

1,25(OH)₂D₃ blocks TNF-induced monocytic tissue factor expression by inhibition of transcription factors AP-1 and NF-κB

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An essential coagulation factor, tissue factor (TF), is rapidly expressed by human monocytes when exposed to a variety of agonists, such as lipopolysaccharide or tumor necrosis factor (TNF). We previously found that 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and its potent synthetic analogs downregulate TF and upregulate thrombomodulin expression on monocytic cells, counteracting the effects of TNF at the level of transcription. The human TF gene has characteristic binding sequences for activator protein-1 (AP-1) (c-Jun/c-Fos), nuclear factor-κB (NF-κB), Sp-1, and early growth response factor-1 (Egr-1). In this study, we investigated the regulatory mechanisms by which 1,25(OH)₂D₃ inhibits TNF-induced TF expression in human monocytic cells. 1,25(OH)₂D₃ reduced basal and TNF-induced TF activities. Gel-shift assay and luciferase assay with the respective reporter vectors showed that 1,25(OH)₂D₃ reduced basal and TNF-induced activities of the nuclear proteins AP-1 and NF-κB, but not Egr-1. 1,25(OH)₂D₃ inhibited TNF-induced phosphorylation of c-Jun without affecting phosphorylation of the other pathways. On the other hand, 1,25(OH)₂D₃ directly inhibited nuclear binding and activities of NF-κB in the nucleus without affecting phosphorylation of the NF-κB activation pathway. These results indicate that 1,25(OH)₂D₃ suppresses basal and TNF-induced TF expression in monocytic cells by inhibition of AP-1 and NF-κB activation pathways, but not of Egr-1. Our results may help to elucidate the regulatory mechanisms of 1,25(OH)₂D₃ in TF induction, and may have physiological significance in the clinical challenge to use potential 1,25(OH)₂D₃ analogs in antithrombotic therapy as well as immunomodulation and antineoplastic therapy of leukemia.

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Tissue factor (TF) is a membrane-bound glycoprotein that is essential for activation of the extrinsic coagulation pathway. TF forms complexes with factor VII or activated factor VII (VIIa). These complexes activate factors IX and X, which then contribute to the generation of thrombin on the surface of both endothelial cells and monocytes. Although synthesis of TF in vascular monocytes is tightly regulated, its expression can be induced by a variety of agonists, such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), phorbol ester, and oxidized low-density protein (LDL).¹ Induction of TF expression by endothelial cells and monocytes is associated with thrombotic disorders, such as disseminated intravascular coagulation (DIC), a rapidly fatal disorder in humans. Downregulation of pathological expression of TF appears to be a critical strategy to prevent and treat various

thrombotic disorders. In addition to its role in coagulation, the TF-dependent signaling pathways contribute to a variety of pathophysiologic processes, including inflammation, atherosclerosis, angiogenesis, and tumor metastasis.

The promoter region of the TF gene contains two activator protein-1 (AP-1) sites, a nuclear factor-κB (NF-κB) site, and three early growth response factor-1 (Egr-1) sites, which serve as binding sites for transcription factors AP-1 (c-Jun/c-Fos), NF-κB (c-Rel/p65), and Egr-1.¹ Functional interactions between these transcription factors are required for maximal induction of TF gene transcription in monocytes by a variety of agonists, such as LPS,² TNF,³ and phorbol ester.⁴

TNF stimulation in various cells leads to phosphorylation of SAPK/ERK kinase (SEK), Jun N-terminal kinase (JNK), and c-Jun and this in turn enhances the AP-1 activation

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pathway. SEK is a protein kinase that activates JNK and functions in a stress-activated protein kinase cascade. Phosphorylated and activated JNK in turn phosphorylates and activates several transcription factors, including c-Jun at Ser 63/73, which then increases its trans-activating potential and DNA-binding activity to the AP-1 sites in the TF gene.⁵

NF-κB transcription factors are present in the cytosol in an inactive state complexed with inhibitor κB (IκB) protein. After stimulation with TNF, IκB is rapidly phosphorylated by IκB kinase (IKK), which in turn targets IκB for degradation. This signal induction then releases NF-κB (c-Rel/p65), which translocates into the nucleus and binds to target sites of responding genes.⁶

p42/44 ERK (ERK1/2) is known to be activated by TNF stimulation, and one of the downstream targets of the ERK pathway is the transcription factor Elk-1. Phosphorylation of Elk-1 by the ERK pathway induces Egr-1 expression.⁷

A recent study demonstrated that PI3K–Akt may play a pivotal role in cytokine-induced transcriptional activation of NF-κB- and AP-1-dependent gene expression.^{8,9}

The active form of vitamin D₃, 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), influences the expression of various genes, whose products are involved in calcium homeostasis, cell differentiation, and regulation of the immune response.¹⁰ The expression of these genes is mediated by binding to its nuclear vitamin D receptor (VDR), a transcription factor, which dimerizes with either itself or other nuclear receptors, such as the retinoid X receptor (RXR).^{11,12} We have previously found that 1,25(OH)₂D₃ and its synthetic analogs downregulate TF and upregulate thrombomodulin (TM) expression in monocytic cells, counteracting the effects of TNF and oxidized LDL.^{13,14} Pathogenic expression of TF mRNA in monocytic cells was markedly downregulated by 1,25(OH)₂D₃ and its synthetic analogs. Several subsequent studies have reported that the vitamin D/VDR system plays a physiological role *in vivo* in the maintenance of antithrombotic homeostasis. Aihara *et al.*¹⁵ demonstrated that upregulation of TF and downregulation of TM in the aorta, liver, and kidney were related to enhanced thrombogenicity in VDR knockout mice. 1,25(OH)₂D₃ was effective against DIC in a rat model induced by LPS.¹⁶

However, the mechanism and signaling pathway of 1,25(OH)₂D₃ that downregulate TNF-induced TF gene expression are not known. In this study, we investigated the regulatory mechanisms by which 1,25(OH)₂D₃ inhibits TNF-induced TF gene expression in several pathways related to TF gene transcription in human monocytic cells.

MATERIALS AND METHODS

Reagents

1,25(OH)₂D₃ (Sigma, St Louis, MO, USA) was dissolved in absolute ethanol and then added to growth media at the indicated concentrations. The final ethanol concentration in the culture media was less than 0.1%. Ethanol at a concentration less than 0.1% has no effect on cell growth or

differentiation of each cell line.¹⁷ For the untreated control, the same amount of ethanol was added to the culture medium. TNF was from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals were reagent-grade products and were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise indicated.

Cell Culture

As the anticoagulant effects of 1,25(OH)₂D₃ have been detected in monocytic leukemia cell line U937 as well as in human peripheral monocytes,¹⁴ we primarily used monocytic leukemia cell line THP-1 cells and partly U937 cells in this study, both of which were provided by Health Science Research Bank. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 0.2% sodium carbonate, 2 mM glutamine, and 50 U/ml penicillin–streptomycin.

Cell Surface TF Activity

A portion of the cells (2×10^6) suspended in phosphate-buffered saline (PBS) was added to 0.1 ml of pooled human normal plasma. After incubation at 37°C for 2 min, 0.1 ml of 25 mM calcium chloride was added and the plasma recalcification time was measured by a CA-100 semiautomatic coagulator (Sysmax, Kobe, Japan). The procoagulant activity associated with the surface of THP-1 cells was attributable to the occurrence of TF expression^{13,14} and thus the prolongation of the recalcification time is mainly due to downregulation of TF expression. TF cofactor activity was quantitatively measured by reference to a standard curve constructed with human placental TF, and the amount of TF activity that yielded a 50-s recalcification time was defined as 1 U/ml.

Mobility Gel Shift Assay

The electrophoretic gel-shift assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals) for binding of nuclear proteins to digoxigenin (DIG)-labeled oligonucleotides containing AP-1 (5'-GGTTGAATCACTGGGGTGAGTCATCC-3'), NF-κB (5'-TCCC GGAGTTTCCTAC-3'), and Egr-1 (5'-GGGCCGGGGCG GGGAGT-3') sites (underlined) of the TF promoter. The relative signal intensity of bands was determined and standardized using Scion Image Software (Scion, Frederick, MD, USA). The binding specificity of nuclear proteins to the DIG-labeled oligonucleotide was determined by competition with an excess of unlabeled oligonucleotide or the respective antibodies, anti-phospho(Ser63)-c-Jun (anti-P-c-Jun), anti-p65, and anti-Egr-1.

Plasmids

The following plasmids were used: p(AP-1)₄-LUC contains two copies of two AP-1 sites (AP-1_D and AP-1_P),¹ p(NF-κB)₄-LUC contains four copies of the NF-κB site, and p(Egr-1)₄-LUC contains two copies of the Egr-1 site

(Sp1_I and Sp1_{II}). These sites were cloned upstream of the minimal simian virus SV40 promoter expressing the firefly luciferase (LUC) reporter gene in pGL3-Enhancer from Promega (Madison, WI, USA). The pRL-SV40 control vector was compared with pGL3-Enhancer vector cloned target plasmids.

Transfection

THP-1 cells were transfected using DEAE-dextran Transfection Reagent (Promega)^{2,18} with p(AP-1)₄SV40-LUC, p(NF-κB)₄SV40-LUC, and p(Egr-1)₄SV40-LUC in each. Cells were cotransfected with a control reporter vector, pRL-SV40, which encoded *Renilla* (Sea Pansy luciferase), and two expression plasmids coding either human VDR or RXRα. Expression vectors for human VDR¹⁹ and RXRα²⁰ cDNAs were kindly provided by Dr Umesono K and Dr Kakizuka A, respectively (Kyoto University). Dr RM Evans (Howard Hughes Medical Institute, CA, USA) generously supported the use of the cDNAs. After transfection, the THP-1 cells were preincubated with 0.1 μM 1,25(OH)₂D₃ for 1 h before stimulating with 1 nM TNF for 5 h at 37°C. Cell lysates were then assayed for LUC activity using the Dual-Luciferase Reporter Assay System (Promega), and LUC assays were sequentially analyzed in a Lumat (Berthold), as previously reported.¹³ LUC activity data are expressed as means ± s.d.

Immunoblotting Analysis

TNF-stimulated cells (2 × 10⁶), at the indicated time with or without 1,25(OH)₂D₃, were suspended in sampling buffer, and lysed by sonication using Bioruptor (Cosmo Bio, Tokyo, Japan). The phosphorylated states of c-Jun, JNK, SEK, and IκB in cell lysates were determined according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). Phosphorylated states of ERK1/2, Elk-1, Egr-1, and Akt in cell lysates were determined according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative signal intensity of bands was determined and standardized using Scion Image Software.

Immunoprecipitation Assay

For immunoprecipitation experiments, cells were lysed in buffer (0.05 M Tris-HCl, 0.15 M NaCl, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride), and sequentially immunoprecipitated using an excess of anti-VDR antibody (Santa Cruz Biotechnology), and then subjected to immunoblotting analysis using anti-c-Jun antibody (Cell Signaling Technology) for detection as described previously.²¹

Statistical Analysis

Data were expressed as mean ± s.d. Statistical analysis was performed by nonparametric Wilcoxon signed rank test for paired data. For nonparametric multiple comparisons, we performed Steel's test or the Steel-Dwass test using KyPlot 4.0

(Kyens Lab Inc., Tokyo, Japan). A *P*-value of >0.05 was considered to represent a statistically nonsignificant change.

RESULTS

Effect of 1,25(OH)₂D₃ on Cell Surface TF Activity

We examined whether 1,25(OH)₂D₃ could suppress TNF-induced TF activity in THP-1 cells (Figure 1). TF activity was upregulated 1.7-fold by TNF. Preincubation with 1,25(OH)₂D₃ before TNF stimulation for 1 h suppressed the upregulation of TF activity by TNF from 778 ± 167 to 277 ± 63 mU/ml (*P* = 0.03, *n* = 5; *). In addition, basal TF activity was inhibited from 458 ± 148 to 73 ± 30 mU/ml by 1,25(OH)₂D₃. These studies indicate that 1,25(OH)₂D₃ reduces both basal and TNF-induced TF activity in THP-1 cells.

Effects of 1,25(OH)₂D₃ on the Binding Activity of Transcription Factors to the Promoter Region of the TF Gene

To determine whether 1,25(OH)₂D₃ suppressed TF expression by preventing activation of the TF gene by TNF, we examined the binding of nuclear proteins in THP-1 cells pretreated with 1,25(OH)₂D₃ to the oligonucleotide containing sequences of the AP-1, NF-κB, or Egr-1 binding site of the promoter region of the TF gene using gel-shift assays (Figure 2). Nuclear extracts were prepared from THP-1 and U937 cells preincubated with or without 1,25(OH)₂D₃ (0.1 μM) for 1 h, followed by stimulation with TNF (1 nM) for 1 h, since our previous study demonstrated that 1 h stimulation with TNF resulted in the strongest binding activity. The binding specificity of nuclear proteins to the DIG-labeled oligonucleotides was confirmed by competition with an excess of each unlabeled oligonucleotide or the respective antibodies. A remarkable degree of binding of nuclear extracts

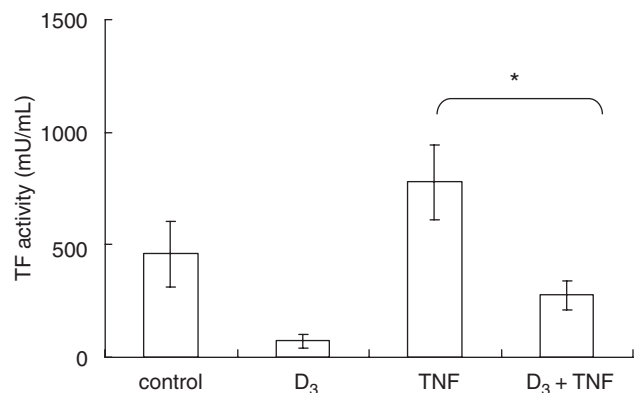


Figure 1 Effects of 1,25(OH)₂D₃ on TF activity. Procoagulant activity of THP-1 cells was measured by recalcification time. The effect of preincubation with 1,25(OH)₂D₃ (0.1 μM) at 37°C for 1 h before stimulation of TNF is shown. THP-1 cells were incubated with or without TNF (1 nM) overnight after preincubation with 1,25(OH)₂D₃ (0.1 μM) for 1 h. Lane 1 represents the control. Results are expressed as means ± s.d. of five experiments.

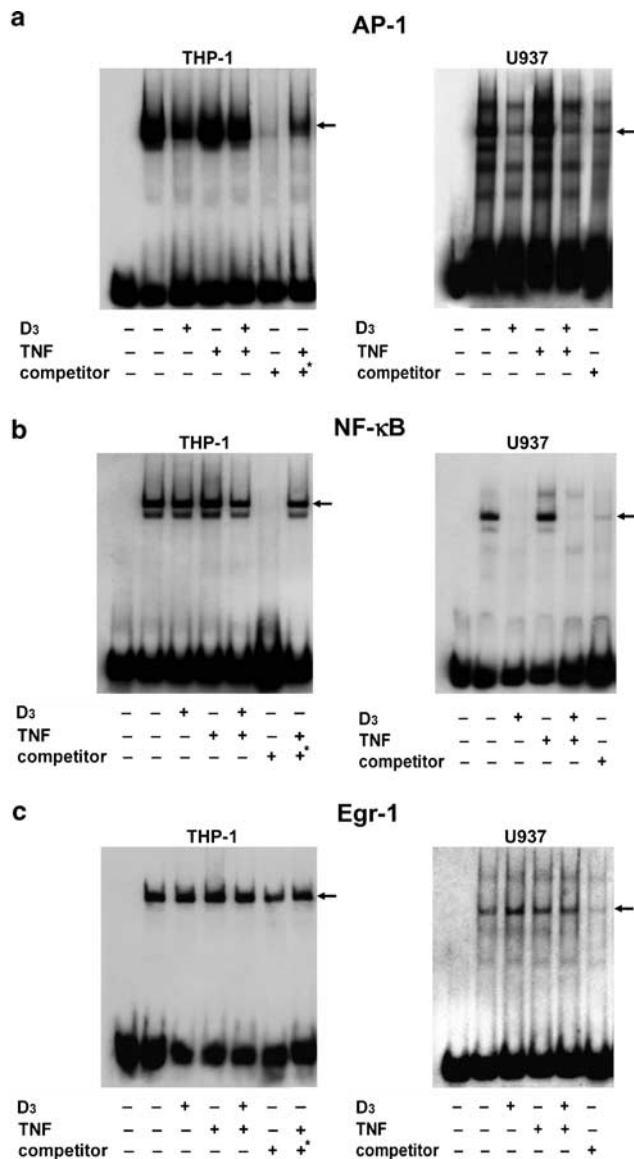


Figure 2 Effects of 1,25(OH)₂D₃ on the binding activity of transcription factors to the promoter region of the TF gene. A probe containing AP-1 (a), NF-κB (b), or Egr-1 sites (c) of the TF promoter was incubated with nuclear extracts isolated from THP-1 or U937 cells. Nuclear extracts were prepared from 5×10^6 cells. Cells were treated with (lane 3) or without (lane 2) 1,25(OH)₂D₃ (0.1 μM) for 120 min. Before the addition of TNF (1 nM) for 60 min, cells were pretreated and maintained with (lane 5) or without (lane 4) 1,25(OH)₂D₃. Gel mobility shift assay was performed using the probe. Lane 1 corresponds to the DIG-labeled probe only. To demonstrate the specificity of protein binding, cold competitor oligonucleotides containing each site or the respective antibodies against transcription factors were used. A 100-fold molar excess of cold competitor oligonucleotides containing each site were included in control samples (lane 6). The respective antibodies (100 μg/ml), anti-c-Jun, anti-p65, and anti-Egr-1, were included in TNF-treated samples (lane 7 indicated by the competitor with an asterisk). Data are representative of six independent experiments using each probe and confirmed by corrected densitometric quantification.

to the oligonucleotides was observed in unstimulated THP-1 and U937 cells. The binding activity of nuclear proteins was increased by stimulation with TNF, and pretreatment of cells

with 1,25(OH)₂D₃ before TNF stimulation markedly suppressed AP-1 and NF-κB binding activity (Figure 2a and b). However, binding activity of Egr-1 increased by TNF was not influenced by 1,25(OH)₂D₃ (Figure 2c) in THP-1 cells. In U937 cells, binding activity of Egr-1 was not decreased by 1,25(OH)₂D₃, but instead increased without TNF stimulation.

The inhibitory effect of AP-1 binding activity by 1,25(OH)₂D₃ was shown in both basal and TNF-induced TF expression. The inhibitory effect was more impressive in U937 cells, probably due to the low basal expression of TF in U937 cells. Coincubation with the anti-VDR antibody did not affect the specific bands, indicating that VDR did not bind to those TF-DNA fragments.

These results indicate that 1,25(OH)₂D₃ suppresses upregulation of TF gene expression by TNF through inhibition of AP-1 and NF-κB binding activity to the TF promoter region.

Effect of 1,25(OH)₂D₃ on TNF-Induced Expression of TF

We performed functional studies to analyze the counteracting effect of 1,25(OH)₂D₃ on transcription factor-dependent expression of TF in THP-1 cells (Figure 3). THP-1 cells were transfected with pGL3E-SV40 vectors containing the sequence of the respective transcription factors and cultured for 60 h. The cells were then preincubated with 1,25(OH)₂D₃ for 1 h before 5 h-stimulation with TNF. 1,25(OH)₂D₃ reduced AP-1- and NF-κB-dependent expression. TNF induced a two-fold increase in LUC activity expressed by p(AP-1)₄SV40-LUC. Preincubation with 1,25(OH)₂D₃ and the following coincubation significantly reduced TNF induction of AP-1-dependent expression (2.14 ± 0.57 -fold vs 1.51 ± 0.33 -fold decrease, $P=0.006$, $n=5$; *) (Figure 3a). Similarly, the LUC activity expressed by p(NF-κB)₄SV40-LUC increased by about 1.45-fold with TNF. 1,25(OH)₂D₃ slightly reduced the TNF induction of NF-κB-dependent expression (1.45 ± 0.25 -fold vs 1.18 ± 0.23 -fold decrease, $P=0.02$, $n=5$; *) (Figure 3b). The LUC activity expressed by p(Egr-1)₄SV40-LUC increased by 1.22-fold. TNF-induced Egr-1-dependent expression was not affected by 1,25(OH)₂D₃ (1.22 ± 0.09 -fold vs 1.11 ± 0.15 -fold, $P=2.26$, $n=5$) (Figure 3c). Cotransfection of expression plasmids coding human VDR and RXRα was necessary to detect statistically significant differences in expression. A negative control, to determine the baseline LUC activity from the vector alone, showed only $2.8 \pm 1.2\%$ activity. These results indicate that 1,25(OH)₂D₃ suppresses TNF-induced TF expression by inhibition of AP-1- and NF-κB-dependent expression, but not of Egr-1.

Effects of 1,25(OH)₂D₃ on TNF-Induced Phosphorylation of c-Jun, JNK, and SEK

We examined whether 1,25(OH)₂D₃ could suppress phosphorylation of the AP-1 pathway induced by TNF with immunoblotting of c-Jun, JNK, and SEK (Figure 4). In unstimulated THP-1 cells, c-Jun was minimally phosphory-

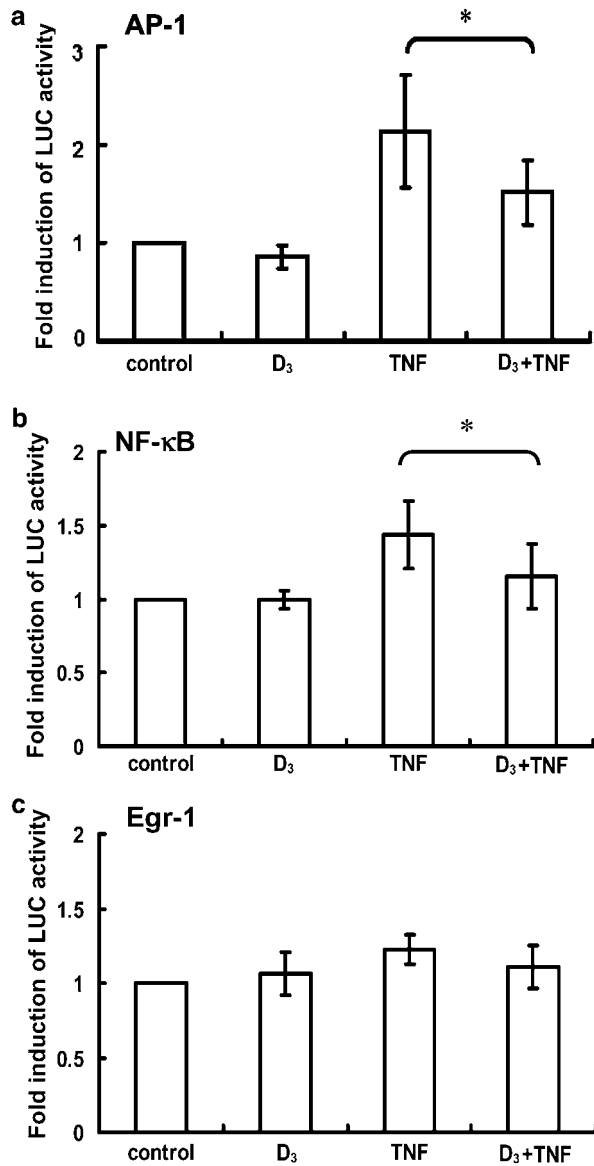


Figure 3 Effect of 1,25(OH)₂D₃ on TNF-induced activation of the TF gene. THP-1 cells were transfected with the pGL3 reporter vector containing p(AP-1)₄SV40LUC (a), p(NF-κB)₄SV40LUC (b), or p(Egr-1)₄SV40LUC (c). As a control vector, the pRL-SV40 vector was cotransfected, which expresses *Renilla* luciferase. pCMX-hVDR and pCl-RXRα were cotransfected into cells to detect statistically significant differences in expression. After transfection for 60 h, cells were divided into four equal portions. Cells were preincubated with (column 2) or without (column 1) 1,25(OH)₂D₃ (0.1 μM) for 6 h. Alternatively, cells were incubated with TNF (1 nM) for 5 h in the presence (column 4) or absence (column 3) of a 1-h pretreatment with 1,25(OH)₂D₃ (0.1 μM). LUC activity was used to determine fold changes (means ± s.d.) in six experiments.

lated. After stimulation with TNF, c-Jun was phosphorylated, peaking at 15 min. Preincubation with 1,25(OH)₂D₃ for 1 h and the following coincubation blocked the phosphorylation of c-Jun by TNF at 15 and 30 min (Figure 4a). Next, we examined whether 1,25(OH)₂D₃ could inhibit phosphorylation of JNK, being located upstream of c-Jun in the signal

pathway. Phosphorylation of JNK occurred after 5 min of TNF stimulation, but 1 h preincubation with 1,25(OH)₂D₃ did not inhibit the phosphorylation of JNK at 5 and 15 min (Figure 4b). Phosphorylated SEK, which induced phosphorylation and activation of JNK, was also detected after 5 min of TNF stimulation, but again was not affected by pretreatment and the following cotreatment with 1,25(OH)₂D₃ at 5 and 15 min (Figure 4c). These results indicate that 1,25(OH)₂D₃ suppresses the AP-1 activation pathway by inhibiting phosphorylation of c-Jun.

Effects of 1,25(OH)₂D₃ on TNF-Induced Phosphorylation of IκB- and PI3K-Akt Pathways

We next examined whether 1,25(OH)₂D₃ could suppress phosphorylation of the NF-κB pathway, induced by TNF, with immunoblotting of IκB and Akt (Figure 5). TNF induced phosphorylation of IκB, peaking at 5 min after stimulation. However, preincubation with 1,25(OH)₂D₃ for 1 h and the following coincubation did not inhibit IκB phosphorylation (Figure 5a). Since 1,25(OH)₂D₃ blocks NF-κB activation without interfering with IκB phosphorylation, we next investigated the effect of 1,25(OH)₂D₃ on PI3K-Akt signaling, as this pathway has been proposed as an alternative molecular mechanism in the regulation of NF-κB activation. As shown in Figure 5b, TNF induced phosphorylation of Akt, peaking at 15 min and 30 min, but preincubation and the following coincubation with 1,25(OH)₂D₃ had no effect on phosphorylation of Akt. These results indicate that 1,25(OH)₂D₃ has no effect on phosphorylation of the NF-κB activation pathway.

Effects of 1,25(OH)₂D₃ on TNF-Induced Phosphorylation of MEK-ERK1/2 Pathway

Since 1,25(OH)₂D₃ had less effect on binding activity and activation of Egr-1 in gel-shift and LUC assays, we next examined whether 1,25(OH)₂D₃ had an effect on TNF-induced phosphorylation of the Egr-1 pathway with immunoblotting of ERK1/2, Elk-1, and Egr-1 (Figure 6). TNF induced phosphorylation of ERK1/2 (p44/42), peaking at 5 min after stimulation. Preincubation with 1,25(OH)₂D₃ for 1 h and the following coincubation had no effect on the phosphorylation of ERK1/2 (Figure 6a and b). Phosphorylation of Elk-1 by TNF occurred after 10 min and peaked at 15 min, but again was not affected by pretreatment with 1,25(OH)₂D₃ (Figure 6a and b).

Egr-1 was induced by phosphorylation and activation of Elk-1, and was detected after 1 h of TNF stimulation and peaked at 2 h. However, preincubation and the following coincubation with 1,25(OH)₂D₃ did not inhibit the increase of Egr-1 (Figure 6c and d). These results indicate that 1,25(OH)₂D₃ has no effect on phosphorylation of MEK-ERK1/2 pathway.

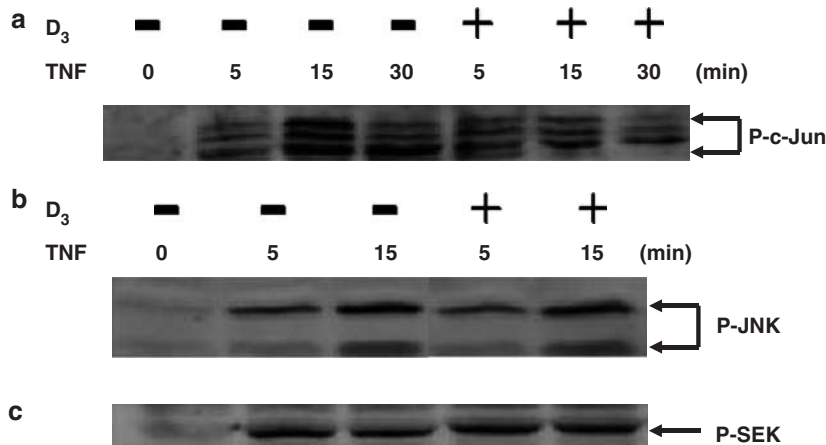


Figure 4 Effects of 1,25(OH)₂D₃ on TNF-induced phosphorylation of c-Jun, JNK, or SEK. THP-1 cells were pretreated with or without 1,25(OH)₂D₃ (0.1 μM) for 1 h and stimulated with TNF (1 nM) for the indicated times. Cell lysates were subjected to Western blot analysis with anti-phospho-c-Jun (a), anti-phospho-JNK (b) or anti-phospho-SEK (c), as indicated. Data are representative of five independent experiments and are confirmed by densitometric quantification.

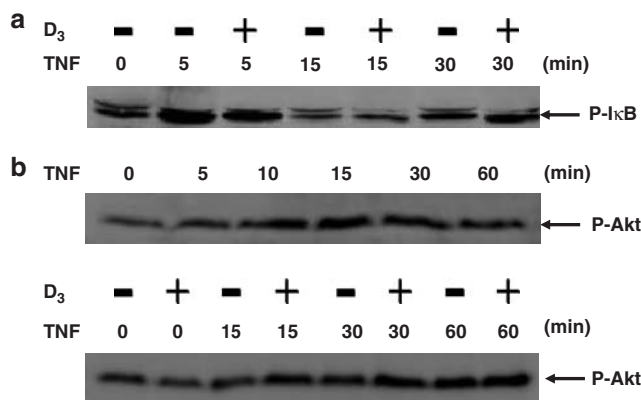


Figure 5 Effects of 1,25(OH)₂D₃ on TNF-induced phosphorylation of IκB and Akt. THP-1 cells were pretreated with or without 1,25(OH)₂D₃ (0.1 μM) for 1 h and stimulated with TNF (1 nM) for the indicated times. Cell lysates were subjected to Western blot analysis with anti-phospho-IκB (a) or anti-phospho-Akt (b).

Effects of 1,25(OH)₂D₃ and the VDR Complex on Phosphorylation of c-Jun

Since 1,25(OH)₂D₃ only has an effect on the phosphorylation of c-Jun in various pathways, we then examined whether 1,25(OH)₂D₃ could repress TF expression through forming a complex of activated VDR with AP-1 by immunoprecipitation with anti-VDR antibody, followed by immunoblotting analysis with anti-c-Jun antibody (Figure 7). Compared with untreated cells, the c-Jun band at 43 kDa was slightly increased when cells were treated with 1,25(OH)₂D₃ for 1 h. After stimulation with TNF for 15 min, the band was markedly decreased and appeared to be shifted upward. This shifted band represents phosphorylated c-Jun complexed with VDR. Pretreatment with 1,25(OH)₂D₃ for 1 h before TNF stimulation and the following incubation prevented the shift and preserved the band at 43 kDa, as observed without TNF. The immunoblot analysis with anti-P-c-Jun antibody after the immunoprecipitation using anti-VDR antibody confirmed the findings (data not shown). The band

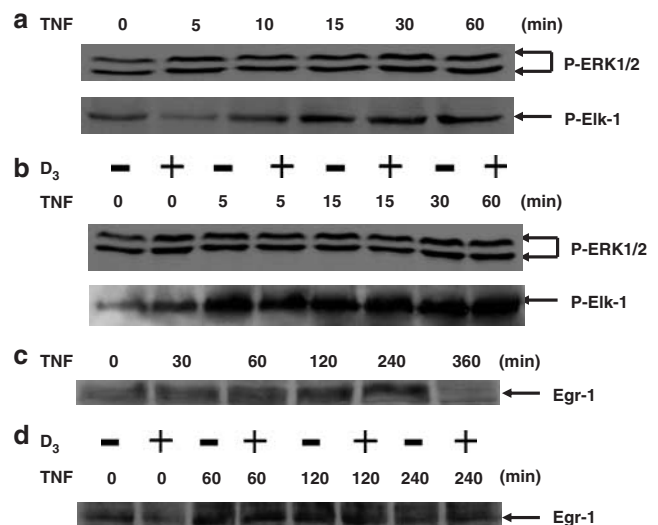


Figure 6 Effects of 1,25(OH)₂D₃ on TNF-induced phosphorylation of ERK1/2, Elk-1, or Egr-1. THP-1 cells were pretreated with or without 1,25(OH)₂D₃ (0.1 μM) for 1 h and stimulated with TNF (1 nM) for the indicated times. Cell lysates were subjected to Western blot analysis with anti-phospho-ERK1/2 (a and b), anti-phospho-Elk-1 (a and b), or anti-Egr-1 (c and d) antibody, as indicated.

of P-c-Jun was recognized in unstimulated cells. After stimulation with TNF, the band was markedly increased. Pretreatment with 1,25(OH)₂D₃ markedly decreased the bands of P-c-Jun in both unstimulated and TNF-stimulated cells. These results indicate that the 1,25(OH)₂D₃ and VDR complex inhibits induction of c-Jun phosphorylation by TNF by forming a complex with c-Jun.

DISCUSSION

In this study, we have shown that 1,25(OH)₂D₃ impairs basal and TNF-induced TF expression in monocytic cells by inhibition of TF gene activation. Since the promoter region of the TF gene has characteristic sequences for the binding sites of transcription factors, we paid attention to the transcription factor sites in this study.

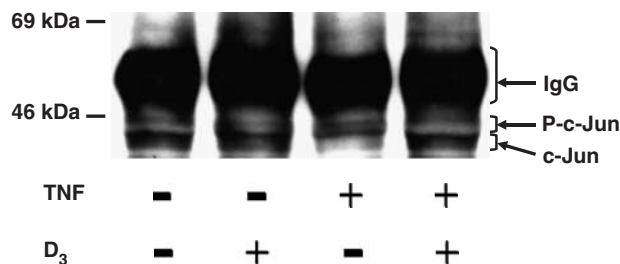


Figure 7 Effect of 1,25(OH)₂D₃ and VDR complex on phosphorylation of c-Jun. THP-1 cells were preincubated with 1,25(OH)₂D₃ (0.1 μM) (lanes 2 and 4) for 1 h, followed by stimulation with TNF (1 nM) for 15 min (lanes 3 and 4). Lane 1 shows untreated THP-1 cells as a control. After treatment, cells were lysed and immunoprecipitated, and the immunoprecipitates were immunoblotted using anti-c-Jun antibody as described in Materials and methods. Data are representative of three independent experiments.

In gel-shift and LUC assays, we demonstrated that 1,25(OH)₂D₃ reduced basal and TNF-induced binding activity of AP-1 and NF-κB to their response elements, and consequently inhibited AP-1- and NF-κB-dependent TF expression in monocytic cells. However, 1,25(OH)₂D₃ did not reduce the binding activity and transcription activity of Egr-1. As U937 cells have low transfection efficiency, we primarily used another monocytic leukemic cell line, namely THP-1 cells, in this study. Since THP-1 and U937 cells are monocytic leukemic cells with malignant transformation, constitutive expression of TF with basal AP-1 and NF-κB activation was documented in both cell lines. In gel-shift assays, the inhibitory effect was more impressive in U937 cells, probably due to the lower basal TF transcriptional activity in U937 cells. In U937 cells, binding activity of Egr-1 was instead increased by 1,25(OH)₂D₃ without TNF stimulation. Upregulation of Egr-1 by 1,25(OH)₂D₃ has been documented in another myeloid leukemia cell line HL60.¹² The sensitivity of the gel-shift assay and that of the LUC assay seemed to be slightly different in terms of TNF-induced TF induction or 1,25(OH)₂D₃-induced TF inhibition.

1,25(OH)₂D₃ suppressed the AP-1 activation pathway by inhibiting phosphorylation of c-Jun. Phosphorylation of JNK upstream of c-Jun or SEK, which is located upstream of JNK in the signal pathway, was not inhibited. Since the biologic effects of 1,25(OH)₂D₃ are mainly mediated by an intranuclear receptor, specifically VDR,¹³ we supposed that the VDR/1,25(OH)₂D₃ complex would block TNF-induced phosphorylation of c-Jun, which has been reported to exist in the nuclear milieu. Therefore, next we examined whether 1,25(OH)₂D₃ could repress TF expression through the binding of activated VDR to the transcriptional activation factor AP-1 by immunoprecipitation using an anti-VDR antibody, followed by immunoblotting analysis using anti-c-Jun and anti-P-c-Jun antibodies. Since the c-Jun band at 43 kDa was recognized in unstimulated cells, c-Jun may constitutively bind to VDR without the ligand binding in the nuclear milieu. After stimulation with 1,25(OH)₂D₃, the c-Jun band at 43 kDa was slightly increased, which indicated

that ligand-bound VDR increased binding activity with c-Jun, and consequently suppressed TF gene expression. After stimulation with TNF, the band was markedly decreased and appeared to shift upward. This shifted band may represent phosphorylated c-Jun complexed with VDR. Pretreatment with 1,25(OH)₂D₃ before TNF stimulation prevented the shift and preserved the band at 43 kDa, as observed without TNF. In the immunoblot analysis with anti-P-c-Jun antibody after the immunoprecipitation using anti-VDR antibody, the P-c-Jun band was recognized in unstimulated cells, and was compatible with basal activation of AP-1 in THP-1 cells. After stimulation with TNF, the band was markedly increased. Pretreatment with 1,25(OH)₂D₃ markedly decreased the P-c-Jun band in both unstimulated and TNF-stimulated cells. These results indicated that the 1,25(OH)₂D₃/VDR complex inhibited the induction of c-Jun phosphorylation and TF induction by TNF stimulation through forming complexes with c-Jun, which suggests a potential direct protein-protein interaction between VDR and c-Jun. A similar inhibitory mechanism with a nuclear receptor was reported in negative regulation of AP-1 responsive genes by retinoic acid and the retinoic acid receptor α complex.²²

Activation of NF-κB has long been thought to be controlled mainly by binding of NF-κB to an inhibitor protein, IκB, which keeps the transcription factor complex in an inactive form in the cytoplasm.²³ PI3K-Akt may also play a pivotal role in TNF-induced NF-κB activation. PI3K-Akt may also cooperate with other TNF-inducible signals to activate NF-κB in HepG2 cells and U937 cells.⁹ On the other hand, LPS activation of PI3K-Akt negatively regulates IKK and glycogen synthase kinase-3β, which reduce the transactivational activity of p65 in THP-1 cells.²⁴ Suppression of the NF-κB activation pathway by 1,25(OH)₂D₃ was not explained by inhibition of phosphorylation of the IKK- and PI3K-Akt-pathways, as TNF-induced phosphorylation of IκB and Akt was not influenced by 1,25(OH)₂D₃ treatment. Sandur *et al* demonstrated that plumbagin suppressed NF-κB activation by directly interfering with the binding of NF-κB to DNA.²⁵ These results indicate that suppression of the NF-κB activation pathway by 1,25(OH)₂D₃ is induced by direct inhibition of the binding activity of NF-κB transcription factors in the promoter region of the TF gene, without suppressing phosphorylation of the NF-κB activation pathway. A mechanism for the direct inhibition is not yet known, as direct binding between VDR and c-Rel/p65 has not been documented (data not shown). Additional mechanisms controlling NF-κB activation may exist in distinct cell types. 1,25(OH)₂D₃ may upregulate expression of another factor(s), which then contributes to the impaired NF-κB translocation to the nucleus and the inhibition of NF-κB binding.^{26,27} The inhibition of the NF-κB pathway may be related to the modulation of the immune system and the differentiation of leukemia cells by 1,25(OH)₂D₃. Further studies are necessary to elucidate the intricate pathways that NF-κB and AP-1 play in TF gene expression.

The pharmacological concentration of 1,25(OH)₂D₃ is 0.2–1 nM in serum, and the physiological concentration of 1,25(OH)₂D₃ (0.1 nM) markedly decreased the expression of TF mRNA.¹⁴ We used 0.1 μM of 1,25(OH)₂D₃ in this study to reveal the effect on TF expression. The more potent 1,25(OH)₂D₃ analogs, which have far stronger binding affinity to VDR and which lack an adverse effect of hypercalcemia, have been synthesized.¹⁴ Such potent analogs may serve as agents for preventing and treating cytokine-mediated thrombotic diseases.

In conclusion, the present study demonstrated that 1,25(OH)₂D₃-mediated inhibition of TF induction by TNF in monocytic cells is due to inhibition of binding activity to the promoter region of the TF gene and transcriptional activity of AP-1 and NF-κB. Of note, 1,25(OH)₂D₃ inhibition of the AP-1 activation pathway is due to a direct interaction between ligand-bound VDR and AP-1 and suppressed phosphorylation of c-Jun, decreasing the transcriptional potential of AP-1 and DNA-binding activity at the AP-1 sites in the TF gene. Suppression of the NF-κB activation pathway by 1,25(OH)₂D₃ is induced by direct inhibition of binding activity of NF-κB transcription factors in the TF gene promoter without suppressing the phosphorylation of NF-κB activation pathway. Our results may help to elucidate the regulatory mechanisms of 1,25(OH)₂D₃ in TF induction, and have physiological significance in the clinical challenge to use potential 1,25(OH)₂D₃ analogs in antithrombotic therapy as well as immunomodulation and antineoplastic therapy of leukemia.

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