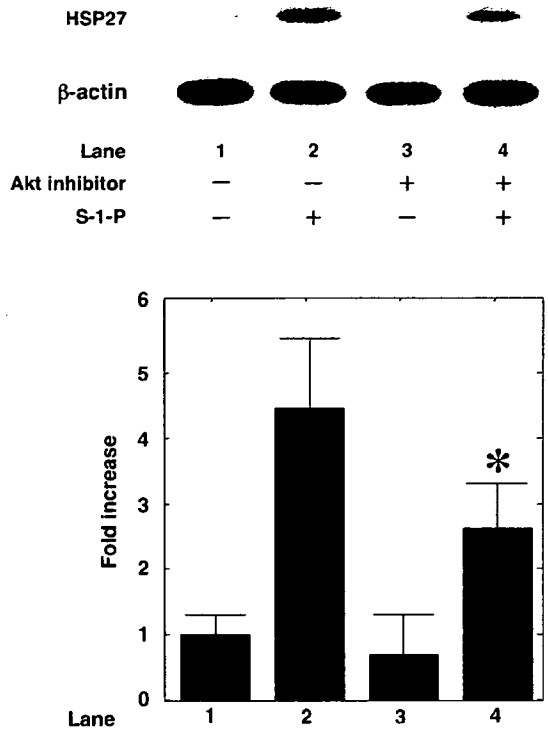

Fig. 2. Effect of Akt inhibitor on the sphingosine 1-phosphate (S-1-P)-induced levels of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 μM of S-1-P or vehicle for 6 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27 or β-actin. The histogram shows quantitative representations of the levels of S-1-P-induced HSP27 after normalization to levels of β-actin. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P < 0.05$, compared to the value of S-1-P.

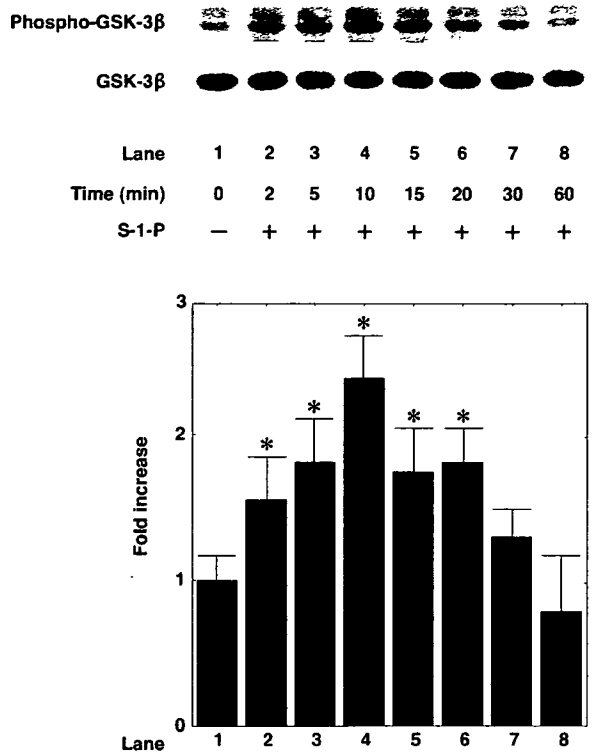

Fig. 3. Effect of sphingosine 1-phosphate (S-1-P) on the phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were stimulated with 30 μM S-1-P for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P < 0.05$, compared to the value of control.

the Akt substrates [Cross et al., 1995]. We found that GSK-3β was time dependently phosphorylated by sphingosine 1-phosphate (Fig. 3). In addition, Akt inhibitor attenuated the sphingosine 1-phosphate-induced phosphorylation of GSK-3β, suggesting that the Akt-mediating pathway actually functions in sphingosine 1-phosphate-stimulated MC3T3-E1 cells (Fig. 4). Akt inhibitor (50 μM) caused about 50% reduction in the sphingosine 1-phosphate-effect.

Effects of LY294002 and Wortmannin on the Sphingosine 1-Phosphate-Induced Phosphorylation of Akt in MC3T3-E1 Cells

In order to clarify whether PI3K acts at a point upstream from Akt, we examined the effect of LY294002, a specific inhibitor of PI3K [Vlahos et al., 1994], on the sphingosine 1-phosphate-induced phosphorylation of Akt. LY294002 dose dependently suppressed the sphingosine 1-phosphate-induced Akt phosphorylation (Fig. 5A). LY294002 (10 μM) caused almost complete reduction in the sphingosine 1-phosphate-effect. Wortmannin, another PI3K inhibitor [Arcaro and Wymann, 1993], also suppressed the phosphorylation of Akt (Fig. 5B). Wortmannin (10 μM) caused about 40% reduction in the sphingosine 1-phosphate-effect. However, LY294002 did not affect the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (data not shown). In addition, SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995] failed to affect the sphingosine 1-phosphate-induced phosphorylation of Akt (data not shown).

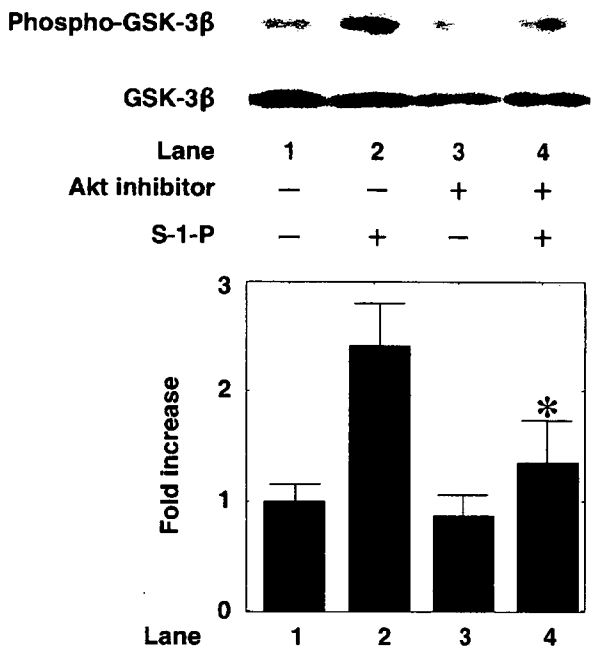

Fig. 4. Effect of Akt inhibitor on the sphingosine 1-phosphate (S-1-P)-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $*P < 0.05$, compared to the value of S-1-P.

Effects of LY294002 and Wortmannin on the Sphingosine 1-Phosphate-Stimulated Induction of HSP27 in MC3T3-E1 Cells

LY294002 significantly suppressed the sphingosine 1-phosphate-stimulated induction of HSP27 in a dose dependent manner between

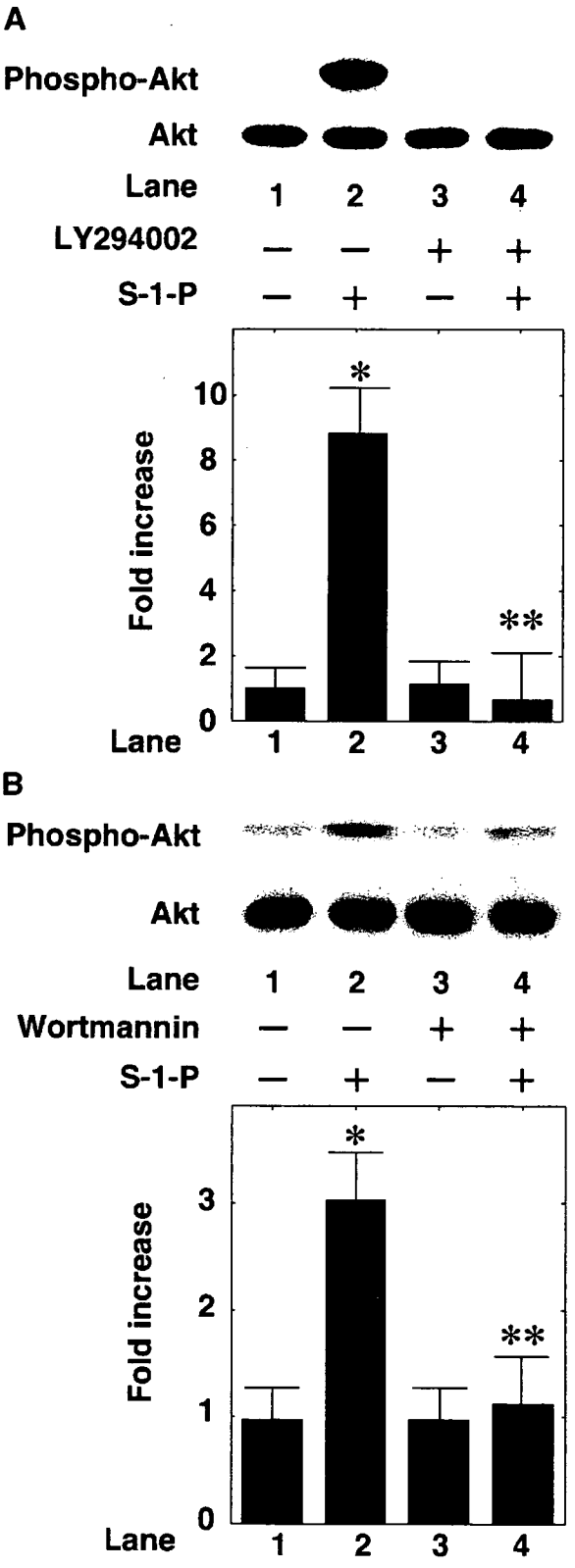

Fig. 5. Effects of LY294002 or wortmannin on the sphingosine 1-phosphate (S-1-P)-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM LY294002 (**A**) or 10 μM wortmannin (**B**) for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $*P < 0.05$, compared to the value of control (without agonist and inhibitor). $**P < 0.05$, compared to the value of S-1-P alone.

10 and 50 μM (Fig. 6A). Additionally, wortmannin markedly reduced the induction of HSP27 similarly to LY294002 (Fig. 6B).

DISCUSSION

We have previously shown that sphingosine 1-phosphate stimulates induction of HSP27 in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase takes a part in the sphingosine 1-phosphate-effect [Kozawa et al., 1999]. In the present study, we first demonstrated that sphingosine 1-phosphate stimulated the phosphorylation of Akt in a time-dependent manner in MC3T3-E1 cells. In addition, we showed that PI3K inhibitors such as LY294002 and wortmannin, suppressed the sphingosine 1-phopshate-induced phosphorylation of Akt, suggesting that Akt functions at a point downstream from PI3K in these cells. PI3K is recruited upon growth factor receptor activation and produces 3′ phosphoinositide lipids [Dudek et al., 1997; Katso et al., 2001]. The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins. These events constitute the start of a complex signaling cascade, which ultimately results in the mediation of cellular activities such as proliferation, differentiation, chemotaxis, and survival. The PI3K/Akt signaling pathway is currently considered to play a critical role in mediating survival signals in a wide range of cell types. The recent identification of a number of substrates for the serine/threonine kinase Akt suggests that it blocks cell death by both impinging on the cytoplasmic cell death machinery and by regulating the expression of genes involved in cell death and survival. In addition, recent experiments suggest that Akt may also use metabolic pathways to regulate cell survival [Brunet et al., 2001; Masuyama et al., 2001].

Therefore, we next examined the correlation between the sphingosine 1-phosphate-stimulated induction of HSP27 and PI3K/Akt in osteoblast-like MC3T3-E1 cells. In the present study, the sphingosine 1-phosphate-stimulated HSP27 induction was reduced by Akt inhibitor. As for Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [Hu et al., 2000], we found that it blocked the phosphorylation of GSK-3β, one of the Akt substrates [Cross et al., 1995]. In addition, we showed that PI3K inhibitors also suppressed

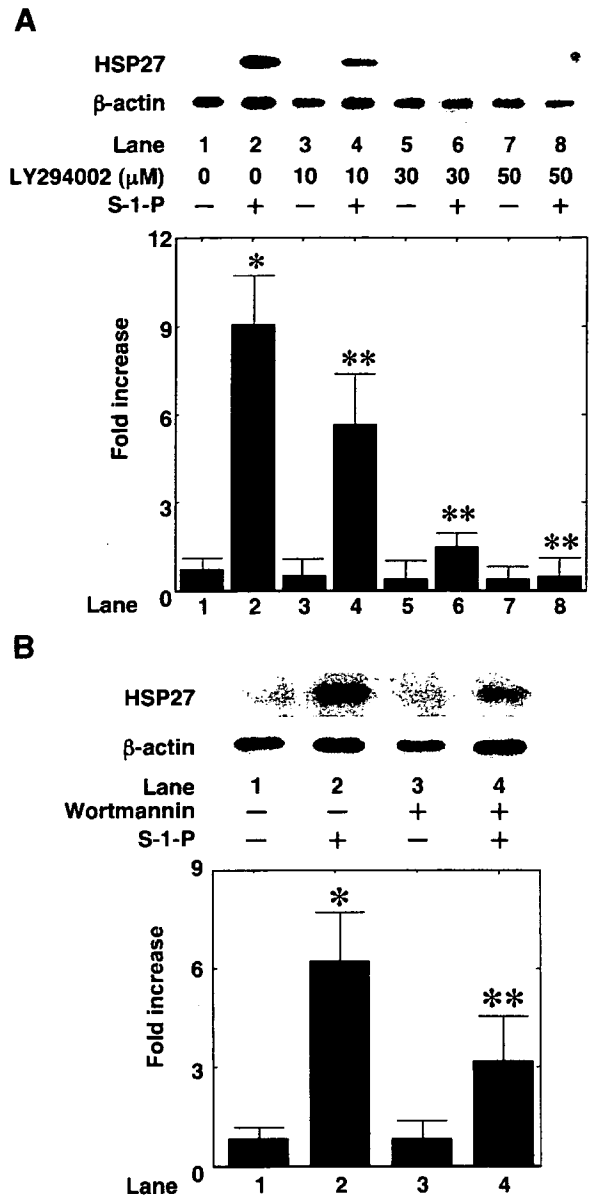

Fig. 6. Effects of LY294002 or wortmannin on the sphingosine 1-phosphate (S-1-P)-induced levels of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or 50 μM wortmannin (**B**) for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 6 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27 or β-actin. The histogram shows quantitative representations of the levels of S-1-P-induced HSP27 after normalization to levels of β-actin. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $^{*}P < 0.05$, compared to the value of control. $^{**}P < 0.05$, compared to the value of S-1-P alone.

the sphingosine 1-phosphate-stimulated HSP27 induction thorough the reduction of the sphingosine 1-phosphate-induced Akt phosphorylation. Therefore, based on our findings, it is most likely that the sphingosine 1-phosphate-stimulated induction of HSP27 is regulated by PI3K/Akt in osteoblast-like MC3T3-E1 cells.

We have previously reported that the activation of p38 MAP kinase is involved in HSP27 induction by sphingosine 1-phosphate in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1999]. Therefore, we investigated to clarify the relationship between p38 MAP kinase and PI3K/Akt in these cells. Akt inhibitor and PI3K inhibitor, LY294002 [Vlahos et al., 1994], failed to influence the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells, and p38 MAP kinase inhibitor, SB203580 [Cuenda et al., 1995], had little effect on the sphingosine 1-phosphate-induced phosphorylation of Akt. In addition, the inhibitory effect of Akt inhibitor or wortmannin on the sphingosine 1-phosphate-stimulated HSP27 induction was partial. We have previously shown that the suppressive effect of SB203580 on the HSP27 induction was partial [Kozawa et al., 1999]. These findings suggest that PI3K/Akt pathway plays a role at least in part in addition to p38 MAP kinase pathway in the sphingosine 1-phosphate-stimulated HSP27 induction in MC3T3-E1 cells. Taking these results into account as a whole, it is most likely that sphingosine 1-phosphate stimulates the induction of HSP27 probably via two independent pathways, PI3K/Akt and p38 MAP kinase, in osteoblast-like MC3T3-E1 cells.

It is recognized that HSP27 is present at two forms, an aggregated form and a dissociated small form in unstressed conditions [Benjamin and McMillan, 1998]. It has been shown that HSP27 is constitutively expressed at high levels in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells [Benjamin and McMillan, 1998]. Post-translational modifications such as phosphorylation and oligomerization are crucial regulators of its functions [Benjamin and McMillan, 1998]. In our previous study [Kato et al., 1994], we have reported that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27. In addition, we have shown that conversion from the non-phosphorylated, aggregated form of HSP27 to the phosphorylated, dissociated form results in decreased tolerance to heat stress [Kato et al., 1994]. It has been shown that estrogen-induced resistance to osteoblast apoptosis is associated with increased HSP27 expression [Cooper et al., 2000]. We speculate that expression of HSP27 via p38 MAP kinase and PI3K/Akt in osteoblasts might be related to the maintenance of the number of viable osteoblasts in bone tissue. Interestingly, sphingosine 1-phosphate reportedly prevents apoptosis in primary rat osteoblasts and human osteosarcoma SaOS-2 cells [Grey et al., 2002]. Taking our findings into account, it is probable that sphingosine 1-phosphate directly affects osteoblasts through the induction of HSP27 through PI3K/Akt and p38 MAP kinase. However, the physiological significance of HSP27 in osteoblasts has not yet been precisely clarified. Further investigations are necessary to clarify the exact roles of HSP27 in osteoblasts.

In conclusion, these results strongly suggest that sphingosine 1-phosphate stimulates the induction of HSP27 via PI3K/Akt pathway in addition to p38 MAP kinase in osteoblasts.

ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aid for Scientific Research (16590873 and 16591482) for the Ministry of Education, Science, Sports and Culture of Japan, the Research Grants for Longevity Sciences (15A-1 and 15C-2), Research on Proteomics and Research on Fracture and Dimentia from Ministry of Health, Labour and Welfare of Japan.

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Involvement of p44/p42 MAP kinase in insulin-like growth factor-I-induced alkaline phosphatase activity in osteoblast-like-MC3T3-E1 cells

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Received 26 January 2006; accepted 17 February 2006

Abstract

It has been shown that insulin-like growth factor-I (IGF-I) stimulates the activity of alkaline phosphatase, a marker of mature osteoblast phenotype, in osteoblasts. In the present study, we investigated the involvement of the mitogen-activated protein (MAP) kinase superfamily in the IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. IGF-I-stimulated alkaline phosphatase activity dose dependently in the range between 1 nM and 0.1 μM. IGF-I induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase but not stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK). PD98059 and U0126, specific inhibitors of the upstream kinase that activates p44/p42 MAP kinase, significantly suppressed the IGF-I-induced alkaline phosphatase activity. On the contrary, SB203580 and PD169316, specific inhibitors of p38 MAP kinase, failed to affect the activity induced by IGF-I. Specific inhibitors for phosphatidylinositol 3-kinase (PI3K)/Akt pathway (LY294002 and wortmannin) also had no significant effect on IGF-I-induced p44/p42 MAP kinase phosphorylation. The phosphorylation of p44/p42 MAP kinase induced by IGF-I was reduced by U0126. These results strongly suggest that p44/p42 MAP kinase among the MAP kinase superfamily plays a role in the IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells.

Keywords: IGF-I; Alkaline phosphatase; p44/p42 MAP kinase; Osteoblast

1. Introduction

It is well recognized that insulin-like growth factor-I (IGF-I) plays a crucial role in the regulation of growth and bone metabolism (Conover, 2000; Olney, 2003). IGF-I, which is mainly synthesized and secreted from liver, mediates a variety of the actions of growth hormone that is secreted from pituitary gland under the control of the hypothalamus. Accumulating evidence suggests that IGF-I is necessary for fracture healing (Trippel, 1998). Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption (Nijweide et al., 1986). As for osteoblasts, it has been reported that IGF-I stimulates the proliferation of these cells and synthesize bone matrix proteins (Conover, 2000). We have previously demonstrated that IGF-I induces DNA synthesis synergistically with protein kinase C activation in osteoblast-like MC3T3-E1 cells (Kozawa et al., 1989). In addition, IGF-I reportedly stimulates alkaline phosphatase activity, a marker of mature osteoblast phenotype (Robinson et al., 1973), in osteoblasts (Schmid et al., 1984). It is recognized that IGF-I is also produced by osteoblasts (Olney, 2003). In a previous study (Kozawa et al., 1992b), we have shown that osteoblast-like MC3T3-E1 cells secrete IGF-I resulting in inducing mineralization, and protein kinase C activation suppresses the secretion of IGF-I. Based on these findings, there is no doubt that IGF-I secreted from osteoblasts plays a pivotal role in the regulation of bone metabolism.

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doi:10.1016/j.mce.2006.02.014

The mitogen-activated protein (MAP) kinase superfamily is well recognized to play crucial roles in the intracellular signaling of variety of agonists (Widmann et al., 1999). Three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), are known as central elements used by mammalian cells to transduce the various messages (Widmann et al., 1999). It has recently been reported that IGF-I up-regulated expression of core binding factor α1 through MAP kinase pathway in osteoblast-like MC3T3-E1 cells (Pei et al., 2003). However, the exact role of the MAP kinase superfamily in IGF-I-effect on osteoblasts has not yet been clarified.

In the present study, we investigated whether the MAP kinase superfamily is involved in the IGF-I-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. We here show that IGF-I activates p44/p42 MAP kinase and p38 MAP kinase in these cells, and that p44/p42 MAP kinase plays a part in the IGF-I-stimulated alkaline phosphatase activity.

2. Materials and methods

2.1. Materials

IGF-I was purchased from R&D Systems, Inc. (Minneapolis, MN). Specific inhibitors for MEK (PD98059 and U0126), p38 MAP kinase (SB203580 and PD169316) and phosphatidylinositol 3-kinase (PI3K) (LY294002 and wortmannin) were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from New England BioLabs, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. 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 alkaline phosphatase activity or the analysis of MAP kinases.

2.2. 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., 1992a). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. Assay for alkaline phosphatase activity

The cultured cells were pretreated with PD98059, U0126, SB203580 or PD169316 for 60 min, and then were stimulated by IGF-I in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. At the end of the incubation, the cells were harvested by scraping with a rubber policeman into 1 ml of 0.2% Nonidet P-40 and disrupted by sonication. After centrifugation at 1500 × *g* for 5 min of the homogenate, alkaline phosphatase activity of the supernatant was measured by the method of Lowry et al. (1954).

2.4. Analysis of mRNA expressions

The cultured cells were stimulated by IGF-I or vehicle in α-MEM containing 0.3% FCS for 0, 12, 24, 36, 48 and 60 h. The expression levels of mRNAs were estimated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) according to the method previously described by Nakashima et al. (2005) and Ishisaki et al. (2004). In brief, at the end of each incubation period, the total RNA was extracted from the cells using Isogen (Nippongene, Toyama, Japan) and complementary DNA was synthesized with Omniscript reverse transcriptase (Qiagen, Valencia, CA) using an oligo$(dT)_{15}$ primer (1 μM) according to the manufacturer's instructions. Each PCR reaction was carried out in 50 μl of mixture containing 1 μl of cDNA, 5 μl of 10× Qiagen PCR buffer, 10 μl of 5× Q-solution PCR buffer, 1 μl of 10 mM each deoxynucleotide triphosphate mix, 0.1 μM each sense and antisense primers and 0.25 μl of Taq DNA polymerase (Qiagen). Each reaction consisted of initial denaturation at 94 °C for 3 min followed by three-step cycling: denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 1 min. Amplification was stopped within linear range and the reaction underwent a final extension at 72 °C for 10 min. Twenty-five cycles were undergone. Amplification products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining followed by UV light illumination. Equal loading of RNA samples were checked by amplification of GAPDH cDNA. The primer sequences used for PCR amplifications, annealing temperature, and expected fragment size were as follows: osteocalcin, forward primer 5′-CTGAGTCTGACAAAGCCTTC-3′, reverse primer 5′-GCTGTGACATCCATACTTGC-3′, 55 °C and 312 bp; osteopontin, forward primer 5′-CGACGATGATGACGATGATGAT-3′, reverse primer 5′-CTGGCTTTGGAACTTGCTTGAC-3′, 60 °C and 495 bp; Runx2, forward primer 5′-AGCAACAGCAACAACAGCAG-3′, reverse primer 5′-GTAATCTGACTCTGTCCTTG-3′, 55 °C and 470 bp; collagen α1(I), forward primer 5′-TCTCCACTCTTCTAGTTCCT-3′, reverse primer 5′-TTGGGTCATTTCCACATGC-3′, 51 °C and 269 bp; glyceraldehydes-3-phosphate dehydrogenase (GAPDH), forward primer 5′-TTCATTGACCTCAACTACATG-3′, reverse primer 5′-GTGGCAGTGATGGCATGGAC-3′, 60 °C and 443 bp, respectively.

2.5. Analysis of MAP kinases

The cultured cells were stimulated by IGF-I 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 (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK 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 PD98059, U0126, PD169316, LY294002 or wortmannin for 60 min.

2.6. Determinations

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 comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean ± S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of IGF-I on alkaline phosphatase activity in MC3T3-E1 cells

It has been shown that IGF-I induces alkaline phosphatase activity in osteoblasts (Schmid et al., 1984). We found that IGF-I significantly stimulates the activity of alkaline phosphatase in a dose-dependent manner in the range between 1 nM and 0.3 μM (Fig. 1). The maximum effect of IGF-I on the activity was observed at 0.3 μM. In addition, we investigated the effect of IGF-I treatment on the several known osteoblast differentiation markers (osteocalcin, osteopontin, Runx2 and collagen α1(I)) in MC3T3-E1 cells. GAPDH mRNA was served as a control. We found that IGF-I had no significant effect on up-regulation of these mRNA expressions at a dose (0.1 μM) that significantly induces ALP activation (data not shown).

3.2. Effects of IGF-I on the phosphorylation of the p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in MC3T3-E1 cells

To investigate whether IGF-I activates the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells, we examined the effects of IGF-I on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK. Stimulation of IGF-I significantly induces the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase (Fig. 2). The phosphorylation of both MAP kinases reached the peak at 10 min. On the contrary, IGF-I did not substantially affect the phosphorylation of SAPK/JNK (data not shown).

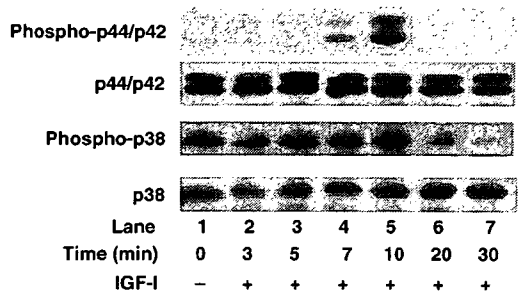

Fig. 2. Effects of IGF-I on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM IGF-I for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase or p38 MAP kinase. Similar results were obtained with two additional and different cell preparations.

3.3. Effects of PD98059 and U0126 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells

To investigate whether p44/p42 MAP kinase is involved in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells, we examined the effect of PD98059, a highly specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi et al., 1995), on the alkaline phosphatase activity. PD98059, which alone hardly affected the basal level of alkaline phosphatase activity, significantly reduced the IGF-I-induced alkaline phosphatase activity (Fig. 3). The maximum inhibitory effect of PD98059 at 30 μM caused about 65% reduction in the IGF-I-effect. In addition, U0126, another inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Favata et al., 1998), dose dependently reduced the alkaline phosphatase

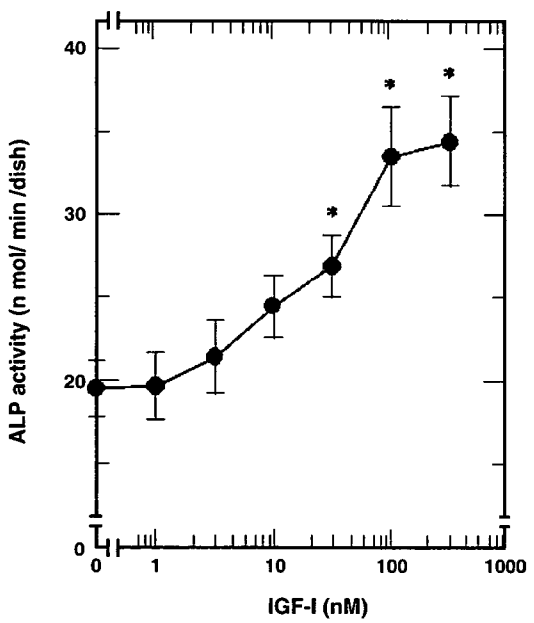

Fig. 1. Effect of IGF-I on alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were stimulated by various doses of IGF-I for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the control value.

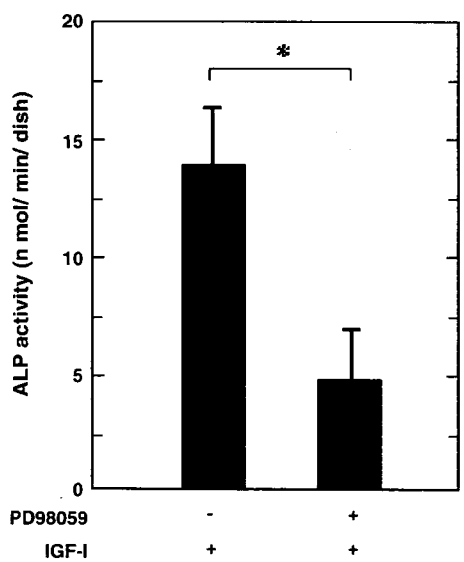

Fig. 3. Effect of PD98059 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 48 h. Values for IGF-I-unstimulated cells were subtracted to produce each data point. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{**}p < 0.05$, compared to the value of IGF-I alone.

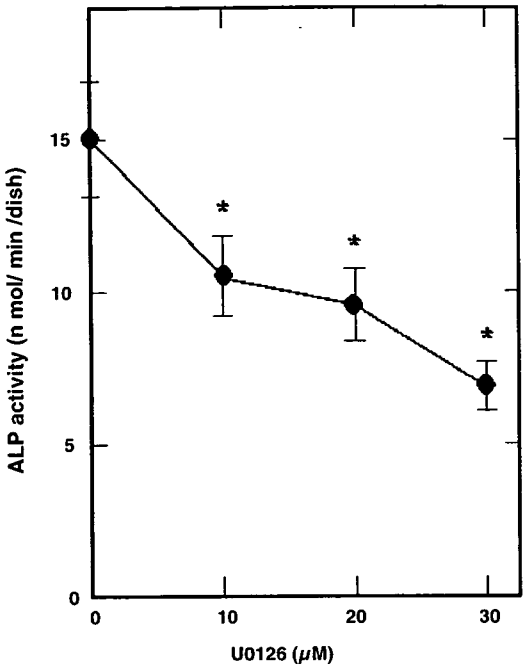

Fig. 4. Dose-dependent effect of U0126 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with various doses of U0126 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 48 h. Values for IGF-I-unstimulated cells were subtracted to produce each data point. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of IGF-I alone.

activity stimulated by IGF-I as well as PD98059 (Fig. 4). The maximum inhibitory effect of U0126 at 30 μM caused about 55% reduction in the IGF-I-effect.

3.4. Effects of PD98059 and U0126 on the phosphorylation of p44/p42 MAP kinase induced by IGF-I in MC3T3-E1 cells

We found that PD98059 truly suppressed the phosphorylation of p44/p42 MAP kinase induced by IGF-I in a dose-dependent manner (Fig. 5). According to the densitometric analysis, PD98059 at 30 μM caused about 75% reduction of the IGF-I-effect on the p44/p42 MAP kinase phosphorylation. Furthermore, U0126 markedly inhibited the IGF-I-induced phosphorylation of p44/p42 MAP kinase (data not shown). According to the densitometric analysis, U0126 caused almost complete reduction of the IGF-I-effect on the activity.

3.5. Effects of SB203580 and PD169316 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells

To investigate whether p38 MAP kinase is involved in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells, we examined the effects of SB203580 and PD169316, highly specific inhibitors of p38 MAP kinase (Cuenda et al., 1995; Kummer et al., 1997), on the alkaline phosphatase activity. However, SB203580 or PD169316 hardly affected the IGF-I-induced alkaline phosphatase activity (15.5 ± 1.6 nmol/min/dish for IGF-I alone; 16.0 ± 1.7 nmol/min/dish for IGF-I with 30 μM SB203580; and 15.1 ± 1.4 nmol/min/dish for IGF-I with 30 μM PD169316, as measured during the stimulation for 48 h, values for IGF-I-unstimulated cells were subtracted to produce each data point).

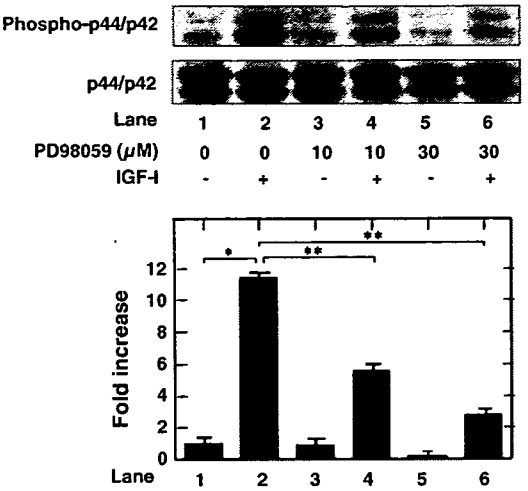

Fig. 5. Effect of PD98059 on the IGF-I-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent 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 IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control; $^{**}p < 0.05$, compared to the value of IGF-I alone.

3.6. Effect of PD169316 on the IGF-I-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells

We found that PD169316 markedly inhibited the phosphorylation of p38 MAP kinase induced by IGF-I (Fig. 6). According to the densitometric analysis, PD169316 caused about 40% reduction of the IGF-I-effect on the p38 MAP kinase phosphorylation.

3.7. Effect of LY294002 or wortmannin on the IGF-I-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells

We have recently reported that in MC3T3-E1 cells, IGF-I-induced alkaline phosphatase is PI3K/Akt dependent (Noda et al., 2005). To test the interaction between PI3K/Akt and p44/p42 MAPK pathway, we examined the effect of PI3K/Akt blockade on the IGF-I-induced p44/p42 MAPK phosphorylation in MC3T3-E1 cells. LY294002 (Vlahos et al., 1994), a specific inhibitor for PI3K/Akt pathway have no significant effect on the IGF-I-induced p44/p42 MAPK phosphorylations in MC3T3-E1 cells (Fig. 7). Wortmannin (Arcaro and Wymann, 1993), an another inhibitor for PI3K/Akt pathway, also had no significant

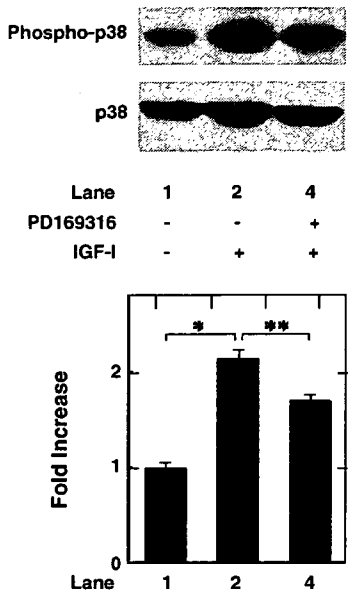

Fig. 6. Effect of PD169316 on the IGF-I-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD169316 or vehicle for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control; $^{**}p < 0.05$, compared to the value of IGF-I alone.

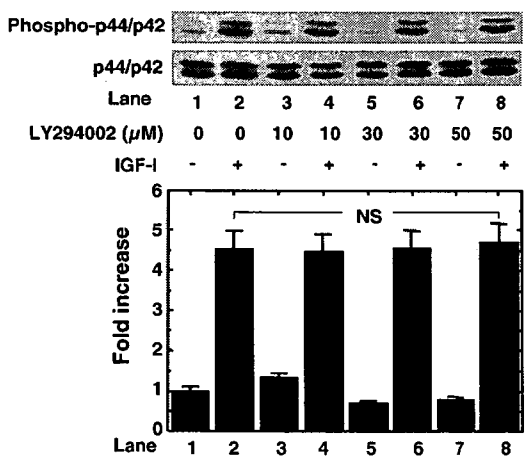

Fig. 7. Effect of LY294002 on the IGF-I-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent 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 IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

effect (data not shown). As shown in our previous study, the inhibitor concentrations used were sufficient to block the IGF-I-induced ALP activation in these cells (~50 μM for LY294002 and ~30 nM for wortmannin).

4. Discussion

In the present study, we demonstrated that IGF-I induces the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase but not SAPK/JNK in osteoblast-like MC3T3-E1 cells. It is currently understood 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 (Widmann et al., 1999). Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK, are known as central elements used by mammalian cells to transducer the diverse messages (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, taking these results into account, it is most likely that IGF-I induces both the activation of p44/p42 MAP kinase and p38 MAP kinase without activating SAPK/JNK in osteoblast-like MC3T3-E1 cells.

We next investigated the involvement of p44/p42 MAP kinase in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells. Herein, we showed that PD98059 (Alessi et al., 1995) and U0126 (Favata et al., 1998) significantly suppressed the IGF-I-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. In addition, we found that these inhibitors truly attenuated the phosphorylation of p44/p42 MAP kinase induced by IGF-I, suggesting that the activation of p44/p42 MAP kinase is involved in IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. Therefore, our findings indicate that p44/p42 MAP kinase participates at least in part in the IGF-I-stimulated alkaline phosphatase activity.

In order to clarify whether p38 MAP kinase plays a role in the IGF-I-stimulated alkaline phosphatase activity, we examined the effect of specific inhibitors of p38 MAP kinase such as SB203580 (Cuenda et al., 1995) and PD169316 (Kummer et al., 1997) on the alkaline phosphatase activity. However, either SB203580 or PD169316 failed to affect the IGF-I-induced activity of alkaline phosphatase. We found that PD169316 truly reduced the IGF-I-induced phosphorylation of p38 MAP kinase. Based on these results, it seems unlikely that p38 MAP kinase is involved in IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells.

We have recently reported that in MC3T3-E1 cells, IGF-I-induced alkaline phosphatase is PI3K/Akt dependent (Noda et al., 2005). To confirm the interaction between PI3K/Akt pathway and p44/p42 MAP kinase pathway, we examined the effect of PI3K/Akt pathway blockade on the IGF-I-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells, and found that LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wymann, 1993) had no significant effect on the IGF-I-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells. It is likely that these two pathways independently regulate IGF-I-

induced ALP induction, or at least, PI3K/Akt pathway does not exert its effect at a point upstream of p44/p42 MAP kinase in IGF-I-induced ALP activation.

It is well known that alkaline phosphatase is a biochemical marker of bone formation (Robinson et al., 1973; Seibel, 2000). During progression from an immature progenitor cell to a mature osteoblast resulting in mineralization, expression of alkaline phosphatase occurs (Aubin and Liu, 1996). Thus, it is generally recognized that alkaline phosphatase plays an important role in mineralization although the precise function of the enzyme is not clarified. It has been recently reported that PD98059 blocks IGF-I-induced expression of core binding factor α1 in osteoblast-like MC3T3-E1 cells, suggesting involvement of p44/p42 MAP kinase in the expression (Kummer et al., 1997). Expression of core binding factor α1 is the earliest osteoblast-specific event identified in the osteoblast differentiation pathway and critical for osteoblastogenesis (Komori et al., 1997). We here showed that p44/p42 MAP kinase plays a role in the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. It is most likely that p44/p42 MAP kinase activation has an important role in IGF-I-induced bone formation. In response to various physiological agonists, such as estrogen and parathyroid hormone, the expression of IGF-I is known to be increased in osteoblasts (Conover and Rosen, 2002), suggesting that IGF-I is an autocrine/paracrine regulator of osteoblast functions. Thus, it is likely that IGF-I-stimulated p44/p42 MAP kinase plays a crucial role in the regulation of bone metabolism under the physiological conditions. In addition, we investigated the effects of IGF-I treatment on the several known osteoblast differentiation markers (osteocalcin, osteopontin, Runx2 and collagen α1(I)) in osteoblast-like MC3T3-E1 cells. We found that IGF-I had no significant effect on up-regulation of these mRNA expressions at a dose that significantly induces ALP activation. It seems unlikely that the involvement of IGF-I signaling is common in differentially expressed osteoblast lineage markers. Mineralization defects have reportedly seen in ALP knockout mouse (Fedde et al., 1999). Taking these findings into account, as a whole, it is possible that the blockade of IGF-I signaling including p44/p42 MAP kinase might result in the impairment of mineralization. Further investigations would be required to clarify the details.

In conclusion, these results strongly suggest that p44/p42 MAP kinase among the MAP kinase superfamily plays a crucial role in the IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells.

Acknowledgements

We all thank Dr. Masato Tamura at Hokkaido University, for useful discussion and technical assistance. 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 (16590873 and 16591482), the Research Grants for Longevity Sciences (15A-1 and 15C-2), Health and Labour Sciences Research Grants for Research on Dimentia and Fracture and Research on proteomics from Ministry of Health, Labour and Welfare of Japan.

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Journal of Cellular Biochemistry 99:1564–1571 (2006)

Phosphatidylinositol 3-Kinase/Akt Auto-Regulates PDGF-BB-Stimulated Interleukin-6 Synthesis in Osteoblasts

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Abstract It has been reported that platelet-derived growth factor (PDGF)-BB stimulates the synthesis of interleukin (IL)-6 in osteoblasts. In the present study, we investigated whether the phosphatidylinositol 3-kinase (PI3K)/Akt is involved in the PDGF-BB-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. PDGF-BB markedly induced the phosphorylation of Akt and GSK-3β. Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-O-octadecylcarbonate, significantly amplified the synthesis of IL-6 by PDGF-BB. The PDGF-BB-induced GSK-3β phosphorylation was suppressed by the Akt inhibitor. The IL-6 synthesis stimulated by PDGF-BB was markedly enhanced by LY294002 and wortmannin, inhibitors of PI3K. Wortmannin and LY294002 suppressed the PDGF-BB-induced phosphorylation of Akt and GSK-3β. Taken together, these results strongly suggest that PI3K/Akt negatively regulates the PDGF-BB-stimulated IL-6 synthesis in osteoblasts. J. Cell. Biochem. 99: 1564–1571, 2006. © 2006 Wiley-Liss, Inc.

Key words: platelet-derived growth factor (PDGF); interleukin-6 (IL-6); phosphatidylinositol 3-kinase; Akt; osteoblast

It is generally known that platelet-derived growth factor (PDGF) is a mitogenic factor, which mainly acts on connective tissue cells [Heldin and Westermark, 1999; Heldin et al., 2002]. PDGF occurs as five different isoforms [Heldin et al., 2002]. PDGF isoforms were originally isolated from platelets, but have been shown to be produced and released from a variety of cell types including osteosarcoma [Heldin et al., 1986; Heldin and Westermark, 1999]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. As for stimulation of biological activities in bone cells, PDGF-BB is a potent stimulator and induces osteoblast proliferation and collagen synthesis [Canalis et al., 1992]. It is recognized that PDGF, released during platelet aggregation, has a pivotal role in fracture healing as a systemic factor and that PDGF also regulates bone remodeling as a local factor [Canalis et al., 1992]. PDGF receptor has an intrinsic protein tyrosine kinase activity and associates with SH-2 domain-containing substrates such as phospholipase C and phosphatidylinositol 3-kinase (PI3K) [Heldin and Westermark, 1999]. We have previously reported that PDGF-BB activates phosphatidylcholine-hydrolyzing phospholipase D via tyrosine kinase activation, resulting in protein kinase C activation in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1995]. However, the exact role of PDGF in osteoblasts is not precisely known.

Grant sponsor: The Ministry of Education, Science, Sports and Culture of Japan; Grant numbers: 16590873, 16591482; Grant sponsor: The Ministry of Health, Labour and Welfare of Japan; Grant numbers: 15A-1, 15C-2, 17A-3.

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Received 1 March 2006; Accepted 28 April 2006
DOI 10.1002/jcb.21007
Published online 30 June 2006 in Wiley InterScience (www.interscience.wiley.com).

Interleukin-6 (IL-6) is a multifunctional cytokine that has important physiological effects on a wide range of functions, such as promoting B cell differentiation, T cell activation, and inducing acute phase proteins [Akira et al., 1993; Heymann and Rousselle, 2000; Kwan Tat et al., 2004]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and promote osteoclast formation [Ishimi et al., 1990; Roodman, 1992; Heymann and Rousselle, 2000; Kwan Tat et al., 2004]. It has been reported that bone resorptive agents such as tumor necrosis factor-α (TNF-α) and IL-1 stimulate IL-6 synthesis in 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. It has been shown that PDGF-BB induces the transcription of IL-6 through the activator protein-1 complex and activating transcription factor-2 in primary cultured rat osteoblasts [Franchimont et al., 1999]. However, the exact mechanism underlying PDGF-BB-stimulated IL-6 synthesis in osteoblasts has not yet been precisely clarified.

It is currently known that Akt, also called protein kinase B, is a serine/threonine protein kinase that plays crucial roles in mediating intracellular signaling of variety of agonists including insulin-like growth factor-I, PDGF, and cytokines [Coffer et al., 1998]. It has been shown that Akt regulates biological functions, such as gene expression, survival, and oncogenesis [Coffer et al., 1998]. Accumulating evidence suggests that PI3K functions at a point upstream from Akt [Chan et al., 1999; Cantley, 2002]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of PI3K and activated. As for osteoblasts, it has been reported that TNF-α and PDGF induce translocation of Akt to the nucleus [Borgatti et al., 2000]. In addition, Akt is reportedly activated by cyclic stretch [Danciu et al., 2003] or androgen [Kang et al., 2004]. However, the exact role of Akt in PDGF-BB-effect on osteoblasts has not yet been clarified.

In the present study, we investigated whether PI3K/Akt is involved in the PDGF-BB-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that PI3-kinase/Akt activated by PDGF-BB plays an inhibitory role in the IL-6 synthesis in these cells.

MATERIALS AND METHODS

Materials

PDGF-BB and mouse IL-6 enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002 and wortmannin were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, and GSK-3β antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Akt inhibitor, wortmannin or LY294002 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect 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., 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 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. The plating density of the cells was about 8×10^5 cells/dish for 35-mm diameter dish, or 7×10^6 cells/dish for 90-mm diameter dish. The media were changed again when the various inhibitors and/or PDGF-BB were subsequently added.

IL-6 Assay

The cultured cells were stimulated by various dose of PDGF-BB in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pre-treated with Akt inhibitor, wortmannin or LY294002 for 60 min. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration was measured by ELISA kit.

Western Blot Analysis

The cultured cells were stimulated by PDGF-BB 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 [1970] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [Kato et al., 1996] by using phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, or GSK-3β antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

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).

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 the mean ± SD of triplicate determinations. Each experiment was repeated three times with similar results.

RESULTS

Effect of PDGF-BB on the Phosphorylation of Akt in MC3T3-E1 Cells

We examined the effect of PDGF-BB on the phosphorylation of Akt in order to investigate whether PDGF-BB activates Akt in osteoblast-like MC3T3-E1 cells. PDGF-BB time-dependently stimulated the phosphorylation of Akt up to 120 min (Fig. 1). The maximum effect of PDGF-BB on the phosphorylation of Akt was observed at 20 min after the stimulation.

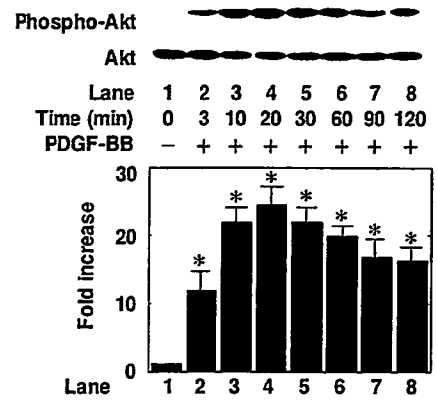

Fig. 1. Effect of PDGF-BB on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated by 50 ng/ml PDGF-BB for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

Effect of Akt Inhibitor on the IL-6 Synthesis by PDGF-BB in MC3T3-E1 Cells

It has been reported that PDGF-BB induces IL-6 transcription in osteoblasts from fetal rat calvariae [Franchimont et al., 1999]. We found that PDGF-BB stimulated IL-6 synthesis in a dose-dependent manner between 10 ng/ml and 70 ng/ml in osteoblast-like MC3T3-E1 cells (data not shown). In order to clarify the involvement of Akt pathway in the PDGF-BB-stimulated IL-6 synthesis in these cells, we first examined the effect of Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [Hu et al., 2000], on the IL-6 synthesis. The Akt inhibitor, which by itself hardly affected the IL-6 levels, significantly enhanced the PDGF-BB-stimulated synthesis of IL-6 (Fig. 2). The amplifying effect of the Akt inhibitor on the IL-6 synthesis was dose-dependent between 1 μM and 3 μM (Fig. 2). The Akt inhibitor at 10 μM caused about 100% enhancement in the PDGF-BB-effect.

Effect of Akt Inhibitor on the Phosphorylation of GSK-3β Induced by PDGF-BB in MC3T3-E1 Cells

It is generally known that GSK-3β is a critical downstream target molecule of the Akt, and its

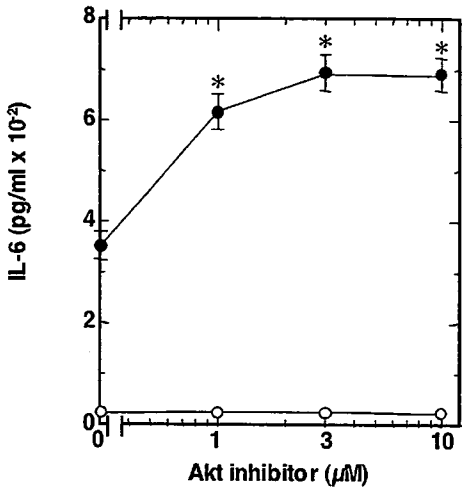

Fig. 2. Effect of Akt inhibitor on the PDGF-BB-stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of Akt inhibitor for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 24 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of PDGF-BB alone.

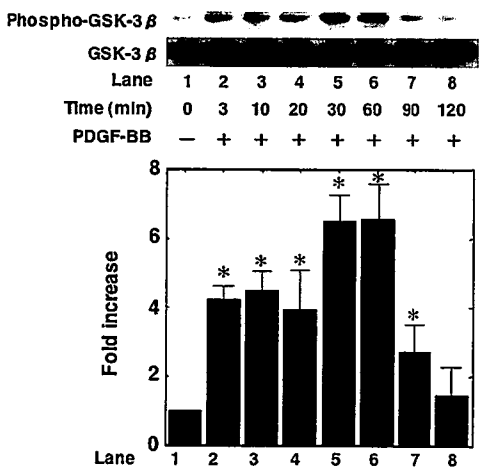

Fig. 3. Effect of PDGF-BB on the phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were stimulated by 50 ng/ml PDGF-BB for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of control.

activity can be inhibited by Akt-mediated phosphorylation of GSK-3β at Ser9 [Cross et al., 1995; Srivastava and Pandey, 1998]. We found that PDGF-BB truly induced the Akt-mediated phosphorylation of GSK-3β in a time-dependent manner in MC3T3-E1 cells (Fig. 3). The maximum effect of PDGF-BB on the phosphorylation of GSK-3β was observed at 60 min after the stimulation. We next examined the effect of Akt inhibitor on the phosphorylation of GSK-3β induced by PDGF-BB in these cells. Akt inhibitor markedly reduced the PDGF-BB-induced phosphorylation of GSK-3β (Fig. 4).

Effects of LY294002 or Wortmannin on the PDGF-BB-Induced Phosphorylation of Akt in MC3T3-E1 Cells

To clarify whether or not PI3K functions at a point upstream from Akt in MC3T3-E1 cells, we examined the effect of LY294002, a specific inhibitor of PI3-kinase [Vlahos et al., 1994], on the Akt phosphorylation induced by PDGF-BB.

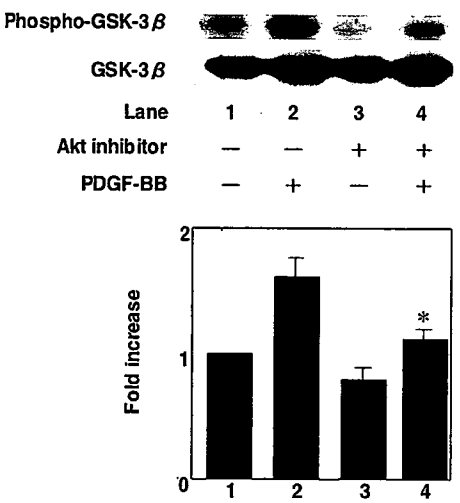

Fig. 4. Effect of Akt inhibitor on the PDGF-BB-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pre-treated with 50 μM Akt inhibitor for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 60 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of PDGF-BB alone.

LY294002 suppressed the PDGF-BB-induced phosphorylation of Akt (Fig. 5). The inhibitory effect of LY294002 was dose-dependent between 10 μM and 50 μM. In addition, the PDGF-BB-induced phosphorylation of Akt was markedly attenuated by wortmannin, another PI3-kinase inhibitor [Arcaro and Wymann, 1993], (Fig. 6).

Effects of LY294002 or Wortmannin on the PDGF-BB-Stimulated IL-6 Synthesis and Phosphorylation of GSK-3β in MC3T3-E1 Cells

LY294002, which by itself hardly affected the IL-6 levels, significantly amplified the PDGF-BB-stimulated synthesis of IL-6 (Fig. 7). The amplifying effect of LY294002 was dose-dependent between 1 μM and 10 μM. The LY294002 at 10 μM caused about 80% enhancement in the PDGF-BB effect. Wortmannin, as well as LY294002, also enhanced the PDGF-BB-stimulated synthesis of IL-6 without affecting IL-6 synthesis alone (data not shown). In addition, we found that the phosphorylation of GSK-3β

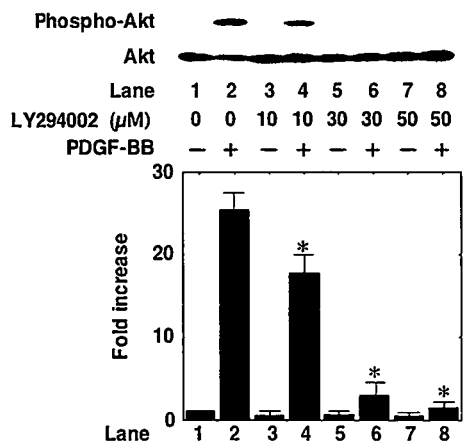

Fig. 5. Effect of LY294002 on the PDGF-BB-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of LY294002 for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

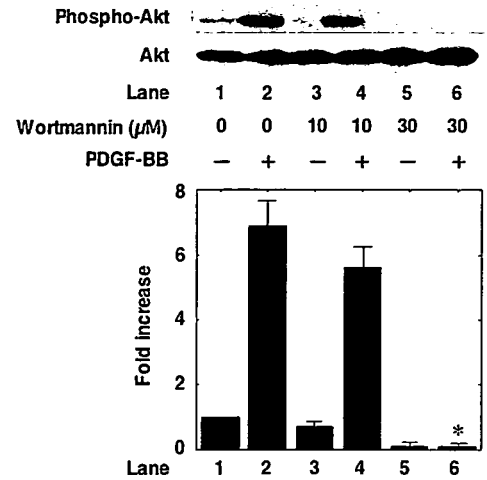

Fig. 6. Effect of wortmannin on the PDGF-BB-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of wortmannin for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

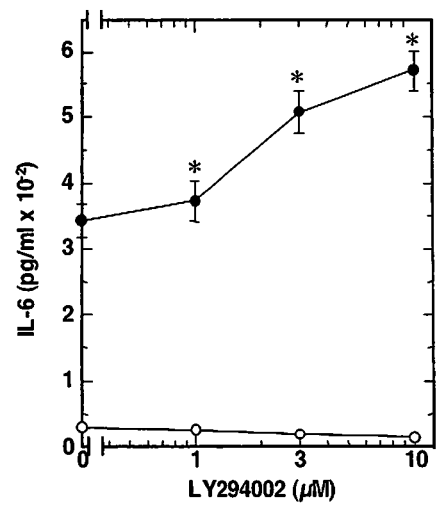

Fig. 7. Effect of LY294002 on the PDGF-BB-stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of LY294002 for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 24 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

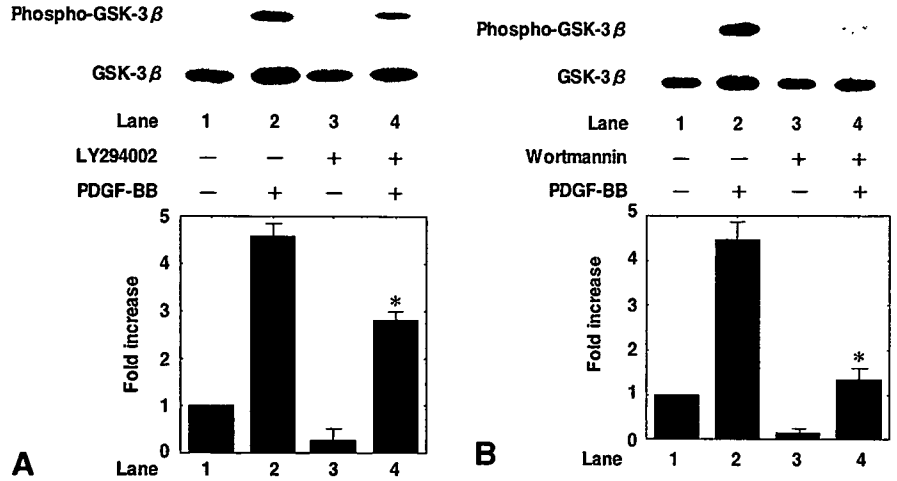

Fig. 8. Effects of LY294002 or wortmannin on the PDGF-BB-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pre-treated with 30 μM of LY294002 (**A**), 30 μM wortmannin (**B**) or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 60 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

induced by PDGF-BB was significantly reduced by LY294002 or wortmannin (Fig. 8A,B).

DISCUSSION

In the present study, we found that PDGF-BB time-dependently induced the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells. It has been reported that Akt is activated by PDGF-BB in osteoblastic cells [Chaudhary and Hruska, 2001]. In addition, we demonstrated that PI3K inhibitors, such as LY294002 [Vlahos et al., 1994] and wortmannin [Arcaro and Wymann, 1993] attenuated the PDGF-BB-induced phosphorylation of Akt in MC3T3-E1 cells. It has been shown that Akt is activated by phosphorylation of threonine and serine residues [Coffer et al., 1998; Chan et al., 1999]. The Akt signaling pathway is currently recognized to play a critical role in mediating survival signals in a wide range of cell types [Chan et al., 1999]. Therefore, these results suggest that PDGF-BB activates Akt via PI3K in osteoblast-like MC3T3-E1 cells.

We investigated whether or not PI3K/Akt functions in the PDGF-BB-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. First, Akt inhibitor [Hu et al., 2000] significantly enhanced the PDGF-BB-stimulated synthesis of IL-6. It is generally known that GSK-3β is a downstream target of Akt and phosphorylated by Akt [Cross et al., 1995; Srivastava and Pandey, 1998]. We confirmed that the PDGF-BB-induced phosphorylation of GSK-3β was truly reduced by the Akt inhibitor. These results suggest that the activated Akt has an inhibitory effect on IL-6 release by PDGF-BB in osteoblast-like MC3T3-E1 cells. In addition, we found that wortmannin [Arcaro and Wymann, 1993] and LY294002 [Vlahos et al., 1994] markedly amplified the PDGF-BB-induced IL-6 synthesis. Furthermore, the PDGF-BB-induced phosphorylation of GSK-3β was suppressed by wortmannin or LY294002. Therefore, taking our results into account as a whole, it is most likely that PDGF-BB activates the PI3K/Akt pathway, resulting in the reduction of IL-6 synthesis. It is probable that the PI3K/Akt signaling pathway activated by PDGF-BB limits the PDGF-BB-stimulated IL-6 synthesis. To the best of our knowledge, our present finding is probably the first report to show that the activation of PI3K/Akt leads to the negative-feedback regulation of IL-6 synthesis in osteoblasts.

The PI3K/Akt pathway is recognized to play a crucial role in several cellular functions, such as proliferation and cell survival in a variety of cells [Coffer et al., 1998]. Our present results indicate that the PI3K/Akt pathway in

osteoblasts has an important role in the regulatory mechanism of the production of IL-6 in bone metabolism. As for the PDGF-induced IL-6 production, it has been reported that three isoforms of PDGF including PDGF-BB induces the expression of IL-6 gene, resulting in the proliferation of human fibroblasts, vascular smooth muscle cells, and mesangial cells [Roth et al., 1995]. In addition, hypoxia-induced IL-6 production associated with the cell proliferation is reportedly mediated by PDGF in human lung-derived fibroblasts and vascular smooth muscle cells [Tamm et al., 1998]. However, the role of PI3K/Akt pathway in the PDGF-induced IL-6 production has not yet been clarified. As far as we know, this is probably the first report showing the role of PI3K/Akt as a negative regulator in the IL-6 production elicited by PDGF-BB. It is well recognized that IL-6 is a potent bone resorptive agent and induces osteoclast formation [Kwan Tat et al., 2004]. Therefore, our present results lead us to speculate that PDGF-BB-activated PI3K/Akt signaling acts as a negative regulator against bone resorption. It is possible that the PI3/Akt pathway in osteoblasts might be considered as a new candidate as a molecular target of bone resorption concurrent with various bone diseases. The physiological significance of the auto-regulation by PI3K/Akt of PDGF-BB effect remains still unclear. Further investigation is necessary to clarify the exact role of PI3K/Akt in osteoblasts.

In conclusion, our results strongly suggest that PI3K/Akt plays a crucial role in the regulation of PDGF-BB-stimulated IL-6 synthesis in osteoblasts, and may serve as a negative-feedback mechanism to avoid the over-synthesis of IL-6.

ACKNOWLEDGMENTS

We are very grateful to Yoko Kawamura and Seiko Sakakibara for their skillful technical assistance.

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