1α,25-Dihydroxyvitamin D₃ and Its Potent Synthetic Analogs Downregulate Tissue Factor and Upregulate Thrombomodulin Expression in Monocytic Cells, Counteracting the Effects of Tumor Necrosis Factor and Oxidized LDL

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Background—We have recently found that a hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], exerts anticoagulant effects by upregulating the expression of an anticoagulant glycoprotein, thrombomodulin (TM), and downregulating the expression of a critical coagulation factor, tissue factor (TF), in monocytic cells including human peripheral monocytes. In this study, we investigated the counteracting effects of 1,25(OH)₂D₃ and its potent analogs on TF induction and TM downregulation by tumor necrosis factor and oxidized LDL in monocytic cells and the modulatory effects of potent analogs on TF and TM expression.

Methods and Results—Effects of 1,25(OH)₂D₃ and its potent synthetic analogs (22R)-22-methyl-20-epi-1,25(OH)₂D₃ (KY3) and 22-oxacalcitriol on TF and TM antigen levels, cell surface activities, and mRNA levels in monocytic cells were examined. 1,25(OH)₂D₃ and its potent analogs showed anticoagulant effects in monocytic cells by downregulating TF and upregulating TM expression, counteracting the effects of tumor necrosis factor and oxidized LDL. KY3 was most potent in its regulatory effect on TF and TM expression.

Conclusions—Because KY3 has the highest affinity for vitamin D receptor, our findings suggest that TF and TM regulation by 1,25(OH)₂D₃ analogs is also mediated by vitamin D receptor. The 1,25(OH)₂D₃ analogs KY3 and 22-oxacalcitriol may have the potential to serve as an agent for preventing and treating atherosclerotic and other cytokine-mediated thrombotic diseases and as a tool for studying the molecular mechanisms of TF and TM regulation.

Key Words: anticoagulants • coagulation • thrombosis

Vitamin D metabolites influence the expression of various genes whose products are involved in calcium homeostasis, cell differentiation, and regulation of the immune response. Expression of these genes is mediated by the nuclear vitamin D receptor (VDR), which belongs to the same family as the steroid and retinoid receptors. Although the molecular mechanisms of gene regulation are still under investigation, it is clear that binding of vitamin D to the VDR initiates a sequence of events resulting in the activation or repression of transcription. Thus, the VDR–vitamin D complex is most directly relevant to the ultimate biological effect.

We have recently found that a hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], as well as vitamin A derivatives, retinoic acids, exerts anticoagulant effects by upregulating the expression of an anticoagulant glycoprotein, thrombomodulin (TM), and downregulating the expression of a critical coagulation factor, tissue factor (TF), in monocytic cells including human peripheral monocytes. Because 1,25(OH)₂D₃ derivatives are expected to be adjunctive antithrombotic agents, its analogs are promising as useful therapeutic agents without adverse effects.

22-Oxa-1,25(OH)₂D₃ (22-oxacalcitriol, maxacalcitol; OCT) (Figure 1) is a so-called “noncalcemic” vitamin D analog with accentuated differentiation-inducing/antiproliferative properties and reduced ability to cause hypercalcemia. Its affinity to vitamin D–binding protein is 500 times lower than that of 1,25(OH)₂D₃, which means that OCT can achieve higher cellular levels for a much shorter duration. (22R)-22-Methyl-20-epi-1,25(OH)₂D₃ (KY3) (Figure 1) was recently synthesized and found to have the highest VDR binding affinity so far known. KY3 has ~20 times higher

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affinity for VDR than 1,25(OH)_{2}D_{3} and does not bind to the transport protein vitamin D–binding protein.\textsuperscript{6}

In cultured endothelial cells, expression of TF procoagulant activity and/or mRNA is induced and expression of TM anticoagulant activity and/or mRNA is suppressed by tumor necrosis factor (TNF), lipopolysaccharides (LPS), and oxidized LDL (ox-LDL).\textsuperscript{7–9} Similar results have been found in monocytic cells, in which expression of TF procoagulant activity and/or mRNA is stimulated by LPS and ox-LDL.\textsuperscript{7} On the other hand, all-trans-retinoic acid (ATRA) downregulates the induction of TF by TNF or LPS in vascular endothelial cells\textsuperscript{11} or monocytes.\textsuperscript{12}

In this study, we investigated the counteracting effects of 1,25(OH)_{2}D_{3} and its potent analogs on TF induction and TM downregulation by TNF and ox-LDL in monocytic cells and the modulatory effects of potent analogs on TF and TM expression.

**Methods**

**Reagents**

Human thrombin (1400 NIH U/mg protein) and ATRA were purchased from Sigma. Human placenta–derived TF (Thromborel S) was from Behringwerke AG. The chromogenic substrate S2266 was from Chromogenix. Human protein C (inactivated and activated) was from Behringwerke AG. The chromogenic substrate S2266 was kindly provided by Chugai Pharmaceutical Co Ltd. OCT was synthesized as described previously.\textsuperscript{6} OCT was kindly provided by Chugai Pharmaceutical Co Ltd. All other chemicals were reagent-grade products and were purchased from Wako Pure Chemicals unless otherwise indicated.

**Cell Culture**

Because anticoagulant effects have been detected in human peripheral monocytes as well as in monocytic leukemia cell lines,\textsuperscript{2} we mainly used U937 cells in this study as a model of monocytic cells. Monoblastic leukemia cell lines U937 and THP-1 were provided by Health Science Research Resources Bank, Osaka, Japan. U937 and THP-1 cells were cultured for the indicated periods in RPMI medium supplemented with 10% FCS, sodium carbonate, glutamine, penicillin, and streptomycin with vitamin D derivatives at the indicated concentrations. Vitamin D derivatives were dissolved in absolute ethanol and then added to the growth media at the desired final concentration. The final concentration of ethanol in the culture media was <0.1%. At this concentration, the cells exhibited no signs of damage. The culture media without vitamin D derivatives, used for the control cells, contained the same concentration of ethanol as the culture media used for the treated cells. All of the procedures involving vitamin D derivatives were performed under subdued light.

Whole blood was collected from healthy donors and treated with heparin (10 U/mL blood) to prevent coagulation. The mononuclear cell fraction was freshly isolated from 4 different healthy donors as previously described.\textsuperscript{4} The percentage of monocytes was between 15% and 25% before adhesion. The isolated cells were cultured as U937 cells.

**Measurement of Levels of TF and TM Antigens**

Leukemic cells were incubated with vitamin D derivatives for 24 hours and then washed with PBS 3 times. Cell numbers were determined and adjusted. To prepare cell lysates, the cells were then treated with 0.5% Triton X-100 in PBS for 30 minutes at 4°C. Cellular debris was removed by centrifugation at 12 000g for 20 minutes. The cell lysates were stored at −80°C until assay. Total levels of TF and TM antigens in cell lysates were measured by ELISA, with the IMUBIND Tissue Factor Factor ELISA Kit, American Diagnostica Inc, and the EIA TM kit Teijin, Teijin Inc, according to the manufacturer’s instructions.

**Cell Surface TF Cofactor Activity: Analysis of Procoagulant Activity in Clotting Assays**

Suspensions of U937, THP-1, and freshly isolated mononuclear cells were prepared in PBS. The U937 cell suspension was adjusted to 1×10\textsuperscript{6} cells/mL in PBS. A portion of the cell suspension (10\textsuperscript{6} cells) was added to 0.1 mL of pooled human normal plasma. After incubation at 37°C for 3 minutes, 0.1 mL of 25 mmol/L calcium chloride was added and the plasma recalcification time was determined with a CA-100 semiautomatic coagulator (Sysmex). Our previous study showed that the procoagulant activity associated with the surface of U937 cells determined as described above was attributable to the occurrence of TF expression\textsuperscript{2} and thus the prolongation of the recalcification time is mainly due to downregulation of TF expression by vitamin D\textsubscript{3} derivatives. TF cofactor activity was quantitatively measured by reference to standard curves (log-log plot) constructed with human placental TF, and the amount of TF activity that yielded a 50-second recalcification time was defined as 1 U/mL.

**Measurement of Cell Surface TM Cofactor Activity**

Cell surface TM cofactor activity was measured as previously described.\textsuperscript{2} In this assay, exogenous protein C (0.16 μmol/L) was activated by intact cells in the presence of thrombin (0.83 NIHU/mL, 6.5 nmol/L) and Ca\textsuperscript{2+} (1.3 mmol/L). After completely inactivating the thrombin activity by treatment with hirudin, cleavage of the small molecular weight substrate S2266 by activated protein C was measured with a spectrophotometer. The results were expressed as the changes in optical density at 405 nm per minute or as the percentage of the initial velocity of activated protein C formation (with 100% taken to be the rate in the case of cell surface TM under basal conditions). Controls with cells in the absence of thrombin and protein C were treated similarly, and no activation of protein C was observed.

**Quantitative Reverse Transcription Polymerase Chain Reaction and Densitometric Analysis**

TF and TM mRNA levels in U937 cells were measured by performing quantitative reverse transcription polymerase chain reaction (RT-PCR) assays with the Super-Script Preamplification System, Life Technologies, Inc, as described previously.\textsuperscript{2} Relative signal intensity was determined on Scion Image Software and standardized by use of the relative density of glyceraldehyde-3-dehydrogenase (GAPDH) signal. TM mRNA half-life was calculated with actinomycin D. Briefly, U937 cells were exposed to 0.1 μmol/L 1,25(OH)\textsubscript{2}D\textsubscript{3} for 1 hour; 10 μg/mL actinomycin D was then added, and total RNA was extracted at 0, 2.5, and 5 hours later. RT-PCR and the determination of signal intensity were performed as well.

**Results**

**Effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and Its Potent Synthetic Analogs on Total Levels of TF and TM Antigen in U937 Cells**

U937 cells were incubated for 24 hours with various concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} and its synthetic analogs, as indicated...
in Figure 2. Both KY3 and OCT decreased the total level of TF antigen in a dose-dependent manner (up to 1 nmol/L) (Figure 2A) while increasing the total level of TM antigen dose-dependently (up to 0.1 μmol/L) (Figure 2B). The changes in the levels of TF and TM antigen resembled those observed in the case of 1,25(OH)_{2}D_{3}. KY3 was more efficient in regulation of the levels of TF and TM antigen at a lower concentration than 1,25(OH)_{2}D_{3} or OCT.

**RT-PCR Analysis of TF and TM mRNA Levels in U937 Cells**

To examine the changes in TF and TM mRNA levels, U937 cells were treated with 0.1 nmol/L 1,25(OH)_{2}D_{3}, KY3, or OCT. The pharmacological concentration of 1,25(OH)_{2}D_{3} is 0.1 nmol/L in serum. Both KY3 and OCT markedly decreased the expression of TF mRNA and increased the expression of TM mRNA, as observed in the case of 1,25(OH)_{2}D_{3} (Figure 3). These effects were in parallel with the changes in TF and TM antigen levels. TM mRNA half-life was not different in the absence and presence of 1,25(OH)_{2}D_{3} (3.0 ± 0.42 and 2.75 ± 0.75 hours, n = 3), which indicates that TM gene is regulated at the level of transcription.

**Counteracting Effects of 1,25(OH)_{2}D_{3} and Its Potent Analogs on Upregulation of TF and Downregulation of TM by TNF or Ox-LDL in Monocytic Cells**

We next examined whether 1 nmol/L 1,25(OH)_{2}D_{3} or its analogs could suppress the induction of TF activity in U937 cells treated with 1 nmol/L TNF or 50 μg/mL ox-LDL (Figure 4A) and the decrease in TM activity caused by TNF (Figure 4B). TF activity on the surface of U937 cells was upregulated by both ox-LDL and TNF (Figure 4A, lanes 5 and 9), whereas TM activity was downregulated by TNF (Figure 4B, lane 9). Induction of TF activity was more
marked when the cells were treated with TNF. Ox-LDL mildly upregulated TM activity as reported in the case of THP-1 cells, another monocytic cell line (Figure 4B, lane 5). Preincubation with 1,25(OH)₂D₃, KY3, or OCT for 1 hour counteracted the upregulation of TF activity by ox-LDL (Figure 4A, lanes 6 to 8) and TNF (Figure 4A, lanes 10 to 12) and the downregulation of TM activity by TNF (Figure 4B, lane 10 to 12). TF activity induced by TNF was suppressed to 11% by KY3. Although somewhat weaker, similar effects were observed when U937 cells were treated with 1,25(OH)₂D₃, 1 hour after the start of incubation with TNF or ox-LDL (data not shown). Similar results were obtained in experiments using THP-1 cells (data not shown) or peripheral mononuclear cells (Figure 4C and D) rather than U937 cells. KY3 suppressed TF activity induced by TNF in mononuclear cells weaker than in U937 cells.

Levels of TF and TM mRNA in U937 cells treated with ox-LDL, TNF, and/or 1,25(OH)₂D₃ were measured by the RT-PCR method (Figure 5). The increase in TF mRNA levels induced by ox-LDL (Figure 5A, lane 2) or TNF (Figure 5B, lane 5) as determined after 5 hours of incubation was antagonized by 1,25(OH)₂D₃ (Figure 5A, lane 3 and Figure 5B, lane 6) in cells preincubated with 1,25(OH)₂D₃ for 1 hour. TM mRNA levels were markedly increased when the cells were preincubated with 1,25(OH)₂D₃ (Figure 5C, lane 3) as compared with incubation with ox-LDL alone (Figure 5C, lane 2). The decrease in TM mRNA levels induced by TNF as determined after 5 hours of incubation (Figure 5D, lane 5) was markedly counteracted by 1,25(OH)₂D₃ (Figure 5D, lane 6) in cells preincubated with 1,25(OH)₂D₃ for 1 hour.

**Discussion**

In this study, we have shown that 1,25(OH)₂D₃, and its potent analogs, KY3 and OCT, exert anticoagulant effects in monocytic cells by downregulating TF expression and upregulating TM expression, counteracting the effects of TNF and ox-LDL. KY3 was most potent in its regulatory effect on TF and TM expression. This is compatible with the findings described in our recent report indicating that KY3 shows stronger activity than 1,25(OH)₂D₃ in VDR binding, stimulation of transcription of the osteopontin gene, promotion of the differentiation of HL60 cells, and inhibition of the proliferation of MCF-7 cells and keratinocytes, because KY3 has the highest affinity for VDR.¹³ Our findings support the view that TF downregulation and TM upregulation by 1,25(OH)₂D₃ and its analogs are mediated by VDR.
Monocyte TF activity is induced by a variety of agents, such as LPS in bacterial infection, cytokines TNF and interleukin-1, antigen-antibody complexes and complement fragments in inflammatory diseases, and ox-LDL in atherosclerotic diseases. Furthermore, LPS and TNF downregulate TM expression in monocytes. In patients with infections or inflammatory diseases, upregulation of TF and downregulation of TM expression in endothelial cells and monocytes/macrophages are major mechanisms involved in triggering thrombosis.

Monocytes/macrophages in atheromatous plaques display TF activity, and it is well established that these cells have thrombogenic activity. Monocytes in patients with coronary ischemic syndrome bear TF antigen. Ox-LDL has been shown to be present in human atherosclerotic lesions, and it enhances the progression of these lesions. Ox-LDL induces macrophage foam cell generation, smooth muscle cell proliferation, platelet adhesion, and aggregation as well as triggering thrombosis by inducing TF expression.

In this context, upregulation of TM and downregulation of TF expression in monocytic cells by 1,25(OH)₂D₃ analogs may be an intriguing novel strategy to prevent thrombosis in inflammatory and atherosclerotic diseases.

Enhancement of TM mRNA by 1,25(OH)₂D₃ analogs is not due to higher stability of mRNA but due to an increase in the transcription of the TM gene. The more potent effect of KY3 on TM mRNA expression may be due to its higher affinity to VDR. 1,25(OH)₂D₃ analogs may mediate upregulation of TM expression at least in part by the retinoic acid-responsive element (RARE) of the TM gene. The TM RARE is in a manner a vitamin D–responsive element, which suggests a cross-talk between the cellular RAR and VDR pathways. Whereas ATRA upregulates TM expression in both monocytic and vascular endothelial cells, 1,25(OH)₂D₃ does not in the latter. Such a cell-specific response in terms of TM regulation may be associated with cell- or tissue-specific expression and regulation of VDR. On the other hand, the mechanism of TF gene repression is not yet clear. The TF promoter contains 2 activator protein-1 (AP-1)-binding sites and a nuclear factor-κB (NF-κB) site, which serve as binding sites for c-Fos/c-Jun and c-Rel/p65 heterodimers, respectively. Functional interactions between these transcription factors are required for maximal induction of TF gene transcription by TNF in vascular endothelial cells. Similar interactions are necessary for induction of TF gene transcription by LPS in monocytic cells such as THP-1 cells. It has been reported that 1,25(OH)₂D₃ inhibits interleukin-12 production by activated THP-1 cells through downregulation of NF-κB activation. On the other hand, ATRA selectively inhibits LPS induction of TF gene expression in THP-1 cells by a mechanism that does not involve repression of AP-1 and NF-κB-mediated transcription. Effects of 1,25(OH)₂D₃ analogs on AP-1 and NF-κB activation are now under investigation.

In contrast with downregulation of TM expression in endothelial cells, ox-LDL induces mild upregulation of TM expression in human monocytic leukemia cell line, U937 and THP-1 cells, by a mechanism that is not yet known.

It has been shown also that increased expression of TF in human malignant cells such as melanoma cells promotes metastasis by enhancing angiogenesis or by other mechanisms. On the other hand, diminished expression of TM
appears to play an important role in the metastatic process of esophageal\textsuperscript{10} and hepatic cancers.\textsuperscript{21} Upregulation of TM and downregulation of TF expression by 1,25(OH)\textsubscript{2}D\textsubscript{3} or ATRA have also been observed in several cancer cell lines (our unpublished observations). Thus, 1,25(OH)\textsubscript{2}D\textsubscript{3} analogs may be novel agents capable of preventing cancer cell metastasis through induction of TM expression and suppression of TF expression.

In summary, 1,25(OH)\textsubscript{2}D\textsubscript{3} analogs, with a potent capacity to suppress TF expression and to induce TM expression, are expected to serve as adjunctive antithrombotic agents in treatment of inflammatory and atherosclerotic diseases. 1,25(OH)\textsubscript{2}D\textsubscript{3} analogs such as OCT, already in use in clinical trials as agents for treatment of secondary hyperparathyroidism and psoriasis, and KY3 should be examined further in in vivo models of thrombotic disease. OCT and KY3 are expected to avoid an adverse effect of hypercalcemia. Although KY3 is twice as hypercalcemic in vivo than 1,25(OH)\textsubscript{2}D\textsubscript{3}, its binding affinity to VDR or biological activities are far stronger (20-fold or \(>100\)-fold) than 1,25(OH)\textsubscript{2}D\textsubscript{3}.\textsuperscript{13} The 1,25(OH)\textsubscript{2}D\textsubscript{3} analogs examined in this study may have the potential to serve as an agent for preventing and treating atherosclerotic and other cytokine-mediated thrombotic diseases and as a tool for studying the molecular mechanisms of TF and TM regulation.

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References
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