Retinoic Acid Inhibits Serum-stimulated Activator Protein-1 *via* Suppression of *c-fos* and *c-jun* Gene Expressions during the Vitamin-induced Differentiation of Mouse Osteoblastic Cell Line MC3T3-E1 Cells

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Abstract: Retinoic acid (RA), an active metabolite of vitamin A, is an important nutrient regulating bone formation and mineralization; however, the mechanisms of RA action remain yet to be elucidated. Osteoblasts play a central role in bone formation and mineralization by synthesizing bone matrix proteins during differentiation into mature cells. In addition, several studies showed that a transcriptional factor activator protein-1 (AP-1) is concerned with the cellular differentiation. Thus, by using mouse osteoblastic cell line MC3T3-E1 cells, we investigated effect of RA on the differentiation of the cells and on the formation of AP-1 in them. RA inhibited DNA synthesis of MC3T3-E1 cells in a dose-dependent manner. In contrast, it markedly increased alkaline phosphatase activity, a marker of osteoblastic differentiation. Also, we observed that RA induced the gene expression of osteopontin, which is a osteoblast phenotype marker and a bone matrix protein. Furthermore, in MC3T3-E1 cells, pretreatment with RA inhibited fetal bovine serum-induced expression of *c-fos* and *c-jun* genes, whose products are components of AP-1. The results of an electorophoretic mobility shift assay showed that RA dramatically depressed specific binding of nuclear proteins to oligonucleotide sequence containing 12-tetra-decanoyl phorbol 13-acetate-responsive element, a consensus sequence for AP-1 binding, in the cells. These results show that RA strongly inhibited the formation of AP-1 by suppressing *c-fos* and *c-jun* gene expression during the vitamin-induced differentiation of osteoblastic MC3T3-E1 cells.

Key words: vitamin A, retinoic acid, activator protein-1, osteoblast, differentiation, retinoic acid receptor, retinoid X receptor

レチノイン酸は，マウス骨芽細胞 **MC3T3-E1** 細胞の分化誘導を強く誘導し，血清誘導性転写因子 **activator protein-1** を ***c-fos*** と ***c-jun*** 遺伝子発現を抑制することによって阻害する

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要旨：ビタミン A の活性型代謝物であるレチノイン酸（RA）は，骨形成と石灰化に密接に関与する栄養素の一つである．しかし，その RA の生物活性の機構は，詳細に解明されていない．骨組織の形成と石灰化において，骨芽細胞は，より成熟した段階の細胞に分化し，骨基質成分を産生することによって，中心的な役割を果たしている．さらに，幾つかの研究は，転写因子 activator protein-1（AP-1）が，本細胞の分化に関与することを報告している．そこで，私どもは，マウス骨芽細胞様細胞 MC3T3-E1 細胞を用いて，RA が，本細胞の分化と AP-1 の活性化を制御する可能性について検討した．RA は，MC3T3-E1 細胞の DNA 合成を，その濃度依存的に阻害した．しかし，RA は，骨芽細胞分化のマーカーであるアルカリフォスファターゼ活性を強く増加した．また，RA は，骨芽細胞のマーカーで，骨基質成分の一つである osteopontin の遺伝子発現を誘導することを確認した．さらに，MC3T3-E1 細胞において，RA は牛胎仔血清（fetal bovine serum ; FBS）の刺激によって誘導される AP-1 構成成分である *c-fos* と *c-jun* の遺伝子発現を阻害した．Electorophoretic mobility shift assay の結果から，RA は，FBS によって誘導される AP-1 が，その結合配列である 12-tetra-decanoyl-phorbol 13-acetate-responsive element へ結合することを強力に抑制した．これらの結果から，RA は，MC3T3-E1 細胞の分化を誘導し，さらに，その分化した細胞において，*c-fos* と *c-jun* 遺伝子発現を阻害することによって AP-1 形成を強く抑制することが明らかとなった．

索引用語：ビタミン A，レチノイン酸，activator protein-1，骨芽細胞，細胞分化，retinoic acid receptor, retinoid X receptor

Introduction

It is well known that retinoic acid (RA), an active metabolite of vitamin A, is an essential nutrient regulating human development ; and moreover, the vitamin is used clinically in the treatment for night blindness, epidemic disorders, malignancies, and arthritis[1–3]. Thus, it has been established that RA maintains health and supports growth in human. Recently, it was demonstrated that RA plays important roles in proliferation and differentiation of various types of cells and that the vitamin exerts biological effects transcriptionally through the function of 2 distinct classes of receptors, retinoic acid receptor (RAR ; RARα, RARβ, and RARγ) and retinoid X receptor (RXR ; RXRα, RXRβ, and RXRγ), that bind to their respective target DNA sequence[4, 5].

Vitamin A toxicity is caused by excessive and/or long-term intake of RA, and hypervitanosis A is known to alter bone and mineral metabolism[6–9]. For example, a high intake of dietary RA is associated with reduced bone mineral density and increased risk for hip fracture in human[6–11]. Furthermore, it has been showed that RA stimulates bone resorption by osteoclasts *in vivo* and *in vitro*[12–14]. On the other hand, hypervitaminosis A induces hyperostosis in laboratory animals[15–19]. Moreover, after long-term-administration of RA to human patients, hyperostosis is observed[6–9, 20]. In addition, a hyperostoic patient who had not been treated with RA had high levels of serum retinol and retinol-binding proteins[21]. Therefore, RA is involved in bone formation and mineralization ; however, little understood about the mechanisms for the RA action.

Bone homeostasis is maintained by a balance between bone resorption by osteoclasts and bone formation by osteoblasts[22–27]. Osteoblasts play a central role in bone formation by synthesizing multiple bone matrix proteins, such as type I collagen, fibronectin, osteonectin, osteopontin, and osteocalcin[28–33]. With progression of differentiation of osteoblasts into mature cells, not only osteoblast markers, such as alkaline phosphatase and collagenase-3, but also matrix pro-

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teins are produced to a much greater extent than in early-stage osteoblasts[28–35]. Therefore, it is interesting to examine whether RA is indeed able to induce the differentiation of osteoblasts.

Activator protein (AP)-1, which is a transcriptional factor formed by Fos proteins (c-fos, FosB, Fra-1, Fra-2) and Jun proteins (c-jun, JunB, JunD), has been implicated in a large variety of biological processes including cell differentiation, proliferation, apoptosis, and oncogenic transformation[36, 37]. Recently, both morphological and functional studies on c-fos transgenic and knockout mice have strongly suggested that AP-1 plays an important role in bone remodeling and metabolism[38, 39]. In fact, AP-1 activity can be induced by transforming growth factor (TGF)-β, parathyroid hormone, and 1α25(OH)$_2$D$_3$, which are potent regulators of the differentiation and proliferation of osteoblasts[40–42]. During osteoblast proliferation, high levels of all Fos and Jun proteins are detected ; and subsequently, during the period of extracellular matrix production and mineralization, the mRNA levels of only c-fos and c-jun decrease[36, 37, 43–45]. In fully differentiated osteoblasts, Fra-2 and JunD become the principal components of the AP-1 complex ; and AP-1 activity is suppressed[43–45].

Therefore, in the present study, we investigated whether RA is able to stimulate the differentiation of mouse osteoblastic MC3T3-E1 cells, and examined effect of RA on AP-1 formation in the cells.

Material and Methods

Reagents

RA (all-*trans*-retinoic acid) was obtained from Sigma (St Louis, MO, USA). RA was dissolved in 95% ethanol in a dark room under flow of nitrogen. A stock solution (10^{-3} M) was prepared and stored in −70°C in the dark until use. α-MEM came from Flow Lab (McLean, VA, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). 5'-[α-^{32}P]-dCTP megaprimed DNA labeling system and [γ-^{32}P]ATP were from Amersham Biosciences (Piscataway, NJ, USA).

Cell culture

Clonal osteoblastic MC3T3-E1 cells derived from C 57 BL/6 mouse calvaria were cultured in α-MEM with 10% FBS at 37°C in plastic dishes in humidified atmosphere of 5% CO^2 in air and subcultured every 3 days as previously described[41, 42, 46]. The cells (3.0×10^5 cells) were cultured at 37°C in α-MEM containing 10% FBS in 90-mm plastic dishes until nearly confluent.

Assay of DNA synthesis and cell growth

MC3T3-E1 cells were seeded into each flat-bottomed well of a microculture plate (Falcon®, Becton Dickinson Labwere, Oxford, CA, USA) in α-MEM containing 10% FBS, and cultured for 24 h. Thereafter, the cells were washed with serum-free α-MEM ; and then they were treated with RA (10^{-8}～10^{-6} M) in serum-free α-MEM for 24 h. After the treatment, the cells were further incubated in medium containing various concentration of FBS for 24 h, and were labeled with 18.5 kBq of ^{3}H-thymidine (New England Nuclear, Boston, MA, USA) for the final 6 h of the incubation[46].

Assay of alkaline phosphatase activity

The cells were cultured under the same culture conditions as described for the cell growth assay. MC3T3-E1 cells were cultured in 35-mm culture dish in α-MEM containing 10% FBS for 24 h. After the culture, the cells were washed with serum-free α-MEM ; and then they were treated with RA (10^{-8}～10^{-6} M) for the indicated time. The cells were washed with phosphate-buffered saline and lysed with 2% Nonidet P-40 (Sigma). The lysate was centrifuged and the supernatant was used for measurement of alkaline phosphatase activity. The activity was measured with *p*-nitrophenylphosphate as a substrate according to the method of Lowry *et al*[46]. One unit of enzyme was defined as the activity which liberated 1 nmol of product per min[46]. Protein content was measured by the method of Bradford[48].

Northern blotting analysis

Total cellular RNA was extracted by the guanidine

isothiocyanate procedure[41]. As previously described[49], the RNA was subjected to 1% agarose electrophoresis, and blotted onto a nylon membrane (MSI Magnagraph, Westboro, MA, USA). The membranes were subsequently baked, prehybridized, and then hybridized with the desired cDNA probe that had been labeled with 5'-[α-^{32}P]-dCTP by use of the megaprimed DNA labeling system. After hybridization, the membranes were washed, dried, and exposed to X ray film (Eastman Kodak Co, Rochester, NY, USA) at –70°C. β-Actin was used as an internal standard for quantification of total mRNA in each lane of the gel.

Preparation of nuclear extracts

Confluent monolayers in 20-cm-diameter dishes were treated with test samples as indicated in the figure legends, and then their nuclei were isolated as described above[49]. Protein concentration was measured by the method of Bradford[48].

Electrophoretic mobility shift assay (EMSA)

This assay was carried out as described previously[49]. Binding reactions were performed for 20 min on ice with 5 μg 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, 1 μg poly(dI-dC)] containing 20,000 cpm. of ^{32}P-labeled oligonucleotide in the absence or presence of nonlabeled oligonucleotide. Poly(dI-dC) and nuclear extract were first incubated at 4°C for 10 min before adding the labeled oligodeoxynucleotide. Thirty mer double-stranded oligonucleotides containing AP-1 binding site (-TGACTCA-, Oncogene Science, Manhasset, NY, USA) were end-labeled by the oligonucleotide 5' end labeling system-[γ-^{32}P]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.

Statistic analysis

Means of groups were compared by analysis of variance, and significance of differences was determined by post-hoc testing using Bonferroni's method.

Results

Inhibitory effect of RA on DNA synthesis of mouse osteoblastic cell line MC3T3-E1 cells.

Serum (FBS) induced DNA synthesis, measured as ^{3}H-thymidine uptake, in MC3T3-E1 cells in a dose-dependent manner, and RA inhibited the induction of DNA synthesis by the serum (**Fig 1**). Under the FBS-free condition, although RA at concentrations tested tended to show an inhibitory effect, the inhibition was not significant. RA at 10^{-6} M strongly inhibited DNA synthesis at the various concentration of FBS ; and the inhibitory effect of RA at each serum level was dose dependent. Furthermore, we comfirmed that RA was able to inhibit 1% FBS-induced growth of MC3T3-E1 cells as determined by direct measurement of cell number (data not shown). Therefore, RA indeed significantly inhibits MC3T3-E1 cell growth.

RA induced alkaline phosphatase activity in MC3T3-E1 cells.

Alkaline phosphatase activity is a marker of osteoblasts[50], and thus the activity increases during dif-

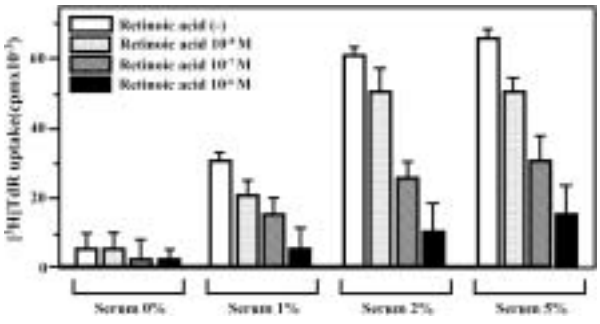

Fig 1 Inhibitory effect of RA on DNA synthesis of mouse osteoblastic cell line MC3T3-E1 cells. MC3T3-E1 cells were cultured for 24 h in α-MEM conteining 10% FBS. Then, the cells were treated with various doses of RA for 24 h. Thereafter, the cells are incubated without or with indicated concentrations of FBS. The cultures were incubated for 24 h, and pulsed with ^{3}H-thymidine for the final 6 h of the incubation. DNA synthesis was the quantified in terms of ^{3}H-thymidine uptake. The results of the DNA synthesis assays are expressed as the mean±standard deviation of 3 cultures.

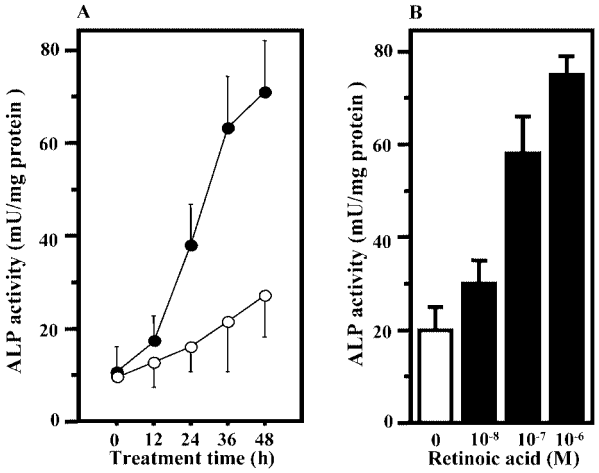

Fig 2 RA induced alkaline phosphatase activity in mouse osteoblastic cell line MC3T3-E1 cells. *A*, MC3T3-E1 cells were cultured in α-MEM containing 10% FBS for 24 h. Then, the cells were treated with 10^{-6} M of RA (●) or not (○) in α-MEM containing 1% FBS for indicated times. *B*, the cells were treated with various concentration of RA for 48 h. After the treatments, the cells were assessed for their alkaline phosphatase activity and protein content. The results of the assay of alkaline phosphatase activity are expressed as the mean±standard deviation of 3 cultures.

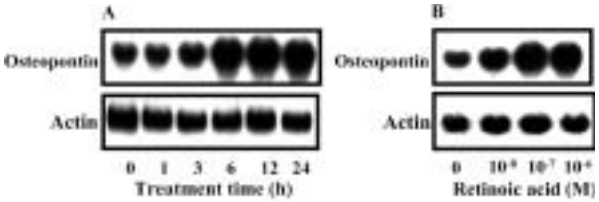

Fig 3 Stimulative effect of RA on gene expression of osteopontin in MC3T3-E1 cells. *A*, MC3T3-E1 cells were incubated in the presence or absence of 10^{-6} M RA for various times, and then total RNA was prepared. *B*, the cells were treated with various doses of RA for 24 h, and then RNA was prepared. Northern blot analysis was performed with osteopontin and β-actin cDNAs used as probes.

ferentiation of osteoblasts. So next, we examined the effect of RA on alkaline phosphatase activity in MC3T3-E1 cells. As shown in **Fig 2A**, alkaline phosphatase activity of MC3T3-E1 cells was markedly increased by RA in a culture time-dependent manner. The stimulatory effect was observed as early as 24 h after treatment with RA (**Fig 2A**). This increase was also dose dependent (**Fig 2B**). These data suggest that RA induces the differentiation of osteoblastic cell line MC3T3-E1 cells.

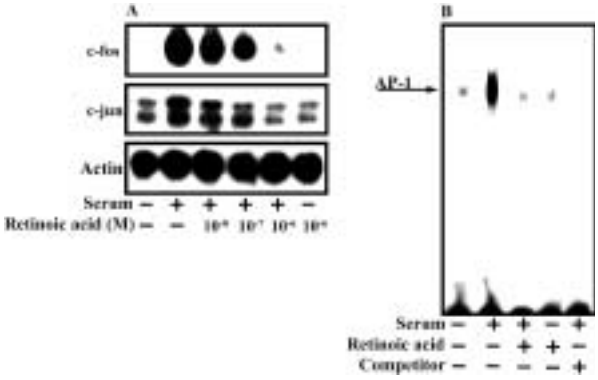

Fig 4 RA inhibits expression of *c-fos* and *c-jun* genes and formation of AP-1 in FBS-treated MC3T3-E1 cells. *A*, MC3T3-E1 cells were incubated in the presence or absence of various dose of RA for 24 h. Then, the cells were treated or not with 1% FBS, and total RNA was prepared 0.5 h later. Northern blot analysis was performed with *c-fos*, *c-jun* and β-actin cDNA used as probes. *B*, MC3T3-E1 cells were incubated in the presence or absence of 10^{-6} M RA for 24 h, and the cells were incubated in the presence or absence of FBS (1%) for 1 h ; and then the nuclear proteins were prepared. The gel mobility shift assay was performed with ^{32}P-labeled oligonucleotide containing the TRE sequence in the presence of the nuclear proteins.

Effect of RA on the gene expression of osteopontin in MC3T3-E1 cells.

Osteopontin is one of bone matrix proteins, and this non-collagenous protein is thought to be involved in mineralization[29, 30]. Moreover, it is another marker of osteoblast differentiation. To confirm that RA could induce differentiation of MC3T3-E1 cells, we examined its effect on expression of this gene in the cells. RA strongly induced expression of this gene in the cell, and the significant induction was observed at 6 hr after the start of 10^{-6} M RA treatment (**Fig 3A**). Additionally, this induced expression was dose dependent (**Fig 3B**). Taken together with the results about alkaline phosphatase activity, the data suggested that RA is able to induce the differentiation of the MC3T3-E1 cells.

RA suppressed *c-fos* and *c-jun* gene expression and inhibited formation of AP-1 in MC3T3-E1 cells incubated with serum (FBS).

c-Fos plays an important role in differentiation and proliferation of osteoblasts[38–40], and resent studies showed that c-fos and c-jun expression is suppressed

in mature osteoblasts[43–45]. Therefore, we next investigated whether RA could inhibit FBS-induced expression of *c-fos* and *c-jun* genes in MC3T3-E1 cells. As shown in **Fig 4A**, RA dose-dependently inhibited the FBS-induced expression of these oncogenes in the cells incubated in 1% FBS-containing medium (**Fig 4A**). Since it is well known that the c-fos/c-jun heterodimer (AP-1) binds to 12-tetra-decanoyl phorbol 13-acetate-responsive element (TRE) consensus sequence in promorter region of various genes[36–39], we suspected that the inhibitory effect of RA on FBS-induced *c-fos* and *c-jun* gene expressions results in decrease in AP-1 binding to TRE in FBS-treated cells. Therefore, we examined this point by conducting EMSA. As shown in **Fig 4B**, RA indeed inhibited AP-1 binding activity to TRE in nuclear extracts of FBS-treated cells. The data suggest that the vitamin-suppressed expression of *c-fos* and *c-jun* genes causes the decrease in AP-1 formation in MC3T3-E1 cells.

Discussion

The molecular mechanisms of RA action on bone cell are still incompletely understood. The present study demonstrated that RA is one of differentiation factors promoting the differentiation of osteoblast-like MC3T3-E1 cells. First, we showed that RA inhibited the DNA synthesis, but stimulated alkaline phosphatase activity, a strong marker of osteoblast differentiation. We also observed that RA promoted the mRNA expression of osteopontin, a bone matrix protein and another marker of differentiated osteoblasts. Osteopontin has been reported to be expressed during pre-osteoblastic cells into mature osteoblasts[33]. Our data are consistent with the results of a recent study that found that RA induced this matrix protein in MC3T3-E1 cells[32]. Moreover, Nagasawa *et al*[32] demonstrated that RA also stimulates the expression of osteocalcin, which is a potent bone matrix protein expressed in mature osteoblastic cells, in a culture of MC3T3-E1 cells. Therefore, as MC3T3-E1 cells are pre-mature osteoblastic cells, RA was able to induce their cellular differentiation into mature cells. Thus RA appears to be a differentiation factor for osteoblasts and may play functional roles in bone formation and mineralization.

We previously showed that MC3T3-E1 cells expressed the genes of both RARs (RAR-α, RAR-β, and RAR-γ) and RXRs (RXR-α and RXR-β). RXR, an auxiliary protein, forms a heterodimer with RAR[41, 49]; and this heterodimer, acting as a transcriptional factor, binds to the target DNA sequence. Thus, the heterodimers of RARs and RXRs play a functional role in RA signal transduction in various cells[4, 5]. Vitamin D/retinoic acid response element is present in promoter region of mouse osteopontin and osteocalcin genes[31], and it has been demonstrated that RA induces the expression of both of these genes in MC3T3-E1 cells[32]. Furthermore, we have observed that RA stimulates binding activity of nuclear proteins toward direct repeat-5 (DR-5), the core response element for RARs-RXRs, in the cells (data not shown). Therefore, we suggest the hetrodimers of RARs and RXRs, as transcriptional factors, to be closely involved in RA-induced differentiation of MC3T3-E1 cells.

In the case of gene expression of collagenase and stromelysin, RA transcriptionally represses the expression of these genes by decreasing the DNA binding activity of AP-1, a dimeric transcriptional activator protein consisting of c-fos and c-jun[51, 52]. AP-1 plays a functional role in proliferation and differentiation of osteoblasts[31, 38–40]. Our previous studies[49, 53] showed that RA suppresses *c-fos* gene expression but not *c-jun* gene expression and inhibits formation of AP-1 in TNF-α-treated MC3T3-E1 cells, These results promoted us to examine whether RA would inhibit FBS-induced *c-fos* and *c-jun* gene expression in the osteoblasts. Consistently with this speculation, we found that RA inhibited the induction of not only *c-fos* but also *c-jun* in the FBS-treated cells. The reason for the difference in *c-jun* expression between the present and the earlier studies is not yet clear. Busam *et al*[54] showed that RA inhibits serum-induced *c-fos* and *c-jun* gene expression in melanoma cells, which study supports our results. FBS includes several osteogenic growth factors such as TGF-β, insulin like growth factor (IGF)-I, IGF-II, fibroblast growth factor (FGF)-2; and these growth factors are able to stimulate expression of the both oncogenes in osteoblastic

cells[38–42]. This evidence raises the possibility that RA inhibits the signaling pathways of these growthfactors, resulting in decrease in the expression of *c-fos* and *c-jun* genes. This possibility remains to be examined. In this present study, we observed that treatment with RA decreased FBS-induced TRE binding activity, suggesting that this decrease might be influenced by RA suppression of *c-fos* and *c-jun* gene expression. Owen *et al*[31] proposed the suppression of AP-1 to precede osteoblast differentiation, because AP-1 inhibits osteocalcin gene expression. In fact, during the differentiation of MC3T3-E1 cells and of rat primary osteoblasts, the levels of *c-fos* and *c-jun* gene expression were shown to decline, causing decreased DNA binding activity of AP-1[43, 44]. Presently, we observed that RA inhibited the FBS-induced formation of AP-1 via suppression of *c-fos* and *c-jun* gene expression in MC3T3-E1 cells, thus suggesting that RA-mediated inhibition of c-fos and c-jun expression is one of functional mechanisms that strongly contribute to RA-induced differentiation of osteoblasts.

Moreover, it is widely recognized that osteoblasts are able to regulate differentiation, activation, and proliferation of osteoclastic cells through osteoblast-produced osteoclastogenic factors, such as receptor activator of nuclear factor κB ligand (RANKL), osteoprotegrin, prostaglandin E_2, monocyte chemoattractant protein-1, macrophage-colony stimulating factor, osteopontin, TGF-β, IGF-I, IGF-II, FGF-2, interleukin-6, and interleukin-1[55–59]. Interestingly, Hiura *et al*[60] showed that the conditioned-medium from differentiated MC3T3-E1 cells supported osteoclast formation better than that from cultures of undifferentiated cells. Furthermore, in human osteosarcoma cell line MG-63, RA increases synthesis of osteocalcin, stimulates expression of RANKL (a stimulator of osteoclast differentiation), and suppresses expression of osteoprotegrin (an inhibitor of osteoclast formation)[61, 62]. In fact, several *in vivo* studies[17–19] demonstrated that excessive of RA induces osteopenia after hyperostosis. These observations suggest to us a possibility that RA-induced osteoblast differentiation is related to bone resorption via osteoclasts.

In conclusion, our present study has demonstrated that RA inhibits AP-1 formation by suppressing *c-fos* and *c-jun* gene expression during the vitamin-induced differentiation of mouse osteoblastic cell line MC3T3-E1 cells. Further, our results suggest that RA-induced osteoblast differentiation may be regulated *via* repression of AP-1 responsive genes.

We thank Dr Yoshiyuki Hakeda for his helpful discussion. Also, we gratefully acknowlege Ms Junko Okubo for her help in the manuscript preparation.

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(Received October 31, 2006 ; accepted November 22, 2006)