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 signaling of thyroid hormone, it has very 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 crucial roles in intracellular signaling of a variety of agonists (Widmann et al., 1999). The three MAP kinases, p38 MAP kinase, p44/p42 MAP kinase, and SAPK (stress-activated protein kinase)/JNK (c-*Jun* N-terminal kinase) are known as central elements used by mammalian cells to transduce the diverse messages (Widmann et al., 1999). In our recent study (Kozawa et al., 2001), we have shown that the T_3-stimulated alkaline phosphatase activity is negatively regulated by p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells (Kozawa et al., 2001). In the present study, we investigated the mechanism behind the thyroid hormone-stimulated synthesis of osteocalcin in MC3T3-E1 cells. We herein show that p38 MAP kinase but not p44/p42 MAP kinase is involved in the T_3-stimulated osteocalcin synthesis in these cells.

2. Materials and methods

2.1. Materials

T_3 was obtained from Sigma Chemical Co. (St. Louis, MO). Osteocalcin radioimmoassay (RIA) kit was obtained from Biomedical Technologies Inc. (Stoughton, MA). PD98059, U0126, SB203580 and PD169316 were purchased from Calbiochem-Novabiochem (La Jolla, CA). IGF-I was obtained from R&D Systems Inc. (Minneapolis, MN). Phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were obtained from New England BioLabs (Beverly, MA). Phospho-specific myelin basic protein (MBP) antibodies and MBP antibodies were obtained from Serotec Ltd. (Oxford, UK). 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. PD98059, U0126, SB203580 and PD169316 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.

Primary cultured mouse osteoblasts were prepared as previously described (Daci et al., 1999), with a minor modification. In brief, the calvarias of neonatal balb/c mice were aseptically dissected from neonatal mice and the soft tissues were carefully removed in phosphate-buffered saline (PBS). The calvarias were then divided into small pieces and sequentially digested with 5 ml of PBS containing 0.1% collagenase (Sigma, MO) for 5 min at 37 °C. The cells isolated in fractions 2–6 were pooled and seeded into 90 mm dishes in α-MEM containing 10% FCS at 37 °C in a humidified atmosphere of 5% CO2/95% air. The medium was exchanged at every 3 days. Confluence was reached by 5 days, and the cells were then subcultured. After trypsinization using trypsin–EDTA (0.05%/0.53 mM), the cells were rinsed three times in 10 ml of PBS. The cells (10^5) were seeded into 35 mm dishes in 2 ml of α-MEM containing 10% FCS. After the confluent, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. We confirmed that the cultured cells possessed the high alkaline phosphatase activity, a mature osteoblast phenotype (Robinson et al., 1973).

2.3. Assay for osteocalcin

The cultured cells were pretreated with PD98059 or U0126 for 60 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 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 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, and was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (Laemmli, 1970) in 10% polyacrylamide gels. To determine the endogenous phosphorylation of p38 MAP kinase or MBP induced by T_3, Western blotting was performed as described previously (Kato et al., 1996) by using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific MBP antibodies or MBP 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 SB203580, PD169316 or PD98059 for 60 min.

2.5. 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).

2.6. 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.D. of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effects of PD98059 or U0126 on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

We found that T_3 dose dependently stimulated the synthesis of osteocalcin in the range between 1 pM and 10 nM and that IGF-I by itself did not affect the levels of osteocalcin (data not shown). We have previously reported that T_3 activates p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells (Kozawa et al., 2001). To clarify the involvement of p44/p42 MAP kinase in the thyroid hormone-stimulated osteocalcin, we examined the effect of PD98059, a specific inhibitor of upstream kinase that activates p44/p42 MAP kinase (Alessi et al., 1995), on the synthesis. However, PD98059 did not affect the levels of osteocalcin (Fig. 1A). In addition, U0126, another inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Favata et al., 1998), had little effect on the T_3-stimulated osteocalcin synthesis (Fig. 1B).

3.2. Effect of T_3 on the phosphorylation of p38 MAP kinase or MBP in MC3T3-E1 cells

In order to clarify whether T_3 activates p38 MAP kinase, we examined the effect of T_3 on the phosphorylation of p38 MAP kinase. T_3 time dependently phosphorylated p38 MAP kinase in a time dependent manner (Fig. 2). Additionally, we found that T_3 induced the phosphorylation of MBP in the same experiment (Fig. 2).

3.3. Effect of SB203580 or PD169316 on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells and mouse primary cultured osteoblasts

To clarify whether p38 MAP kinase is involved in the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells, we examined the effect of SB203580, a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), on the synthesis. SB203580, which alone did not affect the basal level of osteocalcin, significantly suppressed the osteocalcin synthesis stimulated by T_3 (Fig. 3A). The inhibitory effect of SB203580 on the osteocalcin synthesis by T_3 was dose

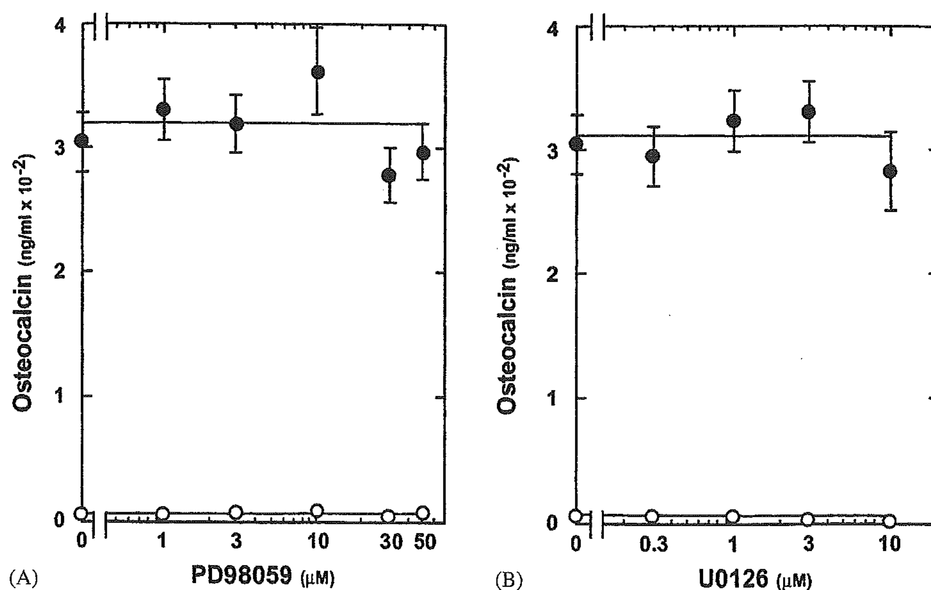


Fig. 1. Effects of PD98059 or U0126 on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 (A) or U-0126 (B) for 60 min, and then stimulated by various doses of T_3 for 72 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

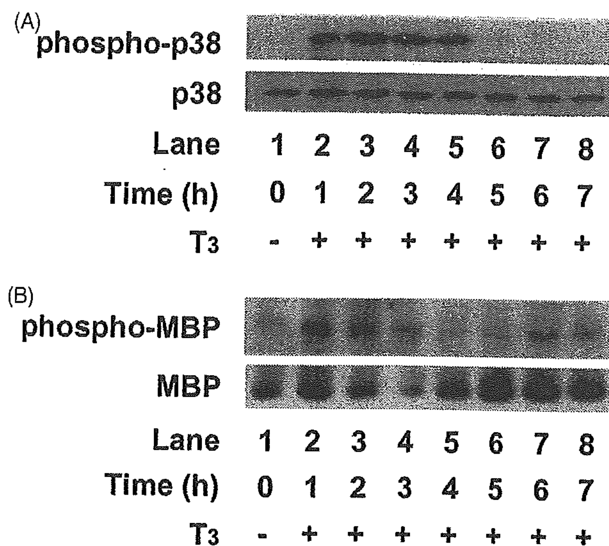


Fig. 2. Effects of T_3 on the phosphorylation of p38 MAP kinase and MBP in MC3T3-E1 cells. The cultured cells were incubated with 10 nM T_3 for the indicated periods. (A) The extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. (B) The extracts of cells were subjected to SDS–PAGE against phospho-specific MBP antibodies or MBP antibodies.

Table 1
Effects of SB203580 or PD169316 on the T_3-stimulated osteocalcin synthesis in mouse primary cultured osteoblasts

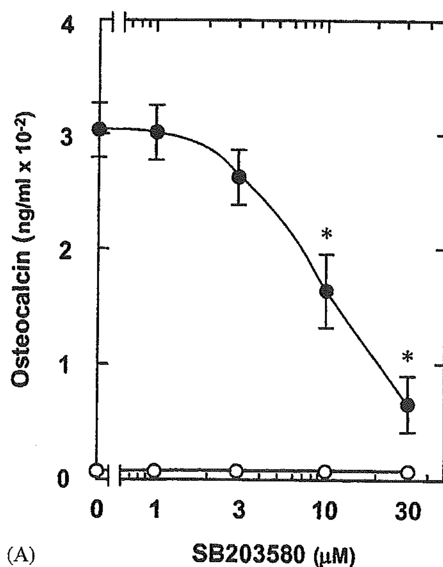
SB203580 (μM)	PD169316 (μM)	T_3	Osteocalcin (ng/ml)
0	0	−	<12.5
0	0	+	4,533 ± 514
3	0	−	<12.5
3	0	+	1,427 ± 500*
10	0	−	<12.5
10	0	+	1,093 ± 227*
30	0	−	<12.5
30	0	+	389 ± 56*
0	1	−	<12.5
0	1	+	1,227 ± 669*
0	3	−	<12.5
0	3	+	461 ± 86*
0	10	−	<12.5
0	10	+	485 ± 206*

The cultured cells were pretreated with various doses of SB203580 or PD169316 for 60 min, and then stimulated with 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.D. 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.

dependent in the range between 1 and 30 μM. The maximum effect of SB203580 was observed at 30 μM, a dose that caused about 80% reduction in the T_3-effect. In addition, the effect of PD169316, another inhibitor of p38 MAP kinase (Kummer et al., 1997), on the osteocalcin synthesis was examined. PD169316 that had little effect on the basal level of osteocalcin, reduced the T_3-stimulated osteocalcin synthesis as well as SB203580 (Fig. 3B).

We further examined the effects of SB203580 or PD169316 on the synthesis of osteocalcin induced by T_3 in mouse primary cultured osteoblasts. SB203580 significantly reduced the osteocalcin synthesis induced by T_3 (10 nM) in a dose-dependent manner in the range between 3 and 30 μM (Table 1). In addition, PD169316 dose dependently suppressed the T_3-stimulated osteocalcin synthesis in the range between 1 and 10 μM (Table 1).



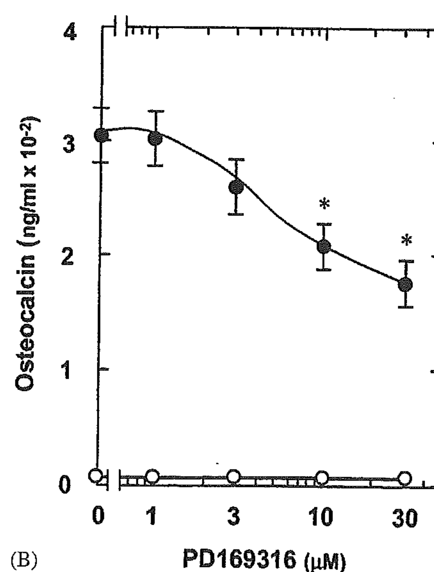


Fig. 3. Effect of SB203580 or PD169316 on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 (A) or PD169316 (B) for 60 min, and then stimulated by 10 nM T_3 for 72 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. (*) $P < 0.05$, compared with the value of T_3 alone.

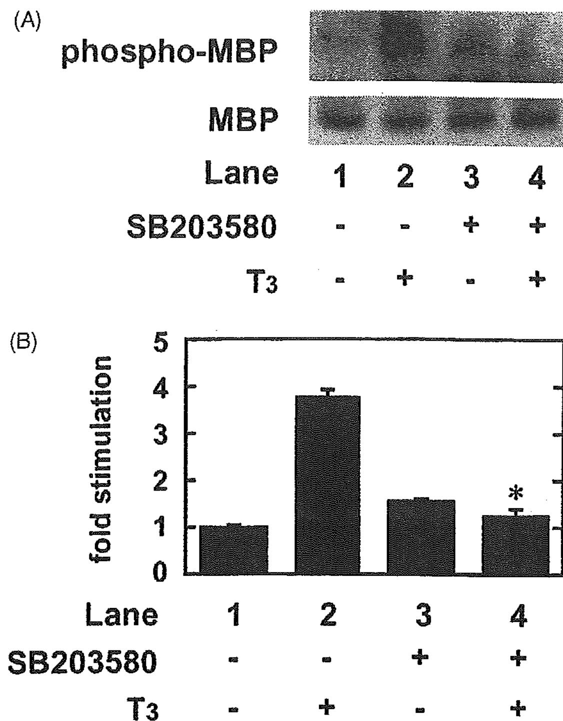


Fig. 4. Effect of SB203580 on T_3-induced phosphorylation of MBP in MC3T3-E1 cells. (A) The cultured cells were pretreated with 10 μM SB203580 for 60 min, and then stimulated by 10 nM T_3 for 1 h. The extracts of cells were subjected to SDS–PAGE against phospho-specific MBP antibodies or MBP antibodies. (B) The histogram shows quantitative representations of the levels of T_3-induced MBP phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. 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.

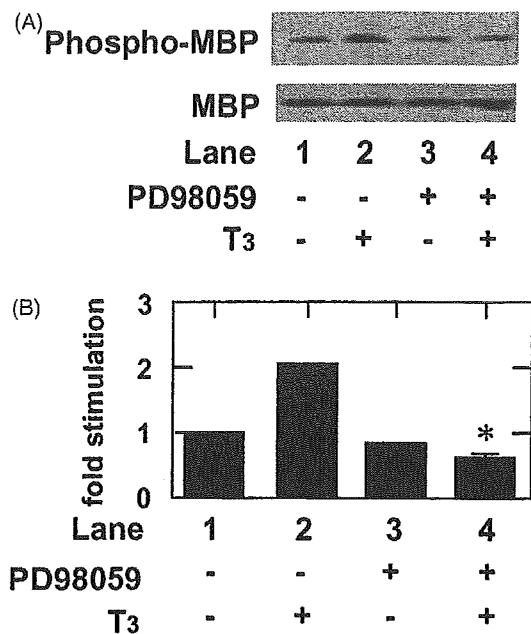


Fig. 5. Effect of PD98059 on T_3-induced phosphorylation of MBP in MC3T3-E1 cells. (A) The cultured cells were pretreated with 30 μM PD98059 for 60 min, and then stimulated by 10 nM T_3 for 1 h. The extracts of cells were subjected to SDS–PAGE against phospho-specific MBP antibodies or MBP antibodies. (B) The histogram shows quantitative representations of the levels of T_3-induced MBP phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.D. 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.

3.4. Effects of SB203580, PD169316 or PD98059 on T_3-induced phosphorylation of MBP in MC3T3-E1 cells

We found that SB203580 truly suppressed the T_3-induced phosphorylation of MBP (Fig. 4). According to the densitometric analysis, SB203580 caused about 65% reduction in the T_3-effect (Fig. 4). Furthermore, the T_3-stimulated phosphorylation of MBP was reduced by PD169316 (data not shown). In addition, we examined the effect of PD98059 on the phosphorylation of MBP induced by T_3. PD98059 significantly reduced the T_3-stimulated phosphorylation of MBP (Fig. 5).

4. Discussion

We have previously demonstrated that T_3 activates p44/p42 MAP kinase and the p44/p42 MAP kinase activated by T_3 limits T_3 itself-induced alkaline phosphatase activity, a mature osteoblast phenotype (Kasono et al., 1988), in osteoblast-like MC3T3-E1 cells (Kozawa et al., 2001). Thus, we first examined the involvement of p44/p42 MAP kinase in the T_3-induced osteocalcin synthesis in these cells. However, PD98059 (Alessi et al., 1995), failed to affect the T_3-induced synthesis. In addition, U-0126 (Favata et al., 1998), had little effect on the osteocalcin synthesis by T_3. We already showed that U-0126 suppresses the T_3-induced phosphorylation of p44/p42 MAP kinase. Taking our findings into account, it seems unlikely that p44/p42 MAP kinase is involved in thyroid hormone-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells.

In the present study, we showed that T_3 induced the phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. It is well recognized that MAP kinases are activated by phosphorylation of tyrosine and threonine residues by dual-specificity MAP kinase (Raingeaud et al., 1995; Widmann et al., 1999). Therefore, our results suggest that T_3 activates p38 MAP kinase in addition to p44/p42 MAP kinase in MC3T3-E1 cells. To the best of our knowledge, our present study probably represents the first report showing the activation of p38 MAP kinase by thyroid hormone in osteoblasts.

Thyroid hormone reportedly stimulates osteocalcin synthesis, a marker of osteoblast phenotype, independently of IGF-I production in osteoblasts including MC3T3-E1 cells (Kasono et al., 1988; Huang et al., 2000). On the other hand, it has been shown that exogenously added IGF-I induces osteocalcin gene expression in human osteosarcoma MG-63

cells and rat osteosarcoma ROS17.2.8 cells (Kudo et al., 1998; Boguslawski et al., 2000). In the present study, we confirmed that IGF-I failed to increase osteocalcin synthesis in MC3T3-E1 cells. Thus, it is possible that the discrepancy is due to the differences of cell species and their stage of differentiation. We next examined whether p38 MAP kinase is involved in the thyroid hormone-induced osteocalcin synthesis in osteoblast-like MC3T3-E1 cells. First, SB203580, a well-known inhibitor of p38 MAP kinase (Cuenda et al., 1995), significantly suppressed the T_3-stimulated synthesis of osteocalcin. These results suggest that activation of p38 MAP kinase is involved in the T_3-stimulated osteocalcin synthesis in osteoblast-like MC3T3-E1 cells. As for the specificity of SB203580, it has been reported that SB203580 at 10 to 30 μM affects c-*Jun*N-terminal kinase (JNK) or protein kinase B pathways (Lali et al., 2000). In the present study, we showed that 10 μM SB203580 significantly reduced the synthesis of osteocalcin induced by T_3. In addition, we previously reported that SB203580 fails to suppress the bFGF-induced activation of p44/p42 MAP kinase or JNK (Tokuda et al., 2000, 2003). Thus, it is probable that the inhibitory effect of SB203580 on the T_3-stimulated osteocalcin synthesis is due to the specific suppression of p38 MAP kinase in MC3T3-E1 cells. Furthermore, we showed that the T_3-stimulated synthesis of osteocalcin was reduced by PD169316, another inhibitor of p38 MAP kinase (Kummer et al., 1997). It is most likely that p38 MAP kinase is involved in the osteocalcin synthesis induced by T_3 in MC3T3-E1 cells. We confirmed that SB203580 or PD169316 significantly reduced the osteocalcin synthesis in primary cultured mouse osteoblasts. Therefore, it is probable that p38 MAP kinase is involved in T_3-stimulated osteocalcin synthesis in osteoblasts in common. In addition, it is generally recognized that MAP kinases phosphorylate MBP (Widmann et al., 1999). Herein, we showed that T_3 elicited the phosphorylation of MBP in osteoblast-like MC3T3-E1 cells. We found that the T_3-induced phosphorylation of MBP was truly inhibited by SB203580, PD169316 or PD98059, suggesting that p38 MAP kinase as well as p44/p42 MAP kinase is involved in the phosphorylation of MBP induced by T_3 in MC3T3-E1 cells. We showed here that the phosphorylation of p38 MAP kinase induced by T_3 stayed increasing after 5 h from the stimulation, however, the T_3-stimulated phosphorylation of MBP was decreased after 3 h. On the other, we previously reported that the phosphorylation of p44/p42 MAP kinase induced by T_3 is sustained up to 5 h after the stimulation (Kozawa et al., 2001). Thus, our finding suggest that MBP phosphorylation induced by T_3 is stimulated at least by both p38 MAP kinase and p44/p42 MAP kinase in MC3T3-E1 cells. In addition, it is generally recognized that protein phosphorylation is regulated by several kinases and phosphatases. Therefore, it is possible that several unknown phosphatases for MBP function after 3 h of T_3-stimulation in these cells. Further investigations would be required to clarify the details. Based on these findings as a whole, it is most likely that T_3 activates p38 MAP kinase, resulting in positively regulating the osteocalcin synthesis in osteoblasts.

These results strongly suggest that p38 MAP kinase but not p44/p42 MAP kinase takes part in the thyroid hormone-stimulated osteocalcin synthesis in osteoblasts.

Acknowledgements

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Interleukin (IL)-17 Enhances Tumor Necrosis Factor-α-Stimulated IL-6 Synthesis via p38 Mitogen-Activated Protein Kinase in Osteoblasts

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Abstract Inflammatory cytokines are well known to play crucial roles in the pathogenesis of rheumatoid arthritis. Among them, interleukin (IL)-17 is a cytokine that is mainly synthesized by activated T cells and its receptors are present in osteoblasts. The synthesis of IL-6, known to stimulate osteoclastic bone resorption, is reportedly responded to bone resorptive agents such as tumor necrosis factor-α (TNF-α) in osteoblasts. It has been reported that IL-17 enhances TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We previously showed that sphingosine 1-phosphate (S1-P) mediates TNF-α-stimulated IL-6 synthesis in these cells. In the present study, we investigated the mechanism of IL-17 underlying enhancement of IL-6 synthesis in MC3T3-E1 cells. IL-17 induced phosphorylation of p38 mitogen-activated protein (MAP) kinase. SB203580 and PD169316, specific inhibitors of p38 MAP kinase, significantly reduced the enhancement by IL-17 of TNF-α-stimulated IL-6 synthesis. IL-17 also amplified S1-P-stimulated IL-6 synthesis, and the amplification by IL-17 was suppressed by SB203580. Anisomycin, an activator of p38 MAP kinase, which alone had no effect on IL-6 level, enhanced the IL-6 synthesis stimulated by TNF-α. SB203580 and PD169316 inhibited the amplification by anisomycin of the TNF-α-induced IL-6 synthesis. Taken together, our results strongly suggest that IL-17 enhances TNF-α-stimulated IL-6 synthesis via p38 MAP kinase activation in osteoblasts. J. Cell. Biochem. 91: 1053–1061, 2004. © 2004 Wiley-Liss, Inc.

Key words: IL-17; IL-6; TNF-α; p38 MAP kinase; osteoblast

Rheumatoid arthritis is characterized by a chronic inflammation of the synovial joints and infiltration by activated T cells, macrophages, and plasma cells [Feldmann et al., 1996]. It is well known that bone resorption is increased in patients suffering from rheumatoid arthritis [Flescher et al., 1990], and that inflammatory cytokines play a crucial role in the pathogenesis of rheumatoid arthritis [Feldmann et al., 1996]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. The formation of bone structures and bone remodeling results from the coupling of bone resorption by activated osteoclasts and subsequent deposition of new matrix by osteoblasts. It is recognized that inflammatory cytokines act as autacoids in bone metabolism [Gowen, 1991; Mundy, 1993; Manolagas, 1995]. Among them, interleukin (IL)-17 is a cytokine that is mainly synthesized by activated T cells and its receptors are present in osteoblasts including osteoblast-like MC3T3-E1 cells [Yao et al., 1995; Spriggs, 1997; Bezooijen et al., 1999]. It has recently been reported that IL-17 in synovial fluids from patients with rheumatoid arthritis acts as a

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potent stimulator of osteoclastgenesis via osteoblasts [Kotake et al., 1999]. However, the exact mechanism of IL-17 in bone metabolism and osteoblasts has not yet been precisely clarified.

IL-6 is a multifunctional cytokine that has important physiological effects such as promoting B cell differentiation, T cell activation, and acute phase proteins induction [Akira et al., 1990; Snick, 1990]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and induce osteoclast formation [Ishimi et al., 1990; Roodman, 1992]. It has been reported that bone resorptive agents such as tumor necrosis factor-α (TNF-α) and IL-1 stimulate IL-6 synthesis by osteoblasts [Helle et al., 1988; Ishimi et al., 1990; Littlewood et al., 1991]. Thus, accumulating evidence indicates that IL-6 secreted from osteoblasts plays a key role as a downstream effector of bone resorptive agents. In previous studies [Kozawa et al., 1997a,c], we have shown that TNF-α stimulates IL-6 synthesis via sphingosine 1-phosphate (S1-P) produced from sphingomyelin turnover in osteoblast-like MC3T3-E1 cells. It has recently been reported that IL-17, which alone had no effect on basal IL-6 level, increases TNF-α-stimulated IL-6 synthesis in these cells [Bezooijen et al., 1999]. In the present study, we investigated the mechanism of IL-17 in mediating the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. Herein, we show that IL-17 amplifies TNF-α-induced IL-6 synthesis via p38 mitogen-activated protein (MAP) kinase activation in these cells.

MATERIALS AND METHODS

IL-17 and IL-6 ELISA kit were purchased from R&D Systems (Tokyo, Japan). TNF-α and S1-P were obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). SB203580, PD169316, and anisomycin were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) and p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) were purchased from New England BioLabs, Inc. (Beverly, MA). The ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. S1-P, SB203580, PD169316, and anisomycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which had no effect on the assay for IL-6 or Western blot analysis.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1997b]. Briefly, the cells were seeded into 35 mm (5×10^4) or 90 mm (3×10^5) diameter dishes in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (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. The pretreatment of IL-17 (0, 1, 3, 10, 30, or 100 μM) was performed for 60 min.

Measurement of IL-6

The cultured cells were stimulated by TNF-α (0 or 10 ng/ml) or S1-P (0 or 10 μM) in 1 ml of α-MEM containing 0.3% FCS, and then incubated for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was then measured by an ELISA kit. When indicated, the cells were pretreated with SB203580 (0, 0.1, 0.3, 1, 3, 10, or 30 μM) or PD169316 (0, 0.1, 0.3, 1, 3, 10, or 30 μM) for 60 min. The pretreatment of anisomycin (0, 1, 3, 10, 30, or 100 μM) was performed for 20 min.

Western Blot Analysis

Cultured cells were stimulated by IL-17 (0 or 100 μM) or anisomycin (0 or 100 μM) in serum-free α-MEM 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 the 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 gel. Western blotting analysis was performed as previously described [Miwa et al., 1999] 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 secondary antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of ECL Western blotting detection

system. When indicated, the cells were pretreated with PD169316 (0 or 30 μM) for 60 min.

Determination

The absorbance of ELISA samples was measured at 450 nm with SLT-Labinstruments EAR 340 AT. Absorbance was correlated with various concentrations. The densitometric analysis was performed using Molecular Analyst/ Macintosh (Bio-Rad Laboratories, Hercules, CA).

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 ± SEM of triplicate determinations.

RESULTS

Effect of IL-17 on p38 MAP Kinase Phosphorylation in MC3T3-E1 Cells

The MAP kinase superfamily mediates intracellularsignaling of a variety of agonists and plays pivotal roles in cellular functions [Widmann et al., 1999]. In order to clarify whether IL-17 activates p38 MAP kinase in MC3T3-E1 cells, we first examined the effect of IL-17 on the phosphorylation of p38 MAP kinase. IL-17 markedly induced the phosphorylation of p38 MAP kinase in a time-dependent manner (Fig. 1). The phosphorylation was clearly detectable after 60 min.

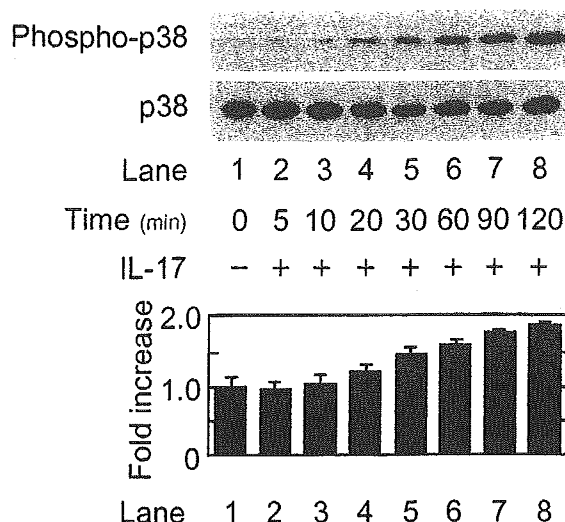

Fig. 1. Effect of interleukin (IL)-17 on the phosphorylation of p38 mitogen-activated protein (MAP) kinase in MC3T3-E1 cells. The cultured cells were stimulated by 100 μM IL-17 for 5 min (**lane 2**), 10 min (**lane 3**), 20 min (**lane 4**), 30 min (**lane 5**), 60 min (**lane 6**), 90 min (**lane 7**), and 120 min (**lane 8**). Cell extracts were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. **Lane 1**, unstimulated cells. The histogram shows quantitative representations of the levels of IL-17-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

Effects of SB203580 or PD169316 on Enhancement by IL-17 of TNF-α-Induced IL-6 Synthesis in MC3T3-E1 Cells

IL-17 reportedly increases TNF-α-induced IL-6 synthesis in MC3T3-E1 cells [Bezooijen et al., 1999]. We confirmed that IL-17 (100 μM), which alone had no effect on IL-6 synthesis, caused about 17-fold amplification in the TNF-α (10 ng/ml)-effect in these cells (Fig. 2). To investigate whether p38 MAP kinase is involved in the enhancement by IL-17 of TNF-α-induced IL-6 synthesis, the effect of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995], on the synthesis of IL-6 was examined. SB203580, which alone had little effect on IL-6 synthesis, significantly suppressed the enhancement by IL-17 of TNF-α-induced IL-6 synthesis (Fig. 2). The inhibitory effect of SB203580 on the TNF-α-induced IL-6 synthesis was dose-dependent between 0.1 and 30 μM, and the maximum effect of SB203580 was observed at 30 μM, a dose that caused about 95% reduction in the TNF-α-effect. The enhancement by IL-17 of the TNF-α-stimulated IL-6 synthesis was also reduced by PD169316, another specific inhibitor of p38 MAP kinase [Kummar et al., 1997], (Fig. 3). PD169316 caused about 95% reduction in the effect of TNF-α.

Effect of PD169316 on IL-17-Stimulated Phosphorylation of p38 MAP Kinase in MC3T3-E1 Cells

We next examined the effect PD169316 on the phosphorylation of p38 MAP kinase induced by IL-17 in MC3T3-E1 cells. PD169316, which alone little affected the phosphorylation of p38 MAP kinase, had little effect on the phosphorylation of p38 MAP kinase induced by IL-17 (Fig. 4).

Effect of IL-17 on S1-P-Stimulated IL-6 Synthesis in MC3T3-E1 Cells

We previously reported that TNF-α stimulates IL-6 synthesis via S1-P produced from

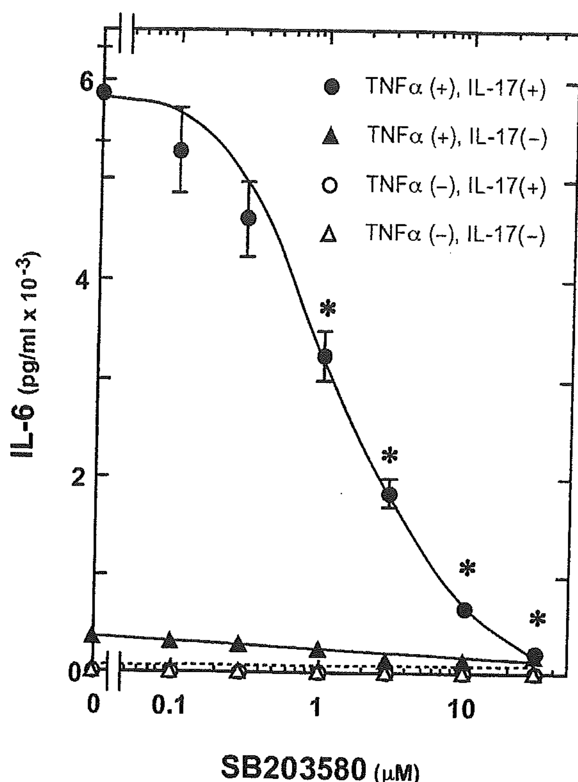

Fig. 2. Effect of SB203580 on the enhancement by IL-17 of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 for 60 min, and then stimulated by 10 ng/ml TNF-α (closed symbols) or vehicle (open symbols) in the presence of 100 μM IL-17 (circles) or vehicle (triangles) for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of TNF-α with IL-17 without SB203580.

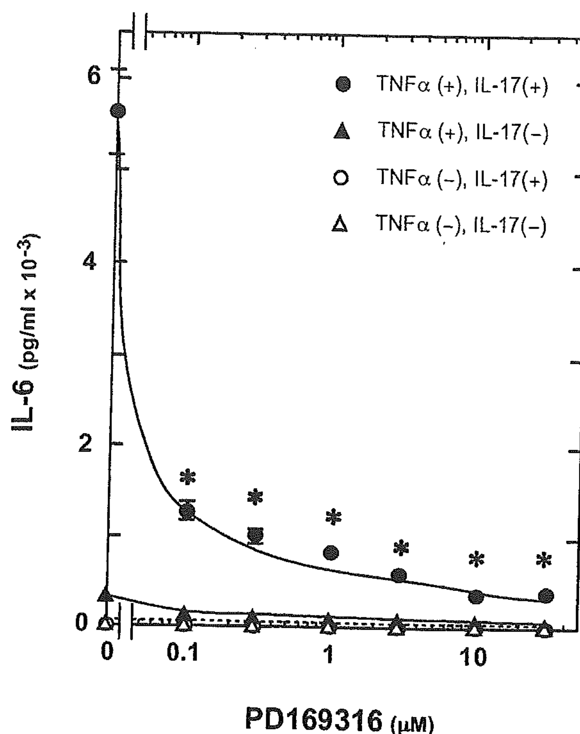

Fig. 3. Effect of PD169316 on the enhancement by IL-17 of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD169316 for 60 min, and then stimulated by 10 ng/ml TNF-α (closed symbols) or vehicle (open symbols) in the presence of 100 μM IL-17 (circles) or vehicle (triangles) for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of TNF-α with IL-17 without PD169316.

sphingomyelin turnover in MC3T3-E1 cells [Kozawa et al., 1997a,c]. Thus, we examined the effect of IL-17 on the IL-6 synthesis stimulated by S1-P. IL-17, which by itself had little effect on the level of IL-6, significantly amplified the S1-P-induced IL-6 synthesis in a dose-dependent manner in the range between 1 and 100 μM (Fig. 5).

Effect of SB203580 on the Amplification by IL-17 of S1-P-Induced IL-6 Synthesis in MC3T3-E1 Cells

To clarify whether p38 MAP kinase is involved in the amplification by IL-17 or not in MC3T3-E1 cells, we investigated the effect of SB203580 on the IL-17-induced amplification of S1-P-stimulated IL-6 synthesis. SB203580, which alone suppressed the S1-P-stimulated IL-6 synthesis, dose-dependently reduced the amplification in the range between 10 and 30 μM (Fig. 6). The maximum inhibitory effect of SB203580 on the amplification of IL-6 synthesis was observed at 30 μM, which caused about 90% reduction of the amplification.

Effect of Anisomycin on the Phosphorylation of p38 MAP Kinase in MC3T3-E1 Cells

Anisomycin is known to be an activator of p38 MAP kinase [Mahadevan and Edwards, 1991]. Thus, we examined the effect of anisomycin on the phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. Anisomycin time-dependently induced the p38 MAP kinase phosphorylation (Fig. 7). The maximum stimulatory effect of anisomycin on the level of p38 MAP kinase phosphorylation was observed at 20 min after the stimulation of anisomycin.

Effect of Anisomycin on TNF-α-Induced IL-6 Synthesis in MC3T3-E1 Cells

To determine whether the activation of p38 MAP kinase amplifies the IL-6 synthesis

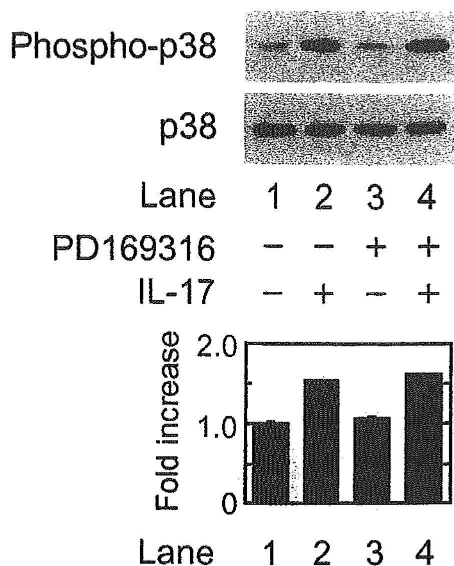

Fig. 4. Effect of PD169316 on the IL-17-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD169316 or vehicle for 60 min, and then stimulated by 100 μM IL-17 or vehicle for 120 min. Cell extracts were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of IL-17-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

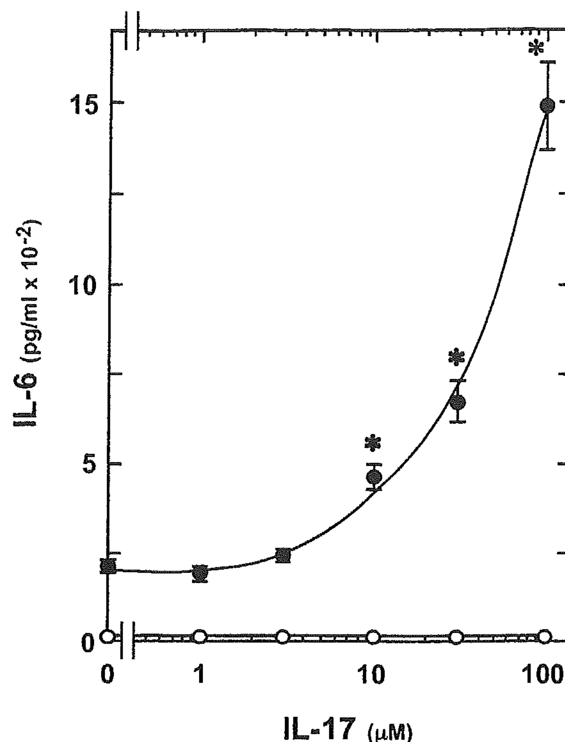

Fig. 5. Effect of IL-17 on the sphingosine 1-phosphate (S1-P)-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM S1-P (closed symbols) or vehicle (open symbols) in the presence of various doses of IL-17 for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of S1-P alone.

induced by TNF-α or not in MC3T3-E1 cells, we tested the effect of anisomycin on the TNF-α-induced IL-6 synthesis. Anisomycin, which alone had little effect on the IL-6 level, significantly enhanced the TNF-α-induced IL-6 synthesis in a dose-dependent manner in the range between 1 and 100 μM (Fig. 8). S1-P-induced IL-6 synthesis, as well as TNF-α-stimulated IL-6 synthesis, was also amplified by anisomycin (data not shown).

Effects of SB203580 or PD169316 on the Enhancement by Anisomycin of TNF-α-Induced IL-6 Synthesis in MC3T3-E1 Cells

We further examined the effects of SB203580 or PD169316 on the anisomycin-induced enhancement of TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. SB203580 dose-dependently reduced the enhancement by anisomycin in the range between 10 and 30 μM (Fig. 9). The maximum inhibitory effect of SB203580 on the enhancement was observed at 30 μM, which caused about 70% reduction. PD169316 also inhibited the enhancement of IL-6 synthesis (Table I). PD169316 (30 μM) caused about 60% reduction in the anisomycin-effect.

DISCUSSION

In the present study, we showed that IL-17 induced phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. The MAP kinase superfamily exists in ubiquitous cells, and plays crucial roles in cellular functions [Widmann et al., 1999]. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [Raingeaud et al., 1995; Widmann et al., 1999]. Therefore, our findings suggest that IL-17 activates p38 MAP kinase in MC3T3-E1 cells. It has been reported that IL-17 activates MAP kinases in human chondrocytes and macrophages [Shalom-Barak et al., 1998; Martel-Pelletier et al., 1999; Laan et al., 2001]. To our knowledge, we are the first to report the activation of p38 MAP

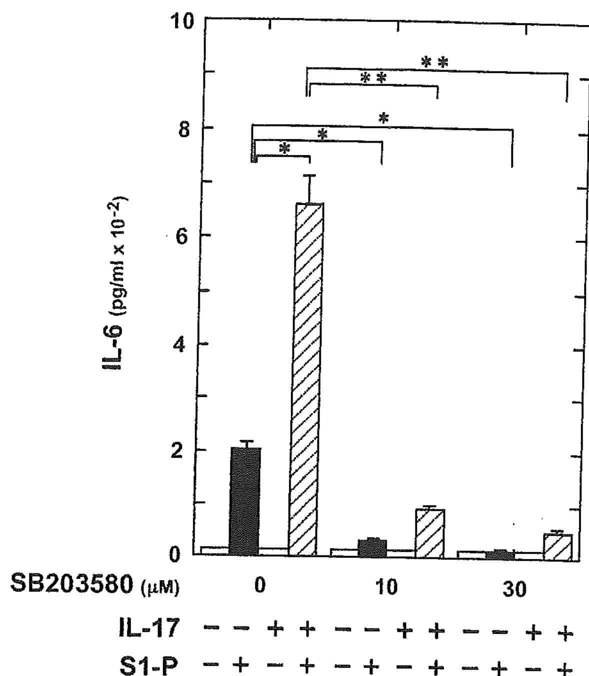

Fig. 6. Effect of SB203680 on the enhancement by IL-17 of S1-P-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 10 μM S1-P or vehicle in the presence of 100 μM IL-17 or vehicle for 24 h. *$P < 0.05$, compared to the value of S1-P alone, *$P < 0.05$, compared to the value of S1-P with IL-17.

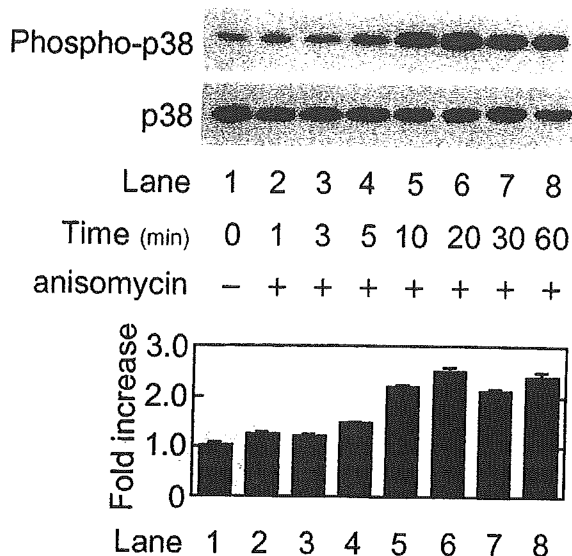

Fig. 7. Effect of anisomycin on the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 100 μM anisomycin for 1 min (**lane 2**), 3 min (**lane 3**), 5 min (**lane 4**), 10 min (**lane 5**), 20 min (**lane 6**), 30 min (**lane 7**) and 60 min (**lane 8**). Cell extracts were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. **Lane 1**, unstimulated cells. The histogram shows quantitative representations of the levels of anisomycin-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

kinase by IL-17 in osteoblasts. It is possible that the activation of p38 MAP kinase plays a role in the modulation of osteoblast cell functions by IL-17.

It has recently been reported that IL-17 enhances TNF-α-induced IL-6 synthesis in osteoblasts-like MC3T3-E1 cells [Bezooijen et al., 1999]. We previously reported that TNF-α induces sphingomyelin hydrolysis resulting in the formation of S1-P in MC3T3-E1 cells, and S1-P subsequently stimulates IL-6 synthesis [Kozawa et al., 1997a,c]. Thus, we investigated the involvement of p38 MAP kinase in the IL-17-induced amplification of the IL-6 synthesis in these cells. Herein, we showed that SB203580 or PD169316 reduced the enhancement by IL-17 of TNF-α-induced IL-6 synthesis. In addition, we demonstrated that IL-17 amplified the S1-P-induced IL-6 synthesis in these cells, and that the S1-P-stimulated IL-6 synthesis and the amplification by IL-17 were also inhibited by SB203580. Therefore, our results suggest that p38 MAP kinase activated by IL-17 positively affects TNF-α-induced IL-6 synthesis at the point downstream from the sphingomyelin hydrolysis in MC3T3-E1 cells. SB203580 or PD169316 is a specific inhibitor not for the upstream kinase of p38 MAP kinase, but for p38 MAP kinase itself [Cuenda et al., 1995; Kummar et al., 1997]. This is a probable reason why PD169316 failed to affect the phosphorylation of p38 MAP kinase induced by IL-17 in MC3T3-E1 cells. Furthermore, we showed here that anisomycin enhanced both TNF-α- and S1-P-induced IL-6 synthesis, and that SB203580 or PD169316 reduced the enhancement by anisomycin of TNF-α-induced IL-6 synthesis in these cells. We also demonstrated that anisomycin induced the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. These results suggest that activation of p38 MAP kinase by itself successfully enhanced the IL-6 synthesis stimulated by TNF-α in osteoblast-like MC3T3-E1 cells. Based on our findings as a whole, it is most likely that IL-17 enhances TNF-α-induced IL-6 synthesis through the activation of p38 MAP kinase in osteoblasts.

Under the pathological conditions characterized by the presence of activated T cells, such as rheumatoid arthritis, it is generally known to be

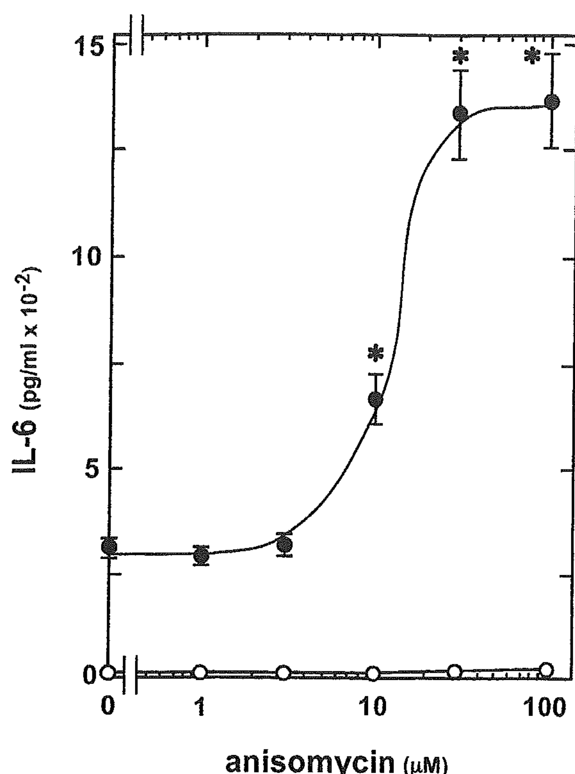

Fig. 8. Effect of anisomycin on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were stimulated by 10 ng/ml TNF-α (closed symbols) or vehicle (open symbols) in the presence of various doses of anisomycin for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of TNF-α alone.

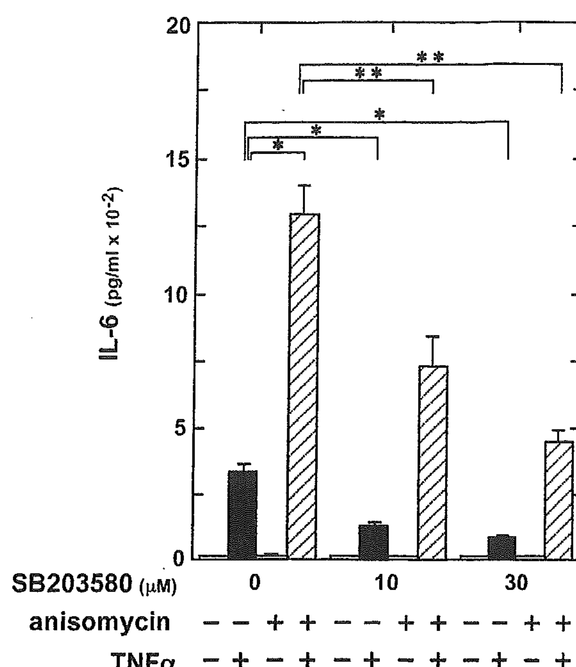

Fig. 9. Effect of SB203680 on the enhancement by anisomycin of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with SB203580 or vehicle for 60 min, and then stimulated by 10 ng/ml TNF-α or vehicle in the presence of 100 μM anisomycin or vehicle for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of TNF-α alone, **$P < 0.05$, compared to the value of TNF-α and anisomycin without SB203580.

associated with increased osteoclastic bone resorption [Flescher et al., 1990]. Activated T cells produce multiple cytokines including TNF-α, which promotes bone resorption, and they are also the exclusive source of IL-17 [Yao et al., 1995; Fossiez et al., 1996]. It seems that the IL-17-induced enhancement of TNF-α-stimulated IL-6 synthesis by osteoblasts is a worsening cycle promoting bone resorption in inflammatory bone diseases. The levels of IL-17 in synovial fluids are reportedly elevated in rheumatoid arthritis patients [Kotake et al., 1999]. Thus, it is probable that the enhancement by IL-17 of TNF-α-induced IL-6 synthesis through the activation of p38 MAP kinase in osteoblasts shown here plays a crucial role in pathological bone resorption in inflammatory bone diseases such as rheumatoid arthritis. The regulation of p38 MAP kinase activation in osteoblasts might be an important molecular target of therapeutic agent for inflammatory bone resorption.

It is well known that receptor activator of nuclear factor κB ligand (RANKL) expressed on osteoblastic cells responding to bone resorbing agents binds to its specific receptor RANK,

TABLE I. Effect of PD169316 on the Enhancement by Anisomycin of TNF-α-Induced IL-6 Synthesis in MC3T3-E1 Cells

PD169316	Anisomycin	TNF-α	IL-6 (pg/ml)
−	−	−	<15.6
−	−	+	322 ± 25
−	+	−	22 ± 3
−	+	+	1415 ± 113*
+	−	−	<15.6
+	−	+	42 ± 5*
+	+	−	<15.6
+	+	+	513 ± 81**

The cultured cells were pretreated with 30 μM PD169316 or vehicle for 60 min, and then stimulated by 10 ng/ml TNF-α or vehicle in the presence of 100 μM anisomycin or vehicle for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of TNF-α alone, **$P < 0.05$, compared to the value of TNF-α and anisomycin without PD169316.

expressed on osteoclast precursor, providing the signals necessary for osteoclast differentiation [Suda et al., 1999]. A combination of IL-6 and soluble IL-6 receptor reportedly induces RANKL expression in UAMS-32 stromal/osteoblastic cell line [O'Brien et al., 1999]. In has been reported that RANK expression is markedly increased by TNF-α in bone marrow cells containing osteoclast precursors [Komine et al., 2001]. Furthermore, it has recently been reported that overexpression of IL-17 promotes RANKL and RANK expression in synovium, resulting in increase of osteoclastic bone resorption and bone erosion in collagen-induced arthritis [Lubberts et al., 2003]. Taking these findings into account, it is likely that the enhancement by IL-17 of TNF-α-induced IL-6 synthesis in osteoblasts acts as a potent positive regulating mechanism of osteoclastic bone resorption cooperatively with RANKL/RANK pathway in the inflammatory bone resorption. Further in vivo and ex vivo investigations using osteoprotegerin, a decoy receptor of RANK which blocks the RANKL signals to osteoclast, or the antibodies for RANKL would be required to clarify the exact mechanism of pathological bone resorption in inflammatory bone diseases.

In conclusion, our present results strongly suggest that IL-17 stimulates TNF-α-induced IL-6 synthesis via p38 MAP kinase activation in osteoblasts.

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PPAR-γ ligands up-regulate basic fibroblast growth factor-induced VEGF release through amplifying SAPK/JNK activation in osteoblasts

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Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) and p44/p42 mitogen-activated protein (MAP) kinase resulting in the stimulation of vascular endothelial growth factor (VEGF) release in osteoblast-like MC3T3-E1 cells and that FGF-2-activated p38 MAP kinase negatively regulates the VEGF release. In the present study, we investigated the effects of ciglitazone and pioglitazone, peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands, on the VEGF release by FGF-2 in MC3T3-E1 cells. The FGF-2-induced VEGF release was significantly enhanced by ciglitazone. The amplifying effect of ciglitazone was dose-dependent between 0.1 and 10 μM. Pioglitazone had a similar effect on the VEGF release. GW9662, an antagonist of PPAR-γ, reduced the effects of ciglitazone and pioglitazone. Ciglitazone or pioglitazone markedly enhanced the phosphorylation of SAPK/JNK induced by FGF-2 without affecting both the FGF-2-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase. GW9662 markedly reduced the amplification by ciglitazone of the SAPK/JNK phosphorylation. Taken together, these results strongly suggest that PPAR-γ ligands up-regulate FGF-2-stimulated VEGF release resulting from amplifying activation of SAPK/JNK in osteoblasts.
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Keywords: PPAR-γ; FGF-2; VEGF; MAP kinase; Osteoblast

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone receptor superfamily [1]. PPAR-γ is a ligand-activated transcription factor that binds to specific sequences in the promoters of target genes [1]. Mesenchymal cells differentiate into several types of cells such as adipocytes and osteoblasts. It is well known that PPAR-γ plays a pivotal role in the regulation of adipocyte differentiation. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption [2]. It has been shown that PPAR-γ is expressed in osteoblasts and the activation of PPAR-γ modulates osteoblast function [3–5]. However, the exact role of PPAR-γ in osteoblasts has not been fully clarified.

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Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells [6]. It is well recognized that VEGF, which is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells [6]. As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate [7]. Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors [6,8–10]. During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. It is currently recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism [11]. Thus, there is no doubt that VEGF secreted from osteoblasts plays an important role in the regulation of bone metabolism. However, the mechanism behind VEGF synthesis in osteoblasts has not yet been fully clarified.

Basic fibroblast growth factor (FGF-2) is found in bone matrix, and osteoblasts synthesize FGF-2 [12,13]. FGF-2 expression in osteoblasts is detected during fracture repair [14]. Thus, it is recognized that FGF-2 plays a crucial role in fracture healing and bone remodeling. In our previous studies [15,16], we have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily [17], p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) participate at least partly in the VEGF release while p38 MAP kinase limits the VEGF release. In the present study, we investigated the effects of PPAR-γ ligands on the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. We here show that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release via enhancing SAPK/JNK activation in these cells.

Materials and methods

Materials. FGF-2 and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems (Minneapolis, MN). Ciglitazone, pioglitazone, and GW9662 were obtained from Calbiochem–Novabiochem (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p38 MAP kinase antibodies, and p38 MAP kinase antibodies were purchased from New England BioLabs (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Ciglitazone, pioglitazone, and GW9662 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [18] were maintained as previously described [19]. 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.

VEGF assay. The cultured cells were stimulated by FGF-2 in 1 ml α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with ciglitazone or pioglitazone for 8 h. The pretreatment of GW9662 was performed for 60 min before the addition of ciglitazone or pioglitazone. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of MAP kinases. The cultured cells were stimulated by FGF-2 in α-MEM containing 0.3% FCS 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/HCl, 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 Laemmli [20] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [21] by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, 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 means of the ECL Western blotting detection system. When indicated, the cells were pretreated with ciglitazone or pioglitazone for 8 h. The pretreatment of GW9662 was performed for 60 min before the addition of ciglitazone or pioglitazone.

Determination. The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Winooski, VT). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

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

Results

Effects of ciglitazone or pioglitazone on the VEGF release by FGF-2 in MC3T3-E1 cells

We have previously shown that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells [15]. To clarify whether PPAR-γ ligands' activation affects FGF-2-stimulated VEGF release in osteoblast-like

MC3T3-E1 cells, we first examined the effect of ciglitazone on the VEGF release. Ciglitazone, which alone had little effect on the VEGF levels, significantly enhanced the FGF-2-stimulated release of VEGF (Fig. 1). The amplifying effect of ciglitazone was dose-dependent between 1 and 10 μM (Fig. 1). Ciglitazone at 10 μM caused about 110% enhancement in the FGF-2 alone. Pioglitazone, another PPAR-γ ligand, had a similar enhancing effect on the FGF2-stimulated VEGF release (data not shown).

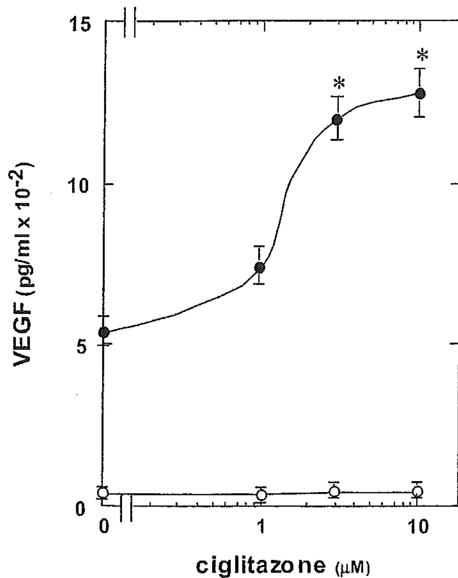

Fig. 1. Effect of ciglitazone on FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of ciglitazone for 8 h, and then stimulated by 30 ng/ml FGF-2 (closed circle) or vehicle (open circle) for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of FGF-2 alone.

Table 1
Effect of GW9662 on the enhancement by ciglitazone of the FGF-2-stimulated VEGF release in MC3T3-E1 cells

GW9662	Ciglitazone	FGF-2	VEGF (pg/ml)
–	–	–	23 ± 10
–	–	+	565 ± 51
–	+	–	45 ± 10
–	+	+	1170 ± 85*
+	–	–	25 ± 10
+	–	+	523 ± 67
+	+	–	20 ± 10
+	+	+	727 ± 49**

The cultured cells were pretreated with 20 μM GW9662 or vehicle for 60 min and then incubated by 3 μM ciglitazone for 8 h. The cells were stimulated by 30 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $p < 0.05$, compared to the value of FGF-2 alone.

** $p < 0.05$, compared to the value of FGF-2 with ciglitazone pretreatment.

Effect of GW9662 on the enhancement by ciglitazone of FGF-2-stimulated VEGF release in MC3T3-E1 cells

To investigate whether the amplifying effect of ciglitazone or pioglitazone on FGF-2-induced VEGF release is mediated through PPAR-γ in MC3T3-E1 cells, we examined the effect of GW9662, a highly specific antagonist of PPAR-γ [22], on the enhancement by ciglitazone. GW9662, which alone hardly affected the basal level of VEGF or the FGF-2-stimulated VEGF release, significantly reduced the enhancement by ciglitazone of FGF-2-induced VEGF release (Table 1). The pioglitazone-enhanced VEGF release was suppressed by GW9662 as well as ciglitazone (data not shown).

Effects of ciglitazone on the phosphorylation of p44/p42 MAP kinase and SAPK/JNK induced by FGF-2 in MC3T3-E1 cells

We have previously shown that FGF-2 stimulates VEGF release at least in part via p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells [15,16]. In order to investigate whether PPAR-γ-effect on the FGF-2-stimulated VEGF release is mediated

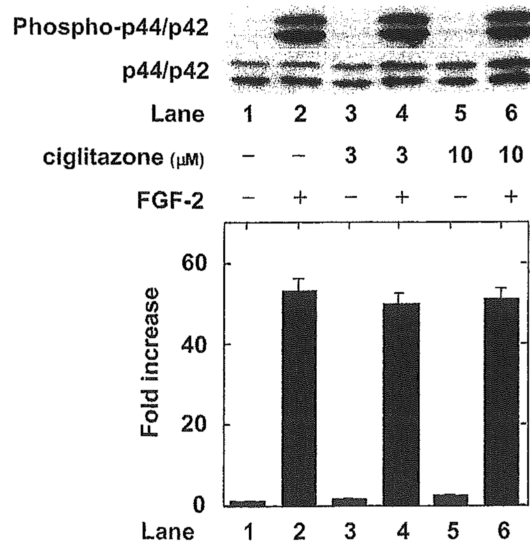

Fig. 2. Effect of ciglitazone on the phosphorylation of p44/p42 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

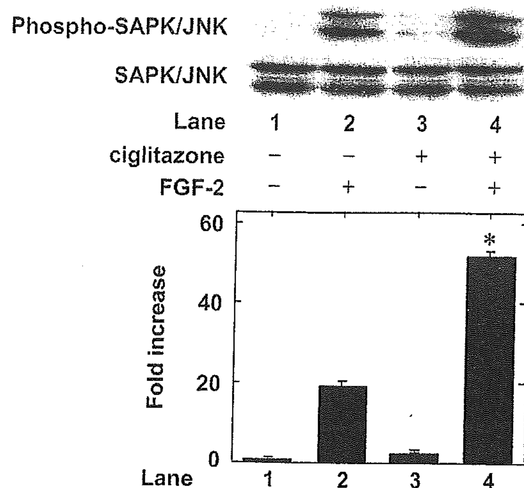

Fig. 3. Effect of ciglitazone on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 3 μM ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$, compared to the value of FGF-2 alone.

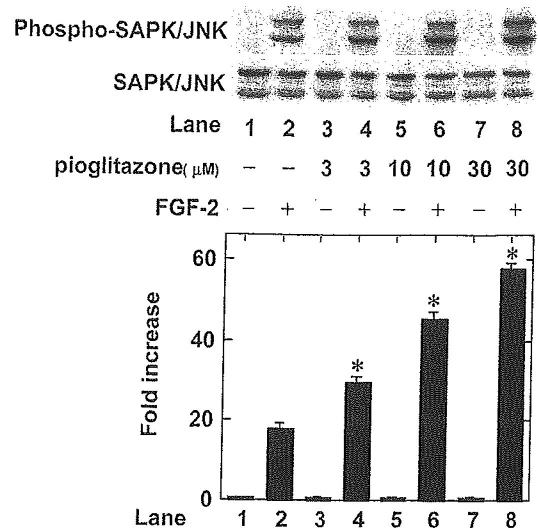

Fig. 4. Effect of pioglitazone on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of pioglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$, compared to the value of FGF-2 alone.

through p44/p42 MAP kinase activation or SAPK/JNK in MC3T3-E1 cells, we next examined the effect of ciglitazone on the FGF-2-induced phosphorylation of p44/p42 MAP kinase. However, ciglitazone failed to affect the phosphorylation of p44/p42 MAP kinase induced by FGF-2 (Fig. 2). On the other hand, the FGF-2-induced SAPK/JNK phosphorylation was markedly amplified by ciglitazone (Fig. 3). According to the densitometric analysis, ciglitazone (3 μM) caused about 70% enhancement of the FGF-2-effect on the SAPK/JNK phosphorylation. In addition, pioglitazone enhanced the SAPK/JNK phosphorylation in a dose-dependent manner (Fig. 4). According to the densitometric analysis, pioglitazone (30 μM) caused about 80% enhancement of the FGF-2-effect on the SAPK/JNK phosphorylation.

Effect of ciglitazone on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells

We have previously reported that the FGF-2-stimulated VEGF release is negatively regulated by FGF-2-activated p38 MAP kinase in MC3T3-E1 cells [15]. In order to investigate whether PPAR-γ-effect on the FGF-2-stimulated VEGF release is mediated through p38 MAP kinase activation in MC3T3-E1 cells, we next

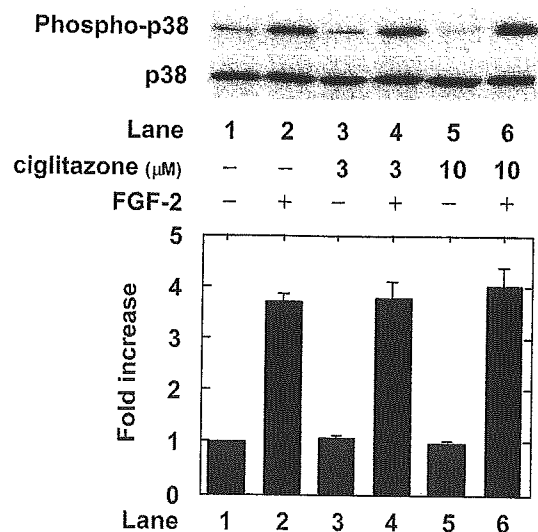

Fig. 5. Effect of ciglitazone on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

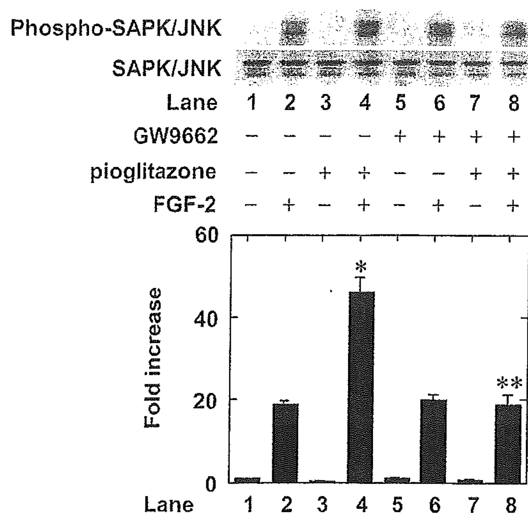

Fig. 6. Effect of GW9662 on the enhancement by pioglitazone of the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM GW9662 or vehicle for 60 min, and then incubated by 10 μM pioglitazone for 8 h. The cells were stimulated by 30 ng/ml FGF-2 or vehicle for 24 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$, compared to the value of FGF-2 alone. $**p < 0.05$, compared to the value of FGF-2 with pioglitazone pretreatment.

examined the effect of ciglitazone on the FGF-2-induced phosphorylation of p38 MAP kinase. However, ciglitazone hardly affected the FGF-2-induced phosphorylation of p38 MAP kinase (Fig. 5).

Effect of GW9662 on the enhancement by pioglitazone of FGF-2-induced SAPK/JNK phosphorylation in MC3T3-E1 cells

To clarify whether the amplifying effect of ciglitazone or pioglitazone on FGF-2-induced SAPK/JNK phosphorylation is mediated through PPAR-γ in MC3T3-E1 cells, we examined the effect of GW9662 on the enhancement by pioglitazone. GW9662, which alone hardly affected the basal level of VEGF or the FGF-2-induced SAPK/JNK phosphorylation, suppressed the enhancement by pioglitazone almost completely to the levels of FGF-2 alone (Fig. 6).

Discussion

In the present study, we demonstrated that ciglitazone, a PPAR-γ-ligand, which by itself did not affect the levels of VEGF, significantly enhanced the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. In addition, pioglitazone, another ligand of PPAR-γ, amplified the VEGF release as well as ciglitazone. Thus, our findings suggest that the FGF-2-stimulated VEGF release is enhanced via the activation of PPAR-γ. To clarify whether the effects of ciglitazone and pioglitazone are mediated through PPAR-γ activation in MC3T3-E1 cells, we next examined the effect of GW9662, a PPAR-γ antagonist [22], on the amplification by ciglitazone. GW9662 significantly suppressed the ciglitazone-induced enhancement of VEGF release while it failed to affect the FGF-2-stimulated VEGF release. Taking these findings into account, it is most likely that activation of PPAR-γ amplifies the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells.

It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and cell death in a variety of cells [17]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce the diverse messages [17]. In our previous studies [15,16], we have shown that FGF-2 activates three MAP kinases in osteoblast-like MC3T3-E1 cells, and p44/p42 MAP kinase and SAPK/JNK act as positive regulators in FGF-2-induced VEGF release while p38 MAP kinase negatively regulates the VEGF release. In the present study, we showed that ciglitazone did not affect the FGF-2-induced phosphorylation of p44/p42 MAP kinase. Thus, it seems unlikely that ciglitazone amplified the FGF-2-induced VEGF release through up-regulating the activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. In addition, ciglitazone had little effect on the FGF-2-induced p38 MAP kinase phosphorylation. Therefore, it seems unlikely that the ciglitazone-induced enhancement of FGF-2-stimulated VEGF release is due to the inhibition of p38 MAP kinase activation.

On the contrary, we showed that the FGF-2-induced SAPK/JNK phosphorylation was markedly amplified by ciglitazone. Furthermore, pioglitazone dose-dependently strengthened the SAPK/JNK phosphorylation as well as ciglitazone. These results suggest that the PPAR-γ activation up-regulates the FGF-2-stimulated activation of SAPK/JNK. We next demonstrated that GW9662 [22] did not affect the SAPK/JNK phosphorylation induced by FGF-2 alone, but markedly suppressed the enhancement by pioglitazone almost to the levels of FGF-2 alone. Therefore, it is probable that the amplification in the SAPK/JNK phosphorylation is mediated through the activation of PPAR-γ. Based on our findings as a whole, it is most likely that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release through enhancing the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. Further