1α25(OH)₂D₃ interferes with retinoic acid-induced inhibition of c-fos gene expression for AP-1 formation in osteoblastic cells

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Abstract: Our previous studies demonstrated retinoic acid (RA) inhibition of activation protein-1 (AP-1) formation in TNF-α-treated osteoblastic MC3T3-E1 cells via c-fos suppression. In the present study, we observed that 1α25(OH)₂D₃ was able to interfere at the transcriptional level with RA inhibition of TNF-α-induced c-fos gene expression in cells when the cells were incubated with the vitamin for 24 hr before the RA treatment. 22-Oxa-1,25(OH)₂D₃ (OCT), an analog derivative of 1α25(OH)₂D₃, having high affinity for the vitamin D₃ receptor (VDR), also interfered with the RA-induced inhibition of c-fos gene expression in the TNF-α-treated cells. In contrast, this was not the case for 24,25(OH)₂D₃. Moreover, we observed that the interfering effect was clearly blocked by pretreatment with VDR antisense oligonucleotide. 1α25(OH)₂D₃ interfered with RA inhibition of the TPA-response element binding activity of AP-1 in the cytokine-treated cells. Furthermore, 1α25(OH)₂D₃ actually blocked the AP-1-mediated gene expression of monocyte chemotactic protein JE/MCP-1 induced in the cytokine-treated cells. The present study suggests a regulatory interference by 1α25(OH)₂D₃ for RA inhibition of TNF-α-induced AP-1 activity in osteoblasts. (J. Oral Sci. 44, 27-34, 2002)

Key words: 1α25(OH)₂D₃; osteoblasts; retinoic acid; AP-1; MCP-1.

Introduction

Retinoic acid (RA) is an important regulator of the proliferation and differentiation of a variety of human and mouse cells (1). Furthermore, it is well known that RA is an anti-inflammatory and anti-tumor agent (2-4). In this regard, several studies (4-8) have demonstrated that RA functions as a negative regulator of AP-1, a transcriptional activator protein. Recently, we found that RA suppressed AP-1 binding to 12-tetra-decanoyl-phorbol-13-acetate-response element (TRE) via inhibition of c-fos gene expression in a mouse osteoblastic cell line (9). Since the pleiotropic effects of RA are likely to be mediated by specific inhibitory retinoic acid receptor (RAR)s, this RA effect on the c-fos gene in our system may be mediated by RARs.

RAR, vitamin D₃ receptor (VDR), and thyroid hormone receptors belong to the same family of nuclear receptors (10,11). After binding to its specific ligand, each receptor regulates the expressions of many genes via binding to the hormone response element, a direct repeat consisting of the AGGTCA consensus motif (12,13). Several interesting studies (14-16) have demonstrated that these receptors form heterodimers with auxiliary nuclear proteins known as retinoid X receptors (RXRs), and consequently function as powerful transcriptional factors in the expression of many genes. Therefore, it is of great interest to examine interference among these hormones for gene expressions as their cellular responses.

In the present study, we examined the effect of 1α25(OH)₂D₃ on RA-mediated inhibition of TNF-α-induced c-fos gene expression in osteoblastic MC3T3-E1 cells. We found that 1α25(OH)₂D₃ interfered with RA inhibition of c-fos gene expression for AP-1 formation in the cytokine-treated osteoblastic cells.
Materials and Methods

Reagents

Human recombinant TNF-α (specific activity of 2.0 × 10^6 units/mg of protein) was provided by the Suntory Institute for Biomedical Research (Osaka, Japan) and purified to homogeneity (>98.9%, as determined by SDS-polyacrylamide gel electrophoresis analysis). 1α25(OH)2D3, 24,25(OH)2D3, and 22-oxa-1,25(OH)2D3 (OCT) were kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). All-trans-retinoic acid was purchased from Sigma Co., Ltd. (St. Louis, MO). α-MEM was obtained from Flow Lab (McLean, VA). Fetal calf serum was purchased from Hyclone (Logan, UT). The 5'-[α-32P]-dCTP megaprimer DNA labeling system and [γ-32P]ATP were purchased from Amersham Japan (Tokyo). 5'-[32P]-UTP and T4 polynucleotide kinase were from New England Nuclear (Boston, MA).

Cell culture

Clonal osteoblastic MC3T3-E1 cells derived from C57BL/6 mouse calvaria were cultured in α-MEM with 10% fetal calf serum in plastic dishes at 37°C and 5% CO2 in air and subcultured every 3 days as previously described (17,18). The osteoblastic cells were kindly provided by Dr. H. Kodama (Bayer Yakuhin, Ltd. Kyoto, Japan). The cells (3.0 × 10^5 cells) were cultured at 37°C under an atmosphere of 5% CO2 in air in α-MEM with 10% fetal calf serum in 90-mm plastic dishes until nearly confluent. Then the cells were washed, incubated for 24 hr in serum-free α-MEM, and treated for the desired periods in serum-free α-MEM with or without test samples at various concentrations.

Northern blotting analysis

Total cellular RNA was extracted by the guanidine isothiocyanate procedure (19). As previously described (17,18), the RNA was subjected to 1% agarose electrophoresis, and blotted onto a nylon membrane (MSI Magnagraph, Westboro, MA), and the membranes were subsequently baked, prehybridized, and then hybridized with each cDNA probe that had been labeled with 5'-[α-32P]-dCTP by use of the megaprimer DNA labeling system. After hybridization, the membranes were washed, dried, and exposed to X-ray film (Eastman Kodak Co., Rochester, NY) at -70°C. β-Actin was used as an internal standard for quantification of total mRNA in each lane of the gel.

Nuclear transcriptional (Run on) assay

This assay was performed according to the method of Groudine et al. (20) as described previously (17,18). Nuclei were prepared essentially as described by Dignam et al. (21). In brief, cells (5.0 × 10^7 cells) in a plate were treated or not with 1α25(OH)2D3. After 24 hr, the cells were next treated or not for 24 h with RA and then TNF-α for 20 min or 1 hr, scrapped into PBS, and centrifuged. Next, 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'-[α-32P]-UTP, and the RNA was isolated and hybridized to slot-blotted cDNA probes (5 μg/slot). Blots were hybridized for 72 h and autoradiographed for 3 days. The β-actin gene was utilized as an internal standard.

Preparation of nuclear extracts

Confluent monolayers in 15-cm-diameter dishes were treated with test samples as indicated in the figure legends, and then their nuclei were isolated as described above. Then the nuclear extracts were prepared as described previously (9,22). Protein concentration was measured by the method of Bradford (23).

Gel mobility shift assay

This assay was carried out as described previously (9,22). Binding reactions were performed for 20 min on ice with 5μg of nuclear protein in 20 μl of binding buffer (2 mM HEPES[pH 7.9], 8 mM NaCl, 0.2 mM EDTA, 12%[v/v] glycerol, 5 mM DDT, 0.5 mM PMSF, 1μg poly[dl-dC]) containing 20,000 c.p.m. of 32P-labeled oligonucleotide in the absence or presence of nonlabeled oligonucleotide. Poly(dl-dC) and nuclear extract were first incubated at 4°C for 10 min before adding the labeled oligodeoxynucleotide. Thirty mer double-stranded oligonucleotides containing -TGACTCA- (Oncogene Science, Inc) of the AP-1 binding site were end-labeled by the oligonucleotide 5’ end-labeling system-[γ-32P]ATP method. Reaction mixtures for the binding were incubated for 15 min at room temperature after adding the labeled oligonucleotide. The 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]). Finally, the gels were vacuumed, dried, and exposed to Kodak X-ray film at -70°C.
Preparation of VDR antisense or sense oligonucleotide

VDR antisense (5'-GCT-GGC-TGC-CAT-TGC-CTC-3') phosphorothioate oligodeoxynucleotide was synthesized and purified as described previously (24). These nucleotide sequences were complementary to the first 18 bases following the AUG sequence of mouse VDR mRNAs. Also, the corresponding sense oligonucleotide was prepared and used as a control. These oligonucleotides penetrated into the cells without any treatment, as reported earlier by Loke et al. (25).

Results

Our previous study (9) showed that TNF-α-induced expression of the c-fos gene was inhibited markedly when MC3T3-E1 osteoblastic cells were pretreated for 24 h with RA at 1 μM. Therefore, in this study, we investigated 1α25(OH)2D3 interference of RA-induced inhibition of c-fos gene expression for AP-1 formation in the cells under these experimental conditions.

1α25(OH)2D3 interferes with RA-mediated inhibition of TNF-α-induced expression of the c-fos gene in MC3T3-E1 cells

Firstly, we examined whether 1α25(OH)2D3 was able to affect the RA-mediated inhibition of TNF-α-induced expression of the c-fos gene in the cells. The cells were incubated for the desired time in the presence or absence of 1α25(OH)2D3 at 10 pM, pretreated or not for 24 h with RA, and subsequently treated or not for 30 min with TNF-α at 100 ng/ml. As shown in Fig. 1 A and 1B, 1α25(OH)2D3 blocked the RA inhibition of TNF-α-induced expression of the c-fos gene in treatment time- and dose-dependent manners. However, such RA inhibition was not observed with respect to the cytokine-induced c-jun gene expression, as described previously (9).

1α25(OH)2D3 interferes at the transcriptional level with RA inhibition of TNF-α-induced expression of the c-fos gene in MC3T3-E1 cells

Next we examined, using the Run-on assay, whether 1α25(OH)2D3 interfered transcriptionally with the RA-mediated inhibition of TNF-α-induced expression of the c-fos gene in the cells. Fig. 2 shows that RA transcriptional inhibition of the cytokine-induced expression c-fos gene was lessened by the 1α25(OH)2D3 treatment.

These results suggest strongly that 1α25(OH)2D3 interfered at the transcriptional level with RA inhibition of TNF-α-induced c-fos gene expression in the cells.
Interfering effect of 1α25(OH)₂D₃ analogs on RA-mediated inhibition of c-fos gene expression in TNF-α-treated MC3T3-E1 cells

Several recent studies (24,26-30) have suggested the presence of vitamin D₃ receptor (VDR)-dependent (genomic) and independent (non-genomic) pathways in the biological action of 1α25(OH)₂D₃. In this regard, several of them (24,27-32) showed that OCT, an analog derivative of 1α25(OH)₂D₃, had a high affinity for the VDR and that its biological action was a genomic one. In contrast, 24,25(OH)₂D₃ is a ligand that has very low affinity for the VDR (27,31,32). Therefore, using these analogs we examined whether the 1α25(OH)₂D₃ interference of the RA-mediated inhibition of c-fos was VDR dependent or independent. As shown in Fig. 3 A and 3B, OCT also blocked the RA-induced inhibition of c-fos gene expression in the TNF-α-treated cells. In contrast, this was not the case for 24,250. These results suggest this hormone interference to be VDR dependent.

Involvement of endogenous VDR in 1α25(OH)₂D₃ interference toward RA-mediated inhibition of TNF-α-induced c-fos gene expression in MC3T3-E1 cells

As described above, our data suggested involvement of an endogenous VDR in the 1α25(OH)₂D₃ interfering effect on RA-mediated inhibition of TNF-α-induced expression of the c-fos gene in MC3T3-E1 cells. In addition, we used mouse VDR antisense oligonucleotide to investigate the functional role of VDR in this interference. Fig. 4A and 4B shows that VDR antisense, but not the sense, oligonucleotide (10μM) pre-treatment clearly inhibited the interference action of 1α25(OH)₂D₃, thus suggesting a functional role for endogenous VDR in the interfering effect of 1α25(OH)₂D₃ on RA-mediated inhibition of TNF-0-induced c-fos gene expression in MC3T3-E1 cells.

Fig. 3 Effect of 1α25(OH)₂D₃ analogs on RA-mediated inhibition of TNF-α-induced c-fos gene expression in MC3T3-E1 cells. (A) Cells were incubated for 24 hr in the presence or absence of 1α25(OH)₂D₃ or its analogs at 10 pM, washed, and pretreated or not for 24 hr with RA (1 μM). Thereafter, the cells were washed and treated or not with TNF-α (100 ng/ml). Then the total RNA was prepared at 30 min after the initiation of the cytokine treatment. Northern blot analysis was performed with c-fos, c-jun, and β-actin cDNAs used as probes. (B) Quantification of the c-fos gene expression in panel (A) was done by densitometry, and is expressed as a % of the maximum. An identical experiment independently performed gave similar results.

Fig. 4 Involvement of endogenous VDR in 1α25(OH)₂D₃ interference towards RA-mediated inhibition of TNF-α-induced c-fos gene expression in MC3T3-E1 cells. (A) Cells were pretreated or not for 12 h with VDR antisense or sense oligonucleotide (10 μM) and subsequently treated or not with 1α25(OH)₂D₃ at 10 pM, washed, and pretreated or not for 24 h with RA (1 μM). After a 24-h incubation with the vitamin, the cells were treated or not for 30 min with TNF-α(100 ng/ml). Thereafter their total RNA was prepared. Northern blot analysis was performed with c-jun and β-actin cDNAs used as probes. (B) Quantification of the c-fos gene expression in panel (A) was done by densitometry, and is expressed as a % of the maximum. An identical experiment independently performed gave similar results.

1α25(OH)₂D₃ interferes with RA-induced inhibition of TRE binding activity of AP-1 in TNF-α-treated MC3T3-E1 cells

Our previous study (9) showed that in the RA-induced inhibition of the c-fos gene expression there was a marked decrease in the binding of AP-1 to TRE in TNF-α-treated
cells. These observations suggested that 1α25(OH)₂D₃ may block this RA effect on the binding of AP-1 to TRE in the cytokine-treated cells. Therefore, we employed the gel mobility shift assay to obtain more evidence for 1α25(OH)₂D₃ interference. Fig. 5 shows that 1α25(OH)₂D₃ dramatically reduced the inhibition by RA of AP-1 binding to TRE in the TNF-α-treated cells. We also observed that the cytokine-stimulated binding of AP-1 was completely inhibited by the unlabeled oligonucleotide containing TRE (data not shown). These results show that 1α25(OH)₂D₃ was able to interfere with RA-induced inhibition of AP-1 formation in the cytokine-treated cells.

1α25(OH)₂D₃ interferes with RA-induced transcriptional inhibition of AP-1-mediated JE/MCP-1 gene expression in TNF-α-treated MC3T3-E1 cells

We previously demonstrated AP-1-mediated expression of the monocyte chemoattractant JE/MCP-1 gene in TNF-α-treated MC3T3-E1 cells (18). Moreover, our study (9) showed that RA suppression of the c-fos gene inhibits expression of TNF-α-induced the chemokine in the cells. Therefore, we examined whether the hormone actually would affect the RA-induced transcriptional inhibition of AP-1-mediated expression of the JE/MCP-1 gene in the cytokine-treated cells. As shown in Fig. 6 A, 1α25(OH)₂D₃ clearly prevented this inhibition. Furthermore, the RA-induced inhibition of the JE/MCP-1 gene transcription was also blocked by the vitamin (Fig. 6 B).

Finally, we examined 1α25(OH)₂D₃ analogs for their effect on RA-induced inhibition of AP-1-mediated expression of the JE/MCP-1 gene in the cytokine-treated cells. As shown in Fig. 6C, such interference of the RA-induced inhibition was also observed with OCT treatment, but not with 24,25(OH)₂D₃ treatment. These results suggested the involvement of VDR in this interference.

Fig. 5 1α25(OH)₂D₃ blocks RA-induced inhibition of TRE binding of AP-1 in TNF-α-treated MC3T3-E1 cells. Cells were incubated for 24 h in the presence or absence of 1α25(OH)₂D₃ at 10 pM, washed, and then pretreated or not for 24 h with RA (1 μM). Then, the cells were washed and treated or not for 1 hr with TNF-α (100 ng/ml), after which the nuclear proteins were prepared. Gel mobility shift assay was performed with 32P-labeled oligonucleotide containing the TRE sequence in the presence of the nuclear proteins. The arrow indicates the position of DNA-protein complexes. An identical experiment independently performed gave similar results.

Fig. 6 1α25(OH)₂D₃ interferes with RA inhibition of AP-1-mediated JE/MCP-1 gene expression in TNF-α-treated MC3T3-E1 cells. (A) Cells were incubated for 24 h in the presence or absence of 1α25(OH)₂D₃ at 10 pM, washed, and pretreated or not for 24 h with RA (1 μM). Thereafter, the cells were washed, and treated or not with TNF-α (100 ng/ml). Total RNA was prepared at 1 hr after the initiation of the cytokine treatment. Northern blot analysis was performed with JE/MCP-1 and β-actin cDNAs used as probes. (B) Cells were treated under the conditions described in “A.” Then their nuclei were incubated for 30 min in the presence of 5'-[α-32P]UTP, after which the RNA was isolated. Transcriptional activity assay was performed with JE/MCP-1 and β-actin cDNAs, pBR322, the vector plasmid, was used as a negative control. (C) Cells were incubated for 24 h in the presence or absence of 1α25(OH)₂D₃ or its analogs at 10 pM, washed, and pretreated or not for 24 h with RA (1 μM). Thereafter, the cells were washed, treated or not with TNF-α(100 ng/ml). Then, total RNA was prepared 1 h after the initiation of the cytokine treatment. Northern blot analysis was performed with JE/MCP-1 and β-actin cDNAs used as probes. Quantification of the JE/MCP-1 gene expression was done by densitometry, and is expressed as a % of the maximum. An identical experiment independently performed gave similar results.
Discussion

The present study showed that 1α25(OH)2D3 eliminated RA-induced inhibition of AP-1-mediated transcriptional activity via its suppression of TNF-α-induced c-fos gene expression in mouse osteoblastic MC3T3-E1 cells. To our knowledge, this study is the first to show that 1α25(OH)2D3 interferes with RA-mediated inhibition of AP-1 formation in osteoblastic cells. These data allow us to propose an important role for 1α25(OH)2D3 as a negative regulator of RA action in AP-1-mediated gene expression in osteoblastic cells.

We observed that 1α25(OH)2D3 eliminated RA-mediated inhibition at the transcriptional level of c-fos gene expression induced by TNF-α in osteoblastic cells. The vitamin D3 interference was dose dependent and pretreatment time dependent. Since the c-fos gene together with the c-jun gene encodes the AP-1, these observations suggest that vitamin D3 acted to reduce RA-induced inhibition of the level of AP-1 binding to TRE in the cells. This suggestion was verified by the results of the gel mobility shift assay showing that vitamin D3 blocked RA-induced inhibition of the binding of AP-1 to TRE. It is of interest to determine whether the 1α25(OH)2D3 interference towards RA suppression of TNF-α-induced AP-1 transcriptional activity actually operates under physiological conditions of the osteoblastic cells. TNF-α-induced expression of the JE/MCP-1 gene in MC3T3-E1 cells is mediated via AP-1 (18). And, in fact, it has been demonstrated that a TRE sequence is located on the promoter region of the murine chemokine gene (33). Therefore, we examined the hormone interference toward RA suppression of TNF-α-induced JE/MCP-1 gene in the cells. We observed that 1α25(OH)2D3 was actually able to interfere with RA-mediated inhibition of the transcription of the AP-1-dependent JE/MCP-1 gene in the cells.

Several studies (24,26-32) have suggested two pathways for the mode of action of 1α25(OH)2D3. One is a VDR-mediated 'genomic action', and the other is the VDR-independent or non-genomic action. For analysis of the mode of action, we used 2 vitamin D3 analogs to determine which mode was involved in the elimination of RA-induced inhibition of AP-1 formation. OCT, an analog that exerts VDR-dependent action (28-32), prevented RA-induced inhibition of c-fos gene expression in TNF-α-treated MC3T3-E1 cells. Also, we observed that the 1α25(OH)2D3 interfering effect was reduced by pretreatment of VDR with antisense oligonucleotide. These data taken together with those on 1α25(OH)2D3 indicate that the elimination of RA-induced inhibition of AP-1 formation may be mediated via VDR-dependent ‘genomic action’. The competition between RAR and VDR for formation of heterodimers with RXR is suggested as a possible mechanism for this elimination of RA-mediated inhibition. Therefore, in further experiments, we will assess this possible mechanism by using cells transfected with a VDR expression vector.

In conclusion, our present study demonstrated 1α25(OH)2D3 to interfere as a negative regulator in RA-induced inhibition of AP-1 formation in mouse osteoblastic cells.

Acknowledgments

We thank Dr. S. Hanazawa for his helpful discussion. We also wish to thank Chugai Pharmaceutical Co., Ltd. for providing 1α25(OH)2D3 and its analogs.

References

