Effects and action mechanism of Diospyros kaki on the differentiation of human leukemia HL-60 cells

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Abstract. Diospyros kaki Thunb. (Ebenaceae) is widely distributed in North-East Asian countries. Almost all parts of this plant have been traditionally used as medicine. 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). Combination of low doses of ATRA or 1,25-(OH)₂D₃ that do not induce toxicity with another drug is a useful strategy for acute promyelocytic leukemia therapy. Our main aim was to investigate the effect of an acetone extract of D. kaki leaves (KV-1) on HL-60 cell differentiation in combination of ATRA or 1,25-dihydroxyvitamin D₃. Treatment of HL-60 cells with zero to 100 μg/ml of KV-1 for 72 h induced a small increase in cell differentiation. Surprisingly, a synergistic induction of differentiation was observed when the HL-60 cells were treated with ATRA or 1,25-(OH)₂D₃ and the extract. The inhibitors of protein kinase C (PKC) (α and β₁) and extracellular signal-regulated kinase (ERK), but not of phosphoinositide 3-kinase (PI3-K) and c-Jun N-terminal kinase (JNK) inhibited the differentiation, presumably with less morbidity than that associated with treatment with the cytodestructive agents (8). 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] and all-trans retinoic acid (ATRA) are well known to be able to induce terminal differentiation in leukemic cell lines, such as HL-60 and U-937, as well as in short-term cultured acute promyelocytic leukemia (APL) cells in human (9,10). However, an APL treatment by 1,25-(OH)₂D₃ and ATRA at a high dose results in side effects including acquisition of drug resistance and hypercalcemia. Therefore, the current approach in solving this problem involves the introduction of a second chemical which enhances the differentiation-inducing effects of 1,25-(OH)₂D₃ or ATRA at a lower, non-toxic concentration. HL-60 cells, extensively used as a model, differentiate into neutrophils.

Introduction

Diospyros kaki Thunb. (Ebenaceae), commonly called persimmon, is widely distributed throughout Korea, Japan, China and other northeast Asian countries. The crude plant extract of D. kaki is a very complex mixture containing compounds such as p-coumaric, gallic acid, catechin, flavonoids and tannin (1). The phytochemicals present in D. kaki have been used for therapeutic purposes such as scavenging of hypercholesterolemia, antioxidant and free radicals; detoxification of snake venom and toxic substances produced by microorganisms; and inhibition of human lymphoid leukemia cells (2). Vasorelaxant activity was also reported from D. kaki leaf extract (3). Major constituents of D. kaki leaves responsible for pharmacological effect are tannins, flavonoids/coumarins and terpenoids (1). Astragalin, a major constituent of flavonoids present in persimmon leaf extract, inhibited dermatitis development and IgE elevation in mice (4). Four flavonoids compound isolated from persimmon leaves inhibited angiotensin-converting enzyme activity in dose-dependent fashion (5). Similarly, five triterpenoids (6) and five flavonoids (7) compounds isolated from Diospyros kaki leaves have been reported to suppress stimulus-induced superoxide generation and tyrosyl phosphorylation in human neutrophils.

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 biochemical agents, indicating that the malignant state can be reversible. Leukaemia may eventually prove treatable with agents that induce terminal differentiation, presumably with less morbidity than that associated with treatment with the cytodestructive agents (8). 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] and all-trans retinoic acid (ATRA) are well known to be able to induce terminal differentiation in leukemic cell lines, such as HL-60 and U-937, as well as in short-term cultured acute promyelocytic leukemia (APL) cells in human (9,10). However, an APL treatment by 1,25-(OH)₂D₃ and ATRA at a high dose results in side effects including acquisition of drug resistance and hypercalcemia. Therefore, the current approach in solving this problem involves the introduction of a second chemical which enhances the differentiation-inducing effects of 1,25-(OH)₂D₃ or ATRA at a lower, non-toxic concentration. HL-60 cells, extensively used as a model, differentiate into...
Materials and methods

Materials. Diospyros kaki Thumb. leaves were collected from Jeonnam area, in October 2006. The plants were authenticated by the Department of Pharmacognosy, Chosun University. First, 2 litres of acetone/water (80:20, v/v) solution was added to 1 kg wet leaves and left at room temperature. After 2 years, residue was removed by centrifugation and the resulting acetone-aqueous solution was evaporated with a rotary evaporator at 40°C. The resulting thick residue was washed three times with hexane and then dissolved in dichloromethane before evaporation. The final residue, KV-1, was dissolved in dimethylsulfoxide (DMSO) to generate a 100 mg/ml stock solution. The solution was diluted at least 1,000-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. Manipulations were conducted under subdued light condition. The HL-60 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). 1,25-(OH)2D3, ATRA, phorbol 12-myristate 13-acetate (PMA), 2-[4-Morpholinyl]-8-phenyl-1-[4H]-benzopyran-4-one (LY 294002), wortmannin, anthrapyrazolone (SP 600125), a Giemsa staining solution, methanol-free paraformaldehyde and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chelerythrine, 1-(5-isouquinolinesulfonyl)-2-methylpyrazoline dihydrochloride (H 7) and 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) were purchased from Tocris Cookson Ltd. (UK). Stock solution of 1 mM ATRA was dissolved in DMSO.

Preparation of cell lysates and Western blot analysis. Cell viability was determined by the trypsin blue exclusion assay, as previously described (12). Viability was calculated as the percentage of live cells in the total cell population. Cell differentiation was assessed by the nitroblue tetrazolium (NBT) reduction assay as previously described (13), which is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For the assay, cells were harvested by centrifugation at 3,000 x g 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 NBT-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

Determination of cell viability and differentiation. Cell viability was determined by the trypan blue exclusion assay, as previously described (12). Viability was calculated as the percentage of live cells in the total cell population. Cell differentiation was assessed by the nitroblue tetrazolium (NBT) reduction assay as previously described (13), which is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For the assay, cells were harvested by centrifugation at 3,000 x g 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 NBT-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

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

Results

Effect of KV-1 on 1,25-(OH)2D3 and ATRA-induced HL-60 cell differentiation. The effect of an acetone extract of D. kaki leaves (KV-1) on 1,25-(OH)2D3 or ATRA-induced HL-60 cell differentiation was examined. HL-60 leukemia cells were treated with KV-1 in combination with either 1,25-(OH)2D3 or ATRA and cellular differentiation was assessed by NBT reduction assay. Cells treated with KV-1 alone were taken as controls. Treatment with KV-1 alone induced a relatively small increase of HL-60 cell differentiation by ~10%, whereas the treatment with a suboptimal concentration of ATRA (50 nM) or 1,25-(OH)2D3 (5 nM) resulted in a slight increase in the
concentrations of KV-1 or in combinations with either 5 nM 1,25-(OH)2D3 or 50 nM ATRA, confirming that KV-1 potentiated 1,25-(OH)2D3- or ATRA-induced HL-60 cell differentiation (Fig. 2A).

To determine the differentiation pathway following treatment with KV-1 and ATRA or 1,25-(OH)2D3, the expression of CD14 antigen was cytofluorometrically analyzed on the treated HL-60 cells with KV-1 alone or in combination with either 1,25-(OH)2D3 or ATRA. The CD14 antigen is exclusively expressed when cells are differentiated into monocytes (15). HL-60 cells treated with a mixture of KV-1 and 1,25-(OH)2D3, reacted very strongly with anti-CD14 mAb (Fig. 2B). Cells treated with 1,25-(OH)2D3 alone, also reacted with the anti-CD14 mAb, but to a lesser extent than did the cells treated with a mixture of KV-1 and 1,25-(OH)2D3. These results indicate that KV-1 stimulated 1,25-(OH)2D3-induced HL-60 cell differentiation along the monocytic pathway. In contrast, HL-60 cells treated with a mixture of KV-1 and ATRA showed little staining with anti-CD14 mAb, although synergistic induction of cell differentiation was observed, as shown by NBT reduction assay. Alternatively, the HL-60 cell treated with a mixture of KV-1 and ATRA stained strongly with a monoclonal antibody against HL-60 cell differentiation marker CD11b (Fig. 2A), indicating that KV-1 stimulated ATRA induced HL-60 cell differentiation along the granulocytic pathway.

Effects of KV-1 and 1,25-(OH)2D3 or ATRA on differentiation pathways of HL-60 cells. Cytocfluorometric analysis was performed to determine the expression of specific surface antigens on HL-60 cells. HL-60 leukemia cells express a cell surface marker, CD11b, when the cells are differentiated into monocytes and granulocytes (14). Treatment with KV-1 markedly increased the number of cells with high fluorescence intensity and also synergistically increased the number of the CD11b-positive cells when combined with either 5 nM 1,25-(OH)2D3 or 50 nM ATRA, confirming that KV-1 potentiated 1,25-(OH)2D3- or ATRA-induced HL-60 cell differentiation (Fig. 2A).

To investigate the involvement of PKC in HL-60 cell differentiation enhanced by KV-1, the HL-60 cells were analyzed by flow cytometry to determine the expression of specific PKC isoforms. In this study, we focused on the conventional PKC isoforms such as α, βI, βII, as these PKC isoforms are known to be the most abundantly expressed in HL-60 leukemia cells and the expression levels have been correlated closely with cell differentiation in HL-60 cells (16). KV-1 increased protein levels of total PKC, in particular, KV-1 increased protein level of PKCo and PKC βI, but not that of

Figure 1. Effects of KV-1 on 1,25-(OH)2D3- or ATRA-induced HL-60 cell differentiation. HL-60 cells were simultaneously treated with various concentrations of KV-1 or in combinations with either 5 nM 1,25-(OH)2D3 or 50 nM ATRA for 72 h. (A) The cellular differentiation was assessed by the NBT reduction assay. Each value represents the mean ± SEM (n=3). *P<0.05 relative to the 5 nM of 1,25-(OH)2D3-treated group; **P<0.05 relative to the 5 nM 1,25-(OH)2D3-treated group. (B) The treated HL-60 cells were assessed by morphological analysis using Giemsa stain.
PKC βII (Fig. 4B). To further elucidate the involvement of ERK, the protein levels of ERK was determined by Western blot analysis. The levels of pERK increased by the treatment with KV-1 alone and KV-1 plus ATRA or 1,25-(OH)_{2}D_{3} (Fig. 5B).

**Discussion**

In the present study, we demonstrated that KV-1 enhanced 1,25-(OH)_{2}D_{3} and ATRA-induced differentiation in HL-60 leukemia cells, which are widely used as a model system for...
Figure 4. Involvement of PKC in HL-60 cell differentiation induced by KV-1 in combination with 1,25-(OH)_2D_3 or ATRA. (A) HL-60 cells were treated with various concentrations of PKC inhibitors (GF 109203X, chelerythrine, H7 and PKC peptide inhibitor) for 1 h, followed by incubation with 100 μg/ml KV-1 in combination with either 5 nM 1,25-(OH)_2D_3 or 50 nM ATRA. The cellular differentiation was assessed by the NBT assay. Each value represents the mean ± SEM (n=3). (B) HL-60 cells were treated with 100 μg/ml KV-1 alone or in combination with either 5 nM 1,25-(OH)_2D_3 or 50 nM ATRA for 1 h. The protein level of total PKC and each of conventional PKC isoforms were determined by Western blot analysis. The experiment was repeated twice with similar results.

Figure 5. Involvement of ERK and a proposed action mechanism in KV-1-mediated enhancement of HL-60 cell differentiation. (A) HL-60 cells were treated with various concentrations of ERK inhibitor (PD 98059) for 1 h, followed by incubation with 100 μg/ml KV-1 in combination with either 5 nM 1,25-(OH)_2D_3 or 50 nM ATRA. The cellular differentiation was assessed by the NBT assay. Each value represents the mean ± SEM (n=3). (B) HL-60 cells were treated with 100 μg/ml KV-1 alone or in combination with either 5 nM 1,25-(OH)_2D_3 or 50 nM ATRA for 1 h. The protein level of ERK and phospho-ERK were determined by Western blot analysis. The experiment was repeated twice with similar results. (C) A proposed mechanism of the KV-1-treated HL-60 cell differentiation in combination with 1,25-(OH)_2D_3 or ATRA.
studies of differentiation. HL-60 cells were synergistically differentiated into monocytes and granulocytes when treated with KV-1 in combination with a low dose of 1.25-(OH)\(_2\)D\(_3\) and ATRA, respectively. ATRA combinations with histone deacetylase inhibitors (17) and gefitinib (18) exerted synergistic effects on HL-60 cell differentiation. Similarly, ascorbate (19), capsaicin (20) and cyclooxygenase inhibitors (21) enhanced the HL-60 cell differentiation produced by a low level of 1.25-(OH)\(_2\)D\(_3\).

Previous studies have revealed that PKC activation is necessary for the differentiation of HL-60 cells (16,22) and mitogen-activated protein kinases (MAPKs) are downstream elements in the PKC signaling pathway of HL-60 cells (23). The c-Jun N-terminal kinase (JNK) signaling module is also known to participate in myeloid cell differentiation (24,25). Phosphatidylinositol 3-kinase (PI3-K) activity plays an essential role in differentiation of HL-60 cells (26). Here, a combination of KV-1 with 1.25-(OH)\(_2\)D\(_3\) or ATRA contributed to the activation of the PKC and ERK, but not that of PI3-K or JNK-mediated pathways, as demonstrated by Western blot analyses and kinase inhibitor studies, although protein levels of the conventional PKC isofoms \(\alpha\) and \(\betaI\) increased at different extent (Figs. 3-5). Taken together, the results suggest that KV-1 potentiates ATRA- and 1.25-(OH)\(_2\)D\(_3\) induced HL-60 cell differentiation through distinct pathways through PKC(\(\alpha\), \(\betaI\))/ERK without involvement of PI3-K and JNK (Fig. 5C). Recently, we have shown that a methanol extract of Panax ginseng synergistically induced differentiation potential of ATRA and 1.25-(OH)\(_2\)D\(_3\), with the involvement of PKC/ERK, but not that of PI3-K (27).

Phytochemicals present in \(D.\) kaki have several pharmacological effects including potential inhibitory effects on human lymphoid leukemia cells (1-4). Major constituents of \(D.\) kaki leaves responsible for pharmacological effect are flavonoids and terpenoids (1,4-7). Therefore, although we could not pinpoint the exact active compound present in acetone extract of KV-1, flavonoid and/or terpenoid compound might be responsible for ATRA and 1.25-(OH)\(_2\)D\(_3\)-induced HL-60 differentiation. Epidemiological studies suggest that there is a low risk of many kinds of cancer to the people who eat large amounts of fruits and vegetables (28). Although the underlying reason is largely unclear, various dietary chemicals such as curcuminoids, and carotenoids may possibly prevent human cancer in part by synergizing endogenously produced differentiation stimulators such as retinoic acids and 1.25-(OH)\(_2\)D\(_3\).

In summary, an acetone extract of \(D.\) kaki leaves markedly increased the HL-60 cell differentiation in combination of a low dose of 1.25-(OH)\(_2\)D\(_3\) or ATRA via PKC(\(\alpha\), \(\betaI\))/ERK pathways. The results suggest that treatment of acute promyelocytic leukemia patients with combinations of acetone extract of \(D.\) kaki leaves and ATRA or 1.25-(OH)\(_2\)D\(_3\) may provide a greater therapeutic response than 1.25-(OH)\(_2\)D\(_3\) or ATRA alone.

References


