Enhancement of 1,25-Di hydroxyvitamin D₃- and All-trans Retinoic Acid-Induced HL-60 Leukemia Cell Differentiation by Panax ginseng

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Ginseng (Panax ginseng C.A. Meyer) has a wide range of therapeutic uses including cancer treatment. Human promyelocytic leukemia cells differentiate into monocytes or granulocytes when treated with 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] or all-trans retinoic acid (ATRA). Treatment of HL-60 cells with zero to 100 μg/ml of a methanol extract of ginseng for 72 h induced a small increase in cell differentiation. Surprisingly, a synergistic induction of differentiation was observed when HL-60 cells were treated with ATRA or 1,25-(OH)₂D₃ and the extract. The inhibitors of protein kinase C (PKC) and extracellular signal-regulated kinase (ERK), but not of phosphoinositide 3-kinase (PI3-K), inhibited the HL-60 differentiation induced by the extract in combination with ATRA or 1,25-(OH)₂D₃, signifying that PKC and ERK were involved in the cell differentiation enhancement by the extract. These results suggest that the ability of a methanol extract of ginseng to enhance the differentiation potential of ATRA or 1,25-(OH)₂D₃ may improve the ultimate outcome of acute promyelocytic leukemia therapy.

Key words: all-trans retinoic acid; HL-60 cells; leukemia; 1,25-dihydroxyvitamin D₃; Panax ginseng

Panax ginseng C.A. Meyer (Araliaceae) is a well-known medicinal herb native to Korea and China. The root of this herb is commonly known as ginseng, which has been widely used in Asia, especially in China and Korea for more than 2000 years, with the belief that it is a tonic and panacea that can promote longevity.¹⁻³ The use of ginseng continues to grow and it has become one of the most preferred medicines throughout the world. In Korea, Panax ginseng is classified into fresh, white and red ginseng.² The use of ginseng continues to grow and it has become one of the most preferred medicines throughout the world. In Korea, Panax ginseng is classified into fresh, white and red ginseng.² The phytochemicals present in ginseng exert such pharmacological effects as antioxidative, anti-carcinogenic, anti-mutagenic, and anti-tumor. However, the underlying mechanisms of action as anti-cancer agents and the other activities of ginseng are largely unclear.⁵ The ingredients found in ginseng are ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, vitamins, minor elements and enzymes. The pharmacological effects of ginseng are attributed to ginsenosides or ginseng saponin which have been well documented in a large body of literature.¹⁻⁵ More than 40 ginsenosides have been identified; based on their structural differences, they can be classified into three categories: The panaxadiol group (e.g., Rb1), panaxatriol group (e.g., Rg1) and oleanolic acid group (e.g., Ro).⁵ Besides the various therapeutic uses of ginseng, ginsenosides Rk1, Rh2, Rg3 and Rg5 have been reported as potent anticancer compounds.²⁻⁵ A Korean red ginseng extract has recently been reported to induce apoptosis and to decrease telomerase activity in U937 human leukemia cells.⁵

Most cancer cells are defective in their capacity to mature into non-replicating adult cells, thereby existing in a highly proliferating state and thus outgrowing their normal cellular counterparts. Leukaemia cells can be induced to undergo terminal differentiation by a variety of biological and chemical agents, signifying that the malignant state is not irreversible. Leukaemia may eventually prove treatable with agents that induce terminal differentiation, most likely with less morbidity than that associated with treatment by cytodestructive agents.⁹ All-trans retinoic acid (ATRA) and 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] are known to induce terminal differentiation in such leukemic cell lines as HL-60 and U-937, as well as in short-term cultured acute promyelocytic leukemia (APL) cells in humans.¹⁰,¹¹ Human HL-60 leukemia cells differentiate into monocytes when treated with 1,25-(OH)₂D₃, and into granulocytes when treated with ATRA.¹⁰,¹² However, an APL treatment with 1,25-(OH)₂D₃ and ATRA

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Abbreviations: 1,25-(OH)₂D₃; 1,25-dihydroxyvitamin D₃; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase; NBT, nitroblue tetrazolium; PD 98059, 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one; PI3-K, phosphoinositide 3-kinase; PE, phycocerythin; PKC, protein kinase C; PI, PKC peptide inhibitor; PMA, phorbol 12-myristate 13-acetate
at high doses may result in side effects such as the acquisition of drug resistance, hypercalcemia and retinoic acid syndrome. Consequently, the introduction of a second biological or chemical agent which can potentiate the differentiation-inducing ability of ATRA or 1,25-(OH)2D3 at a low, non-toxic concentration could be a useful strategy to solve the underlying problems.

In this paper, the term ginseng represents the dried root of red ginseng (Panax ginseng C.A. Meyer). We present here the enhancing effect of a methanol extract of ginseng (RG-1) on HL-60 leukemia cell differentiation in combination with a low dose of 1,25-(OH)2D3 or ATRA. ATRA and 1,25-(OH)2D3 were selected for the present study because they are the endogenous stimulators which are extensively used in HL-60 cell differentiation.

Materials and Methods

Materials. A six year old red ginseng was purchased from Korea Punngi Ginseng Agricultural Co., Korea. A methanol/water (30:70, v/v) solution (150 ml) was added to 20 g of red ginseng powder and left at room temperature. After 3 d, the residue was removed by filtration followed by centrifugation at 4,000 × g for 30 min. Ethyl acetate (150 ml) was added to the resulting supernatant and mixed vigorously. The upper ethyl acetate layer was then separated and evaporated with a rotary evaporator at 30 °C. The final dried residue (150 mg) from the methanol extract of red ginseng (RG-1) was stored at −20 °C until needed. RG-1 was dissolved in dimethylsulfoxide (DMSO) to generate a 100 mg/ml stock solution. The solution was diluted at least 1000-fold in the growth medium, such that the final DMSO concentration had no effect on the differentiation and proliferation behavior of the HL-60 cells. All manipulation was conducted under subdued light. The HL-60 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in an RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Chelerythrine was purchased from Tocris Cookson (UK). ATRA, 1,25-(OH)2D3, phorbol 12-myristate 13-acetate (PMA), a Giemsa staining solution, described previously. Viability was determined by the trypan blue exclusion assay as the percentage of cells to produce superoxide upon stimulation with PMA. For this assay, the cells were harvested by centrifugation at 3,000 × g for 30 min and incubated with an equal volume of 1% NBT dissolved in phosphate-buffered saline (PBS) containing 200 ng/ml of freshly diluted PMA at 37 °C for 30 min in the dark. Cytosin slides were prepared and examined for a blue-black nitroblue diformazan deposit, indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

Morphological studies. Single-cell suspensions were prepared and loaded into a cytofunnel and spun at 1,050 × g in a cytosin centrifuge. The slides were fixed with methanol and dried. The slides were then stained with a Giemsa staining solution for 20 min, before being rinsed in deionized water, air-dried, and observed under a microscope with a camera. The stained cells were assessed for their size, regularity of the cell margin, and morphological characteristics of the nuclei.

Immunofluorescent staining and cytofluorometric measurements. The expression of cell surface molecules on cells was cytofluorometrically analyzed with a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Briefly, a single-cell suspension was collected from each of the various cultures and washed twice with ice-cold PBS (pH 7.4). Afterwards, phycoerythrin (PE)-conjugated anti-human CD11b or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 monoclonal antibodies (BD Bioscience) were added, before incubating at 4 °C for 1 h. After this incubation, the cells were washed with PBS and fixed in PBS containing 1% paraformaldehyde, and the cytofluorometric analysis was performed. Background staining was determined by staining the cells with PE- or FITC-conjugated isotype control monoclonal antibodies. One-parameter fluorescence histograms were generated by analyzing at least 1 × 106 cells.

Statistical analyses. Student’s t-test and a one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical significance of differences between the values of the various experimental and control groups. A P value of <0.05 is considered to be significant.

Results

Effect of RG-1 on 1,25-(OH)2D3- and ATRA-induced HL-60 cell differentiation

The effect of the methanol extract of ginseng (RG-1) on 1,25-(OH)2D3- or ATRA-induced HL-60 cell differentiation was examined. HL-60 leukemia cells were treated with RG-1 in combination with either 1,25-(OH)2D3 or ATRA, and cellular differentiation was assessed by an NBT reduction assay, taking cells treated with RG-1 alone as controls. Treatment with RG-1 alone induced a relatively very small increase (<5%) in the differentiation of HL-60 cells. The treatment with a suboptimal concentration of ATRA (50 nm) or 1,25-(OH)2D3 (5 nm) resulted in a small increase in the degree of cell differentiation by 18% and 15%, respectively (Fig. 1A). Interestingly, RG-1 synergistically potentiated 1,25-(OH)2D3- or ATRA-induced HL-60 cell differentiation, which was found to increase with increasing RG-1 concentration. The effect of 5 nm 1,25-(OH)2D3 or 50 nm ATRA in combination with 100 μg/ml RG-1 was markedly higher (>70%) than the effect observed with the individual treatment. The cell proliferation and viability for each treatment group were determined. As shown in Fig. 1B, treatment with 100 μg/ml of RG-1 inhibited cell proliferation by 23%, as determined by the MTT assay. Treatment with RG-1 in combination with 1,25-(OH)2D3 or ATRA inhibited cell proliferation by approximately 26%–48%. With all the treatments, throughout the incubation period, the viability of the cells was greater than 95%, as demonstrated by the trypan blue exclusion assay (data not shown).

The morphological phenotypes of HL-60 cells were analyzed in order to further determine the cell differentiation enhanced by RG-1. As depicted in Fig. 2, Giemsa-stained undifferentiated control HL-60 cells (upper left) were predominantly promyelocytes with round and regular cell margin, and large nuclei, suggesting that the cells were highly active in DNA synthesis and rapidly proliferated. Treatment with 100 μg/ml of RG-1, 5 nm 1,25-(OH)2D3 or 50 nm ATRA cells exhibited a relatively small change in cell morphology such as irregular cell margins. The com-
Combined treatment of HL-60 cells with 5 nm 1,25-(OH)_2D_3 or 50 nm ATRA alone, or in combination with various concentrations of a methanol extract of ginseng (RG-1) for 72 h. Subsequent HL-60 cell differentiation and proliferation were evaluated via an NBT reduction assay (A) and MTT assay (B). Data are expressed as the mean ± standard deviation of triplicate determinations from one representative experiment. *P < 0.01, relative to the untreated group; **P < 0.01, relative to the group treated with 5 nm 1,25-(OH)_2D_3 alone; ***P < 0.01, relative to the group treated with 50 nm ATRA alone.

Fig. 1. Effects of RG-1 on 1,25-(OH)_2D_3- or ATRA-Induced HL-60 Cell Differentiation and Growth.

HL-60 leukemia cells were treated with either 5 nm 1,25-(OH)_2D_3 or 50 nm ATRA alone, or in combination with various concentrations of a methanol extract of ginseng (RG-1) for 72 h. Subsequent HL-60 cell differentiation and proliferation were evaluated via an NBT reduction assay (A) and MTT assay (B). Data are expressed as the mean ± standard deviation of triplicate determinations from one representative experiment. *P < 0.01, relative to the untreated group; **P < 0.01, relative to the group treated with 5 nm 1,25-(OH)_2D_3 alone; ***P < 0.01, relative to the group treated with 50 nm ATRA alone.

Fig. 2. Morphological Analysis of RG-1-Treated HL-60 Leukemia Cells.

HL-60 leukemia cells were treated for 72 h with the medium alone, 5 nm 1,25-(OH)_2D_3 or 50 nm ATRA alone (top panel); 100 µg/ml of RG-1 alone, 100 µg/ml of RG-1 plus 5 nm 1,25-(OH)_2D_3, or 50 nm ATRA (bottom panel). The morphology of the cells was assessed by using Giemsa stain. The data are representative of three independent experiments.

No treatment 1,25-(OH)_2D_3 ATRA

RG-1 (100 µg/ml)

A cytofluorometric analysis was performed to determine the expression of specific surface antigens on the HL-60 cells. HL-60 leukemia cells express the cell surface marker, CD11b, when differentiated into monocytes and granulocytes. Treatment with RG-1 markedly increased the number of cells showing a high fluorescence intensity and also synergistically increased the number of CD11b-positive cells when combined with either 5 nm 1,25-(OH)_2D_3 or 50 nm ATRA, confirming that RG-1 enhanced 1,25-(OH)_2D_3- or ATRA-induced HL-60 cell differentiation (Fig. 3A).

Effects of RG-1 and 1,25-(OH)_2D_3 or ATRA on the differentiation pathway in HL-60 leukemia cells

To determine the differentiation pathway following the treatment with RG-1 and 1,25-(OH)_2D_3 or ATRA, the expression of the CD14 antigen was cytofluorometrically analyzed on HL-60 cells treated with RG-1 alone or in combination with either ATRA or 1,25-(OH)_2D_3. The CD14 antigen is exclusively expressed when cells are differentiated into monocytes. To determine the differentiation pathway after the treatment with RG-1 and 1,25-(OH)_2D_3- or ATRA, a cytofluorometric analysis was performed on HL-60 cells treated with RG-1 alone or in combination with 1,25-(OH)_2D_3- or ATRA. As shown in Fig. 3B, HL-60 cells treated with a mixture of RG-1 and 1,25-(OH)_2D_3 reacted very strongly with the anti-CD14 monoclonal antibody. The cells treated with 1,25-(OH)_2D_3 alone also reacted with the anti-CD14 monoclonal antibody, but to a lesser extent than did the cells treated with a mixture of RG-1 and 1,25-(OH)_2D_3. These results indicate that RG-1 stimulated 1,25-(OH)_2D_3-induced HL-60 cell differentiation along the monocytic pathway. In contrast, HL-60 cells treated with a mixture of RG-1 and ATRA were not stained with the anti-CD14 monoclonal antibody. On the other hand, the cells treated with a mixture of RG-1 and ATRA stained more strongly than with ATRA alone with a monoclonal antibody against the HL-60 cell differentiation marker, CD11b (Fig. 3A), indicating that RG-1 stimulated ATRA-induced HL-60 cell differentiation along the granulocytic pathway.
Sensitizing effect of RG-1 on HL-60 cell differentiation

Some differentiation-enhancing agents sensitize leukemia cells to other differentiation-inducing agents, resulting in a strong increase in cell differentiation. HL-60 cells were pre-, co-, or post-treated with RG-1, followed by 1,25-(OH)<sub>2</sub>D<sub>3</sub> or ATRA. As shown in Fig. 4, the pretreatment (before 24 h) and simultaneous treatment (at the same time) with RG-1 significantly increased the 1,25-(OH)<sub>2</sub>D<sub>3</sub>- or ATRA-induced HL-60 cell differentiation, while post-treatment (after 24 h) showed a relatively smaller effect. Moreover, removing RG-1 before treating with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or ATRA also had a relatively smaller differentiation effect than pretreatment and simultaneous treatment. These results indicate that RG-1 not only potentiated leukemia cell differentiation, but also sensitized the cells to ATRA or 1,25-(OH)<sub>2</sub>D<sub>3</sub> for enhancing the cell differentiation.

Effect of inhibitors of PKC, ERK or PI3-K on the HL-60 cell differentiation induced by RG-1 in combination with ATRA or 1,25-(OH)<sub>2</sub>D<sub>3</sub>

To determine if any relationship existed between the effect of RG-1 on 1,25-(OH)<sub>2</sub>D<sub>3</sub>- or ATRA-induced cell differentiation and PKC, ERK and PI3-K activation, HL-60 cells were pretreated with a specific inhibitor and then incubated for 72 h in the presence of RG-1 alone or in combination with either 1,25-(OH)<sub>2</sub>D<sub>3</sub> or ATRA. The degree of cell differentiation was then assessed by an NBT assay. As shown in Fig. 5, the PKC inhibitors (GF 102903X, chelerythrine, and the PKC peptide inhibitor) and ERK inhibitor (PD 98059) significantly inhibited HL-60 cell differentiation when treated with RG-1 in combination with ATRA or 1,25-(OH)<sub>2</sub>D<sub>3</sub>, whereas the PI3-K inhibitor (LY 294002) did not.

Discussion

The present study was aimed to search for a natural source that can be useful in APL therapy either alone or in the combination with well-known differentiation agents. Panax ginseng C.A. Meyer is a highly valued medicinal herb used under a variety of pathological conditions and illnesses such as hypodynamia, anorexia, shortness of breath, palpitation, insomnia, impotence, hemorrhage and diabetes. Additionally, ginseng is one of the most sought-after medicines throughout the world because of the active constituent, ginsenosides, which have such pharmacological properties as anticancer, antioxidantive, antiapoptotic, immunostimulative, neuroprotective, and immunomodulative effects. Various types of ginsenosides have been reported from red ginseng; among them, Rg3, Rg5, Rk1, and Rh2 have been reported to have anti-cancer effects. A Korean
RG1, RG3, Rh2 and Rk1, were assumed as possible human leukemia cells. Among a variety of saponins apoptosis and decrease telomerase activity in U-937 red ginseng extract has recently been shown to induce (Figs. 1A and 2). HL-60 leukemia cells were synergistically differentiated into a granulocyte and monocyte lineage when treated with RG-1 in combination with a low dose of ATRA and 1,25-(OH)2D3, respectively (Fig. 3). The differentiation potentials of such inducing agents as 1,25-(OH)2D3 and ATRA have previously been reported to synergistically enhance with other additional agents. ATRA combinations with histone deacetylase inhibitors, gefitinib, and carnosic acid have exerted synergistic effects on HL-60 leukemia cell differentiation. In the same way, capsaicin, ascorbate, and cyclooxygenase inhibitors have enhanced the HL-60 cell differentiation produced by a low level of 1,25-(OH)2D3. Such analogues of vitamin D3 as 1,25-(OH)2D3 are used clinically for the treatment of psoriasis. ATRA, an active derivative of vitamin A, is widely used for the treatment of APL patients with a high rate of complete remission. Despite its advantage in leukemia treatment, ATRA has such side effects as retinoic acid syndrome and hypercalcaemia.

The induction of differentiation in HL-60 cells requires the activation of a variety of signal transduction pathways, including PKC, PKC and MAPK. Moreover, several studies have suggested that ginsenoside affected PKC activation. In our study, the inhibitors of PKC or ERK kinase significantly inhibited the HL-60 cells differentiation induced by RG-1 in combination with ATRA or 1,25-(OH)2D3, suggesting that the potentiation of cell differentiation by RG-1 may be, at least in combination with ATRA or 1,25-(OH)2D3, via the PKC- and ERK-mediated signaling pathways. In contrast, the PI3-K inhibitor did not decrease the enhanced cell differentiation by RG-1 in the presence of a low level of 1,25-(OH)2D3 or ATRA. These results indicate that RG-1 potentiated ATRA- and 1,25-(OH)2D3-induced HL-60 cell differentiation via the PKC/ERK signaling pathways. Further studies need to be carried out concerning the detailed action mechanism for HL-60 cell differentiation enhanced by RG-1 by the activation of conventional, novel or atypical PKC isoforms, and/or other kinases, which is the aim of our future experiments.

Epidemiological studies have suggested that people who eat large amounts of fruit and vegetables have a lower risk of many kinds of cancer. Although the reason behind it is yet to be explained, it may be possible that various dietary chemicals such as carotenoids, curcuminoids, and tocopherols, and the constituents of other edible plants can prevent human cancer in part by synergizing with endogenously produced differentiation stimulators such as retinoic acids and 1,25-(OH)2D3. The results suggest the possible use of a ginseng extract in combination with a low dose of ATRA or 1,25-(OH)2D3 in APL therapy, which may
produce a greater therapeutic response than ATRA or 1,25-(OH)$_2$D$_3$ alone, presumably with fewer side effects such as retinoic acid syndrome.

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References