Ginsenoside Rh2 inhibits proliferation of human promyelocytic HL-60 leukemia cells via G_0/G_1 phase arrest and induction of differentiation

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Summary

1 The present work was performed to investigate the effects of ginsenoside Rh2 on proliferation, cell cycle-regulation and differentiation of human leukemia HL-60 cells as well as the underlying mechanisms for these effects.

2 Ginsenoside Rh2 potently inhibited the proliferation of HL-60 cells in both a dose- and time-dependent manner with an IC_{50}, 20 μM.

3 DNA flow-cytometry indicated that ginsenoside Rh2 markedly induced a G_1 phase arrest of HL-60 cells.

4 Among the G_1 phase cell cycle-related proteins, the levels of cyclin-dependent kinase (CDK)4, 6 and cyclin D1, cyclin D2, cyclin D3 were reduced by ginsenoside Rh2, whereas the steady-state levels of CDK2 and cyclin E were unaffected.

5 The protein levels of a CDK inhibitor p16, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ were markedly increased by ginsenoside Rh2.

6 Ginsenoside Rh2 markedly enhanced the binding of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ with CDK2 and CDK6, resulting in the reduced activity of both kinases and the hypophosphorylation of Rb protein.

7 We furthermore suggest that ginsenoside Rh2 is a potent inducer of the differentiation of HL-60 cells, based on observations such as a reduction of the nitroblue tetrazolium level, an increase in the esterase activities and phagocytic activity, morphology changes, and the expression of CD11b, CD14, CD64 and CD66b surface antigens.

8 In conclusion, the onset of ginsenoside Rh2-induced the G_0/G_1 arrest of HL-60 cells prior to the differentiation is linked to a sharp up-regulation of the $p21^{CIP1/WAF1}$ level and a decrease in the CDK2, CDK4 and CDK6 activities. This is the first report demonstrating that ginsenoside Rh2 potently inhibits the proliferation of human promyelocytic HL-60 cells via the G_1 phase cell cycle arrest and differentiation induction.

Keywords: ginsenoside Rh2; cell cycle arrest; differentiation; p21

Introduction

Ginseng (the root of Panax ginseng C.A. MEYER, family Araliaceae) is frequently used as a crude substance taken orally in Asian countries as a traditional medicine and has been reported to have many physiological and pharmacological effects (Baek et al. 1995; Kim et al. 1996). The major components of ginseng are ginsenosides, which contain an aglycone with a dammarane skeleton (Tanaka *et al*., 1972; Shibata et al., 1963). These ginsenosides have been reported to have an effect on cultured cancer cells by reducing cell proliferation (Popov, 2002), blocking cell cycle (Lee *et al*., 1996), inducing apoptosis, and altering membrane permeability and integrity (Popovich & kitts, 2002). Among them, ginsenoside Rh2 (Rh2) is a plant glycoside with a dammarane skeleton (Figure 1) resembling a steroid skeleton, as an aglycone and it has been suggested to have a cell- differentiating effect on various cancer cells (Lee *et al*., 1996; Xia & Han, 1996; Kim *et al*., 1998). Ginsenoside Rh2 is thought to be a rare ginsenoside that is found only in red ginseng; red ginseng differs from white ginseng as a result of applying a thermal process procedure to extend shelf-life (Kim et al., 2000). Rh2 has also recently been found to be a breakdown product formed by the addition of thermal energy to the extraction of ginsenosides. The main effect of cytotoxic ginsenosides, such as Rh2, on cultured cancer cells is thought to be the result of nonspecific changes to the cell membrane that eventually leads to cell death (Popov, 2002). However, characterization of the type of cell death via either apoptotic or necrotic pathways has not been definitively established.

The essential molecules that regulate cell cycle progression are cyclin-dependent kinases (CDKs) and cyclins. In the early G1 phase, cyclin D is expressed in response to a growth-promoting mitogen and subsequently binds to CDK4/6. The cyclin D/CDK4/6 complex is then activated by Cdk-activating kinase (CAK) which leads to the phosphorylation of the Rb protein.

Phosphorylation of the Rb protein disrupts its association with the E2F family of transcription factors. The hypophosphorylated Rb proteins bind to the E2F family of transcription factors, an important regulator of the cell cycle-progression, and keep them inactive during the M and G_0 phase. The released E2F triggers the expression of several essential proteins for cell cycle progression, such as cyclin E, cyclin A, and thymidine kinase (Ohtani, 1999; Tamraka *et al*., 2000). The activity of the Rb proteins is modulated by the sequential phosphorylation by CDK4/6-cyclin D and CDK2-cyclin E complexes (Lundberg & Weinberg, 1998). Cyclin E forms a complex with CDK2 and the activated cyclin E/CDK2 complex then completes the process by phosphorylating the Rb protein on additional sites. Cyclin/CDK complexes regulate progression through the G1 phase and the initiation of DNA synthesis or entry into the S phase. Cyclin/CDK complex activity is constrained by CDK inhibitors (CKIs). The CKIs that govern these events are classified into two families based on their structures and CDK targets. There are two known classes of mammalian CKIs. One group is the CIP/KIP family, including p21 (CIP/WAF1), p27 (KIP1) and p57 (KIP2), with a broad specificity (Sherr & Roberts, 1999; Morgan, 1997), and the other is the INK 4 family including p15 (INK4B), p16 (INK4A), p18 (INK4C) and p19 (INK4D), which target CDK4 and CDK6 (Sherr & Roberts, 1999). The primary substrates of CDK4/6 and CDK2 in G_1 progression are members of the retinoblastoma protein family, Rb, p107 and p130 (Yu *et al*., 2001; Adams, 2001). Therefore, the growth arrest associated with differentiation could be achieved by several mechanisms including the down-regulation of CDKs, cyclins or the up-regulation of the CKIs or both.

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, provides a useful model system for studying the cellular and molecular events involved in the differentiation process (Drexler *et al*., 1995). Several compounds including dimethyl sulfoxide, retinoic acid, phorbol ester and 1,α,25-dihydroxy vitamin D_3 were known to induce HL-60

promyelocytic leukemia cells to differentiate toward mature cells (brackman *et al*., 1995). The terminal differentiation of HL-60 cells can be monitored by the changes in the morphological, biochemical and immunological properties. The differentiated HL-60 phenotype is characterized by a growth inhibition, an increased adherence, a loss of the cell surface transferring receptors, an increase in the level of the monocytic surface markers, the induction of α-naphthyl acetate (non-specific) esterase and certain patterns of protein phosphoryl-ation (Yam *et al*., 1971).

Therefore, as a part of our screening program to evaluate the chemopreventive potential effect of natural compounds, we investigated the effect of ginsenoside Rh2 (Figure 1) on HL-60 cell growth and induction of differentiation.

Results

Effects of ginsenoside Rh2 on growth inhibition of HL-60 cells

This study was initiated by examining the cytotoxicities of the ginsenoside Rh2 using a MTT assay on the various cancer cells (Table 1). The ginsenoside Rh2 showed different degrees of cytotoxicity on these cells as judged by the IC_{50}, and its values ranged from 38.5 to >200 μM. Among the tested cancer cell lines, the HL-60 cells were the most vulnerable to ginsenoside Rh2. In addition, the effect of sinsenoside Rh2 on the proliferation of HL-60 cells was examined. The cell growth of HL-60 was inhibited in a concentration-and time-dependent manner (Figure 2), suggesting that this chemical has an antiproliferative activity. The inhibitory effect became apparent at a concentration of 20 μM ginsenoside Rh2 , and no cytocidal effects were observed under these conditions. Thus, these concentrations were used throughout the present study.

Effect on Differentiation of HL-60 Cells

After 4 d treatment, the effect of ginsenoside Rh2 on HL-60 cell differentiation was compared with that of $1\alpha,25(OH)_2D_3$, and the results are summarized in Table 2. When HL-60 cells were incubated with ginsenoside Rh2 at concentrations of 20 μM for 4 d, approximately 54.46% of HL-60 cells were stained with NBT, respectively, whereas only 6.71% of the untreated cells were positive (Table 2). $1\alpha,25(OH)_2D_3$ (20 nM) gave 46.18% of NBT-reducible cells. In order to test whether ginsenoside Rh2 HL-60 cells to differentiate into granulocyte and monocyte/macrophage, the esterase activity was measured under the identical conditions. Treatment of HL-60 cells with of 20 μM ginsenoside Rh2 for 4 d resulted in a 26.52% increase of the α-naphthyl acetate esterase activity, respectively, and the effect of ginsenoside Rh2 on the AS-D chloroacetate esterase activity was 24.46%. Moreover, cells treated with these compounds

showed apparent phagocytic activity (Table 2).

In addition, as shown in Figure 3a, 20 μM ginsenoside Rh2 significantly increased the expression of membrane antigens CD11b, CD14, CD64 and CD66b which determine granulocyte and monocyte phenotype. Therefore, we concluded that ginsenoside Rh2 induced differentiation of human promyelocytic leukemia cells to myelocyte/marcrophage and granulocytes. The effect of ginsenoside Rh2 on the cell cycle in HL-60 cells was determined by FACS. As shown in Figure 3b, DNA flow cytometric analysis indicated that a ginsenoside Rh2 treatment for 4 d led to a significant increase in the G1 phase of the cell cycle, whereas a decrease in the G2/M and S phases was detected.

Cell cycle-analysis and expression of cell cycle-regulatory proteins in HL-60 cells

Ginsenoside Rh2 induced the cell cycle arrest in HL-60 cells in a time-dependent manner (Figure 4). The CDK4/6-cyclin D and CDK2-cyclin E protein levels were next examined under the same conditions because CDK4 and CDK6 are believed to be involved in the early G_1, whereas CDK2 is necessary to complete the G_1 phase and initiate the S phase. Ginsenoside Rh2 down-regulated the CDK4, CDK6 and cyclin D1, D2, D3 protein levels, whereas CDK2 and cyclin E were unaffected (Figure 4a). Therefore, these results indicate that the inhibitory effect of ginsenoside Rh2 on cell proliferation is a result of the induction of the G_1 phase arrest of the HL-60 cell cycle through changes in the G_1 phase-regulatory proteins.

Effect of ginsenoside Rh2 on $p16^{INK4a}$, $p21^{CIP/WAF1}$ and $p27^{KIP1}$ expression in HL-60 cells

Because ginsenoside Rh2 induced a G_1 arrest in HL-60 cells, we next examined the change in the $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins, which are the CKIs related with the G_1 phase arrest. The level of $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins were increased in a time-

dependent manner (Figure 4b). The phosphorylation mediated by CDK4/6-cyclin D and CDK2-cyclin E of the Rb protein is required for the cells to progress from G_1 into S phase in cell cycle. Thus we evaluated the degree of phosphorylation of this tumor suppressor protein, Rb. In HL-60 conrol cells, pRb was almost completely hyperphosphorylated, whereas in ginsenoside Rh2 treated cells, a progressive loss of phosphate groups was evident after 2d, as indicated by the shift in the mobility of the pRb bands (figure 4b). The E2F-1, transcription factor, protein levels also decreased (figure 4b).

Effect of ginsenoside Rh2 on the $p21^{CIP/WAF1}$ level of the CDK immune complex and on the CDK-associated kinase activity

Next, we questioned whether or not ginsenoside Rh2-induced $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ would be detected in the complexes with the CDKs during the cell cycle. The CDK2, CDK4 and CDK6 complexes were immunoprecipitated from the HL-60 cells, which were either treated or not treated with ginsenoside Rh2, and the co-immunoprecipitated $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ level in each immune complex was determined by western blot analysis using anti-p16, p21 and p27 antibodies. As shown in Figure 5a, the $p16^{INK4a}$ levels in the CDK4 and CDK6 immune complex of the ginsenoside Rh2-treated cells were distinctively higher than in those of the untreated cells. In addition, the $p21^{CIP1/WAF1}$ levels in the CDK2, CDK4 and CDK6 immune complex and the $p27^{KIP1}$ level in the CDK2 immune complex of the ginsenoside Rh2-treated cells were also distinctively higher than in those of the untreated cells.

Such changes in the $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ levels in each CDK complex was inversely correlated with the *in vitro* CDK kinase activity, which was directly measured by an immune complex using the histone H1 (for CDK2) or GST-Rb fusion protein (for CDK 4 and CDK6) as substrates; the CDK2, CDK4 and CDK6-associated kinase activity dramatically

decreased in the HL-60 cells, which were treated with ginsenoside Rh2 (Figure 5b). In addition, the decrease in the CDK2, CDK4 and CDK6-associated kinase activity was associated with the underphosphorylation of the Rb protein (Figure 5b). Collectively, these results suggest that the $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins might play a key role in the G_1 phase arrest although it increased binding to CDK2, CDK4 and CDK6 in the ginsenoside Rh2-treated HL-60 cells, which leads to the down-regulation of CDK2, CDK4 and CDK6 kinase activity and hence to the cell cycle arrest.