Control of Phenotypic Expression of Cultured B16 Melanoma Cells by Plant Glycosides

Shizuo Odashima, Takahide Ohta, Hiroyuki Kohno, Takeshi Matsuda, Isao Kitagawa, Hiroko Abe, and Shigeru Arichi

Department of Pathology, Kanazawa Medical University, Uchinada-machi, Ishikawa-ken 920-02; [S. O., T. O., H. K., T. M.]; Faculty of Pharmaceutical Sciences, Osaka University, 1-6, Yamada-oka, Suita, Osaka [I. K.]; and The Research Institute of Oriental Medicine, Kinki University, Nishiyama, Sayama-cho, Osaka [H. A., S. A.], Japan

ABSTRACT

The effects of two plant glycosides, ginsenosides Rh₁ and Rh₂, on the growth and differentiation of mouse melanoma (B16) cells in culture were studied. These plant glycosides have a dammarane skeleton resembling a steroid skeleton as an aglycone. Ginsenoside Rh₂ inhibits the growth of B16 melanoma cells, causes morphological alterations, and stimulates melanogenesis at high cellular density. When ginsenoside Rh₂ was removed after 2 or 6 days of treatment, the growth rate recovered slightly but not completely, during the period of observation (4 days after removal). On the other hand, ginsenoside Rh₁ does not inhibit the growth of melanoma cells even at concentrations over 100 µM but stimulates the expression of the melanotic phenotype. Ginsenosides Rh₁ and Rh₂, possessing a glucose molecule at C-6 and C-3, respectively, have very similar chemical structures, but their effects on B16 melanoma cells differ remarkably. While it appears that the degree of differentiation is inversely related to cell growth, the present observations suggest that the differentiation and growth capacity of this B16 melanoma subline are independent phenotypic expressions.

INTRODUCTION

Recently, a number of chemical compounds have been found that inhibit growth and induce phenotypic reversion in certain cancer cells (10, 16), and studies also suggest that several of these compounds have potential value as chemopreventive or therapeutic agents (6, 19). We previously reported that crude ginsenosides extracted from the root of Panax ginseng C. A. Meyer induced phenotypic reverse transformation in cultured Morris hepatoma cells (1, 13). P. ginseng C. A. Meyer, with more than a 4000-year history, occupies a particularly important place among the tonic remedies of Oriental medicine. Pharmacological investigation has shown that crude ginsenosides can increase the nonspecific resistance of an organism to various untoward influences and provide a normalization action, irrespective of the direction of previous pathological shifts (7).

We have isolated 2 new compounds, designated ginsenoside Rh₁ and Rh₂, from the methanol extract of the processed root of this plant. Experiments reported herein demonstrate that Rh₂ causes growth inhibition and stimulates melanogenesis, while Rh₁ stimulates only melanogenesis in cultured B16 melanoma cells.

MATERIALS AND METHODS

Cell Line and Maintenance. The cell line used 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. (15) 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 of 5% CO₂ 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 KCl, 1.15 g Na₂HPO₄, and 0.2 g ·KH₂PO₄ in 1000 ml H₂O, pH 7.2). The doubling time of these cells in culture under the above condition was 20 h, and cell transplatability (2 x 10⁶ cells in the inoculation) to C57BL/6 mice was 80%, as for the parent cell line.

Extraction and Purification of Ginsenosides. The 2 ginsenosides were isolated from the processed root of P. ginseng C. A. Meyer according to the conventional method for isolating plant saponin, with modifications (13). Their chemical structures (Chart 1) were established on the basis of chemical and physicochemical findings (9). Ginsenoside Rh₁ and Rh₂ were dissolved in ethanol and stored at 4°C.

Assay of Growth Inhibition. Cells (10⁵) were plated in 60-mm Falcon dishes in medium containing different Rh₁ or Rh₂ 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. Cell viability was routinely estimated by trypsin blue staining.

Cytotfluorometric Analysis. Cells were cultured for 96 h in 100-mm dishes with 10 ml of the media containing 2% fetal calf serum and various quantities of ginsenoside Rh₂. The cells were then removed from the dish by the trypsin treatment. Single-cell suspensions were fixed in 70% ethanol, treated with a 100-µl 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 (17) with slight modifications. Stained cells were subjected to cytotfluorometric analysis utilizing an Ortho-Cytograf System 50H, with a 500-milliwatt 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 Rh₁ or Rh₂ (µM). Control cultures were treated with 0.1% ethanol. The melanin content in cultures exposed to ginsenosides and ethanol was measured by colorimetric methods (16). Relative melanin contents were expressed as the absorbance at 470 nm/1 x 10⁶ cells. The values represent the ratio of melanin content in treated and control cultures.

Morphology. Changes in the morphology and melanin content of B16 melanoma cells cultured in a medium with Rh₁ or Rh₂ were documented by microphotography.

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2 To whom requests for reprints should be addressed.

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RESULTS AND DISCUSSION

Rh₁ and Rh₂ are plant glycosides with a dammarane skeleton resembling a steroid skeleton as an aglycone, with differences in their chemical structures at the binding site of the glucose molecule as shown in Chart 1. Rh₁ has one molecule of sugar moiety at C-6, while Rh₂ has it at C-3, but despite their chemical similarities their effects on cancer cells are remarkably different.

Chart 2 shows the effect of Rh₁ and Rh₂ on the growth of B16 melanoma cells in vitro. Rh₁ does not inhibit the growth of B16 melanoma cells even at concentrations over 100 μM, while Rh₂ inhibits the cell growth in a dose-dependent manner and causes complete inhibition at concentrations over 15 μM. Interpolation indicates that 10 μM is required for 50% inhibition. The concentration of Rh₂ needed to inhibit growth by 50% in the medium containing 2% fetal calf serum was about 2.5-fold lower than that obtained in medium-supplemented 10% fetal calf serum. This result suggests that serum factors may in some way make ginsenoside Rh₂ less available to melanoma cells. A similar phenomenon has been reported in the study on growth inhibition of melanoma cells caused with vitamin E acid Buccinate (16). As shown in Chart 3, the growth inhibition by Rh₂ was observed 24 h after treatment; when Rh₂ was removed after 2 or 6 days of treatment, the growth rate recovered slightly, but not completely, during the period of observation (4 days after removal of Rh₂). This result indicates that growth inhibition of Rh₂ is persistent and is the result of cytostatic but not cytotoxic effects of Rh₂. This indication was also confirmed by examinations of the cell viability and plating efficiency of B16 melanoma cells treated with Rh₂ (data not shown).

Control cultures in the absence of Rh₂ tended to pile up and form multilayers with a few round cells (Fig. 1). Cells treated with Rh₂ appeared larger and elongated and were arranged in parallel at several locations, but melanin granules were not found in cytoplasms. These cells failed to form multilayers at high cellular density, and these morphological changes did not reverse during the period of observation (4 days after removal of Rh₂).

The cytofluorometric analysis of the culture after 96 h of exposure to Rh₂ revealed that Rh₂ treatment of B16 melanoma cells leads to a marked accumulation of cells in G₁, suggesting that the growth inhibition is the result of a block during G₁ and that such cells do not enter S phase (Chart 4). We found previously that Rh₂ also markedly inhibits insulin-induced proliferation of Morris hepatoma cells (14). B16 melanoma cells used in the present study are known to be insensitive to insulin. Accordingly, Rh₂ could be judged effective on both insulin-dependent and insulin-independent growth.

Table 1 shows the stimulation of melanogenesis by Rh₁ or Rh₂ at high cellular density. The remarkable stimulation of melanogenesis was observed in cultures treated with 10 μM Rh₂. Rh₁ did not stimulate melanogenesis at low concentrations of 5 or 10 μM but stimulated dose dependently at higher concentrations and long incubation as shown in Table 2. These results indicate that plant glycosides Rh₁ and Rh₂ induce the differentiation of
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B16 melanoma cells, since melanin synthesis is considered a specialized function of melanoma cells, including the differentiation in normal melanocytes and in melanoma cells (21).

There are reports that increased melanin synthesis seems to be coordinated with a reduction in cell proliferation rate (10, 20). However, we also found that Rh₂ enhances melanogenesis in B16 melanoma cells but neither stimulates nor inhibits their growth, while Rh₁ greatly inhibits cell growth and enhances melanogenesis. Thus, the stimulatory effect on melanin synthesis in B16 melanoma cells cannot be explained on the basis of growth inhibition alone. Therefore, we believe that the growth capacity and melanogenesis of B16 melanoma cells may repre-

sent completely independent phenotypic expressions, although cancer cell growth is generally thought to have an inverse relation to such differentiation.

It is interesting that the slight difference in molecular structure between Rh₁ and Rh₂ seems to cause a remarkable difference in their biological action against cancer cells. The mechanism by which Rh₁ and Rh₂ exert their regulatory effects on the phenotypic expression of B16 melanoma cells is unknown at present. It has been reported that retinoic acid reduces the rate of cell proliferation in S91 mouse melanoma clone C2 cells and stimulates the expression of their melanotic phenotype (11). Retinoic acid also induces specific changes in cell surface glycoconjugates of S91 mouse melanoma cells, and the changes in the membrane surface may be causally related to growth inhibition (12). Glucocorticoids also induce changes on cell surfaces and affect proliferation (4). It is accepted that plant glucosides have a high affinity for cell membranes, which appear to be a target organelle, and can modify the membrane fluidity and surface charge of various cells (2, 3). The binding sites of Rh₁ and Rh₂ to cell membranes and the subsequent modification of membrane properties are now under investigation.

As mentioned above, steroid hormones and ginsenosides are very similar in their gross molecular shapes, and steroids with glucocorticoid activity are reported to induce differentiation of myeloid leukemia cells into macrophages and granulocytes (18). It has also been shown that exposure to glucocorticoids results in a significant inhibition of cell growth in many cell types, including B16 melanoma cells and human malignant melanoma cells (5, 8, 22). The actual cellular mechanism by which glucocorticoids modulate these phenotypic expressions has not yet been unraveled. Nevertheless, it seems that some chemical compounds having a steroid or dammarane skeleton as aglycone affect the growth capacity and differentiation of cancer cells. The study of the effects of these compounds on cancer cells will be valuable in elucidating the relationship between growth capacity and differentiation and in advancing effective therapies for cancer.

REFERENCES


The table below shows the effects of ginsenosides Rh₁ and Rh₂ on melanogenesis in B16 melanoma cells at high cellular density.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells/dish (10⁶) on Day 4</th>
<th>Relative melanin content (A₄₇₀ (nm)/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.36 ± 0.069</td>
<td>0.079 ± 0.022</td>
</tr>
<tr>
<td>Rh₁ (10 μM)</td>
<td>2.35 ± 0.04</td>
<td>0.062 ± 0.003</td>
</tr>
<tr>
<td>Rh₂ (5 μM)</td>
<td>2.38 ± 0.01</td>
<td>0.074 ± 0.022</td>
</tr>
<tr>
<td>Rh₂ (5 μM)</td>
<td>2.27 ± 0.03</td>
<td>0.067 ± 0.015</td>
</tr>
<tr>
<td>Rh₂ (10 μM)</td>
<td>2.55 ± 0.05</td>
<td>0.346 ± 0.052</td>
</tr>
</tbody>
</table>

* Average ± SE of triplicate cultures.

The table below shows the extent of melanogenesis stimulation in B16 melanoma cells exposed to ginsenoside Rh₁.

<table>
<thead>
<tr>
<th>Rh₁ added (μM)</th>
<th>No. of cells/dish (10⁶) on Day 7</th>
<th>Relative melanin content (A₄₇₀ (nm)/10⁶ cells)</th>
<th>Melanogen-esis stimulation (T/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1.43 ± 0.04</td>
<td>0.031 ± 0.004</td>
<td>1.0</td>
</tr>
<tr>
<td>20</td>
<td>1.59 ± 0.08</td>
<td>0.052 ± 0.006</td>
<td>1.0</td>
</tr>
<tr>
<td>40</td>
<td>1.39 ± 0.05</td>
<td>0.193 ± 0.009</td>
<td>1.0</td>
</tr>
<tr>
<td>60</td>
<td>1.57 ± 0.01</td>
<td>0.167 ± 0.024</td>
<td>1.0</td>
</tr>
<tr>
<td>80</td>
<td>1.70 ± 0.02</td>
<td>0.178 ± 0.016</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Ratios of melanin content in treated (T) and control (C) cultures.
* Average ± SE of triplicate cultures.
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Fig. 1. Phase-contrast micrographs of B16 melanoma cells cultured for 4 days in the absence (a) or presence (b) of Rh2. Cells were grown for 4 days in control medium (a) or in medium containing 12.5 μM ginsenoside Rh2 (b). Original magnification, × 200.
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