1α,25-Dehydroxyvitamin D₃ Synergism toward Transforming Growth Factor-β1-induced AP-1 Transcriptional Activity in Mouse Osteoblastic Cells via Its Nuclear Receptor*

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The present study demonstrates 1α,25-dehydroxyvitamin D₃ (1α,25-(OH)₂D₃) synergism toward transforming growth factor (TGF)-β1-induced activation protein-1 (AP-1) activity in mouse osteoblastic MC3T3-E1 cells via the nuclear receptor of the vitamin. 1α,25-(OH)₂D₃ synergistically stimulated TGF-β1-induced expression of the c-jun gene in the cells but not that of the c-fos gene. We actually showed by a gel mobility shift assay 1α,25-(OH)₂D₃ synergism of TGF-β1-induced AP-1 binding to the 12-tetra-decanoylphorbol-13-acetate response element (TRE). 1α,25-(OH)₂D₃ markedly stimulated the transient activity of TGF-β1-induced AP-1 in the cells transfected with a TRE-chloramphenicol acetyltransferase (CAT) reporter gene. Also, a synergistic increase in TGF-β1-induced CAT activity was observed in the cells cotransfected with an expression vector encoding vitamin D₃ receptor (VDR) and the reporter gene. However, the synergistic CAT activity was inhibited by pretreatment with VDR antisense oligonucleotides. In addition, in a Northern blot assay, we observed 1α,25-(OH)₂D₃ synergism of TGF-β1-induced expression of the c-jun gene in the cells transfected with the VDR expression vector and also found that the synergistic action was clearly blocked by VDR antisense oligonucleotide pretreatment. The present study strongly suggests a novel positive regulation by 1α,25-(OH)₂D₃ of TGF-β1-induced AP-1 activity in osteoblasts via "genomic action."

TGF-β1 is locally produced by osteoblasts and accumulates abundantly in bone matrix tissue (1–3). This local cytokine plays an important role as a "coupling factor" in bone remodeling (4–7). We (8, 9) previously demonstrated that TGF-β1 plays an important role as a "coupling factor" in bone remodelling (4–7). We (8, 9) previously demonstrated that TGF-β1, a potent activator of AP-1, a transcriptional factor that is a heterodimer of FOS and JUN proteins, in mouse osteoblastic MC3T3-E1 cells. AP-1 activates the transcription of target genes by binding to specific promoter elements called TRE. In fact, several studies (10–16) have suggested that AP-1 is a potent activator of AP-1, a transcriptional factor that is a heterodimer of FOS and JUN proteins, in mouse osteoblastic cells and also found that the synergistic action is referred to as the "genomic action" of the hormone. On the other hand, other recent studies (27–37) have suggested that the hormone is able to induce several biological activities via protein kinase C, ceramide signaling pathways, and via an increase in the intracellular calcium concentration, which are called "nongenomic action."

Several investigators (21–24) have demonstrated negative regulation between AP-1 and VDR of the transcriptional activity of osteocalcin and collagen genes, both of which are involved in bone formation. However, positive regulation by 1α,25-(OH)₂D₃ of AP-1 transcriptional activity in osteoblastic cells has not been demonstrated in detail. Therefore, it is of interest to explore the possibility of 1α,25-(OH)₂D₃ positive regulation of AP-1 transcriptional activity via "genomic" or "nongenomic" action.

In this regard, we investigated in the present study the regulation by 1α,25-(OH)₂D₃ of TGF-β1-induced AP-1 transcriptional activity in osteoblastic MC3T3-E1 cells. As a result, we demonstrated the presence of 1α,25-(OH)₂D₃ synergism toward TGF-β1-induced AP-1 transcriptional activity via genomic action. This demonstration suggests the presence of a novel positive regulation by 1α,25-(OH)₂D₃ of AP-1 transcriptional activity in osteoblastic cells via the VDR-dependent pathway (genomic action).

MATERIALS AND METHODS

Reagents—Human recombinant TGF-β1 was purified to homogeneity (>98.9%, determined by SDS-polyacrylamide gel electrophoresis analysis: King Brewer, Kakogawa, Japan). 1α,25-(OH)₂D₃, 24,25-(OH)₂D₃, and 22-oxa-1,25-dihydroxyvitamin D₃ (OCT) were kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). α-MEM was obtained from Flow Laboratories (McLean, VA). FCS was from HyClone (Logan, UT). 5'-[α-³²P]dCTP megaprime DNA labeling system and [γ-³²P]ATP were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). 5'-[α-³²P]UTP and T4 polynucleotide kinase were from NEN Life Science Products.

Cell Culture—Clonal osteoblastic MC3T3-E1 cells derived from C57BL/6 mouse calvaria were cultured in α-MEM supplemented with 10% FCS in plastic dishes at 37 °C and 5% CO₂ in air and subcultured every 3 days as described previously (8, 38). The cells (3 × 10⁵ cells) were cultured at 37 °C under an atmosphere of 5% CO₂ in air and subcultured every 3 days as described previously (8, 38). The cells (3 × 10⁵ cells) were cultured at 37 °C under an atmosphere of 5% CO₂ in air and subcultured every 3 days as described previously (8, 38).

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α-MEM, and subsequently treated for the desired periods in serum-free α-MEM with or without test samples at various concentrations.

**Northern Blot Analysis**—Total cellular RNA was extracted by the guanidine isothiocyanate procedure (39). As described previously (9, 38), the RNA was subjected to 1% agarose electrophoresis and blotted onto a nylon membrane (MSI Magnagrapht, Westboro, MA), and the membranes were subsequently baked, prehybridized, and then hybridized with mouse c-fos cDNA (Oncor, Gaithersburg, MD), mouse c-jun cDNA (ATCC, Rockville, MD), and human VDR cDNA (ATCC) probes labeled with 5'-32PdCTP by use of the megaprime DNA labeling system. After hybridization, the membranes were washed, dried, and exposed to x-ray film (Eastman Kodak Co.) at -70 °C. β-Actin was used as an internal standard for quantification of total RNA in each lane of the gel.

**Nuclear Transcriptional (Run-on) Assay**—This assay was performed according to the method of Groudine et al. (40) as described previously (8, 41). Nuclei were prepared essentially as described by Diaignant et al. (42). In brief, cells (5 × 10⁶ cells) were cultured at 37 °C under an atmosphere of 5% CO₂ in α-MEM supplemented with 10% FCS in 15-cm plastic dishes until nearly confluent. Then the cells were washed and incubated for 24 h in serum-free α-MEM. In addition, the cells were next treated or not for 24 h with 1α,25-(OH)₂D₃ and then for 40 min with TGF-β1, scraped into phosphate-buffered saline, and centrifuged. Subsequently, the cell pellet was suspended in a lysis buffer (10 mM Tris (pH 7.4), 3 mM MgCl₂, 10 mM NaCl, 0.5% Nonidet P-40) after which the nuclei were separated from the cytosol by centrifugation at 3,000 × g for 15 min. Transcription initiated in intact cells was allowed to proceed for 30 min at 30 °C in the presence of 5'-[^32P]UTP, and the RNA was isolated and hybridized to slot-blotted cDNA probes (5 μgslot). Blots were hybridized for 72 h and autoradiographed for 3 days. Protein concentration was measured by the method of Bradford (43).

**Gel Mobility Shift Assay**—This assay was carried out as described previously (8, 9). Binding reactions were performed for 20 min on ice with 5 μg of nuclear protein of 20 μl of binding buffer (2 mM HEPES (pH 7.9), 8 mM NaCl, 0.2 mM EDTA, 12% (v/v) glycerol, 5 mM diethiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of poly(dI-dC) containing 20,000 cpm of ^32P-labeled oligonucleotide in the absence or presence of unlabeled oligonucleotide. Poly(dI-dC) and nuclear extract were first incubated at 4 °C for 10 min before addition of the labeled oligonucleotide. 30-Mer double-stranded oligonucleotides containing the -TGAATTC- sequence (Oncogene Science, Inc., Manhasset, NY) of the AP-1 binding site were end-labeled by the oligonucleotide 5'-end-labeling [γ-32P]ATP method. Reaction mixtures for the binding were incubated for 15 min at room temperature after addition of the labeled oligonucleotide. Unlabeled double-stranded oligonucleotide was used as the competitor. DNA-protein complexes were electrophoresed on native 6% polyacrylamide gels in 0.25 × TBE buffer (22 mM Tris, 22 mM boric acid, and 0.5 mM EDTA (pH 8.0)). Gels were vacuumed, dried, and exposed to Kodak x-ray film at -70 °C.

**Plasmid Construction for Transient Expression Assay**—The plasmid pTRE-TK-CAT was constructed by inserting a synthetic oligonucleotide containing the -TGAATTC- motif with HindIII-XbaI sites into the corresponding sites of pTK-CAT, which contains the herpes simplex virus thymidine kinase promoter enhancer region located upstream of the CAT gene. The plasmid pcGL-TK used also contains the herpes simplex virus thymidine kinase promoter enhancer region, but it is located upstream of the luciferase gene, and this plasmid was made from a pGL3-Enhancer Vector (Promega, Madison, WI). pSG-VDR is a rat VDR expression vector.

**Transient Expression Assay**—This assay was performed according to the method of Felgner et al. (44, 45). The cells (1 × 10⁶ cells) were inoculated into 5-cm diameter dishes and incubated for 12 h in 10% FCS containing α-MEM. Then the cells were washed 3 times with Opti-MEM (Life Technologies, Inc.), transfected with a total of 7 μg of DNA by use of 10 μg of LipofectAMINE (Life Technologies, Inc.), and incubated for 6 h in serum-free Opti-MEM. The cells were transfected with 2 μg of reporter plasmid, and the expression vector for the nuclear receptor (4 μg of each expression vector) was used. The assay was performed in the presence of 1 μg of pGL-TK, a luciferase expression plasmid, used as an internal control to normalize for variations in transfection efficiency. BlueScript M13° (Stratagene, La Jolla, CA) was used as a carrier to adjust the total amount of DNA. The cells were washed three times after the transfection, and 1α,25-(OH)₂D₃ at 10⁻⁸ M or its analogs at 10⁻⁸ M in serum-free α-MEM was then added. After a 24-h incubation, the cells were treated for 6 h with TGF-β1. The cellular extracts were prepared by use of Reporter Lysis Buffer (Promega) and subjected to the CAT assay after normalizing luciferase activity. CAT activity was determined by autoradiography of thin layer chromatography (TLC) plates following completion of the CAT reaction using the appropriate concentration of v-threo-[dichloroacetyl-1,1⁴C]chloramphenicol (Amersshm Pharmacia Biotech, Japan) as described previously (46, 47).

**Preparation of VDR Antisense or Sense Oligonucleotide—VDR-antisense (5'-GCT GGC TGC CAT TGC CTC-3') phosphorothioate oligodeoxyribonucleotide was synthesized and purified as described previously (8, 9, 41). These nucleotide sequences were complementary to the first 18 bases following the AUG sequence of mouse VDR mRNA. Also, the corresponding sense oligonucleotide was prepared and used as a control.

**RESULTS**

We (8, 9) previously demonstrated by the gel mobility shift assay that TGF-β1 induces the binding of AP-1 to TRE in osteoblastic MC3T3-E1 cells. Since AP-1 typically appeared when the cells were treated for 3 h with TGF-β1 at 1 ng/ml, in this study we investigated the regulatory action of 1α,25-(OH)₂D₃ on TGF-β₁-induced AP-1 activity in the cells under the experimental conditions.

1α,25-(OH)₂D₃ Synergistically Stimulates Expression of the TGF-β₁-induced c-jun Gene in MC3T3-E1 Cells—First, we examined the effect of 1α,25-(OH)₂D₃ on TGF-β₁-induced expression of the c-jun and c-fos genes in the cells. The cells were treated or not with TGF-β₁ at 1 ng/ml before the vitamin D₃ was added to the cell cultures. As shown in Fig. 1A, 1α,25-(OH)₂D₃ stimulated synergistically TGF-β₁-induced expression of the c-jun gene in the cells. The synergistic action was...
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observed at a physiological concentration (10^-10 M) of the hormone. However, such action was not observed in the expression of the cytokine-induced c-fos gene in the cells. Also, the synergistic effect of 1α-25-(OH)_2D_3 on the expression of the c-jun gene in the cells was observed even when the hormone pre-treatment time was only 1 h before the cells were incubated with TGF-β1 and tended to be pre-treatment time-dependent (Fig. 1B).

1α-25-(OH)_2D_3 Synergistic Effect on TGF-β1-induced Expression of the c-jun Gene in MC3T3-E1 Cells Occurs at the Transcriptional Level—Next we examined, using the run-on assay, whether or not the 1α-25-(OH)_2D_3 synergistic action toward TGF-β1-induced expression of the c-jun gene in the cells operates at the transcriptional level. The cells were pre-treated or not for 24 h with 1α-25-(OH)_2D_3 at 10^-8 M and then were treated or not for 1 h with TGF-β1 at 1 ng/ml. Thereafter, the run-on assay was performed using nuclei isolated from the cells. Fig. 2, A and B, shows that 1α-25-(OH)_2D_3 clearly stimulated the transcriptional activity of the TGF-β1-induced c-jun gene. These results indicate the synergistic action of 1α-25-(OH)_2D_3 at the transcriptional level for TGF-β1-induced expression of c-jun gene in the cells.

Synergistic Effect of 1α-25-(OH)_2D_3 on AP-1 Binding to TRE in TGF-β1-treated MC3T3-E1 Cells—AP-1 is a heterodimer of FOS and JUN proteins and binds to the TRE consensus sequence (48). Synergistic stimulation by 1α-25-(OH)_2D_3 of TGF-β1-induced expression of the c-jun gene in the cells suggested to us that the hormone may remarkably increase AP-1 binding to TRE in the cytokine-treated cells. Therefore, we examined this point by the gel mobility shift assay. As we expected, and as shown in Fig. 3, 1α-25-(OH)_2D_3 caused a synergistic increase in AP-1 binding to TRE in the TGF-β1-treated cells in a dose-dependent manner. The stimulated binding was completely inhibited by the unlabeled oligonucleotide containing TRE (data not shown).

1α-25-(OH)_2D_3 Stimulates TGF-β1-induced AP-1 Transcriptional Activity in MC3T3-E1 Cells—In addition, we explored whether 1α-25-(OH)_2D_3 is able, moreover, to stimulate TGF-β1-induced AP-1 transcriptional activity in the cells. We investigated this possibility by employing the transient CAT assay on cells transfected with plasmids containing with TRE-TK-CAT reporter gene (pTk-TK-CAT). As shown in Fig. 4, A and B, 1α-25-(OH)_2D_3 pretreatment clearly stimulated TGF-β1-induced AP-1 transcriptional activity in the cells. No CAT activity was observed in the cells transfected with the TK-CAT reporter plasmid (pTk-CAT).

Effect of 1α-25-(OH)_2D_3 Analogs on TGF-β1-induced AP-1 Transcriptional Activity in MC3T3-E1 Cells—Several recent studies (27–37) have suggested the presence of VDR-dependent (genomic) and -independent (non-genomic) pathways in 1α-25-(OH)_2D_3 signal transduction. Therefore, it was of interest to determine whether the synergistic action of 1α-25-(OH)_2D_3 toward AP-1 transcriptional activity in TGF-β1-treated cells was VDR-dependent or -independent. In this regard, some studies (34, 49) have shown that OCT, a 1α-25-(OH)_2D_3 derivative, has a high affinity for VDR and that its biological activity is generated via the VDR-dependent (genomic) pathway. In contrast, 24,25-(OH)_2D_3 is an analog that has very low affinity for VDR (29, 49). Therefore, using these two analogs, we exam-
Fig. 5. Effect of 1α-25-(OH)2D3 analogs on TGF-β1-induced c-jun gene expression and AP-1 transcriptional activity in MC3T3-E1 cells. A, cells were incubated for 24 h in the presence or absence of 1α-25-(OH)2D3 or its analogs at 10−8 M, washed, and treated or not with TGF-β1 (1 ng/ml). Then total RNA was prepared at 1.5 h after the initiation of the cytokine treatment. Northern blot analysis was performed with c-fos, c-jun, and β-actin cDNAs used as probes. Quantification of c-jun mRNA level was done by densitometry and is expressed as a percentage of maximum. B, cells were transfected with a reporter plasmid (pTRE-TK-CAT) or control plasmid (pTK-CAT) and were washed three times and then incubated in serum-free α-MEM supplemented with or without 1α-25-(OH)2D3 or its analogs at 10−8 M. After a 24-h incubation, the cells were treated or not for 6 h with TGF-β1 (1 ng/ml). Thereafter, the cellular extracts were prepared and subjected to the CAT assay. All assays were performed in the presence of 1 μg of pGL-TK, a luciferase expression plasmid, used as an internal control to normalize for variations in transfection efficiency. Quantification of the CAT activity was done by densitometry and is expressed as a percentage of maximum. An identical experiment independently performed gave similar results.

Fig. 6. Transfection with a VDR expression vector demonstrates receptor role in 1α-25-(OH)2D3 synergy in c-jun gene expression in TGF-β1-treated MC3T3-E1 cells. A, cells were transfected or not with the VDR expression vector (pSG-VDR) or control vector (pSG-5) and then were washed three times. Thereafter, the cells were incubated in serum-free α-MEM supplemented or not with 1α-25-(OH)2D3 at 10−8 M. After a 24-h incubation, the cells were washed and subsequently were treated or not with TGF-β1 (1 ng/ml). Total RNA was prepared at 1.5 h after the initiation of the cytokine treatment. Northern blot analysis was performed with c-jun and β-actin cDNAs used as probes. B, quantification of c-jun mRNA level in A was done by densitometry and is expressed as a percentage of maximum.

gene expression was analyzed by the Northern blot assay. As shown in Fig. 6, A and B, 1α-25-(OH)2D3 markedly stimulated TGF-β1-induced expression of the c-jun gene in pSG-VDR-transfected cells when the gene expression was compared with that of the control and pSG-5 vector-transfected cells. These results confirm the functional role of endogenous VDR in 1α-25-(OH)2D3 synergy in TGF-β1-induced expression of the c-jun gene in the cells.

Synergistic Stimulation by 1α-25-(OH)2D3 of TGF-β1-induced AP-1 Transcriptional Activity in MC3T3-E1 Cells Is Mediated via VDR—1α-25-(OH)2D3 synergy in TGF-β1-induced expression of the c-jun gene in the cells transfected with the VDR expression vector suggested to us the possibility that 1α-25-(OH)2D3 stimulates synergistically TGF-β1-induced AP-1 transcriptional activity in the cells via VDR. Therefore, we explored this possibility by using a cotransfection system with VDR expression vector (pSG-VDR) and pTREG-TK-CAT. Fig. 7, A and B, shows that TGF-β1-induced AP-1 transcriptional activity in pSG-VDR-transfected cells was approximately 1.5 times that in the cells transfected with pSG-5. In a CAT assay with the TK-CAT reporter plasmid (pTK-CAT), no effect of pSG-VDR or pSG-5 was observed (data not shown). These results suggest strongly that 1α-25-(OH)2D3 pretreatment of TGF-β1-induced AP-1 transcriptional activity in MC3T3-E1 cells in a VDR-dependent manner.

Effect of VDR Expression on 1α-25-(OH)2D3 Synergy in c-jun Gene Expression in TGF-β1-treated MC3T3-E1 Cells—To ensure that 1α-25-(OH)2D3 synergistically enhances TGF-β1-induced AP-1 transcriptional activity in the cells is mediated through VDR, it is significant to understand the precise role of VDR in the hormone synergy toward c-jun gene expression in the cells. Therefore, we explored using a VDR expression vector (pSG-VDR) the functional role of endogenous VDR in this synergy. The cells were transfected with pSG-VDR or control vector pSG-5 and then pretreated or not with 1α-25-(OH)2D3. Subsequently, the cells were treated or not with TGF-β1, and c-jun

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induced expression of the c-jun gene in the cells.

Also, we examined involvement of the endogenous VDR in the cytokine-induced AP-1 transcriptional activity in the cells. The cells were pretreated or not with pTRE-TK-CAT and then pretreated or not for 12 h with VDR sense or antisense oligonucleotide. Thereafter, the cells were treated or not for 24 h with 1α-25-(OH)2D3 at 10−8 M. After a 24-h incubation with the hormone, the cells were treated or not for 1.5 h with TGF-β1 (1 ng/ml). Thereafter their total RNA was prepared. Northern blot analysis was performed with c-jun and β-actin cDNAs used as probes. An identical experiment independently performed gave similar results. Quantification of c-jun mRNA level was done by densitometry and is expressed as a percentage of maximum.

Involvement of endogenous VDR in 1α-25-(OH)2D3 synergy toward TGF-β1-induced AP-1 transcriptional activity in MC3T3-E1 cells. A, cells were cotransfected with the reporter plasmid (pTRE-TK-CAT) and VDR expression vector (pSG-VDR) or control vector (pSG-5) and then were washed three times. The transfected cells were incubated in serum-free α-MEM supplemented or not with 1α-25-(OH)2D3 at 10−8 M. After a 24-h incubation, the cells were treated or not for 6 h with TGF-β1 (1 ng/ml). Thereafter, the cellular extracts were prepared and subsequently subjected to the CAT assay. All assays were performed in the presence of pGL-TK, a luciferase expression plasmid, used as an internal control to normalize for variations in transfection efficiency. B, quantification of CAT activity in A was done by densitometry and is expressed as a percentage of maximum. An identical experiment independently performed gave similar results.

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DISCUSSION

TGF-β1 and 1α-25-(OH)2D3 are important local and systemic regulatory factors in bone remodeling (4–7, 54–56). For osteoblastic cells, both are potent factors in growth and differentiation (7–9, 20–25). We previously demonstrated that TGF-β1-stimulated expression of the osteopontin gene in MC3T3-E1 cells is mediated via AP-1. And, in fact, it has been demonstrated that a TRE sequence is located on the promoter region of the murine osteopontin gene (52, 53). Therefore, finally, we investigated 1α-25-(OH)2D3 synergism toward TGF-β1-stimulated expression of the osteopontin gene in the cells. As we expected, 1α-25-(OH)2D3 pretreatment at 10−8 M stimulated expression of the osteopontin gene in the cytokine-treated cells (Fig. 9A), and a run-on assay (Fig. 9B) showed that 1α-25-(OH)2D3 operates at the transcriptional level in the synergistic action. Also, such synergism was observed with OCT pretreatment (Fig. 9C). These observations allow us to propose that 1α-25-(OH)2D3 actually stimulates AP-1-mediated differentiation of TGF-β1-treated osteoblastic cells.
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1α-25-(OH)₂D₃ may exhibit multiple effects on gene expression in osteoblastic cells via its receptor-dependent mechanism or genomic action. On the other hand, interestingly, recent studies (20–26) have suggested that the hormone exerts several effects on cells by a receptor-independent mechanism, i.e., by nongenomic action. In this regard, it is of interest to investigate whether 1α-25-(OH)₂D₃ action on the AP-1 activity would be genomic or non-genomic in nature. We observed here that TGF-β1-induced AP-1 transcriptional activity in mouse MC3T3-E1 cells. Our interest was whether the 1α-25-(OH)₂D₃ action on the AP-1 activity would be genomic or nongenomic. Of these two mechanisms, we observed that the synergistic action of 1α-25-(OH)₂D₃ toward TGF-β1-induced AP-1 transcriptional activity in the cells occurs via the genomic activity (the receptor-dependent mechanism).

Several studies (10–16, 21–23) have well documented that AP-1 in osteoblasts is an important transcriptional factor in bone formation and resorption. We observed here that TGF-β1-induced expression of the c-jun gene in MC3T3-E1 cells was synergistically stimulated by 1α-25-(OH)₂D₃ pretreatment, although such synergistic effect was not observed for c-fos gene expression. The synergistic action of 1α-25-(OH)₂D₃ toward c-jun gene expression was dose- and pretreatment-dependent. Our run-on assay indicated that the 1α-25-(OH)₂D₃ synergistic action in the c-jun gene expression in TGF-β1-treated cells resulted from stimulation of its transcription. Since these observations suggested the synergistic increase by the hormone of AP-1 binding to TRE in the cells, we explored this point by using the gel mobility shift assay. This assay clearly demonstrated that the cytokine-induced AP-1 binding to TRE in the cells was synergistically increased by 1α-25-(OH)₂D₃ pretreatment. Of more interest was whether the hormone would be actually able to stimulate synergistically AP-1 transcriptional activity. In fact, the TRE-TK-CAT assay showed that 1α-25-(OH)₂D₃ clearly stimulated the AP-1 transcriptional activity in TGF-β1-treated cells. In this regard, Sassone-Corsi and co-workers (48, 57) showed that AP-1 transcriptional activity in mouse embryonal carcinoma F9 cells, which express constitutively c-jun and c-fos genes at a low level, is induced by transfecting the cells with a c-jun autonomous expression vector. We (8, 58) observed that curcumin, a potent inhibitor of c-jun gene expression, completely inhibited the synergistic effect of 1α-25-(OH)₂D₃ on TGF-β1-induced AP-1 transcriptional activity in MC3T3-E1 cells (data not shown). These observations of vitamin D₃ support the notion that the stimulated expression of the c-jun gene provided an important clue in the mechanism of 1α-25-(OH)₂D₃ synergism toward TGF-β1-induced AP-1 transcriptional activity in the cells.

It is well documented that multiple biological actions of 1α-25-(OH)₂D₃ in osteoblastic cells are mediated via interaction of its receptor complex with specific DNA sequences (20–22, 24, 25). On the other hand, recent studies (27–37) have demonstrated that 1α-25-(OH)₂D₃ expresses several biological actions via ceramide and protein kinase C signaling pathways and also via intracellular calcium signals. Thus, it was of interest to demonstrate whether 1α-25-(OH)₂D₃ synergy in the stimulation of AP-1 transcriptional activity of TGF-β1-treated MC3T3-E1 cells is genomic action-dependent or -independent. Therefore, we explored this point by using 1) 1α-25-(OH)₂D₃ analogs having high and low affinities to VDR, 2) transfection assay with VDR expression and TRE-TK-CAT vectors, and 3) VDR antisense oligonucleotide to eliminate production of endogenous VDR. We observed that OCT synergy in the expression of the c-jun gene in TGF-β1-treated MC3T3-E1 cells was the same as that of 1α-25-(OH)₂D₃. Since OCT is a ligand having high affinity for VDR (33, 37), these observations suggested to us that 1α-25-(OH)₂D₃ synergy in the stimulation of AP-1 transcriptional activity of TGF-β1-treated cells may be genomic in nature. We proved this suggestion by using a cotransfection assay with VDR expression and TRE-TK-CAT vec-

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tors. 1α,25-(OH)2D3 or OCT markedly stimulated TGF-β1-induced AP-1 transcriptional activity in the cells when they were cotransfected with VDR expression and TRE-TK-CAT vectors. In addition, VDR antisense oligonucleotide pretreatment inhibited approximately 40% the synergistic action of 1α,25-(OH)2D3 toward TGF-β1-induced AP-1 transcriptional activity in the cells. These results together with the above demonstrate that 1α,25-(OH)2D3 synergism toward TGF-β1-induced AP-1 transcriptional activity in MC3T3-E1 cells is mediated via a VDR-dependent mechanism (genomic action).

Several studies (59–62) have shown that the combination of 1α,25-(OH)2D3 and TGF-β1 functionally regulates bone formation. Our previous study (9) showed that TGF-β1-induced expression of RAR-α, RAR-γ, and retinoic X receptor-α is mediated via the cytokine-induced AP-1 signaling pathway in MC3T3-E1 cells. Most recently, we observed that the cytokine-induced expression of RAR-α, -γ, and retinoic X receptor-α genes were remarkably stimulated by pretreatment with 1α,25-(OH)2D3. In addition, as we also showed in this study, the hormone stimulated synergistically TGF-β1-induced expression of the osteopontin gene at the transcriptional level in the cells. Since osteopontin is one of the extracellular non-collagenous matrix proteins and also is a marker of differentiation of osteoblastic cells (20, 50–53), these observations are significant with respect to bone metabolism.

In further experiments, our interest will be to define the mechanism of 1α,25-(OH)2D3 synergism operating in TGF-β1-induced AP-1-mediated expression of several genes in osteoblastic cells.

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