Plant-Glycoside Modulation of Cell Surface Related to Control of Differentiation in Cultured B16 Melanoma Cells

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ABSTRACT

We have shown that the ginsenosides Rh1 and Rh2, which are plant glycosides with a dammarane skeleton resembling a steroid skeleton as an aglycone, control the phenotypic expression of mouse B16 melanoma cells in different ways. The effects of Rh1 and Rh2 on the cell surface were studied to clarify the relationship between the control of phenotypic expression and modification of the cell surface in B16 melanoma cells. Rh1, which has the capacity to inhibit the growth of and to stimulate melanogenesis in B16 melanoma cells, causes flattening of the cells cultured in a collagen gel, leading to organized, nonoverlapping monolayers. Cell-to-cell adhesiveness and cell-to-substrate adhesiveness were markedly increased in the B16 melanoma cells treated with Rh2. In Rh2-treated cells, the binding of peanut agglutinin on the cell surface was also increased, whereas no marked changes were observed in the binding of concanavalin A or wheat germ agglutinin. In contrast, Rh1, which showed no effect on cell growth, did stimulate melanogenesis, did not cause morphological changes of the cells and exerted no effect on cell adhesiveness or cell surface lectin binding. 1,6-Diphenyl-1,3,5-hexatriene polarization values markedly decreased in cells treated with either Rh1 or Rh2. Rh2 was found to be incorporated in the lipid fraction of the B16 melanoma cell membrane. In contrast, Rh1 was not detected in the lipid fraction of B16 melanoma cells. However, novel lipid components were found.

INTRODUCTION

We previously reported that a crude fraction of ginsenosides extracted from the root of Panax ginseng (C. A. Meyer) induced phenotypic reverse transformation in cultured Morris hepatoma cells (1), and that the ginsenosides Rh1 and Rh2 purified from these crude materials affected the growth of B16 melanoma cells and the expression of their melanotic phenotype (2). Both of these ginsenosides have a dammarane skeleton resembling a steroid skeleton as an aglycone, with differences in their chemical structures at the binding site of the glucose molecule. Rh2 has one sugar moiety at C-6, while in Rh1 the sugar is found at C-3. Despite their chemical similarities their effects on cancer cells are remarkably different. Rh2 has the capacity to inhibit the growth of and to stimulate melanogenesis in B16 melanoma cells, whereas Rh1 stimulates melanogenesis. It is thought that elucidation of the differences in the mechanism of action of these ginsenosides will provide an important clue to the clarification of the regulation mechanisms of cell growth and differentiation.

Cell surface functions are of major importance in determining many features of tumor cell behavior, and it has been shown that agents inducing redifferentiation of cancer cells, such as glucocorticoids, have a capacity to modify the cell surface of the cancer cells. On the other hand, plant glycosides generally contain both hydrophilic and hydrophobic groups in their mol-

cules, and they are considered to have strong affinities for the cell membrane, which appears to be the plant glycosides' target organelle (3, 4). In this current report, the effects of Rh1 and Rh2 on the cell surface and cell membrane of cultured B16 melanoma cells were studied to clarify the relationship between phenotypic expression and surface modification of the cells.

MATERIALS AND METHODS

Cell Line and Culture. Mouse B16 melanoma cells were cultured in a mixture of Ham's F-10 and L-15 (ratio, 3:7), containing 10% fetal calf serum (Flow Laboratory), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Melanoma cells were subcultured every 5 days with one medium change. In the experiments, cells were cultured in the same medium supplemented with 2% fetal calf serum, which enabled B16 cells to grow at half their maximum rate (2).

Extraction and Purification of Ginsenosides. The ginsenosides were isolated from the processed root of Panax ginseng (C. A. Meyer) according to a modified form of the conventional method for isolating plant saponins (1). Their chemical structures were established on the basis of chemical and physicochemical findings (5).

Treatment with Ginsenosides. Ginsenosides Rh1 and Rh2 were dissolved in ethanol and stored at −20°C. Cells were cultured for 24 h before ginsenosides were added to the growth medium. The effects of ginsenosides on B16 melanoma cells were examined using growth medium containing either 40 μM Rh1 or 12.5 μM Rh2. In previous experiments, it was observed that exposure for 4 days to Rh1 at the 40 μM concentration caused maximal stimulation of melanogenesis in B16 melanoma cells, while Rh2 at the 12.5 μM concentration has the capacity to inhibit the growth of and to stimulate melanogenesis in these cells. Ethanol concentration below 0.2% has not been found to affect the cell growth and morphology, and the concentration of ethanol during experiments was kept well under 0.125% (v/v). An equal concentration of ethanol was added to control cultures.

Preparation of Collagen Gels. Collagen gels were prepared according to Katayma et al. (6). Type I collagen solution (0.3% in dilute hydrochloric acid, pH 3.0) was purchased from Nitta Gelatin Co. (Japan). Eight parts of collagen solution were mixed with one part of 10× culture medium and one part of 0.26 M sodium bicarbonate in 0.14 M NaOH and kept on ice. The solution (0.5 ml) was poured into 35-mm dishes and allowed to stand for 5 to 10 min at room temperature to solidify.

Cell Detachment Assay. The rate of cell detachment from the poly-styrene surface of a culture dish was measured. Cells (1 × 10⁵ for control and Rh1 treatment, 2 × 10⁵ for Rh2 treatment) were inoculated onto 60-mm dishes. After 24 h, Rh1 or Rh2 was added and cultured for 48 h; the cells were then washed 3 times with PBS (8.0 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄ in 1000-ml H₂O, pH 7.4) and treated with 0.005% EDTA or 0.01% trypsin solution in PBS at room temperature. At different time intervals, detached cells were collected by gentle rinses with PBS, and the number of cells was counted with a Coulter Counter (Model ZBI; Coulter Electronic, Inc.).

Cell Aggregation Assay. Aggregation of dissociated cells was carried out according to Takeichi et al. (7) with slight modifications. Monolayer
cells treated with ginsenosides for 72 h were rinsed 3 times with PBS and incubated with a solution containing 0.02% EDTA in PBS for 15 to 30 min at 37°C. The cells were collected, washed with culture medium containing 2% fetal calf serum and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, suspended in the same medium, and dissociated by pipetting. Cells (2 to 5 x 10^6 cells/ml) were placed in Falcon bacteriological dishes (35 mm) and incubated at 37°C on a gyratory shaker at 80 rpm. After the indicated time of incubation, the total particle number in a given cell suspension was counted with a Coulter Counter set at a 100-μm aperture, and the distribution curves of relative cell volume were obtained using a Coulter Channelyzer. The degree of aggregation was represented by the ratio of the total particle number at incubation time t (n_t) to the initial single cell number (n_0).

Assay of FITC-Lectin Binding to the Cell Surface. FITC-PNA, FITC-WGA were obtained from Seikagaku Kogyo Co., Ltd., Japan. Cells were harvested with 0.02% EDTA in PBS, washed twice with PBS, and incubated at 4°C for 90 min with occasional agitation, in 0.5 ml of PBS containing FITC-lectin (20 μg/ml). After incubation, the cells were washed twice with ice-cold PBS and fixed with 2% formaldehyde for 10 min at room temperature. The cells were then washed with PBS and analyzed in an Ortho-Cytograf System 50H equipped with a 500 mW argon-ion laser (488 nm).

Measurement of DPH Polarization. The fluorescent hydrocarbon, DPH, was used as a probe (8). One volume of cell suspension (1 x 10^6/ml) or membrane suspension (400 μg protein/ml) was mixed with one volume of DPH (1 x 10^-4 M) dispersed in PBS. After incubation at 37°C for 30 min with gentle shaking, they were washed twice with PBS, and fluorescence polarization was measured at 25°C in the Elscint MV-1 apparatus.

Preparation of Plasma Membrane. Crude plasma membranes were prepared according to Hogeboom et al. (9).Cells (1 x 10^7) were suspended in a hypotonic solution (2 mM MgCl2:10 mM Tris-HCl, pH 7.5), kept for 5 min in an ice bath, and then homogenized in a Potter-Elvehjem homogenizer. After removing the nuclei by low-speed centrifugation, the homogenate was centrifuged at 9,000 x g for 10 min, and then the supernatant was centrifuged at 105,000 x g for 60 min. The final pellets were used as a crude plasma membrane preparation.

TLC of the Lipid Fraction. Lipid extraction from cell membranes was carried out as follows: 3.75 ml of chloroform:methanol (1:2) were added to 1 ml of cell suspension (1 x 10^6 cells) or 1 ml of membrane suspension isolated from 1 x 10^7 cells, and extracted at room temperature for 90 min with occasional shaking. After centrifugation, the supernatant was stored, and the remaining cell pellet was extracted again in the same manner. Chloroform (2 ml) and distilled water (2 ml) were added to the total supernatant, and the mixture was centrifuged at 3000 rpm for 5 min. The lower phase was collected and evaporated under a stream of nitrogen. The extracted lipid was dissolved in a constant volume of chloroform, and aliquots were chromatographed in two dimensions on a high-performance TLC (Silica Gel 60; E. Merck A. G., Darmstadt, West Germany) by the method of Abramson and Blecher (10). The developed plates were sprayed with 10% HSO_3 and heated to obtain the appropriate color. Major spots on the TLC plates were identified by collating the TLC pattern of standard lipids.

**RESULTS**

Cell-Substratum Adhesiveness. B16 melanoma cells were cultured in the presence of 12.5 μM Rh2 for 48 h, and the number of cells that detached in response to 0.005% EDTA was serially determined. While all control cells were detached within 25 min, only 20% of the Rh2-treated cells were detached (Fig. 1A). Treatment with 0.01% trypsin solution produced similar results (Fig. 1B). In contrast, B16 melanoma cells treated with 40 μM Rh2 for 48 h showed no such increase in cell-substratum adhesiveness. Furthermore, when the cells were cultured on collagen gels with or without Rh2 (12.5 μM) for 7 days, a difference in adhesion properties to the collagen was clearly observed between control and Rh2-treated cells. Rh2-treated cells were more flattened and well spread on the collagen gels, while the control cells showed rounded morphology and were poorly spread. The cells treated with Rh2 (40 μM) for 7 days showed similar morphology to the control cells (data not shown).

Cell Agglutinability. B16 melanoma cells treated with 12.5 μM Rh2 for 72 h were dissociated into single cells by treatment with 0.02% EDTA, and the cell agglutinability was determined by measuring the decrease in particle number (single cells). After 120-min incubation, n_t/n_0 in control cells was 0.9, while n_t/n_0 in Rh2-treated cells was 0.6, indicating an increase in cell-cell adhesiveness (Fig. 2A). The increase of cell agglutinability due to treatment with Rh2 was also observed in the particle size (cell aggregation). The increase of particle size as a result of cell aggregation was more prominent in the Rh2-treated cells compared to controls (Fig. 2B). In contrast, the cells treated with 40 μM Rh1 for 72 h showed no such changes in cell agglutinability.

**Binding of Various Lectins to Cell Surface.** The binding of various FITC-lectins showing different carbohydrate specificities was determined by flow cytometry. In Rh2-treated cells, the quantity of bound PNA was markedly increased compared with control cells, whereas no marked differences were observed in the binding of Con A and WGA (Fig. 3). In contrast, Rh2-treated cells showed no change in the binding of any of these lectins. The PNA binding to the Rh2-treated cells was inhibited by 0.1 M p-galactose. This change was presumed to have occurred due to treatment with Rh2.
B16 melanoma cells, Sx IO4 for control and 2 x 10⁶ for Rh; treatment, were inoculated onto 60-mm dishes. After 24 h, Kh., was added and cultured for 48 h still exhibited
curred in the carbohydrate chains of glycoproteins on the cell
surface, since PNA did not bind to the cell surface after treat-
ment of the cells with 0.125% trypsin solution.

**Effects on DPH Polarization.** The effects of ginsenosides on
the membrane lipid fluidity were evaluated by determining
DPH polarization (polarization value). Isolated plasma mem-
branes labeled with DPH were incubated in the presence of
Rh2, and the polarization value was serially determined. The
addition of Rh2 (40 mM) for 96 h. However, novel spots which were not detected in the
lipid fraction of control cells were observed in Rh1-treated cells
data not shown).

**DISCUSSION**

Rh2 caused flattening of cells and led to organized, nonover-
lapping monolayers in B16 melanoma cells anchored to a
collagen gel, which is a more native substratum than a plastic
surface. This suggests that Rh2 alters the receptors for collagen
fibers in these cells. Moreover, Rh2 increased cell adhesiveness
to plastic surfaces and cell agglutinability. These effects of Rh2
on B16 melanoma cells led us to consider that Rh2 affects the
cell surface and membrane, which are involved in the regulation
of phenotypic expression of the cells. It is well known that cell
agglutinability and cell adhesiveness to substratum are impor-
tant in the development of multicellular organisms and are
closely related to the control of morphogenesis, differentia-
tion, and cell growth. We previously reported that Rh2 has the
capacity to inhibit cell growth and to induce phenotypic reverse
transformation of B16 melanoma cells (2). These data suggest
that Rh2 is capable of modulating the surface composition of
B16 melanoma cells.

A striking effect of Rh2 on the cell surface composition is the
marked increase of PNA binding sites in glycoproteins. PNA
has a high specificity and binds preferentially to the disaccha-
idic carbohydrate D-Gal1-3GalNAc (11), which is found in O-glycosidic-type
oligosaccharides (12). Others have found evidence that the
carbohydrate chains of glycoproteins and glycolipids on the cell
surface change in the course of development and differentiation
of embryonal cells. The expression of PNA receptors also
changes during the in vitro differentiation of mouse embryonal
carcinoma cells (13, 14) and the initial (early) development of
amphibia (15). Furthermore, glycoproteins having many O-
glycosidic carbohydrate chains undergo alteration during the
differentiation of human leukemia HL-60 (16).

The polarization value for intact B16 melanoma cells labeled
with DPH rose with increasing culture density up to a maxi-
mum at 1 × 10⁶ cells/cm² (subconfluent). B16 melanoma cells
cultured in the presence of 12.5 μM Rh2 for 48 h still exhibited
this phenomenon, but showed lower polarization values than
those of untreated control cells at equal cell densities (Fig. 5A).
A similar overall decrease in polarization values was observed in
B16 melanoma cells treated with 40 μM Rh1 for 48 h (Fig. 5B).

**Lipid Composition of the Plasma Membranes.** Rh2 was de-
tected in the lipid fraction extracted from crude plasma mem-
branes of B16 melanoma cells treated for 96 h (Fig. 6). In addition, the quantitative changes of ceramidizedihexoside were observed in the lipid components of the cell membrane from
Rh2-treated cells. In contrast, Rh1 was not detected even in the
lipid fraction of whole cells after treatment with Rh1 (40 μM)
for 96 h. However, novel spots which were not detected in the
lipid fraction of control cells were observed in Rh1-treated cells
(data not shown).

**Fig. 5. Effects of Rh2 and Rh1 on density-dependent changes of DPH polari-
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**zation of B16 melanoma cells. B16 melanoma cells were inoculated at various
seeding levels in 100-mm dishes. After 6 h, Rh2 (A) and Rh1 (B) were added and
cultured for 48 h. Cells were harvested with 0.01% EDTA, and DPH polarization
of the cells in control (C) and treated ginsenosides (D) was measured.
Rh₂ inhibits growth of B16 melanoma cells and is capable of modulating the surface composition of adhesion molecules which are important in intercellular recognition and cell adhesion to plastic surfaces. However, Rh₁, which does not inhibit growth, but does stimulate melanogenesis of B16 melanoma cells, had no effects on cell agglutinability, cell adhesion to substratum, or carbohydrate chains on the cell surface. From these data, it is tentatively concluded that growth inhibition of B16 melanoma cells by Rh₂ is closely related to modulation of the surface composition of the cells. Stimulation of melanogenesis in B16 melanoma cells by Rh₂ may be directly related to this growth inhibition which results in accumulation of cells in the G₀ or G₁ phases of the cell cycle, since cultured B16 melanoma cells produce no pigment during the growth phase, but after entering the stationary phase show an abrupt onset of melanin synthesis (17).

A rapid increase of DPH polarization of isolated cell membranes was observed within 2 min after Rh₂ addition. The same results were obtained from the experiments using intact cells labeled with DPH (data not shown). Rh₂ has a lipophilic structure and was incorporated into the cell membranes of B16 melanoma cells. These data indicate that Rh₂ is incorporated into membrane lipid bilayers and makes them more rigid.

On the other hand, a decrease of polarization values was observed in the intact cells during prolonged (48 h) treatment with Rh₂. Labeling of intact cells with DPH is followed by a progressive incorporation of the probe into intracellular organelles and any cytoplasmic lipid inclusions, and the apparent polarization values are a weight average of all labeled lipid domains (18, 19). Whether the DPH polarization of intact cells reflects the fluidity of cell membranes or intracellular lipid components is unclear, but DPH polarization values of intact cells are associated with several biological phenomena, for example, cell growth (20, 21), differentiation (22), phagocytosis (23), and platelet activation (24).

In this study, the elevation of polarization values in proportion to cellular densities in culture was observed in B16 melanoma cells. Such cell density-dependent changes in the DPH polarization have been observed in other normal and malignant culture cells (20). Furthermore, the DPH polarization is known to change according to the cell cycle (25). While the mechanism and significance of these changes remain unknown, they indicate that DPH polarization values reflect the control mechanism of cell growth. B16 melanoma cells cultured in a medium containing Rh₁ (12.5 μM) or Rh₂ (40 μM) for 48 h still exhibited density-dependent changes of polarization value, but showed lower polarization values than those of untreated control cells at the same cellular density. These changes in the DPH polarization caused by Rh₁ or Rh₂ are associated with the differentiation. We are investigating which
cellular components were affected by Rh₁ and Rh₂ during the 48-h treatment.

These results suggest that, after Rh₂ is incorporated into the membrane, it subsequently changes the cell surface and lipid organization of which the functions are related to differentiation. Rh₁, on the other hand, caused no polarization value changes during a brief incubation (90 min) and was not detected in the cell. However, novel lipid components, which were not observed in control cells, appeared in the lipid fraction of the Rh₂-treated cells. These lipid components were not aglycone metabolized from Rhi by the removal of the sugar residue. The properties and functions of these components remain unexplained.

The mechanism of the differentiation-inducing activities of Rh₁ such as stimulation of melanin synthesis, without being incorporated into the cell membrane lipid fraction is unclear. We speculate that novel lipid components present in the membrane of Rh₂-treated cells play an important role in induction of phenotypic reverse transformation of B16 melanoma cells.

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