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Effects of Glycyrrhizin and Glycyrrhetinic Acid on Growth and Melanogenesis in Cultured B16 Melanoma Cells

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Abstract—*The effects of glycyrrhizin (GL) and its aglycone, glycyrrhetinic acid (GA), on the growth and differentiation of mouse melanoma (B16) cells in culture were studied. GA inhibits the growth of B16 melanoma cells, causes morphological alterations and stimulates melanogenesis. GL also resulted in the same changes but only when the concentration was about 20 times more than that needed for GA. When GA was removed after 84 h of treatment, the growth rate recovered slightly, but the doubling time was about twice that of the control. Cytofluorometric analysis showed that the growth inhibition of GA is the result of inhibition of the transfer from G_1 to S phase.*

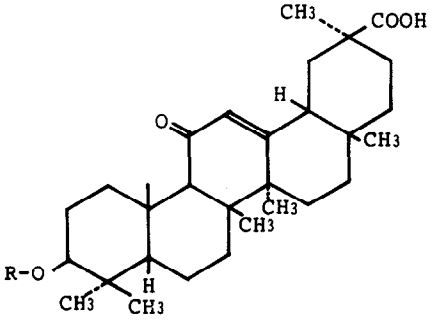
INTRODUCTION

SINCE ancient times, *Glycyrrhizae radix*, commonly called licorice, has been used against gastric ulcers and other diseases in Europe and Asia and its still occupies an important place in traditional Oriental medicine. Glycyrrhizin (GL) and glycyrrhetinic acid (GA) are the components extracted from the roots of *Glycyrrhizae glaba*. GL has been reported to have corticoid-like action [1] and has been used against allergic diseases [2] and hepatic disease [3]. Recently, plant glycosides with a dammarane skeleton resembling a steroid skeleton such as the aglycone have been known to induce phenotypic reverse transformation in cultured cancer cells [4, 5]. In the present study, we examined the effects of GL and GA, which both have oleanane skeletons, on cultured melanoma cells. We found that both of them cause growth inhibition and stimulate melanogenesis and that GA acts more extensively than GL.

MATERIALS AND METHODS

Drugs

Glycyrrhizin (GL) and glycyrrhetinic acid (GA) (Fig. 1) were donated by Minophagen Co., Tokyo.



R=H: Glycyrrhetinic acid
R=2-glucuronic acid: Glycyrrhizin

Fig. 1. Chemical structures of GL and GA.

Cell line and maintenance

The cell line in this study was derived from the B16 melanoma cell line by selection on the basis of its poor capacity for melanogenesis, using the Falcon Microtest plate (No. 3034) method as described by Poste *et al.* [6] with slight modifications. The cells were cultured on 100 mm Falcon plates in a Leibovitz's L-15: Ham's F-10 mixture (7 : 3) supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (50 units/ml). Cultures were incubated at 37°C in a humidified atmosphere at 5% CO_2 and 95% air. The medium was changed twice a week, and subcultures were performed regularly at weekly intervals with 0.01% trypsin solution in phosphate-buffered saline (8.0 g NaCl, 0.2 g

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KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1000 ml H_2O, pH 7.2).

Assay of growth inhibition

Cells (10^5) were plated in 60 mm Falcon dishes in media containing different GL or GA concentrations. The concentration of fetal calf serum in the experimental media was 2.0%, which gave one-half the maximum growth rate of the B16 melanoma cells and is adequate for studying enhancement or inhibition of cell growth. At the termination of the experiments on day 4, the cells were detached with 0.01% trypsin solution in phosphate-buffered saline and counted with an electronic particle counter.

Cytofluorometric analysis

Cells were cultured for 4 days in 100/mm dishes with 10 ml of the media containing 2% fetal calf serum and various quantities of GA. The cells were then removed from the dish by the trypsin treatment. Single-cell suspensions were fixed in 70% ethanol, treated with a 100 μg/ml solution of RNase I (Sigma Chemical Co., St. Louis, MO) in distilled water for 20 min at 37°C, and stained with propidium iodide (0.05 mg/ml) in 0.1% sodium citrate and 0.1% Nonidet P-40 according to the method of Rapaport [7] with slight modifications. Stained cells were subjected to cytofluorometric analysis utilizing an Ortho-Cytograf System 50H, with a 500 mW excitation at 488 nm.

Assay of melanin content

Cells were plated in 35-mm Falcon dishes; after 24 h, triplicate cultures were subjected to various concentrations of GL or GA. Control cultures were treated with 0.1% ethanol. The melanin content was then measured by a colorimetric method [8]. Relative melanin contents were expressed as the absorbance at 470 nm/1 × 10^6 cells.

Preparation of collagen gels

Type I collagen solution (0.3% in dilute hydrochloric acid, pH 3.0) were purchased from Nitta Geratin Co. (Japan). Eight parts of collagen solution was mixed with one part of 10× culture medium and one part of 0.26 M sodium bicarbonate in 0.1 M NaOH, and kept on ice. The solution (0.5 ml) was poured into 35-mm dishes and allowed to stand for 5–10 min at room temperature to solidify.

Morphology

Changes in the morphology and melanin content of B16 melanoma cells cultured in a medium with GL or GA were documented by microphotography.

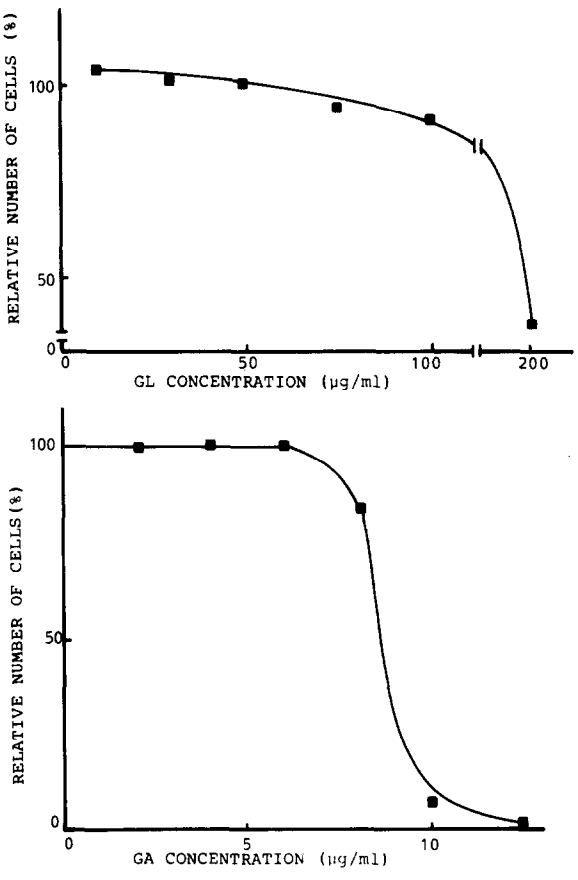

Fig. 2. Effects of GL and GA on growth of B16 melanoma cells. Twenty-four hours after plating, GL or GA at various concentrations was added individually to separate dishes. At the termination of the experiments on day 4, the cells were detached with 0.01% trypsin solution in phosphate-buffered saline and counted with an electronic counter. Each point represents an average of five experiments; bars, S.E.

RESULTS

Effect on cell growth

The inhibitory effects of GL or GA at concentrations in the range 5–200 μg/ml or the range 2–12.5 μg/ml, respectively, were determined after a 3-day incubation. GA inhibited the growth of B16 melanoma cells in a dose-dependent manner and caused complete inhibition at concentrations over 10 μg/ml, while GL did not inhibit cell growth even at concentrations over 100 μg/ml. GL (200 μg/ml) inhibited growth by about 40% (Fig. 2). When B16 melanoma cells were subcultured in the presence of 10 μg/ml GA, the rate of cellular proliferation was progressively reduced over the time of culture. The growth of B16 melanoma cells cultured in a medium containing 12.5 μg/ml of GA was completely inhibited even after 1 day in culture. GL did not show complete inhibition even at a concentration of 200 μg/ml (Fig. 3). When GA was removed after 4 days of treatment, growth did not begin for a period of 24 h. A slight increase in growth was observed at 2 days after removal of GA but the growth rate

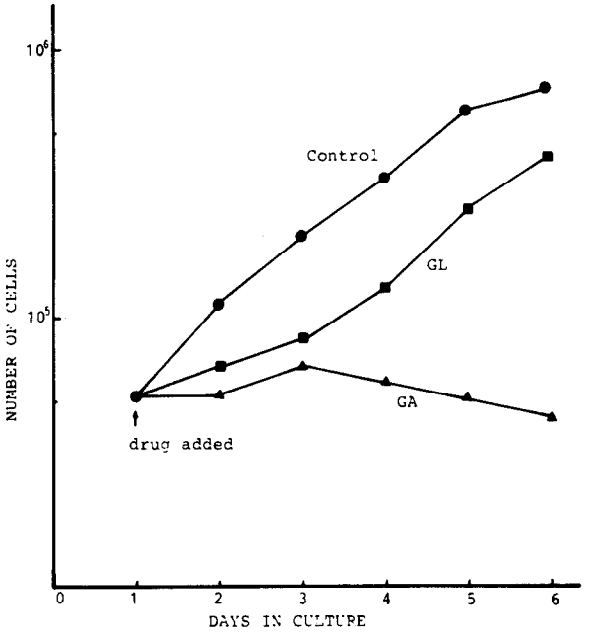

Fig. 3. Growth curves of melanoma cells treated with GL or GA. Cells 5×10^4 *were cultured with either no additions* (●), 200 μg/ml *GL* (■) *or* 12.5 μg/ml *GA* (▲). *At the indicated times cells were harvested from the culture flasks and counted with an electronic particle counter.*

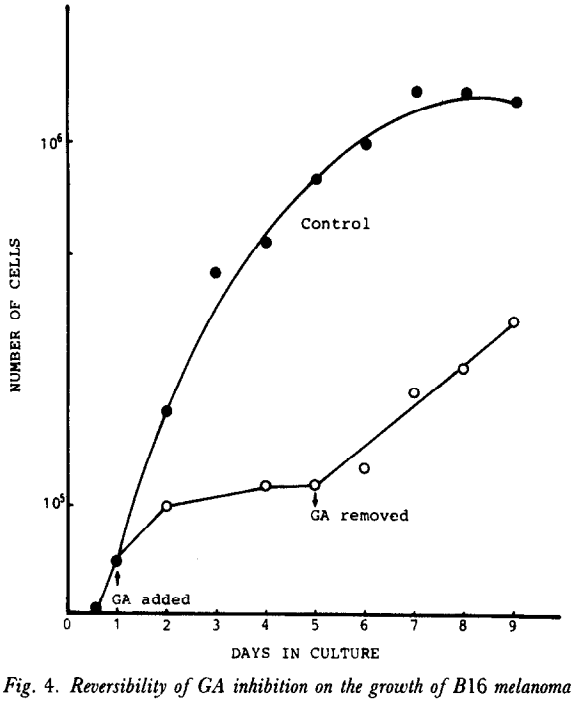

Fig. 4. Reversibility of GA inhibition on the growth of B16 melanoma cells in culture. Cells (5×10^4) *were plated in Falcon dishes* (35 mm), 12 h *after plating, GA* (12.5 μg/ml) *was added to culture. At the indicated day, GA was removed. The number of cells in the control and GA-treated cultures was determined.*

did not recover completely during the period of observation (4 days after removal of GA) (Fig. 4).

The cytofluorometric analysis of the culture after 4 days of exposure to GA (12.5 μg/ml) revealed a marked accumulation of cells in G_1 and a disappearance of cells in S and G_2 -M phase compared with the control culture in the absence of GA (Fig. 5)

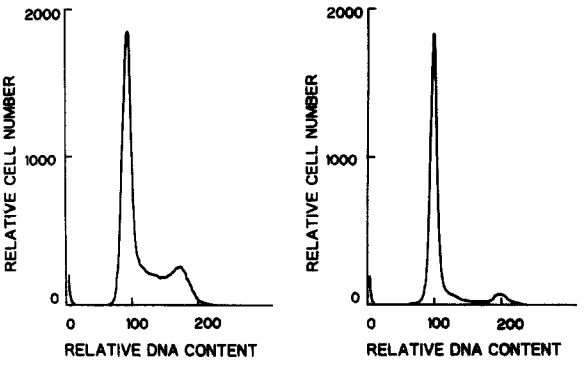

Fig. 5. DNA distribution of B16 melanoma cells treated with GA. Cells were cultured for 96 h *in* 100-mm *dishes in the absence (left) or presence (right) of* 12.5 μg/ml *GA. DNA-fluorescent staining was performed as described in Materials and Methods. The relative DNA content of the cells indicates cell populations in* G_1 *(left peak), S phase (trough) and* G_2 + *M (right peak). Similar results were obtained in three additional independent experiments.*

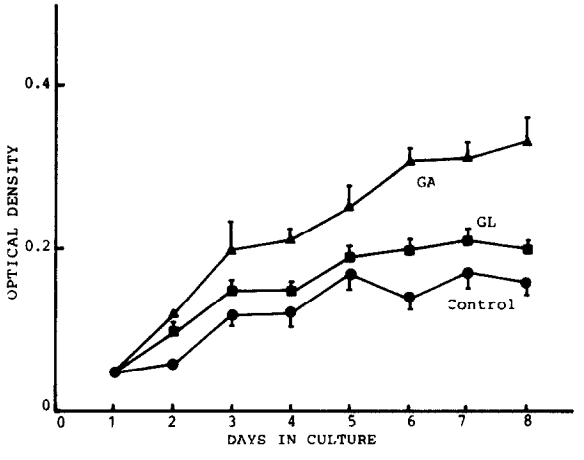

Fig. 6. Time course of GL or GA-induced melanogenesis stimulation. B16 melanoma cells (5×10^4) *were plated in* 35-mm *Falcon dishes. Cultures were subjected to* 200 μg/ml *GL or* 10 μg/ml *GA. Melanin contents were measured by a colorimetric method.*

Effect on melanin content

The melanin content in untreated cultures increased very slightly after culturing. However, GA-treated melanoma cells contained about twice the amount of melanin than those found in the untreated culture at 7 days after treatment. The stimulation of melanogenesis was found also in cells treated with 200 μg/ml of GL but the melanin content was much lower than that in GA-treated cells (Fig. 6).

Effect on morphology

Control melanoma cells in culture exhibited primarily a fibroblastic appearance and tended to pile up and form multilayers with a few round cells

(Fig. 7a). Cells treated with GA appeared larger than untreated cells and have abundant fine granular cytoplasm. The most remarkable change in morphology was the appearance of melanin granules in GA-treated cells (Fig. 7b).

Cells cultured on collagen gels with or without GA (12.5 μg/ml) showed the difference of adhesion property. Control cells showed a round shape and spread poorly on the collagen gels (Fig. 8a), while GA-treated cells were more flattened and well spread (Fig. 8b).

DISCUSSION

Recently, some chemical compounds having a steroid [9–11] or dammarane skeleton [4, 5] as the aglycone have been found to affect the growth capacity and differentiation of cancer cells. In the present study, GL and GA, which both have an oleanane skeleton, were found to reduce the proliferation rate of melanoma cells and to induce a concomitant increase in melanin production.

When GA was removed after 4 days of treatment, a slight increase in growth was observed but the doubling time was still almost twice that of control cells. This result indicates that the growth inhibition of GA is persistent. In addition, we confirmed that growth inhibition of GA is the result of cytostatic but not cytotoxic effects of GA by examinations of the cell viability and plating efficacy of B16 melanoma cells treated with GA. The cytofluorometric analysis suggested that GA causes the accumulation in early G_1 or G_0 phase (data not shown).

The efficacy of growth inhibition and melanogenesis were stronger with GA than with GL. Accordingly, it appears that the effect of these compounds on melanoma cells is to be ascribed mainly to the aglyconic component. The differences in the efficacy of GL and GA have also been reported with respect to other pharmacological and cytological actions [12–14]. Especially, it is noteworthy that the effect on cell membranes is significantly stronger in GA than in GL [15]. The precise mechanism of the induction of phenotypic reversion such as growth inhibition and melanogenesis by plant glycosides is not known. However, the effects of glucocorticoids, retinoic acid and plant glycosides, which control the phenotypic expression of cancer cells, on the cell membrane have been thought to play a very important role in reverse transformation of cancer cells [5, 16, 17]. Accordingly, we suspect that the difference in the effects of GL and GA on the cell membrane must cause the different efficacy in cell growth inhibition and other phenotypic changes. The details of effects of GL and GA on cell membrane are now under investigation.

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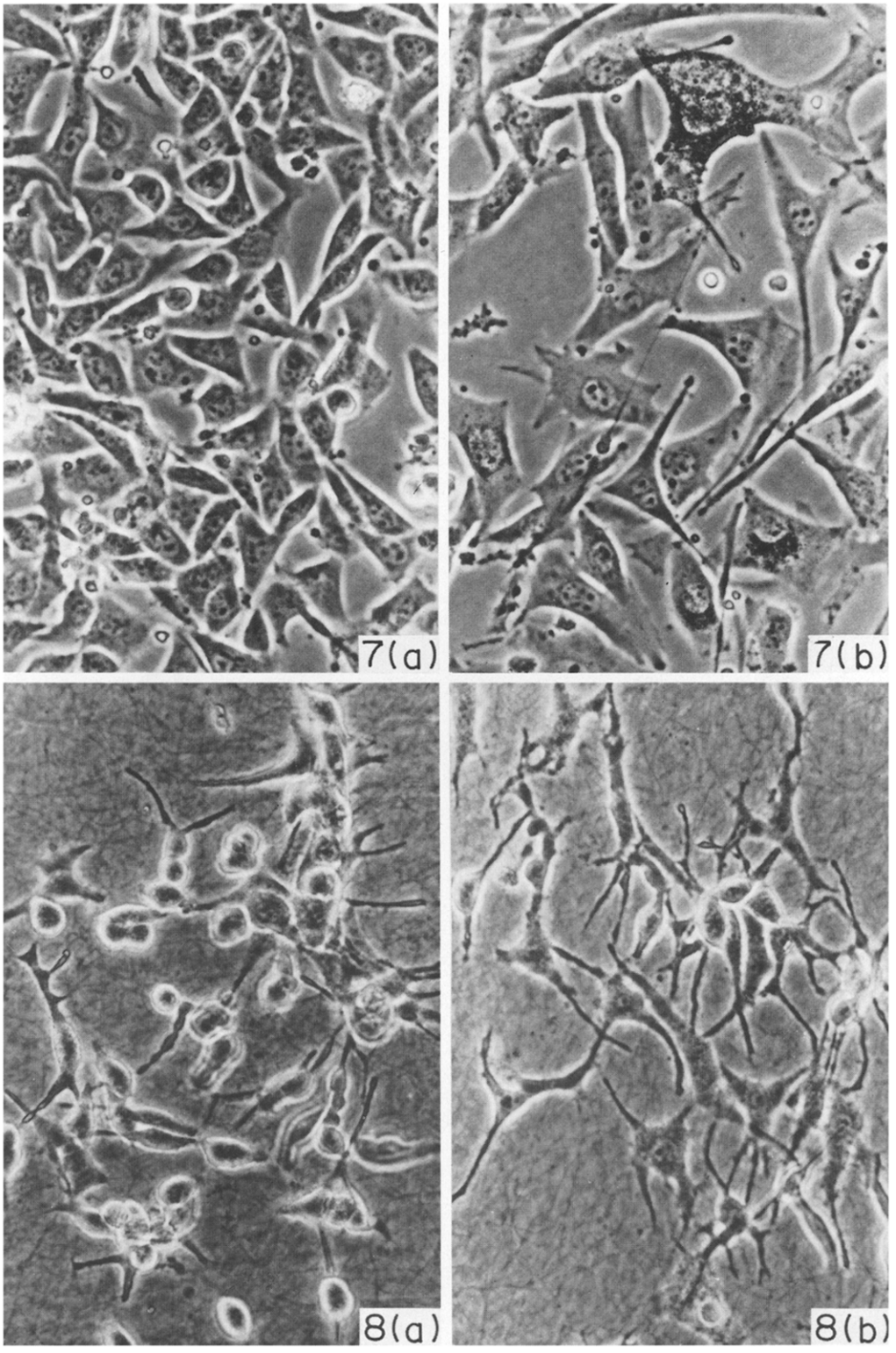

Fig. 7. Phase-contrast micrographs of B16 melanoma cells cultured in the absence (a) or presence (b) on GA. Cells were grown for 3 days in control medium (a) or in medium containing 12.5 μg/ml GA (b). Original magnification, × 180.

Fig. 8. Phase-contrast micrographs of B16 melanoma cells cultured in collagen gels without (a) or with GA (b). Cells were cultured for 5 days in collagen gels without (a) or with GA (12.5 μg/ml) (b). Original magnification, × 180.

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