been reported that transforming growth factor-β (TGF-β) induces the expression of VEGF in osteoblast-like MC3T3-E1 cells [8,9]. However, the mechanism behind VEGF synthesis in osteoblasts has not yet been precisely clarified.

TGF-β is a multifunctional cytokine that regulates cell growth, differentiation and extracellular matrix production [10]. TGF-β, which is abundantly stored in bone matrix tissue, stimulates the recruitment and proliferation of osteoblasts [11]. As for intracellular signaling, TGF-β binds to the TGF-β type II receptor, which activates TGF-β type I receptor [12,13]. Activated type I receptor is known to phosphorylate Smad 2 and Smad 3, resulting in their translocation into the nucleus, where they can bind to DNA in the promoters of TGF-β target genes [14]. In addition to the Smad signaling pathway, other signaling pathways such as the mitogen-activated protein (MAP) kinase superfamily have recently been shown to mediate TGF-β signaling [14]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and c-*Jun* *N*-terminal kinase, are known to be central elements used by mammalian cells to transduce the diverse messages [15]. TGF-β-activated kinase (TAK1), a member of the MAP kinase kinase kinase family, has been identified as an upstream kinase for MAP kinase [16]. TAK1 kinase activity is stimulated by TGF-β in osteoblast-like MC3T3-E1 cells [16]. The p44/p42 MAP kinase pathway has been reported to participate in the upregulation of β1(I) collagen gene expression by TGF-β in ROS17/2.8 osteosarcoma cells [17]. We previously reported that TGF-β activates p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells resulting in VEGF synthesis [18,19]. However, the involvement of SAPK/JNK in TGF-β signaling in osteoblasts has not yet been elucidated.

In the present study, we investigated the involvement of SAPK/JNK in TGF-β-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. Here, we demonstrate that TGF-β activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in these cells, and that SAPK/JNK plays a role as a positive regulator in TGF-β-induced VEGF synthesis.

Materials and Methods

Materials

TGF-β and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). SP600125, SB203580 and PD98059 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phosphospecific 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. SP600125, SB203580 or PD98059 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the VEGF assay or the analysis of SAPK/JNK.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [20] were maintained as previously described [21]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35 mm diameter dishes or 90 mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

VEGF assay

The cultured cells were stimulated by TGF-β in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SP600125, PD98059 or SB203580 for 60 min. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of SAPK/JNK

The cultured cells were stimulated by TGF-β 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 according to Laemmli [22] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [23] by using phosphospecific SAPK/JNK antibodies or SAPK/JNK antibodies with peroxidase-labeled antibodies raised in goat anti-rabbit IgG used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film using the ECL Western blotting detection system. When indicated, the cells were pretreated with SP600125, PD98059 or SB203580 for 60 min.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). 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; $p < 0.05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effect of TGF-β on SAPK/JNK phosphorylation in MC3T3-E1 cells

To clarify whether TGF-β activates SAPK/JNK in osteoblast-like MC3T3-E1 cells, we examined the effect of TGF-β on SAPK/JNK phosphorylation. TGF-β markedly induced SAPK/JNK phosphorylation in a time-dependent manner (Fig. **1**). The maximum effect of TGF-β on phosphorylation was observed at 60 min after stimulation.

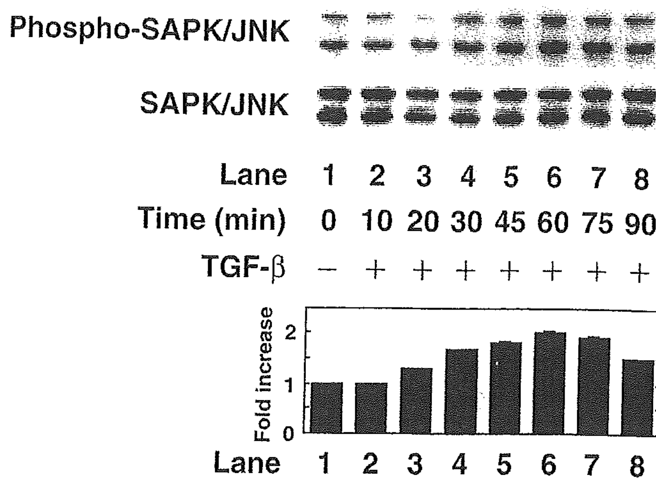


Fig. 1 Effect of TGF-β on SAPK/JNK phosphorylation in MC3T3-E1 cells. The cultured cells were stimulated by 5 ng/ml of TGF-β for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific SAPK/JNK or SAPK/JNK. The upper bands and the lower ones indicated p54 and p46 SAPK/JNK, respectively. Lane 1, control cells.

Effect of SP600125 on TGF-β-induced VEGF synthesis in MC3T3-E1 cells

We have recently shown that TGF-β stimulates VEGF synthesis by activating p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells [19]. To investigate whether SAPK/JNK is involved in TGF-β-induced VEGF synthesis in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of JNK [24], on VEGF synthesis. SP600125, which alone hardly affected basal VEGF levels, significantly reduced TGF-β-stimulated VEGF synthesis (Fig. **2A**). The inhibitory effect of SP600125 on TGF-β-induced VEGF synthesis was dose-dependent in the range between 1 and 30 µM (Fig. **2B**). The maximum inhibitory effect of SP600125 on synthesis was observed at 30 µM, which caused a 44% reduction in the effect of TGF-β.

Effect of SP600125 on SAPK/JNK phosphorylation induced by TGF-β in MC3T3-E1 cells

We found that SP600125 truly inhibited SAPK/JNK phosphorylation induced by TGF-β (Fig. **3**). According to the densitometric analysis, SP600125 caused a reduction of around 50% in the effect of TGF-β on SAPK/JNK phosphorylation.

Effect of PD98059 or SB203580 on TGF-β-induced SAPK/JNK phosphorylation in MC3T3-E1 cells

PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase [25], failed to affect TGF-β-induced SAPK/JNK phosphorylation (Fig. **4a**). Additionally, SB203580, a specific inhibitor of p38 MAP kinase [26], did not reduce TGF-β-induced SAPK/JNK phosphorylation (Fig. **4b**).

Combined effect of SP600125 and PD98059 or that of SP600125 and SB203580 on TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells

We further examined the effect of a combination of SP600125 and PD98059 on TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells. Combining SP600125 and PD98059 significantly reduced TGF-β-stimulated VEGF synthesis in an additive manner (Table **1**). Moreover, a combination of SP600125 and SB203580 also suppressed VEGF synthesis induced by TGF-β in an additive manner (Table **2**).

Discussion

The MAP kinase superfamily is currently known to mediate intracellular signaling of extracellular agonists and play a crucial role in cellular function including proliferation, differentiation, and cell death in a variety of cells [15]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK function as central elements used by mammalian cells to transduce a variety of messages from extracellular agonists to nucleus [15]. In the present study, we demonstrated that TGF-β induces SAPK/JNK phosphorylation in osteoblast-like MC3T3-E1 cells. We have previously shown that TGF-β activates both p44/p42 MAP kinase

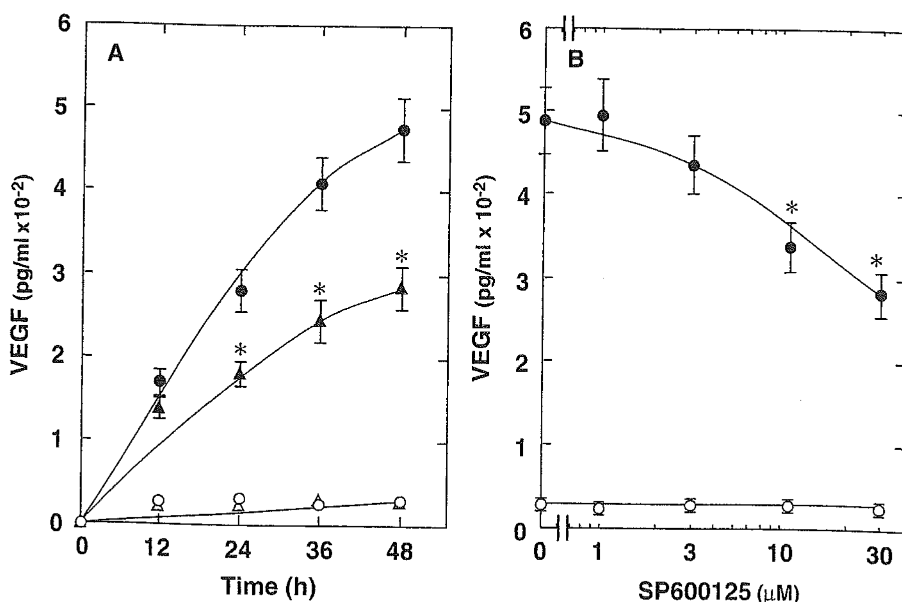


Fig. 2 Effect of SP600125 on TGF-β-induced VEGF synthesis in MC3T3-E1 cells. **a** The cultured cells were pretreated with 30 µM SP600125 (triangles) or vehicle (circles) for 60 min, and then stimulated by 5 ng/ml TGF-β (solid symbols) or vehicle (open symbols) for the indicated periods. **b** The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated by 5 ng/ml TGF-β (solid circles) or vehicle (open circles) for 48 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 with the value of TGF-β alone.

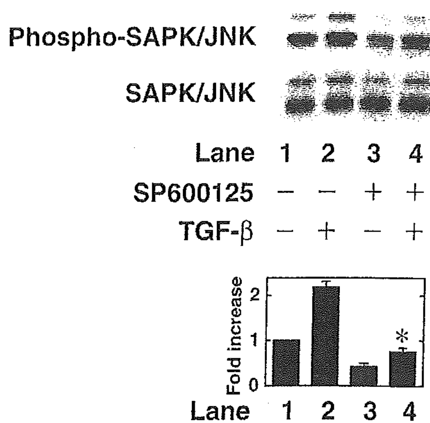
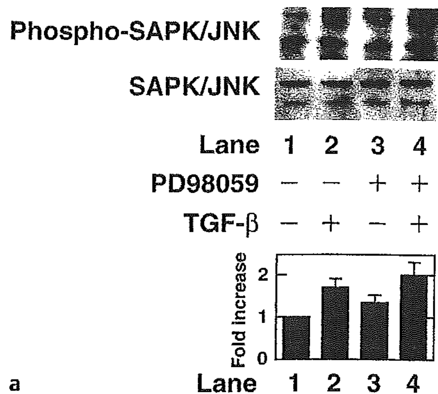


Fig. 3 Effect of SP600125 on TGF-β-induced SAPK/JNK phosphorylation in MC3T3-E1 cells.

The cultured cells were pretreated with 30 µM SP600125 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of TGF-β-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 TGF-β alone.



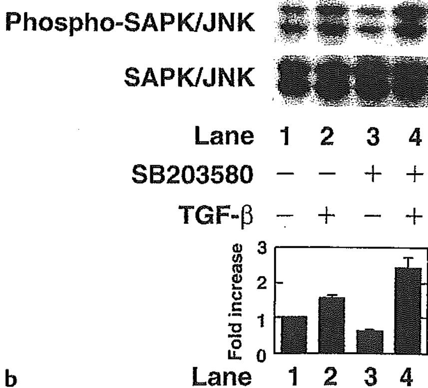


Fig. 4 Effect of PD98059 or SB203580 on TGF-β-induced SAPK/JNK phosphorylation in MC3T3-E1 cells.

a The cultured cells were pretreated with 50 µM PD98059 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 20 min. **b** Cultured cells were pretreated with 30 µM SB203580 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of TGF-β-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.

Table 1 Effect of a combination of SP600125 and PD98059 on TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells

SP600125	*PD98059*	*TGF-β*	*VEGF (pg/ml)*
–	–	–	28 ± 3
–	–	+	487 ± 40
–	+	–	24 ± 3
–	+	+	320 ± 35*
+	–	–	21 ± 2
+	–	+	281 ± 29*
+	+	–	28 ± 5
+	+	+	136 ± 18**

The cultured cells were pretreated with 30 µM SP600125, 10 µM PD98059 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 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 with the value of TGF-β alone. ** $p < 0.05$, compared with the value of SP600125 and TGF-β.

Table 2 Effect of a combination of SP600125 and SB203580 on the TGF-β-stimulated VEGF release in MC3T3-E1 cells

SP600125	*SB203580*	*TGF-β*	*VEGF (pg/ml)*
–	–	–	25 ± 3
–	–	+	505 ± 49
–	+	–	28 ± 3
–	+	+	316 ± 30*
+	–	–	21 ± 2
+	–	+	323 ± 29*
+	+	–	24 ± 5
+	+	+	115 ± 18**

The cultured cells were pretreated with 30 µM SP600125, 10 µM SB203580 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 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 with the value of TGF-β alone. ** $p < 0.05$, compared with the value of SP600125 and TGF-β.

and p38 MAP kinase in MC3T3-E1 cells [18]. MAP kinases are known to be activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [15,27]. Therefore, these results strongly suggest that TGF-β independently activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells.

TGF-β reportedly induces the expression of VEGF in osteoblast-like MC3T3-E1 cells [8,9]. Here, we have shown that SP600125, a specific inhibitor of SAPK/JNK [24], significantly suppresses TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. These results strongly suggest that the activation of SAPK/JNK is involved in TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, we found that SP600125 truly attenuated SAPK/JNK phosphorylation induced by TGF-β in these cells. Thus, it is probable that suppression of TGF-β-stimulated VEGF synthesis by SP600125 is due to the inhibition of SAPK/JNK activation. We have recently reported that activating both p44/p42 MAP kinase and p38 MAP kinase play important

roles as positive regulators in TGF-β-induced VEGF synthesis in MC3T3-E1 cells [19]. In addition, we have demonstrated that PD98059, a specific inhibitor of MEK [25] or SB203580, a specific inhibitor of p38 MAP kinase [26], did not reduce TGF-β-induced SAPK/JNK phosphorylation. Therefore, SAPK/JNK would seem to participate at least partly in TGF-β-stimulated VEGF synthesis, independently of p44/p42 MAP kinase or p38 MAP kinase. Moreover, TGF-β-stimulated VEGF synthesis was suppressed additively by a combination of SP600125 and PD98059 or SP600125 and SB203580. Based on these findings, it is most likely that VEGF synthesis stimulated by TGF-β is mediated through p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK activation in osteoblast-like MC3T3-E1 cells. We have recently reported that SAPK/JNK is involved in prostaglandin E1-induced VEGF synthesis in MC3T3-E1 cells [28]. Interestingly, not p44/p42 MAPK but p38 MAPK is involved in PGE1-induced VEGF synthesis in these cells [29]. In addition, activating p44/p42 MAPK and JNK but not p38 MAPK by TGF-β reportedly negatively regulates Smad3-induced alkaline phosphatase activity and mineralization in MC3T3-E1 cells [30]. Thus, it is likely that each of these MAP kinases plays a separate role and finely regulates the cellular function of osteoblasts. Further investigations on other signaling systems of TGF-β such as Smad 2 and Smad 3 are required to clarify the detail regulatory mechanism of VEGF synthesis induced by TGF-β in osteoblasts. Regarding the involvement of MAP kinases in VEGF synthesis stimulated by TGF-β, we previously reported that p44/p42 MAP kinase and p38 MAP kinase, but not SAPK/JNK, participate in TGF-β-induced VEGF synthesis in aortic smooth muscle A10 cells [31]. The involvement of SAPK/JNK in TGF-β-induced VEGF synthesis may not be a general phenomenon, but specific to osteoblasts.

Capillary network-providing microvasculature expansion is an essential process in bone remodeling [7]. Since VEGF is a specific mitogen of vascular endothelial cells [1], it is probable that VEGF synthesized by osteoblasts acts as an important intercellular mediator between osteoblasts and vascular endothelial cells. It has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse [2], supporting the importance of VEGF in bone metabolism. Flt-1, known as VEGF receptor 1, has been shown as a novel cell surface marker for the lineage of monocyte-macrophages in humans, which was found to differentiate efficiently into multinuclear osteoclasts [32]. Moreover, VEGF has recently been reported to induce osteoclast chemotaxis [33]. These evidences strongly suggest that VEGF secreted from osteoblasts also plays a role in the regulation of osteoclastic bone resorption. On the other hand, TGF-β is know to be produced by osteoblasts, stored abundantly in bone matrix in the latent form, and activated in the bone microenviroment [11]. During bone resorption, there is speculation that TGF-β in extracellular bone matrix is released, affecting osteoblasts and osteoprogenitor cells existing in the neighborhood. In addition, a recent study has reported that TGF-β expression is regulated by IGF-I receptor signaling in Ewing's osteosarcoma cells, suggesting the involvement of tumor angiogenesis [34]. Taking these reports into account, it seems that TGF-β-induced VEGF synthesis by osteoblasts plays a pivotal role in the process of bone remodeling through regulating the capillary endothelial cells proliferation and osteoclastic bone resorption. Further investigations would be necessary to clarify the exact role of VEGF in bone metabolism.

In conclusion, our present results strongly suggest that TGF-β activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblasts, and that SAPK/JNK plays at least a contributory role as a positive regulator in TGF-β-induced VEGF synthesis.

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Possible Involvement of Phosphatidylinositol 3-Kinase/ Akt Pathway in Insulin-like Growth Factor-I-induced Alkaline Phosphatase Activity in Osteoblasts

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Abstract

In the present study, we investigated whether Akt is involved in insulin-like growth factor-I (IGF-I)-stimulated activity of alkaline phosphatase, a marker of mature osteoblast phenotype, in osteoblast-like MC3T3-E1 cells. IGF-I induced the phosphorylation of Akt in these cells. Akt inhibitor significantly suppressed the IGF-I-stimulated alkaline phosphatase activity. The phosphorylation of Akt induced by IGF-I was reduced by the Akt inhibitor. LY294002 and wortmannin, inhibitors of phosphatidylinositol 3-kinase, significantly suppressed the IGF-I-induced alkaline phosphatase activity. The phosphorylation of Akt induced by IGF-I was markedly reduced by LY294002 and wortmannin. These results strongly suggest that phosphatidylinositol 3-kinase/Akt plays a role in the IGF-I-stimulated alkaline phosphatase activity in osteoblasts.

Key words
IGF-I · Alkaline phosphatase · Akt · Osteoblast

Introduction

Insulin-like growth factor-I (IGF-I) is generally known to play an important role in bone metabolism [1,2]. Mainly synthesized and secreted from liver, IGF-I mediates a variety of the actions of growth hormone secreted from pituitary gland under the control of the hypothalamus. Evidence is accumulating that IGF-I is necessary for bone-fracture healing [3]. Bone metabolism is recognized to be mainly regulated by two functional cells, osteoblasts and osteoclasts, with the former responsible for bone formation and the latter for bone resorption [4]. As for osteoblasts, IGF-I has been reported to stimulate the proliferation of these cells and synthesize bone matrix proteins [1]. We have previously demonstrated that IGF-I induces DNA synthesis synergistically with protein kinase C activation in osteoblast-like MC3T3-E1 cells [5]. In addition, IGF-I reportedly stimulates alkaline phosphatase activity, a marker of mature osteoblast phenotype [6], in osteoblasts [7]. IGF-I is also produced by osteoblasts [2]. In a previous study [8], we have shown that osteoblast-like MC3T3-E1 cells secrete IGF-I resulting in stimulation of mineralization, and protein kinase C activation suppresses the secretion of IGF-I. These findings gave rise to our speculation that IGF-I secreted from osteoblasts may play a crucial role in the regulation of bone metabolism.

Akt, also known as protein kinase B, is a serine/threonine protein kinase that plays a crucial role in mediating intracellular signaling of variety of agonists including IGF-I, platelet derived growth factor (PDGF), and cytokines [9]. Akt has been shown to regulate biological functions such as gene expression, survival and oncogenesis [9]. Akt has been identified as a downstream target of phosphatidylinositol 3-kinase [10,11]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of phosphatidylinositol 3-kinase, and activated. As for osteoblasts, IGF-I and PDGF have been reported to induce translocation of Akt to the nucleus [12]. In addition, cyclic stretch or androgen was recently reported to activate Akt [13,14]. How-

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ever, the exact role of Akt in IGF-I-effect on osteoblasts has not yet been clarified.

In the present study, we investigated whether Akt is involved in the IGF-I-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. In this communication, we will demonstrate that Akt plays a role in the IGF-I-stimulated alkaline phosphatase activity in these cells.

Materials and Methods

Materials

IGF-I was purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor, wortmannin and LY294002 were obtained from Calbiochem. Co. (La Jolla, CA). Phospho-Ser 473 in Akt antibodies and Akt antibodies were purchased from New England BioLabs, Inc. (Beverly, MA). The ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Wortmannin and LY294002 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for alkaline phosphatase activity or the analysis of Akt.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [15] were maintained as previously described [16]. 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.

Assay for alkaline phosphatase activity

The cultured cells were pretreated with Akt inhibitor, wortmannin or LY294002 for 60 min, then stimulated by IGF-I in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. At the end of 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 centrifuging the homogenate at 1,500 × *g* for 5 min, alkaline phosphatase activity of the supernatant was measured by the method of Lowry et al. [17].

Analysis of Western blotting

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 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 125000 × *g* for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [18] in 10% polyacrylamide gel. Western blotting analysis was performed as previously described [19] using phospho-specific Akt antibodies or Akt antibodies with peroxidase-labeled antibodies raised in goat vs. rabbit IgG used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on x-ray film with the ECL Western blotting detection system. Where indicated, the cells were pretreated with Akt inhibitor, LY294002 or wortmannin for 60 min.

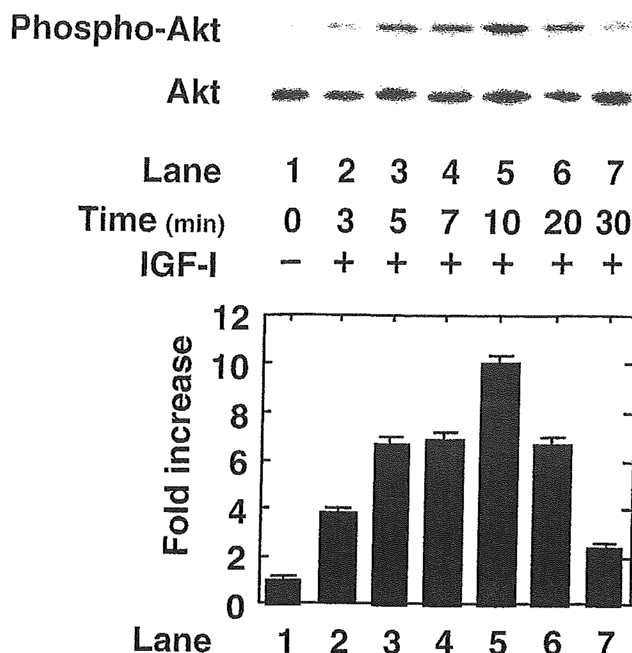

Fig. 1 Effects of IGF-I on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 0.1 µM IGF-I for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific Akt or Akt. The histogram shows quantitative representations of the levels of IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations.

Determinations

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). Densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA). Cell viability was assessed by Trypan blue dye exclusion test.

Statistical analysis

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

Results

Effects of IGF-I on the phosphorylation of Akt in MC3T3-E1 cells

To investigate whether IGF-I activates Akt in osteoblast-like MC3T3-E1 cells, we examined the effects of IGF-I on the phosphorylation of Akt. Stimulation of IGF-I significantly induces the phosphorylation of Akt (Fig. 1). The phosphorylation of Akt reached the peak at 10 min, and decreased from then on.

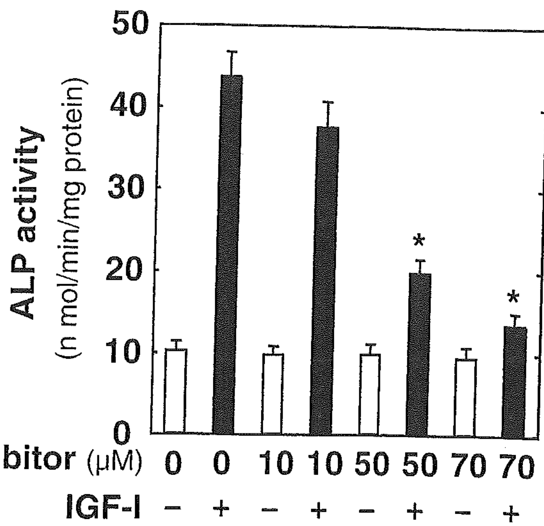

Fig. 2 Effect of Akt inhibitor on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor for 60 min, then stimulated by 0.1 µM IGF-I (filled bars) or vehicle (blank bars) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p < 0.05 compared to the value for IGF-I alone.

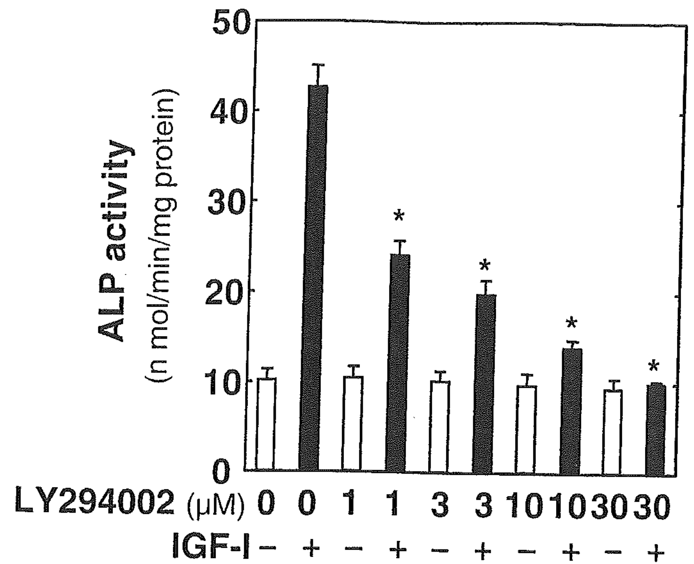

Fig. 4 Effect of LY294002 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 for 60 min, then stimulated by 0.1 µM IGF-I (filled bars) or vehicle (blank bars) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p < 0.05 compared to the value for IGF-I alone.

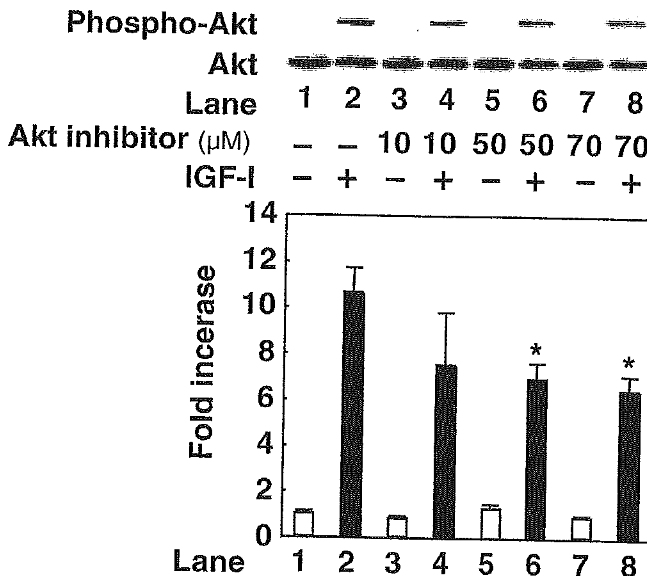

Fig. 3 Effect of Akt inhibitor on the IGF-I-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor for 60 min, then stimulated by 0.1 µM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific Akt or Akt. 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 ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p < 0.05 compared to the value for IGF-I alone.

Effect of Akt inhibitor on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells

To investigate whether Akt is involved in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells, we examined the effect of Akt inhibitor [20] on the alkaline phosphatase activity. Akt inhibitor, which alone hardly affected the basal level of alkaline phosphatase activity, significantly reduced IGF-I-induced alkaline phosphatase activity (Fig. **2**). The maximum inhibitory effect of Akt inhibitor at 70 µM caused a reduction in IGF-I effect of around 90%. Cell counts were hardly changed during treatment with Akt inhibitor for 48 h (data not shown), and cell viability after treatment was more than 90% of the control cells.

Effects of Akt inhibitor on the phosphorylation of Akt induced by IGF-I in MC3T3-E1 cells

We next examined the effect of Akt inhibitor on IGF-I-induced phosphorylation of Akt in these cells. IGF-I-induced phosphorylation of Akt was dose-dependently reduced by Akt inhibitor to between 10 and 70 µM (Fig. **3**). The maximum inhibitory effect of the Akt inhibitor was observed at 70 µM, which caused a reduction in IGF-I effect of about 45%.

Effects of LY294002 or wortmannin on IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells

Akt has been shown to act as a downstream effector of phosphatidylinositol 3-kinase [10,11]. Therefore, we next examined the effect of LY294002, a specific inhibitor of phosphatidylinositol 3-kinase [21], on IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. LY294002 significantly suppressed phosphorylation of Akt induced by IGF-I in a dose-dependent manner (Fig. **4**). We also examined the effect of wortmannin, another inhibitor of phosphatidylinositol 3-kinase [22], on the alkaline phosphatase activity. Wortmannin markedly inhibited IGF-I-induced alkaline phosphatase activity (data not shown). Cell counts were hardly changed during the treatment with LY294002 or wortmannin for 48 h (data not shown), and cell viability after these treatments was more than 90% of control cells.

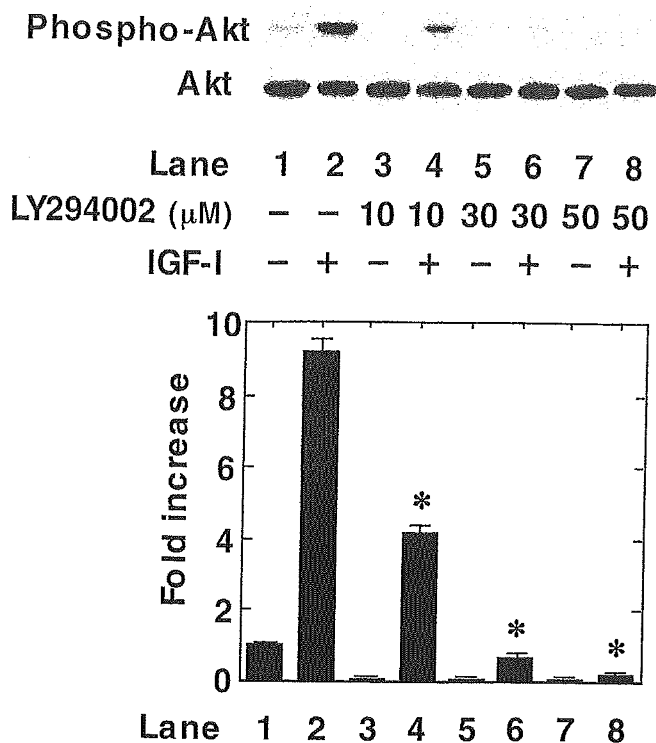

Fig. 5 Effect of LY294002 on the IGF-I-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 for 60 min, then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific Akt or Akt. The histogram shows quantitative representations of the levels of IGF-I-induced phosphorylation obtained from laser densitometric analysis in three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$ compared to the value for IGF-I alone.

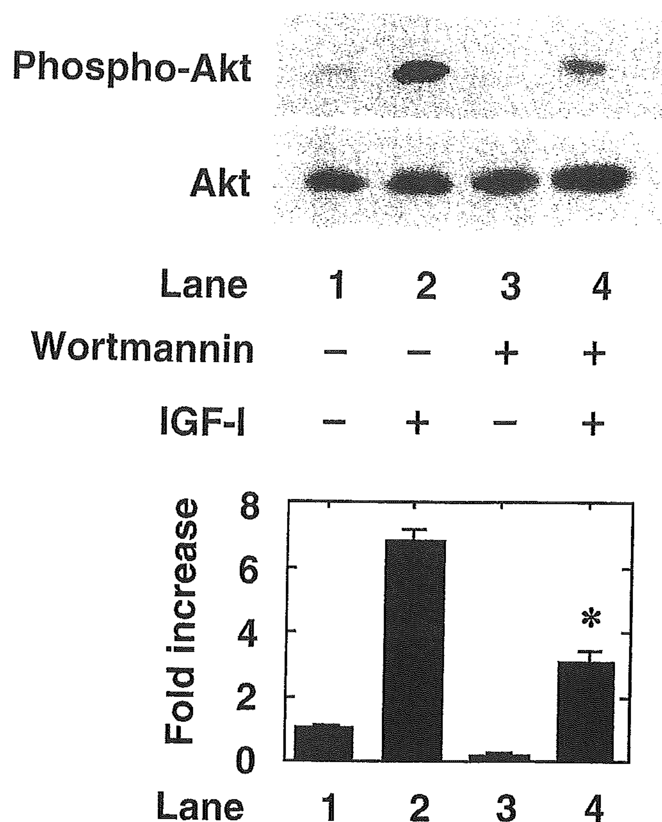

Fig. 6 Effect of wortmannin on the IGF-I-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 10 nM wortmannin for 60 min, then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific Akt or Akt. 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 ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$ compared to the value for IGF-I alone.

Effects of LY294002 or wortmannin on the phosphorylation of Akt induced by IGF-I in MC3T3-E1 cells

We found that LY294002 markedly inhibited the phosphorylation of Akt induced by IGF-I (Fig. 5). The inhibitory effect of LY294002 was dose-dependent at between 10 and 50 μM. According to the densitometric analysis, LY294002 (50 μM) caused an almost complete reduction of the IGF-I effect on Akt phosphorylation.

In addition, Akt phosphorylation induced by IGF-I was significantly attenuated by wortmannin (Fig. 6).

Discussion

In the present study, we have demonstrated that IGF-I time-dependently induces the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells. It is currently recognized that Akt mediates intracellular signaling by extracellular agonists and plays a crucial role in cellular functions such as proliferation and cell survival in a variety of cells [9]. Threonine and serine residues have been shown to activate Akt [9,10]. Therefore, taking these results into account, it is most likely that IGF-I activates Akt in osteoblast-like MC3T3-E1 cells.

IGF-I has been shown to stimulate the activity of alkaline phosphatase, a marker of mature osteoblast phenotype, in osteoblasts [7]. We investigated whether Akt is involved in IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. In this study, we have demonstrated that a specific inhibitor of Akt [20] significantly suppressed IGF-I-induced alkaline phosphatase activity as well as Akt phosphorylation in these cells. Thus, the activation of Akt is likely involved in IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. The effect of the Akt inhibitor on ALP activity that appeared to be induced by IGF-I only became apparent at concentrations that were considerably higher than the reported IC50 for Akt inhibition [20]. This could indicate either an unspecific action of the inhibitor or the requirement of higher concentrations to inhibit Akt in the cells studied. We examined the effect of Akt inhibitor in living MC3T3-E1 cells. Thus, it is probable that higher doses are required for inhibition of Akt in intact cells than in enzymological studies. In addition, Akt is known to be a downstream target of phosphatidylinositol 3-kinase [10,11]. We next examined the effects of phosphatidylinositol 3-kinase inhibitors on the IGF-I-stimulated activity of alkaline phosphatase in MC3T3-E1 cells. We found that wortmannin [22] significantly

reduced the alkaline phosphatase activity induced by IGF-I. In addition, wortmannin markedly attenuated the IGF-I-induced Akt phosphorylation. These findings suggest that phosphatidylinositol 3-kinase is implicated in IGF-I-stimulated alkaline phosphatase activity through Akt in MC3T3-E1 cells. We also showed that alkaline phosphatase activity induced by IGF-I was significantly suppressed by LY294002 [21]. LY294002 dose-dependently reduced IGF-I-stimulated Akt phosphorylation. Taking our results into account, Akt most likely plays a crucial role in IGF-I-stimulated alkaline phosphatase activity at a point downstream from phosphatidylinositol 3-kinase in osteoblast-like MC3T3-E1 cells. Here, LY294002 was sufficient to inhibit ALP to about 10% at a dose of 10 μM LY294002, while the phosphorylation of Akt is still about 40% at the same concentration of LY294002. Taking account of these findings, it is possible that a different PI3-kinase/Akt-independent mechanism(s) is involved in ALP-regulation by IGF-I, if not the non-specific effects of the inhibitor. Further investigation would be required to clarify the details.

Alkaline phosphatase is a well-known biochemical marker of bone formation [6,23]. Expression of alkaline phosphatase occurs during progression from an immature progenitor cell to a mature osteoblast, resulting in mineralization [24]. Thus, alkaline phosphatase is recognized as playing a pivotal role in mineralization, although the precise function of the enzyme is not clarified. We have demonstrated here that phosphatidylinositol 3-kinase/Akt plays a role in IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. It appears that IGF-I stimulated phosphorylation of Akt peaks within minutes, returning to basal values after only 30 min. On the other hand, we have observed significant upregulation of IGF-I-stimulated ALP activity after 48 h. Akt, which is activated by phosphatidylinositol 3-kinase, is recognized as an early step molecule of intracellular signal transduction [10,11]. Upregulation of ALP stimulated by IGF-I requires several cellular events, such as mRNA transcription and protein synthesis, which occur after the early signaling steps such as phosphatidylinositol 3-kinase/Akt. Thus, it seems reasonable that short-term activation of IGF-I-induced phosphatidylinositol 3-kinase/Akt is involved in the long-term upregulation of ALP activity in osteoblast-like MC3T3-E1 cells. Taking these findings as a whole, it is most likely that phosphatidylinositol 3-kinase/Akt activation has an important role in IGF-I-induced bone formation. Further investigations would be necessary to clarify the exact role of Akt in bone metabolism.

In conclusion, these results strongly suggest that phosphatidylinositol 3-kinase/Akt plays a crucial role in IGF-I-stimulated alkaline phosphatase activity in osteoblasts.

Acknowledgements

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