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Induction of Phenotypic Reverse Transformation by Ginsenosides in Cultured Cancer Cells

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Introduction

Crude ginsenosides extracted from the root of *Panax ginseng* C. A. Meyer inhibit the proliferation and colony-forming ability of Morris hepatoma cells in soft agar culture, and stimulate serum protein synthesis by these cells, thus converting the cell characteristics both functionally and morphologically to those resembling original normal liver cells[1]. Similar alterations of cancer cell properties have been induced by retinoids, glucocorticoids, tocopherol, and dibutyryl cAMP. We have called such phenomena "reverse transformation", in which cancer cells resulting from malignant transformation are transformed again to near normal cells both functionally and morphologically.

In this report, the results of our recent investigation are described with particular reference to reverse transformation of B16 melanoma cells induced by ginsenoside Rh_2 (Rh_2). Furthermore, the action mechanism of ginsenoside Rh_2 is discussed.

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, penicillin, and streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. In the experiments, cells were cultured in the same medium supplemented with 2% fetal calf serum.

Ginsenosides

Purified ginsenosides were kindly provided by Dr. Isao Kitagawa, Faculty of Pharmaceutical Science, Osaka University, Osaka, Japan.

Assay of growth inhibition, measurement of melanin content, cell cycle analysis, collagen gel culture, cell detachment assay, cell aggregation assay, assay of FITC-lectin binding, measurement of DPH polarization, and TLC of the lipid fraction

These procedures were performed as previously described[2,3].

Staining of actin stress fiber

Cells grown on glass coverslips were rinsed briefly in phosphate-buffered saline (PBS) fixed for 10 min in 3.7% formaldehyde in PBS at room temperature and washed extensively in PBS. After fixation, cells were dehydrated in absolute acetone for 4 min at −20°C and air dried. NBD-phallacidin was applied to the cells at a concentration of 0.165 $\mu g/ml$ in PBS and allowed to react for 20 min at room temperature. The cells were washed with PBS twice and mounted on microscope slides in a mixture of 30% glycerol and 70% PBS (v/v). Fluorescence photomicrographs were taken using epifluorescent illumination.

Results and Discussion

Phenotypic reverse transformation of B16 melanoma cells by ginsenoside Rh_2 in culture

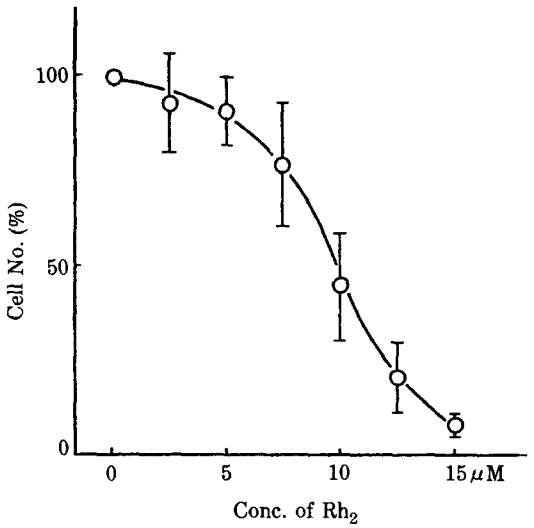

Fig. 1. Effect of ginsenoside Rh_2 on growth of B16 melanoma cells. Cells were plated in 60 mm dishes. 24 h after plating, Rh_2 at various concentrations was added. At the termination of the experiments on Day 4, the cells were detached and counted with an electronic particle counter.

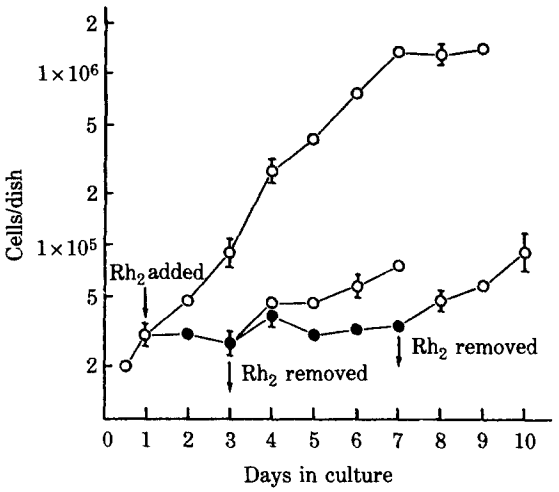

Fig. 2. Reversibility of Rh_2 inhibition on the growth of B16 melanoma cells in culture. Cells were plated in 35 mm dishes. 12 h after plating, Rh_2 (12.5 μM) was added to the cultures. Growth media and Rh_2 were changed on Days 3 and 7. On the indicated days, Rh_2 was removed from one set of cultures; one half of the cells received fresh growth media (○) and the other half received 12.5 μM Rh_2 (●).

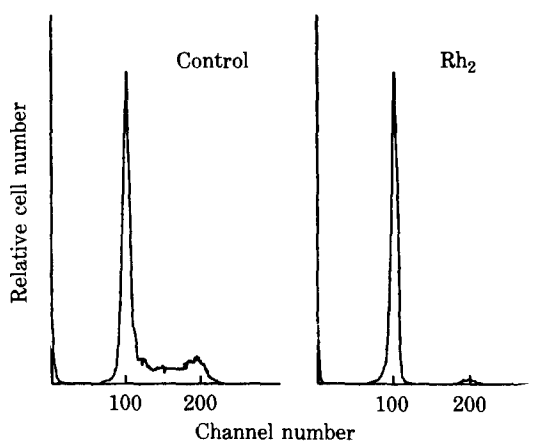

Fig. 3. DNA histograms of B16 melanoma cells treated with Rh_2. Cells were cultured for 96 h in the absence (left) or presence (right) of 12.5 μM Rh_2. Then the cells were harvested and DNA was stained with propidium iodide. Stained cells were analyzed by flow cytometry.

Effects of Rh_2 on growth and melanin synthesis in B16 melanoma cells[2]

Reverse transformation of cancer cells can be characterized mainly by the suppression of proliferation and the restoration of differentiation. Accordingly, we examined the effects of ginsenoside Rh_2 on the growth and melanin synthesis of B16 melanoma cells. Melanin synthesis is a specialized function of melanocytes, and the increase of melanin synthesis indicates the differentiation of normal melanocytes and melanoma cells.

Rh_2 suppressed the growth of B16 melanoma cells dose-dependently at concentrations over 5 μM (Fig. 1). Rh_2 at the concentration of 12.5 μM was used in the following experiments. This inhibition is not due to the cell-killing effect of Rh_2, because the growth of these cells was completely suppressed by 12.5 μM of Rh_2 but recovered when Rh_2 was removed from the culture medium (Fig. 2). However, the rate of proliferation was notably lower, suggesting that the growth inhibiting effect of Rh_2 on these cells persists.

We examined the effect of Rh_2 on the cell cycle progression of B16 melanoma cells. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry (Fig. 3). The majority of the Rh_2-treated cell population was in the G1 phase, indic-

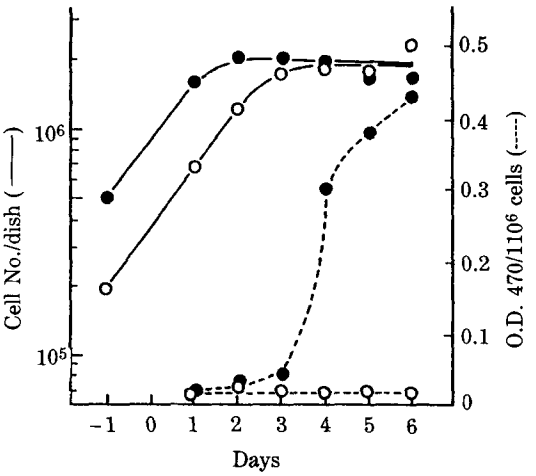

Fig. 4. Promotion of melanin synthesis by Rh_2 in B16 melanoma cells. Cells were plated in 35 mm dishes. After 24 h, cultures were subjected to 12.5 μM of Rh_2 (●) o 0.1% ethanol as a control (○). Cell numbers (solid lines) were counted with an electronic particle counter, and melanin contents (broken lines) were measured by a colorimetric method.

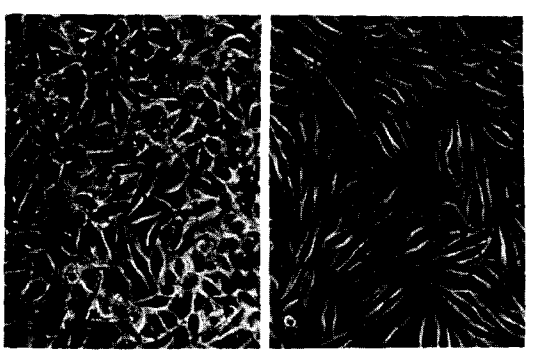

Fig. 5. Phase-contrast micrographs of B16 melanoma cells cultured for 4 days in the absence (A) or presence (B) of 12.5 μM Rh_2. The bar indicates 100 μm.

ting that Rh_2 inhibits the cell cycle progression from the G_1 to S phase in B16 melanoma cells.

Rh_2 promoted the melanin synthesis of B16 melanoma cells. The melanin content of the control cells was very low throughout the culture period, whereas the melanin content of the Rh_2-treated cells increased dramatically at 4 days after Rh_2 addition (Fig. 4).

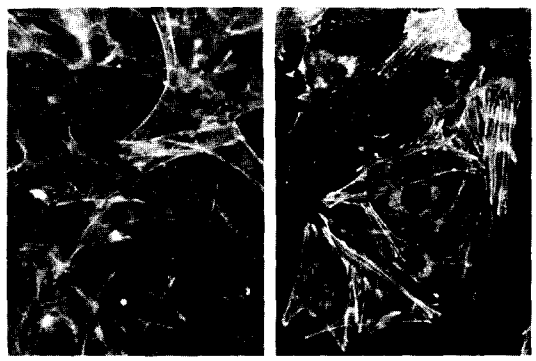

Fig. 6. Actin stress fibers of control (left) and Rh_2-treated cells (right). Cells were cultured with Rh_2 12.5 μM for 4 days, and actin stress fibers were stained with NBD-phallacidin.

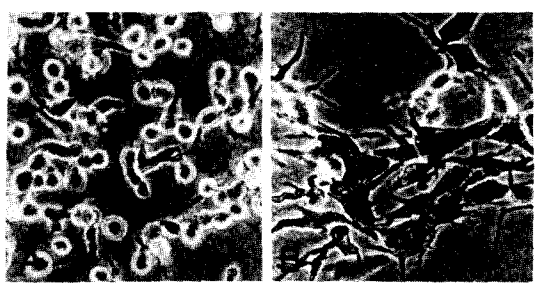

Fig. 7. Phase-contrast micrographs of B16 melanoma cells cultured in collagen gel for one week in the absence (A) or presence (B) of 12.5 μM Rh_2. Type I collagen gels were prepared as previously described (3). The bar indicates 100 μm.

Effects of Rh_2 on the cell morphology and the cytoskeleton of B16 melanoma cells

The morphology of the Rh_2 treated cells is shown in figure 5. The cells were treated with Rh_2 for 4 days. The orientation of the control cells was random, and these cells overlapped, whereas the Rh_2-treated cells oriented well and did not overlap.

Figure 6 shows the morphology of actin stress fiber stained with NBD-phallacidin. The control cells had poorly organized stress fiber, whereas the Rh_2-treated cells had thicker and more organized stress fiber than the control cells. Furthermore, there were many adhesion plaques in the Rh_2-treated cells.

Generally, cultured cells express a more intact

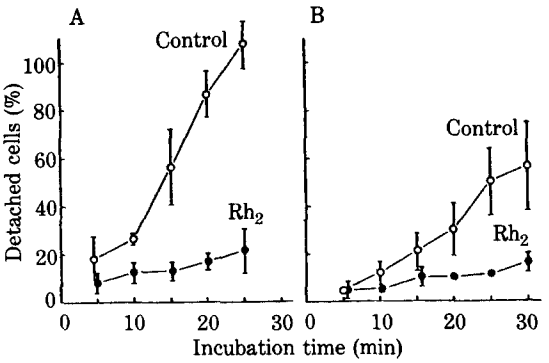

Fig. 8. Kinetics of detachment of B16 melanoma cells from the substratum with EDTA and trypsin. Cells were treated with Rh_2 12.5 μM for 2 days. Then, the detachment of cells treated with 0.005% EDTA (A) or 0.01% trypsin (B) at room temperature was measured.

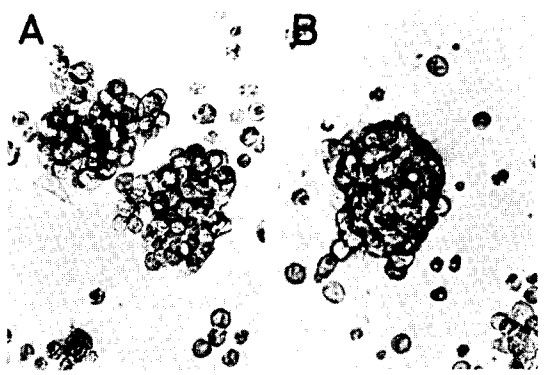

Fig. 9. Phase-contrast micrograph of aggregates of B16 melanoma cells in suspension culture. Cells were cultured for 4 days without (A) or with (B) Rh_2 12.5 μM.

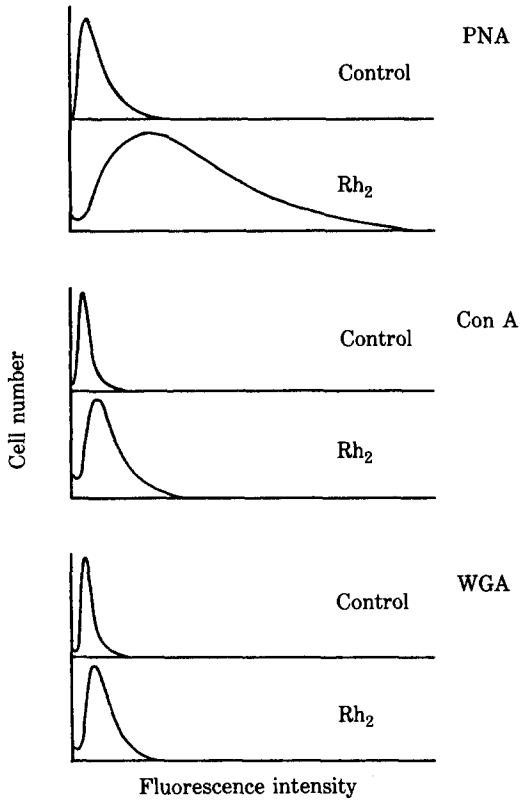

Fig. 10 Effect of Rh_2 on FITC-lectin binding to the cell surface of B16 melanoma cells. Cells were treated with Rh_2 12.5 μM for 3 days and were stained with FITC-lectins. Stained cells were analyzed by flow cytometry.

morphology in collagen gel culture. Therefore, we cultured B16 melanoma cells in collagen gel with 12.5 μM of Rh_2 for 1 week (Fig. 7). The control cells were round in shape and less melanotic, while the Rh_2-treated cells were extended and highly melanotic[3].

These traits of Rh_2-treated cells in culture seem to resemble those of the untransformed phenotype.

Mechanism of reverse transformation by ginsenoside Rh_2

The above data suggest that ginsenoside Rh_2 can induce the reverse transformation of B16 melanoma cells. To clarify the mechanism of this reverse transformation by ginsenoside Rh_2, we examined the effects of Rh_2 on the cell surface and on the expression of oncogenes. Both the cell surface and the oncogene are known to play important roles in proliferation and differentiation.

Effects of Rh_2 on cell surface properties[2,3]

As shown in Figure 8, the Rh_2-treated cells were more resistant to detachment by EDTA and trypsin, indicating that Rh_2 enhanced cell adhesiveness to culture dishes. Figure 9 shows the morphology of aggregates of B16 melanoma cells in suspension culture. The aggregates of Rh_2-treated cells were more compact than those of the controls, and the surface of these aggregates was smoother. These resulting indicate that Rh_2 increases the cell-sub-

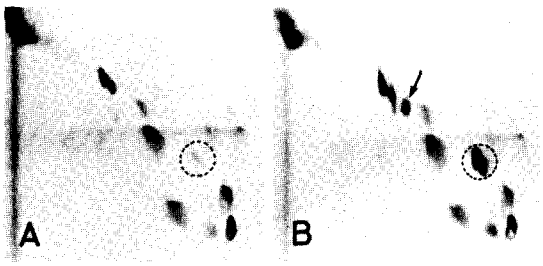

Fig. 11. HPTLC analysis of the lipid extract from B16 melanoma cells. Cells were treated without (A) or with (B) Rh_2 12.5μM for 4 days. Then, the total lipids were extracted from whole cells and analyzed by high performance thin layer chromatography (HPTLC). The arrow indicates Rh_2, and the broken circles indicate ceramidedihexoside (CDH).

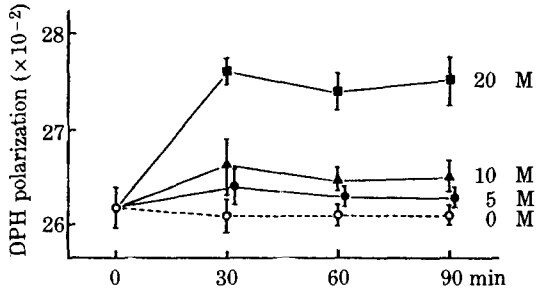

Fig. 12. Effect of Rh_2 on the 1,6-diphenyl-1,3,5-hexatriene (DPH) polarization of B16 melanoma cells. Rh_2 was added to the cells labeled with DPH, and the DPH polarization was measured at 25°C in the Elscint MV-1a apparatus.

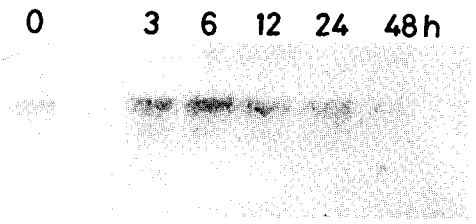

Fig. 13. Effect of Rh_2 on c-myc mRNA expression of B16 melanoma cells. Cells were treated with Rh_2 for 0-48 h, and total RNA was prepared from each sample. Total RNA was size fractionated in 0.75% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labelled human c-myc cDNA.

strate and cell-cell adhesiveness of B16 melanoma cells.

The lectin recognizes a specific sugar chain structure. Rh_2 altered the binding of PNA to the cell surface but did not change that of Con A and WGA (Fig. 10), suggesting that Rh_2 can change the specific sugar chain structure on the cell surface.

Effects of Rh_2 on the membrane lipid of B16 melanoma cells[2,3]

Because ginsenoside Rh_2 has both hydrophilic and hydrophobic groups, it is considered to have strong affinity for the cell membrane lipid bilayers. Therefore, we supposed that the primary target site of Rh_2 may be a cell membrane lipid bilayer. We could detect Rh_2 in the lipid fraction obtained from the Rh_2 treated cells (Fig. 11). Similar results were obtained when erythrocyte ghosts were treated with Rh_2. Furthermore, ceramidedihexoside (CDH), which is an important intermediate glycolipid in ganglioside metabolism, increased in the Rh_2-treated B16 melanoma cells (Fig. 11).

1,6-Diphenyl-1,3,5-hexatriene (DPH) polarization reflects the fluidity of the membrane lipid bilayer. We labeled B16 melanoma cells with DPH and measured DPH polarization after Rh_2 addition. Rh_2 changed the DPH polarization of B16 melanoma cells within 30 min (Fig. 12).

These findings suggest that Rh_2 is rapidly incorporated into the cell membrane and changes the nature of the lipid bilayer, and then changes various cell surface-related characteristics.

We examined the effects of various ginsenosides on DPH polarization and found that only Rh_2 could change DPH polarization. Furthermore, the aglycones, both protopanaxadiol and protopanaxatriol, could also change DPH polarization. These three molecules could inhibit cell growth. These observations suggest that the absence of sugar molecules at C-6 and C-20 is important for growth inhibition.

Effect of Rh_2 on the expression of c-myc oncogene in B16 melanoma cells

Oncogenes are known to play important roles in cell proliferation and differentiation. Therefore, it would be of interest to know whether Rh_2 affects

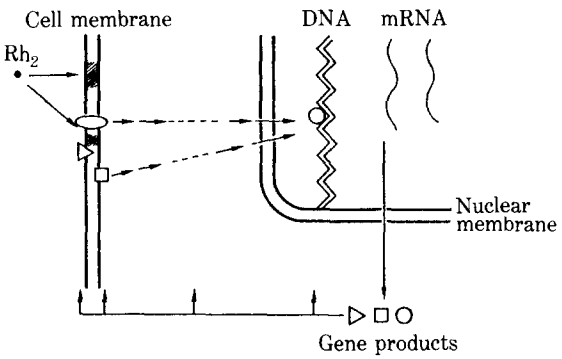

Fig. 14. Model of the action mechanism of ginsenoside Rh_2. See text for detail.

the expression of oncogenes. We examined the effect of Rh_2 on the expression of c-myc oncogene in B16 melanoma cells. Figure 13 shows the c-myc mRNA expression of Rh_2-treated B16 melanoma cells. Rh_2 decreased the c-myc mRNA expression at 24 to 48 h after Rh_2 addition. This change occurred later than the other changes, such as the cell cycle arrest and the increase of DPH polarization by Rh_2, suggesting that the effect of Rh_2 on c-myc mRNA expression is indirect.

From these data we speculated as to the mechanism of the reverse transformation by ginsenoside Rh_2 (Fig. 14). Since Rh_2 is considered to have strong affinity for the lipid bilayer, Rh_2 is rapidly incorporated into the cell membrane lipid bilayer, and affects the various functional molecules on the cell surface by changing the nature of the lipid bilayer. For example, glycosidase, glycosyltransferase, receptor protein, and adhesion protein, may be affected. Furthermore, the signals originating from these molecules or the transmission of these signals to the nucleus is changed by Rh_2. As a result, the expression of certain genes, such as c-myc oncogene, change. Finally, Rh_2-treated cancer cells come to express a phenotype closer to that of their normal counterpart.

References

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