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Induction of Phenotypic Reverse Transformation by Ginsenosides in Cultured Morris Hepatoma Cells

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Abstract—*Ginsenosides (oligoglycosides) extracted from the root of Panax Ginseng, C. A. Meyer, are found to induce the reverse transformation of Morris hepatoma cells (MH_1C_1) in vitro.*

After 24 subcultures of growth medium containing ginsenoside, MH_1C_1 cells were morphologically changed. These cells were relatively large and polygonal in shape with abundant fine granular cytoplasm and clear intercellular space, and resembled normal hepatocyte-like cells.

These cells reversely transformed, by ginsenosides, showed much less ability to grow in a 0.33% soft agar suspension culture in comparison to control MH_1C_1 cells, but the doubling time of these cells was 30 hr which is similar to that of original Morris hepatoma cells.

In these reversely transformed cells, a remarkable increase in uptake of L-^{3}H-ornithine in an arginine deficient medium, an increase of activity of succinate-cytochrome c reductase and a decrease in 5′-nucleotidase activity were observed.

INTRODUCTION

EXTRACT from the root of Panax Ginseng, C. A. Meyer, has been empirically used for thousands of years in Asian countries as an analeptic, stomachic and erythropoietic.

Pharmacological investigations [1, 2] have shown that the basic action of these extracts is to increase non-specific residence of the organism to various untoward influences and also to produce normalization which reveals itself irrespective of the direction of the previous pathological shift.

Recently, the chemical structure and the biological effects of these extracts have been widely studied, and it has been revealed that ginsenosides which are the effective substances extracted from Panax Ginseng roots stimulate various metabolic reaction of liver cells *in vivo* [3–5].

Previous work [6] in this laboratory has shown that ginsenosides induce morphological reverse transformation of cultured Morris hepatoma cells. Attempts have been made to elucidate the characteristics of Morris hepatoma cells redifferentiated by ginsenosides.

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MATERIALS AND METHODS

1. *Extraction and purification of ginsenosides*

Ginsenosides were isolated from the roots of Panax Ginseng, C. A. Meyer, according to the usual method [7, 8] of isolation of plant saponin with modification. The roots of Panax Ginseng, C. A. Meyer, were minced and extracted for 3 hr with boiling methanol. The combined extract was shaken with ether to remove the oily portion and the precipitate shaken three times with *n*-butanol saturated with water. The butanol layer was shaken with one-tenth volume of water and evaporated under vacuum to give crude ginsenosides. This crude ginsenoside was dissolved in methanol and ether (20 × by volume) was added to the methanol solution stirring gently. The mixture was left overnight and the precipitate was dried at less than 60°C.

The mixture was determined by thin-layer chromatography to contain two groups of glycosides, glycosides with an oleanane type of

triterpenoid and with a dammarane type of triterpenoid as the aglycone. The major components of the mixture used in this experiment are oligoglycosides with 20S-protopanaxadiol and 20S-protopanaxatriol as aglycone. These chemical structures are shown in Fig. 1.

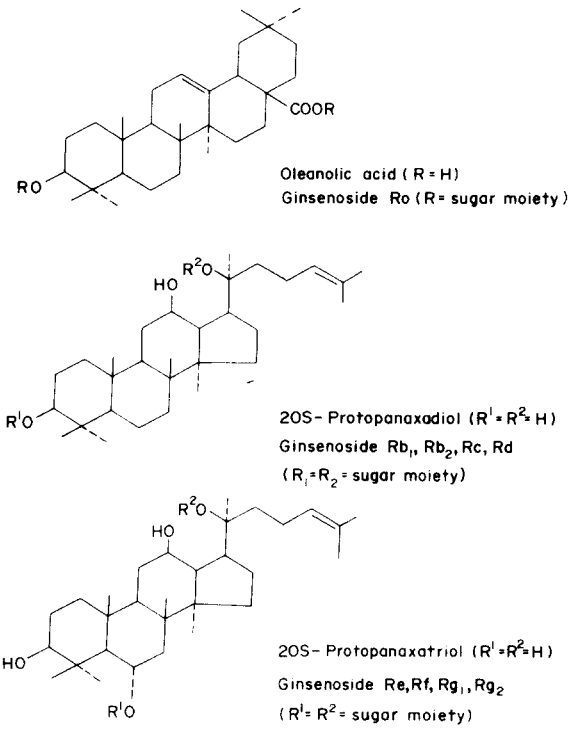

Fig. 1. Structure of ginsenosides extracted from the root of Panax Ginseng, C. A. Meyer. Ginsenosides are divided into two groups, glycosides with oleanane type of triterpenoid, and those with a dammarane type of triterpenoid, based on the chemical structure of aglycone. The ginsenosides used in this study consist mainly of oligoglycosides with 20S-protopanaxadiol and 20S-protopanaxatriol as the aglycone, which are less polar glycosides than glycoside of oleanolic acid.

2. *Cells and growth media*

The MH_1C_1 cells were derived from transplantable Morris rat hepatoma tissue. They were established as a clonal strain by Richardson *et al.* [9]. The cells were grown in Falcon plastic dishes in Leibovitz L-15 medium supplemented with 10% fetal calf serum, 50 Units/ml of penicillin and 50 μg/ml of streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed twice a week and subculture regularly performed at weekly intervals with a 0.01% trypsin solution in phosphate buffered saline.

The 3T3 cells used as control in the experiment on uptake of L-^{3}H-ornithine were kindly donated by Dr. S. Nishikawa.

The effects of ginsenosides on the MH_1C_1 cells were examined using growth media containing 20 or 100 μg/ml of ginsenosides under the same condition as stated above. These concentrations of ginsenosides have been confirmed to show no cytotoxicity to various cultured cells in a preliminary experiment.

3. *Agar suspension culture*

The ability of the cells to grow in 0.33% soft agar medium was examined using the method of Macpherson *et al.* [10]. Cells were seeded at a density of 10^3 cells per 60 mm Petri dish in soft agar made up with growth medium. After 21 days of seeding the dishes were photographed and the number of colonies counted.

4. *Incorporation of* L-^{3}H-*ornithine*

The incorporation of L-^{3}H-ornithine into trichloracetic acid (TCA) precipitable material from cultured cells was determined by the method of Odashima *et al.* [11]. The uptake of L-^{3}H-ornithine by 3T3 fibroblasts which are deficient in a urea cycle was measured under the same condition as the control.

5. *Enzyme assay and protein determination*

Glucose-6-phosphatase activity was measured according to the method of Swanson [12], using the glucose-6-phosphate disodium salt. Succinate-cytochrome c reductase activity was measured by the method of King [13]. 5'-nucleotidase activity was determined by the method of Wright [14]. Protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as standard.

6. *Chemicals*

Leibovitz L-15 medium and fetal calf serum were supplied by Flow Lab., Inc. U.S.A. L-^{3}H-ornithine was obtained from the New England Nuclear, U.S.A. Bact-agar was purchased from Difco Lab., Detroit, Michigan, U.S.A. Bovine serum albumin and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. All cultured dishes were the products of Falcon.

RESULTS

1. *Morphological appearance of Morris hepatoma cells cultured with growth medium containing ginsenosides*

The border of the colony of MH_1C_1 cells used in this experiment is characteristically smooth and the cells grow in a compact mass. These MH_1C_1 cells are relatively small ($18.95 \pm 0.78 \times 11.33 \pm 0.58$ μm), rounded in shape

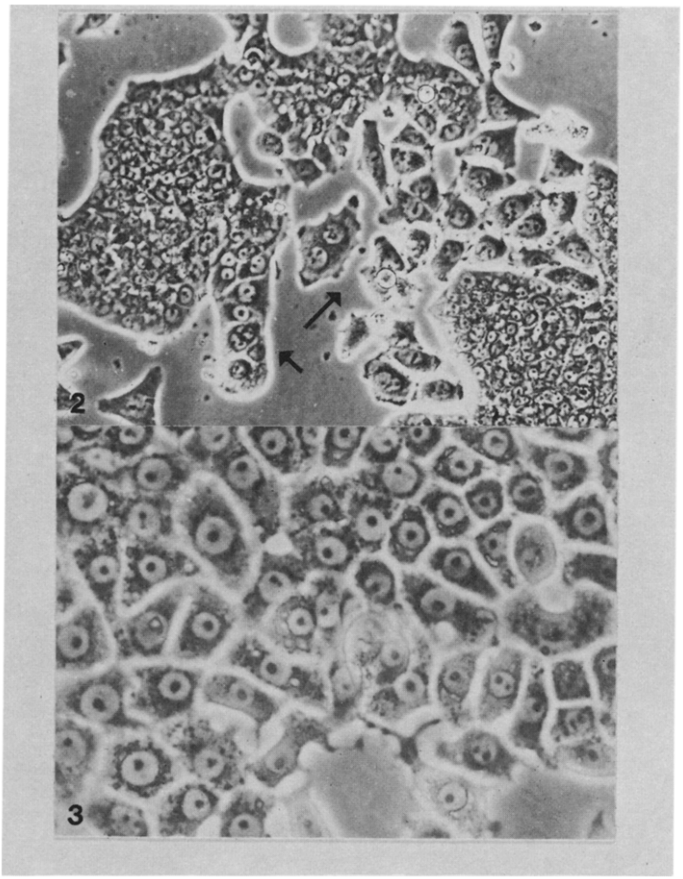

Fig. 2. *Phase-contrast micrograph showing Morris hepatoma cells* (MH_1C_1) *cultured for* 24 *subcultures in a medium with* 20 μg/ml *of ginsenosides. Arrows indicate transformed hepatoma cells* (× 200).

Fig. 3. *Phase-contrast micrograph showing hepatoma cells transformed by ginsenosides.*
The cells are relatively large, form the typical epitherial pattern and the cytoplasm is granular (× 400).

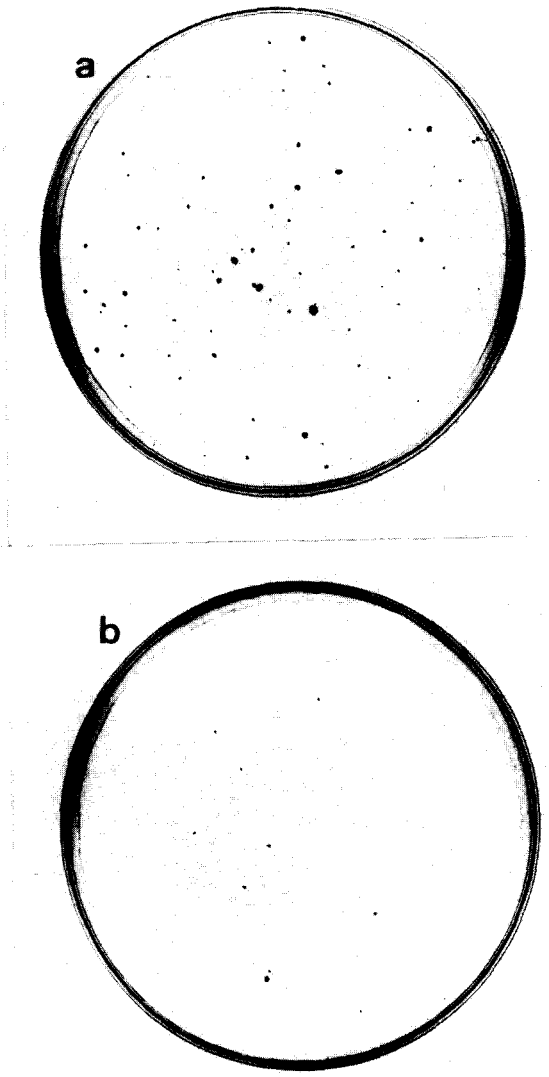

Fig. 4. *Colony formation of hepatoma cells in soft agar suspension culture* (*a*) *After* 21 days *of seeding* 1×10^3 *hepatoma cells, cultured in a standard medium* (*b*) *After* 21 days *of seeding* 1×10^3 *hepatoma cells, cultured in a medium with ginsenosides of* 100 μg/ml.

and have a prominent nucleus containing one or two nucleoli. The cytoplasm is scanty and the border of each cell is unclear under light microscopic observation.

The MH_1C_1 cells grown in the medium containing 20 or 100 μg/ml of ginsenosides for 24 subcultures (about 170 days) are much larger than the original MH_1C_1 cells; the cell length and width are 32.68 ± 1.08 and 21.53 ± 0.65 μm, respectively. The large cells are polygonal in shape and have abundant fine granular cytoplasm and the intercellular space of these cells is clearly recognizable (Figs. 2 and 3).

These cells formed a complete monolayer in the typical pattern of normal hepatocyte. Initially five or ten of these redifferentiated cells can be seen in a given field, and a few weeks later the whole field of a dish becomes occupied by redifferentiated cells. Experiments were performed three times with similar results.

These cells redifferentiated by ginsenosides did not reverse to the original MH_1C_1 cells even when cultured in a standard medium without ginsenosides for the same 24 subcultures.

2. *Growth in agar suspension culture*

One of the assays required for neoplastic transformation of cells is their ability to grow in soft agar suspension culture. The doubling time of these reversely transformed cells by ginsenosides is 30 hr which is similar to that of original MH_1C_1 cells, but the ability of these cells to grow in 0.33% soft agar suspension culture is remarkably lower (Fig. 4). That is, when seeded at a density of 10^3 cells per dish with 0.33% agar made up with growth medium, the number of colonies of the original MH_1C_1 cells was 87.2 ± 25.8 after 21 days of seeding, however the number of colonies of the cells cultured in a medium with 20 and 100 μg/ml of ginsenosides was 16.7 ± 7.2 and 15.2 ± 8.1, respectively.

3. *Incorporation of ornithine*

The uptake of L-^{3}H-ornithine by MH_1C_1 cells was 620.3 ± 156.6 dis/min/10^5 cells in a complete medium and 2573.3 ± 405.5 dis/min/10^5 cells in an arginine deficient medium. By the reversely transformed cells by ginsenosides, the uptake of L-^{3}H-ornithine was slightly higher in a complete medium and significantly higher in an arginine deficient medium as shown in Table 1. The uptake of L-^{3}H-ornithine by 3T3 cells which was measured for comparison did not increase in an arginine deficient medium.

4. *Enzyme activities*

The marker enzyme activities for mitochondria, endoplasmic reticulum and plasma membranes were measured on homogenates of both MH_1C_1 cells and the reversely transformed cells by ginsenosides.

The activity of succinate-cytochrome c reductase of redifferentiated cells cultured in a medium with 20 and 100 μg/ml of ginsenosides was increased 51 and 68%, respectively. The 5'-nucleotidase activity of these cells decreased by approximately 60%. However, in glucose-6-phosphatase, neither an increase nor a decrease was observed (Table 2).

DISCUSSION

Ginsenosides extracted from Panax Ginseng, C. A. Meyer, have been known to promote protein synthesis and to activate the lipid or sugar metabolism of liver tissue *in vivo* [3–5].

The present experiments were designed to assess the direct effects of ginsenosides on Morris hepatoma cells. The results of these experiments indicate that ginsenosides induce reverse transformation or redifferentiation of Morris hepatoma cells, since it appeared to reverse the formation of many of the characteristics assumed by cells treated with malignant transforming agents.

MH_1C_1 cells cultured in growth medium with ginsenosides are relatively large and normal hepatocyte-like cells with the characteristics of epitherial cells. The size of these cells is very similar to that of cultured normal liver cells [11]. The doubling time of these reversely transformed cells is not changed in comparison to the original cells but their ability to grow in 0.33% soft agar is remarkably decreased.

Macpherson *et al.* [10] have reported that an ability to grow in soft agar is an effective assay for neoplastic transformation. Therefore, the remarkable decrease of colony formation of the reversely transformed cells by ginsenosides supports the conclusion that ginsenosides induce phenotypic reverse transformation.

In general, fast growing or poorly differentiated hepatoma cells are deficient in the urea synthesis which is unique to liver cells [16]. But the Morris hepatoma cells used in this experiment retained the capacity of urea syn-

Table 1. *Incorporation of* L-^{3}H-*ornithine into cultured cells in media with and without arginine*

Medium	MHC*	MH20†	MH100‡	3T3§
Complete medium	620.3 ± 156.6 (23,846.2 ± 6020.2)	1057.5 ± 232.5 (28,961.0 ± 6367.3)	1376.0 ± 346.2 (37,702.0 ± 9485.8)	581.4 ± 45.6 —
Arginine deficient medium	2573.3 ± 405.5 (98,969.0 ± 15,595.5)	5772.9 ± 1223.0 (158,152.0 ± 330,504.8)	7258.2 ± 668.4 (198,869.2 ± 18,313.7)	667.7 ± 97.2 —

Radioactivity determined from TCA precipitable material from cell cultures. Each value represents mean dis/min/10^{5} cells ± standard error (mean dis/min/mg protein ± standard error) from 3 aliquots of cells.
*Control MH_1C_1 cells.
†MH_1C_1 cells cultured in a medium with 20 μg/ml of ginsenosides.
‡MH_1C_1 cells cultured in a medium with 100 μg/ml of ginsenosides.
§3T3 cells which were examined for comparison.

Table 2. Effects of ginsenosides on succinate-cytochrome c reductase, 5′-nucleotidase and glucose-6-phosphatase activities

	MHC*	MH20†	MH100‡
Succinate-cyt. c reductase (μmole/mg protein/hr)	0.188±0.035	0.284±0.016	0.316±0.022
5′-nucleotidase (μmole/mg protein/hr)	0.540±0.081	0.203±0.046	0.211±0.025
Glucose-6-phosphatase (μmole/mg protein/hr)	0.619±0.052	0.569±0.051	0.572±0.079

*Control MH_1C_1 cells.
†MH_1C_1 cells cultured in a medium with 20 μg/ml of ginsenosides.
‡MH_1C_1 cells cultured in a medium with 100 μg/ml of ginsenosides.

thesis. In the MH_1C_1 cells cultured with a medium containing ginsenosides the uptake of L-^{3}H-ornithine in an arginine deficient medium was much higher than control MH_1C_1 cells and as high as that of primary liver cells.

In addition, the activities of succinate-cytochrome c reductase and 5′-nucleotidase calculated as values per mg of protein were remarkably changed in these reversely transformed cells. These results indicated that ginsenosides do not cause only morphological redifferentiation but also functional redifferentiation. However, the changes of these enzyme activities which are represented in values per mg of protein may require further detailed examination, since these reversely transformed cells synthesis serum proteins and their capacity for protein synthesis is much higher than original MH_1C_1 cells [17, 18].

Many chemicals by which neoplastic transformation is induced have been extensively investigated but there has not been found any chemical substance which can induce well differentiation of cancer cells. The study of the effects of ginsenosides on cancer cells will be valuable in understanding cell differentiation and in advancing effective therapies for cancer.

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