Combined treatment of leukemia cells with vitamin K$_2$ and 1α,25-dihydroxy vitamin D$_3$ enhances monocytic differentiation along with becoming resistant to apoptosis by induction of cytoplasmic p21$^{CIP1}$

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Abstract. Vitamin K$_2$ (VK$_2$) effectively induces apoptosis in leukemia cell lines, including HL-60 and U937. However, combined treatment of cells with VK$_2$ plus 1α,25-dihydroxy-vitamin D$_3$ (VD$_3$) resulted in suppression of VK$_2$-inducing apoptosis and pronounced induction of monocytic differentiation as compared with that by VD$_3$ alone. After achieving monocytic differentiation by pre-exposure to VK$_2$ and VD$_3$, the cells became resistant to various apoptotic stimuli including VK$_2$- and H$_2$O$_2$-treatment and serum deprivation. Accumulation of cytoplasm p21$^{CIP1}$ along with disappearance of nuclear p21$^{CIP1}$ was detected in cells in response to 96-h treatment with VK$_2$ plus VD$_3$. A stable transfec tant, U937-ANLS-p21$^{CIP1}$, which lacked the nuclear localization signal of p21$^{CIP1}$ and showed overexpression of cytoplasm p21$^{CIP1}$ without monocytic differentiation, was resistant to apoptosis. These data suggest that a change of intracellular distribution of p21$^{CIP1}$ from nucleus to cytoplasm along with differentiation appears to be anti-apoptotic. Clinical benefits of using VK$_2$ for treatment of patients with leukemia and myelodysplastic syndrome (MDS) have been reported. Our data suggest that VK$_2$ plus VD$_3$ may be an effective combination for differentiation-based therapy for leukemia and also MDS whose cytopenias are mediated though apoptosis.

Introduction

Normal hematopoiesis is regulated by a balance of cell growth, differentiation and apoptosis, which results in dynamic turnover and homeostasis of blood cells in vivo (1). Deregulation of this balance favoring cell growth, differentiation, or apoptosis results in various hematopoietic disorders, including leukemia and myelodysplastic syndrome (MDS) (2,3). Acute myeloid leukemia (AML) is usually caused by gain in cell growth and loss of differentiation and apoptotic ability (2,4). MDS is a group of clonal stem cell disorders caused by an acquired genetic anomaly, which is characterized by cytopenias via enhanced apoptosis of hematopoietic progenitors and blood cells and a variable risk of transformation to AML (3). A growing body of evidence suggests that cell growth and differentiation are inversely regulated in hematopoietic cells. Differentiating cells lose growth potency and telomerase activity (5-7). Therefore, therapeutic attempts to induce differentiation of leukemia cells have been carried out with various reagents (7,8). Differentiation therapy in patients with acute promyelocytic leukemia (APL) using all-trans-retinoic acid (ATRA) has now been well established as an effective treatment strategy (9,10). Although there are other potent differentiation-inducing agents including 1,25(OH)$_2$-vitamin VD$_3$ (VD$_3$) for leukemia cells in vitro, few of the inducers known to date have therapeutic value because of severe adverse effects or the unusually high concentrations required (11).

We have originally reported that vitamin K$_2$ (menaquinone: VK$_2$) has a potent apoptosis-inducing effect in leukemia cell lines and primary leukemia cells (12,13). Several case studies have shown clinical benefits of using VK$_2$ for the treatment of patients with AML and MDS (14-17). Since VK$_2$ has much lower toxicity than other anti-cancer agents and can be administered long-term, it may open up novel strategies for AML therapy and also for chemoprevention for the management of patients with MDS. VK$_2$ also induces differentiation of acute myeloid leukemia cell lines such as HL-60 and U937 (18). In HL-60-bcl-2 cells, which overexpress BCL-2, VK$_2$ induces monocytic differentiation but, in HL-60-neo cells with a lower BCL-2 expression level, VK$_2$ induces apoptosis (19). Although HL-60-bcl-2 became almost completely resistant to apoptosis induction by VK$_2$, monocytic differentiation and G0/G1 arrest was still observed.
This observation suggests that VK2 promotes not only apoptosis but also has a differentiation-inducing effect in those leukemia cells that are resistant to apoptosis. However, comparing the effects of VK2 for apoptosis and differentiation-inductions in leukemia, apoptosis induction appears to be more prominent (19). In contrast to VK2, VD3 has been reported to induce differentiation rather than apoptosis in leukemia cell lines (20-22). In the present study, we therefore focused on the combined effects of VK2 and VD3 on leukemia cells for determining cell growth, differentiation, and apoptosis.

Our data demonstrate that a combination of VD3 and VK2 enhances induction of monocytic differentiation in leukemia cell lines. We also report that the sensitivity against apoptotic stimuli including VK2 and monocytic differentiation are inversely regulated by a change in the intracellular distribution of p21CIP1.

**Materials and methods**

**Reagents and antibodies.** Menaquinon-4, a vitamin K2 analog, was donated by Eisai Co. Ltd. (Tokyo, Japan). 1α,25(OH)2-vitamin VD3 was supplied by Chugai Pharmaceutical Co. (Tokyo, Japan) (12). Fluorescein isothiocyanate (FITC) conjugated anti-human CD14 and phycoerythrin (PE) conjugated AP02.7 monoclonal antibody (mAb), PE conjugated anti-human CD11c mAb, phycoerythrin cyanin 5 (PC5) conjugated anti-human CD14 and AP02.7 mAbs were all obtained from Immunotech (Marseille, France). Anti-JNK1 mAb, anti-JNK2 mAb, and anti-phospho-JNK1/2 mAbs were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-actin mAb was purchased from Chemicon International (Temecula, CA).

**Cell lines and primary cultures.** Acute myeloid leukemia cell lines, HL-60 and U937, obtained from the American Type Culture Collection (Rockville, MD), were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (100 μg/ml). Exponentially growing cells were used for further experiments.

**Flow cytometric analysis.** Cellular antigens, viable cell numbers, and cell cycle analysis were analyzed by flow cytometry using EPICS XL II (Beckman-Coulter Japan, Tokyo, Japan). Before staining with antibodies, cells were pre-incubated with human γ-globulin (Venoglobulin-H, Welfide Co. Osaka, Japan), for 30 min at room temperature for blocking antibody binding to Fc receptors. Thereafter, cells were incubated with FITC-, PE-, or PC5-conjugated mAbs for 30 min at 4°C. After washing three times with PBS, fluorescent intensities were analyzed.

For the assessment of the viable cell count, the cells treated with or without VK2 or VD3 were stained with a solution containing 1% (w/v) propidium iodide (PI) (Sigma Chemical Co.) for 30 min at 4°C. First, the gating area of a cytogram for detecting the viable cells was established from the PI negative area (indicating viable cells) and the forward- and side-scatter intensities. Then, the cell cultures were pipetted gently to obtain a uniform cell suspension, and were introduced to a flow cytometer. The number of cells in the gating area for viable cells was determined for 60 sec. The number relative to the cells treated with a control medium was well-correlated with the results obtained from a Cell Counting Kit (Dojin East, Tokyo, Japan), with absorption measurements at 450 nm (12).

**Assessment of apoptosis.** Cells undergoing apoptosis were determined by flow cytometry using PCS-conjugated AP02.7 mAb (clone 2.7; Immunotech). AP02.7 is directed against the 38 kDa mitochondrial membrane protein (7A6 antigen) specifically expressed by cells undergoing apoptosis (23). Detection of the percentage of AP02.7-positive cells is consistent with the results of the TUNEL method and DNA ladder formation (13). Caspase-3 activity was examined by flow cytometry using a substrate reagent kit containing PhiPhiLux-G6D2, a rhodamine-containing specific substrate with amino acid sequence GDEVDGDI (Oncolimmunin, College Park, MD) as previously described (24). After treatment with or without VK2 and VD3, cells were washed with PBS and incubated with the substrate reagent at 37°C for 60 min. Fluorescence of the caspase-3 cleaved substrate by activated caspase-3 was analyzed by flow cytometry at FL2 channel with excitation at 488 nm.

**Assessment of cell differentiation.** Differentiation of leukemia cell lines was assessed by morphology and cell surface antigen expressions using flow cytometry as described above. For morphologic observations, the cell suspension was introduced in Shandon Cytospin 2 (Shandon Inc., Pittsburgh, PA), and the preparations were stained with May-Giemsa (19).

**Cell cycle analysis.** Cells were fixed and stained with a solution containing 1% PI, 100 μg/ml digitonin, 0.01% NaNO3, 200 mg/ml RNAase (Sigma), and 2.5% FCS for 10 min at room temperature. Cells were analyzed by flow cytometry with a cell cycle analysis program, MultiCycle AV (Phoenix Flow Systems Inc., San Diego, CA) (19).

**Immunoblotting.** Immunoblotting was performed as previously described in detail (19). Briefly, the cells treated with VK2 and/or VD3 were lysed with lysis buffer containing 1% NP-40 (9). Total protein content of the lysate was determined by a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA). The same amount of cellular protein was loaded and separated by 12.5% SDS-PAGE. Proteins were electrophoretically transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). After blocking with Tris-buffer saline (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl plus 0.05% Tween-20) (TBST) containing 5% bovine albumin (Sigma) for 2 h, the blots were incubated with primary mAb for 2 to 4 h at room temperature. The membranes were washed 5X with TBST, then incubated with peroxidase-conjugated second Ab. Immunoreactive proteins were detected by a chemiluminescent system (ECS; Amersham) (19).

**Subcellular localization of p21CIP1.** To assess the intracellular distribution and expression of p21CIP1, immunofluorescence assay was performed. Cells were fixed with 3.7% formaldehyde in PBS for 20 min and then blocked and permeabilized.
with 10% FBS in 0.1% Triton X-100 for 30 min at 23°C, and further incubated with murine anti-p21\textsuperscript{Clp1} mAb for 2 h at room temperature. After washing 3X with TBS, FITC-labeled goat anti-murine IgG was used as a second antibody. Immunoreactive p21\textsuperscript{Clp1} was detected with fluorescent microscopy using the AcuaCosmos System (Hamamatsu Photonix, Shizuoka, Japan).

A stable transfectant for ANLSp21\textsuperscript{Clp1} (aal-140) gene in U937 cells. A stable transfectant of U937 cells with the ANLSp21\textsuperscript{Clp1} (aal-140) gene, which lacks a nuclear localizing signal, was established as previously described (20).

Statistical analyses. Data are given as the mean ± SD. Comparisons between two groups were assessed with the Mann-Whitney U test.

Results

Combined treatment with VK2 plus VD3 synergistically enhances monocytic differentiation in leukemia cell lines. VK2 treatment induces apoptosis of leukemia cells and cell lines (12,13,18). However, VK2 also shows some differentiation-inducing effect toward some leukemia cells that express a high level of BCL-2 (19). In contrast, VD3 has been shown to induce monocytic differentiation in HL-60 and U937 cells (20-22). Therefore, we first examined the combined effects of VK2 plus VD3 on differentiation in HL-60 and U937. When the cells were exposed to more than 1 nM of VD3, the cell surface expression of CD14, a monocyte-lineage specific antigen, was increased in a dose-dependent manner and leveled off at more than 10 nM of VD3 (data not shown). Combined treatment with VK2 and VD3 enhanced induction of CD14, far exceeding the effect of VD3 alone (Fig. 1A). Morphological studies revealed that HL-60 and U937 cells both underwent almost complete monocytic maturation, with condensed nuclear chromatin and spread cytoplasm. In contrast, when the cells were treated with VK2 alone, the typical microscopic features of apoptotic cells were observed, i.e. they were shrunken and had fragmented nuclei (Fig. 1B). These data suggest that the combined treatment of VD3 plus VK2 synergistically enhances monocytic differentiation of leukemia cell lines, as previously reported in HL-60 cells treated with 22-oxa-1,25-dihydroxyvitamin VD3, a vitamin VD3 derivative, and VK2 (26). Cell-cycle analysis also demonstrated the enhanced accumulation of cells in the G0/G1 phase after 24-h-treatment with VD3 plus VK2 (Fig. 2).
Combined treatment with VK2 plus VD3 inhibits apoptosis and JNK activity along with monocytic differentiation in HL-60 and U937 cells. Since VK2 induces apoptosis rather than differentiation in leukemia cells, we next examined apoptosis induction by combined exposure to VK2 and VD3. After 96-h exposure to the drugs, apoptotic cells were assessed by flow cytometry using APO2.7 mAb (Fig. 3). VK2 alone induced apoptosis of HL-60 cells, whereas VD3 alone had no apoptotic effect. However, combined treatment with VK2 plus VD3 resulted in suppression of VK2-induced apoptosis. Inhibition of VK2-induced caspase-3 activation was also detected when the cells were cultured in the presence of VD3 (Figs. 1B and 3).

These data raise the question of whether VD3 inhibits apoptosis directly, or whether monocytic differentiation triggered by the combined treatment with VK2 and VD3 results in apoptosis suppression. It appeared also important to determine whether this inhibition extends to other stimuli of apoptosis. To answer these questions, we pre-treated HL-60 and U937 cells with VK2 plus VD3 for 96 h in order to achieve almost complete monocytic differentiation. The cells were then washed to remove VK2 and VD3, and exposed to various apoptotic stimuli such as serum depletion, H2O2, and VK2-treatment. As shown in Fig. 4, the control cells without pretreatment with VK2 and VD3 underwent apoptosis in response to these stimuli. However, once cells had differentiated by pre-treatment with VK2 plus VD3, they became resistant to apoptosis. Sequential treatment for 48 h by VK2 followed by 48 h with VD3 did not inhibit apoptosis in HL-60 and U937 cells (data not shown).

Many lines of evidence suggest that various apoptosis stimuli converge on the activation of JNK for apoptosis induction (27-29). Immunoblotting with anti-JNK1 and }

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**Figure 2.** Cell-cycle analysis of HL-60 and U937 cells after exposure to 1α,25(OH)2VD3 and/or VK2 in HL-60 cells. After 48-h exposure to either VK2 (10 μM), 1α,25(OH)2VD3 (10 nM) or both, cell cycle analysis was performed by flow cytometry as described in Materials and methods. Results are one of the representative results from three separate experiments.

**Figure 3.** Induction of apoptosis after exposure to VK2 and/or 1α,25(OH)2VD3 in HL-60 and U937 cells. After treatment of HL-60 cells with either VK2 (10 μM), 1α,25(OH)2VD3 (10 nM) or both for 72 h, caspase-3 activities and the apoptotic cells were assessed by flow cytometry as described in Materials and methods. The number in each panel indicates the percentage of the cells positive for APO2.7 mAb, a mAb specifically detecting the cells undergoing apoptosis and the fluorescence substrate cleaved by activated caspase-3. This is one of the representative results from four separate experiments. afa, auto-fluorescent activity.
Figure 4. Inhibition of apoptosis induction after monocytic differentiation in HL-60 and U937 cells. HL-60 cells (1x10^5 cells/ml) were pretreated with VK2 (10 μM) plus 1α,25(OH)₂VD3 (10nM) for 96 h for the induction of mature monocytic differentiation. After washing twice with PBS, the cells were treated with the medium containing either VK2 10 μM with 10% FCS for 72 h, FCS-depleted medium for 72 h, or 300 μM of H₂O₂ for 2 h, respectively, for induction of apoptosis. As for controls, the cells without pre-exposure to VK2 plus VD3 were treated under the same conditions. Then APO2.7 expression was assessed by flow cytometry as described in Materials and methods. Data are from one representative result from three separate experiments.

Figure 5. JNK expression and activity in HL-60 cells after treatment with VK2, VD3 or both. (A) HL-60 cells were treated with 10 μM of VK2 for various lengths of time. Thereafter, the cells were separated by 7.5% SDS-PAGE and immunoblotted with anti-JNK1, anti-JNK2, and anti-phospho JNK1/2 mAbs, respectively. Transferred membrane was also immunoblotted with anti-actin mAb as an internal control. (B) After treating HL-60 cells with either VK2 (10 μM), 1α,25(OH)₂VD3 (10 nM) or both for 18 h, the cellular proteins were separated and immunoblotted with either anti-JNK1, anti-JNK2, anti-phospho JNK1/2 mAbs, and anti-actin mAb, respectively. Anti-phospho JNK1/2 mAb specifically recognizes the endogenous, active forms of JNK1 and JNK2 phosphorylated at threonine 183 and tyrosine 185.

Monocytic differentiation of leukemia cell-line exchanges of subcellular distribution of p21^CIP1. Our data show that combined treatment of leukemia cells with VK2 plus VD3: i) induces monocytic differentiation and G0/G1 arrest and ii) renders cells resistant to apoptosis, probably via inhibition of JNK activation. Based on these results, we further examined the intracellular distribution of p21^CIP1. p21^CIP1 is an inhibitor of cycline-dependent kinases (CDKs). It effectively blocks cell-cycle progression at the G1/S transition (30,31). For monocytic differentiation of U937 cells by VD3, however, induction of p21^CIP1 and subsequent G0/G1 cell-cycle arrest are required (32,33). Intracellular distribution of p21^CIP1 changes from nucleus to cytoplasm along with monocytic differentiation. This appears to lead to an apoptosis-resistant phenotype, mediated by binding to and inhibition of the stress-activated apoptosis signal-regulating kinase (ASK1) (20).

As shown in Fig. 6, immunofluorescence assay with p21^CIP1 mAb revealed changes of intracellular p21^CIP1 distribution in HL-60 and U937 cells after treatment with VK2 and VD3. After 24-h exposure to both vitamins, p21^CIP1 was condensed in the nucleus. After 96-h exposure, and almost complete monocytic differentiation, p21^CIP1 was mostly detected in the cytoplasm. Cytoplasmic p21^CIP1 was not detected in cells treated with VK2 alone. In a stable transfectant of U937, termed U937-ΔNLS-p21^CIP1, expressing a p21^CIP1 that lacks a nuclear localizing signal, expression of p21^CIP1 was found in the cytoplasm without monocytic differentiation (20). U937-ΔNLS-p21^CIP1 cells were resistant to apoptosis induced by VK2 (Fig. 7).
These data suggest that an enhanced change in the intracellular distribution of $p21^{CIP1}$ from nucleus to cytoplasm, along with prominent monocytic differentiation, appears to be functioning as anti-apoptotic behavior, as previously reported in U937 treated with VD3 alone (22).

**Discussion**

VK2 shows a potent apoptosis-inducing activity toward leukemia cells (12,13,19,24). In the present study, we have demonstrated that combined treatment with VK2 plus VD3 results in synergistic enhancement of induction of monocytic differentiation in HL-60 cells along with enhanced accumulation in G0/G1 (Figs. 1-3). In addition, the cells became resistant to apoptotic stimuli, including VK2 treatment (Fig. 4). To explain this phenomenon, we focused on a cyclin-dependent kinase inhibitor, $p21^{CIP1}$.

It was reported that $p21^{CIP1}$ protects cells from apoptosis initiated by stress or p53 (31). VD3 contributes to cell-cycle arrest and leads to monococyte/macrophage differentiation of U937 cells by induction of $p21^{CIP1}$ (32). A sustained expression of $p21^{CIP1}$ is also essential for the survival of differentiating neuroblastoma cells (34). An NH2-terminal domain of $p21^{CIP1}$ inhibits cyclin-CDK kinase and a COOH-terminal domain of $p21^{CIP1}$ inhibits proliferation cell nuclear antigen (35,36). These cell cycle inhibitory activities of $p21^{CIP1}$ are correlated with nuclear localization (36,37). However, previous studies provide evidence that $p21^{CIP1}$ has other biological activities in the cytoplasm (37). During monocytic maturation, $p21^{CIP1}$ shifts from nucleus to cytoplasm (20). This results in direct interaction with ASK1 and inhibition of its kinase activity. Inhibition of ASK-1 blocks the JNK apoptosis signaling pathway in U937 cells (20). Moreover, $p21^{CIP1}$ forms a complex with procaspase-3 to suppress Fas-mediated apoptosis (38). In the present study, the phosphorylation of JNK-(activated JNK) and caspase-3-activation induced by VK2 treatment was significantly suppressed in the presence of VD3 (Figs. 3 and 5). In addition, a significant accumulation of cytoplasmic $p21^{CIP1}$ along with the disappearance of nuclear $p21^{CIP1}$ was detected after 96-h exposures to VK2 and VD3 (Fig. 6). Ectopic expression of cytoplasmic $p21^{CIP1}$ in U937 cells resulted in resistance to apoptosis (Fig. 7). These data are correlated with the enhanced monocytic differentiation of leukemia cells treated with VK2 plus VD3. Accumulation of cytoplasmic $p21^{CIP1}$ along with enhanced monocytic differentiation by combined treatment of both VD3 and VK2 appears rather to function as protection against apoptotic stimuli.

**Figure 6.** Subcellular localization of $p21^{CIP1}$ in HL-60 cells after treatment with VK2, VD3 or both. (A) HL-60 cells were treated with either VK2 (10 μM), 1α,25(OH)2VD3 (10 nM) or both for 96 h. Then the cells were fixed and intracellular $p21^{CIP1}$ expression was assessed by immunofluorescence technique using FITC-conjugated anti-human $p21^{CIP1}$ mAb. This is one of the representative results from four separate experiments. (B) HL-60 cells were treated with VK2 (10 μM) and 1α,25(OH)2VD3 (10 nM) for 12 to 96 h. Intracellular $p21^{CIP1}$ expression was assessed. This is one of the representative results from four separate experiments.
The molecular mechanism underlying the monocytic
differentiation of leukemia cells by combined treatment with both vitamins is still unclear. Although a nuclear receptor for VK2 may exist (39-41), the specific nuclear binding protein for VK2 has not been identified. The mechanism of VK2 action in bone formation and activation of coagulant factors is thought to involve its natural activities as an essential co-factor for γ-carboxylation of bone matrix protein and coagulant factors (42,43). Recent evidence suggests that VK2 has a transcriptional regulatory function in bone homeostasis (44). VK2 binds to and activates the orphan nuclear receptor, SXR, and induces expression of the SXR target gene, CYP3A4, identifying VK2 as a bona fide SXR ligand (44). Further investigations of interactions and modulations of SXR and RXR and their co-transcriptional factors involved in the activities of VD3 may elucidate the mechanism of the enhanced differentiation-inducing activity.

Several case reports demonstrate the clinical benefits of VK2 in the treatment of patients with AML (14-16). The effect appears to be based on the induction of apoptosis in leukemia cells. However, our data indicate that combined treatment with VK2 and VD3 induces differentiation and resistance to apoptosis in leukemia cells. Resistance to apoptosis may be an unfavorable therapeutic effect. However, leukemia cells lose their growth ability as they differentiate. Extended culture over 7 days resulted ultimately in apoptosis of well-differentiated HL-60 cells (data not shown). Complete monocytic differentiation of HL-60 cells induced by both vitamins also leads to a dramatic decrease of telomerase activity (Miyazawa and Ohhashiki, unpublished data). The therapeutic benefits of the combined vitamin treatment may extend to patients with refractory anemia in MDS. Protection against apoptosis, along with enforced differentiation, may explain improvements of cytopenia in refractory anemia (RA), since cytopenia is mediated though apoptosis during differentiation and maturation of blood cells in the bone marrow (45). VK2 with or without VD3 has been used for the treatment of patients with RA (14,15). However, to explain the improvement of cytopenias in all lineages, including anemia and thrombocytopenia, in RA patients in response to VK2, further studies will be required to prove the anti-apoptotic effects during enforced induction of differentiation in erythroid and megakaryocytic lineages as well as in monocytic differentiation.

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