Transcriptional Regulation by Transforming Growth Factor β of the Expression of Retinoic Acid and Retinoid X Receptor Genes in Osteoblastic Cells Is Mediated through AP-1*

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We now report that transforming growth factor β1 (TGF-β1), a potent regulatory cytokine of bone remodeling, is a powerful stimulator for gene expression of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in osteoblastic MC3T3-E1 cells. TGF-β1 transcriptionally stimulated the expression of RARα, RARγ, and RXRα genes, but did not do so for RARβ, RXRβ, and RXRγ genes. We also observed that AP-1, a transcriptional factor, plays an important role in the signal pathway for expression of RARα, RARγ, and RXRα genes stimulated by TGF-β1 because stimulation of the expression of these genes in the cytokine-treated cells was markedly inhibited by a mixture of antisense c-fos and c-jun. A gel mobility shift assay demonstrated that TGF-β1 is able to increase, in a dose-dependent manner, the binding of nuclear proteins to direct repeat 5, a consensus sequence with high affinity for RAR-RXR heterodimers. The mobility shift assay, using specific antibody for each receptor, showed that direct repeat 5-binding proteins may be RAR and RXR isoforms. The stimulated binding to direct repeat 5 was inhibited strongly by H-7, an inhibitor of serine/threonine kinase, and by curcumin, an inhibitor of AP-1. The present study suggests a novel pathway for TGF-β1 action in osteoblastic cells via stimulation of RAR-RXR transcriptional activity in a ligand-dependent fashion.

Bone remodeling is regulated by a network of local and systemic cytokines and hormones (1–4). Of them, TGF-β is produced by osteoblastic cells in bone tissues, and this factor accumulates abundantly in bone matrix tissues. Thus, this cytokine may play a central role in the cytokine network of bone remodeling. In fact, many studies (5–12) have demonstrated a functional role for this cytokine in the regulation of the proliferation and differentiation of osteoblastic and osteoclastic cells. RA is an important regulatory hormone for the proliferation and differentiation of a variety of human and mouse cells (13–16). RA exerts its biological effects transcriptionally through the function of two distinct classes of receptors, RAR (RARα, RARβ, and RARγ) and RXR (RXRα, RXRβ, and RXRγ), that bind to their respective target DNA sequences. Also, more recent studies (17–21) have demonstrated that RXRs, as an auxiliary protein, forms heterodimers with vitamin D3 and thyroid hormone and may even affect signaling of the steroid hormone receptor family. Therefore, RXRs play an important role in the regulation of signal transduction of RA and of vitamin D3 and thyroid hormone. In the present study, we examined whether TGF-β1 regulates gene expression of RARs and RXRs in osteoblastic MC3T3-E1 cells. We show here that TGF-β1 regulates positively, at the transcriptional level, RARα, RARγ, and RXRα genes. These results also suggest that TGF-β1 may effect positive regulation of RA in osteoblasts via the stimulation of its nuclear receptors.

Materials and Methods

Reagents—Human platelet-derived TGF-β1, purified to homogeneity (>98%, determined by SDS-polyacrylamide gel electrophoresis), was purchased from King Brewer (Kakogawa, Japan). α-MEM was obtained from Flow Laboratories (McLean, VA) and fetal calf serum from HyClone (Logan, UT). [α-32P]dCTP and megaprimed DNA labeling system were from Amersham Japan (Tokyo, Japan), and [α-32P]UTP and oligonucleotide 5'-end-labeling system were from DuPont NEN. γ-[32P]ATP was purchased from Amersham Japan. Anti-mouse RARα and RARβ and RXRα, RXRβ, and RXRγ antisera was kindly supplied by Dr. P. Chambon (Institut de Chimie Biologique Faculté de Medecine, France). The characteristics and specificity of these antisera were described previously (22).

The osteoblastic cell line and its culture were as follows. Cells of clonal osteoblastic MC3T3-E1 cell line (provided kindly by Dr. H. Kodama, Chuo University, Koryama, Japan) were cultured to the subconfluent state in α-MEM containing 10% fetal calf serum at 37°C and 5% CO2 in air, washed, and then incubated overnight in serum-free α-MEM. They were then washed further and treated with test samples at various concentrations.

cDNA Hybridization Probe—Each plasmid containing the mouse RAR α, RAR β, or RXRα cDNA sequences was provided by Dr. P. Chambon. Plasmid containing β-actin cDNA was obtained from Oncor (Gaithersburg, MA). The methods used for plasmid preparation were described earlier (23).

Northern Blotting Analysis—Total cellular RNA was extracted by the guanidine isothiocyanate procedure (24). The RNA was subjected to 1% agarose electrophoresis and blotted onto a nylon membrane (MSI Magnagraph, Westboro, MA). The membranes were baked, prehybridized, and then hybridized to each cDNA probe labeled with [α-32P]dCTP by use of a 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—Nuclei were prepared essentially as described by Dignam et al. (25), and a "run-on" assay was performed according to the method of Groudine et al. (26) as described previously (27, 28). In brief, the cells were or were not treated with TGF-β1. After 2.5 h, the cells were scraped from the dishes and suspended in pellet lysis buffer (10 mm Tris, pH 7.4, 3 mm MgCl2, 10 mm NaCl, and 0.5% Nonidet P-40). The nuclei were separated from the cytosol by centrifugation. Transcription initiated in intact cells was allowed to proceed for 30 min at 30°C in the presence of [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.
TGF-β Stimulates RAR and RXR Gene Expression

**RESULTS**

**TGF-β Stimulates Expression of RARα and RARγ Genes in MC3T3-E1 Cells**—We first examined the possible regulatory effect of TGF-β1 on gene expression of α, β, and γ subclasses of RAR in MC3T3-E1 cells. As shown in Fig. 1A, the cytokine at 0.05 ng/ml stimulated gene expression of both RARα and RARY. The stimulated expression of both genes was dose-dependent. On the other hand, we observed that the RARβ gene was not expressed constitutively in the cells and that the gene expression was not induced by the cytokine (data not shown).

We next examined the time course of gene expression of RARα and RARY stimulated by TGF-β1. Fig. 1B shows that the cytokine clearly stimulated expression of both genes, maximally at 3 h after initiation of the cytokine treatment. Although the stimulated expression of the RARα gene was short lived, the RARY gene expression continued to increase in a treatment-time-dependent manner. These results show that TGF-β1 positively regulates expression of the RARαs and RARY genes of MC3T3-E1 cells.

**TGF-β1 Also Stimulates Expression of RXRα Gene in MC3T3-E1 Cells**—We furthermore examined whether TGF-β1 also regulated gene expression of RXRα, the other class of RAR, in MC3T3-E1 cells. As shown in Fig. 2A, although the cytokine at 0.5 ng/ml stimulated RXRα gene expression, alteration of RXRβ gene expression was not observed with the doses tested. We also found that the RXRγ gene was not expressed at all in the cells (data not shown). Fig. 2B shows the time course of the expression of the RXRγ gene stimulated by TGF-β1. Peak expression of the RXRα gene was observed at 1 h after initiation of the cytokine treatment, and the expression decreased quickly thereafter, reaching base line by 3 h. These results suggest that TGF-β1 also stimulates expression of the RXRα gene but not that of the RXRβ in MC3T3-E1 cells.
TGF-β Stimulates RAR and RXR Gene Expression

Fig. 3. TGF-β1 stimulates transcription of RARα, RARγ, and RXRα genes in MC3T3-E1 cells. The cells were or were not treated for 2.5 or 0.5 h with TGF-β1 (1 ng/ml) for RARα, RARγ, or RXRα genes, respectively, and then their nuclei were incubated for 30 min in the presence of [α-32P]UTP, after which the RNA was isolated. Transcriptional activity assay (run-on assay) was performed with RARα (A), RARγ (A), and RXRα (B) cDNAs and β-actin cDNA. pBR322, the vector plasmid, was used as a negative control. An identical experiment independently performed gave similar results.

Fig. 4. Inhibitory effect of curcumin on TGF-β1-stimulated expression of RARα, RARγ, and RXRα genes. A, the cells were or were not treated with curcumin in the presence or absence of TGF-β1 (1 ng/ml), and then total RNA was prepared at 3 h after the initiation of the treatment. Northern blot analysis was performed with RARα, RARγ, and β-actin cDNAs used as probes. B, the cells were or were not treated with curcumin in the presence or absence of TGF-β1 (1 ng/ml), and then total RNA was prepared at 1 h after the initiation of the treatment. Northern blot analysis was performed with RXRα and β-actin cDNAs used as probes. An identical experiment independently performed gave similar results.

does not affect TPA-induced c-fos gene expression. Therefore, we examined, using curcumin, the probable involvement of AP-1 in RARα, RARγ, and RXRα gene expressions in the cells. Fig. 4, A and B show that curcumin strongly inhibited the stimulated expression of RARα, RARγ, and RXRα genes in the cytokine-treated cells.

Since these observations indicated a role for AP-1 in the expression of these genes in the cells, we looked, using antisense c-fos and c-jun oligonucleotides, to see whether either one of the c-fos and c-jun protooncogenes or both are necessary for these expressions. The cells were pretreated for 3 h with each antisense and/or sense oligonucleotide (2.5 μM) and thereafter treated with TGF-β1 (1 ng/ml). The gene expressions were then analyzed by the Northern blot assay. As shown in Fig. 5, A-D, simultaneous addition of c-fos and c-jun antisense oligonucleotides to the cells inhibited TGF-β1-stimulated RARα, RARγ, and RXRα gene expressions. On the other hand, neither sense oligonucleotide inhibited the expression of these genes.

Furthermore, to verify the AP-1 induction in the cells treated with TGF-β1, we employed the gel mobility shift assay to look for its presence in the cytokine-treated cells. The cytokine stimulated the binding of nuclear proteins to the TRE sequence in a dose-dependent manner. The stimulated appearance of the nuclear proteins was eliminated by the use of unlabeled competitor (Fig. 6). These results imply that AP-1 plays a functional role as a transcriptional factor in RARα, RARγ, and RXRα gene expressions in TGF-β1-treated MC3T3-E1 cells.

TGF-β Stimulates Binding of Nuclear Proteins to DR5 in MC3T3-E1 Cells—As demonstrated by several studies (32–34), the members of nonsteroid nuclear receptor families can recognize the common consensus half-site sequence AGGTCA in the form of a direct repeat. And RAR-RXR heterodimers bind efficiently to the DR5 sequence that encodes two copies of -AGGTCA- in directed repeat separated by 5 nucleotides and promote transcriptional activation in a ligand-dependent manner. Thus it is very important to demonstrate whether TGF-β1 stimulates binding of nuclear proteins to the DR5 in MC3T3-E1 cells. We prepared nuclear proteins from TGF-β1-treated and -untreated cells and examined by the gel mobility shift assay to determine whether the cytokine stimulates the binding of nuclear proteins to DR5 in the cells. As shown in Fig. 7A, TGF-β1 increased the binding of protein (presumably RAR-RXR heterodimers) in vitro in a dose-dependent manner. The TGF-β1-stimulated binding was eliminated when an unlabeled DR5 oligonucleotide was used as a competitor. These results suggest the possibility that the cytokine may stimulate RAR-RXR binding to DR5 in the MC3T3-E1 cells.

To confirm the specificity of RARα, γ, and RXRα stimulation in the cytokine-treated cells, we also performed the gel mobility shift assay using antibodies to each receptor. As shown in Fig. 7B, the DNA and protein complexes were shifted to a position indicating slower migration when the extracts were treated with specific antibody against each receptor. However, such a shift in protein complexes was not detected when normal rabbit serum was used. Thus, these observations strongly suggest that these DR5-binding proteins are RARα, RARγ, and RXRα.

TGF-β1-stimulated Binding of Nuclear Proteins to DR5 in MC3T3-E1 Cells Is Inhibited by H-7 and Curcumin—As recent studies (35–39) have demonstrated that the TGF-β receptor has an inner domain expressing serine/threonine kinase activity, a serine/threonine kinase may be involved in signal transduction of the cytokine. In fact, we observed that H-7 (10 μM), a potent inhibitor of serine/threonine kinases, especially of protein kinase C, also inhibited expression of RARα, RARγ, and RXRα genes in the cells (Fig. 8). Therefore, to understand the signal pathway of TGF-β1-stimulated expression of these genes, we examined the effect of H-7 and curcumin on the
performed with 32P-labeled oligonucleotide containing the DR5 se-

6 h, the nuclear proteins were prepared. Gel mobility shift assay was performed with 32P-labeled oligonucleotide containing the TRE sequence in the presence of their nuclear proteins. Unlabeled oligonucleotide containing the AP-1 sequence was used as a competitor. An identical experiment independently performed gave similar results. The arrow indicates position of DNA-protein complexes.

FIG. 6. TGF-β1 stimulates binding of AP-1 to TRE in MC3T3-E1 cells. The cells were treated or not with TGF-β1. After 3 h, 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. Unlabeled oligonucleotide containing the AP-1 sequence was used as a competitor. An identical experiment independently performed gave similar results. The arrow indicates position of DNA-protein complexes.

binding of nuclear proteins to DR5 in TGF-β1-treated MC3T3-E1 cells. Fig. 9 shows that either H-7 or curcumin, at 10 μM, markedly inhibited the binding of nuclear proteins to DR5. These results indicate that TGF-β1 activates H-7-sensitive protein kinase and stimulates the binding of nuclear proteins to DR5 via induction of AP-1.

FIG. 7. TGF-β1 stimulates binding of nuclear proteins to DR5 in MC3T3-E1 cells. A, the cells were treated or not with TGF-β1. After 6 h, the nuclear proteins were prepared. Gel mobility shift assay was performed with 32P-labeled oligonucleotide containing the DR5 sequence in the presence of the nuclear proteins. Unlabeled oligonucleotide containing the DR5 sequence was used as a competitor. An identical experiment independently performed gave similar results. The arrow indicates position of DNA-protein complexes. B, the nuclear extracts prepared as described in panel A were or were not treated with specific antibody against RARα, RARγ, or RXRα. The gel mobility shift assay was performed with 32P-labeled oligonucleotides containing the DR5 sequence in the presence of their nuclear proteins. Lower arrow indicates the position of the DNA-protein complexes. Upper arrow indicates the shifted complex formed in the presence of each antibody to RARα, RARγ, or RXRα.

TGF-β is a multifunctional growth factor that often has opposite effects on cellular responses. This cytokine regulates bone remodeling by acting like a “coupling factor.” It is well known that RA is also a potent regulator of osteoblasts and osteoclasts (28, 40–42). Biological actions of this hormone are mediated by its nuclear receptors, which act as transcriptional factors. It is thus of much interest to determine whether TGF-β1 regulates expression of the RAR and RXR genes in osteoblastic cells. In the present study, we sought to demonstrate the regulatory effect of the cytokine on RAR and RXR gene expressions and the signal pathway for their receptor expressions. We showed here that TGF-β1 transcriptionally stimulates the expression of RARα, RARγ, and RXRα genes in osteoblastic MC3T3-E1 cells via AP-1 and increases the binding of nuclear proteins to DR5, whose sequence has a high affinity for RAR-RXR heterodimers.

We had an interest in determining which among the subclasses of RAR and RXR genes in MC3T3-E1 cells are regulated negatively and which are regulated positively by TGF-β1 because an interesting study (43) has shown that RARγ plays an important role with RA in murine bone formation and also because RXRs are auxiliary proteins that heterodimerize with RA, vitamin D₃, and thyroid hormone receptors, all of which are potent regulators of bone remodeling. TGF-β1 stimulated RARα, RARγ, and RXRα gene expressions in the cells. However, RARβ and RXRγ genes were not induced by the cytokine. On the other hand, a constitutive RXRβ gene expression of high intensity was observed although the gene expression was not altered by the cytokine. These observations suggest that RXRα may play a functional role as the predominant receptor in heterodimer formation with RA, vitamin D₃, and thyroid hormone receptors in response to TGF-β1 stimulation.

Recently, the TGF-β-type I receptor has been identified as a serine/threonine kinase receptor that is related to the type II receptor family (35–39). Thus, these type I and II heterometric complexes play an important role in the cytokine signaling. We actually showed that TGF-β1-stimulated expressions of RARα,
RARγ, and RXRα genes in MC3T3-E1 cells were inhibited by H-7, a potent inhibitor of serine/threonine kinases. Therefore, these results suggest involvement of an H-7-sensitive protein kinase, probably a serine/threonine kinase receptor, in the cytokine transducing pathway for these gene expressions. This possibility is also supported by our observation that TGF-β-stimulated binding of nuclear proteins to DR5 was inhibited markedly by H-7 treatment.

It was also of interest to us to understand which transcriptional factor is involved in TGF-β-induced expression of RAR and RXR genes in MC3T3-E1 cells. Thus, we examined by using actinomycin D and the run-on assay whether the cytokine stimulates the expression of RARα, RARγ, and RXRα genes at the transcriptional level. Actinomycin D inhibited these gene expressions in TGF-β-treated cells (data not shown), and the run-on assay showed stimulation of the transcriptional rate of these genes in the cytokine-treated cells. Since it has been shown in MC3T3-E1 cells that TGF-β1 is able to strongly induce the expression of c-jun and c-fos genes, which encode AP-1, it was reasonable to examine the involvement of AP-1 in the cytokine-stimulated expression of each RA receptor.

We observed that TGF-β-stimulated expression of RARα, RARγ, and RXRα genes was inhibited by antisense c-fos and c-jun oligonucleotides. And an inhibitor of AP-1, curcumin, markedly inhibited these genes stimulated by TGF-β1. These observations suggest the involvement of AP-1 in the cytokine stimulation for expression of these genes. In fact, our gel mobility shift assay actually showed that the cytokine increased, in a dose-dependent manner, the AP-1 binding to TRE.

For the nonsteroid members of the receptor superfamily, the hormone response elements consist of a minimal core consensus sequence, -AGGTTCA-, that can be configured into a variety of structured motifs. Several studies (32–34) have shown that heterodimers of RAR-RXR bind to DR5, which encodes two copies of AGGTTCA in direct repeat separated by 5 nucleotides. Since the binding of the heterodimers to DR5 activates transcription from DNA containing DR5, it was very important to examine by the gel mobility shift assay the binding activity of nuclear proteins to DR5 in TGF-β1-treated cells. In doing so, we observed that the cytokine increased, in a dose-dependent manner, the binding of nuclear proteins to DR5. We also demonstrated by the gel mobility shift assay, using antibody specific for each receptor, the specificity of RARα, RARγ, and RXRγ stimulation in the cytokine-treated cells. These results suggest the possibility that TGF-β1 may stimulate the RAR-RXR transcriptional activity in a ligand-dependent manner through induction of their mRNA expression. Furthermore, we observed that curcumin strongly inhibited the stimulated binding of nuclear proteins to DR5 in TGF-β1-treated MC3T3-E1 cells. Thus, in consideration of all of these observations, we suggest here that TGF-β1 induces AP-1 by activation of H-7-sensitive protein kinase and stimulates RARα, RARγ, and RXRα gene expressions via AP-1 in MC3T3-E1 cells.

In conclusion, this study supports a novel pathway of TGF-β1 action for proliferation and differentiation of osteoblast cells via stimulation of RAR-RXR transcriptional activity in a ligand-dependent fashion.

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