DIFFERENTIATION MECHANISM OF GINSENOSIDES IN CULTURED MURINE F9 TERATOCARCINOMA STEM CELLS

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ABSTRACT

The effects of total ginseng saponin, extracts of *Panax ginseng* C.A. Meyer, on the differentiation of F9 teratocarcinoma stem cells were studied. F9 stem cells cultured in the presence of ginseng saponin together with dibutyric cAMP became parietal endoderm-like cells. Moreover, the expressions of differentiation marker genes, laminin, type Ⅳ collagen, and retinoic acid receptor-β(RARβ) were increased after treatment of ginseng saponin. Among various ginsenosides purified from crude ginseng saponin, Rh_1 and Rh_2 caused the differentiation of F9 cells most effectively. Since ginsenosides and steroid hormone show resemblance in chemical structure, we studied the possibility of the involvement of a steroid receptor in the differentiation process induced by ginsenosides. According to Southwestern blot analysis, a 94 kDa protein regarding as a steroid receptor was detected in F9 cells cultured in the medium containing ginseng saponin. Based on these data, we suggest that ginseng saponin, especially ginsenosides Rh_1 and Rh_2, cause the differentiation of F9 cells and the effects of ginsenosides might be exerted *via* binding with a steroid receptor or its analogous nuclear receptor.

INTRODUCTION

Cancer is a disease involving loss of cellular growth control and disruption of cell differentiation. Recent approaches to therapy for various types of cancer have focused on drugs that induce the differentiation of maturation resistant cells causing the disease(1). These compounds are expected to be a new type of anticancer agent because they induce neoplastic cells to differentiate into normal cells(2, 3). This new conceptual strategy for the prevention and treatment of cancer is called as differentiation therapy. However, only a few studies have been reported on differentiation inducers. The representative differentiation agent, retinoic acid(RA), has recently demonstrated promising results in several specific clinical trials for the prevention of cancers. However, several papers concerning the cytotoxic activities of RA have been reported (3, 4), and we therefore have searched for naturally occurring substances that induce the differentiation of cancer cells as the RA. We used F9 teratocarcinoma stem cells as a model system because the differentiation of F9 stem cells results in the loss of their tumorigenicity and the differentiation response of F9 cells is irreversible and fairly synchronous (5, 6). Moreover, several differences in gene expression have been documented and serve as convenient markers of differentiation of F9 stem cells(7, 8, 9).

In the course of the study, we have identified that ginsenosides have the ability to induce the differentiation of F9 stem cells. Ginsenosides is obtained from *Panax ginseng* C.A. Meyer, an important plant in the oriental medicine which has a history reaching back more than 4000 years. Recently, the chemical structure of ginsenosides and its biological effects have been widely studied, and it has been reported that ginsenosides exhibited anticancer activities and anti-inflammatory effects (10, 11).

The present study examines the effects of total ginsenosides and compares the activity of various ginsenosides purified from crude ginsenosides on the differentiation of F9 teratocarcinoma stem cells. In addition, the effect of steroid receptor on the differentiation of F9 cells were studied to clarify the relationship between ginsenosides and steroid receptor during the differentiation process, because ginsenosides are plant glycosides with molecular structures remarkably similar to the steroid hormone. Experiments reported herein demonstrate that ginsenosides, especially Rh_1 and Rh_2, causes the differentiation of F9 cells and the effects of ginseng saponin are probably exerted by forming a complex with the steroid receptor.

MATERIALS AND METHODS

Cell culture. F9 stem cells(ATCC CRL 1720) were grown on gelatinized plasticware in DMEM, supplemented with 10% FBS, penicillin(100 U/ml) and streptomycin(100 μg/ml) in humidified atmosphere of 5% CO_2 at 37℃. In the differentiation experiments, the cells were cultured in the DMEM supplemented with 2% FBS. The medium was changed twice a week, and subculture were performed every 5 days with one medium change.

Differentiation of F9 Stem Cells. For differentiation, F9 stem cells were trypsinized and seeded onto gelatinized T75 tissue culture flask containing 25 ml of culture medium and incubated 24 hr prior to the addition of drugs. When differentia-

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tion was induced by RA, 1 X 10^{-6} M all trans-RA, 5 X 10^{-4} M dibutyryl cyclic AMP (dbcAMP) and 2.5 X 10^{-4} M theophylline (RACT) were added to F9 stem cells plated in T75 culture flask. In the case of ginsenoside-induced differentiation, F9 stem cells were treated with 40 μg/ml total ginsenoside, 5 X 10^{-4} M dbc AMP and 2.5 X 10^{-4} M theophylline(GSCT). The effects of various ginsenoside derivatives on F9 cells were examined using medium containing 50 μM ginsenoside derivatives, 5 X 10^{-4} M dbc AMP and 2.5 X 10^{-4} M theophylline. RA was prepared as a 10^{-3} M stock in ethanol, dbcAMP was prepared as 10^{-1} M aqueous solution and theophylline was a 10^{-1} M stock in 0.1 N NaOH, and total ginsenoside was 10^{-1} M aqueous solution. Various ginsenoside fractions purified from total ginsenoside were prepared as 20~40 mM in ethanol. Ethanol concentration below 0.2% did not affect the cell growth, and the concentration of ethanol during experiments kept well under 0.125% (v/v). Stock solutions were sterilized by filteration and diluted directly into the culture medium. After the differentiation, the cell morphology was judged under a phase-contrast microscopy.

RNA preparation and Northern Blot Analysis. Total cellular RNA was prepared according to the procedure of the acid-guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method (12). The total RNAs were quantitated by absorbance at 260 nm and purity was assessed by determination in the ratio of absorbance at 260 nm to that of 280 nm. RNA samples were resolved on 1% agarose formaldehyde gels under denaturing conditions, and transferred to nylon membranes (Zeta-Probe, Bio-Rad, CA). After baking at 80℃ for 2 hr, blots were hybridized in a solution containing 50% deionized formamide, 0.25 M $NaHPO_4$, 7% SDS, and 1 mM EDTA at 42℃ in the presence of ^{32}P-labeled cDNA probes for 24 hrs. Blots were then washed in 2 X SSC and 0.1% SDS at room temperature and autoradiographed at -80℃. Then, the X-ray films were developed. The expression of the gene was analyzed by microdensitometer tracings of autoradiograms.

Southwestern Blot Analysis of the DNA binding Activity in Protein Extracts of F9 Cells. The Southwestern hybridization technique were adopted from modified method of Silva *et al*(13). F9 stem cells were cultured as described above. Approximately 2~4 X 10^8 cells were incubated in the medium containing either 50 μM total ginsenosides or 25 μM dexamethasone for 2 hr at 0℃ with gentle agitation. Then the cells were harvested, resuspended in 100 μl of 5 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 5 mM 2-mercaptoethanol, 0.1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, and then sonicated. The mixture was immediately centrifuged at 13,000 g for 10 min at 0℃ and the supernatant was transferred to a fresh tube. Protein concentration was measured by the method of Bradford (20). Aliquots of denatured proteins were electrophoresed on preparative slab gels consisting of 3.0% acrylamide stacking gel and 7.5% acrylamide resolving gel. After electrophoresis the proteins were electrophoretically transferred onto Hybond C-extra nylon membrane. Then the membrane was washed for 24 hr in 200 ml of renaturation buffer (10 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 1 mM DTT, 10% (v/v) glycerol, and 5% (w/v) nonfat milk powder) with gentle agitation. The membrane was placed into heat-sealable plastic bags containing binding buffer (10 mM HEPES, 0.1 mM EDTA, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 10% glycerol and 0.25% nonfat milk opwder) and 2 μg of poly [dI-dC], and pretreated for 1 hr with gentle agitation to block nonspecific DNA binding. The binding buffer was then added with 6 X 10^6 cpm of ^{32}P-DNA per ml, and the filter was incubated at 4℃ overnight. After DNA binding, the filter was washed for two 20 min in 50-100 ml binding buffer, air dried, and exposed to X-ray films at -70℃.

RESULTS

Morphological Observations in Ginsenosides-Treated F9 Teratocarcinoma Stem Cells. To examine the effects of ginsenosides on the differentiation of F9 cells, the morphology of F9 cells were first tested after treating with total ginsenosides. F9 teratocarcinoma stem cells grew in culture as closely packed colonies, and it was difficult to distinguish cell-cell boundaries(fig. 1-A). F9 cells cultured in the presence of GSCT for 10 days formed a flat round cell (Fig. 1-C), and resembles F9 cells differentiated by RACT in shape (Fig. 1-B).

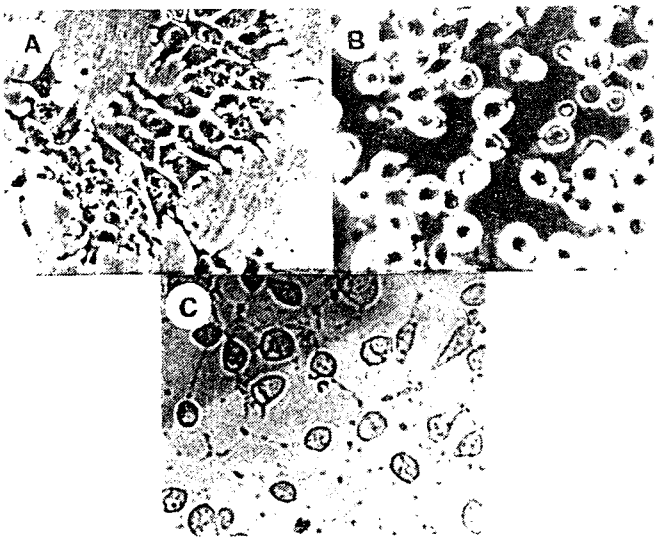

Fig. 1. Phase-contrast photomicrographs of F9 cells cultured in the presence of retinoic acid or ginseng saponin.
(A) F9 cells growing exponentially in the undifferentiated state
(B) F9 cells cultured in the presence of 1 μM of retinoic acid, 500 μM of dibutylyl-cAMP (dbcAMP), and 250 μM of theophylline (RACT) for 6 days.
(C) F9 cells cultured in the prescence of 40 μg/ml of total ginseng saponin, 500 μM of dbcAMP, and 250 μM of theophylline (GSCT) for 10 days.

Comparison of the Steady State mRNA Level of Differentiation - Specific Genes between Ginsenosides - and RA - treated F9 Cells. Here various aspects of the GSCT - treated F9 cell cultures have been analyzed to determine whether ginsenosides have differentiation - inducing activity. Firstly, the expressions of laminin B1, type Ⅳ collagen, and RARβ were examined during manipulations of differentiation with GSCT in F9 cells by Northern blot analysis and the mRNA levels were compared with those of F9 cells treated with RACT. As shown in Fig. 2, the mRNA levels of laminin B1, type Ⅳ collagen, and RARβin F9 cells treated with GSCT were increased rapidly and the levels were slightly lower than those of differentiated F9 cells induced by RACT (Fig. 2 - A, B, c). The mRNA level of β - actin was detected as a control and it remains the same in all three cases(fig. 2 - D). These findings suggested that basement membrane components, such as laminin and type Ⅳ collagen, were related with in the differentiation of F9 teratocarcinoma stem cell induced by ginsenosides.

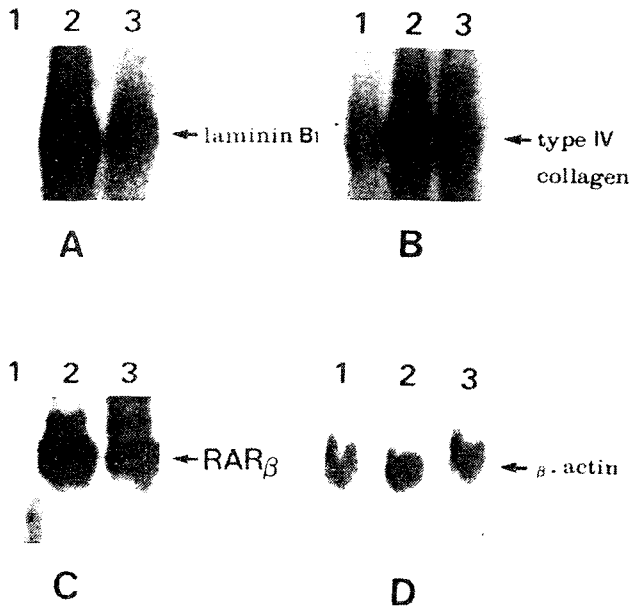

Fig. 2. Comparision of the steady state level of laminin Bi (A), type Ⅳ collagen (B), RARβ(C), and β - actin (D) mRNA in F9 cells during manipulation of differentiation by RACT or GSCT.

Total cellular RNAs from the F9 stem cells (lane 1), RACT - treated F9 cells (lane 2), and GSCT - treated F9 cells (lane 3) were isolated, electrophoresed, and transferred onto nylon membrane. Then the RNAs were hybridized with the 464 bp laminin B1 cDNA, 2 kb type Ⅳ collagen (α) **cDNA, 1.2 kb** β - RAR, and 1.35 kb fibronectin cDNA probes.

Effects of purified ginsenosides on the differentiation of F9 cells. We have shown that a crude fraction of ginsenosides extracted from *Panax ginseng*(C.A. Meyer) induced the differentiation of F9 cells as indicated in fig. 1 and 2. To obtain more information on the ginsenoside derivatives which have differentiation inducing - activity, F9 cells were cultured in the medium containing 14 ginsenoside derivatives {Rb_2, Rc, Rd, Re, Rf, Rh_1, Rh_2, Rg_1, Rg_3, Rg_3', Ro, panaxatriol (PT), panaxadiol (PD), protopanaxadiol (PPD)} with dbcAMP and then the morphological change and expression of type Ⅳ collagen was examined and analyzed by microdensitometer tracing of autoradiogram. Among them, 11 derivatives (Rb_2, Rc, Rd, Re, Rf, Rh_1, Rh_2, Rg_1, Rg_3, Rg_3', PT, and PD) induced the morphological alteration of F9 cells(data not shown) and 10 derivatives (Rb_2 Rc, Rd, Re, Rf, Rh_1, Rh_2, Rg_1, Rg_3, PT, and PD) increased the expression of type Ⅳ collagen (Fig. 3). Particularly, transcripts of type Ⅳ collagen was detected strongly in F9 cells treated with ginsenosides Rh_1 or Rh_2. These results indicate that ginsenosides induce the differentiation of F9 cells and among them ginsenosides Rh_1 and Rh_2 are most effective differentiation agents.

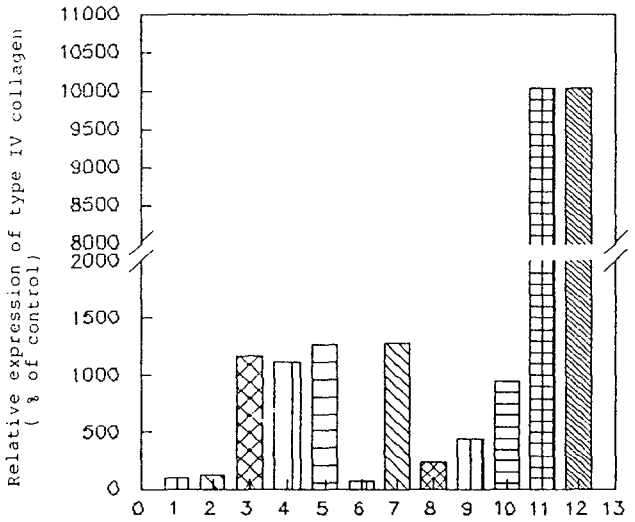

Fig. 3. Effects of purified ginsenosides on the expression of type Ⅳ collagen of F9 cells.
F9 stem cells, control (Lane 1), F9 cultured in the presence of dbcAMp with, Rb_2 (lane2), Rc(lane 3), Rd (lane 4), Re (lane 5), Rg_3' (lane 6), Rf (lane 7), PD (panaxadiol) (lane 8), PT (panaxatriol) (lane 9), Rg_3 (lane 10), Rh_1 (lane 11), and Rh_2 (lane 12) were isolated, hybridized with the type Ⅳ zcollagen cDNA, and analyzed by microdensitometer.

Southwestern blot analysis. With to inspection that the chemical structure of ginsenosides were very similar to that of steroid hormone, we have interested in the role of steroid receptor during the differentiation of F9 teratocarcinoma stem cells induced by ginsenosides. It is well known that steroid hormone binds tightly and specifically with the steroid receptor, increase its affinity for the nucleus (14), and thus the hormone - receptor complex may function in the control of differential patterns of gene expression (15). We therefore considered

the possibility that ginsenosides exert differentiation - inducing effects *via* steroid receptor. To investigate this possibility, we examined F9 cell proteins which selectively bind with the glucocorticoid responsive element (GRE) sequence. When protein extracts of F9 cells treated with 40 μg/ml total ginsenoside or 25 μM of dexamethasone was probed with ^{32}P - labeled PEPCK promoter cDNA containing the GRE sequence, both a high molecular weight protein (94 kDa) band and a low molecular weight protein (45 kDa) band were detected (Fig. 4). It was previously reported that a 97 kDa and a 45 kDa glucocorticoid receptor protein was present in HeLa S3 cells (16). Moreover, glucocorticoid receptor protein is very unstable and its 40 kDa proteolytic form was reported (17). According to these data, the 94 kDa protein in F9 cells treated with total ginsenoside or dexamethasone can be identified as a steroid receptor protein and the 45 kDa protein might be a proteolytic form of steroid receptor.

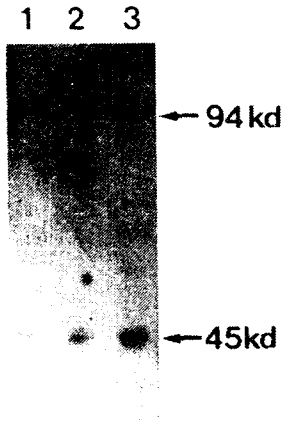

Fig. 4. Southwestern blot analysis of the glucocorticoid responsive element binding activity.
Total proteins from F9 cells (lane 1), incubated with total ginseng saponin (lane 2), and dexamethasone (lane 3) were electrophoresed, transferred onto nylon membrane and probed with ^{32}P - labeled phosphoenol - pyruvate carboxykinase promoter cDNA.

DISCUSSION

In many respect, cancer is fundamentally a disease of abnormal cell differentiation (3), and it may be controlled by agents which control cell differentiation rather than kill cells. Based on such considerations, we have searched for candidates of tumor cell differentiation agents utilizing F9 teratocarcinoma stem cells as a model system, because F9 stem cells, which does not undergo significant spontaneous differentiation, can be induced to differentiate upon the addition of differentiation agent like RA into a endoderm - like cells, which begin to synthesize basement membrane proteins, including laminin and type Ⅳ collagen (18, 19). In an effort to find naturally occurring subtances which may be utilized as differentiation inducing agents, we have identified that ginsenosides obtained from *Panax ginseng* C.A. Meyer induce the differentiation of F9 cells. F9 cells cultured in the medium containing 40 μg/ml crude ginsenosides extracted from *Panax ginseng* together with 500 μM of dbcAMP (GSCT) became flat round (data not shown). This is strikingly resembled with the parietal endoderm - like F9 cells differntiated by RA, a typical differentiation inducer. These results represent a possibility that ginsenosides have the ability to change the morphology of F9 stem cells into the differentiated form. To determine whether the morphological alteration of F9 stem cells into the differentiated form. To determine whether the morphological alteration of F9 stem cells into the differentiated form. To determine whether the morphological alteration of F9 cells is the result of the differentiation, the modulation of laminin B1, type Ⅳ collagen, and RARβ characterized as differentiated phenotype, by exogenous addition of ginsenosides as examined in F9 cells. The mRNA level of laminin B1, type Ⅳ collagen, and RARβ in parietal endoderm - like F9 cells was increased upon exposure to GSCT, and the levels were slightly lower than those of RACT - treated F9 cells. The effects of various ginsenoside fractions isolated from crude ginsenoside were examined using 14 ginsenoside fractions. Among them, 7 fractions (Rc, Rd, Re, Rf, Rh_1, Rh_2, and Rg_3) induced the differentiation of F9 cells. Furthermore, ginsenosides Rh_1 and Rh_2 were most effective during the differentiation of F9 cells in terms of the induction of morphological change and gene expression.

These results suggest that ginsenosides act as a differentiation agent like RA and it affects mainly the expression of basement membrane proteins such as laminin and type Ⅳ collagen. In contrast, RA is responsible of the various gene expressions involved in differentiation and proliferation. According to these results, the biosynthesis of basement membrane proteins, laminin and type Ⅳ collagen, are most strongly regulated by ginsenosides at the differentiation of F9 teratocarcinoma stem cells. Actions of ginsenosides on cancer cells have been reported (22, 23) but the detailed mechanisms of action of these plant glycosides are still unknown. We therefore speculate that ginsenosides bind the steroid receptor or their analogs because steroid hormones and ginsenosides are very similar in their gross molecular shapes and steroids with glucocorticoid activity are reported to induce the differentiation of myeloid leukemia cells into macrophages and granulocytes (24). According to the Southwestern blot analysis using probes containing GRE, a 94kDa protein was detected in F9 cells treated with 40 μg/ml crude ginsenosides. Several lines of previous published evidence indicate that the 94kDa protein with which the GRE interacts is the glucocorticoid receptor (13). These results suggest ginsenosides have differentiation inducing effects through binding with steroid receptor. But actual cellular mechanism by which ginsenosides modulate phenotypic expressions has not yet been unraveled. Moreover, how can individual ginsenosides elicit such a different response is unknown as yet and identification of the receptor contacted by ginsenosides is required. Nevertheless, it seems

that some chamical compounds having steroid skeleton affect the differentiation of cancer cells. The study of the effects of ginsenosides on cancer cells will be valuable in illuminating the relationship between growth capacity and differentiation and in advancing effective therapies for cancer.

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