1α-Dihydroxyvitamin D₃ and Retinoic Acid Increase Nuclear Vitamin D Receptor Expression in Monocytic THP-1 Cells

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Abstract. Background: 1α,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] and retinoic acid, such as all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9cRA), are known to induce differentiation of myeloid leukemia cells. Combined treatment effectively enhances the differentiation effect, particularly in monocytic leukemia cells. The underlying mechanism of this combined effect remains unknown. Materials and Methods: THP-1 monocytic leukemia cells were treated with 1,25(OH)₂D₃ in combination with 9cRA, ATRA or selective synthetic ligand for retinoic acid receptor (RAR) or retinoid X receptor (RXR), and the nuclear expression and function of vitamin D receptor (VDR) were examined. Results: Combined treatment with 1,25(OH)₂D₃ and RAR ligand, not RXR ligand, effectively increased nuclear VDR expression and induced expression of the VDR target gene cathelicidin antimicrobial peptide (CAMP) in a gene-selective manner. Conclusion: Combination of 1,25(OH)₂D₃ plus RAR ligand is effective in induction of nuclear VDR expression and of target gene.

The vitamin D receptor (VDR) belongs to the nuclear receptor superfamily and mediates biological actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], in calcium and bone metabolism, cellular growth and differentiation, immunity and cardiovascular function (1, 2). 1,25(OH)₂D₃ and its derivatives have been shown to inhibit the proliferation and induce the differentiation of various types of malignant cells, including myeloid leukemia cells (3). The administration of 1,25(OH)₂D₃ and its analogs was shown to have therapeutic effects in a mouse model of myeloid leukemia (4), and VDR ligands can induce the differentiation of various myeloid leukemia cells (5). Upon ligand binding, VDR undergoes a conformational change that results in interaction with the retinoid X receptor (RXR) and exchange of cofactor complexes (6). Ligand binding enhances nuclear localization of VDR to exert its transcriptional regulatory activity on specific target genes (7, 8). The VDR–RXR heterodimer binds preferentially to a consensus element that consists of a two-hexanucleotide (AGGTCA or a related sequence) motif separated by three nucleotides, called the direct repeat 3 (9). Such response elements are located in the VDR target gene promoters, including CYP24A1 and CAMP, which encode vitamin D 24-hydroxylase and cathelicidin antimicrobial peptide, respectively. Ligand-bound VDR also mediates transrepression of genes, such as CYP27B1 and TNF, which encode 25-hydroxyvitamin D 1α-hydroxylase and tumor necrosis factor α, respectively, through a poorly characterized mechanism. Although anti-leukemia effects of 1,25(OH)₂D₃ have been reported to be mediated by transactivation and transrepression, non-genomic actions are possible and the characterization of a detailed mechanism remains unclear (3, 5).

While 1,25(OH)₂D₃ induces monocytic differentiation of myeloid leukemia cells, all-trans retinoic acid (ATRA) induces their granulocytic differentiation (4, 10-12). ATRA also induces monocytic differentiation of monoblastic leukemia cells (13, 14). Combined treatment with 1,25(OH)₂D₃ and ATRA, or another natural retinoid 9-cis retinoic acid (9cRA), induces the differentiation of monoblastic leukemia cells to the monocyte/macrophage-lineage cells more effectively than does 1,25(OH)₂D₃ alone (15, 16). Interestingly, 9cRA plus 1,25(OH)₂D₃ induces M2 macrophage markers in differentiated monocytic leukemia cells (17). ATRA binds to the nuclear receptor retinoic acid receptor (RAR), while 9cRA acts as a ligand for both RAR and RXR (18). Although 1,25(OH)₂D₃ activates the VDR–RXR heterodimer effectively, this heterodimer does...
not allow RXR ligand activation (19). The combination of 1,25(OH)₂D₃ with a selective RAR ligand induces differentiation of myeloid leukemia cells more potently than that with a selective RXR ligand (16). These findings indicate that retinoid signaling enhances differentiation induced by 1,25(OH)₂D₃ through a mechanism different from activation of VDR–RXR heterodimer. In this study, we examined the effect of 1,25(OH)₂D₃ in combination with retinoids on nuclear VDR expression.

Materials and Methods

Compounds. 1,25(OH)₂D₃, ATRA and 9cRA were purchased from Wako Pure Chemical Industries (Osaka, Japan). A synthetic RAR agonist, Am80 (4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid), and a synthetic RXR agonist, HX630 (4-[2,3-(2,5-dimethyl-2,5-hexano)dibenzo[b,f][1,4]-thiazepin-11-yl] benzoic acid), were kindly provided by Dr. Koichi Shudo of Research Foundation ITSUU Laboratory (Tokyo, Japan).

Cell culture. THP-1 human myeloid leukemia cells (RIKEN Cell Bank, Tsukuba, Japan) were culture in RPMI-1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Suspensions of cells (10⁵ cells/ml) were cultured with vehicle control (ethanol), 1,25(OH)₂D₃, retinoid (9cRA, ATRA, Am80, or HX630), or their combination for 24, 48, or 72 h.

Western blot analysis. Nuclear extracts from cells were prepared as described previously (20). The proteins were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a membrane for immunoblotting. Western blot analysis was performed using antibody to VDR and antibody to lamin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), visualized with an alkaline phosphatase conjugate substrate system as reported previously (21).

Reverse transcription and quantitative real-time polymerase chain reaction. Total RNA from cells was prepared by the acid guanidium thiocyanate-phenol/chloroform method (22). cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega Corporation, Madison, WI, USA). Real-time polymerase chain reaction (PCR) was performed on the ABI PRISM 7000 Sequence Detection System (Life Technologies Corporation, Rockville, MD, USA) with Power SYBR Green PCR Master Mix (Life Technologies Corporation). Primers were as follows: CYP24A1 (GenBank accession no. NM_009996), 5′-TGG AGA CGA CCG CAA ACA G-3′ and 5′-CAG CAC GCT CTG TAT T-3′; CAMP (GenBank accession no. NM_009921), 5′-GCG TGA TTC TTC GAT C-3′ and 5′-ACC CAA TCT TCT TCT CAC G-3′; VDR (GenBank accession no. NM_009504), 5′-CCGCCA CAG AGT TCT TTT TCT GCC CAC G-3′ and 5′-AGG CAC ATT CCG GTC AAA GTC-3′. For relative mRNA expression, the mRNA values were normalized to the mRNA levels of β-actin as reported previously (17).

Statistics. All quantitative data were analyzed by one-way factorial ANOVA followed by Tukey’s post hoc test using Prism 6 (Graphed Software, La Jolla, CA, USA).

Results

Previous studies have shown that 9cRA is more potent than ATRA in inducing differentiation of monocytic leukemia cells (15-17, 23). We previously reported that 1,25(OH)₂D₃ treatment increases nuclear VDR expression at 24 hours but levels decrease at 48 h in myeloid leukemia HL60 cells (21). We examined the effect of 1,25(OH)₂D₃ with/without 9cRA...
on nuclear VDR expression in THP-1 monocytic leukemia cells. Similar to the previous report (21), 1,25(OH)2D3 treatment increased nuclear VDR expression at 48 h (Figure 1A). Interestingly, combined treatment with 1,25(OH)2D3 and 9cRA markedly increased VDR expression, while 9cRA alone had a weak effect. Nuclear VDR expression decreased at 72 hours in cells treated with 1,25(OH)2D3, but was maintained at a high level with the combination of 1,25(OH)2D3 plus 9cRA. A similar effect on nuclear VDR expression was observed with the combination of 1,25(OH)2D3 and ATRA (Figure 1B).

9cRA is a ligand for both RAR and RXR. Although ATRA is a ligand for RAR not for RXR, cells treated with ATRA have RXR activity after conversion to 9cRA (24, 25). In order to clarify which receptor is involved in increased nuclear VDR expression, we compared the effects of synthetic RAR agonist (Am80) and RXR agonist (HX630) in combination with 1,25(OH)2D3. In the absence of 1,25(OH)2D3, 9cRA, ATRA and Am80 slightly increased VDR expression (Figure 2). Combined treatment with 1,25(OH)2D3 and these retinoids effectively increased nuclear VDR protein levels. The RXR agonist HX630 was not effective in combination with 1,25(OH)2D3. Cytosolic VDR protein levels were not changed under any of the treatment conditions (data not shown). Thus, activation of RAR, not RXR, is involved in increased nuclear VDR expression.

Finally, we examined whether increased VDR expression affects VDR target-gene expression in cells treated with 1,25(OH)2D3 plus retinoic acid. We previously reported that the combination of 1,25(OH)2D3 and RAR ligand effectively induces mRNA expression of CD14, which is a VDR target gene and is a marker of monocytic differentiation (17). We examined the expression of other VDR target genes, CYP24A1 and CAMP, which have a consensus direct repeat 3 element in the promoters. ATRA increased CAMP expression but reduced CYP24A1 expression in cells treated with 1,25(OH)2D3, while ATRA alone had no effect (Figure 3). The combined effect of 1,25(OH)2D3 and ATRA may be gene selective. 1,25(OH)2D3 tended to increase VDR mRNA levels but combined treatment with ATRA did not further significantly increase its expression (Figure 3).

**Discussion**

We showed that combined treatment with 1,25(OH)2D3 and RAR ligand effectively increases nuclear VDR protein expression and expression of the VDR target gene CAMP in human monocytic leukemia cells. RAR ligand alone did not increase mRNA or protein expression of VDR, consistent with previous reports (23, 26, 27). The combination of 1,25(OH)2D3 and RAR ligand increased nuclear VDR protein expression but not VDR mRNA expression, as reported previously (23). Thus, increased nuclear VDR expression is mediated by a translational or post-translational mechanism. The selective RAR agonist Am80 exhibited a similar effect to ATRA and 9cRA on enhanced VDR expression, while the selective RXR agonist HX630 did not, indicating that the effect of retinoic acid on nuclear VDR expression is not mediated by RXR activation in the VDR–RXR heterodimer. Because treatment with RAR
ligand alone was not effective, RAR activation does not directly induce mRNA or protein expression of VDR. The combination of 1,25(OH)₂D₃ and RAR ligand was not effective in increasing VDR mRNA level or cytosolic VDR protein level. A small proportion of VDR may translocate from the cytosol to the nucleus in cells, and the change of cytosolic VDR expression may be difficult to detect. Nuclear VDR expression is regulated by several mechanisms, including nuclear import and export (8, 28) and protein degradation (21, 29). Protein modification, including phosphorylation, is also involved in VDR expression (21, 30). RAR signaling may increase nuclear VDR expression by enhancing its import or reducing its export or degradation. Further studies are needed to elucidate the detailed mechanism.

Combined treatment with 1,25(OH)₂D₃ and ATRA increased CAMP mRNA expression. Vitamin D signaling plays an important role in innate immune responses in monocytes and keratinocytes through the VDR-dependent induction of CAMP (31). Vitamin A is also essential in immune homeostasis, particularly in gut mucosal immunity (32). RAR ligands, ATRA and Am80, are used in the treatment of acute promyelocytic leukemia (33). Since application of 1,25(OH)₂D₃ and its derivatives in the treatment of non-calcemic diseases, such as leukemia, autoimmune/inflammatory disease, and infection, has a risk of hypercalcemia, a principal physiological effect of vitamin D, the combination of VDR ligand with other drugs, such as retinoid acid, may be useful in reducing adverse effects (34). ATRA treatment increased CAMP expression but not CYP24A1 expression, indicating a gene-selective effect of RAR signaling on VDR target-gene expression. Therefore, combined treatment with VDR and RAR ligands may be useful for enhancement of selective VDR function, specifically in the immune response.

Conflicts of Interests
The Authors declare no conflicts of interest in regard to this study.

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