investigations are required to elucidate the precise mechanism of PPAR-γ activation in the amplification of VEGF release.

It is generally recognized that the expansion of capillary network providing microvasculature is an essential process of bone remodeling [11]. Since VEGF is a specific mitogen of vascular endothelial cells [6], it is speculated that VEGF synthesized by osteoblasts acts as a crucial intercellular mediator between osteoblasts and vascular endothelial cells. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse [7], supporting the importance of VEGF in bone metabolism. On the other hand, it is well known that PPAR-γ transcription factor determines the differentiation of progenitors into adipocytes [1]. In addition, a mesenchymal stem cell gives rise to adipocytes, osteoblasts, endothelial cells, and chondrocytes [23]. It has recently been reported that PPAR-γ is expressed also in osteoblasts including MC3T3-E1 cells [3]. Taking our results into account, it is probable that PPAR-γ ligand-enhanced VEGF release in osteoblasts plays an important role in the process of bone remodeling through regulating the capillary endothelial cells proliferation.

As for effects of PPAR-γ ligands on osteoblasts, it has recently been shown that PPAR-γ activators modulate osteoblast maturation such as alkaline phosphatase activity, Cbfa1 activity, and the expression of osteocalcin [3–5]. Therefore, it is probable that activation of PPAR-γ in osteoblasts functions as a pivotal role in bone metabolism. Further investigations are necessary to clarify the exact role of PPAR-γ activation in osteoblasts.

In conclusion, our present results strongly suggest that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release via enhancing activation of SAPK/JNK among the MAP kinase superfamily in osteoblasts.

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Adenylyl cyclase-cAMP system inhibits thyroid hormone-stimulated osteocalcin synthesis in osteoblasts

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Abstract

It is generally recognized that thyroid hormone modulates osteoblast cell function. We have previously shown that triiodothyronine (T_3) activates p38 mitogen-activated protein (MAP) kinase, resulting in the synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the effect of the adenylyl cyclase-cAMP system on thyroid hormone-stimulated osteocalcin synthesis in these cells. Dibutyryl-cAMP (DBcAMP) reduced the osteocalcin synthesis stimulated by T_3. Forskolin and cholera toxin suppressed the osteocalcin synthesis while dideoxyforskolin, a forskolin derivative that does not activate adenylyl cyclase, had little effect on the synthesis. KT5720, a selective inhibitor of protein kinase A, reversed the inhibitory effect of forskolin or DBcAMP. DBcAMP and forskolin markedly reduced the phosphorylation of p38 MAP stimulated by T_3. Pituitary adenylate cyclase-activating polypeptide (PACAP) significantly inhibited the T_3-stimulated osteocalcin synthesis. These results strongly suggest that the adenylyl cyclase-cAMP system has an inhibitory role in thyroid hormone-stimulated osteocalcin synthesis via suppression of p38 MAP kinase activation in osteoblasts.

Keywords: Triiodothyronine; Osteocalcin; cAMP; Osteoblast

1. Introduction

Thyroid hormone is an important regulator of skeletal function, resulting in modulating bone metabolism and hyperthyroidism causes secondary osteoporosis (Khosla and Melton, 1995). In hyperthyroidism, the serum levels of alkaline phosphatase and osteocalcin, markers of osteoblast phenotype, and the excretion of pyridinoline and hydroxypyridinoline cross-link, which reflects bone resorption, are elevated (Stern, 1996). It is well known that both increased bone resorption and decreased bone formation contribute to the loss of bone mass by hyperthyroidism (Stern, 1996). Two functional cells, osteoblasts and osteoclasts mainly regulate bone metabolism (Nijweide et al., 1986). The former cells are responsible for bone formation and the latter for bone resorption. The receptor for triiodothyronine (T_3) has been shown to exist on osteoblasts (Stern, 1996). It has been reported that thyroid hormone stimulates alkaline phosphatase activity and secretion of osteocalcin and insulin-like growth factors in osteoblasts and that it modulates proliferation of osteoblasts (Stern, 1996; Rizzoli et al., 1986; Kasono et al., 1988). In a previous study (Tokuda et al., 1998), we have demonstrated that T_3 modulates interleukin-6 synthesis at two points in osteoblast-like MC3T3-E1 cells as follows; one is exerted at the point between adenylyl cyclase and protein kinase A, and the other is at a point downstream from protein kinase C activation. However, the exact mechanism of thyroid hormone in osteoblasts has not been fully clarified.

It is generally recognized that the receptor of thyroid hormone belongs to the nuclear hormone receptor superfamily (Evans, 1988). The effects of thyroid hormone, as well as

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other steroid hormones, are exerted through binding to its specific intracellular receptors and subsequently activating the expression of the gene network (Evans, 1988). As for the intracellular signal transduction of thyroid hormone, it has recently been reported that the activation of p44/p42 mitogen-activated protein (MAP) kinase, which belongs to the MAP kinase superfamily (Widmann et al., 1999), is involved in the regulation of thyroid hormone-inhibited p53 transcriptional activity in human kidney cells (Shih et al., 2001). The MAP kinase superfamily plays pivotal roles in intracellular signaling of a variety of agonists to transduce the diverse messages (Widmann et al., 1999). In our recent studies (Kozawa et al., 2001; Ishisaki et al., 2004), we have demonstrated that the T_3 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase plays a part in T_3-stimulated synthesis of osteocalcin, an osteoblastic-specific phenotype marker (Ducy et al., 1996).

cAMP is produced from ATP by adenylyl cyclase and then activates cAMP-dependent protein kinase (protein kinase A) (Defer et al., 2000). It is generally recognized that the adenylyl cyclase-cAMP system plays a crucial role in osteoblast differentiation and proliferation (Siddhanti and Quarerles, 1994). We previously showed that pituitary adenylate cyclase-activating polypeptide (PACAP), a physiological agent for osteoblasts, stimulates cAMP production while having no effect on protein kinase C activation or Ca^{2+} mobilization in osteoblast-like MC3T3-E1 cells (Suzuki et al., 1994). In the present study, we investigated whether adenylyl cyclase-cAMP system affects the thyroid hormone-stimulated synthesis of osteocalcin in MC3T3-E1 cells. We herein show that adenylyl cyclase-cAMP system has an inhibitory role in T_3-stimulated osteocalcin synthesis in these cells.

2. Materials and methods

2.1. Materials

T_3, forskolin, dideoxyforskolin, dibutyryl-cAMP (DB-cAMP), cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO). Osteocalcin radioimmuassay (RIA) kit was obtained from Biomedical Technologies Inc. (Stoughton, MA). PACAP was obtained from Peptide Institute, Inc. (Minoh, Japan). KT5720 was purchased from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were obtained from New England BioLabs (Beverly, MA). An enhanced chemiluminescence (ECL) Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. T_3 was dissolved in 0.1 M NaOH. Forskolin, dideoxyforskolin, KT5720 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for osteocalcin and Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells, which have been derived from newborn mouse calvaria (Sudo et al., 1983), were maintained as previously described (Kozawa et al., 1992). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35 mm-diameter dishes or 90 mm-diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. Assay for osteocalcin

The cultured cells were pretreated with forskolin, DB-cAMP, KT5720 or PACAP for 20 min, and then stimulated by T_3 in 1 ml of α-MEM containing 0.3% FCS, and then incubated for the indicated periods. The pretreatment with cholera toxin was performed for 2 h. The conditioned medium was collected, and osteocalcin in the conditioned medium was then measured by an osteocalcin RIA kit.

2.4. Western blot analysis

The cultured cells were pretreated with forskolin or DB-cAMP for 20 min, and then stimulated by T_3 for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris–Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 × g for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (Laemmli, 1970) in 10% polyacrylamide gels. Western blotting was performed as described previously (Kato et al., 1996) by using phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by use of an ECL Western blotting detection system. When indicated, the cells were pretreated with DBcAMP or forskolin for 20 min.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized as follows: 1 μg of DNase-treated total RNA together with 0.5 μg of oligo dT12-18 (Pharmacia, Uppsala, Sweden) in a total volume of 11 μl were heated to 70 °C for 10 min and then chilled on ice. A mix consisting of 4 ml of five times first strand cDNA buffer (Gibco-BRL, Gaithersburg, MD), 2 μl of 100 mM dithiothreitol, 1 μl of 10 mM dNTPs and 1 μl of RNase block (40 U/μl, Stratagene, La Jolla, CA) was added to the tube and heated at

42 °C for 2 min. SuperScriptk II RNase H-Reverse Transcriptase (Gibco-BRL) (200 U) was then added and the reaction was continued at 42 °C for 50 min. After a 15 min inactivation step at 70 °C, the cDNA was stored at −20 °C until use. RT-PCR was performed by co-amplification of the gene in question using cDNA template generated as described. The primer sequences for osteocalcin were as follows: 5′-TgC gCT CTg TCT CTC TgA CC-3′(sense) and 5′-CTg TgA CAT CCA TAC TTg Cag g-3′(antisense). PCR was carried out in a total volume of 10 μl containing 0.5 μl of cDNA solution, 0.5 U of Taq DNA polymerase (Sigma), 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of sense and antisense primers, 10 mM Tris–HCl, pH 8.3, and 50 mM KCl in a Robot Thermal Cycler (Stratagene) as follows: initial denaturation for 5 min at 94 °C, 29–35 cycles with denaturation at 94 °C for 30 s, annealing at 56–61 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension step for 5 min at 72 °C. After PCR, 7 μl of products was run on an agarose gel, stained with ethidium bromide and the intensity of bands was quantified using 1-D Image Analysis Software (Kodak Digital Sciencek, USA).

2.6. Determination

The radioactivity of radioimmunoassay samples was determined using a Wallac 1480 WIZARD3" automatic gamma counter (Turk, Finland). The densitometric analysis was performed using Molecular Analysis/Macintosh (Bio-Rad Laboratories, Hercules, CA).

2.7. Statistical analysis

The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a $P < 0.05$ was considered significant. All data are presented as the mean ± S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of DBcAMP on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

We have previously reported that T_3 activates p38 MAP kinase in osteoblast-like MC3T3-E1 cells, resulting in the synthesis of osteocalcin (Ishisaki et al., 2004). To clarify the role of the adenylyl cyclase-cAMP system in the thyroid hormone-stimulated osteocalcin in MC3T3-E1 cells, we examined the effects of each direct activator of the adenylyl cyclase-cAMP system on the osteocalcin synthesis. DBcAMP, a permeable analogue of cAMP, which alone did not affect the levels of osteocalcin in MC3T3-E1 cells, significantly suppressed the osteocalcin synthesis stimulated by T_3 (Fig. 1A). The inhibitory effect of DBcAMP on the T_3-stimulated osteocalcin synthesis was dose dependent in the range between 0.1 and 3 mM (Fig. 1B). The effect of DBcAMP was observed at 3 mM, a dose that caused about 80% reduction in the T_3-effect (Fig. 1B). We also examined the effect of DBcAMP on the T_3-induced mRNA for osteocalcin in MC3T3-E1 cells. DBcAMP markedly suppressed T_3-induced mRNA for osteocalcin (Fig. 1C).

Table 1
Effect of dideoxyforskolin on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

	T_3	Osteocalcin (ng/ml)
–	–	5.0 ± 0.7
–	+	305.0 ± 28.2*
Dideoxyforskolin	–	4.3 ± 1.6
Dideoxyforskolin	+	301.2 ± 43.2
Forskolin	–	4.6 ± 3.5
Forskolin	+	122.0 ± 15.4**

The cultured cells were pretreated with 10 μM dideoxyforskolin, 10 μM forskolin or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared with the value of T_3 alone.

** $P < 0.05$, compared with the value of T_3 with forskolin-pretreatment.

3.2. Effects of forskolin or dideoxyforskolin on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

Forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981), which by itself had little effect on the levels of osteocalcin, significantly reduced the T_3-stimulated osteocalcin synthesis (Fig. 2A). The inhibitory effect of forskolin on the T_3-stimulated osteocalcin synthesis was dose dependent in the range between 0.1 and 50 μM (Fig. 2B). The effect of forskolin was observed at 50 μM, a dose that caused about 80% reduction in the T_3-effect. We also examined the effect of forskolin on the T_3-induced mRNA for osteocalcin in MC3T3-E1 cells. Forskolin markedly suppressed T_3-induced mRNA for osteocalcin (Fig. 2C).

On the contrary, dideoxyforskolin (10 μM), a forskolin derivative that does not activate cAMP (Seamon et al., 1984), failed to suppress the osteocalcin synthesis while forskolin (10 μM) significantly reduced the synthesis (Table 1).

3.3. Effect of cholera toxin on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

Cholera toxin, which alone did not affect the levels of osteocalcin, significantly inhibited the T_3-stimulated synthesis of osteocalcin (Table 2).

3.4. Effect of KT5720 on the inhibition by forskolin or DBcAMP in T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

To clarify whether cAMP-dependent protein kinase (protein kinase A) mediates the inhibitory effect of forskolin

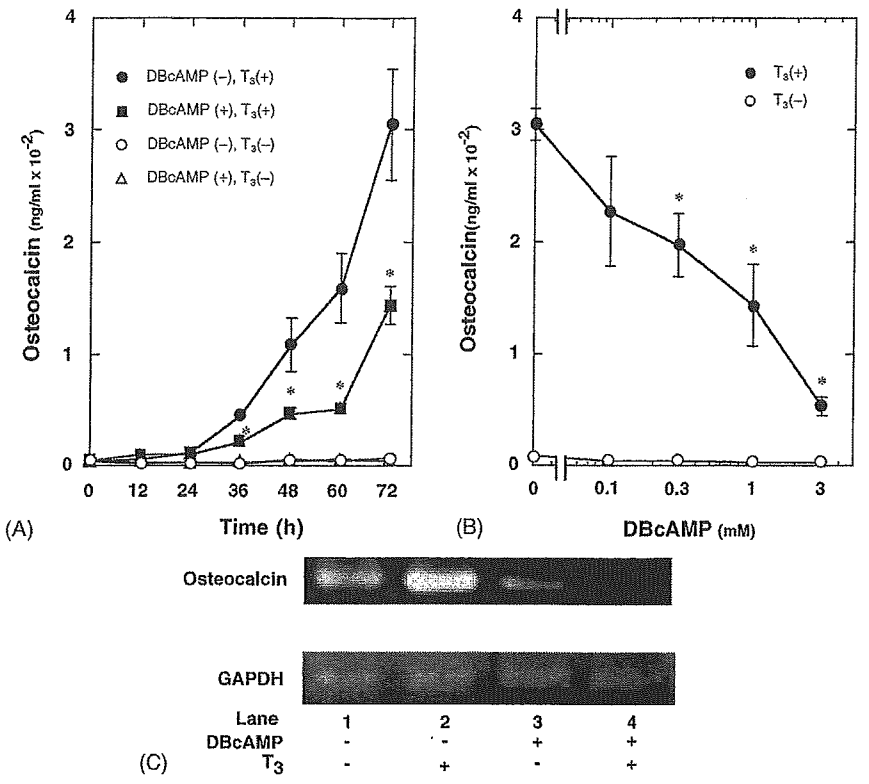

Fig. 1. Effect of DBcAMP on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells. (A) The cultured cells were pretreated with 1 mM DBcAMP (triangles) or vehicle (circles) for 20 min, and then stimulated by 10 nM T_3 (closed symbols) or vehicle (open symbols) for the indicated periods. (B) The cultured cells were pretreated with various dose of DBcAMP for 20 min, and then stimulated by 10 nM T_3 (closed circles) or vehicle (open circles) for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of T_3 alone. (C) Effect of DBcAMP on the T_3-induced levels of the mRNA for osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with 1 mM DBcAMP or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 12 h.

or DBcAMP in osteoblast-like MC3T3-E1 cells, we next examined the effect of KT5720, a specific inhibitor of protein kinase A (Kase et al., 1987), on the inhibition by forskolin in the T_3-stimulated synthesis of osteocalcin. KT5720 significantly reversed the suppressive effect of forskolin on the T_3-stimulated synthesis of osteocalcin (Table 3). Additionally, the inhibition by DBcAMP of the osteocalcin synthesis was reversed by KT5720 (data not shown).

Table 2
Effect of cholera toxin on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

Cholera toxin	T_3	Osteocalcin (ng/ml)
−	−	6.5 ± 0.4
−	+	304.6 ± 28.6*
+	−	4.1 ± 1.3
+	+	63.6 ± 11.5**

The cultured cells were pretreated with 1 μg/ml cholera toxin or vehicle for 6 h, and then stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared with the value of T_3 alone.

** $P < 0.05$, compared with the value of T_3 with cholera toxin-pretreatment.

3.5. Effect of PACAP on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

We previously showed that PACAP induces cAMP production in osteoblast-like MC3T3-E1 cells (Suzuki et al., 1994). Therefore, we investigated whether a physiological

Table 3
Effect of KT5720 on the inhibition by forskolin in the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

KT5720	Forskolin	T_3	Osteocalcin (ng/ml)
−	−	−	4.8 ± 2.4
−	−	+	306.2 ± 36.9
−	+	−	7.0 ± 3.2
−	+	+	34.6 ± 5.2*
+	−	−	6.3 ± 2.9
+	−	+	266.4 ± 28.1
+	+	−	3.8 ± 3.1
+	+	+	90.0 ± 6.6**

The cultured cells were pretreated with 10 μM KT5720 or vehicle for 20 min, and then incubated with 10 μM forskolin for 20 min. The cells were stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared with the value of T_3 alone.

** $P < 0.05$, compared with the value of T_3 with forskolin-pretreatment.

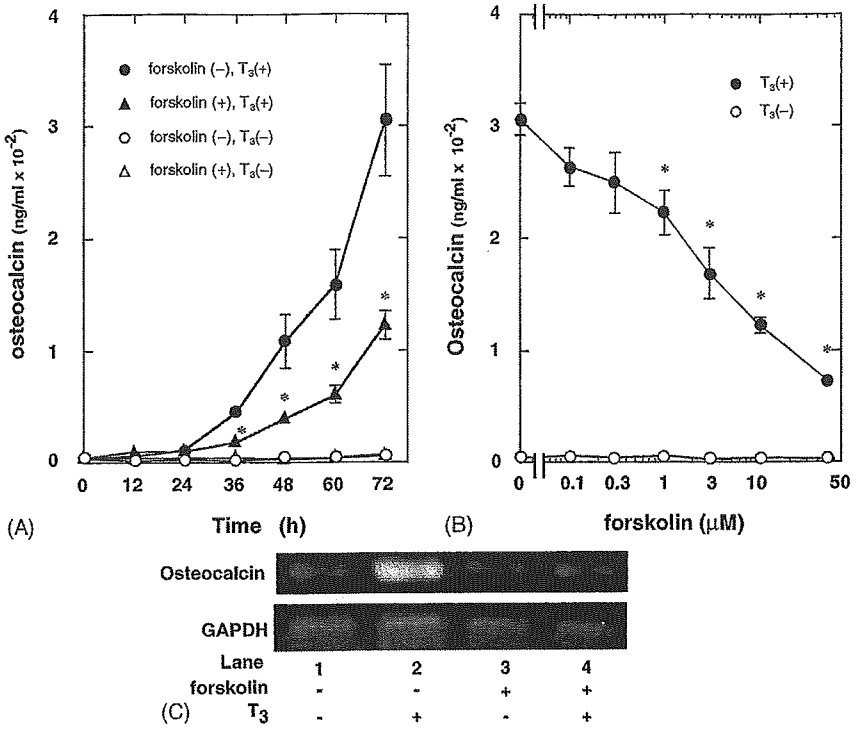

Fig. 2. Effect of forskolin on T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells. (A) The cultured cells were pretreated with 10 μM forskolin (triangles) or vehicle (circles) for 20 min, and then stimulated by 10 nM T_3 (closed symbols) or vehicle (open symbols) for the indicated periods. (B) The cultured cells were pretreated with various dose of forskolin for 20 min, and then stimulated by 10 nM T_3 (closed circles) or vehicle (open circles) for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P<0.05$, compared with the value of T_3 alone. (C) Effect of forskolin on the T_3-induced levels of the mRNA for osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM forskolin or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 12 h.

agonist such as PACAP that stimulates cAMP production affects the T_3-stimulated synthesis of osteocalcin in these cells. PACAP actually significantly reduced the osteocalcin synthesis stimulated by T_3 (Table 4). PACAP (0.1 μM) caused a 35% reduction in the effect of T_3.

3.6. Effects of DBcAMP and forskolin on the T_3-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells

We previously showed that p38 MAP kinase acts as positive regulator in the T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells (Ishisaki et al., 2004). We already demonstrated that T_3 time dependently induces p38 MAP kinase phosphorylation in these cells (Ishisaki et al., 2004). Thus, we next investigated whether adenylyl cyclase-cAMP system affects the T_3-induced activation of p38 MAP kinase. DBcAMP markedly reduced the T_3-induced phosphorylation of p38 MAP kinase (Fig. 3A). In addition, forskolin suppressed the p38 MAP kinase phosphorylation by T_3 (Fig. 3B).

Table 4
Effect of PACAP on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

PACAP	T_3	Osteocalcin (ng/ml)
–	–	9.2 ± 4.4
–	+	305.2 ± 24.6*
+	–	8.5 ± 4.1
+	+	198.6 ± 39.8**

The cultured cells were pretreated with 0.1 μM PACAP or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P<0.05$, compared with the value of T_3 alone.

** $P<0.05$, compared with the value of T_3 with PACAP-pretreatment.

4. Discussion

We have previously demonstrated that T_3 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase is involved in T_3-stimulated synthesis of osteocalcin (Kozawa et al., 2001; Ishisaki et al., 2004). In the present study, we showed that forskolin suppressed T_3-stimulated synthesis of osteocalcin in these cells. It is well known that forskolin is a direct activator of adenylyl cyclase (Seamon and Daly, 1981) and that we have shown that forskolin truly stimulates cAMP accumulation in MC3T3-E1 cells (Kozawa et al., 1992). In addition, dideoxyforskolin, which is a biologically inactive forskolin analogue (Seamon et al., 1984), failed to suppress the T_3-stimulated synthesis of osteocalcin. Thus, it seems that cAMP inhibits T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells. We next demonstrated that cholera

toxin, a direct and continuous activator of Gs (Gilman, 1987), reduced the T_3-stimulated synthesis of osteocalcin in these cells. It is well known that Gs, a heterotrimeric GTP-binding protein that mediates stimulative signals from the receptor to adenylyl cyclase, and the activation of adenylyl cyclase results in the production of cAMP (Gilman, 1987). Therefore, these results suggest that cAMP produced by adenylyl cyclase activation suppresses the osteocalcin synthesis stimulated by T_3 in MC3T3-E1 cells. Moreover, DBcAMP, a permeable analogue of cAMP, reduced the T_3-stimulated synthesis of osteocalcin. In a previous study (Suzuki et al., 1994), we have reported that PACAP stimulated cAMP accumulation by various doses in osteoblast-like MC3T3-E1 cells, and the effect of PACAP reached a maximum value at 10 nM. PACAP stimulated in the cAMP production without affecting the activity of protein kinase C in these cells. In addition, we found that PACAP had little effect on Ca^{2+} influx in these cells. Thus, we investigated whether PACAP, a physiological agonist for osteoblasts and aselective stimulator of cAMP production, extracellulary affects the synthesis of osteocalcin stimulated by T_3 in these cells. We demonstrated that PACAP truly reduced the T_3-stimulated synthesis of osteocalcin. Taking our findings into account, it is most likely that the adenylyl cyclase-cAMP system has an inhibitory role in T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells.

Parathyroid hormone (PTH) functions as a major mediator of bone remodeling as well as an essential regulator of calcium homeostasis (Strewler et al., 1987). PTH affects both cortical and cancellous bone mass and architecture, and PTH leads to increased bone resorption. PTH also activates rat osteocalcin transcription via cAMP-dependent protein kinase A pathway (Yu and Chandrasekhar, 1997). Thus, PTH is probably the most important agent that acts on osteoblasts through the cAMP signaling system. Therefore, we investigated that the effect of PTH on T_3-induced osteocalcin expression. However, PTH did not have an effect on T_3-stimulated synthesis of osteocalcin (data not shown). It is possible that PTH-dependent cAMP may have little effect on T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells.

In the present study, we showed that KT5720, a specific inhibitor of protein kinase A (Kase et al., 1987), salvaged the suppressive effect of forskolin on the T_3-stimulated osteocalcin synthesis. Thus, it seems that the inhibitory effect of forskolin is mediated through protein kinase A in MC3T3-E1 cells. In addition, we found that the suppression by DBcAMP of the T_3-stimulated synthesis of osteocalcin was reversed by KT5720. Therefore, our results strongly suggest that protein kinase A activated by cAMP negatively

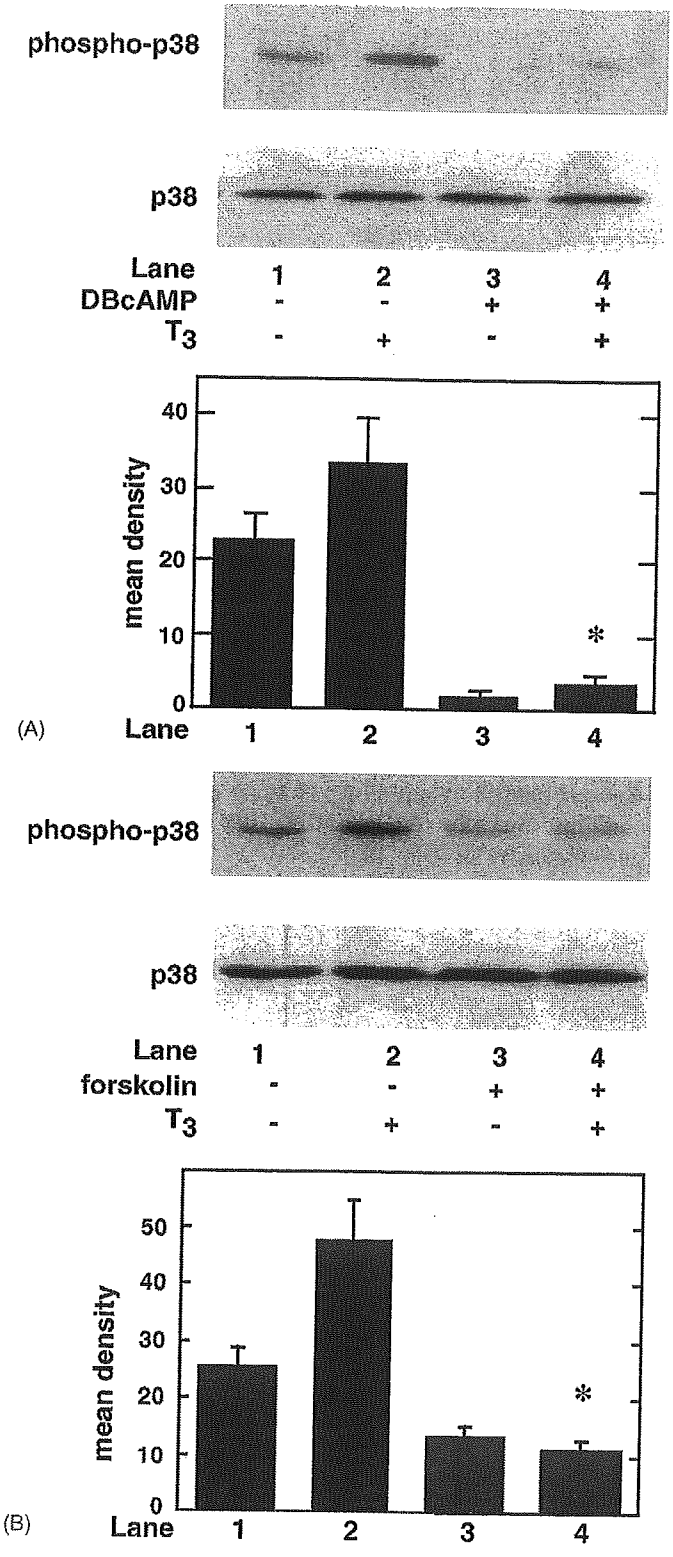

Fig. 3. Effects of DBcAMP or forskolin on T_3-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with (A) 1 mM DBcAMP or (B) 10 μM forskolin for 20 min, and then stimulated by 10 nM T_3 for 3 h. The extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. The histogram shows quantitative representations of the levels of T_3-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of T_3 alone.

regulates T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells.

We have shown that not p44/p42 MAP kinase but p38 MAP kinase among the MAP kinase superfamily plays a part in the T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells (Ishisaki et al., 2004). To investigate whether the adenylyl cyclase-cAMP system affects T_3-induced p38 MAP kinase activation in these cells, we examined the effect of forskolin on the p38 MAP kinase phosphorylation stimulated by T_3. We showed that forskolin markedly reduced the phosphorylation of p38 MAP kinase induced by T_3. It is well known that MAP kinase is activated by phosphorylation on tyrosine and threonine by dual specificity MAP kinase (Raingeaud et al., 1995). These findings suggest that the adenylyl cyclase-cAMP system inhibits the T_3-induced activation of p38 MAP kinase at a point upstream from p38 MAP kinase in MC3T3-E1 cells. In addition, we found that the T_3-induced phosphorylation of p38 MAP kinase was truly inhibited by DBcAMP. Based on our results as a whole, it is most likely that T_3-stimulated osteocalcin synthesis is negatively regulated by protein kinase A at a point upstream from p38 MAP kinase in osteoblast-like MC3T3-E1 cells.

Osteocalcin is well known as an osteoblastic-specific marker, which is a γ-carboxylated, calcium-binding protein produced and secreted only by mature osteoblasts (Ducy et al., 1996; Stein and Lian, 1993). The levels of initially expressed osteocalcin are low and then up-regulated with time in cultured osteoblasts (Franchesi and Iyer, 1992). Osteocalcin is one of the most abundant noncollagenous proteins in the extracellular matrix of bone, and it contains glutamic acid residues (Shearer, 1997). Vitamin K is essential for carboxylation of glutamic acid residues, and Vitamin K promotes osteocalcin accumulation and mineralization in human osteoblasts (Koshihara et al., 1996; Koshihara and Hoshi, 1997). Osteocalcin-deficient mice develop hyperostosis (Ducy et al., 1996), suggesting that γ-carboxyglutamic acid (Gla)-containing osteocalcin promotes normal bone mineralization. In vitro and in vivo data provide conflicting insights into the role of osteocalcin in the regulation of biomineralization. When γ-carboxylated by a Vitamin K-dependent carboxylase, osteocalcin binds to bone mineral (apatite) with high affinity (Poser and Price, 1979). This affinity enables it to regulate the rate of hydroxyapatite crystal growth in solution (Boskey et al., 1985; Hauschka et al., 1983; Hunter et al., 1996; Romberg et al., 1986), suggesting that osteocalcin can affect mineral formation and mineral crystal growth. In culture, osteocalcin is chemotactic for osteoclasts (Malone et al., 1982), implying that it is involved in bone remodeling. Taking our findings into account, osteocalcin synthesis is negatively regulated by adenylyl cyclase-cAMP system. It has probable a crucial role as a regulator of bone metabolism.

In conclusion, these results strongly suggest that the adenylyl cyclase-cAMP system has an inhibitory role in thyroid hormone-stimulated osteocalcin synthesis via suppression of p38 MAP kinase activation in osteoblasts.

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Methotrexate Suppresses Inflammatory Agonist Induced Interleukin 6 Synthesis in Osteoblasts

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ABSTRACT. ***Objective.*** Interleukin 6 (IL-6) is a pleiotropic cytokine that plays a crucial role in the pathogenesis of rheumatoid arthritis (RA). In bone metabolism, it is known that IL-6 is produced and secreted by osteoblasts, and that IL-6 induces osteoclast formation and stimulates bone resorption. Various bone inflammatory agonists such as tumor necrosis factor-α (TNF-α), IL-1α, prostaglandin D_2 (PGD_2), PGE_2, and $PGF_{2\alpha}$, which play important roles in the pathogenesis of RA, induce IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Low dose methotrexate (MTX) is currently used for treatment of patients with RA. We investigated the effect of MTX on IL-6 synthesis induced by these agents in MC3T3-E1 cells.

Methods. Cultured cells were pretreated with various doses of MTX, and then stimulated by these inflammatory agonists. The IL-6 in the conditioned medium was measured by IL-6 enzyme immunoassay.

Results. MTX significantly suppressed IL-6 synthesis stimulated by these agonists in a dose-dependent manner, although MTX alone had no effect on the levels of IL-6. In addition, MTX significantly inhibited the enhancement by IL-17 of TNF-α-stimulated IL-6 synthesis. MTX reduced the levels of IL-6 induced by 12-*O*-tetradecanoylphorbol 13-acetate, a direct activator of protein kinase C (PKC), suggesting that MTX inhibits PKC signals for IL-6 synthesis.

Conclusion. MTX suppresses IL-6 synthesis stimulated by various inflammatory agonists in osteoblasts. (J Rheumatol 2005;32:787–95)

Key Indexing Terms:
METHOTREXATE INTERLEUKIN 6 OSTEOBLAST
RHEUMATOID ARTHRITIS INFLAMMATION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflamed synovial hyperplasia and cartilage destruction with excessive inflammatory cell infiltration[1]. In RA, various cytokines[2] or arachidonates[3] act as mediators and/or modulators of inflammation and joint destruction. Among them, tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) are well recognized as inflammatory cytokines in the pathogenesis of RA[2]. Recent therapeutic interventions, including TNF-α antibodies and IL-1 receptor antagonists, strongly support the importance of these cytokines in RA[2]. It has been reported that IL-17, a T cell derived cytokine, contributes to the pathogenesis of RA, and shows additive or even synergistic effects with TNF-α and IL-1 in inducing joint pathology[4]. It is generally recognized that prostaglandins (PG) act as local modulators in osteoblasts and play a crucial role in the regulation of bone metabolism[5]. PG have been shown to be important mediators for inflammatory joint disorders such as RA[3,6].

Several disease modifying antirheumatic drugs are used for patients with RA[1]. Recently, methotrexate (MTX) has gained an important place among them for RA[7]. MTX, as a folate antagonist, was developed for the treatment of malignancies[8], and is useful for autoimmune inflammatory diseases such as RA in low dosage[9,10]. Antiinflammatory mechanisms for MTX have been reported mainly in the synovial cells and inflammatory cells: i.e., reduction of immunoglobulin[11], suppression of neutrophil chemotaxis[12], inhibition of IL-1 activity[13], increased adenosine release[14], inhibition of cyclooxygenase-2 (COX-2) activation[15], and suppression of lymphocyte proliferation[16]. Recently, it was reported that MTX inhibited IL-6 production by lipopolysaccharide (LPS) activated peripheral blood mononuclear cells (PBMC) obtained from patients with juvenile RA[17]. However, the mechanism underlying MTX induced suppression of IL-6 synthesis has not been precisely clarified; as well, the effect of MTX on osteoblasts around the diseased joint in RA remains to be elucidated.

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Bone metabolism is strictly regulated by osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively[5]. It is well known that receptor activator nuclear factor-κB ligand (RANKL) expressed on osteoblasts plays a pivotal role to transduce an essential differentiation signal to osteoclast lineage cells through binding to its receptor, RANK, expressed on the latter cells[18]. In addition, it has been reported that cytokines stimulating osteoclastogenesis, such as IL-1, IL-6, IL-11, IL-17, and TNF-α, increase the expression of RANKL with decrease of osteoprotegerin expression in osteoblast/stromal lineage cells[18].

IL-6 is a pleiotropic multifunctional cytokine that regulates diverse cell functions[19,20], and it has been reported that IL-6 stimulates bone resorption and induces osteoclast formation[21,22]. We have shown that TNF-α[23], IL-1[24], PGD_2[25], PGE_2[26], and $PGF_{2\alpha}$[27] stimulate IL-6 production and its secretion in cultured osteoblasts. Thus, accumulating evidence suggests that IL-6 secreted from osteoblasts plays an important role in bone resorption as a downstream effector of a variety of bone resorptive agents.

We investigated the effect of MTX on the synthesis of IL-6 induced by the various agonists such as TNF-α, IL-1α, PGD_2, PGE_2, and $PGF_{2\alpha}$ that have been shown to strongly affect the pathogenesis of RA in osteoblast-like MC3T3-E1 cells. We observed that, although MTX has little effect on IL-6 synthesis, it suppresses IL-6 synthesis induced by these agents.

MATERIALS AND METHODS

MTX was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). TNF-α and IL-1α were purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). PGD_2, PGE_2, $PGF_{2\alpha}$, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse IL-6 and vascular endothelial growth factor (VEGF) enzyme immunoassay kits and IL-17 were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Other materials and chemicals were obtained from commercial sources. PGD_2, PGE_2, and $PGF_{2\alpha}$ were dissolved in ethanol. TPA was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO was 0.1%, which did not affect the assay for IL-6 and VEGF.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria[28] were maintained as described[29]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4 cells) were seeded into 35 mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml α-MEM containing 0.3% FCS. Cells were used for experiments after 48 h.

Primary culture mouse osteoblasts were prepared from neonatal balb/c mouse as described[30]. In brief, primary osteoblastic cells were prepared from calvariae of 2-day-old neonatal balb/c mice by digesting them with an enzyme solution containing 0.1% collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.2% dispase (Godo Shusei, Tokyo, Japan). The isolated cells were pooled and seeded into 90 mm dishes in α-MEM containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4 cells) were seeded into 35 mm dishes in 2 ml α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

Assay for IL-6. The cultured cells were pretreated with various doses of MTX for 1 h, and then stimulated by the indicated doses of various agonists or vehicle in 1 ml α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was measured using an IL-6 enzyme immunoassay kit.

Assay for VEGF. Cultured cells were pretreated with various doses of MTX for 1 h, and then stimulated by the indicated doses of various agonists or vehicle in 1 ml α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and VEGF in the medium was measured by VEGF enzyme immunoassay kit.

Assay for cell viability. Cell viability was assessed as a function of NADH content using a TetraColor One [5 mM (2-(2-methoxy-4-nytrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt); 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate; and 150 mM NaCl]-based assay according to the manufacturer's instructions (Seikagaku Inc., Nihonbashi, Tokyo, Japan)[31]. The cells (1.5×10^3 cells/well) were seeded into 96-well tissue culture plates in 100 µl of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 100 µl of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. Then some cells were pretreated with 1 µM of MTX for 1 h and then stimulated by 10 µM PGD_2 for 48 h; the other cells were not. After that, each well was washed once with α-MEM, and then 100 µl of α-MEM was added to each well without any supplement and incubated for 16 h. Finally, 10 µl of Tetracolor One solution was added to each well, and the cells were incubated for 1.5 h. A well for the negative control was prepared as described above without cells. The absorbance of each well was then determined at a wavelength of 450 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression. Cells were treated with 1 µM MTX for 1 h. Total RNA was isolated from the cells using Isogen (Nippon Gene, Tokyo, Japan), then a 1 µg sample was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Using 4% of the reverse-transcribed mix, cDNA fragments of test genes were amplified within the linear range by PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The specific primers were synthesized according to motifs: TTC ACA AGT CCG GAG AGG AG (IL-6, sense), TGG TCT TGG TCC TTA GCC AC (IL-6, antisense), TTC ATT GAC CTC AAC TAC ATG (GAPD), sense), and GTG GCA GTG ATG GCA TGG AC (GAPDH, antisense). PCR amplification of IL-6 cDNA (488 bp) for 33 cycles was 94°C denaturation (60 s), 55°C annealing (60 s), and 72°C extension (60 s). PCR amplification of GAPDH cDNA (443 bp) for 20 cycles was 94°C denaturation (60 s), 60°C annealing (60 s), and 72°C extension (60 s). Following these cycles of PCR amplifications, the amplified cDNA were further extended by additional incubation at 72°C for 10 min. Then equal amounts of each reaction were fractionated on 1% agarose gel in 1× TAE buffer, and the agarose gel was soaked in 1× TAE buffer containing ethidium bromide for 15 min with gentle agitation. The amplified cDNA fragments in the agarose gel were then visualized on an UV transilluminator and photographed.

Absorbance measurement. The absorbance of ELISA samples was measured at 450 nm with an EL340 Bio Kinetic Reader (Bio-Tek Instruments, Winooski, VT, USA).

Statistical analysis. Each experiment was repeated 3 times with similar results. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs. $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

RESULTS

Effect of MTX on TNF-α induced IL-6 synthesis in MC3T3-E1 cells. We previously reported that TNF-α significantly induced synthesis of IL-6 in a time-dependent manner up to

48 h[23]. We investigated the effect of MTX on TNF-α induced IL-6 synthesis. MTX (1 μM), which alone had little effect on IL-6 synthesis, significantly inhibited the TNF-α induced IL-6 synthesis in a time-dependent manner. The inhibitory effect of MTX reached a maximum at 48 h after stimulation (Figure 1A). The effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX on TNF-α induced IL-6 synthesis was observed at 1.0 μM, which caused about 30% decrease in the TNF-α effect (Figure 1B).

Effect of MTX on amplification by IL-17 of TNF-α induced IL-6 synthesis in MC3T3-E1 cells. It is well known that IL-17 is expressed in the synovium of patients with RA and contributes to the pathogenesis of arthritis, and expresses additive or even synergistic effects with TNF-α in inducing joint pathology[4]. We have reported that IL-17 markedly enhances the TNF-α induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells[32]. Here, we investigated the effect of MTX on the amplification by IL-17 of TNF-α induced IL-6 synthesis in these cells. MTX significantly suppressed the enhancement by IL-17 of TNF-α stimulated IL-6 synthesis in a dose-dependent manner in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM, which caused about 30% inhibition in the IL-17 enhanced TNF-α effect (Figure 2).

Effect of MTX on IL-1α induced IL-6 synthesis in MC3T3-E1 cells. We have reported that IL-1α induces IL-6 synthesis in a time-dependent manner up to 24 h in osteoblast-like MC3T3-E1 cells[24]. We examined the effect of MTX on the IL-1α induced IL-6 synthesis in osteoblasts. MTX (1 μM) decreased the IL-1α induced IL-6 synthesis in a time-dependent manner. The inhibiting effect by MTX reached a maximum at 24 h after stimulation (Figure 3A). The effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM. MTX caused about 35% decrease in the IL-1α effect (Figure 3B).

Effect of MTX on PGD_2 induced IL-6 synthesis in MC3T3-E1 cells. We previously showed that PGD_2 stimulates IL-6 synthesis in MC3T3-E1 cells[25]. To clarify whether MTX affects the PGD_2 induced level of IL-6, we examined the effect of MTX on PGD_2 induced IL-6 synthesis. MTX (1.0 μM) significantly suppressed the PGD_2 stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (data not shown). MTX significantly suppressed PGD_2 stimulated IL-6 synthesis in a dose-dependent manner, and the maximum effect of MTX was observed at 1.0 μM. MTX caused about 60% decrease in the PGD_2 effect (Figure 4A).

Effect of MTX on PGE_2 induced IL-6 synthesis in MC3T3-

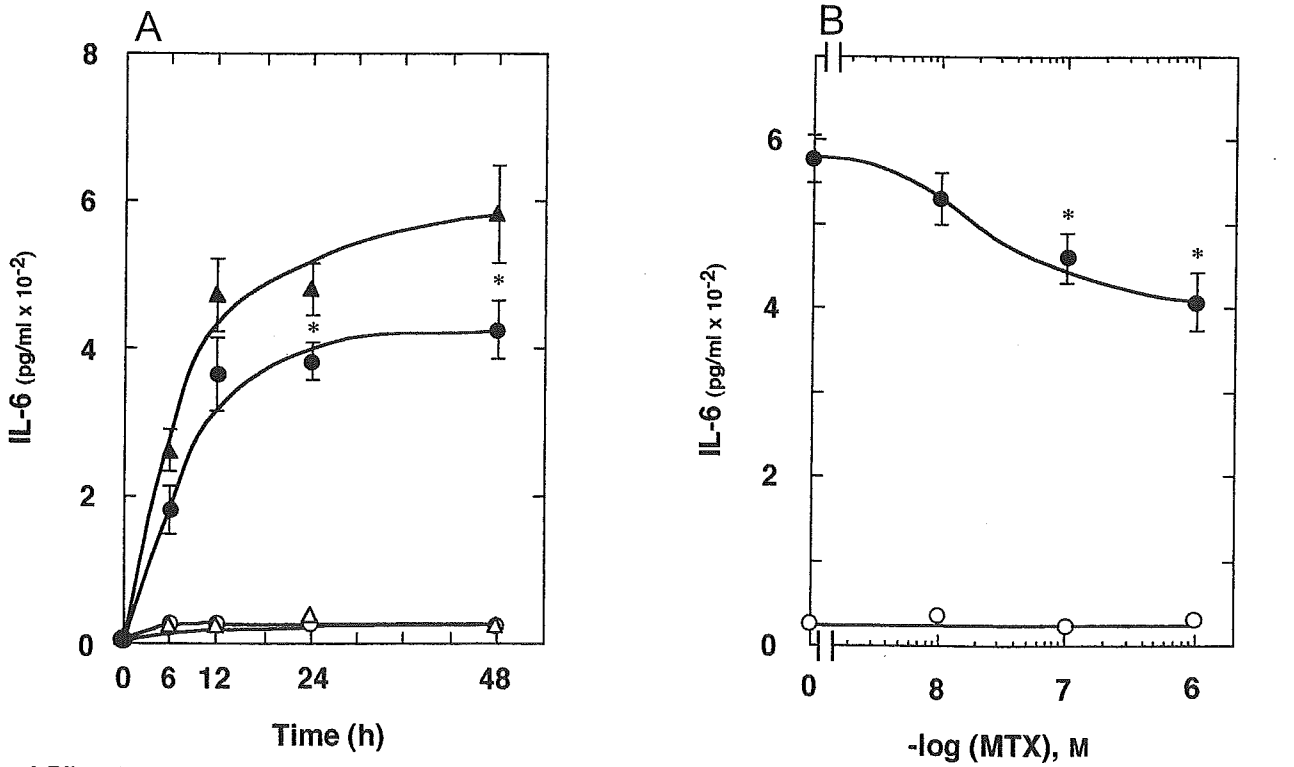

Figure 1. Effect of MTX on TNF-α induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, and then stimulated with 10 ng/ml TNF-α (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TNF-α alone. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TNF-α without MTX.

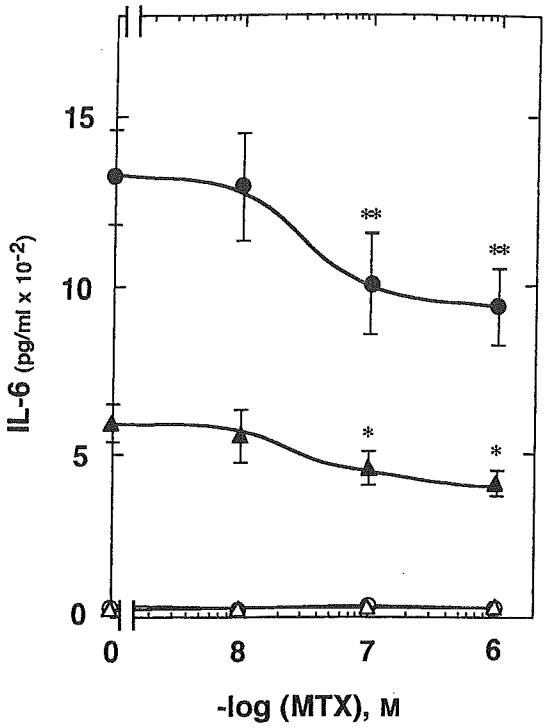

Figure 2. Effect of MTX on IL-17 amplification of TNF-α induced IL-6 synthesis in MC3T3-E1 cells. Cultured cells were pretreated with indicated doses of MTX for 1 h; cells were next treated with 10 ng/ml IL-17 (●, ○) or vehicle (▲, △) for 1 h, then stimulated with 10 ng/ml TNF-α (●, ▲) or vehicle (○, △) for 48 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TNF-α without IL-17 and MTX. **$p < 0.05$ vs TNF-α with IL-17 without MTX.

E1 cells. It is generally accepted that PGE_2 promotes inflammation and participates in destructive mechanisms in the rheumatoid joint[33]. In addition, it has been reported that PGE_2 and $PGF_{2\alpha}$ levels in the synovial fluid of patients with RA are significantly higher than the values obtained in patients with osteoarthritis (OA)[3]. As reported[26], PGE_2 stimulates IL-6 synthesis through both EP_1 receptor and EP_2 receptor in osteoblast-like MC3T3-E1 cells. We next examined the effect of MTX on the PGE_2 induced IL-6 synthesis in these cells. MTX (1.0 μM) inhibited PGE_2 stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (data not shown). The effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM and caused about 25% reduction in the PGE_2 effect (Figure 4B).

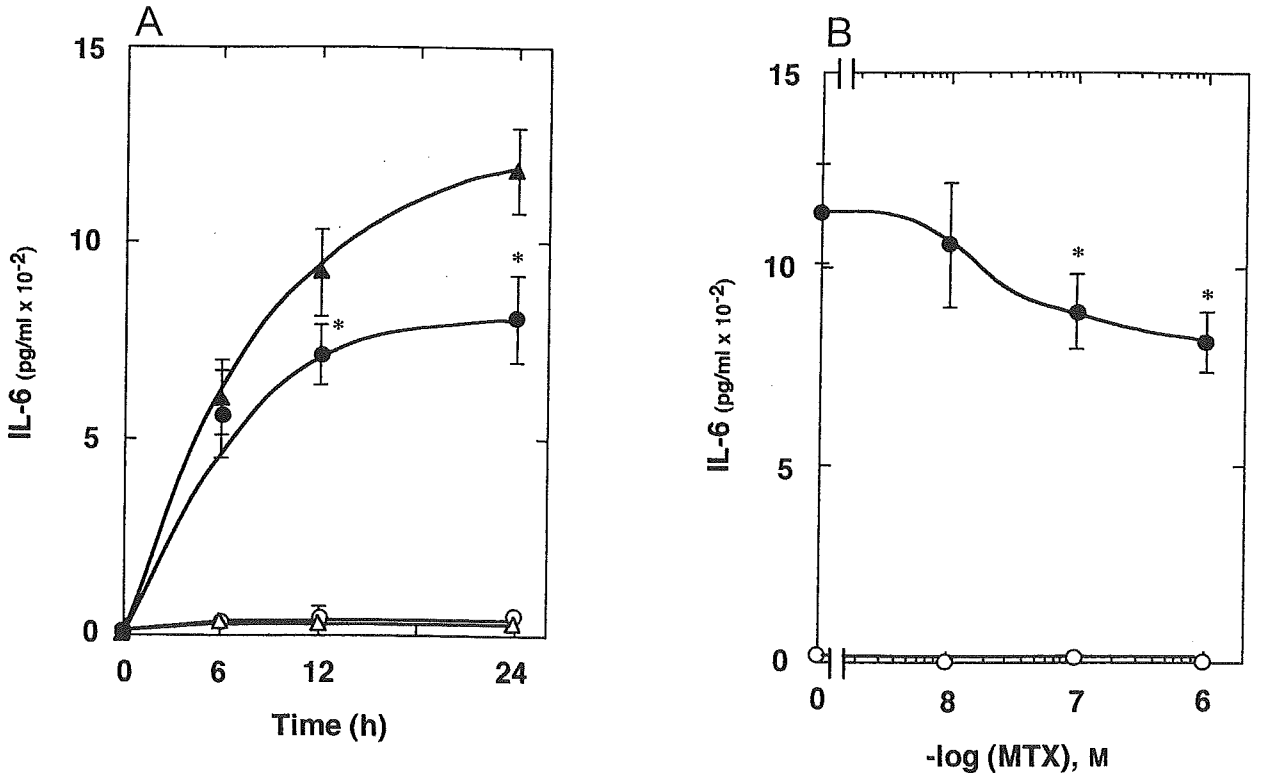

Figure 3. Effect of MTX on IL-1α induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, and then stimulated with 30 ng/ml IL-1α (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs IL-1α alone. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 30 ng/ml IL-1α (●) or vehicle (○) for 24 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs IL-1α without MTX.

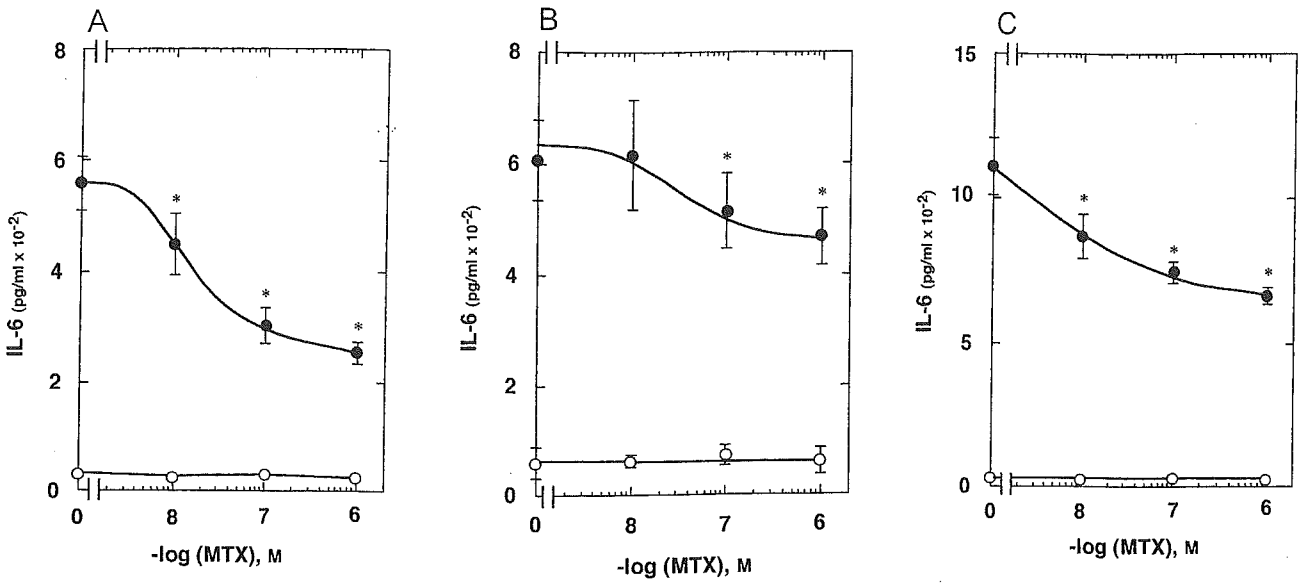

Figure 4. Effect of MTX on PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGD_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGD_2 without MTX. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGE_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGE_2 without MTX. (C) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs $PGF_{2\alpha}$ without MTX.

Effect of MTX on $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. We have demonstrated[27,34] that $PGF_{2\alpha}$ induces IL-6 synthesis in MC3T3-E1 cells, and that protein kinase C (PKC) activation is involved in the mechanism. To clarify whether MTX affects the $PGF_{2\alpha}$ stimulated IL-6 synthesis, we examined the effect of MTX on $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. MTX (1.0 μM) significantly suppressed the $PGF_{2\alpha}$ stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (data not shown). MTX significantly suppressed $PGF_{2\alpha}$ stimulated IL-6 synthesis in a dose-dependent manner in the range between 0.01 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM. MTX caused about 40% decrease in the $PGF_{2\alpha}$ effect (Figure 4C).

Effect of MTX on TPA induced IL-6 synthesis in MC3T3-E1 cells. PKC is known to play a pivotal role in the regulation of various cellular functions[35]. We have demonstrated that activation of PKC directly activated by TPA, a PKC-activating phorbol ester[35], induces IL-6 synthesis in MC3T3-E1 cells[34]. We investigated the effect of MTX on IL-6 synthesis induced by TPA in these cells. MTX (1.0 μM) significantly suppressed TPA stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (Figure 5A). The inhibitory effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM, which caused about 30% decrease in the TPA effect (Figure 5B).

Effect of MTX on VEGF synthesis induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. It is recognized that angiogenesis is an important process in the development and perpetuation of RA[36]. VEGF is known as an essential mediator of angiogenesis[37]. It is reported that VEGF levels are markedly higher in the serum and synovial fluids of patients with RA than in patients with OA and healthy controls[38]. In osteoblasts, it has been reported that PGE_2 induces VEGF synthesis[39]. We have shown that $PGF_{2\alpha}$ induces VEGF synthesis[40]. We investigated the effect of MTX on VEGF synthesis induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. MTX alone had no effect on VEGF synthesis in these cells. Further, MTX did not affect the concentrations of VEGF induced by $PGF_{2\alpha}$ (Figure 6).

Effects of MTX on PGD_2, PGE_2, and $PGF_{2\alpha}$ induced IL-6 synthesis in primary osteoblastic cells. To clarify whether these effects of MTX are specific to MC3T3-E1 cells, we next investigated the effect of MTX on PGD_2, PGE_2, and $PGF_{2\alpha}$ induced IL-6 synthesis in primary mouse osteoblastic cells. MTX (0.1 or 1.0 μM) significantly suppressed IL-6 synthesis stimulated by these inflammatory agonists in a dose-dependent manner. The maximum effect of MTX was observed at 1.0 μM in each case (Figure 7).

Effect of PGD_2 with MTX treatment on viability of MC3T3-E1 cells. As shown in Figure 8, cell viability of PGD_2 with MTX treated cells was not lower than that of the untreated cells, and was instead significantly higher. Thus, it seems that PGD_2 with MTX treatment has no toxic effects on MC3T3-E1 cells.

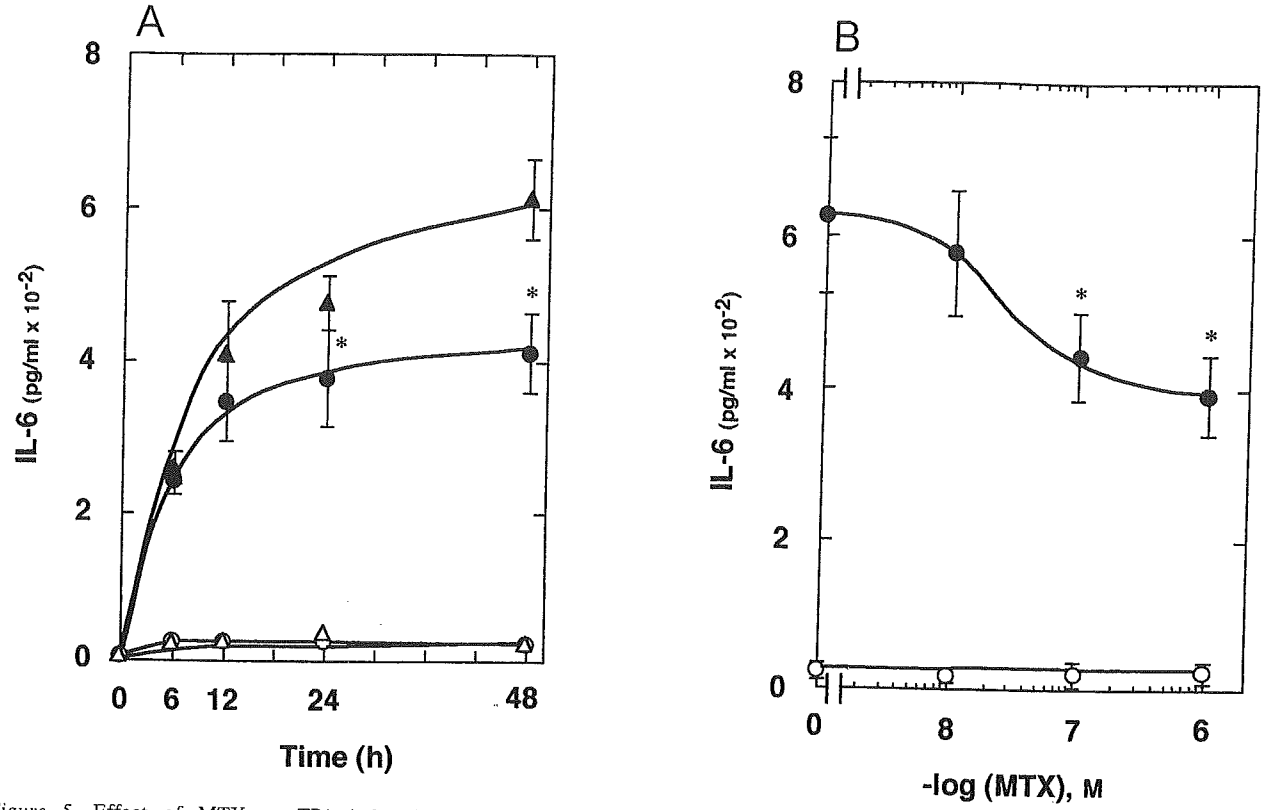

Figure 5. Effect of MTX on TPA induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, then stimulated with 0.1 μM TPA (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TPA alone. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 0.1 μM TPA (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TPA without MTX.

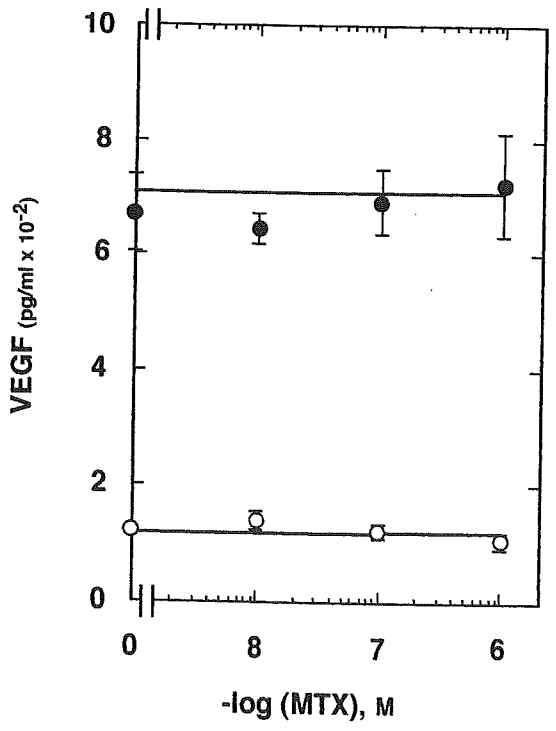

Figure 6. Effect of MTX on $PGF_{2\alpha}$ induced VEGF synthesis in MC3T3-E1 cells. Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations.

DISCUSSION

RA is one of the most common inflammatory diseases, and various inflammatory cytokines play crucial roles in its pathogenesis[2]. Among them, TNF-α and IL-1 are pivotal proinflammatory cytokines that have been shown to contribute to the clinical manifestations of RA[2,33,41]. TNF-α and IL-1 are reported to stimulate osteoblasts directly, and increase the expression of RANKL in these cells[18]. It is recognized that RANKL-RANK interaction is essential for osteoclast differentiation; TNF-α and IL-1 partially regulate osteoclastogenesis through osteoblast-osteoclast interaction[18,42]. RA is often complicated by generalized osteopenia due to increased bone resorption by osteoclasts[43]. It is known that TNF-α antibody[44] and IL-1 receptor antagonists[43] prevent bone loss caused by inflammatory arthritis, such as RA. PG are well known as important mediators of inflammation and joint destruction in RA[33]. COX-2

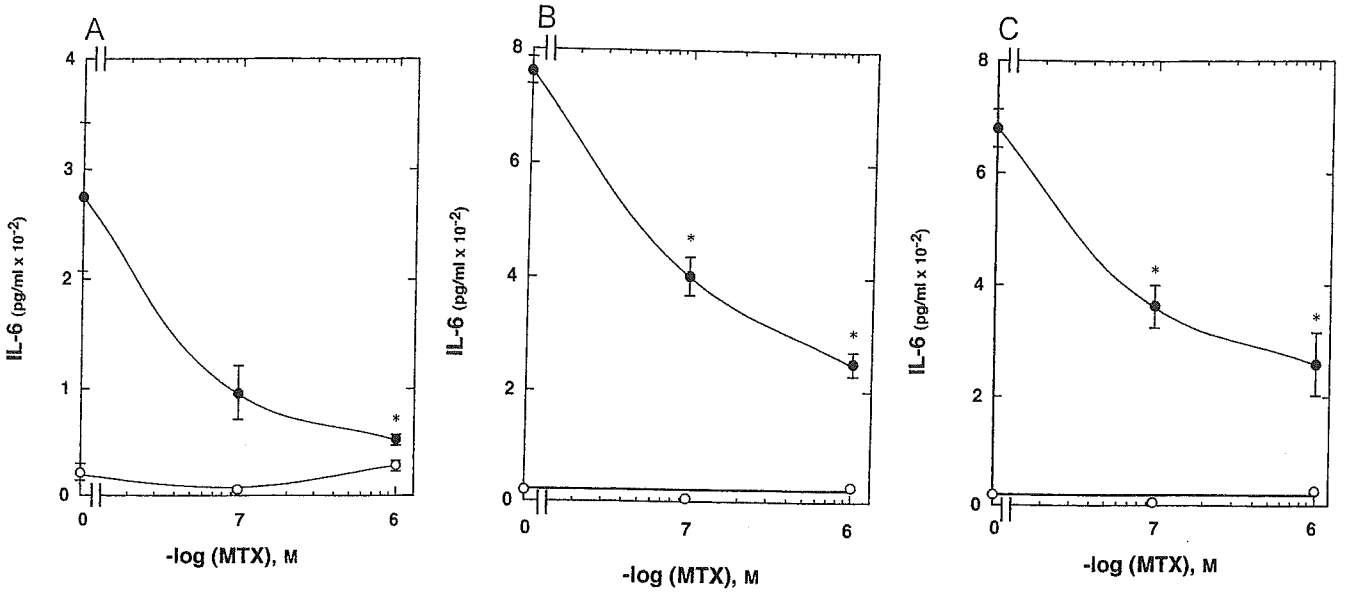

Figure 7. Effects of MTX on PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in primary osteoblastic cells. (A) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGD_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGD_2 without MTX. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGE_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGE_2 without MTX. (C) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs $PGF_{2\alpha}$ without MTX.

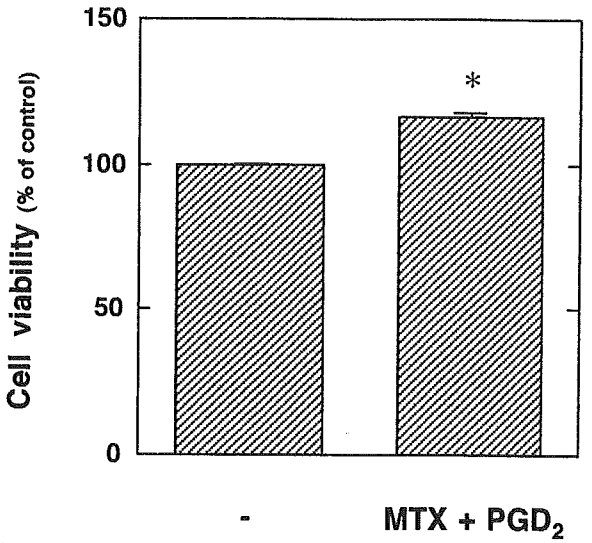

Figure 8. Effect of PGD_2 with MTX treatment on viability of MC3T3-E1 cells. Cells were treated first with MTX (1 μM) for 1 h and then with PGD_2 (10 μM) for 48 h (MTX + PGD_2, right bar), or not (left bar). After that, each culture was washed once with α-MEM, supplemented with 100 μl fresh α-MEM with no supplements, and then incubated 16 h. Finally, cell viability in each culture was assessed as a function of NADH content using TetraColor One solution. Absorbance of each well was determined at 450 nm. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.01$ vs untreated cells.

inhibitor, one of the most common nonsteroidal antiinflammatory drugs routinely used in RA, blocks the conversion of arachidonic acid to PGH_2, subsequently converting to other PG, which results in preventing the inflammation of RA[45].

It is recognized that IL-6 stimulates bone resorption by inducing osteoclast formation, and IL-6 secreted from osteoblasts plays a key role in osteoclastogenesis[21,22]. In osteoblasts, we have shown that TNF-α[23], IL-1α[24], PGD_2[25], PGE_2[26], and $PGF_{2\alpha}$[27] stimulate IL-6 synthesis. In addition, we demonstrated that IL-17 enhanced TNF-α induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells[32].

It has recently been reported that MTX inhibits IL-6 production by LPS stimulated PBMC obtained from patients with juvenile RA[17]. However, the mechanism underlying MTX induced suppression of IL-6 synthesis is unknown. In addition, the effect of MTX on osteoblasts around the diseased joint in RA remains to be clarified. In this study, we showed that MTX significantly suppressed TNF-α stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells, although MTX by itself had little effect on IL-6 levels. It is likely that MTX significantly decreased IL-1α, PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in these cells. Moreover, MTX significantly suppressed the PG stimulated IL-6 synthesis in primary osteoblastic cells as observed in osteoblast-like MC3T3-E1 cells. Therefore, it is probable that this observation is common in osteoblastic lineage cells. These results strongly suggest that MTX suppresses inflammatory agonist induced IL-6 synthesis in osteoblasts.

We examined the level of IL-6 mRNA in MC3T3-E1 cells 1 h after addition of MTX alone using RT-PCR analysis. The level of IL-6 mRNA was not affected 1 h after addition of 1.0 μM MTX (data not shown), suggesting that MTX

might inhibit IL-6 accumulation at protein levels. Further investigations are required to examine specifically how MTX suppresses the IL-6 accumulation in osteoblast-like cells.

The suppressive effects of MTX on inflammatory agonist induced IL-6 synthesis at clinically relevant concentrations are not large (20%–30%). However, we have shown that MTX suppressed IL-6 synthesis stimulated by various bone inflammatory agonists, such as TNF-α, IL-1α, PGD_2, PGE_2, and $PGF_{2\alpha}$, that play important roles in the pathogenesis of RA in osteoblast-like MC3T3-E1 cells. In addition, MTX significantly inhibited the enhancement by IL-17 of TNF-α stimulated IL-6 synthesis. These results strongly suggest that MTX generally suppresses IL-6 synthesis stimulated by various inflammatory agonists in osteoblasts, although the effect is not large. Intriguingly, Nowak, *et al* recently reported that MTX in combination with prednisone decreases blood levels of IL-1ß and IL-6 and inhibits the intensity of free radical mediated processes in patients with RA[46]. Therefore, it is possible that prednisone may enhance the suppressive effect of MTX on IL-6 synthesis induced by various inflammatory agonists as described above in MC3T3-E1 cells. If so, such results justify the utility that the combination of MTX and prednisone decreases levels of IL-6 in patients with RA. We will next examine whether the combination of MTX and prednisone decreases IL-6 synthesis by the inflammatory agonists described above.

Previously, we showed that PGD_2 stimulates PKC activation through phosphoinositide hydrosis by phospholipase C (PLC) in osteoblast-like MC3T3-E1 cells[47]. Recently, we reported that PGD_2 stimulates HSP27 induction through p38 mitogen activated protein (MAP) kinase, p44/p42 MAP kinase, and stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and that PKC acts at a point upstream from these MAP kinases[48,49]. Additionally, we have reported that MTX enhances PGD_2 stimulated heat shock protein 27 (HSP27) induction at a point downstream from MAP kinases in MC3T3-E1 cells[49]. MTX by itself had no effect on the formation of inositol phosphates, on activation of PKC, or on activation of the MAP kinases. Taking these results into account, it is most likely that MTX enhances the level of PGD_2 stimulated HSP27 at a point downstream from the 3 MAP kinases. On the other hand, we previously described that TNF-α and PGD_2 upregulate the level of IL-6 synthesis through activation of PKC[23,25], and that $PGF_{2\alpha}$ upregulates the level of IL-6 synthesis through p44/p42 MAPK[27] in MC3T3-E1 cells. In addition, MTX inhibited TPA induced IL-6 synthesis in osteoblasts. Thus, it is probable that MTX suppresses inflammatory agonist induced IL-6 synthesis at a point downstream from the MAP kinases in MC3T3-E1 cells. We also tried to examine how cycloheximide, an inhibitor of protein synthesis[50], affects the inhibitory activity of MTX on PGD_2 induced IL-6 accumulation. Pretreatment with 1.0 μM cycloheximide for 20 min completely suppressed PGD_2 induced IL-6 accumulation (data not shown). We were not able to evaluate how cycloheximide affects the inhibitory action of MTX on PGD_2 induced IL-6 accumulation. In addition, these results suggest that PGD_2 stimulates *de novo* synthesis of IL-6 protein in MC3T3-E1 cells.

MTX did not affect levels of VEGF induced by $PGF_{2\alpha}$, suggesting that MTX is unlikely to correlate with VEGF synthesis, and that the suppressive effect of MTX on IL-6 synthesis is not due to a cytotoxic effect of MTX on these cells, but to its specific inhibitory action on IL-6 synthesis in these cells.

In general, MTX is administered weekly in low doses (5–20 mg/week) to patients with RA[10]. It has been reported that weekly low dose pulse MTX therapy was performed routinely in patients with RA, resulting in serum concentrations of MTX up to 0.58 ± 0.2 μM[51]. Our results suggest that MTX, in the therapeutic doses for RA, suppresses IL-6 synthesis induced by all of these inflammatory agonists in osteoblast-like MC3T3-E1 cells. Taking these findings into account, it is probable that therapeutic dose MTX induced inhibition of IL-6 synthesis in osteoblasts takes part in the preventive effect of MTX on bone resorption by osteoclasts.

Our results suggest that low dose MTX therapy may prevent bone resorption by inhibiting IL-6 synthesis induced by various inflammatory agents (TNF-α, IL-1α, PGD_2, PGE_2, and $PGF_{2\alpha}$) in osteoblasts, resulting in suppression of osteoclast formation in the diseased joint in RA. These results raise the possibility that one of the therapeutic mechanisms of MTX for RA may inhibit osteopenia through the suppression of IL-6 synthesis induced by inflammatory agents.

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SAPK/JNK Plays a Role in Transforming Growth Factor-β-induced VEGF Synthesis in Osteoblasts

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Original Basic

Abstract

We previously reported that transforming growth factor-β (TGF-β) activates p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase, resulting in the stimulation of vascular endothelial growth factor (VEGF) synthesis in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the involvement of stress-activated protein kinase/c-*Jun N*-terminal kinase (SAPK/JNK), another member of the MAP kinase superfamily, in TGF-β-induced VEGF synthesis in these cells. TGF-β markedly induced SAPK/JNK phosphorylation. SP600125, a specific inhibitor of SAPK/JNK, markedly reduced TGF-β-induced VEGF synthesis. SP600125 suppressed TGF-β-induced SAPK/JNK phosphorylation. PD98059, an inhibitor of upstream kinase of p44/p42 MAP kinase and SB203580, an inhibitor of p38 MAP kinase, each failed to reduce TGF-β-induced SAPK/JNK phosphorylation. A combination of SP600125 and PD98059 or SP600125 and SB203580 suppressed TGF-β-stimulated VEGF synthesis in an additive manner. These results strongly suggest that TGF-β activates SAPK/JNK in osteoblasts, and that SAPK/JNK plays a role in addition to p42/p44 MAP kinase and p38 MAP kinase in TGF-β-induced VEGF synthesis.

Key words
TGF-β · VEGF · SAPK/JNK · Osteoblast

Introduction

Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells [1]. VEGF, which is produced and secreted from a variety of cell types, is known to increase capillary permeability and stimulate proliferation of endothelial cells [1]. As for bone metabolism, inactivation of VEGF has been shown to cause complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate [2]. Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors such as insulin-like growth factor I and vitamin D_3 [1,3–5]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts; the former is responsible for bone formation and the latter for bone resorption [6]. During bone remodeling, capillary endothelial cells provide the microvasculature as well as osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, and migrate into the resorption lacuna. Osteoblast, osteoclast and capillary endothelial cell activity is recognized to be closely coordinated via humoral factors and direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism [7]. Thus, there is no doubt that VEGF secreted from osteoblasts plays an important role in bone metabolism regulation. It has recently

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