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.

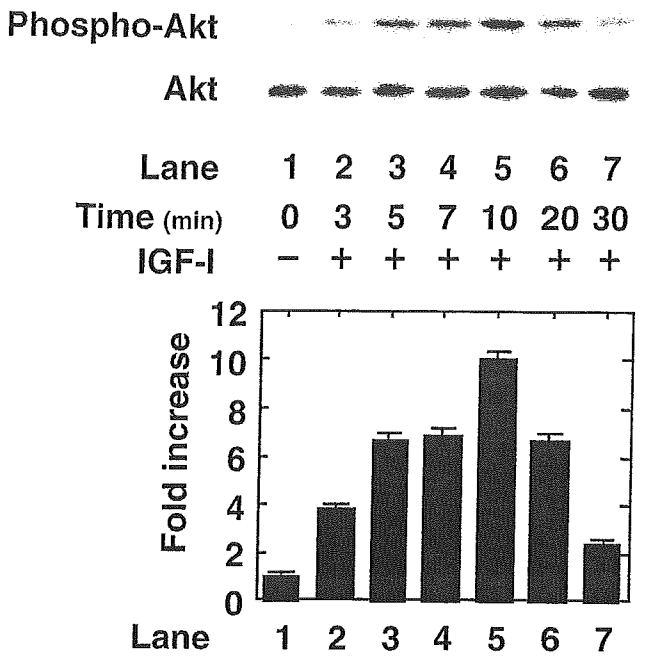

Fig. 1 Effects of IGF-I on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 0.1 μM IGF-I for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific Akt or Akt. The histogram 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.

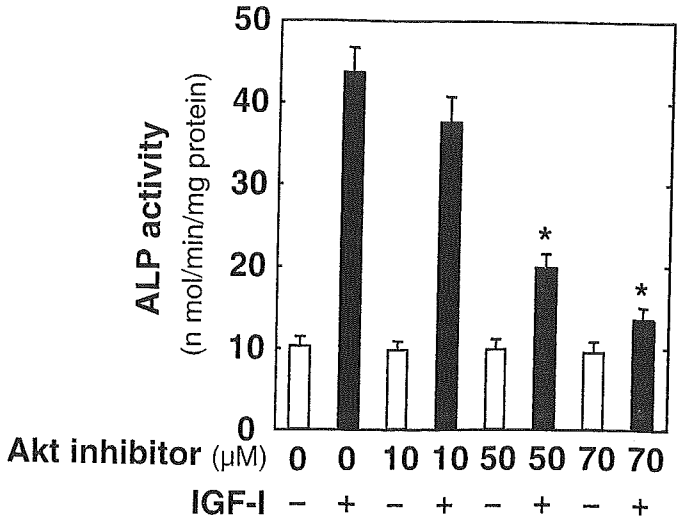

Fig. 2 Effect of Akt inhibitor on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor for 60 min, then stimulated by 0.1 μM IGF-I (filled bars) or vehicle (blank bars) for 48 h. Each value represents the mean ± SEM 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.

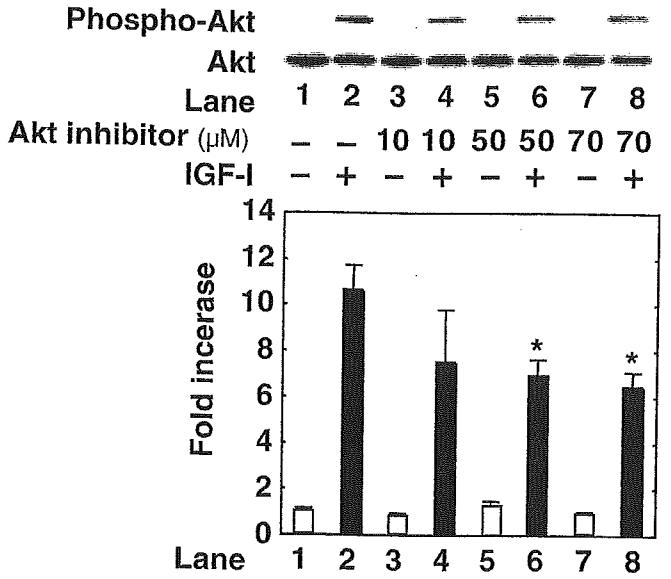

Fig. 3 Effect of Akt inhibitor on the IGF-I-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor for 60 min, then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western 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.

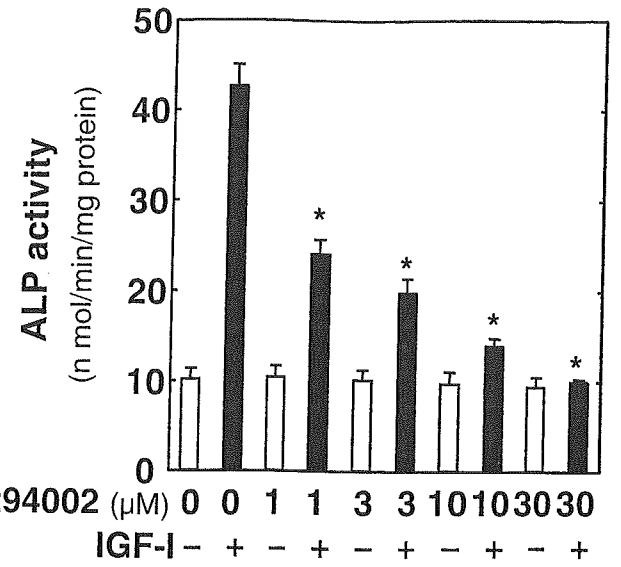

Fig. 4 Effect of LY294002 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 for 60 min, then stimulated by 0.1 μM IGF-I (filled bars) or vehicle (blank bars) for 48 h. Each value represents the mean ± SEM of triplicate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

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.

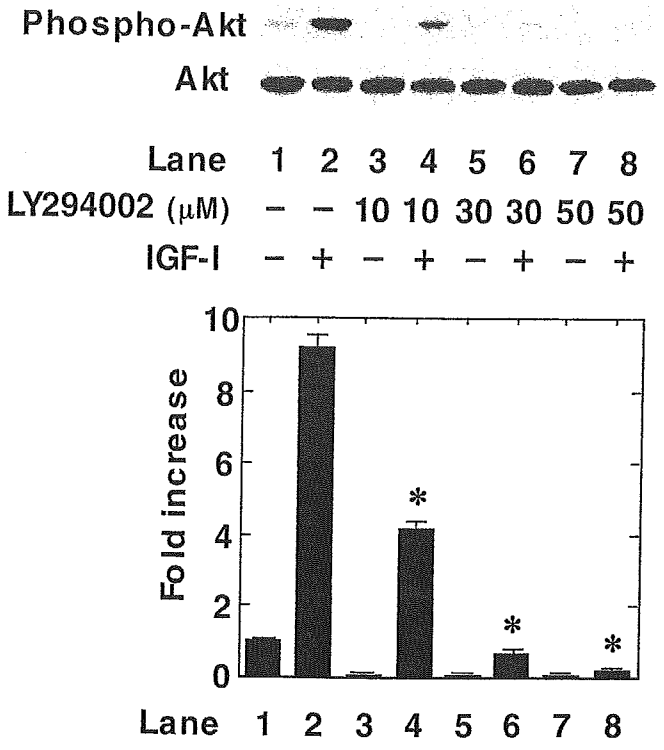

Fig. **5** Effect of LY294002 on the IGF-I-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 for 60 min, then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting 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.

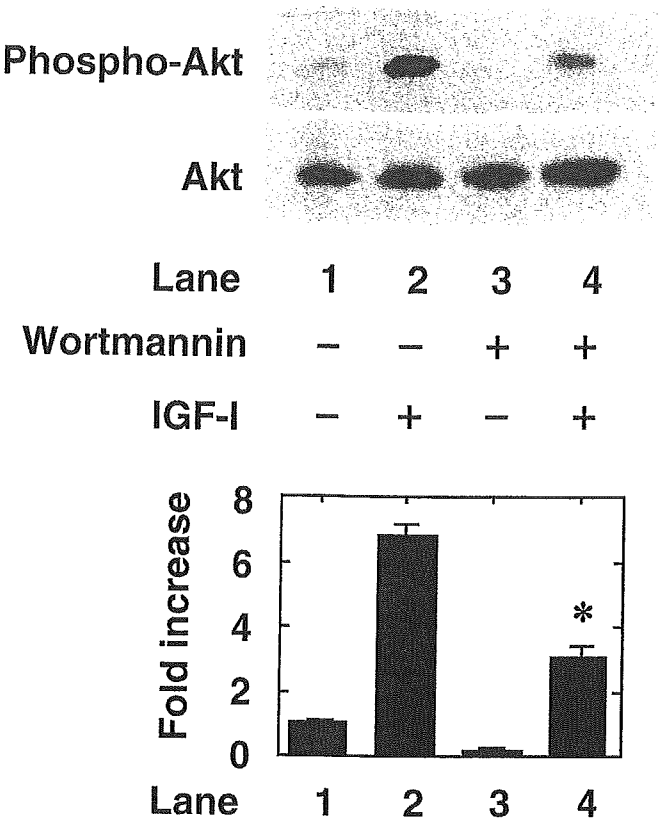

Fig. **6** Effect of wortmannin on the IGF-I-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 10 nM wortmannin for 60 min, then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting 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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