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

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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 modifications. Stained cells were subjected to Cytofluorometric analysis utilizing an Ortho-Cytograf System 50H, with a 500-milliwatt excitation cone. Ginsenoside Rh2 inhibits the growth of B16 melanoma cells in culture under the above condition was 20 h, and cell transplantability (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 growth inhibition was assessed by trypsinization when the cells reached 90% confluence. A single-cell suspension was fixed with 70% ethanol for 24 h, washed with PBS, and stained with 0.1% crystal violet in 40% ethanol. The cells were counted after 24 h with a hemocytometer. Relative cellular death rates were expressed as the ratio of control cultures.

Assay of Melanin Content. Cells in 35-mm Falcon dishes were treated with 0.1% ethanol for 24 h, trypsinized, adjusted to 107 cells/ml, and treated with 1 mM 3-methyladenine for 24 h. Melanin content was extracted according to the method of Rapoport (17) with slight modifications. The cells were then fixed with 15% formaldehyde and mounted on glass slides. Melanin content was determined spectrophotometrically at 470 nm after extraction with 5 N NaOH. Relative melonin contents were expressed as the absorbance at 470 nm of treated cultures against that of control cultures.

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 transplantability (2 x 106 cells in the inoculation) to C57BL/6 mice was 80%, as for the parent cell line.

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RESULTS AND DISCUSSION

Rhi 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. Rhi 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...
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 illuminating the relationship between growth capacity and differentiation and in advancing effective therapies for cancer.

REFERENCES

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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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