growth factor β stimulate the induction of HSP27 in osteoblast-like MC3T3-E1 cells [6,7]. However, the exact mechanism behind the HSP27 induction in osteoblasts and its roles have not yet been precisely clarified.

Prostaglandin (PG) $F_{2\alpha}$, known as a potent bone resorptive agent, stimulates the proliferation and inhibits the differentiation of osteoblasts [8]. We have previously reported that $PGF_{2\alpha}$ activates phosphoinositide-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase D (PC-PLD) in osteoblast-like MC3T3-E1 cells, resulting in the protein kinase C activation [9,10]. In addition, we have reported that $PGF_{2\alpha}$ stimulates the induction of HSP27 through the PKC-dependent activation of p44/p42 mitogen-activated protein (MAP) kinase in these cells [11]. In the present study, we investigated the involvement of SAPK/JNK in the $PGF_{2\alpha}$-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. We here show that SAPK/JNK in addition to p44/p42 MAP kinase acts as a positive regulator in $PGF_{2\alpha}$-induced HSP27 in these cells.

2. Materials and methods

2.1. Materials

$PGF_{2\alpha}$ was purchased from Sigma (St. Louis, MO). SP600125 and PD98059 were obtained from Calbiochem–Novabiochem Co. (La Jolla, CA). Phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies were obtained from New England BioLabs (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham, Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SP600125 and PD98059 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect immunoassay for HSP27, Northern blot analysis or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells which have been derived from newborn mouse calvaria [12] were maintained as previously described [13]. 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 (5×10^4 cells/dish) or 90-mm diameter dishes (5×10^5 cells/dish) 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. When indicated, the cells were pretreated with SP600125 or PD98059 for 60 min.

2.3. Immunoassay of HSP27

The concentration of HSP27 in soluble extracts of the cells was determined by means of a sandwich-type enzyme immunoassay, as described previously [14]. The cultured cells were stimulated by $PGF_{2\alpha}$ for the indicated periods in 1 ml of α-MEM containing 0.3% FCS. The cells were washed twice with 1 ml of phosphate-buffered saline and frozen at −80°C for a few days before analysis. The frozen cells on each dish were collected and suspended in 0.3 ml of phosphate-buffered saline, and then each suspension was sonicated and centrifuged at 125,000 *g* for 20 min at 4°C. The supernatant was used for the immunoassay that employs polystyrene balls (3.2 mm in diameter; Immuno Chemicals, Okayama, Japan) carrying immobilized $F(ab')_2$ fragments of antibody and the same Fab′ fragments labeled with β-D-galactosidase from *Escherichia coli*. A polystyrene ball carrying antibodies was incubated either with the purified standard for HSP27 or with an aliquot of the samples. The incubation was carried out at 30°C for 5 h in a final volume of 0.5 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl, 0.5% hydrolyzed gelatin, 0.1% bovine serum albumin (BSA), 1 mM $MgCl_2$, and 0.1% NaN_3. After being washed, each ball was incubated at 4°C overnight with 1.5 mU of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside.

2.4. Isolation of RNA and Northern blotting analysis of HSP27

The cultured cells were stimulated by $PGF_{2\alpha}$ in serum-free α-MEM for the indicated periods. Total RNA was isolated using a QuickPrep Total RNA Extraction kit (Pharmacia Biotech, Tokyo, Japan). Next, 20 μg of total RNA were subjected to electrophoresis on a 0.9% agarose-2.2 M formaldehyde gel and were blotted onto a nitrocellulose membrane. For Northern blot analysis, membrane was allowed to hybridize with a cDNA probe that had been labeled with a multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously [15]. A *Bam*HI–*Hind*III fragment of cDNA for mouse HSP27 [14] was kindly provided by Dr. L.F. Cooper of the University of North Carolina.

2.5. Western blot analysis of SAPK/JNK and p44/p42 MAP kinase

The cultured cells were stimulated by $PGF_{2\alpha}$ 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 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 [16] in 10% polyacrylamide gels. Western blotting was performed as described previously [15] by using phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies or p44/p42 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.

2.6. Determination

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

2.7. Statistical analysis

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

3. Results

3.1. Effect of $PGF_{2\alpha}$ on the phosphorylation of SAPK/JNK in MC3T3-E1 cells

To clarify whether $PGF_{2\alpha}$ activates SAPK/JNK in osteoblast-like MC3T3-E1 cells, we examined the effect of $PGF_{2\alpha}$ on the SAPK/JNK phosphorylation. $PGF_{2\alpha}$ markedly induced the phosphorylation of SAPK/JNK in a time-dependent manner up to 30 min (Fig. 1). The maximum effect of $PGF_{2\alpha}$ on the SAPK/JNK phosphorylation was observed at 20 min after the stimulation.

3.2. Effect of SP600125 on the $PGF_{2\alpha}$-stimulated HSP27 accumulation in MC3T3-E1 cells

To investigate whether SAPK/JNK is involved in the $PGF_{2\alpha}$-stimulated HSP27 induction in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of SAPK/JNK [17], on the accumulation of HSP27. SP600125, which alone did not affect the HSP27 accumulation, significantly suppressed the $PGF_{2\alpha}$-stimulated HSP27 accumulation (Fig. 2). The inhibitory effect of SP600125 on the $PGF_{2\alpha}$-stimulated HSP27 accumulation was dose-dependent in the range between 0.1 and 30 μM (Fig. 3). The maximum effect of SP600125 on the HSP27 accumulation was observed at

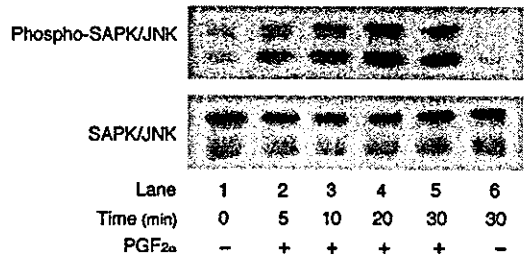

Fig. 1. Effect of $PGF_{2\alpha}$ on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. Lane 1, unstimulated cells.

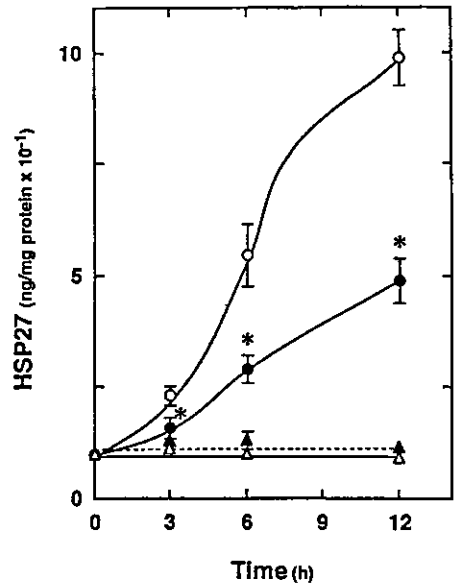

Fig. 2. Effect of SP600125 on the $PGF_{2\alpha}$-stimulated accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 (closed symbols) or vehicle (open symbols) for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (circles) or vehicle (triangles) for the indicated periods. 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 $PGF_{2\alpha}$ alone.

30 μM, which caused about 60% reduction in the $PGF_{2\alpha}$-effect.

3.3. Effect of SP600125 on $PGF_{2\alpha}$-increased level of HSP27 mRNA in MC3T3-E1 cells

We previously showed that the maximum effect of $PGF_{2\alpha}$ on the level of HSP27 mRNA is observed at 2 h after the stimulation [11]. We next examined the effect of SP600125 on the $PGF_{2\alpha}$-increased level of mRNA for HSP27 in MC3T3-E1 cells. SP600125 markedly reduced the $PGF_{2\alpha}$-increased level of HSP27 mRNA (Fig. 4).

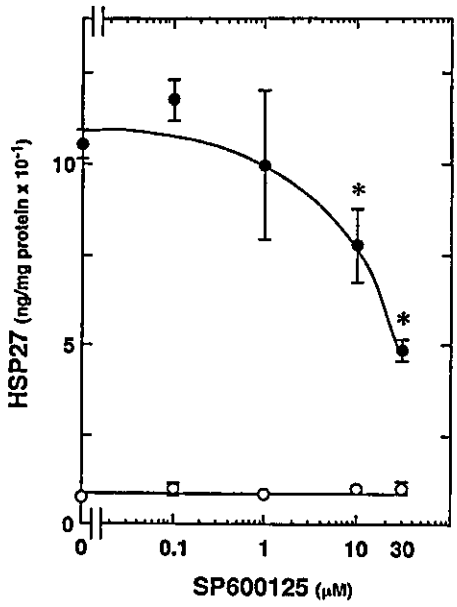

Fig. 3. Dose-dependent effect of SP600125 on the $PGF_{2\alpha}$-stimulated accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (closed symbols) or vehicle (open symbols) for 12 h. Each value 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 $PGF_{2\alpha}$ alone.

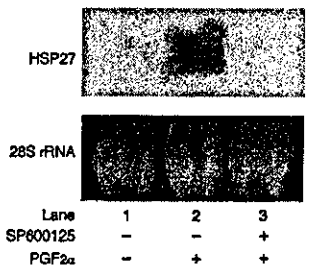

Fig. 4. Effect of SP600125 on the $PGF_{2\alpha}$-increased levels of the mRNA for HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 2 h. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with cDNA probe for HSP27. Bands of 28S rRNA are shown for reference.

3.4. Effects of SP600125 on the phosphorylation of SAPK/JNK or p44/p42 MAP kinase induced by $PGF_{2\alpha}$ in MC3T3-E1 cells

We found that SP600125 truly inhibited the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$ (Fig. 5). According to the densitometric analysis, SP600125 almost completely reduced the $PGF_{2\alpha}$-effect on the SAPK/JNK phosphorylation. However, SP600125 had little effect on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase (Fig. 6).

3.5. Effects of PD98059 on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells

PD98059, an inhibitor of the upstream kinase of p44/p42 MAP kinase [18], significantly reduced the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase (Fig 7). On the other hand, PD98059 had little effect

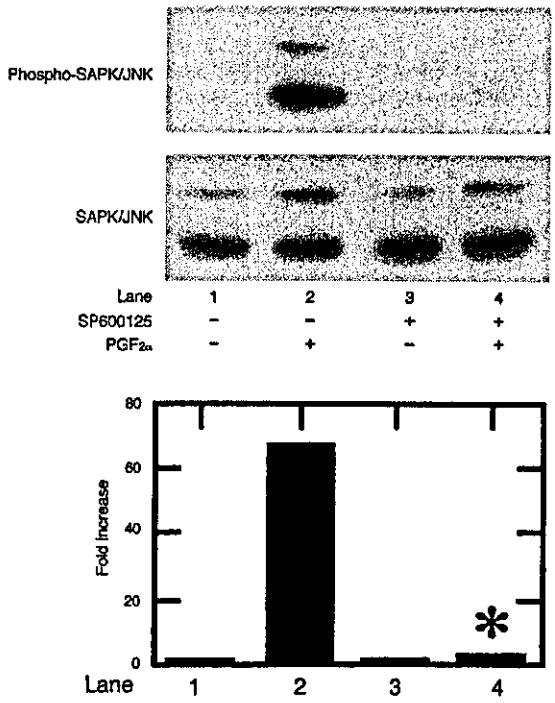

Fig. 5. Effect of SP600125 on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-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 with the value of $PGF_{2\alpha}$ alone.

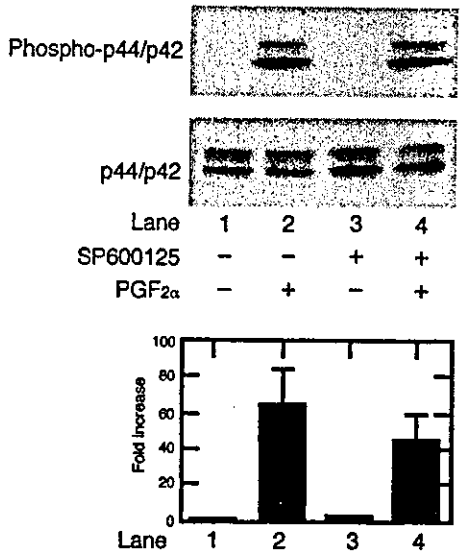

Fig. 6. Effect of SP600125 on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to 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 $PGF_{2\alpha}$-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.

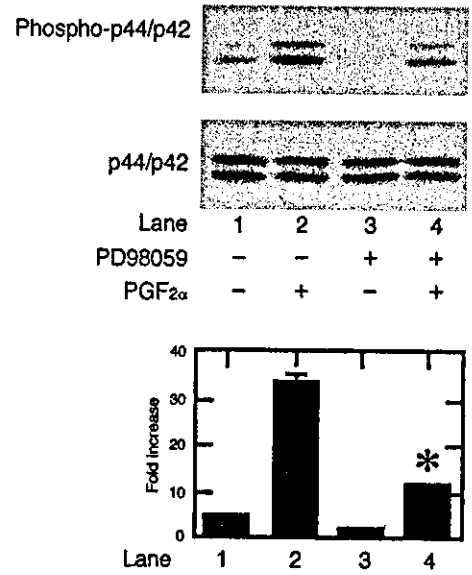

Fig. 7. Effect of PD98059 on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE 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 $PGF_{2\alpha}$-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 with the value of $PGF_{2\alpha}$ alone.

on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK (Fig. 8).

4. Discussion

It is well known that the MAP kinase superfamily mediates intracellular signaling of various agonists and plays pivotal role in cellular functions including proliferation, differentiation and cell death in a variety of cells [19]. Three major MAP kinases such as p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are recognized to transduce signals in mammalian cells [19]. We have previously reported that $PGF_{2\alpha}$-stimulates HSP27 induction through the activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells [11]. Therefore, we conducted the present study to investigate whether SAPK/JNK plays a role in $PGF_{2\alpha}$-stimulated HSP27 induction in these cells.

Herein, we demonstrated that $PGF_{2\alpha}$ elicited the phosphorylation of SAPK/JNK in osteoblasts-like MC3T3-E1 cells. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [19, 20]. Thus, it is most likely that $PGF_{2\alpha}$ activates SAPK/JNK in MC3T3-E1 cells. This is probably the

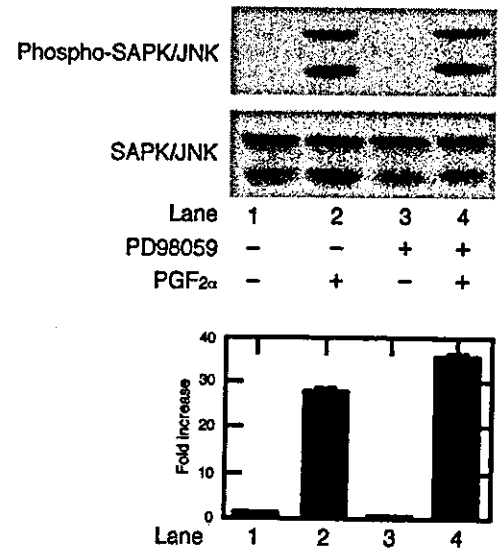

Fig. 8. Effect of PD98059 on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-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.

first report showing the activation by $PGF_{2\alpha}$ of SAPK/JNK in osteoblasts-like cells as far as we know. We also showed that SP600125 [17] significantly reduced the $PGF_{2\alpha}$-stimulated accumulation of HSP27 in MC3T3-E1 cells. Therefore, our findings suggest that SAPK/JNK is involved in the HSP27 induction by $PGF_{2\alpha}$ in these cells. In addition, we demonstrated that the phosphorylation induced by $PGF_{2\alpha}$ was truly suppressed by SP600125 in these cells. Furthermore, we found that SP600125 hardly affected the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ in these cells. Thus, it is probable that SP600125 suppressed the accumulation of HSP27 induced by $PGF_{2\alpha}$ via the attenuation of not p44/p42 MAP kinase but SAPK/JNK in MC3T3-E1 cells. Additionally, the $PGF_{2\alpha}$-increased level of HSP27 mRNA was suppressed by SP600125. Taking our findings into account, it is most likely that SAPK/JNK plays a part in the $PGF_{2\alpha}$-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells.

We previously reported that PD98059 [18] attenuates the $PGF_{2\alpha}$-induced HSP27 accumulation in these cells. However, we found that PD98059, which significantly reduced the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$, did not affect the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$. Our results suggest that the attenuation by PD98059 of the $PGF_{2\alpha}$-induced HSP27 accumulation results from the suppression of not SAPK/JNK but p44/p42 MAP kinase activation in osteoblasts-like MC3T3-E1 cells. Based on our results as a whole, it is most likely that the HSP27 induction stimulated by $PGF_{2\alpha}$ is mediated through the activation of both p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. It is well known that the low-molecular-weight HSPs such as HSP27, as well as the high-molecular-weight HSPs such as HSP90 and HSP70, act as molecular chaperones in protein folding, oligomerization and translocation [1]. It is possible that $PGF_{2\alpha}$-induced accumulation of HSP27 plays a role in the changes of cellular function such as secretion, proliferation or differentiation in osteoblasts. To coordinate them, SAPK/JNK and p44/p42 MAP kinase seems to regulate cooperatively the induction of HSP27. In addition, it has been reported that the upregulation of HSP27 plays a role in cell survival pathways, resulting in increase of the resistance to apoptosis [21]. Taking these findings into account, it is most likely that $PGF_{2\alpha}$-increased HSP27 through the activation of SAPK/JNK in addition to p44/p42 MAP kinase finely coordinates the $PGF_{2\alpha}$-provocated cellular events to minimize the damage in osteoblasts. Further investigations would be required to clarify the exact roles of HSP27 in osteoblasts.

In conclusion, our present results strongly suggest that $PGF_{2\alpha}$-activated SAPK/JNK in addition to p44/p42 MAP kinase plays a part in $PGF_{2\alpha}$-induced HSP27 in osteoblasts.

Acknowledgements

This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Y. Okamoto for her skillful secretarial assistance.

References

[1] I.J. Benjamin, D.R. McMillan, Stress (heat shock) proteins molecular chaperones in cardiovascular biology and diseases, Circ. Res. 83 (1998) 117–132.
[2] P.J. Nijweide, E.H. Burger, J.H. Feye, Cells of bone: proliferation, differentiation, and hormonal regulation, Physiol. Rev. 66 (1986) 855–886.
[3] H. Yasuda, N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, N. Takahashi, T. Suda, Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastgenesis-inhibitory factor and is identical to TRANCE/RANKL, Proc. Natl. Acad. Sci. USA 95 (1998) 3597–3602.
[4] A.R. Shakoori, A.M. Oberdorf, T.A. Owen, L.A. Weber, E. Hickey, J.L. Stein, J.B. Lian, G.S. Stein, Expression of heat shock genes during differentiation of mammalian osteoblasts and promyelocytic leukemia cells, J. Cell. Biochem. 48 (1992) 277–287.
[5] L.F. Cooper, K. Uoshima, Differential estrogenic regulation of small M(r) heat shock protein expression in osteoblasts, J. Biol. Chem. 269 (1994) 7869–7873.
[6] H. Kawamura, T. Otsuka, H. Matsuno, M. Niwa, N. Matsui, K. Kato, T. Uematsu, O. Kozawa, Endothelin-1 stimulates heat shock protein 27 induction in osteoblasts: involvement of p38 MAP kinase, Am. J. Physiol. 277 (1999) E1046–E1054.
[7] D. Hatakeyama, O. Kozawa, M. Niwa, H. Matsuno, H. Ito, K. Kato, N. Tatematsu, T. Shibata, T. Uematsu, Upregulation by retinoic acid of transforming growth factor-β-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases, Biochim. Biophys. Acta. 1589 (2002) 15–30.
[8] C.C. Pilbeam, J.R. Harrison, L.G. Raisz, Prostaglandins and bone metabolism, in: J.P. Bilezikian, L.G. Raisz, G.A. Rodan (Eds.), Principles of Bone Biology, Academic Press, San Diego, 1996, pp. 715–728.
[9] M. Miwa, H. Tokuda, K. Tsushita, J. Kotoyori, Y. Takahashi, N. Ozaki, O. Kozawa, Y. Oiso, Involvement of pertussis toxin-sensitive GTP-binding protein in prostaglandin $F_{2\alpha}$-induced phosphoinositide hydrolysis in osteoblast-like cells, Biochem. Biophys. Res. Commun. 171 (1990) 1229–1235.
[10] O. Kozawa, A. Suzuki, J. Kotoyori, H. Tokuda, Y. Watanabe, Y. Ito, Y. Oiso, Prostaglandin $F_{2\alpha}$ activates phospholipase D independently from activation of protein kinase C in osteoblast-like cells, J. Cell. Biochem. 55 (1994) 373–379.
[11] O. Kozawa, H. Tokuda, M. Miwa, H. Ito, H. Matsuno, M. Niwa, K. Kato, T. Uematsu, Involvement of p44/p42 mitogen-activated protein kinase in prostaglandin $F_{2\alpha}$-stimulated induction of heat shock protein 27 in osteoblasts, J. Cell. Biochem. 75 (1999) 610–619.
[12] H. Sudo, H. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, J. Cell Biol. 96 (1983) 191–198.

[13] O. Kozawa, H. Tokuda, M. Miwa, J. Kotoyori, Y. Oiso, Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells, Exp. Cell Res. 198 (1992) 130–134.

[14] Y. Inaguma, S. Goto, H. Shinohara, K. Ohshima, K. Kato, Physiological and pathological changes in levels of the two small stress proteins, HSP27 and αB-crystallin in rat hindlimb muscles, J. Biochem. 114 (1993) 378–384.

[15] K. Kato, H. Ito, K. Hasegawa, Y. Inaguma, O. Kozawa, T. Asano, Modulation of the stress-induced synthesis of HSP27 and αB-crystallin by cyclic AMP in C6 rat glioma cells, J. Neurochem. 66 (1996) 946–950.

[16] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[17] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase, Proc. Natl. Acad. Sci. USA 98 (2001) 13681–13686.

[18] D.R. Alessi, A. Cuenda, P. Cohen, D.T. Dudley, A.R. Saltiel, PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo, J. Biol. Chem. 270 (1995) 27489–27494.

[19] C. Widmann, S. Gibson, M.B. Jarpe, G.L. Johnson, Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human, Physiol. Rev. 79 (1999) 143–180.

[20] J. Raingeaud, S. Gupta, J.S. Rogers, M. Dickens, J. Han, R.J. Ulevitch, R.J. David, Pro-inflammatory cytokines and enviromental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine, J. Biol. Chem. 270 (1995) 7420–7426.

[21] M.J.D. Wagstaff, Y. Collaco-Moraes, J. Smith, J.S. de Belleroche, R.S. Coffin, D.S. Latchman, Protection of neuronal cells from apoptosis by HSP27 derived with a herpes simplex virus based vector, J. Biol. Chem. 274 (1999) 5061–5069.

ELSEVIER

Molecular and Cellular Endocrinology 214 (2004) 189–195

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Activation of p38 mitogen-activated protein kinase mediates thyroid hormone-stimulated osteocalcin synthesis in osteoblasts

A. Ishisaki [a], H. Tokuda [a,b], M. Yoshida [a], K. Hirade [a], K. Kunieda [a], D. Hatakeyama [a,c], T. Shibata [c], O. Kozawa [a,*]

[a] *Division of Cellular and Molecular Biology, Department of Pharmacology, Gifu University School of Medicine, Gifu 500-8705, Japan*
[b] *Department of Internal Medicine, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Aichi, Japan*
[c] *Division of Organ Pathobiology, Department of Oral and Maxillofacial Sciences, Gifu University School of Medicine, Gifu, Japan*

Received 29 January 2003; accepted 14 October 2003

Abstract

It is well known that thyroid hormone modulates osteoblast cell function. We have previously shown that triiodothyronine (T_3) activates p44/p42 mitogen-activated protein (MAP) kinase, which limits T_3-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether p44/p42 MAP kinase or p38 MAP kinase is involved in the thyroid hormone-stimulated osteocalcin synthesis in these cells. T_3 markedly induced the phosphorylation of p38 MAP kinase in addition to p44/p42 MAP kinase. PD98059 and U0126, inhibitors of the upstream kinase that activates p44/p42 MAP kinase, had little effect on the T_3-induced synthesis of osteocalcin. On the contrary, the T_3-induced osteocalcin synthesis was significantly reduced by SB203580 and PD169316, inhibitors of p38 MAP kinase. SB203580, PD169316 or PD98059 suppressed the T_3-phosphorylation of myelin basic protein. T_3-induced osteocalcin synthesis was significantly reduced by SB203580 or PD169316 also in primary cultured mouse 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.

Keywords: Thyroid hormone; Osteocalcin; Mitogen-activated protein kinase; Osteoblasts

1. Introduction

It is generally known that thyroid hormone is a crucial regulator of skeletal function, resulting in modulating bone metabolism and hyperthyroidism is a major cause of 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 recognized that both increased bone resorption and decreased bone formation contribute to the loss of bone mass by hyperthyroidism (Stern, 1996). Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts (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 shown that thyroid hormone stimulates alkaline phosphatase activity and secretion of osteocalcin and insulin-like growth factors (IGFs) in osteoblasts and that it modulates proliferation of osteoblasts (Rizzoli et al., 1986; Kasono et al., 1988; Stern, 1996). IGF-I production is reportedly essential for the mitogenic effect of thyroid hormone but not for the stimulatory effects of thyroid hormone on alkaline phosphatase activity and osteocalcin synthesis in osteoblasts (Huang et al., 2000). In a previous study (Tokuda et al., 1998), we have reported 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 precisely clarified.

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

* Corresponding author. Tel.: +81-58-267-2233; fax: +81-58-267-2959.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

0303-7207/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2003.10.049

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

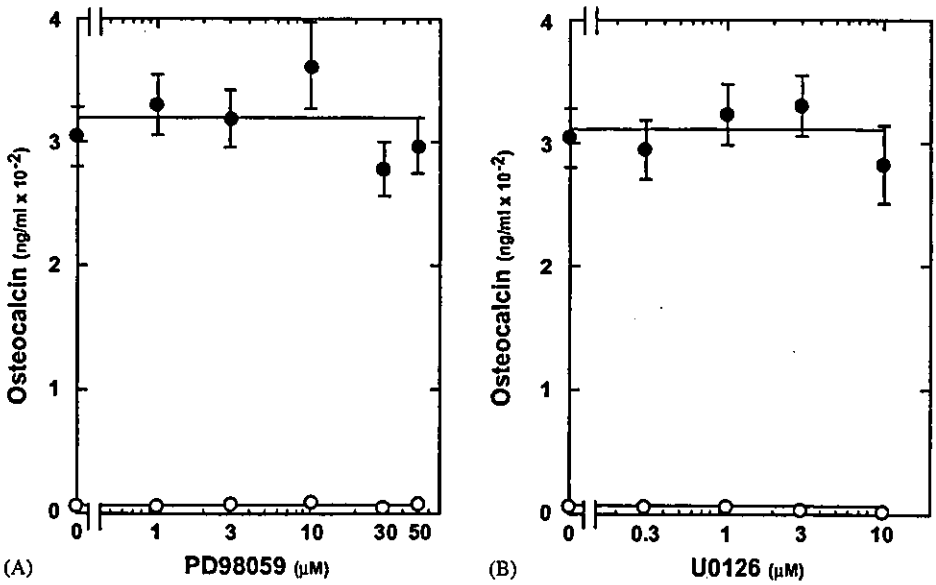

Fig. 1. Effects of PD98059 or U0126 on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 (A) or U-0126 (B) for 60 min, and then stimulated by various doses of T_3 for 72 h. Each value represents the mean ± S.D. of triplicate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

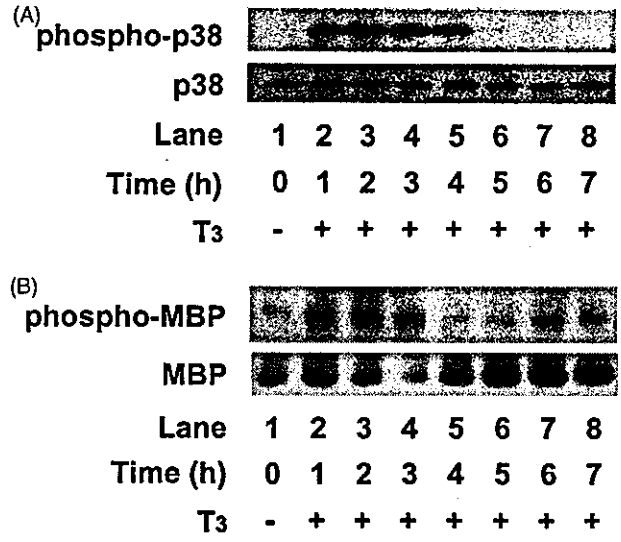

Fig. 2. Effects of T_3 on the phosphorylation of p38 MAP kinase and MBP in MC3T3-E1 cells. The cultured cells were incubated with 10 nM T_3 for the indicated periods. (A) The extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies.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

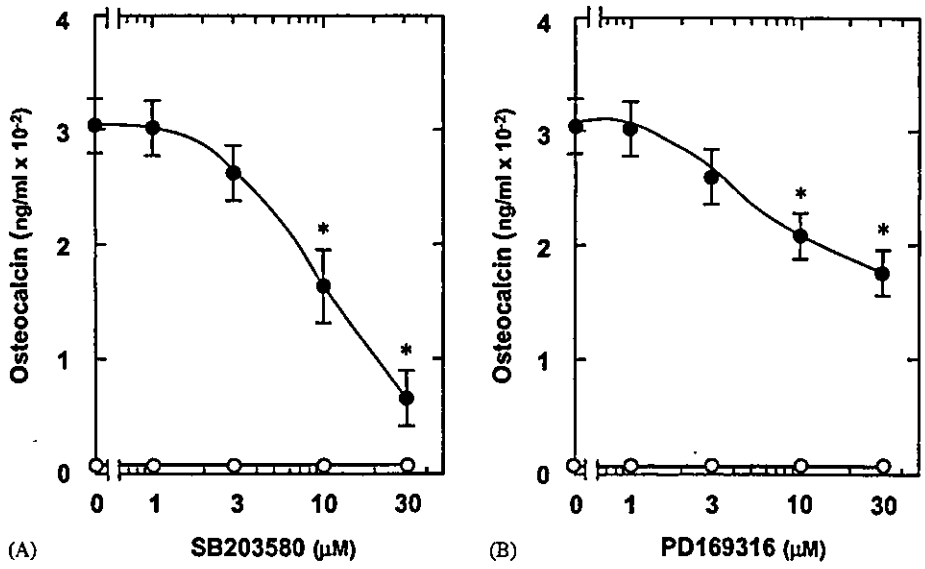

Fig. 3. Effect of SB203580 or PD169316 on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 (A) or PD169316 (B) for 60 min, and then stimulated by 10 nM T_3 for 72 h. Each value represents the mean ± S.D. of triplicate determinations.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

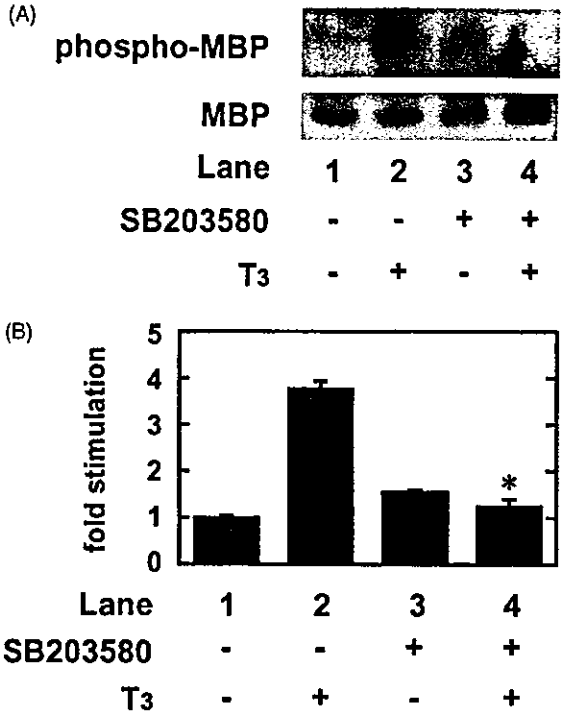

Fig. 4. Effect of SB203580 on T_3-induced phosphorylation of MBP in MC3T3-E1 cells. (A) The cultured cells were pretreated with 10 μM SB203580 for 60 min, and then stimulated by 10 nM T_3 for 1 h. The extracts of cells were subjected to SDS–PAGE against phospho-specific MBP antibodies or MBP antibodies. (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.

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

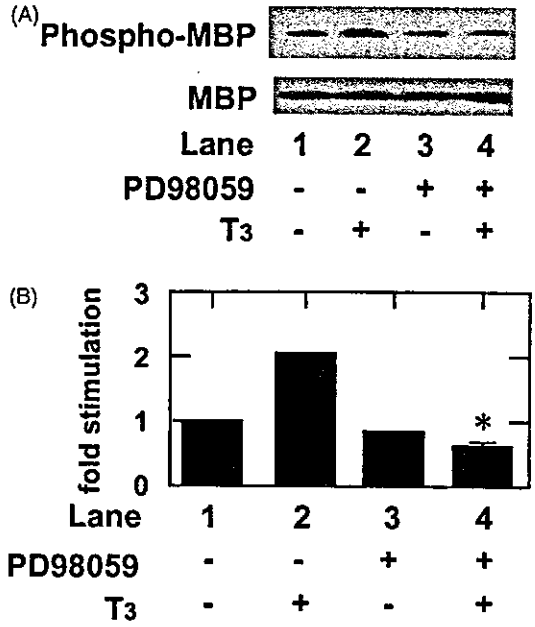

Fig. 5. Effect of PD98059 on T_3-induced phosphorylation of MBP in MC3T3-E1 cells. (A) The cultured cells were pretreated with 30 μM PD98059 for 60 min, and then stimulated by 10 nM T_3 for 1 h. The extracts of cells were subjected to SDS–PAGE against phospho-specific MBP antibodies or MBP antibodies. (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.

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

This work was supported in part by a Grant-in-Aid for Scientific Research (09671041, 12470015) from the Ministry of Education, Science, Sports and Culture of Japan.

References

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., Saltiel, A.R., 1995. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J. Biol. Chem. 270, 27489–27494.

Boguslawski, G., Hale, L.V., Yu, X.-P., Miles, R.R., Onyia, J.E., Santerre, R.F., Chandraekhar, S., 2000. Activation of osteocalcin transcription involves interaction of protein kinase A- and protein kinase C-dependent pathways. J. Biol. Chem. 275, 999–1006.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., Lee, J.C., 1995. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. 364, 229–233.

Daci, E., Udagawa, N., Martin, T.J., Bouillon, R., Carmeliet, G., 1999. The role of the plasminogen system in bone resorption in vitro. J. Bone Miner. Res. 14, 946–952.

Evans, R.M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889–895.

Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A., Trzaskos, J.M., 1998. Identification of a novel inhibitor of mitogen-activated protein kinase. J. Biol. Chem. 273, 18623–18632.

Huang, B.K., Golden, L.A., Tarjan, G., Madison, L.D., Stern, P.H., 2000. Insulin-like growth factor I production is essential for anabolic effects of thyroid hormone in osteoblasts. J. Bone Miner. Res. 15, 188–197.

Kasono, K., Sato, K., Han, D.C., Fujii, Y., Tsushima, T., Shizume, K., 1988. Stimulatory effect of alkaline phosphatase activity by thyroid hormone in mouse osteoblast-like (MC3T3-E1): a possible mechanism of hyperalkaline phosphatasia in hyperthyroidism. Bone Miner. 4, 355–363.

Kato, K., Ito, H., Hasegawa, K., Inaguma, Y., Kozawa, O., Asano, T., 1996. Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 rat glioma cells. J. Neurochem. 66, 946–950.

Khosla, S., Melton III, L.J., 1995. Osteoporosis. In: Riggs, L.G., Melton III, L.J. (Eds.), Etiology, Diagnosis and Management, second ed. Lippincott–Raven Publishers, Philadelphia, pp. 183–204.

Kozawa, O., Hatakeyama, D., Yoshida, M., Kamiya, Y., Kondo, C., Matsuno, H., Uematsu, T., 2001. Activation of p44/p42 mitogen-activated protein kinase limits triiodothyronine-stimulated alkaline phosphatase activity in osteoblasts. Biochem. Biophys. Res. Commun. 286, 1140–1143.

Kozawa, O., Tokuda, H., Miwa, M., Kotoyori, J., Oiso, Y., 1992. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp. Cell Res. 198, 130–134.

Kudo, Y., Iwashita, M., Takeda, Y., Muraki, T., 1998. Evidence for modulation of osteocalcin containing γ-carboxyglutamic acid residues

synthesis by insulin-like growth factor-I and vitamin K_2 in human osteosarcoma cells line MG-63. Eur. J. Endocrinol. 138, 443–448.

Kummer, J.L., Rao, P.K., Heidenreich, K.A., 1997. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. J. Biol. Chem. 282, 20490–20494.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lali, F.V., Hunt, A.E., Turner, S.J., Foxwell, B.M.J., 2000. The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. J. Biol. Chem. 275, 7395–7402.

Nijweide, P.J., Burger, E.H., Feyen, J.H.M., 1986. Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol. Rev. 66, 855–886.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., Davis, R.J., 1995. Pro-inflammatory cytokines and environmntal stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation of tyrosine and threonine. J. Biol. Chem. 270, 7420–7426.

Rizzoli, R., Poser, J., Burgi, U., 1986. Nuclear thyroid hormone receptor in cultured bone cells. Metabolism 35, 71–74.

Robinson, R.J., Doty, S.B., Cooper, R.R., 1973. Electron micrography of mammalian bone. In: Zipkin, I. (Ed.), Biological Mineralization. Academic Press, New York, pp. 257–296.

Shih, A., Lin, H.Y., Davis, F.B., Davis, P.J., 2001. Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. Biochemistry 40, 2870–2878.

Stern, P.H., 1996. Thyroid hormone and bone. In: Bilezikian, J.P., Raisz, L.G., Rodan, G.A. (Eds.), Principles of Bone Biology. Academic Press, San Diego, pp.521–531.

Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., Kasai, S., 1983. *In vivo* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell. Biol. 96, 191–198.

Tokuda, H., Kozawa, O., Harada, A., Isobe, K., Uematsu, T., 1998. Triiodothyronine modulates interleukin-6 synthesis in osteoblasts: inhibition in protein kinase A and C pathways. Endocrinology 139, 1300–1305.

Tokuda, H., Kozawa, O., Uematsu, T., 2000. Basic fibroblast growth factor stimulates vascular endothelial growth factor release in osteoblasts: divergent regulation by p42/p44 mitogen-activated protein kinase and p38 mitogen-activated protein kinase. J. Bone Miner. Res. 15, 2371–2379.

Tokuda, H., Hirade, K., Wang, X., Oiso, Y., Kozawa, O., 2003. Involvement of SAPK/JNK in basic fibroblast growth factor-induced vascular endothelial growth factor release in osteoblasts. J. Endocrinol. 177, 101–107.

Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 79, 143–180.

Journal of Cellular Biochemistry 91:1053–1061 (2004)

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

Haruhiko Tokuda,[1,2] Yosuke Kanno,[2,4] Akira Ishisaki,[2] Motoki Takenaka,[2] Atsushi Harada,[3] and Osamu Kozawa[2]*

[1]Department of Internal Medicine, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Japan
[2]Department of Pharmacology, Gifu University School of Medicine, Gifu, Japan
[3]Department of Orthopaedics, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Japan
[4]Institute of Biological Science, University of Tsukuba, Tsukuba, Japan

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.

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

Grant sponsor: Ministry of Education, Science, Sports and Culture of Japan; Grant sponsor: Grant-in-Aid for Scientific Research.

*Correspondence to: Osamu Kozawa, Department of Pharmacology, Gifu University School of Medicine, Gifu 500-8705, Japan. E-mail: okozawa@cc.gifu-u.ac.jp

Received 20 August 2003; Accepted 13 November 2003
DOI 10.1002/jcb.20004
Published online 25 February 2004 in Wiley InterScience (www.interscience.wiley.com).

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.

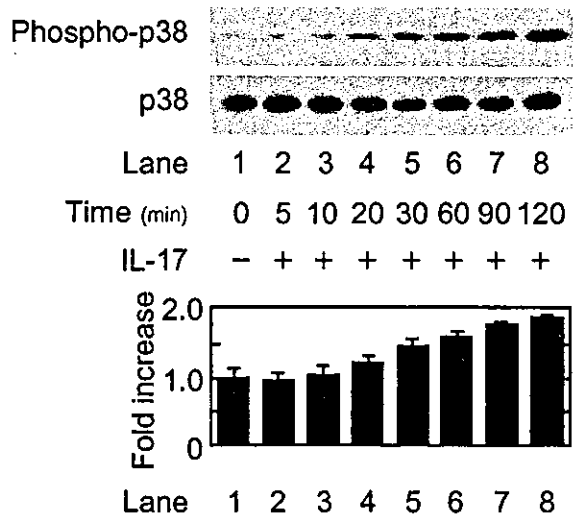

Fig. 1. Effect of interleukin (IL)-17 on the phosphorylation of p38 mitogen-activated protein (MAP) kinase in MC3T3-E1 cells. The cultured cells were stimulated by 100 μM IL-17 for 5 min (**lane 2**), 10 min (**lane 3**), 20 min (**lane 4**), 30 min (**lane 5**), 60 min (**lane 6**), 90 min (**lane 7**), 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

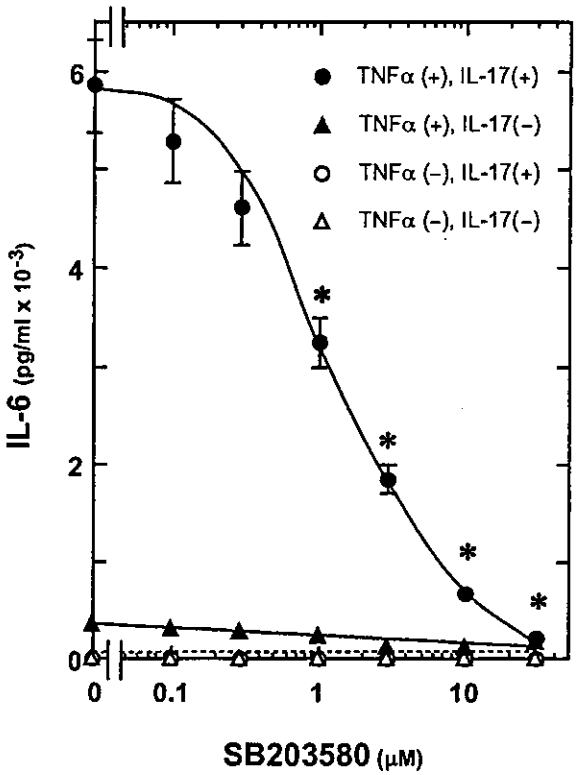

Fig. 2. Effect of SB203580 on the enhancement by IL-17 of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 for 60 min, and then stimulated by 10 ng/ml TNF-α (closed symbols) or vehicle (open symbols) in the presence of 100 μM IL-17 (circles) 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.

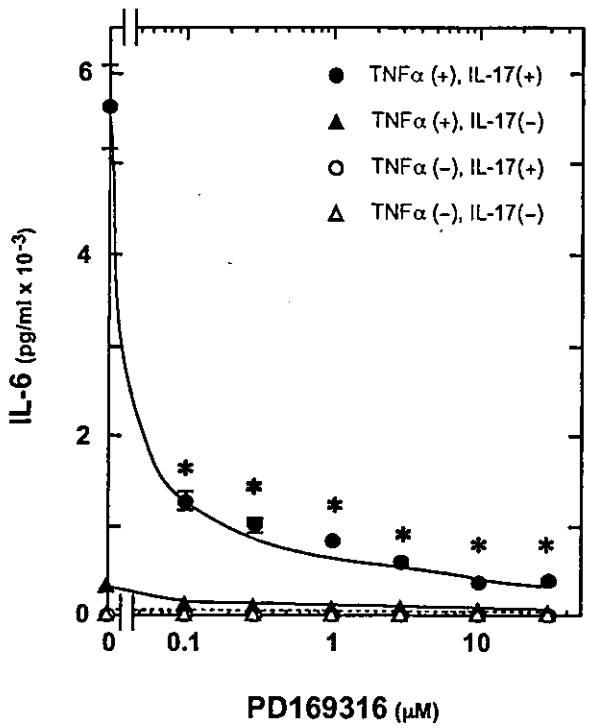

Fig. 3. Effect of PD169316 on the enhancement by IL-17 of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD169316 for 60 min, and then stimulated by 10 ng/ml TNF-α (closed symbols) or vehicle (open symbols) in the presence of 100 μM IL-17 (circles) 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

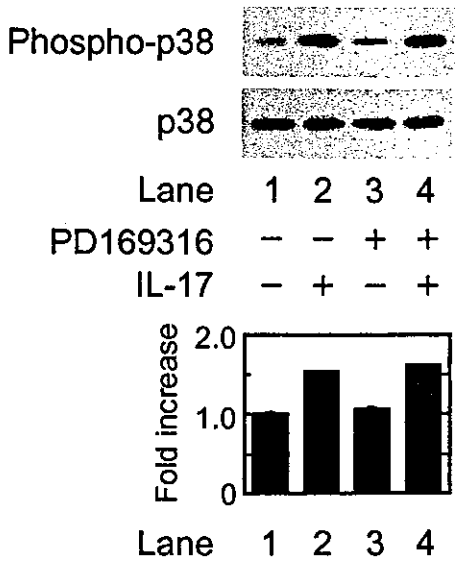

Fig. 4. Effect of PD169316 on the IL-17-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD169316 or vehicle for 60 min, and then stimulated by 100 μM IL-17 or vehicle for 120 min. Cell extracts were subjected to SDS–PAGE with subsequent Western 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.

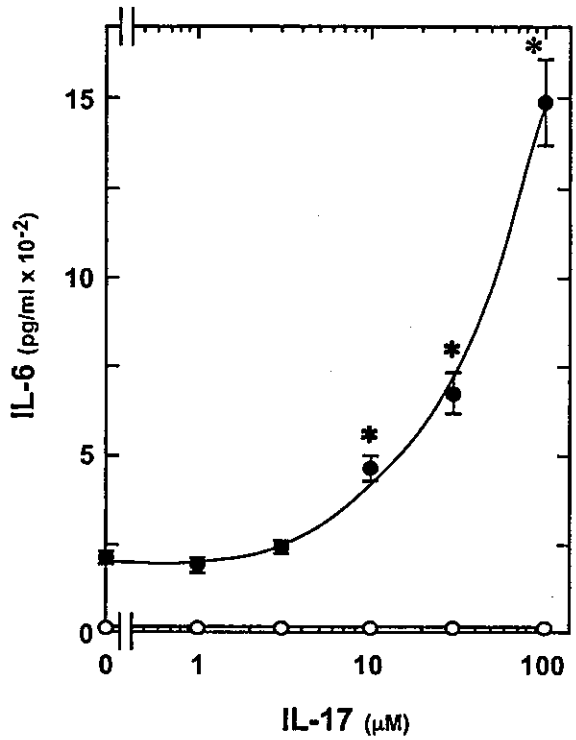

Fig. 5. Effect of IL-17 on the sphingosine 1-phosphate (S1-P)-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM S1-P (closed symbols) or vehicle (open symbols) in the presence of various doses of IL-17 for 24 h. Each value represents the mean ± SEM of triplicate determinations. 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

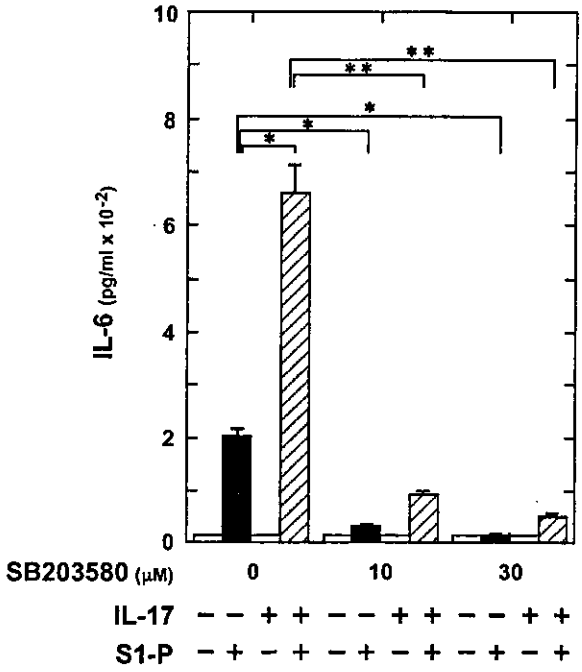

Fig. 6. Effect of SB203680 on the enhancement by IL-17 of S1-P-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 10 μM S1-P or vehicle in the presence of 100 μM IL-17 or vehicle for 24 h. *$P < 0.05$, compared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

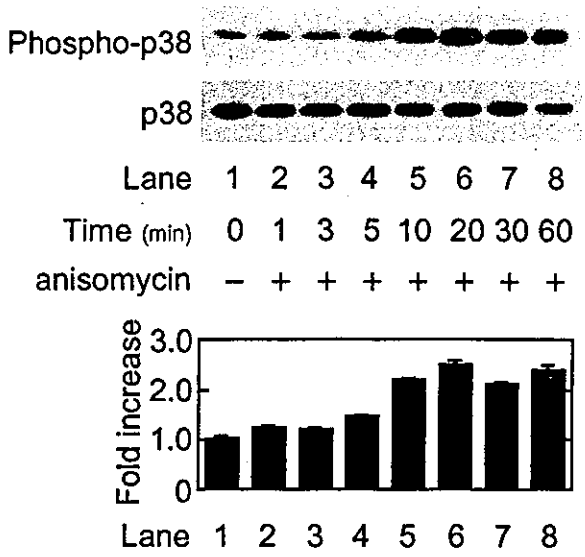

Fig. 7. Effect of anisomycin on the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 100 μM anisomycin for 1 min (**lane 2**), 3 min (**lane 3**), 5 min (**lane 4**), 10 min (**lane 5**), 20 min (**lane 6**), 30 min (**lane 7**) and 60 min (**lane 8**). Cell 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; Kumar 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

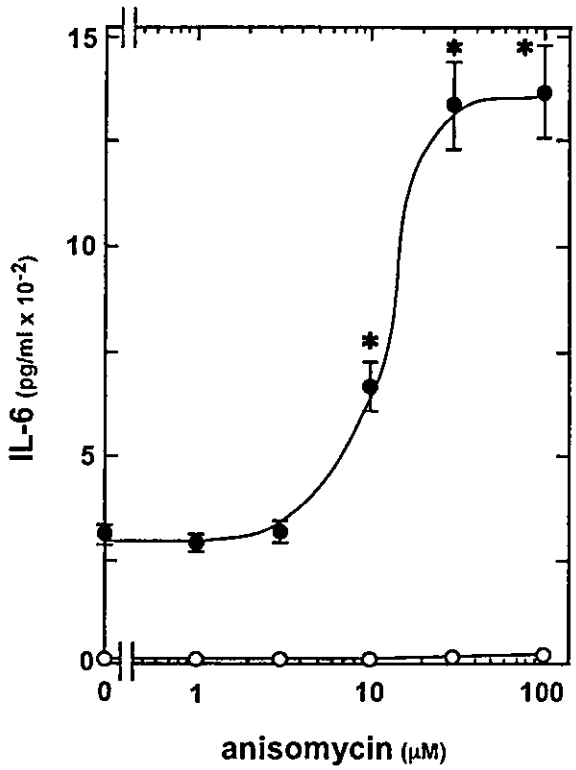

Fig. 8. Effect of anisomycin on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were stimulated by 10 ng/ml TNF-α (closed symbols) or vehicle (open symbols) in the presence of various doses of anisomycin for 24 h. Each value represents the mean ± SEM of triplicate determinations.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

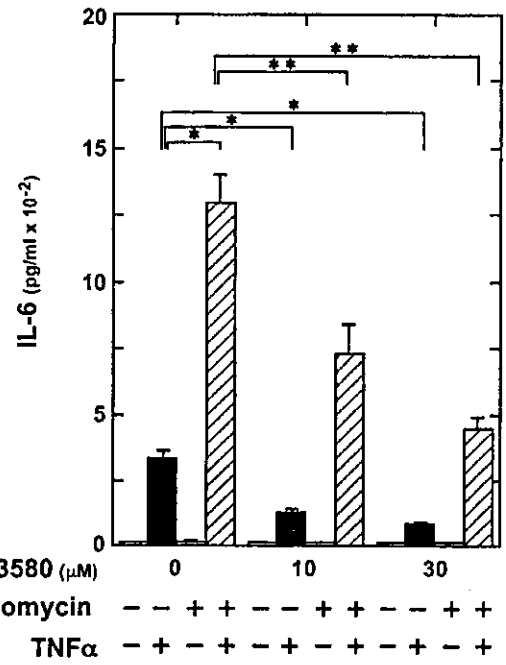

Fig. 9. Effect of SB203680 on the enhancement by anisomycin of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with SB203580 or vehicle for 60 min, and then stimulated by 10 ng/ml TNF-α or vehicle in the presence of 100 μM anisomycin or vehicle for 24 h. Each value 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.