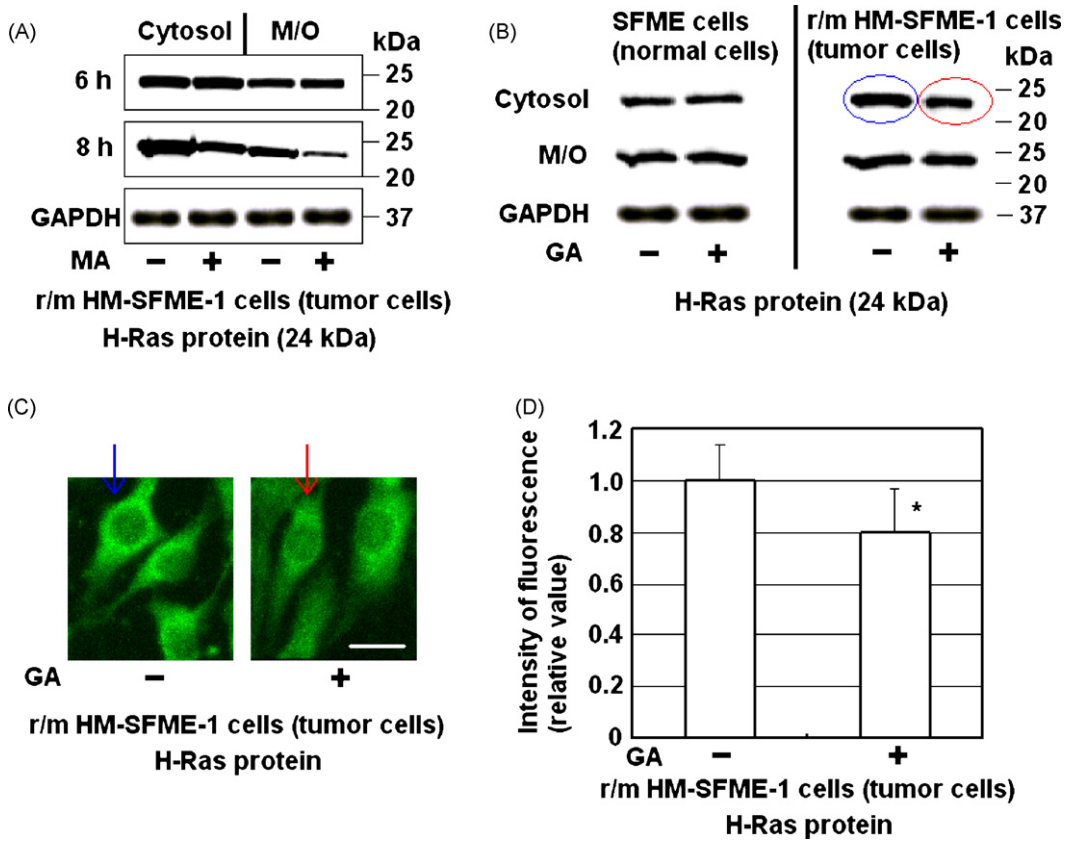

Fig. 5. GA downregulates H-Ras in the cytosol of tumor cells. Normal SFME and tumorigenic r/m HM-SFME-1 cells were treated with MA or GA at IC_{50} values for 6 h or 8 h. (A) Expression of H-Ras protein in the cytosolic and M/O fractions of MA-treated tumor cells. Exposure to MA for 8 h, but not 6 h, decreases H-Ras protein in both fractions. GAPDH was used as a loading control. (B) Expression of H-Ras protein in the cytosolic and M/O fractions of GA-treated cells. H-Ras protein is downregulated in both fractions, especially the cytosolic fraction, in the tumor cells. GAPDH was used as a loading control. Blue circle, control tumor cells. Red circle, GA-treated tumor cells. (C) Fluorescence staining of H-Ras protein and confocal laser scanning microscopic observation of the tumor cells. H-Ras protein is downregulated in the cytosol in the GA-treated tumor cells. Blue arrow, control tumor cells. Red arrow, GA-treated tumor cells. Bar, 20 μm. (D) Relative intensities of the immunofluorescence for H-Ras. H-Ras expression is significantly downregulated in the GA-treated tumor cells. Each point is the mean ± SD of at least six experiments. $^*P < 0.05$ by Student's *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

initiated between 6 and 8 h of treatment. We also treated the normal and tumor cells with GA for 8 h, and found that H-Ras protein was slightly upregulated in the cytosolic fraction in the normal cells (Fig. 5B). On the contrary, H-Ras protein was downregulated in both fractions, especially the cytosolic fraction, in the tumor cells. Downregulation of H-Ras protein in the cytosol of the GA-treated tumor cells was also detected by fluorescence staining and confocal laser scanning microscopy (Fig. 5C and D).

4. Discussion

Triterpenoids, which are biosynthesized in plants by cyclization of squalene, are widely distributed throughout the vegetable kingdom and are the major components of many medicinal plants used in Asian countries (Martin et al., 2007). There is growing interest in the elucidation of the biological and pharmacological roles of triterpenoids in analgesic, anti-inflammatory, antitumor, hepatoprotective and immunomodulatory effects (Martin et al., 2007), and we have been focusing our attention on certain triterpenoids as multifunctional agents for the prevention and treatment of cancer. Recently, we found that UA was selectively toxic to tumor cells (Yamaguchi et al., 2008). In the present study, we analyzed normal and tumor cells from the same lineage in the CNS and found that the selectivity of GA against the tumor cells was more potent than that of MA. 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 toxicity. Ras is known to confer growth and survival advantages on tumor cells through the Ras/MEK/ERK and Ras/PI3K/Akt signaling pathways (Sears et al., 2008). Our present study showed that, upon GA treatment, there was a difference in the H-Ras expression levels between the normal and tumor cells, suggesting that downregulation of H-Ras contributes to the selective toxicity against the tumor cells. A previous study revealed potent antitumor activities of GA derivatives through upregulation of Kruppel-like factor-4 (KLF-4) (Chintharlapalli et al., 2007). Although the antiproliferative effect of KLF-4 is neutralized in the presence of Ras/cyclin D1 signaling, KLF-4 simultaneously represses p53 levels and induces $p21^{CIP1}$, resulting in cell cycle arrest (Rowland et al., 2005). Our present study has revealed H-Ras downregulation by GA in the tumor cells, which could enhance the antiproliferative effect of KLF-4, thereby leading to cell cycle arrest. Ras pathways are also targeted for the development of chemotherapeutic interventions against tumor cells by interfering with cytoskeletal proteins such as actin (Bijman et al., 2008) and tubulin (Wasylyk et al., 2008). In the present study, the GA-treated tumor cells exhibited H-Ras downregulation, suggesting that GA affects Ras pathways and possibly the cytoskeleton. As mentioned above, it is likely that the GA-induced tumor cell death involves quite complicated mechanisms. Nevertheless, this characteristic of multiple mechanisms can be advantageous for cancer preven-

tion and therapy by allowing the possibility of targeting several pathways that inhibit tumor growth and proliferation. Although the underlying mechanisms implicated in the tumor cell-selective toxicity by GA have only been preliminarily investigated, it is possible that multiple factors, such as cell cycle arrest and cell growth inhibition through Ras downregulation, cytoskeletal disruption, induction of apoptosis by other unidentified factors, are synergistically involved.

In conclusion, selectivity is an important issue for cancer prevention and therapy. However, only a few studies, such as those on β-phenylethyl isothiocyanate (Trachootham et al., 2006) and lupeol (Saleem et al., 2008), 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 through H-Ras downregulation, and that its selectivity is 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 (de Groot et al., 1988), 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.

Conflict of interest

No conflict of interest is declared.

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