Pergamon

European Journal of Cancer Vol. 32A, No. 8, pp. 1420–1428, 1996

Printed in Great Britain
0959-8049/96 $15.00+0.00

PII: S0959-8049(96)00102-5

Original Paper

In Vitro Induction of Differentiation by Ginsenosides in F9 Teratocarcinoma Cells

Y.-N. Lee,[1] H.-Y. Lee,[1] H.-Y. Chung,[2] S.-I. Kim,[3] S.-K. Lee,[4] B.-C. Park[5] and K.-W. Kim[1]

Departments of [1]Molecular Biology and [2]Pharmacy, Pusan National University, Pusan; [3]Korea Ginseng and Tobacco Research Institute, Daejeon; [4]College of Pharmacy, Seoul National University, Seoul; and [5]Kosin Cancer Research Institute, Kosin University, Pusan, Korea

The aim of this study was to determine the ability of the ginsenosides, extracts of *Panax ginseng* C.A. Meyer, to cause differentiation of F9 teratocarcinoma stem cells as a model system. F9 stem cells cultured in the presence of the ginsenosides together with dibutyryl cyclic AMP (dbcAMP) became parietal endoderm-like cells. Moreover, the expression of differentiation marker genes, such as laminin B1 and type IV collagen, was increased after treatment with the ginsenosides. Among the various purified ginsenosides, Rh_1 and Rh_2 were the most effective at causing differentiation of F9 cells. Since ginsenosides and glucocorticoid hormone have similar chemical structures, we examined the possibility of the involvement of a glucocorticoid receptor (GR) in the differentiation process induced by the ginsenosides. According to Southwestern blot analysis, a 94 kDa protein regarded as a GR was detected in F9 cells cultured in the medium containing the ginsenosides Rh_1 or Rh_2. In addition, F9 stem cells treated with the ginsenosides Rh_1 or Rh_2 and with RU486, a glucocorticoid antagonist with a high affinity for the GR, did not differentiate into endoderm cells morphologically, and the expression of laminin B1 gene was not induced in these cells. In a gel mobility shift assay, protein factors capable of binding to the glucocorticoid responsive element (GRE) specifically were detected in nuclear extracts of the ginsenoside-treated F9 cells. Moreover, overexpression of GR by cotransfection of GR expression vector and GRE-luciferase vector enhanced the transactivation activity of GRE promoter in the presence of ginsenosides Rh_1 or Rh_2 and was further augmented by dbcAMP. In addition, ginsenosides Rh_1 and Rh_2 bound to a GR assessed by whole-cell binding assay, even though the specific binding affinity was weaker compared to dexamethasone. Based on these data, we suggest that the ginsenosides Rh_1 and Rh_2 cause the differentiation of F9 cells and the effects of ginsenosides might be exerted via binding with a GR or its analogous nuclear receptor.

Key words: ginsenosides, differentiation, F9 teratocarcinoma cells
Eur J Cancer, Vol. 32A, No. 8, pp. 1420–1428, 1996

INTRODUCTION

THERE HAS been an increasing interest in compounds that inhibit growth and induce differentiation of cancer cells which, compared with normal cells, tend to be less differentiated [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]. A representative differentiation agent, retinoic acid (RA), has recently demonstrated promising results in several clinical trials for the prevention of cancer. RA appears to play a major role in embryogenesis and homeostasis [4]. However, several observations concerning the cytotoxic activities of RA have been reported [3, 5], and we therefore have searched for naturally occurring non-toxic substances that induce the differentiation of cancer cells. 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, in addition, the differentiation response of F9 cells is irreversible and relatively synchronous [6, 7]. Moreover, several differences in gene expression have been documented and serve as convenient markers of differentiation of F9 stem cells [8–10].

Correspondence to K.-W. Kim.
Received 10 Apr. 1995; revised 18 Jan. 1996; accepted 23 Jan. 1996.

Ginsenosides are obtained from *Panax ginseng* C.A. Meyer, an important plant in oriental medicine. Recently, the chemical structure and biological effects of ginsenosides have been widely studied, and it has been revealed that extracts of *Panax ginseng* have been known to stimulate RNA and protein synthesis in rat liver and kidney *in vivo* [11]. The ginsenosides Rh_1 and Rh_2, purified from these crude materials, affected the growth of B16 melanoma cells and the expression of their phenotype [12, 13]. Ginsenosides Rh_1 and Rh_2, 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. Odashima and associates [14] reported that Rh_2 causes growth inhibition and stimulates melanogenesis in the cultured B16 melanoma cells, whereas Rh_1 simply stimulates melanogenesis.

This study examined the effects of ginseng extracts and compared the activity of various purified ginsenosides on the differentiation of F9 teratocarcinoma stem cells. Because ginsenosides are plant glycosides with molecular structures remarkably similar to the glucocorticoid hormone, the effect of glucocorticoid receptors (GR) on the differentiation of F9 cells was studied to clarify the relationship between ginsenosides and GR during the differentiation process. Experiments reported herein demonstrate that ginsenosides, especially Rh_1 and Rh_2, cause the differentiation of F9 cells and the effects of ginsenosides are probably exerted by forming a complex with the GR.

MATERIALS AND METHODS

Materials

F9 cells were obtained from the American Type Culture Collection (ATCC CRL 1720). RA was purchased from Sigma, and Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco Laboratories. Tissue culture flasks were from Costar, restriction endonucleases were purchased from Promega and nitrocellulose papers and ^{32}P-dCTP were from Amersham. Guanidinium thiocyanate and P60 gel were from Bethesda Research Laboratories. RU486 was kindly provided by Roussel, UCLAF (France). Other chemicals were purchased from Sigma.

Cell culture

F9 stem cells were grown on gelatinised plastic flasks in DMEM, supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. For the induction of differentiation, the cells were cultured in a DMEM supplemented with 2% FBS. The medium was changed twice a week, and subcultured every 5 days with one medium change. Cells used for differentiation experiments were between passages 1 and 7.

Purification of ginsenosides

Fourteen fractions of ginsenosides were purified according to the method described by Kitagawa and associates [15]. Powdered red ginseng (steamed and dried roots of *Panax ginseng* C.A. Meyer, Araliaceae which was produced by the Monopoly Bureau of Korea) was refluxed with methanol. The extract, concentrated to dryness, was suspended in water and extracted with *n*-butanol (saturated with water). The extract was dried, and this dried powder was used as total ginsenosides. To purify each ginsenoside, total ginsenosides powder was dissolved in methanol. To the methanol solution of total ginsenosides, diethylether was added with stirring to provide an ether-soluble and ether-insoluble portion. Thus, repeated chromatography of the ether-soluble portion using Bondapek C_{18} column eluting with methanol–water (1:1 → 7:3) and silica gel eluting with $CHCl_3$-methanol (10:1) provided ginsenosides Rh_1 and Rh_2. The ether-insoluble portion was submitted to column chromatography on silica gel eluting with $CHCl_3$–methanol–water (65:35:10, the low phase) and *n*-butanol–ethylacetate–methanol–water (4:2:1:1, the low phase), and Bondapek C_{18} eluting with methanol–water (1:1→7:3) to give ginsenoside Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_3, $Rg_{3'}$, Ro, panaxatriol, panaxadiol and protopanaxadiol. The purity of each ginsenoside was determined by melting point, optical rotation, and pos. FAB-MS spectra. The melting points were determined by Fisher-John Apparatus, optical rotations were measured with Jasco DIP-370 Instrument, and pos FAB-MS spectra were determined with VG-VSEQ (type EBqQ).

In vitro *induction of differentiation in F9 cells*

For differentiation, F9 stem cells were trypsinised and seeded on to gelatinised T75 tissue culture flasks containing 25 ml of culture medium and incubated for 24 h prior to the addition of drugs. For induction of differentiation by RA, 1×10^{-6} M all *trans*-RA, 5×10^{-4} M dibutyryl cyclic AMP (dbcAMP) and 2.5×10^{-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 ginsenosides, 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (GSCT). The effects of various ginsenosides on F9 cells were examined using medium containing 5×10^{-5} M ginsenosides, 5×10^{-4} M dbcAMP and 2.5×10^{-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 ginsenosides were a 10^{-1} M aqueous solution. After differentiation, cell morphology was judged by phase-contrast microscopy. To test the effect of the synthetic antiglucocorticoid drug, RU486, on the differentiation of F9 cells, F9 cells were plated in T75 culture flasks, and 5×10^{-5} M ginsenoside Rh_1 plus 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (Rh_1CT) together with 1.5×10^{-5} M RU486 were added 1 day after plating. Cells pretreated with 1.5×10^{-5} M RU486 for 3 days to occupy GR completely were also treated with Rh_1CT. At the end of treatment, cells were examined under the phase-contrast photomicroscopy.

Preparation of radiolabeled DNA probes

The 464 bp *Eco*RI fragment of mouse laminin B1, 2.0 kb *Pst*1 fragment of mouse type IV collagen (α2), 0.6 kb *Pst*1 fragment of mouse β-actin, 200 bp *Bst*EII-*Sac*I fragment of glucocorticoid responsive element (GRE) present in pMTV-CAT cDNA and 274 bp *Eco*RI-*Hind*III fragment of phosphoenol pyruvate carboxykinase (PEPCK) promoter cDNA, were used as DNA probes. They were labelled by the random primer labelling method [16].

RNA preparation and Northern blot analysis

Total cellular RNA was isolated according to the procedure of the acid-guanidinium thiocyanate–phenol–chloroform (AGPC) extraction method [17]. Equal amounts (20 μg) of denatured RNA were fractionated on 1% agarose gels

containing 1.0 M formaldehyde, and transferred to nylon membranes (Zeta-Probe, Bio-Rad, California, U.S.A.). After baking at 80°C for 2 h, blots were hybridised in a solution containing 50% deionised formamide, 0.25 M $NaHPO_4$, 7% sodium dodecyl sulphate (SDS), and 1 mM EDTA at 42°C in the presence of ^{32}P-labelled cDNA probes for 24 h. Blots were washed three times for 5 min with 2 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at room temperature, followed by two 15-min washes in 0.1 × SSC and 0.1% SDS at 55°C. The filters were autoradiographed at −80°C. Then, the X-ray films were developed.

Southwestern blot analysis of the DNA binding activity in protein extracts of F9 cells

The Southwestern hybridisation technique was adopted from the modified method of Silva and associates [18]. Approximately 2–4 × 10^8 F9 cells were incubated in the medium containing either 5 × 10^{-5} M ginsenoside Rh_1 or 2.5 × 10^{-5} M dexamethasone (Dex) for 2 h at 0°C 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 dithiothreitol (DTT), 1 mM phenylmethylsulphonyl-fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin, and then sonicated. The mixtures were immediately centrifuged at 13000*g* for 10 min at 0°C and the supernatant was transferred to a fresh tube. Protein concentration was measured by the method of Bradford [19]. 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 on to Hybond C-extra nylon membrane. Then the membrane was washed for 24 h 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) non-fat 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% non-fat milk powder) and 2 μg of poly(dI-dC) (Pharmacia), and pretreated for 1 h with gentle agitation to block non-specific DNA binding. The binding buffer was then added with 6 × 10^6 cpm of ^{32}P-DNA per ml, and the filter was incubated at 4°C overnight. After DNA binding, the filter was washed for 2 × 20 min in 50–100 ml binding buffer, air dried, and exposed to X-ray films at −70°C.

Gel mobility shift assay

Nuclear extracts from F9 stem cells and F9 cells treated with 5 × 10^{-5} M ginsenoside Rh_1 or 2.5 × 10^{-5} M Dex as a control were prepared as described by Dignam and associates [20]. The double-stranded oligonucleotides for the GRE sequences were 5′-end labelled with [α-^{32}P]ATP using T4 polynucleotide kinase (Promega) and purified by ethanol precipitation. Indicated amounts of F9 nuclear extracts preincubated with 2 μg of poly(dI-dC) at 0°C for 15 min were incubated with labelled oligonucleotide (approximately 5 fmol, 10000 cpm) for 20 min at room temperature in buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10 mM $MgCl_2$, 10% (v/v) glycerol, 0.05% NP40. In gel shift competition assay, 10 μg of F9 nuclear extracts were preincubated with 2 μg of poly(dI-dC) and 25× or 50× unlabelled oligonucleotide at 0°C for 15 min, and then further reacted with the probe for 20 min at room temperature. Protein-DNA complexes were separated from protein-free DNA by non-denaturing 4% polyacrylamide (29:1, acrylamide: bisacrylamide) gel electrophoresis in buffer containing 50 mM Tris–HCl, 0.5 M glycine, 2 mM EDTA, pH 8.5 at 4°C. Gels were run at constant voltage of 200 V. After suitable separation was achieved, the gel was vacuum dried for autoradiography and exposed to Kodak XAR-5 film.

Transient transfection assay

Transient transfections were performed in F9 stem cells (5 × 10^5 cells/60 mm dish) using the calcium phosphate technique with 5 μg of human glucocorticoid receptor expression plasmid (RShGRα), 5 μg of GRE-luciferase reporter plasmid (pGL2-GRE), and 1 μg of β-galactosidase containing plasmid (pMMuLV-SV-lacZ) as described previously [21]. Cells were exposed to the precipitate for 12–24 h and washed by fresh medium and then cultured in the absence or presence of 2.5 × 10^{-5} M dexamethasone, 5 × 10^{-5} M ginsenosides Rh_1 or Rh_2 alone or plus 5 × 10^{-4} M dibutyryl cyclic AMP (dbcAMP) and 2.5 × 10^{-4} M theophylline (DexCT, Rh_1CT, Rh_2CT). Cell extracts were prepared after 30–36 h and assayed for luciferase enzyme activity using luciferase assay system (Promega, U.S.A.). To normalise for transfection efficiency, luciferase activities were corrected for β-galactosidase activity. Each transfection was performed in triplicate, and the fluorescence of each sample was measured with a luminometer.

Whole-cell ligand binding assay

The receptor binding assay was carried out as described previously [22]. F9 cells (5 × 10^6) were cultured for 1 h at 37°C with 10 nM of [1,2,4,6,7-^{3}H]dexamethasone (83 Ci/mmol, Amersham, U.K.) in the absence or presence of 10-fold, 100-fold and 500-fold excess of unlabelled dexamethasone, ginsenosides Rh_1 or Rh_2 as a competitor. Then the cells were washed three times in ice-cold PBS to remove free ligand. Cell pellets were solubilised in a toluene-based, Triton X-100-containing scintillation cocktail. Radioactivity in whole-cell pellets was assayed by a liquid scintillation counter.

RESULTS

Morphological observations in ginsenosides-treated F9 *teratocarcinoma stem cells*

F9 teratocarcinoma stem cells usually grow in culture as closely packed colonies, and it is difficult to distinguish cell–cell boundaries (Figure 1a). F9 cells cultured in the presence of GSCT for 10 days formed a flat round cell (Figure 1b), and resembled F9 cells differentiated by RACT in shape (Figure 1c).

Effects of purified ginsenosides on the differentiation of F9 *cells*

To exclude the possibility that the differentiation inducing effects of total ginsenosides are mediated partly through co-purified retinoids present in these extracts, we repeated the differentiation-inducing experiments with purified ginsenosides. The purity of preparations of ginsenosides was confirmed by melting point [Mp(°C)], optical rotation ($[\alpha]_D$), and mass spectra (pos. FAB-MS $[M + 1]^+$) as shown in Table 1. F9 cells were cultured in the medium containing 14 ginsenosides [Rb_2, Rc, Rd, Re, Rf, Rh_1, Rh_2, Rg_1, Rg_3, $Rg_{3'}$, Ro, panaxatriol

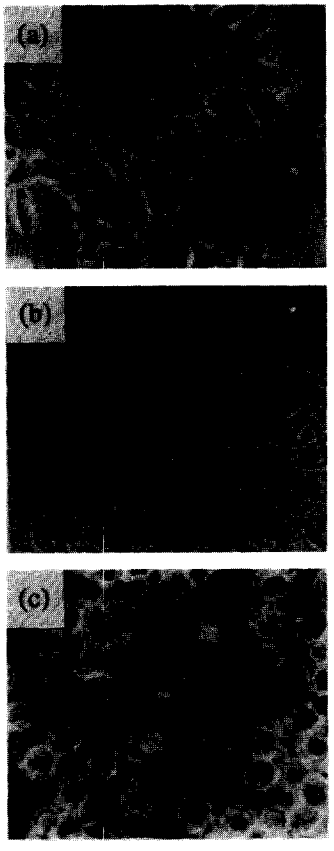


Figure 1. Phase-contrast photomicrographs of F9 cells cultured in the presence of retinoic acid or ginsenosides. (a) F9 cells growing exponentially in the undifferentiated state. (b) F9 cells cultured in the presence of 40 μg/ml crude ginsenosides, 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (GSCT) for 10 days. (c) F9 cells cultured in the presence of 1×10^{-6} M retinoic acid, 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (RACT) for 6 days.

(PT), panaxadiol (PD), protopanaxadiol (PPD)], respectively, with dbcAMP and theophylline (CT) and then the morphological change and expression of marker gene (laminin B1 and type IV collagen) were examined. Among them, 11 ginsenosides (Rb_2, Rc, Rd, Re, Rf, Rh_1, Rh_2, Rg_3, $Rg_{3'}$, PT, and PD) induced the morphological alteration of F9 cells (data not shown). Particularly, transcripts of laminin B1 and type IV collagen were detected strongly in F9 cells treated with ginsenosides Rh_1 (Figure 2a) or Rh_2 (Figure 2b). The mRNA levels of laminin B1 in the Rh_1CT-treated F9 cells were much higher than the CT-treated cells and almost the same as the RACT-treated F9 cells (Figure 2b).

Southwestern blot analysis

With the observation that the chemical structure of ginsenosides is very similar to that of glucocorticoid hormone as shown in Table 2, we became interested in the role of GR during the differentiation of F9 teratocarcinoma stem cells induced by ginsenosides. We therefore considered the possibility that ginsenosides exert differentiation-inducing effects via GR. 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 5×10^{-5} M ginsenoside Rh_1 or 2.5×10^{-5} M Dex was probed with ^{32}P-labelled 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 (Figure 3a). Ginsenoside Rh_2 showed a similar result (data not shown). In addition, a 94 kDa protein band was also detected when probed with ^{32}P-labelled GRE cDNA (Figure 3b). It has been previously reported that a 97 kDa and a 45 kDa GR protein is present in HeLa S3 cells [18]. Moreover, GR protein is very unstable and its 45 kDa proteolytic form has been reported [23]. According to these data, the 94 kDa protein in F9 cells treated with ginsenoside Rh_1 or Dex can be identified as a GR protein and the 45 kDa protein might be a proteolytic form of GR.

Tests for the effects of RU486 on the differentiation of F9 cells

To confirm that ginsenosides bind with GR, we examined the action of a glucocorticoid antagonist, RU486 (Figure 4a), on the differentiation of F9 cells by ginsenosides Rh_1 or Rh_2. The result with Rh_2 is similar with that of Rh_1, hence, only the data of Rh_1 is shown here. Figure 4bii shows that F9 cells treated with Rh_1CT alone became round. Most of F9 cells pretreated with 1.5×10^{-5} M RU486 for 3 days and then treated with Rh_1CT did not acquire the differentiated morphology (Figure 4biii). In addition, it was observed that treatment with 1.5×10^{-5} M RU486 alone showed no increase in laminin B1 mRNA and exposure to Rh_1CT induced expression of laminin B1 in F9 cells. RU486 inhibited the expression of laminin B1 in F9 cells either pretreated with 1.5×10^{-5} M RU486 and then induced to differentiate by Rh_1CT or treated with 1.5×10^{-5} M RU486 and Rh_1CT at the same time. However, the mRNA level of laminin B1 was not changed in RACT plus RU486 treated F9 cells (Figure 4c). These results suggest that ginsenosides Rh_1 and Rh_2 might have agonistic activity of GR in F9 cells.

Gel mobility shift assay

A gel mobility shift assay was used to investigate the binding of GRE to nuclear protein extracts of F9 cells treated with ginsenosides to examine whether the MMTV DNA binding protein of 94 kDa was fortuitous or was, in fact, indicative of a selective interaction between GR and GRE. Nuclear extracts of F9 cells treated with 5×10^{-5} M ginsenosides Rh_1 or Rh_2 (data not shown) or 2.5×10^{-5} M Dex as a control were incubated at 0°C for 15 min in the presence of poly(dI-dC) to decrease non-specific binding, and then for 20 min at room temperature with the ^{32}P-labelled GRE oligonucleotide. The sequence of GRE oligonucleotide was 5′-TCG ACT GTA CAG GAT GTT CTA GCT ACT-3′ and was purchased from Promega. As shown in Figure 5a, the nuclear proteins of Dex-treated F9 cells formed a complex with GRE, whereas no complex was detected with the untreated F9 cells. With increasing concentrations of the nuclear proteins of Rh_1-treated F9 cells, a single band with increasing density, migrating slower than the uncomplexed DNA probe, was detected (Figure 5b). Since these results indicate that the nuclear factors form a DNA–protein complex, competition experiments were carried out to define the specificity of the complex. We allowed the complex to form in the presence of unlabelled GRE oligonucleotide. A very weak retarded complex was observed when unlabelled GRE oligonucleotide was added to the reaction mixture, and we found that increasing amounts of unlabelled GRE increasingly blocked the complex formation which was almost complete at a 50-fold molar excess of competitor DNA (Figure 5c).

Table 1. *Purity of preparations of ginsenosides*

Ginsenosides	Properties	Mp(°C)	$[\alpha]_D$	pos. FAB-MS $[m + 1]^+$	Formula
Rb_2	White powder	201~203	$[\alpha]_D^{22}$ 4.1° (C = 1.28, MeOH)	1079	$C_{53}H_{90}O_{22}$
Rc	White powder	199~202	$[\alpha]_D^{22}$ 2.1° (C = 1.73, MeOH)	1079	$C_{53}H_{90}O_{22}$
Rd	White powder	206~208	$[\alpha]_D^{22}$ 21.5° (C = 1.63, MeOH)	947	$C_{48}H_{82}O_{18}$
Re	White powder	201~203	$[\alpha]_D^{15}$ 1.2° (C = 1.06, MeOH)	947	$C_{48}H_{82}O_{18}$
Rf	White powder	197~198	$[\alpha]_D^{15}$ 1.2° (C = 0.98, MeOH)	801	$C_{42}H_{72}O_{14}$
Rg_3	White powder	298~300	$[\alpha]_D^{24}$ − 15.1° (C = 0.86, C_5H_5N)	785	$C_{42}H_{72}O_{13}$
$Rg_{3'}$	White powder	187~195	$[\alpha]_D^{25}$ 5.9° (C = 1.25, MeOH)	767	$C_{42}H_{70}O_{12}$
Rh_1	White powder	159~161	$[\alpha]_D^{25}$ 22.2° (C = 1.17, MeOH)	639	$C_{36}H_{62}O_9$
Rh_2	White powder	218~220	$[\alpha]_D^{19}$ 20.8° (C = 1.16, MeOH)	623	$C_{36}H_{62}O_8$
Panaxadiol	White powder	237~238	$[\alpha]_D^{25}$ 21.4° (C = 1.25, $CHCl_3$)	461	$C_{30}H_{52}O_3$
Panaxatriol	White powder	238~239	$[\alpha]_D^{25}$ 15.1° (C = 1.72, $CHCl_3$)	476	$C_{30}H_{52}O_4$

Transactivation studies using GRE–luciferase construct

To determine whether ginsenosides Rh_1 and Rh_2 act on GR-mediated gene transcription, we investigated transcriptional activity of GR through GRE in the presence of Rh_1 or Rh_2 (5×10^{-5} M) and with or without 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (Rh_1CT, Rh_2CT), and the activity was compared with dexamethasone (2.5×10^{-5} M). F9 cells were transfected with GR expression plasmid and GRE-luciferase reporter plasmid, and the luciferase activity of the cells was monitored. As shown in Figure 6a, ginsenosides Rh_1 or Rh_2 doubled luciferase activity compared with controls and the levels were similar to that produced by dexamethasone. To evaluate whether the inductive effect of ginsenosides was mediated via cAMP as a second messenger, GR-induced transactivation was determined in the presence of dbcAMP and theophylline (CT). As indicated in Figure 6b, dbcAMP revealed a strong additive effect on the GRE-transactivation. Taken together, dbcAMP alone doubled the luciferase activity compared with that of F9 stem cells, whereas Rh_1CT and Rh_2CT increased the activity by approximately 6-fold, and the levels were somewhat lower than DexCT.

Glucocorticoid receptor binding assay

The binding affinities of ginsenosides to GR were estimated by examining their inhibitory effects on the specific binding of [^{3}H]dexamethasone (Figure 7). Ginsenosides Rh_1 and Rh_2 showed somewhat lower binding affinity for GR than dexamethasone. The inhibitory effects were approximately 60% of the specific binding of [^{3}H]dexamethasone when treated with 500-fold excess ginsenoside Rh_1.

DISCUSSION

In many respects, cancer is fundamentally a disease of abnormal cell differentiation [3], and it could be controlled by agents which control cell differentiation rather than kill cells. Differentiation of F9 teratocarcinoma stem cells provides a unique opportunity to explore new differentiation inducers because F9 cells, which do not undergo significant spontaneous differentiation, can be induced to differentiate, upon the addition of agents such as RA, into endoderm-like cells, which begin to synthesise retinoic acid receptor β and basement membrane proteins including laminin and type IV collagen [4, 24]. Thus, the F9 cell is a useful model system for searching for new differentiation inducers.

Recently, a number of chemical compounds that inhibit growth and induce phenotypic reversion in certain cancer cells have been found and studies also suggest that several of these compounds have potential value as chemopreventive or therapeutic agents [25, 26]. In order to find new differentiation agents, we have screened many compounds which have been used as antitumour agents isolated from plant sources using F9 teratocarcinoma stem cells as a model system in this work. We have shown that total ginsenosides obtained from *P. ginseng* C.A. Meyer induce differentiation of F9 cells. F9 cells cultured in the medium containing total ginsenosides together with dbcAMP (GSCT) became flat and round. They strikingly resembled the parietal endoderm-like F9 cells differentiated by RA, a typical differentiation inducer. These results suggest the 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 cells is the result of differentiation, the modulation of

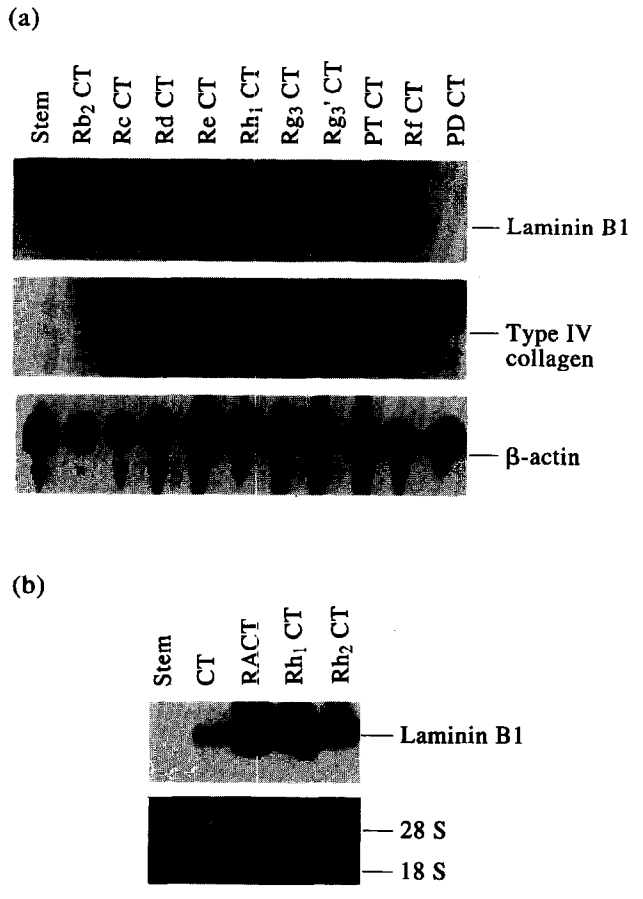
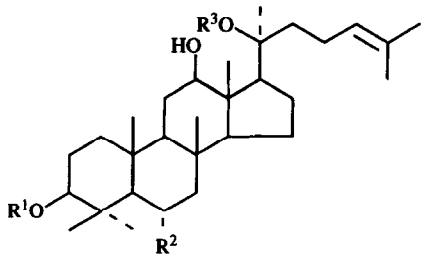


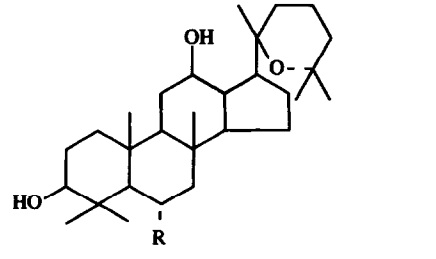
Figure 2. Effects of various ginsenosides on the expression of laminin B1 and type IV collagen of F9 cells. (a) Total cellular RNAs from F9 stem cells, F9 cells cultured in the presence of 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline with 5×10^{-5} M Rb_2, Rc, Rd, Re, Rh_1, Rg_3, Rg_3', PT (panaxatriol), Rf, PD (panaxadiol), respectively, were isolated, and hybridised with ^{32}P-labelled laminin B1, type IV collagen, and β-actin cDNA probes. (b) Total cellular RNAs from F9 stem cells, F9 cells cultured in the presence of 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (CT), 1×10^{-6} M retinoic acid plus 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (RACT), 5×10^{-5} M Rh_1 plus 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (Rh_1CT), 5×10^{-5} M Rh_2 plus 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (Rh_2CT), respectively, were isolated, and hybridised with a ^{32}P-labelled laminin B1 cDNA probe.

laminin B1 and type IV collagen in F9 cells by exogenous addition of ginsenosides was examined using Northern blot analysis. The mRNA levels of laminin B1 and type IV collagen in parietal endoderm-like F9 cells were increased upon exposure to GSCT, and the levels were slightly lower than those of RACT-treated F9 cells. These results suggest that ginsenosides act as a differentiation agent like RA, and that they affect mainly the expression of basement membrane proteins such as laminin and type IV collagen. The effects of various ginsenoside fractions isolated from total ginsenosides were examined using 14 ginsenosides. Among them, ginsenosides Rh_1 and Rh_2 were most effective for differentiation of F9 cells in terms of the induction of morphological change and gene expression. These results can be compared with that of Odashima and associates [14]. They reported that purified Rh_2 caused a reverse transformation of mouse B16 melanoma cells, inhibited their growth and stimulated melanogenesis, and induced two patterns of response, G1 phase arrest and S

Table 2. Structural formulae of ginsenosides purified from Panax ginseng *C.A. Meyer*



20(*S*)-protopanaxadiol group			20(*S*)-protopanaxatriol group		
	R^2 = H			R^1 = H, R^2 = O-R^4	
	R^1	R^3		R^4	R^3
PPD	-Glc^2-Glc	-H	PPT	-H	-H
Rb_2	-Glc^2-Glc	-Glc^6-Ara(p)	Re	-Glc^2-rham	-Glc
Rc	-Glc^2-Glc	-Glc^6-Ara(f)	Rf	-Glc^2-Glc	-H
Rd	-Glc^2-Glc	-Glc	Rh_1	-Glc	-H
Rg_3	-Glc^2-Glc	-H			
Rh_2	-Glc	-H			



R = H Panaxadiol R = OH Panaxatriol

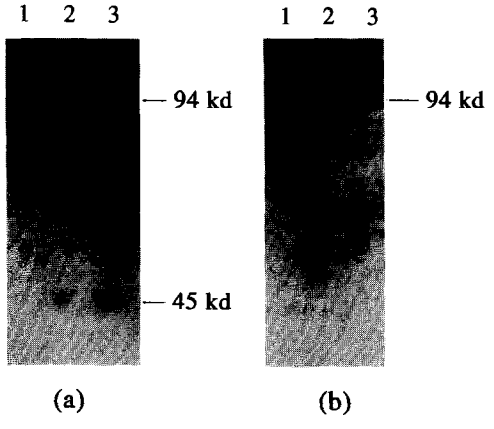


Figure 3. Southwestern blot analysis of the glucocorticoid responsive element binding activity. Total proteins from F9 cells (lane 1), incubated with 5×10^{-5} M ginsenoside Rh_1 (lane 2), or 2.5×10^{-5} M dexamethasone (lane 3) were electrophoresed, transferred on to nylon membrane and probed with ^{32}P-labelled phosphoenol-pyruvate carboxykinase promoter (a) and ^{32}P-labelled pMTV-CAT cDNA (b).

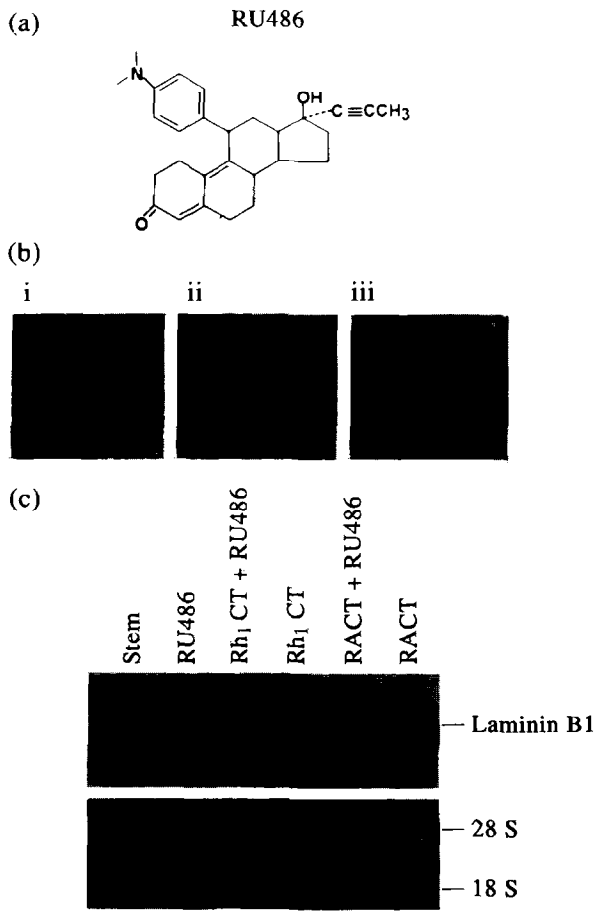


Figure 4. Effects of RU486 on the differentiation of F9 cells. (a) Chemical structure of RU486. (b) Phase-contrast photomicrographs of F9 stem cells (i), F9 cells treated with 5×10^{-5} M ginsenoside Rh_1 plus 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (Rh_1CT) (ii), and F9 cells pretreated with 1.5×10^{-5} M RU486 and then treated with Rh_1CT (iii). (c) Northern blot analysis of laminin B1 mRNA in F9 stem cells, F9 cells treated with 1.5×10^{-5} M RU486, 1.5×10^{-5} M RU486 plus Rh_1CT, Rh_1CT, 1.5×10^{-5} M RU486 plus 1×10^{-6} M retinoic acid, 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (RACT), and RACT.

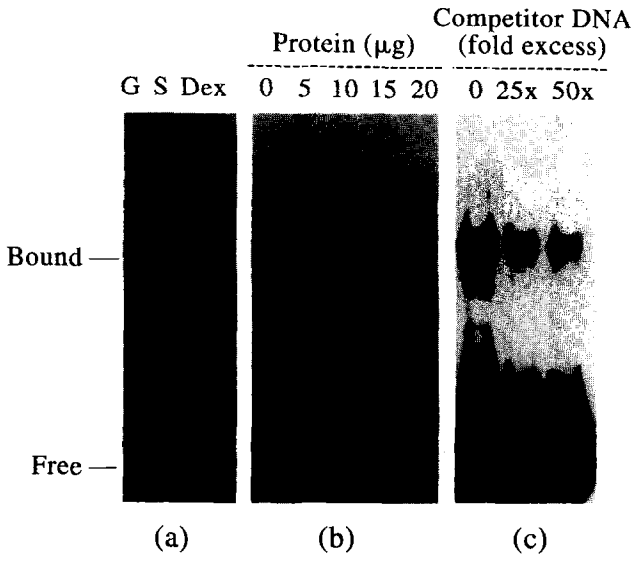


Figure 5. Specific binding of the F9 nuclear proteins to glucocorticoid responsive element (GRE) sequence. (a) Labelled GRE DNA but no extract (G), nuclear extracts from undifferentiated F9 cells as a negative control (S), F9 cells treated with 2.5×10^{-5} M dexamethasone (Dex) as a positive control were reacted with a ^{32}P-labelled GRE. (b) The indicated quantities of nuclear proteins (μg) from the F9 cells treated with 5×10^{-5} M ginsenosides Rh_1 were tested for DNA binding activity with a ^{32}P-labelled GRE oligonucleotide. (c) Nuclear extracts from the F9 cells treated with 5×10^{-5} M ginsenoside Rh_1 were reacted with a ^{32}P-labelled GRE probe in the absence or presence of 25× and 50× unlabelled cold GRE probe.

phase prolongation, in the tumour cell lines [14]. They also showed that Rh_2 is the most effective at inhibiting B16 melanoma cell growth among many ginsenosides with similar chemical structures extracted from *P. ginseng* C.A. Meyer.

In addition, actions of ginsenosides on cancer cells and epidemiological studies on the preventive effect of ginsenosides against various human cancers have been reported [13, 14, 27, 28], but the detailed mechanisms of action of these plant glycosides are still unknown. We therefore speculate that ginsenosides bind the GR or its analogue(s) because glucocorticoid hormones and ginsenosides are very similar in their gross molecular shapes. The GR is a member of the nuclear receptor family of ligand-inducible transcription factors [29–31]. The ligand-activated GR binds to GREs, located close to hormone-regulated genes and, depending on the gene, activates or inhibits gene transcription by a still largely unknown mechanism [32]. It is widely assumed that transcriptional transactivation by the glucocorticoids occurs via contacts between parts of the receptor protein and proteins of the transcriptional apparatus [33]. Based on such considerations, we investigated the involvement of GR during ginsenoside-induced differentiation of F9 cells using Southwestern analysis, gel mobility shift analysis, treatment with the antiglucocorticoid reagent, RU486, transactivation studies using GRE-luciferase construct and GR binding assay.

According to the Southwestern blot analysis using probes containing GRE, a 94 kDa protein was detected in F9 cells treated with ginsenosides Rh_1 or Rh_2. Several lines of evidence indicate that the 94 kDa protein with which the GRE interacts is the GR [18]. Moreover, GR protein is very unstable and its 45 kDa proteolytic form has been reported [23]. Thus, it is likely that the 94 kDa protein in F9 cells treated with ginsenosides Rh_1 or Rh_2 is a GR and the 45 kDa protein is a proteolytic form of GR. Furthermore, F9 cells treated with Rh_1CT and RU486 did not differentiate into endoderm cells in terms of morphological change and laminin B1 gene expression. The steroid analogue RU486 is a potent antagonist of glucocorticoids and it binds with high affinity to GR to compete with the natural ligand [34]. The mechanism of action of RU486 lies in its ability to bind GR with high affinity and to behave as an antagonist in the transcriptional control of a target gene in target cells [35–39]. Therefore, the blocking by RU486 of differentiation of F9 cells induced by ginsenosides Rh_1 or Rh_2 suggests that Rh_1 and Rh_2 may be agonistic to GR in F9 cells. In the gel shift assay, we identified a nuclear protein which is capable of binding to the GRE in the Rh_1- or Rh_2-treated F9 cells. With increasing concentrations of this nuclear protein, a single band with increasing density, migrating slower than the uncomplexed DNA probe, was detected by autoradiography. The specificity of the slower complex was confirmed by competition experiments. A weak complex was formed between F9 nuclear proteins and GRE probe when cold GRE was

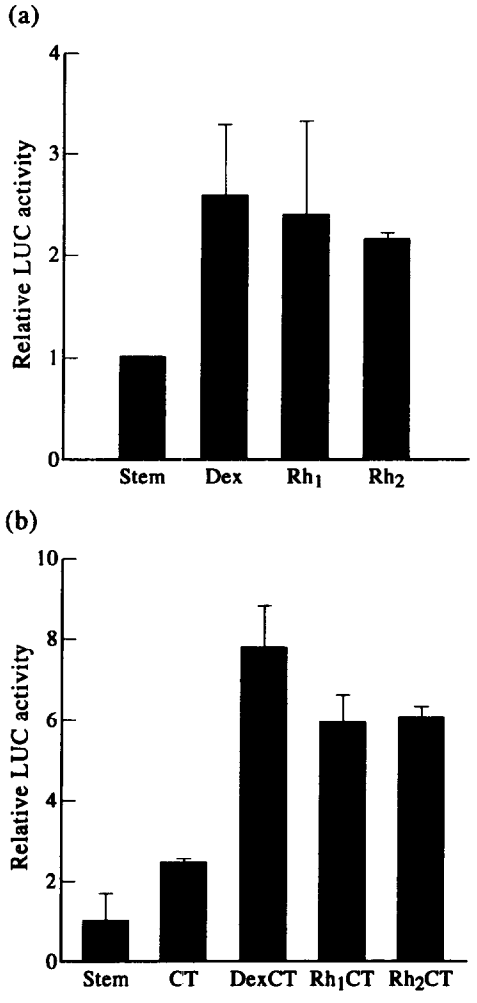


Figure 6. Activation of glucocorticoid receptor by ginsenosides. (a) F9 cells were cotransfected with GRE-luciferase plasmid and GR expression plasmid. The transfected cells were treated with 5×10^{-5} M ginsenosides (Rh_1 or Rh_2) or 2.5×10^{-5} M dexamethasone (Dex) as a positive control. Luciferase activities are presented as fold activation relative to the undifferentiated F9 stem cells. (b) Experiments were performed as described in (a) except that F9 cells treated with ginsenosides (Rh_1 or Rh_2) or Dex in the presence of 5×10^{-4} M dbcAMP and 2.5×10^{-4} M theophylline (CT).

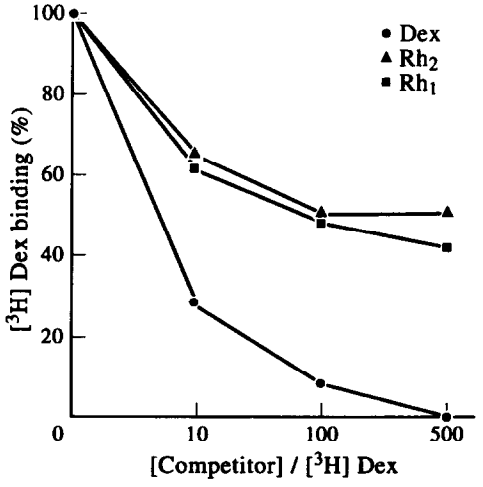


Figure 7. Binding of ginsenosides to glucocorticoid receptor. The inhibitory effects of dexamethasone (Dex), ginsenosides Rh_1, and Rh_2 on the specific binding of [^{3}H]dexamethasone (10 nM) to GR was examined. The percentage of ^{3}H-binding was calculated as a percentage of the specific binding noted when a 500-fold molar excess of unlabelled Dex was used as the competitor. Specific binding of [^{3}H]Dex is shown as 100%. Each point is an average of two sets of experiments.

added to binding reactions. In addition, the GR-mediated transactivation ability of ginsenoside was tested by performing a cotransfection assay. As a result, ginsenosides Rh_1 and Rh_2 induced GRE-transactivation and an additive effect of dbcAMP was found. Using the GR binding assay, it was shown that the relative weak binding affinities of ginsenosides for GR compared to that of dexamethasone were observed, and it suggests that ginsenosides and glucocorticoids may have different binding sites for GR, or other homologous nuclear receptor(s) could be involved in the differentiation inducing mechanism of ginsenosides. Taken together, these results suggest that ginsenosides have differentiation inducing effects through interaction with GR.

The actual cellular mechanism by which ginsenosides modulate phenotypic expression has not yet been unravelled. Moreover, how individual ginsenosides elicit such different responses is still unknown and identification of the receptors for ginsenosides is required. Nevertheless, it seems that some chemical compounds having glucocorticoid-like structures affect the differentiation of cancer cells. The study of the effects of ginsenosides on cancer cells will be valuable in elucidating the relationship between growth capacity and differentiation and in advancing effective therapies for cancer. Thus, the ginsenosides, which are non-toxic natural compounds, should be a subject for further *in vivo* animal studies in order to determine the possibility of clinical trials.

1. Pierce GB, Podesta A, Wells RS. Malignancy and differentiation: the role of the blastocyst in control colony formation. *CSH Conf Cell Proliferation* 1983, **10**, 15–22.
2. Verma IM, Corsi PS. Proto-oncogene *fos*: complex but versatile regulation. *Cell* 1987, **51**, 513–514.
3. Sporn MB, Roberts AB. Role of retinoids in differentiation and carcinogenesis. *Cancer Res* 1983, **43**, 3034–3040.
4. Strickland S, Sawey MJ. Studies on the effect of retinoids on the differentiation of teratocarcinoma cells *in vitro* and *in vivo*. *Dev Biol* 1980, **78**, 76–85.
5. Strickland S, Smith KK, Marotti KR. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cyclic AMP. *Cell* 1980, **21**, 347–355.
6. Martin GR. Teratocarcinomas and mammalian embryogenesis. *Science* 1989, **209**, 768–776.
7. Wang SY, LaRosa GJ, Gudas LJ. Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyryl cyclic AMP in cultured mouse teratocarcinoma cells. *Dev Biol* 1985, **107**, 75–86.
8. Griep AE, Deluca HF. Studies on the relation of DNA synthesis to retinoic acid induced differentiation of F9 teratocarcinoma cells. *Exp Cell Res* 1986, **164**, 223–231.
9. Eriksson U, Hansson E, Jonsson KH, Sundelin J, Peterson E. Increased level of several retinoid binding proteins resulting from retinoic acid-induced differentiation of F9 cells. *Cancer Res* 1986, **46**, 712–722.
10. Hosler BA, LaRosa GJ, Grippo JF, Gudas LJ. Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Mol Cell Biol* 1989, **9**, 5623–5629.
11. Yamamoto H, Katano M, Matsunaga H. Anti-tumor substance from panax ginseng roots. *Korean J Ginseng Sci* 1990, **14**, 244–252.
12. Ota T, Fujikawa-Yamamoto K, Zong ZP, Yamazaki M, Odash-

ima S. Plant-glycoside modulation of cell surface related to control of differentiation in cultured B16 melanoma cells. *Cancer Res* 1987, **47**, 3863–3867.
13. Ota T, Maeda M, Odashima S. Mechanism of action of ginsenoside Rh_2: uptake and metabolism of ginsenoside Rh_2 by cultured B16 melanoma cells. *J Pharmaceut Sci* 1991, **80**, 1141–1146.
14. Odashima S, Ota T, Kohno H, *et al.* Control of phenotypic expression of cultured B16 melanoma cells by plant glycosides. *Cancer Res* 1985, **45**, 2781–2784.
15. Kitagawa I, Yoshikawa M, Yoshihara M, Hayashi T, Taniyama T. Chemical studies on crude drug precession I. On the constituents of Ginseng Radix Rubra(1). *Yakugaku Zasshi* 1983, **103**, 612–622.
16. Feinberg AP, Vogelstein BA. Technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1984, **137**, 255–267.
17. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid-guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987, **162**, 156–159.
18. Silva CM, Tully DB, Petch LA, Jewell CM, Ciduiwski JA. Amplication of a protein-blotting procedure to the study of human glucocorticoid receptor interactions with DNA. *Proc Natl Acad Sci USA* 1987, **84**, 1744–1748.
19. Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
20. Dignam JD, Lebowitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983, **11**, 1475–1489.
21. Sambrook J, Fritsch EF, Maniatis T. Introduction of recombinant vectors into mammalian cells. *Molecular Cloning, A Laboratory Manual*, 2nd edn. New York, Cold Spring Harbor, 1989, 16.30–16.40.
22. Northrop JP, Gametchu B, Harrison RW, Ringold GM. Characterization of wild type and mutant glucocorticoid receptors from rat hepatoma and mouse lymphoma cells. *J Biol Chem* 1985, **260**, 6398–6403.
23. Schüle R, Muller M, Otsuka-Murakami H, Renkawitz R. Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. *Nature* 1988, **332**, 87–90.
24. Marotti KR, Brown GD, Strickland S. Two-stage hormonal control of type IV collagen mRNA levels during differentiation of F9 teratocarcinoma cells. *Dev Biol* 1985, **108**, 26–31.
25. Lotan R, Lotan D. Stimulation of melanogenesis in a human melanoma cell line by retinoids. *Cancer Res* 1980, **40**, 3345–3350.
26. Prasad KN, Edaward-Prasad J. Effects of tocopherol (Vitamin E) acid succinate on morphological alterations and growth inhibition in melanoma cells in culture. *Cancer Res* 1982, **42**, 550–555.
27. Yun TK, Choi SY. A case-control study of ginseng intake and cancer. *Int J Epidemiol* 1990, **19**, 871–876.
28. Yun TK, Choi SY. Preventive effect of ginseng intake against various human cancers: a case-control study on 1987 pairs. *Cancer Epidemiol Biomarkers Prevention* 1995, **4**, 401–408.
29. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988, **240**, 889–895.
30. Beato M. Gene regulation by steroid hormones. *Cell* 1989, **56**, 335–344.
31. Wright APH, Carlstedt-Duke J, Gustafsson J-Å. Ligand-specific transactivation of gene expression by a derivative of the human glucocorticoid receptor expressed in yeast. *J Biol Chem* 1990, **265**, 14763–14769.
32. Zilliacus J, Wright APH, Norinder U, Gustafsson J-Å, Carlstedt-Duke J. Determinants for DNA-binding site recognition by the glucocorticoid receptor. *J Biol Chem* 1992, **267**, 24941–24947.
33. Scheidereit C, Gisse S, Westphal HM, Beato M. The glucocorticoid receptor binds to defined nucleotide sequences near the promotor of mouse mammary tumor. *Nature* 1983, **304**, 749–752.
34. Philibert D. RU38486: an original multifaceted antihormone *in vivo*. In Agarwal MK, ed. *Adrenal Steroid Antagonism*. de Gruyter, Berlin, 1984, 77–101.
35. Baulieu E-E. Contragestion and other clinical applications of RU486, an antiprogesterone at the receptor. *Science* 1989, **245**, 1351–1357.
36. Horwotz KB. The molecular biology of RU486. Is there a role for antiprogestins in the treatment of breast cancer? *Endocr Rev* 1992, **13**, 146–163.
37. Mao J, Regelson W, Kalimi M. Molecular mechanism of RU486 action: a review. *Mol Cell Biochem* 1992, **109**, 1–8.
38. Gronemeyer H, Meyer M-E, Bocquel M-T, Kastner P, Turcotte B, Chambon P. Progestin receptors: isoforms and antihormone action. *J Steroid Biochem* 1991, **40**, 271–278.
39. Kim MY, Lee KY, Lee SK. Inductive effect of ginsenoside-Rg_1 on tyrosine aminotransferase gene expression in rat primary hepatocyte cultures. *Biochem Mol Biol Int* 1994, **34**, 845–851.

Acknowledgements—We thank Dr Ronald M. Evans, La Jolla, California, U.S.A. for providing the GR expression plasmid and pMTV-CAT cDNA. We also appreciate Dr Lloyd A. Culp, Case Western Reserve University, Cleveland, Ohio, U.S.A. for giving us pMMuLV-SV-lacZ gene. This work was supported by the research grant (KOSEF-SRC-56-CRC-94K3-0401-03-01-3) from the Korea Science and Engineering Foundation through the Cancer Research Center.