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Ginsenoside Rh2 induces cell cycle arrest and differentiation in human leukemia cells by upregulating TGF-β expression

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The triterpene saponin ginsenoside Rh2 has been shown to have antiproliferative effects on various cancer cells. However, the effect of Rh2 on the cell cycle and its underlying molecular mechanism in human leukemia cells are not fully understood. In this study, we found that Rh2 inhibited the proliferation of human leukemia cells concentration- and time-dependently with an IC_{50} of ~38 µM. DNA flow cytometric analysis indicated that Rh2 blocked cell cycle progression at the G_1 phase in HL-60 and U937 cells, and this was found to be accompanied by the downregulations of cyclin-dependent kinase (CDK) 4, CDK6, cyclin D1, cyclin D2, cyclin D3 and cyclin E at the protein level. However, CDK inhibitors (CDKIs), such as $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, were gradually upregulated after Rh2 treatment at the protein and messenger RNA (mRNA) levels. In addition, Rh2 markedly enhanced the bindings of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ to CDK2, CDK4 and CDK6, and these bindings reduced CDK2, CDK4 and CDK6 activities. Furthermore, Rh2 induced the differentiation of HL-60 cells as demonstrated by biochemical assays and the expression levels of cell surface antigens. In addition, treatment of HL-60 cells with Rh2 significantly increased transforming growth factor-β (TGF-β) production, and cotreatment with TGF-β neutralizing antibody prevented the Rh2-induced downregulations of CDK4 and CDK6, upregulations of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels and the induction of differentiation. These results demonstrate that the Rh2-mediated G_1 arrest and the differentiation are closely linked to the regulation of TGF-β production in human leukemia cells.

Introduction

Deregulation of the cell cycle is one of the most frequent alterations associated with tumor development. Therefore, the blockade of cell cycle is regarded as a feasible strategy for eliminating cancer cells. In this regard, regulations of the cell cycle and proliferation have been studied extensively during the last few years, and as a result, a consensus mechanism of cell cycle regulation has been developed. According to this mechanism, the retinoblastoma (Rb) protein family functions as a cell cycle master switch. Phosphorylation of Rb by the cyclin-dependent kinases (CDKs) of this family of proteins causes proliferation (1). After Rb phosphorylation by cdk4 and cdk6 complexes during the G_1 phase and CDK2 at the G_1-S interphase, E2F1 proteins are released to promote the transcriptions of genes essential for transition to the S-phase of the cell cycle (2). CDK activity is governed by a complex network of regulatory subunits and phosphorylation events. These kinases are activated by D-type cyclins (D1, D2 and D3) and cyclin E, and it is inhibited by two families of CDK inhibitors (CDKIs), namely the INK ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$) and CIP/KIP families ($p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (3)). This multiplicity of regulatory mechanisms allows cell cycle progression to be responsive to a variety array of external and internal factors, and it prevents cell cycle progression during periods when DNA damage or other cellular conditions make such progression harmful.

HL-60, a human promyelocytic cell line, has been extensively used as an *in vitro* model for studying the effects of factors that regulate the growth and differentiation of hematopoietic cells in general and of myeloid leukemia cells in particular (4). In the presence of all-*trans*-retinoic acid, HL-60 cells undergo differentiation to granulocytes, whereas $1\alpha,25(OH)_2$-vitamin D_3 (Vit D_3) and 12-*O*-tetradecanoylphorbol-13-acetate induce differentiation into monocytes/macrophages (5). Hematopoietic cell growth and differentiation are regulated by a number of cytokines *in vitro* and *in vivo*. Furthermore, transforming growth factor-β (TGF-β), a negative regulator of growth at all stages of hematopoiesis (6), induces differentiation of HL-60 cells to promonocytes and has been shown to act synergistically with Vit D_3, tumor necrosis factor or all-*trans*-retinoic acid plus tumor necrosis factor (7,8). Other studies have shown that the induction of terminal differentiation by retinoids and Vit D_3 requires TGF-β1 as an autocrine mediator, which suggests that endogenous TGF-β1 plays a critical role in the differentiation of leukemia cells (9,10).

The ginsenosides are triterpenes saponins and are considered to be the main bioactive principles of herbal medicines derived from the roots and rhizomes of different *Panax* species (Araliaceae). The ginsenosides can be classified as 20(S)-protopanaxadiol (ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Rh2, Rg3 and others) or 20(S)-protopanaxatriol (ginsenosides Re, Rg1, Rg2, Rh1 and others) compounds (except ginsenoside R0). Among these, ginsenoside Rh2 has been shown to inhibit the growth of a number of types of human cells by inducing cell cycle arrest and apoptosis (11–13). Recently, it was reported that Rh2 treatment blocks cell cycle progression in human breast cancer cells (MCF-7 and MDA-MB-231) at the G_0/G_1 phase boundary by inhibiting the kinase activities of G_1-S-specific CDK/cyclin complexes, reducing the phosphorylation of Rb and suppressing the transcriptional activity of E2F1 by inducing the expressions of $p15^{INK4B}$ and $p27^{KIP1}$ (12). Furthermore, the Rh2-induced apoptosis of neuroblastoma cells was found to be caused by the activations of caspase-1 and -3 and the upregulation of Bax (14). In addition, apoptosis induction resulting from Rh2 exposure in PC-3 and LNCaP human prostate cells was found to be correlated with the modulation of mitogen-activated protein kinases (15). Although the mechanism underlying the induction of apoptosis by Rh2 is known, it has not been established whether Rh2 induces cell cycle arrest and differentiation in human promyelocytic leukemia HL-60 cells. In this study, we investigated the effects of ginsenoside Rh2 on proliferation, cell cycle regulation and differentiation in human leukemia HL-60 cells and the mechanism involved.

Abbreviations: CDK, cyclin-dependent kinase; CDKI, CDK inhibitors; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; Rb, retinoblastoma; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β; Vit D3, 1α,25(OH)2-vitamin D3.

[†]These authors contributed equally to this work.

Materials and methods

Reagents

Rh1 and Rh2 (Figure 1A) used for this study were isolated from the roots of *Panax ginseng,* and structural identities were determined spectroscopically

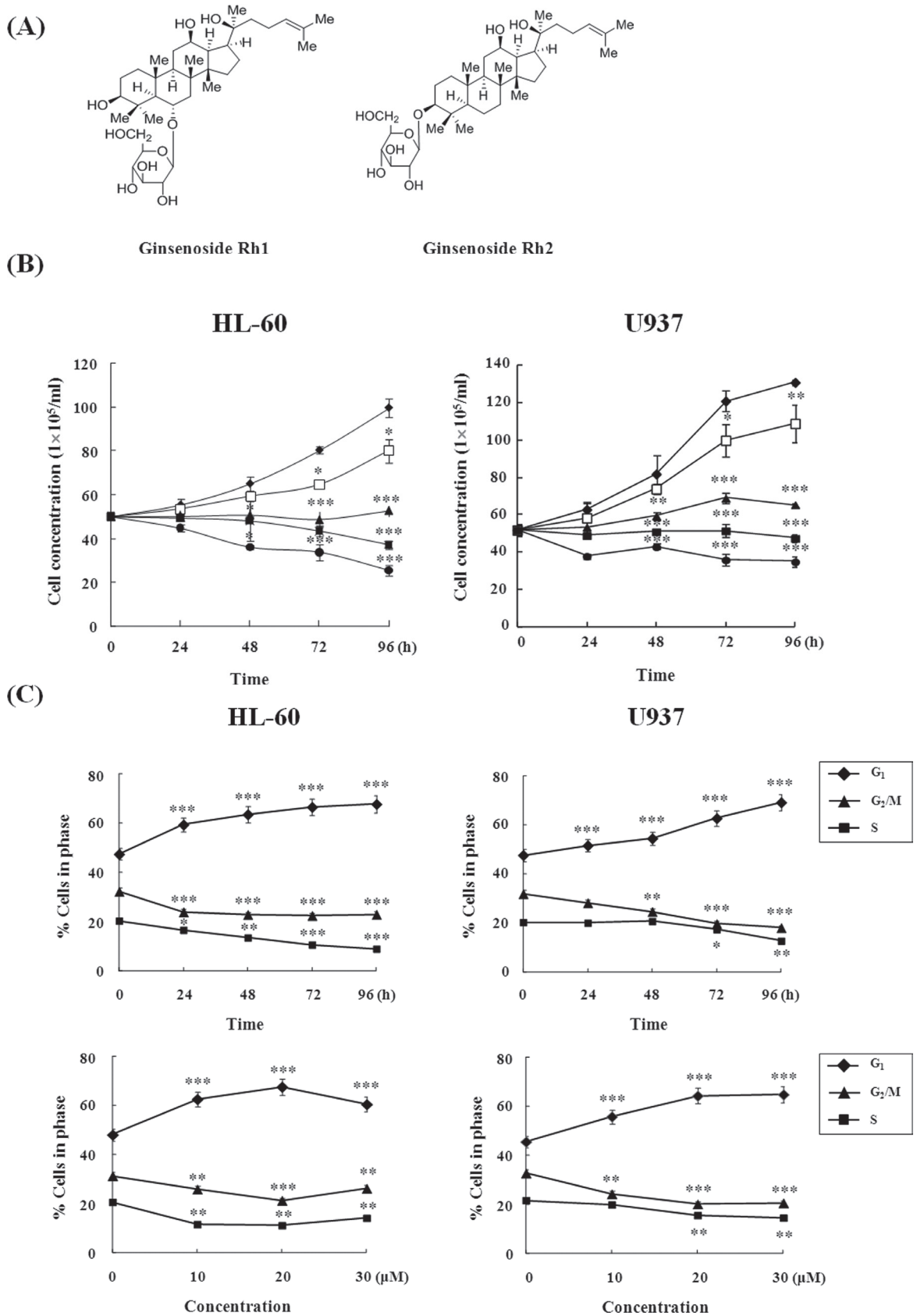


Fig. 1. Effects of ginsenoside Rh2 on the growth inhibition and the cell cycle in human leukemia cells. (**A**) Chemical structures of ginsenoside Rh1 and Rh2. (**B**) Exponentially growing cells were treated with the indicated concentrations of ginsenoside Rh2 for 96 h (closed diamond, control; opened quadrangle, 10 μM; closed triangle, 20 μM; closed quadrangle, 30 μM, closed circles, 40 μM). Cell growth inhibition was assessed using trypan blue exclusion assays, as described in Materials and methods. HL-60 and U937 cell growths were significantly inhibited by ginsenoside Rh2 in a time- and dose-dependent manner. (**C**) Cells were treated with ginsenoside Rh2 for the indicated times and concentrations and then cell cycle analysis was performed as described in Materials and methods. The data shown represent mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control group.

(^{1}H and ^{13}nuclear magnetic resonance (NMR), infrared spectroscopy (IR), mass spectrometry) as described previously (16). The identity of isolated compounds were confirmed by liquid chromatography–mass spectrometry and was found to be >98% pure. RPMI 1640 medium, fetal bovine serum, penicillin and streptomycin were obtained from Life Technologies (Grand Island, NY). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT), propidium iodide, phenylmethylsulfonylfluoride, protease inhibitor cocktails, nitroblue tetrazolium (NBT), Vit D_3, 12-*O*-tetradecanoylphorbol-13-acetate, α-naphthyl acetate esterase kit and naphthol AS-D chloroacetate esterase kit were purchased from Sigma Chemical Co. (St. Louis, MO). CD11b, CD14, CD64 and CD66b were obtained from Pharmingen (San Diego, CA). Antibodies against $p21^{CIP1/WAF1}$, $p27^{KIP1}$, CDK2, CDK4, CDK6, cyclin D1, cyclin D2, cyclin D3, cyclin E, Rb, p-Rb, E2F1, α-tubulin and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-FoxO3a (ser 253), FoxO3a, p-Samd 2 (ser465/467), p-Smad 3 (ser423/425) and Smad 2/3 were purchased from Cell Signaling Technology (Beverly, MA), and TGF-β1 neutralizing antibody was purchased from R&D System (Minneapolis, MN). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat Ig were purchased from Jackson Immunoresearch (West Grove, PA).

Cell culture

HL-60 promyelocytic leukemia, U937 human histocytic lymphoma, A549 human lung adenocarcinoma, HeLa human negroid cervix epitheloid carcinoma, HepG2 human hepatoblastoma, P388 mouse lymphoblast and A431 human epidermoid carcinoma cells were obtained from the Korean cell line bank (KCLB, Seoul, Korea) and cultured in RPMI 1640 or Dulbecco's modified Eagle's minimum essential medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) (Life Technologies). Cells were maintained at 37°C in an atmosphere of 5% CO_2 in air (standard conditions of incubation).

Cytotoxicity test

Cytotoxicity was assessed by MTT assay (17). Briefly, the cells (5×10^4 cells/ml) were seeded in each well containing 100 μl of the RPMI medium supplemented with 10% fetal bovine serum in a 96-well plate. After 24 h, various concentrations of ginsenosides were added. After 96 h, 50 μl of MTT [5 mg/ml stock solution in phosphate-buffered saline (PBS)] was added, and the plates were incubated for an additional 4 h. The medium was discarded, and the formazan blue, which formed in the cells, was dissolved with 100 μl dimethyl sulfoxide. The optical density was measured at 540 nm.

Trypan blue assay

The *in vitro* growth inhibitory effect of Rh2 on the HL-60 and U937 cells was determined by trypan blue dye exclusion. The reduction in viable cell number was assessed for each 96 h. The cells were seeded at a concentration of 2×10^5 cells/ml and were maintained for logarithmic growth by passing them every 48–96 h and incubated for 24–96 h with Rh2 at various concentrations. Rh2 dissolved in dimethyl sulfoxide was added to the medium in serial dilution (the final dimethyl sulfoxide concentration in all assays did not exceeded 0.05%). Cells were loaded on a hemocytometer, and viable cell number was determined based on exclusion of trypan blue dye.

Cell cycle analysis

The cell cycle distribution has been described previously (18). Briefly, the cells were collected by centrifugation at 2500 r.p.m. for 5 min, fixed in 70% ice-cold ethanol at 4°C for 1 h and washed once with PBS and resuspended in 1 ml of PBS containing 2.5 μg/ml ribonuclease and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature and analyzed using fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Becton-Dickinson, Heidelberg, Germany). A total of 10 000 events were acquired for analysis using Cell Quest software.

Cell fractionation and western blot analysis

Rh2-treated cells were harvested and washed twice with ice-cold PBS. Nuclear extracts were prepared as described previously (19). Cell pellets were resuspended in hypotonic buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol (DTT), 10 μg/ml aprotinin] and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at 12 000*g* for 1 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM DTT, 1 mM NaF and 1 mM sodium orthovanadate). For total cell protein extracts, cell pellets were lysed in ice-cold cell lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.1% Triton X-100, 0.5 mM DTT, 5 mM NaF, 0.5 mM Na orthovanadate, 0.1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktails) for 20 min on ice. Cell debris was removed by microcentrifugation (10 000*g*, 5 min), followed by quick freezing of the supernatants. Protein concentration was determined by Bio-Rad protein assay reagent. Protein samples of cell lysate were mixed with an equal volume of 5× sodium dodecyl sulfate (SDS) sample buffer, boiled for 4 min and then separated by 10–12% SDS–polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane. The membranes were incubated for 1 h with blocking solution and then incubated with a 1:1000 dilution of primary antibodies for overnight. Blots were washed three times with Tween 20/Tris-buffered saline and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed again three times with Tween 20/Tris-buffered saline and then developed by enhanced chemiluminescence (Amersham Life Science, London).

RNA preparation and real-time PCR

Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea), according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed from each sample using MuLV reverse transcriptase, 1 mM deoxynucleoside triphosphate and oligo ($dT_{12–18}$) 0.5 μg/μl. Real-time PCR was performed using Thermal Cycler Dice Real-Time PCR System (Takara, Shiga). The primers used for SYBR Green real-time reverse transcription–PCR were as follows: for *TGF-β1*, sense primer: 5′-gtg tga cag atg ggc tct gc-3′ and antisense primer: 5′-gag ggt gca cat aca aac gg-3′; and for glyceraldehyde 3-phosphate dehydrogenase, sense primer: 5′-ggg gct ctc cag aac atc at-3′ and antisense primer: 5′-cag gtc agg tcc acc act ga-3′. A dissociation curve analysis of TGF-β1 and glyceraldehyde 3-phosphate dehydrogenase showed a single peak. PCRs were carried out for 40 cycles using the following conditions: denaturation at 95°C for 5 s, annealing at 57°C for 10 s and elongation at 72°C for 20 s. Mean Ct. of the gene of interest was calculated from triplicate measurements and normalized with the mean Ct. of a control gene, *GAPDH*.

Immunoprecipitation

After harvesting and washing, pellets were lysed in immunoprecipitation buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM ethylenediaminetetraacetic acid, 2.5 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 5 mM NaF, 0.1 mM Na_3VO_4, 1% Triton X-100 and protease inhibitor cocktails) for 15 min on ice. After centrifugation (10 000*g*, 5 min), protein concentration were determined. Equal amount of protein (100 μg) was incubated with anti-CDK2, anti-CDK4 and anti-CDK6 polyclonal antibodies for 12 h at 4°C, followed by incubation with 40 μl protein A-Sepharose beads for 4 h. The protein complex was washed four times with immunoprecipitation buffer and released from the beads by boiling in 6× sample buffer (350 mM Tris, pH 6.8, 10% SDS, 30% β-mercaptoethanol, 6% glycerol, 0.12% bromophenol blue) for 5 min. The reaction mixture was then resolved by a 10–12% SDS–PAGE gel, transferred to nitrocellulose membrane and probed with anti-CDK2, anti-CDK4, anti-CDK6, anti-$p21^{CIP1/WAF1}$ and anti-$p27^{KIP1}$ monoclonal antibody. The blot was developed by enhanced chemiluminescence.

Kinase activity assay

CDK2-associated histone H1 kinase activity was determined as described (20). Briefly, the total lysates (500 μg protein) were prepared and immunoprecipitated with 5 μg each of anti-CDK2, anti-CDK4 and anti-CDK6 polyclonal antibody as described Reagents. Fifty microliter of protein A-Sepharose CL-4B (Amersham Bioscience) prepared at 6 mg/ml in 0.1 M potassium phosphate buffer (pH 8.0) was added to each sample and incubated for 18 h at 4°C. The immunocomplexes were washed three times with lysis buffer and finally once with kinase assay buffer (50 mM Tris–HCl, pH 7.4, 1 mM DTT and 10 mM $MgCl_2$). The kinase reactions were carried out in a final volume of 40 μl containing 20 μM adenosine triphosphate, 25 μCi [γ-^{32} P] adenosine triphosphate, 2 μg histone H1 for CDK2 or 1 μg glutathione S-transferase-Rb for CDK4 and CDK6. The reactions were performed for 20 min at 30°C and quenched by adding an equal volume of a 6× SDS loading buffer. After boiling for 10 min, the reaction products were separated by 12% SDS–PAGE, and the phosphorylated proteins were detected by autoradiography.

Differentiation assay

(i) NBT reduction test: the percentage of HL-60 cells capable of reducing NBT was measured by counting the number of cells containing the precipitated formazan particles after the cells had been incubated with the NBT (1.0 mg/ml) at 37°C for 30 min. 12-*O*-Tetradecanoylphorbol-13-acetate was used to stimulate the formation of formazan. (ii) Esterase activity test: a smear preparation was chemically stained for α-naphthyl acetate esterase and naphthol AS-D chloroacetate esterase using the standard techniques (20). (iii) Phagocytosis test: the HL-60 cells (1×10^6 cells/ml) were suspended in serum-free RPMI 1640 medium containing 0.2% of the latex particles (average diameter, 0.81 μm) and incubated at 37°C for 4 h. After incubation, the

cells were washed once with PBS. The cells containing >10 latex particles were scored as being phagocytic cells (20). (iv) Flow cytometry: the HL-60 cells (2×10^5 cells/ml) exposed to ginsenoside Rh2 were collected and washed twice with ice-cold PBS. The cells were then incubated with the direct fluorescein isothiocyanate-labeled anti-CD11b, anti-CD14, anti-CD64 or anti-CD 66b antibodies (Pharmingen) on ice for 30 min, washed twice with PBS and the level of antibody binding to the cells was quantified using FACS cater-plus flow cytometry (Becton-Dickinson).

Analysis of TGF-β1 protein levels

TGF-β1 protein was measured with Quantikine enzyme-linked immunosorbent assay according to the manufacturer's (R&D Systems, Minneapolis, MN) protocol. This assay employs the quantitative sandwich enzyme immunoassay technique. The cell culture supernatant (100 µl) was mixed with 20 µl of 1 N HCl, incubated at room temperature for 10 min and neutralized by 1.2 N NaOH and 0.5 M HEPES. TGF-β1 protein was measured by enzyme-linked immunosorbent assay using polyclonal antibody.

Statistical analysis

Results are expressed as the mean ± SD of triplicate experiments. Statistically significant values were compared using analysis of variance and Dunnett's *post-hoc* test, and *P*-values of <0.05 were considered statistically significant.

Results

Ginsenoside Rh2 induced the G_1 cell cycle arrest in human leukemia cells

The cytotoxicities of ginsenosides (Rh2 and Rh1) were examined using MTT assays in various cancer cell lines by determining IC_{50} values (Table I). It was found that Rh2 was more potent than Rh1 in cancer cells tested, and that human leukemia cells (HL-60 and U937) were most sensitive to Rh2. Cell numbers were counted after treatment with Rh2 at different concentrations and times using trypan blue dye exclusion assays. Exponentially growing HL-60 and U937 cultures rapidly underwent growth inhibition when treated with Rh2 at concentrations of 10–40 µM (Figure 1B). At 20 µM, Rh2 had a cytostatic effect, but at 30 and 40 µM, it had a cytocidal effect. To investigate whether Rh2 affects cell cycle regulation, HL-60 and U937 cells were cultured with Rh2 at concentrations of 20 µM for 96 h, and DNA contents were then analyzed by DNA flow cytometric analysis. As shown in Figure 1C, Rh2 induced the cell cycle arrest at G_1 phase in a time-dependent manner. In addition, the treatment caused an arrest of 62.6% and 55.8% cells in G_1 phase of the cell cycle at 10 µM, which further increased to 67.7% and 64.8% at 20 µM, whereas this slightly decreased to 60.5% and 64.3% at 30 µM compared with control (48.1% and 45.6%) in HL-60 and U937cells, respectively. This increase in G_1 cell population was accompanied with a concomitant decrease of cell number in S-phase in HL-60 and U937cells (Figure 1C). This result suggests that the growth inhibitory effect of Rh2 was the result of a block of cell cycle at G_1 phase in human leukemia cells.

Rh2 induced the changes in the expression and bindings of cell cycle-related proteins following decreased phosphorylation of Rb and the translocation of E2F1

Because Rh2 induced the G_1 phase arrest in HL-60 and U937 cells (Figure 1), we evaluated the protein levels of the CDKIs $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, which play a key role in G_1 cell cycle regulation, after treatment with Rh2 (20 µM). It was found that Rh2 increased the protein and messenger RNA (mRNA) levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in a time-dependent manner (Figure 2A and Supplementary Figure 1, available at *Carcinogenesis* Online), suggesting that Rh2 modulated the G_1 phase arrest, at least in part, by upregulating $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. In vertebrate cells, G_1 progression and the G_1-S transition are also regulated by D-type cyclins (cyclin D1, D2 and D3) that bind to and activate CDK4 and CDK6 and cyclin E, which results in the activation of CDK2 (3). Treatment of HL-60 and U937cells with Rh2 (20 µM) was found to downregulate CDK4, CDK6 and cyclin D1, cyclin D2, cyclin D3 and cyclin E protein levels, whereas CDK2 was unaffected (Figure 2B). Next, we questioned whether Rh2-induced levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ would be detected in the complexes

Table I. Cytotoxic activities of ginsenoside Rh2 on various cancer cell lines *in vitro*

Cell lines	IC_{50} (µM)[a]	
	Rh1	Rh2
HL-60	>100	38.5 ± 2.4
U937	>100	41.2 ± 1.5
p388	>100	49.6 ± 2.7
A549	>100	59.6 ± 3.2
HeLa	>100	>200
Hep G2	>100	>100
A431	>100	59.6 ± 3.2

[a]IC_{50} is defined as the concentration that results in a 50% decrease in the number of cells compared with that of the control cultures in the absence of an inhibitor. The values represent the mean ± SD of results from three independent experiments with similar patterns.

with the CDK. As shown in Figure 2C, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels in CDK2, CDK4 and CDK6 complexes in Rh2-treated HL-60 cells were obviously higher than in those of the untreated cells. Collectively, these results suggest that $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins play a key role in G_1 phase arrest by binding to CDK2, CDK4 and CDK6 in Rh2-treated HL-60 cells (Figure 2C). In addition, we examined whether increased levels of CDKI and decreased levels of cell cycle regulatory proteins inhibit CDK activity in Rh2-treated HL-60 cells. The CDK activity assays employed the use of histone H1 (for CDK2) or glutathione S-transferase-Rb fusion protein (for CDK4 and CDK6) as substrates. As shown in Figure 2D, following treatment with Rh2, CDK2, CDK4 and CDK6-associated kinase activities were markedly lower in HL-60 cells. CDKIs play important roles in the regulation of G_1-S transition by binding to and inhibiting the kinase activities of CDK/cyclin complexes (21). CDK4/cyclin D1 and CDK6/cyclin D1 complexes hyperphosphorylate Rb protein, leading to its dissociation from transcription factor E2F1, which regulates the expressions of genes necessary for cell cycle progression (1). In order to probe the mechanism of Rh2-induced G_1 phase arrest, we examined the effect of Rh2 on the phosphorylation of Rb and the translocation of E2F1 to the nucleus by immunoblotting. As shown in Figure 2E, Rh2 treatment suppressed Rb phosphorylation and attenuated E2F1 levels in nuclear fractions of HL-60 cells. Taken together, these results indicate that the Rh2-mediated G_1 phase cell cycle arrest is associated with increased recruitment of CDKIs $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ to CDK2, CDK4 and CDK6 and then a reduction in the phosphorylation of Rb leading to inhibition of E2F1 translocation in HL-60 cells.

Rh2 induced the differentiation of HL-60 cells

In order to determine whether growth inhibition via G_1 phase arrest by Rh2 is associated with differentiation, we used a NBT reduction assay, which is a reliable marker of myeloid leukemia cell differentiation (22). As shown in Figure 3A, when HL-60 cells were incubated with 20 µM Rh2 for 96 h, approximately 54.5% of cells were stained with NBT, whereas only 6.7% of the untreated cells were stained. Vit D_3 (20 nm), a positive control, gave 46.2% of NBT-reducible cells. In order to determine whether Rh2 causes HL-60 cells to differentiate into granulocytes and monocytes/macrophages, esterase activities were measured. Treatment of HL-60 cells with 20 µM Rh2 for 96 h caused 22.8% and 31.1% increases in AS-D chloroacetate esterase and α-naphthyl acetate esterase activities, respectively (Figure 3B). Furthermore, cells treated with Rh2 showed apparent phagocytic activity (Figure 3C), which is considered as a criterion of mature macrophages. In addition, Rh2 (20 µM) significantly increased the expressions of the membrane antigens CD11b, CD14, CD64 and CD66b on HL-60 cells, which determine the granulocyte- and monocyte/macrophage-specific phenotypes. Accordingly, these findings show that Rh2 induced the differentiation of human promyelocytic leukemia cells to monocyte/macrophage and granulocyte lineages, whereas the control cells did not express these markers of cell surface antigens (Figure 3D).

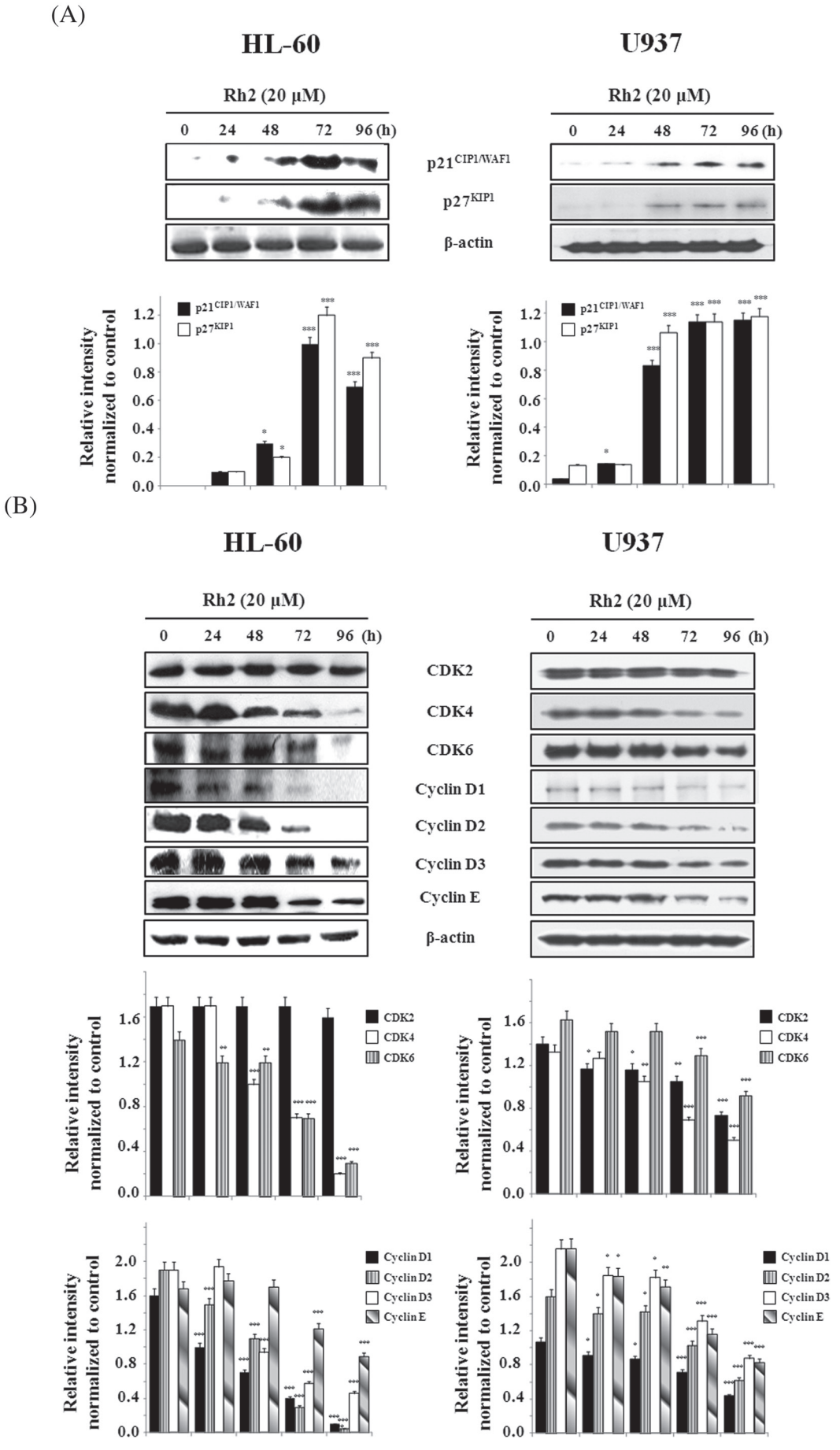


Fig 2. *Continued*

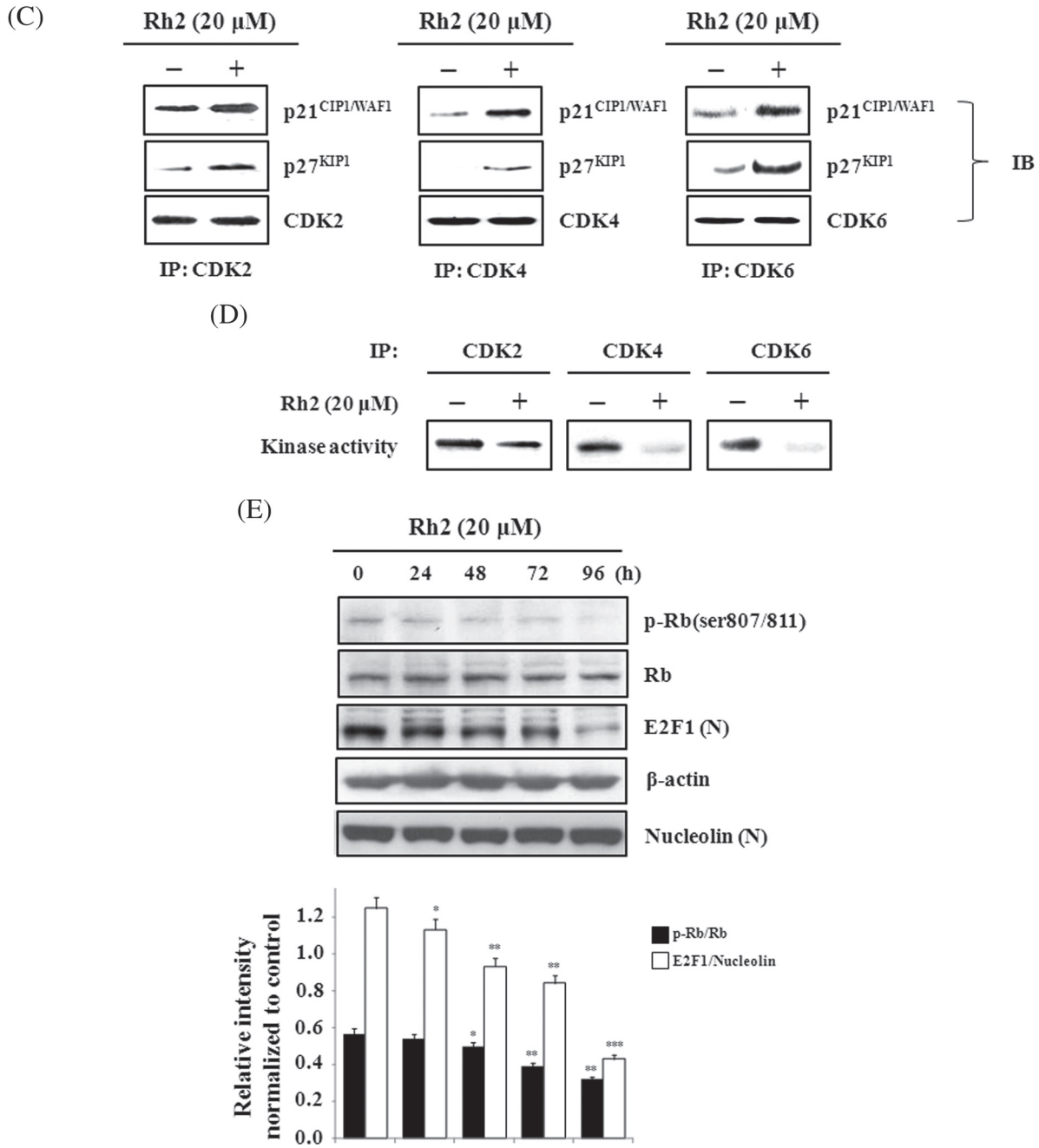
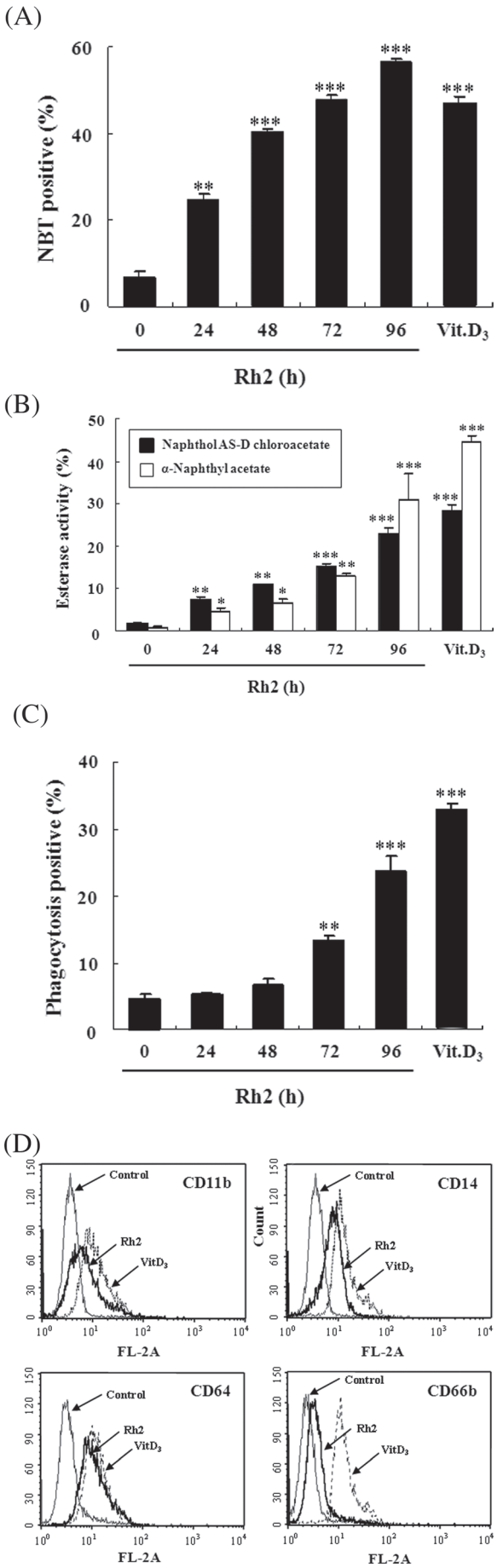


Fig. 2. Effects of ginsenoside Rh2 on the expressions of cell cycle-related proteins and the binding between CDKs and CDKI in human leukemia cells. (**A**) Cells were incubated with or without 20 μM ginsenoside Rh2 for the indicated times and then harvested. The expression levels of CDKI ($p21^{CIP1/WAF1}$ and $p27^{KIP1}$) proteins were determined by western blotting. Experiments were repeated three times, and similar results were obtained. (**B**) The expression levels of CDK and cyclin protein were determined by western blotting using specific antibodies for cell cycle-related proteins. (**C**) CKDI ($p21^{CIP1/WAF1}$ or $p27^{KIP1}$)–CDK association after ginsenoside Rh2 treatment. HL-60 cells were incubated with or without 20 μM ginsenoside Rh2 for 96 h. Total lysates were immunoprecipitated using anti-CDK2, anti-CDK4 or anti-CDK6 antibodies. Levels of bound $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ in each immune complex were determined by western blotting (IP: immunoprecipitation, IB: immunoblotting). (**D**) Kinase activities of CDKs after ginsenoside Rh2 treatment in HL-60 cells. Total cell lysates from control cells and cells treated with 20 μM ginsenoside Rh2 for 96 h were immunoprecipitated using anti-CDK2, anti-CDK4 or anti-CDK6 antibodies. Kinase activities were assayed using histone H1 (for CDK2) and Rb (for CDK4 and CDK6) as substrates. (**E**) The expression levels of p-Rb and translocation of E2F1 protein were determined by western blotting using specific antibodies in HL-60 cells. β-actin and nucleolin were used as internal controls. Densitometric analysis was performed using Bio-Rad Quantity One® Software. The data shown represent mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus control group.

Rh2-regulated cell cycle arrest and differentiation by inducing TGF-β expression in HL-60 cells

Because TGF-β1 has inhibitory effects on cell growth and regulates proliferation, apoptosis and differentiation (6), we raised the question of whether TGF-β1 might be involved in Rh2-induced cell growth inhibition. We addressed this question by immunoassays of TGF-β1 production using culture media from Rh2-treated HL-60 cells. The results obtained revealed that Rh2 time-dependently increased the production of TGF-β1 in HL-60 cells (Figure 4A). Next, we examined whether the effects of Rh2 are regulated at the transcriptional



level by quantifying *TGF-β1* mRNA levels. It was found that Rh2 also time-dependently increased the expression of *TGF-β1* mRNA (Figure 4B). Furthermore, we examined whether the production of TGF-β1 in Rh2-treated HL-60 inhibits cell cycle regulators and differentiation. Treatment of HL-60 cells with recombinant human TGF-β1 (25 ng/ml) was found to upregulate $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ protein levels, whereas CDK2, CDK4 and CDK6 were downregulated (Supplementary Figure 2, available at *Carcinogenesis* Online). In addition, NBT reduction test showed that TGF-β1 (25 ng/ml) induces a significant differentiation of HL-60 cells into monocyte/macrophage and granulocyte lineages (Supplementary Figure 2, available at *Carcinogenesis* Online). The role for TGF-β1 was examined using 20 μM Rh2 in the presence or absence of 1 μg/ml TGF-β1-neutralizing antibody. Cell extracts were analyzed for the cell cycle potentiators, CDK2, CDK4 and CDK6 because they are known to bind to both cyclin D and cyclin E, both of which could be blocked by $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ (Figure 2C). It was found that TGF-β1-neutralizing antibody blocked the Rh2-mediated decrease in the expressions of CDK4 and CDK6 and reduced the expressions of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ (Figure 4C). In addition, pretreating HL-60 cells with TGF-β1-neutralizing antibody dose-dependently inhibited Rh2-induced differentiation (Figure 4D).

It has been reported that TGF-β1 inhibits the proliferation of malignant epithelial cells by inducing cell cycle arrest via the formation of a Smad/FoxO3a complex, which upregulates the cell cycle inhibitory gene $p21^{CIP1/WAF1}$ (23). To confirm that Rh2-mediated growth inhibition and cell cycle arrest are involved in Smad/FoxO3a signaling in response to TGF-β1, we performed western blotting in HL-60 and U937 cells to explore critical molecular changes in this pathway. Rh2 treatment significantly elevated the level of p-Smad2/3 and the nuclear translocation of FoxO3a, whereas Rh2 reduced the level of p-FoxO3a in a time-dependent manner in HL-60 and U937 cells (Figure 4E and Supplementary Figure 3, available at *Carcinogenesis* Online). These findings suggested that Rh2 increases TGF-β1 production, stimulates Smad/FoxO3a signaling and mediates the G_1-S transition phase of the cell cycle and the differentiation of human leukemia cells.

Discussion

Previous studies of the structure–activity relationship of ginsenosides have shown that the activities of panaxadiol compounds are of greater cytotoxicity than those of the panaxatriol compounds, and that the aglycones are more effective than the glycosides (24). Ginsenosides Rh1 and Rh2, possessing sugar moieties at C-6 and C-3, respectively, have similar chemical structures, but the cytotoxic activities of the Rh2 (panaxadiol type) on various cancer cells were also found to be greater than those of Rh1 (panaxatriol type). This study was performed to investigate the effects of ginsenoside Rh2 on proliferation, cell cycle regulation and induction of differentiation in human leukemia HL-60 cells as well as the underlying mechanisms of these effects. Rh2 potently caused cell cycle arrest in human promyelocytic leukemia HL-60 cells and inhibited proliferation and the induction of differentiation. Cell cycle analysis revealed that Rh2 markedly induced G_1 phase arrest in time-dependent manner. Therefore, we investigated the expressions of the G_1 phase-related cell cycle regulators, CDKs, cyclins and CDKIs in Rh2-treated HL-60 cells, and the interaction between CDK/cyclin complex and CDKIs, which is another important regulator of CDK/cyclin activity (25). During cell

Fig. 3. The differentiation-inducing effect of ginsenoside Rh2 in HL-60 cells. (**A**) Cells were incubated with or without 20 μM ginsenoside Rh2 for the indicated times. The NBT reduction test, (**B**) esterase activity test and (**C**) phagocytosis test were perfovrmed as described in Materials and methods. Data represent mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control group. (**D**) Expressions of CD11b, CD14, CD64b and CD66 antigens in HL-60 cells treated with ginsenoside Rh2 (20 μM, 96 h) were determined by FACS analysis. The FACS analysis shown is representative of two separate experiments. Vit D_3 (20 nm) was used as a positive control.

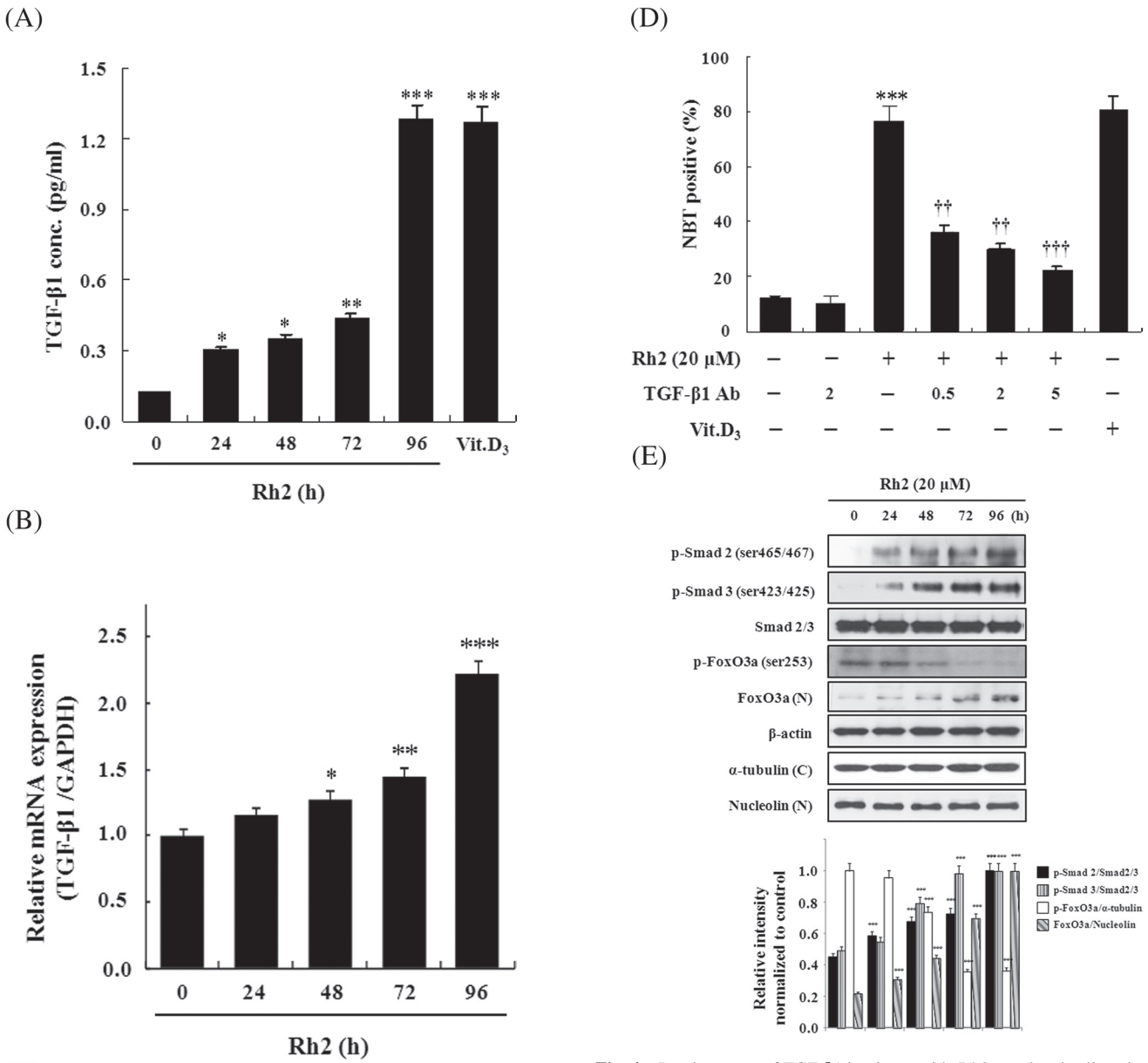


Fig. 4. Involvements of TGF β1 in ginsenoside Rh2-regulated cell cycle arrest and differentiation in HL-60 cells. (**A**) TGF-β1 protein and (**B**) mRNA levels were measured in 20 μM ginsenoside Rh2-treated HL-60 cells for the indicated times. TGF-β1-neutralizing antibody was used to determine whether ginsenoside Rh2 (20 μM, 96 h)-induced changes in cell cycle-related protein levels (**C**) and differentiation (**D**) were due to TGF-β1 treatment. Data represent means ± SD of three independent experiments. ***$P < 0.001$ versus control group, ††$P < 0.01$, †††$P < 0.001$ versus Rh2-treated group. (**E**) Cells were incubated with or without 20 μM ginsenoside Rh2 for the indicated times and then harvested. The expression levels of proteins were determined by western blotting. Experiments were repeated three times, and similar results were obtained. Densitometric analysis was performed using Bio-Rad Quantity One® Software. Data represent mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus control group.

cycle progression, CDKIs $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ often participate in the suppression of CDK activity. Furthermore, the overexpressions of $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ are known to cause cell cycle arrest in the G_1 phase. The targets of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ *in vivo* and *in vitro* are CDK4/cyclin D and CDK2/cyclin E, which physically associate with $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ (1). In this study, G_1 phase arrest by Rh2 in HL-60 cells was found to be associated with marked upregulations of the CDKIs $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, and CDK2, CDK4 and CDK6 are activated in association with the D-type cyclins or cyclin

E during G_1 progression and G_1-S transition (26). This study demonstrates that CDK4, CDK6 and cyclin D1, D2, D3 and cyclin E expressions are reduced time-dependently after Rh2 treatment, and that CDK2 was unaffected. In addition, the accumulation of CDKI proteins in association with G_1 phase arrest was detected largely in complexes with CDK2, CDK4 and CDK6 (27). In this study, levels of CDKs–CDKIs complexes increased after Rh2 treatment, supporting the notion that Rh2 markedly reduced CDK2-, CDK4- and CDK6-associated kinase activities in HL-60 cells. Overall, the blocking of entry into the S-phase from the G_1 phase in Rh2-treated HL-60 cells appears to be mediated by the downregulations of CDK2-, CDK4- and CDK6-associated kinase activities in association with the induction of CDKIs ($p21^{CIP1/WAF1}$ and $p27^{KIP1}$). These results are consistent with those of others who showed that Rh2 induces G_1 phase arrest in other types of human cancer cells (11,28). However, the mechanism involved may be cell line specific. For example, Rh2-mediated cell cycle arrest in MCF-7 cells is accompanied by the downregulations of CDK and cyclins, and reduced interaction between cyclin D1 and CDK4/CDK6 and increased recruitment of $p15^{INK4B}$ and $p27^{KIP1}$ to CDK4/cyclin D1 and CDK6/cyclin D1 complexes (12). In addition, the Rh2-mediated G_0/G_1 phase cell cycle arrest in A549 lung adenocarcinoma cells has been reported to be correlated with suppression of CDK6, cyclin D1 and cyclin E proteins expression, but unlike that observed in MCF-7 cells, levels of CDK4, CDK2 and cyclin A were unaltered (11). This study is the first to issue on the elucidation of the mechanism responsible for Rh2-mediated cell cycle arrest in human leukemia cells.

Rb family proteins play central roles at the restriction point of the cell cycle (1). In mammalian cells, Rb family proteins are believed to ensure cell cycle exit and prevent cells from reentering the cell cycle, predominantly by binding to E2F1 transcription factor, inhibiting the expressions of E2F1 target genes and remodeling chromatin into an inactive state (29,30). In the presence of mitogens, CDK/cyclin complexes phosphorylate Rb family members, which relieve the inhibition of E2F1 targets and enable S-phase entry. The compromised ability of cells with mutations in the Rb pathway to arrest at G_1 is considered to reflect the tumor suppressor activity of this pathway (31). In this study, western blotting showed that Rh2-mediated cell cycle arrest in HL-60 cells is correlated not only with reduced Rb phosphorylation but also with the inhibition of E2F1 translocation to nuclei.

Antiproliferative effects were related to the terminal differentiation of HL-60 cells. Terminal differentiation in diverse cell types, which occurs either spontaneously or as a consequence of a treatment with the specific inducing agents, is correlated with an irreversible loss of proliferative potential (32). Several leukemias are characterized by the breakdown of cell maturation pathways (33). To assist the restoration of normal pathways, some patients are treated with differentiation-inducing agents. Accordingly, it is worthwhile to search for these compounds, which induce differentiation as chemopreventive or therapeutic agents (20). In this respect, Rh2 has been demonstrated to have a differentiation-inducing effect on teratocarcinoma cells, ovarian cancer cells and melanoma cells (34). This study demonstrated that Rh2 (20 μM for 96 h) significantly induced the differentiation in the HL-60 cells using a NBT reduction test, esterase activity assay, phagocytosis test and the expressions of the cell surface antigens (CD11b, CD14, CD64 and CD66b). These results suggest that Rh2 is a new potent inducer of HL-60 differentiation to monocytes/macrophages and granulocytes. Furthermore, these findings are consistent with those of a previous report in which Rh2 was found to induce HL-60 differentiation into granulocytes (35).

The TGF-β family of growth factors plays a central role in the regulations of cell growth, differentiation and migration (36). TGF-β1 has various effects on hematopoietic cells, for example, it enhances the proliferation of granulocytes in response to granulocyte–macrophage colony-stimulating factor (37) and inhibits the responsiveness of progenitors to growth promoting cytokines (38). Furthermore, TGF-β1 has been shown to induce growth arrest and differentiation by upregulating of $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ in multiple cell lines (39). This study shows that the differentiation-inducing activities of Rh2 on leukemia cells involve TGF-β1 signaling. Rh2 treatment was found to increase TGF-β1 production and mRNA expression, stimulate Smad/FoxO3a signaling and then mediate the G_1-S transition phase of the cell cycle and differentiation in human leukemia cells, which suggests that TGF-β1 plays a critical role in the Rh2-mediated growth inhibition. Further mechanistic studies on the effect of Rh2 on TGF-β1 production are needed to provide more information on the relationship between Rh2 and TGF-β1 induction.

In summary, this study demonstrates that Rh2 exerts an antiproliferative effect via cell cycle arrest, and that this cell cycle arrest precedes its differentiation-inducing effect on human promyelocytic HL-60 leukemia cells. Furthermore, the Rh2-induced cell cycle arrest and differentiation in HL-60 cells was found to depend on TGF-β1 signaling. Accordingly, we conclude that ginsenoside Rh2 appears to have the potential treatment for leukemia.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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