

## Activation of phosphatidylinositol 3-kinase/Akt limits FGF-2-induced VEGF release in osteoblasts

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### Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p44/p42 mitogen-activated protein (MAP) kinase, resulting in the release of vascular endothelial growth factor (VEGF) in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the role of Akt/protein kinase B in the FGF-2-stimulated VEGF release in these cells. FGF-2 time-dependently induced the phosphorylation of Akt and GSK-3 $\beta$ , a downstream element of Akt. The Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, significantly amplified the FGF-2-induced VEGF release, in a dose-dependent manner between 1 and 70  $\mu$ M, while it suppressed the FGF-2-induced phosphorylation of GSK-3 $\beta$ . The phosphorylation of Akt induced by FGF-2 was markedly attenuated by wortmannin and LY294002, inhibitors of phosphatidylinositol 3-kinase (PI3-kinase) in osteoblast-like MC3T3-E1 cells. Both wortmannin and LY294002 enhanced the FGF-2-induced VEGF release. In addition, Akt inhibitor had no significant effect on the FGF-2-induced phosphorylation of p44/p42 MAP kinase and SAPK/JNK. Furthermore, the FGF-2-induced Akt phosphorylation was not affected by PD98059, a MEK inhibitor, or SP600125, a SAPK/JNK inhibitor. Taken together, our findings strongly suggest that PI3-kinase/Akt plays an inhibitory role in FGF-2-induced VEGF release in osteoblasts.

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**Keywords:** FGF-2; PI3K/Akt; VEGF; Osteoblast

### 1. Introduction

It is generally known that bone remodeling carried out by osteoclasts and osteoblasts is accompanied by angiogenesis and capillary outgrowth (Brighton, 1978; Brighton and Hunt, 1991; Parfitt, 1994). During bone remodeling, capillary endothelial cells provide the microvasculature. Thus, it is currently recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated and regulate bone metabolism (Erlebacher et al., 1995). These functional cells influence one another via humoral factors as well as by direct cell-to-cell contact. Vascular endothelial growth factor (VEGF) is a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara and Davis-Smyth, 1997). VEGF that is produced and secreted

from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells (Ferrara and Davis-Smyth, 1997). As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate (Gerber et al., 1999). Accumulating evidence indicates that osteoblasts produce and secrete VEGF in response to various humoral factors (Goad et al., 1996; Wang et al., 1996; Ferrara and Davis-Smyth, 1997; Schallaeppi et al., 1997). Therefore, it is thought that VEGF secreted from osteoblasts may play a crucial role in the regulation of bone metabolism (Erlebacher et al., 1995; Zelzer and Olsen, 2005). However, the exact mechanism behind VEGF synthesis and its release in osteoblasts is not fully understood.

Basic fibroblast growth factor (FGF-2) is embedded in bone matrix, and osteoblasts synthesize FGF-2 (Baylink et al., 1993; Hurley et al., 1993). FGF-2 expression in osteoblasts is detected during fracture repair (Bolander, 1992). Therefore, it is

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currently recognized that FGF-2 may play a pivotal role in fracture healing, bone remodeling and osteogenesis (Marie, 2003). We have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily (Widmann et al., 1999), p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) play as positive regulators in the VEGF release (Tokuda et al., 2000, 2003).

It is well recognized 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 FGF-2, insulin-like growth factor I (IGF-I) and platelet-derived growth factor (PDGF) (Coffer et al., 1998). It has been shown that Akt regulates biological functions such as gene expression, cell survival and oncogenesis (Coffer et al., 1998). Akt has been identified as a downstream target of phosphatidylinositol 3-kinase (PI3-kinase) (Chan et al., 1999; Cantley, 2002). As for osteoblasts, FGF-2 reportedly induces the cell survival via PI3-kinase/Akt (Chaudhary and Hruska, 2001; Debais et al., 2004). We have recently shown that IGF-I stimulates activity of alkaline phosphatase, a mature osteoblast phenotype, via Akt in osteoblast-like MC3T3-E1 cells (Noda et al., 2005). These findings gave rise to our speculation that Akt in osteoblasts may play an important role in the regulation of bone metabolism. However, the relationship between VEGF release and PI3-kinase/Akt in osteoblasts remains unclear. In the present study, therefore, we investigated whether Akt is involved in the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. We here show that PI3-kinase/Akt auto-regulates FGF-2-induced VEGF release in these cells.

## 2. Materials and methods

### 2.1. Materials

Mouse VEGF enzyme immunoassay kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-*(R)*-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002, wortmannin, PD98059 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Actinomycin D was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3 $\beta$  antibodies, GSK-3 $\beta$  antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK 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, LY294002, PD98059 and SP600125 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or Western blot analysis.

### 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., 1992). Briefly, the cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cells were seeded into 35 or 90-mm diameter dishes in  $\alpha$ -MEM containing 10% FCS. After 5 days, the medium was exchanged for  $\alpha$ -MEM containing 0.3% FCS. The cells were used for experiments after 24 h.

Freshly isolated osteoblasts were obtained from the calvaria of newborn (1 or 2-day-old) balb/c mice as previously described (Yoshida et al., 2004). They were seeded into 90-mm-diameter dishes ( $25 \times 10^4$  cells) in  $\alpha$ -MEM containing 10% FCS. The medium was changed every 3 days until the cells were reached confluence at about 5 days. Then, the medium was exchanged for  $\alpha$ -MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

### 2.3. VEGF assay

The cultured cells were stimulated by FGF-2 in 1 ml of  $\alpha$ -MEM containing 0.3% FCS for the indicated periods. The cells were pretreated with Akt inhibitor, wortmannin, LY294002 or actinomycin D for 60 min. The reaction was terminated by collecting the medium, and then VEGF in the medium was measured by Quantikine<sup>®</sup> mouse VEGF enzyme immunoassay kit (R&D systems) according to the manufacture's instruction. The assay kit can detect the mouse VEGF in the range between 7.8 and 500 pg/ml. When the samples generate values higher than 500 pg/ml, the samples were adequately diluted with calibrator diluent provided with the kit, and re-assayed.

### 2.4. Western blot analysis

The cultured cells were stimulated by FGF-2 in  $\alpha$ -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  $125,000 \times g$  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-3 $\beta$  antibodies, GSK-3 $\beta$  antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 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 PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

### 2.5. Determination

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

### 2.6. 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  $\pm$  S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

## 3. Results

### 3.1. Effect of FGF-2 on the phosphorylation of Akt in MC3T3-E1 cells

We examined the effect of FGF-2 on the phosphorylation of Akt in order to investigate whether FGF-2 activates Akt in MC3T3-E1 cells. FGF-2 time-dependently induced the phosphorylation of Akt up to 60 min (Fig. 1). The maximum effect of FGF-2 on the phosphorylation of Akt was observed at 10 min after the stimulation.

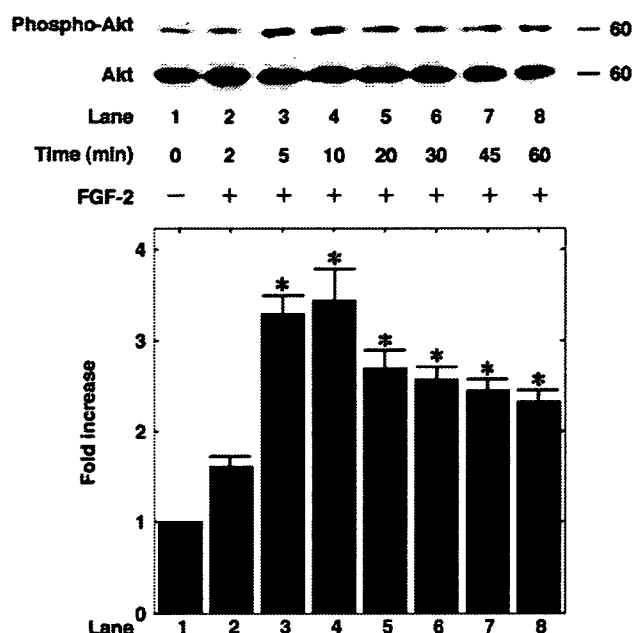


Fig. 1. Effect of FGF-2 on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations. \* $p < 0.05$ , compared to the value of control.

### 3.2. Effects of Akt inhibitor on the VEGF release by FGF-2 or the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells

In our previous studies (Tokuda et al., 2000, 2003), we have demonstrated that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells. In order to clarify whether or not Akt pathway is involved in the FGF-2-stimulated VEGF release 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 VEGF release. The Akt inhibitor, which by itself had little effect on the VEGF levels, significantly amplified the FGF-2-induced release of VEGF (Fig. 2A). The amplifying effect of the Akt inhibitor on the VEGF release was dose-dependent between 1 and 70  $\mu$ M (Fig. 2A). The Akt inhibitor at 70  $\mu$ M caused about 140% enhancement in the FGF-2-effect. We next examined the effect of the Akt inhibitor on the phosphorylation of Akt induced by FGF-2 in MC3T3-E1 cells. The Akt inhibitor failed to affect the FGF-2-induced phosphorylation of Akt (Fig. 2B).

### 3.3. Effect of Akt inhibitor on the VEGF release by FGF-2 in primary culture of osteoblasts

We investigated the effect of Akt inhibitor on the FGF-2-induced VEGF release in primary culture of osteoblasts. We

found that the Akt inhibitor significantly enhanced the FGF-2-induced VEGF release also in primary cultured osteoblasts (Fig. 2C). The amplifying effect of the Akt inhibitor on the VEGF release was dose-dependent in the range between 1 and 30  $\mu$ M (Fig. 2C). The maximum effect of the Akt inhibitor (30  $\mu$ M) caused about 60% enhancement in the FGF-2-effect.

### 3.4. Effect of Akt inhibitor on the phosphorylation of Akt and GSK-3 $\beta$ induced by FGF-2 in MC3T3-E1 cells

It is well recognized that GSK-3 is a critical downstream target molecule of the Akt cell survival pathway, and its activity can be inhibited by Akt mediated phosphorylation of GSK-3 $\alpha$  at Ser21 and GSK-3 $\beta$  at Ser9 (Cross et al., 1995; Srivastava and Pandey, 1998). We found that FGF-2 truly induced the Akt mediated phosphorylation of GSK-3 $\beta$  time-dependent manner in MC3T3-E1 cells (Fig. 3A). Then, we examined the effect of Akt inhibitor on the phosphorylation of GSK-3 $\beta$  induced by FGF-2 in these cells. Akt inhibitor markedly suppressed the FGF-2-induced phosphorylation of GSK-3 $\beta$  (Fig. 3B). The inhibitor (50  $\mu$ M) caused a reduction in FGF-2-effect of about 50%.

### 3.5. Effects of LY294002 or wortmannin on the FGF-2-induced VEGF release in MC3T3-E1 cells

To investigate whether or not PI3-kinase is correlated to Akt, we examined the effect of LY294002, a specific inhibitor of PI3-kinase (Vlahos et al., 1994), on the VEGF release by FGF-2 in MC3T3-E1 cells. LY294002, alone hardly affected the VEGF levels, significantly enhanced the FGF-2-induced VEGF release. The amplifying effect of LY294002 was dose-dependent between 3 and 30  $\mu$ M (Fig. 4A). The LY294002 at 30  $\mu$ M caused about 60% enhancement in the FGF-2-effect. Wortmannin, another PI3-kinase inhibitor (Arcaro and Wymann, 1993), as well as LY294002, also enhanced the FGF-2-induced VEGF release in the range between 1 and 3 nM without affecting VEGF release alone (Fig. 4B). Wortmannin at 3 nM caused about 60% enhancement in the FGF-2-effect.

### 3.6. Effects of wortmannin or LY294002 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells

We next examined the effects of wortmannin or LY294002 on the phosphorylation of Akt in MC3T3-E1 cells. Both wortmannin and LY294002 truly suppressed the FGF-2-induced phosphorylation of Akt (data not shown). Wortmannin (100 nM) caused a reduction in FGF-2-effect of about 80%. In addition, we found that the phosphorylation of GSK-3 $\beta$  induced by FGF-2 was significantly attenuated by wortmannin and LY294002 (Fig. 5A and B).

### 3.7. Effects of Akt inhibitor on FGF-2-induced phosphorylations of p44/p42 MAP kinase and SAPK/JNK in MC3T3-E1 cells

We have previously reported that FGF-2 stimulates VEGF release at least in part via p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2000, 2003).

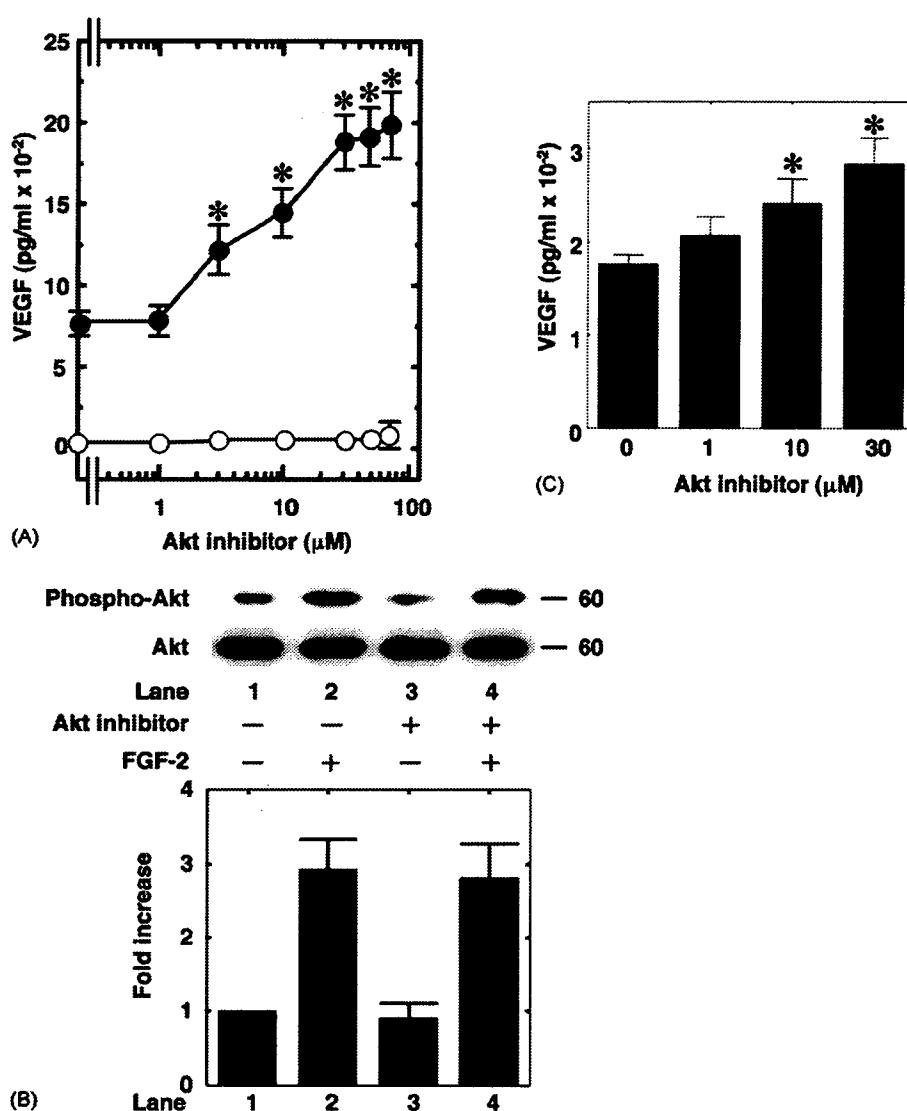


Fig. 2. Effects of Akt inhibitor on the VEGF release by FGF-2 or the FGF-2-induced phosphorylation of Akt. (A) Osteoblast-like MC3T3-E1 cells were pretreated with various doses of Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean  $\pm$  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 FGF-2 alone. (B) Osteoblast-like MC3T3-E1 were pretreated with 50  $\mu\text{M}$  Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. (C) Primary culture of osteoblast were pretreated with various doses of Akt inhibitor, and then stimulated by 30 ng/ml of FGF-2 or vehicle for 24 h. Values for FGF-2-unstimulated cells were subtracted to produce each data point. Each value represents the mean  $\pm$  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.

In order to investigate whether Akt inhibitor-effect on the FGF-2-induced VEGF release is dependent upon the activation of p44/p42 MAP kinase or SAPK/JNK, we next examined the effect of Akt inhibitor on the FGF-2-induced phosphorylations of p44/p42 MAP kinase or SAPK/JNK in these cells. However, Akt inhibitor failed to affect the phosphorylations of p44/p42 MAP kinase or SAPK/JNK induced by FGF-2 (Fig. 6A and B).

### 3.8. Effects of PD98059 or SP600125 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells

On the other hand, PD98059, a highly specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi et

al., 1995), had little effect on the FGF-2-induced Akt phosphorylation (Fig. 7A). Furthermore, we found that the FGF-2-induced phosphorylation of Akt was not affected by SP600125, a highly specific inhibitor of JNK (Bennett et al., 2001) (Fig. 7B).

### 3.9. Effect of actinomycin D on the enhancement by Akt inhibitor of FGF-2-stimulated VEGF release in MC3T3-E1 cells

We examined the effect of actinomycin D, a transcriptional inhibitor (Reich, 1963), on the enhancement by the Akt inhibitor of FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. Actinomycin D, which by itself had no effect on the basal

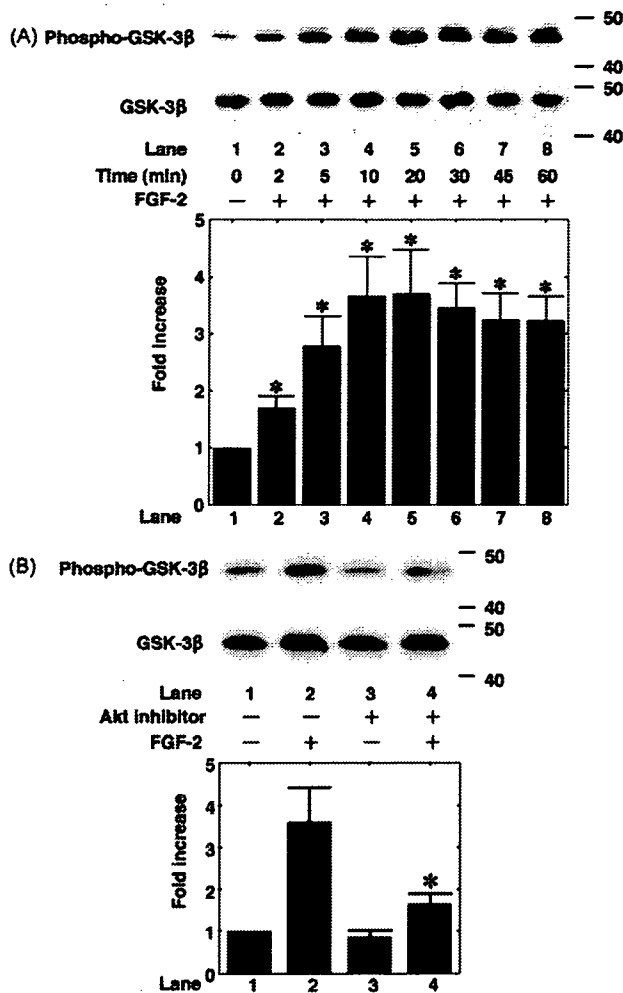


Fig. 3. Effect of FGF-2 on the phosphorylation of GSK-3 $\beta$ , and effect of Akt inhibitor on the FGF-2-induced phosphorylation of GSK-3 $\beta$  in MC3T3-E1 cells. (A) The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. \* $p$ <0.05, compared to the value of control. (B) The cultured cells were pretreated with 50  $\mu$ M Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. \* $p$ <0.05, compared to the value of FGF-2 alone. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3 $\beta$  or GSK-3 $\beta$ . The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations.

levels of VEGF, significantly reduced both the VEGF release induced by FGF-2 and the enhancement by the Akt inhibitor of FGF-2-stimulated VEGF release (Table 1).

#### 4. Discussion

In the present study, we demonstrated that FGF-2 time dependently induced the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells. It is generally known that Akt mediates intracellular signaling of various extracellular agonists and plays a crucial role in cellular functions such as proliferation and cell survival in a variety of cells (Coffer et al., 1998). According to

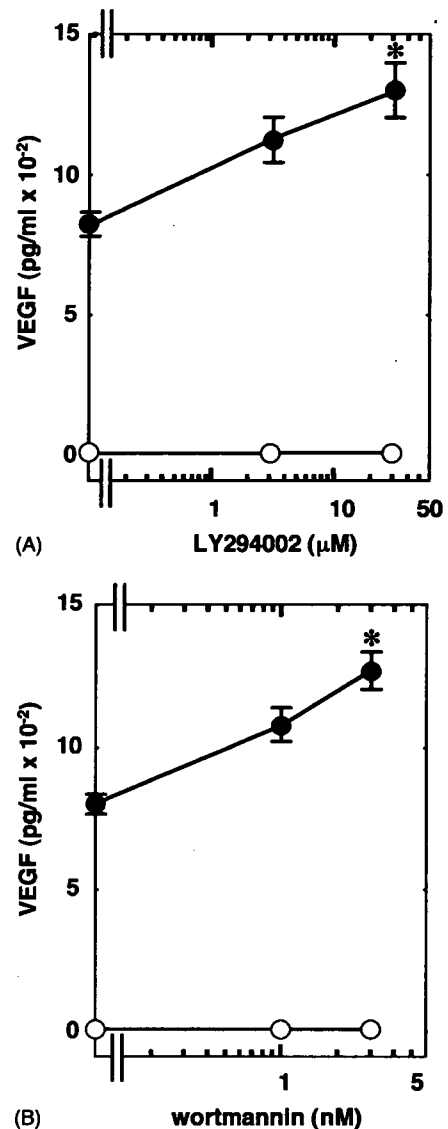


Fig. 4. Effects of wortmannin or LY294002 on the FGF-2-induced VEGF release in MC3T3-E1 cells. Osteoblast-like MC3T3-E1 cells were pretreated with various doses of LY294002 (A) or wortmannin (B) for 60 min, and then stimulated by 30 ng/ml FGF-2 ( $\bullet$ ) or vehicle ( $\circ$ ) for 48 h. Each value represents the mean  $\pm$  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 FGF-2 alone.

the previous reports (Coffer et al., 1998; Chan et al., 1999), Akt is activated by phosphorylation of threonine and serine residues. Therefore, our present result suggests that FGF-2 truly activates Akt in osteoblast-like MC3T3-E1 cells. In addition, we next showed that PI3-kinase inhibitors such as LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wymann, 1993) attenuated the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells. PI3-kinase is recruited upon growth factor receptor activation and produces 3' phosphoinositide lipids (Dudek et al., 1997; Katso et al., 2001). The lipid products of PI3-kinase act as second messengers by binding to and activating diverse cellular target proteins. In addition, it is well known that Akt is a downstream target of PI3-kinase (Chan et al., 1999; Cantley,

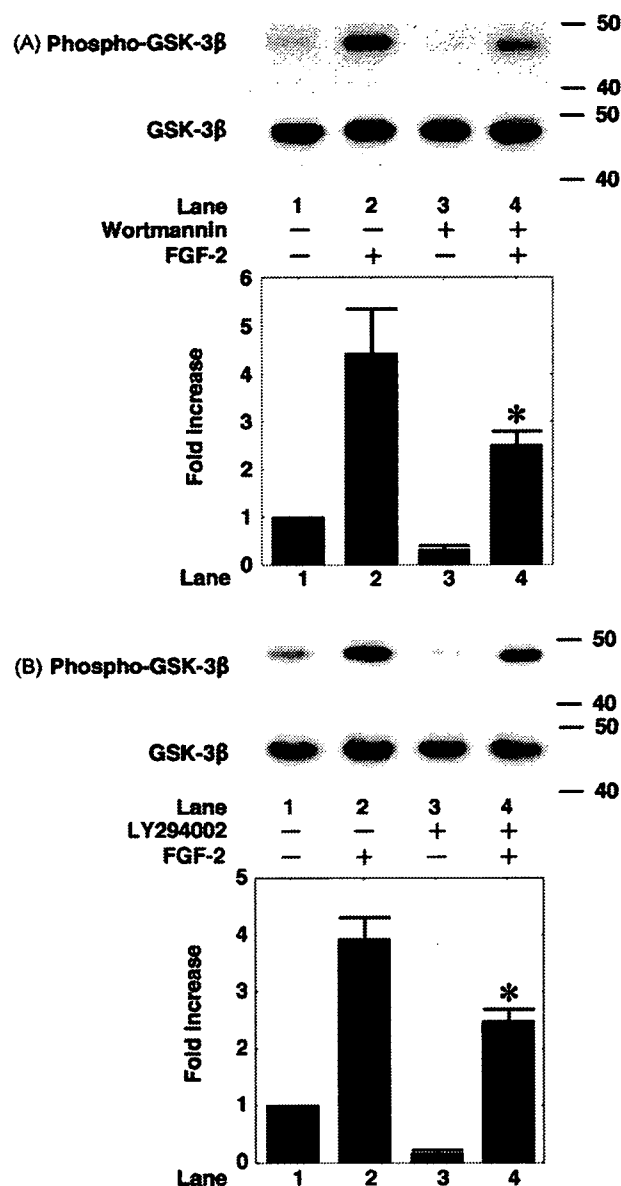


Fig. 5. Effects of wortmannin or LY294002 on the FGF-2-induced phosphorylation of GSK-3 $\beta$  in MC3T3-E1 cells. The cultured cells were pretreated with 0.1  $\mu$ M of wortmannin (A), 30  $\mu$ M of LY294002 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3 $\beta$  or GSK-3 $\beta$ . The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations. \* $p < 0.05$ , compared to the value of FGF-2 alone.

2002). Nowadays, the PI3-kinase/Akt signaling pathway is recognized to play a critical role in mediating survival signals in a wide range of cell types. Taking these findings into account, it is most likely that PI3-kinase/Akt pathway participates in the FGF-2 signaling in osteoblast-like MC3T3-E1 cells.

Hence, we have previously reported that FGF-2 stimulates the release of VEGF in osteoblast-like MC3T3-E1 cells (Tokuda

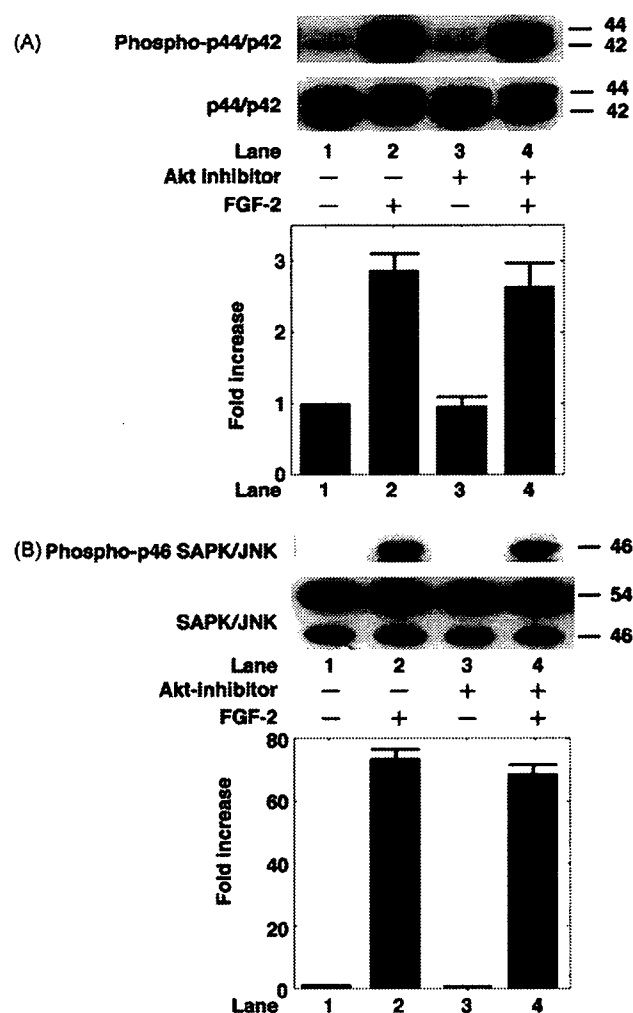


Fig. 6. Effects of Akt inhibitor on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 50  $\mu$ M Akt inhibitor or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (A) phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase, or (B) phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations.

et al., 2000), we next investigated whether PI3-kinase/Akt is involved in the FGF-2-induced VEGF release in MC3T3-E1 cells. First, Akt inhibitor (Hu et al., 2000) significantly enhanced the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. In addition, we found that the Akt inhibitor significantly enhanced the FGF-2-induced VEGF release also in primary cultured osteoblasts. Therefore, it is probable that the negative regulation by Akt of FGF-2-induced VEGF release is a general phenomenon in osteoblasts. Although the phosphorylation of Akt was observed prior to the FGF-2 stimulation, and the Akt inhibitor alone did not show any effect on the VEGF release in osteoblast-like MC3T3-E1 cells. It is generally recognized that

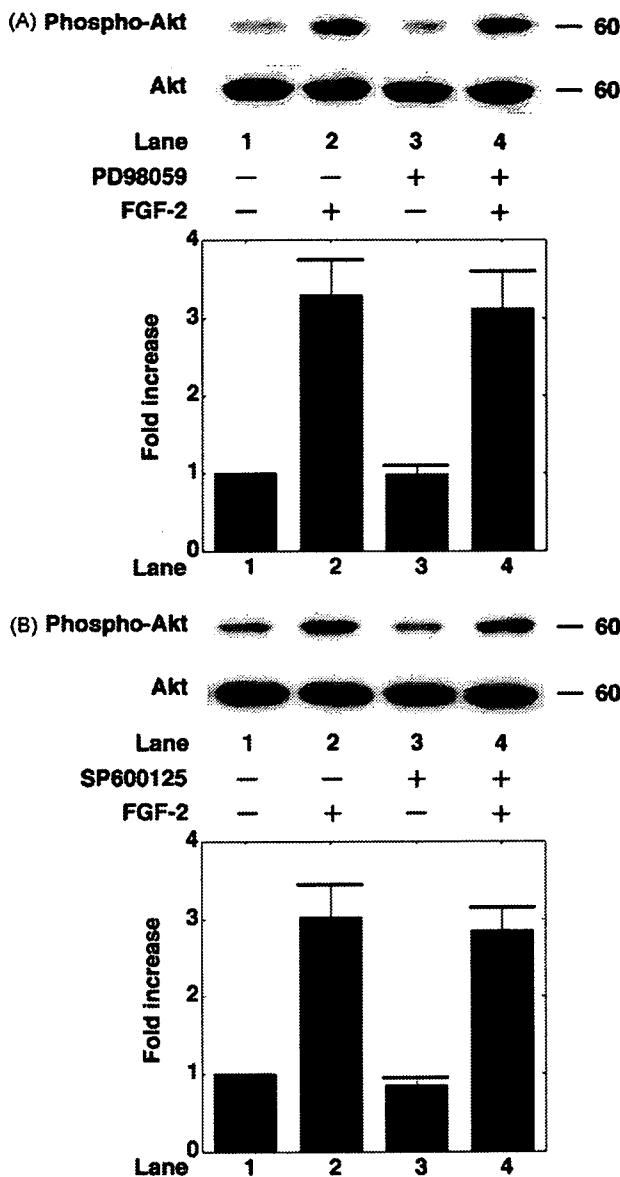


Fig. 7. Effects of PD98059 or SP600125 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 30  $\mu$ M of PD98059 (A), 30  $\mu$ M of SP600125 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells 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 FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  S.E.M. of triplicate determinations. Numbers on the right indicate molecular masses (kDa). Similar results were obtained with two additional and different cell preparations.

Akt plays an important role in regulating the balance between mitogenesis and apoptosis in cell function (Coffer et al., 1998). Thus, it is likely that the activation of Akt is usually required in these cells, and that the inhibition of Akt without the stimulation of FGF-2 is insufficient for VEGF release.

We next confirmed that the FGF-2-induced phosphorylation of GSK-3 $\beta$ , which is well known as a downstream target of

Table 1

Effect of actinomycin D on the enhancement by Akt inhibitor of FGF-2-stimulated VEGF release in MC3T3-E1 cells

Actinomycin D	Akt inhibitor	FGF-2	VEGF (pg/ml)
-	-	-	14 $\pm$ 2
-	-	+	819 $\pm$ 52
-	+	+	1560 $\pm$ 104
+	-	-	12 $\pm$ 2
+	-	+	411 $\pm$ 31*
+	+	+	1169 $\pm$ 68**

The cultured cells were pretreated with 10 ng/ml of actinomycin D, 10  $\mu$ M of Akt inhibitor, or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 48 h. Each value represents the mean  $\pm$  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 FGF-2 alone.

\*\*  $p < 0.05$ , compared to the value of Akt inhibitor and FGF-2.

Akt (Cross et al., 1995; Srivastava and Pandey, 1998), was truly reduced by the Akt inhibitor in MC3T3-E1 cells. These results suggest that FGF-2-induced VEGF release is suppressed by activation of Akt in osteoblast. We also found that wortmannin (Arcaro and Wymann, 1993) and LY294002 (Vlahos et al., 1994) markedly enhanced the FGF-2-induced VEGF release. Additionally, the FGF-2-induced phosphorylation of GSK-3 $\beta$  was suppressed by wortmannin or LY294002. Although Akt inhibitor failed to suppress FGF-2-induced Akt phosphorylation, it seems that the Akt inhibitor affects at the point downstream of Akt phosphorylation. Therefore, it is probable that the Akt inhibitor has no effect on the Akt phosphorylation but suppresses the activity. In addition, the enhancement by Akt inhibitor of FGF-2-induced VEGF release was significantly reduced by actinomycin D, a transcriptional inhibitor (Reich, 1963). Therefore, these results suggest that the regulation by Akt of FGF-2-stimulated VEGF release is at least in part a transcriptional regulation in osteoblasts. Taking our results into account as a whole, it is most likely that FGF-2 activates PI3-kinase/Akt pathway, resulting in attenuating the release of VEGF. It is probable that PI3-kinase/Akt signaling pathway activated by FGF-2 limits the FGF-2-induced VEGF release. To the best of our knowledge, our present results probably represent the first report to show that the activation of PI3-kinase/Akt leads to the negative feedback of VEGF release in osteoblasts.

It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays an important role in cellular functions including proliferation, differentiation and apoptosis 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 transduce the diverse messages (Widmann et al., 1999). In our previous studies (Tokuda et al., 2000, 2003), we have shown that FGF-2 activates p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells, and these MAP kinases act as positive regulators in FGF-2-induced VEGF release. Thus, it is necessary to clarify whether or not the relationship between PI3-kinase/Akt and these MAP kinases exists in the FGF-2-induced VEGF release in these cells. However, Akt inhibitor or PI3-kinase inhibitors failed to affect the

phosphorylation of p44/p42 MAP kinase and SAPK/JNK. Furthermore, we found that PD98059, a MEK inhibitor (Alessi et al., 1995), and SP600125, a JNK inhibitor (Bennett et al., 2001), had little effect on the FGF-2-induced phosphorylation of Akt. It seems unlikely that PI3-kinase/Akt signaling pathway affects the FGF-2-induced VEGF release in a dependent manner upon p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. The relative importance of these three pathways during osteoblast differentiation remains clarified. It is also unclear why these parallel opposing pathways would be physiologically advantageous, however, the complicated regulatory mechanism of FGF-2-induced VEGF release might reflect the importance of the event in osteoblasts.

We showed here that the VEGF release stimulated by FGF-2 is under the strict control of the survival signal, PI3-kinase/Akt in osteoblasts. When bone is damaged, FGF-2 expressed in osteoblasts plays a crucial role in fracture repair, bone remodeling and osteogenesis (Bolander, 1992; Marie, 2003). Bone remodeling is accompanied by angiogenesis and capillary outgrowth (Erlebacher et al., 1995). Since VEGF is a specific mitogen of vascular endothelial cells (Ferrara and Davis-Smyth, 1997), it is probable that adequate levels of VEGF are necessary to regulate vascularization of developing bones. VEGF expressed by osteoblasts could couple angiogenesis to bone formation by adjusting the angiogenic response to osteoblastic activity (Zelzer and Olsen, 2005). Taking these findings into account as a whole, our present results lead us to speculate that FGF-2-activated PI3-kinase/Akt signaling limits over-release of VEGF, resulting in the accommodation of bone microvasculature development that is required for fracture repair and so on. The concentration of FGF-2 stimulating the VEGF release observed in the present study was much higher than the physiological concentrations reported by previous *in vivo* reports (Ii et al., 1993; D'Amore et al., 1994). FGF-2 produced by osteoblast is accumulated in extracellular matrix of bone (Baylink et al., 1993; Hurley et al., 1993), suggesting that the osteoblasts, which make contact with bone matrix are possibly exposed to relatively high doses of FGF-2. Even under the physiological conditions, therefore, it is possible that FGF-2 stimulates the release of VEGF via p44/p42 MAP kinase and SAPK/JNK but regulates the excess of VEGF release through PI3-kinase/Akt signaling cascade in osteoblasts. Further investigations would be required to clarify the details.

In conclusion, our present results strongly suggest that the FGF-2-induced release of VEGF is negatively regulated by PI3-kinase/Akt activated by FGF-2 itself in osteoblasts.

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#### References

- Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., Saltiel, A.R., 1995. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *J. Biol. Chem.* 270, 27489–27494.
- Arcaro, A., Wymann, M.P., 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* 296, 297–301.
- Baylink, D.J., Finkleman, R.D., Mohan, S., 1993. Growth factor to stimulate bone formation. *J. Bone Miner. Res.* 8, S565–S572.
- Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13681–13686.
- Bolander, M.E., 1992. Regulation of fracture repair by growth factors stimulate tyrosine kinase activity *in vivo*. *Proc. Soc. Exp. Biol. Med.* 200, 165–170.
- Brighton, C.T., 1978. Structure and function of the growth plate. *Clin. Orthop. Rel. Res.* 136, 22–32.
- Brighton, C.T., Hunt, R.M., 1991. Early histological and ultrastructural changes in medullary fracture callus. *Am. J. Bone Joint Surg.* 73, 832–847.
- Cantley, L.C., 2002. The phosphoinositide 3-kinase pathway. *Science* 296, 1655–1657.
- Chan, T.O., Rittenhouse, S.E., Tschlis, P.N., 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68, 965–1014.
- Chaudhary, L.R., Hruska, K.A., 2001. The cell survival signal Akt is differently activated by PDGF-BB, EGF, and FGF-2 in osteoblastic cells. *J. Cell Biochem.* 81, 304–311.
- Coffer, P.J., Jin, J., Woodgett, J.R., 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* 335, 1–13.
- Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785–789.
- D'Amore, P.A., Brown Jr., R.H., Ku, P.T., Hoffman, E.P., Watanabe, H., Arahata, K., Ishihara, T., Folkman, J., 1994. Elevated basic fibroblast growth factor in the serum of patients with Duchenne muscular dystrophy. *Ann. Neurol.* 35, 362–365.
- Debiais, F., Lefevre, G., Lemonnier, J., Le Mee, S., Lasmoles, F., 2004. Fibroblast growth factor-2 induces osteoblast survival through a phosphatidylinositol 3-kinase-dependent-beta-catenin-independent signaling pathway. *Exp. Cell Res.* 297, 235–246.
- Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., Greenberg, M.E., 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 275, 661–665.
- Erlebacher, A., Filvaroff, E.H., Gitelman, S.E., Derynck, R., 1995. Toward a molecular understanding of skeletal development. *Cell* 80, 371–378.
- Ferrara, N., Davis-Smyth, T., 1997. The biology of vascular endothelial growth factor. *Endocr. Rev.* 18, 4–25.
- Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., Ferrara, N., 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med.* 5, 623–628.
- Goad, D.L., Rubin, J., Wang, H., Tashjian Jr., A.H., Patterson, C., 1996. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. *Endocrinology* 137, 2262–2268.
- Hu, Y., Qiao, L., Wang, S., Rong, S.B., Meuliet, E.J., Berggren, M., Gallegos, A., Powis, G., Kozikowski, A.P., 2000. 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J. Med. Chem.* 43, 3045–3051.
- Hurley, M.M., Abreu, C., Harrison, J.R., Lichtler, A.C., Raisz, L.G., Kream, B.E., 1993. Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells. *J. Biol. Chem.* 268, 5588–5593.
- Ii, M., Yoshida, H., Aramaki, Y., Masuya, H., Hada, T., Hatanaka, M., Ichimori, Y., 1993. Improved enzyme immunoassay for human basic fibroblast growth factor using a new enhanced chemiluminescence system. *Biochem. Biophys. Res. Commun.* 193, 540–545.



- Kato, K., Ito, H., Hasegawa, K., Inaguma, Y., Kozawa, O., Asano, T., 1996. Modulation of the stress-induced synthesis of hsp27 and  $\alpha$ B-crystallin by cyclic AMP in C6 glioma cells. *J. Neurochem.* 66, 946–950.
- Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., Waterfield, M.D., 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* 17, 615–675.
- Kozawa, O., Tokuda, H., Miwa, M., Kotoyori, J., Oiso, Y., 1992. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E<sub>2</sub> in osteoblast-like cells. *Exp. Cell Res.* 198, 130–134.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Marie, P.J., 2003. Fibroblast growth factor signaling controlling osteoblast differentiation. *Gene* 316, 23–32.
- Noda, T., Tokuda, H., Yoshida, M., Yasuda, E., Hanai, Y., Kozawa, O., 2005. Possible involvement of phosphatidylinositol 3-kinase/Akt pathway in insulin-like growth factor-I-induced alkaline phosphatase activity in osteoblasts. *Horm. Metab. Res.* 37, 270–274.
- Parfitt, A.M., 1994. Osteonal and hemi-osteonal remodeling: the spatial and temporal framework for signal traffic in adult human bone. *J. Cell Biochem.* 55, 273–286.
- Reich, E., 1963. Biochemistry of actinomycins. *Cancer Res.* 23, 1428–1441.
- Schlaeppi, J.M., Gutzwiller, S., Finlenzeller, G., Fournier, B., 1997. 1,25-Dihydroxyvitamin D<sub>3</sub> induces the expression of vascular endothelial growth factor in osteoblastic cells. *Endocr. Res.* 23, 213–229.
- Srivastava, A.K., Pandey, S.K., 1998. Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. *Mol. Cell Biochem.* 182, 135–141.
- Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., Kasai, S., 1983. *In vivo* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.* 96, 191–198.
- Tokuda, H., Kozawa, O., Uematsu, T., 2000. Basic fibroblast growth factor stimulates vascular endothelial growth factor release in osteoblasts: divergent regulation by p44/p42 mitogen-activated protein kinase and p38 mitogen-activated protein kinase. *J. Bone Miner. Res.* 15, 2371–2379.
- Tokuda, H., Hirade, K., Wang, X., Oiso, Y., Kozawa, O., 2003. Involvement of SAPK/JNK in basic fibroblast growth factor-induced VEGF release in osteoblasts. *J. Endocrinol.* 177, 101–107.
- Vlahos, C.J., Matter, W.F., Hui, K.Y., Brown, R.F., 1994. A specific inhibitor of phosphatidylinositol 3-kinase 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269, 5721–5728.
- Wang, D.S., Yamazaki, K., Nohtomi, K., Shizume, K., Ohsumi, K., Shibuya, M., Demura, H., Sato, K., 1996. Increase of vascular endothelial growth factor mRNA expression by 1, 25-dihydroxyvitamin D<sub>3</sub> in human osteoblast-like cells. *J. Bone Miner. Res.* 11, 472–479.
- Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* 79, 143–180.
- Yoshida, M., Niwa, M., Ishisaki, A., Hirade, K., Ito, H., Shimizu, K., Kato, K., Kozawa, O., 2004. Methotrexate enhances prostaglandin D<sub>2</sub>-stimulated heat shock protein 27 induction in osteoblast. *Prostaglandins Leukot. Essent. Fatty Acids* 71, 351–362.
- Zelzer, E., Olsen, B.R., 2005. Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. *Curr. Top. Dev. Biol.* 65, 169–187.



## Rho-kinase regulates endothelin-1-stimulated IL-6 synthesis via p38 MAP kinase in osteoblasts

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### Abstract

We have previously reported that endothelin-1 (ET-1) stimulates interleukin-6 (IL-6), a potent bone resorptive agent, through p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the involvement of Rho-kinase in the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells. ET-1 time-dependently induced the phosphorylation of myosin phosphatase targeting subunit (MYPT-1), a Rho-kinase substrate. Y27632, a specific inhibitor of Rho-kinase, significantly suppressed the IL-6 synthesis induced by ET-1 as well as the MYPT-1 phosphorylation. Fasudil, another inhibitor of Rho-kinase, reduced the ET-1-stimulated IL-6 synthesis. Y27632 as well as fasudil attenuated the ET-1-induced phosphorylation of p38 MAP kinase but not p44/p42 MAP kinase. These results strongly suggest that Rho-kinase regulates ET-1-stimulated IL-6 synthesis through p38 MAP kinase activation in osteoblasts.

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**Keywords:** ET-1; Rho-kinase; IL-6; Osteoblast

It is well recognized that interleukin-6 (IL-6) is a multi-functional cytokine that has crucial effects on a wide range of functions such as promoting B cell differentiation, T cell activation, and inducing acute phase proteins [1–3]. The bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [4]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and promote osteoclast formation [2,3,5,6]. It has been reported that potent bone resorptive agents such as tumor necrosis factor- $\alpha$  and IL-1 stimulate IL-6 synthesis in osteoblasts [5,7,8]. Currently, evidence is accumulating that IL-6 secreted from osteoblasts plays a pivotal role as a downstream effector of bone resorptive agents.

Endothelin-1 (ET-1) is a potent vasoconstrictor produced by endothelial cells [9]. In bone metabolism, it is well recognized that ET-1 plays an important role in the regulation of bone metabolism and acts as a bone resorptive agent [10]. Accumulating evidence suggests that tumor-produced ET-1 mediates bone metastases by stimulating osteoblast proliferation and new bone formation [11]. As for intracellular signaling of ET-1 in osteoblasts, the activities of ET-1 are mediated via ET<sub>A</sub> receptors and ET<sub>B</sub> receptors [11]. We have shown that ET-1 activates phospholipase C and phospholipase D through ET<sub>A</sub> receptors in osteoblast-like MC3T3-E1 cells [12]. Regarding about IL-6 synthesis, we have reported that ET-1 stimulates IL-6 synthesis via p44/p42 MAP kinase and p38 MAP kinase but not stress-activated protein kinase (SAPK)/c-Jun N terminal kinase (JNK) among the MAP kinase superfamily in these cells [13,14]. However, the exact mechanism

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underlying ET-1-stimulated IL-6 synthesis in osteoblasts is not fully known.

Recent studies suggest that Rho and the down-stream effector, Rho-associated kinase (Rho-kinase) play important roles in a variety of cellular functions such as cell motility and smooth muscle contraction [15–17]. As for osteoblasts, it has been demonstrated that Rho and p38 MAP kinase are involved in the ET-1-induced expression of prostaglandin endoperoxide G/H synthase mRNA in osteoblasts [18]. In addition, Rho/Rho-kinase pathway reportedly stimulates osteoblast proliferation whereas it inhibits osteoblast differentiation [19]. However, the exact role of Rho-kinase in osteoblasts has not yet been fully clarified.

In the present study, we further investigated the exact mechanism behind ET-1-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that Rho-kinase regulates ET-1-stimulated IL-6 synthesis through p38 MAP kinase activation in these cells.

## Materials and methods

**Materials.** ET-1 and mouse IL-6 enzyme immunoassay (ELISA) kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Y27632 was obtained from Calbiochem–Novabiochem Co. (LaJolla, CA). Hydroxyfasudil (fasudil) was from Sigma (St. Louis, MO). Phospho-specific MYPT-1 antibodies were purchased from Upstate (Lake Placid, NY). MYPT-1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, and p38 MAP kinase antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. Y27632 was 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 [20] were maintained as previously described [21]. Briefly, the cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cells were seeded into 35- or 90-mm diameter dishes in  $\alpha$ -MEM containing 10% FCS. After 5 days, the medium was exchanged for  $\alpha$ -MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

**IL-6 assay.** The cultured cells were stimulated by various doses of ET-1 in 1 ml of  $\alpha$ -MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with various doses of Y27632 or fasudil 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 pretreated with Y27632 or fasudil for 60 min, and then stimulated by ET-1 in  $\alpha$ -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 125,000g for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [22] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [23] by using phospho-specific MYPT-1 antibodies, MYPT-1 antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the 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 means  $\pm$  SEM of triplicate determinations. Each experiment was repeated three times with similar results.

## Results

### *Effects of ET-1 on the phosphorylation of MYPT-1 in MC3T3-E1 cells*

It is well known that myosin phosphatase targeting subunit (MYPT-1), which is a component of myosin phosphatase, is a down-stream substrate of Rho-kinase [15,24]. To investigate whether ET-1 activates Rho-kinase in osteoblast-like MC3T3-E1 cells, we examined the effect of ET-1 on the phosphorylation of MYPT-1. ET-1 significantly induced the phosphorylation of MYPT-1 in a time-dependent manner (Fig. 1A). The effect of ET-1 on the MYPT-1 phosphorylation reached its peak within 2 min and decreased thereafter (Fig. 1A).

We found that Y27632, a specific inhibitor of Rho-kinase [16], markedly suppressed the ET-1-induced phosphorylation levels of MYPT-1 (Fig. 1B). Additionally, the phosphorylation levels of MYPT-1 induced by ET-1 were attenuated by fasudil, another inhibitor of Rho-kinase [16] (data not shown).

### *Effects of Y27632 or fasudil on the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells*

We previously showed that ET-1 stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells [13]. In order to investigate whether or not Rho-kinase is involved in the ET-1-induced synthesis of IL-6 in MC3T3-E1 cells, we next examined the effect of Y27632 on the synthesis of IL-6 induced by ET-1. Y27632, which alone failed to affect the IL-6 levels, significantly suppressed the ET-1-induced synthesis of IL-6 (Fig. 2). The inhibitory effect of Y27632 was dose-dependent in the range between 0.1 and 10  $\mu$ M. Y27632 at 10  $\mu$ M caused approximately 90% inhibition in the ET-1-effect.

Fasudil as well as Y27632 reduced the ET-1-stimulated IL-6 synthesis in these cells (Table 1). The effect of fasudil on the IL-6 synthesis was dose-dependent in the range between 0.3 and 3  $\mu$ M. Fasudil (3  $\mu$ M) caused approximately 70% inhibition in the ET-1-effect.

### *Effect of Y27632 on the ET-1-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells*

It is generally recognized that three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are known as central elements used by mammalian cells to

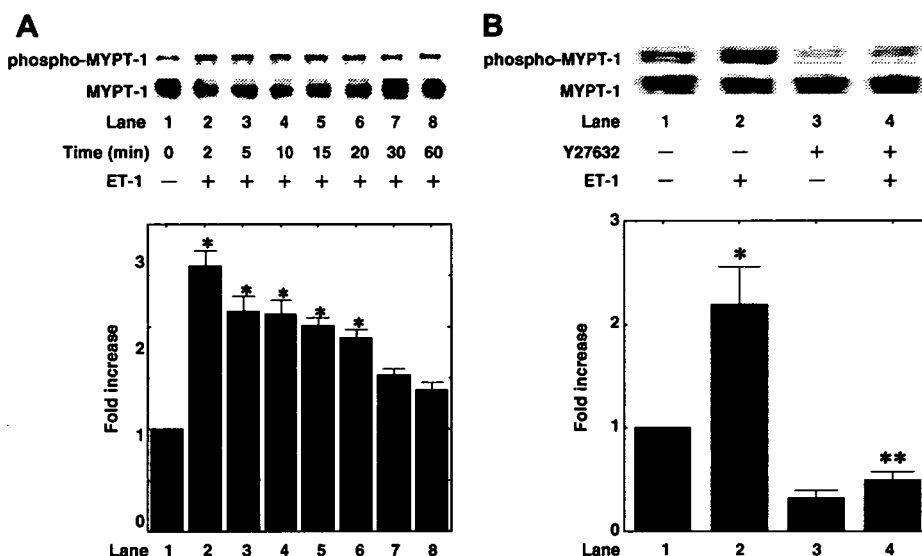


Fig. 1. Effect of ET-1 on the phosphorylation of MYPT-1 and effect of Y27632 on the ET-1-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. (A) The cultured cells were stimulated by 0.1  $\mu$ M ET-1 for the indicated periods. (B) The cultured cells were pretreated with 10  $\mu$ M Y27632 or vehicle for 60 min, and then stimulated by 0.1  $\mu$ M ET-1 or vehicle for 2 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific MYPT-1 or MYPT-1. The histogram shows quantitative representations of the levels of ET-1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  SEM 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 ET-1 alone.

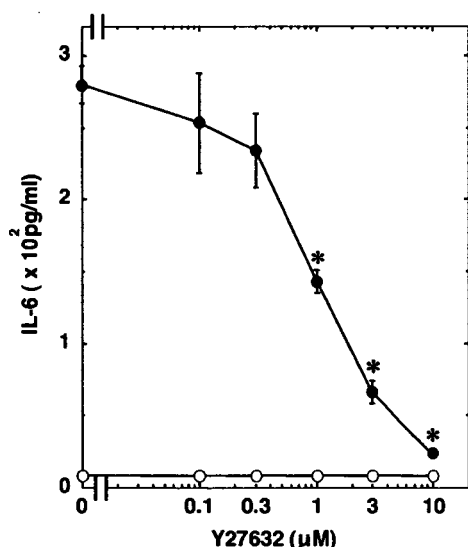


Fig. 2. Effect of Y27632 on the ET-1-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 0.1  $\mu$ M ET-1 or vehicle for 48 h. Each value represents the mean  $\pm$  SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. \* $p$  < 0.05, compared to the value of ET-1 alone.

transduce the various messages of a variety of agonists [25]. In our previous studies [13,14], we have shown that ET-1 stimulates IL-6 synthesis via p44/p42 MAP kinase and p38 MAP kinase but not SAPK/JNK among the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells. In order to investigate whether Rho-kinase-effect on the ET-

Table 1  
Effect of fasudil on the ET-1-stimulated IL-6 synthesis in MC3T3-E1 cells

Fasudil ( $\mu$ M)	ET-1	IL-6 (pg/ml)
0	-	<7.8
0	+	287 $\pm$ 16*
0.3	-	<7.8
0.3	+	261 $\pm$ 6
1	-	<7.8
1	+	171 $\pm$ 5**
3	-	<7.8
3	+	70 $\pm$ 5**

The cultured cells were pretreated with various doses of fasudil for 60 min, and then stimulated by 0.1  $\mu$ M ET-1 or vehicle for 48 h. Each value represents the mean  $\pm$  SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

\*  $p$  < 0.05, compared to the control.  
\*\*  $p$  < 0.05, compared to the value of ET-1 alone.

1-stimulated IL-6 synthesis is dependent upon the activation of p44/p42 MAP kinase or p38 MAP kinase in MC3T3-E1 cells, we next examined the effect of Y27632 on the phosphorylation of p44/p42 MAP kinase by ET-1. However, Y27632 failed to affect the ET-1-induced phosphorylation of p44/p42 MAP kinase (Fig. 3).

*Effects of Y27632 or fasudil on the ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells*

In addition, we examined effect of Y27632 on the ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. Y27632, which itself had little effect on

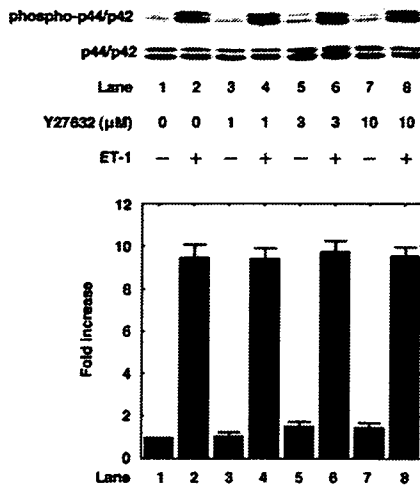


Fig. 3. Effect of Y27632 on the ET-1-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 0.1  $\mu$ M ET-1 or vehicle for 5 min. The extracts of cells were subjected to SDS-PAGE with subsequent 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 ET-1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

the phosphorylation of p38 MAP kinase, markedly suppressed the ET-1-induced phosphorylation of p38 MAP kinase (Fig. 4A). The Y27632-effect on the phosphorylation levels was dose-dependent in the range between 1

and 10  $\mu$ M. Y27632 (10  $\mu$ M) caused about 80% inhibition in the ET-1-effect.

Fasudil attenuated the ET-1-induced levels of phosphorylated-p38 MAP kinase (Fig. 4B). The inhibitory effect of fasudil was dose-dependent in the range between 1 and 10  $\mu$ M. Fasudil (10  $\mu$ M) caused approximately 70% inhibition in the ET-1-effect.

## Discussion

In the present study, we found that ET-1 time-dependently induced the phosphorylation of MYPT-1 in osteoblast-like MC3T3-E1 cells, using phospho-specific MYPT-1 (Thr850) antibodies. It is generally recognized that MYPT is a myosin-binding subunit of myosin phosphatase, which regulates the interaction of actin and myosin, and a downstream target of Rho-kinase [15,24]. In addition, we found that Rho-kinase inhibitors [16] such as Y27632 and fasudil truly reduced the ET-1-induced phosphorylation levels of MYPT-1. Taking our findings into account, it is most likely that ET-1 stimulates the activation of Rho-kinase in osteoblast-like MC3T3-E1 cells.

We next investigated whether Rho-kinase functions in the ET-1-stimulated IL-6 synthesis or not in osteoblast-like MC3T3-E1 cells. The ET-1-stimulated synthesis of IL-6 was significantly suppressed by Y27632, a specific inhibitor of Rho-kinase [16]. We confirmed that the Rho-kinase inhibitor truly reduced the ET-1-induced phosphorylation levels of MYPT-1. It seems that the activated Rho-kinase acts as a positive regulator in the IL-6 synthesis by ET-1 in osteoblast-like MC3T3-E1 cells. In addition, the ET-1-

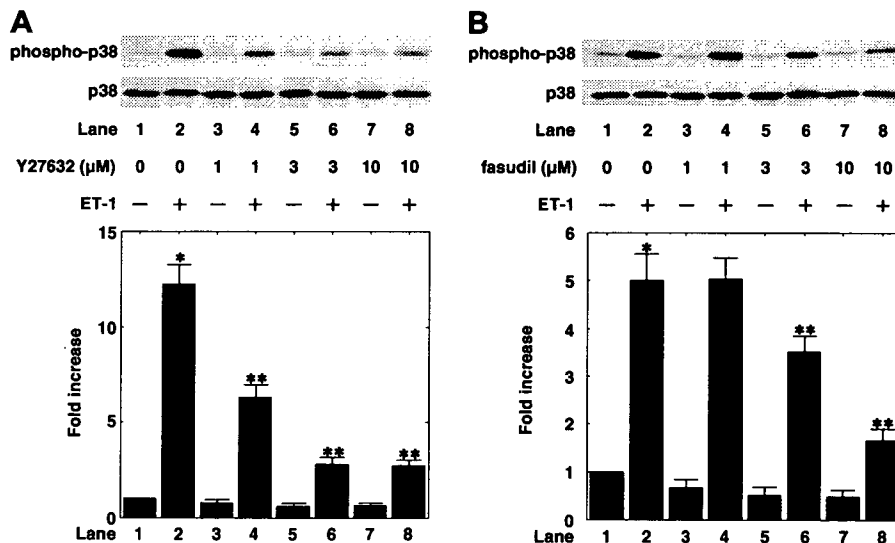


Fig. 4. Effects of Y27632 or fasudil on the ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 0.1  $\mu$ M ET-1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of ET-1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. \* $p$  < 0.05, compared to the control. \*\* $p$  < 0.05, compared to the value of ET-1 alone.

stimulated IL-6 synthesis as well as the phosphorylated levels of MYPT-1 was inhibited by fasudil, another Rho-kinase inhibitor [16]. Therefore, based on our results, it is most likely that ET-1 activates Rho-kinase, resulting in up-regulation of IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

Regarding about IL-6 synthesis, we have previously demonstrated that the ET-1 stimulated IL-6 synthesis is mediated by activation of p44/p42 MAP kinase and p38 MAP kinase but not SAPK/JNK among the MAP kinase superfamily in osteoblast-like MC3T3-E1 cells [13,14]. Additionally, we investigated the relationship between Rho-kinase and these MAP kinases in the IL-6 synthesis in MC3T3-E1 cells. However, Y27632 failed to affect the ET-1-induced phosphorylation levels of p44/p42 MAP kinase. Therefore, it seems unlikely that Rho-kinase affects the ET-1-stimulated IL-6 synthesis through the modulation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. On the contrary, the ET-1-induced phosphorylation levels of p38 MAP kinase were significantly suppressed by Y27632 and fasudil. These results suggest that Rho-kinase regulates the ET-1-stimulated IL-6 synthesis via p38 MAP kinase. In the present study, the maximum effect on the phosphorylation of MYPT-1, a well-known downstream target of Rho-kinase [15], was observed within 2 min after the ET-1 stimulation. In our previous study [26], we have shown that the phosphorylation of p38 MAP kinase reach the peak at 20 min after the stimulation of ET-1 in MC3T3-E1 cells. The time course of the phosphorylation of MYPT-1 stimulated by ET-1 seems to be faster than that of p38 MAP kinase, in turn, ET-1-induced activation of p38 MAP kinase subsequently occurs after the Rho-kinase activation. Taking our findings into account as a whole, it is most likely that Rho-kinase acts at a point upstream from p38 MAP kinase in the ET-1-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

Rho-kinase is currently recognized to play a pivotal role in various cellular functions, especially vascular smooth muscle contraction [15,16]. In bone metabolism, it has been reported that the Rho-kinase pathway acts as a negative regulator of osteoblast differentiation and induces osteoblast proliferation [19]. Our present results indicate that Rho-kinase activation in osteoblasts has an important role in the control of the production of IL-6, one of the key regulators of bone metabolism. It is well known that IL-6 produced by osteoblasts is a potent bone resorptive agent and induces osteoclast formation [3,5]. In addition, it is recognized that ET-1 acts as a bone resorptive agent in bone metabolism [10]. Evidence is recently accumulating that tumor cells produces ET-1 which mediates bone metastasis by stimulating the proliferation of osteoblasts and new bone formation [11]. Therefore, our present findings lead us to speculate that ET-1-activated Rho-kinase acts as a positive regulator of bone resorption via the fine tuning of local cytokine network. Therefore, the Rho-kinase pathway in osteoblasts might be considered to be a new candidate as a molecular therapeutic target of bone resorption

and osteoblastic bone metastases due to breast or prostate cancer. The pathophysiological significance of regulatory mechanism by Rho-kinase in osteoblasts remains still unclear. Further investigation is necessary to clarify the exact role of Rho-kinase in osteoblasts.

In conclusion, our results strongly suggest that Rho-kinase regulates the ET-1-stimulated IL-6 synthesis at a point upstream from p38 MAP kinase in osteoblasts.

#### Acknowledgments

We are very grateful to Yoko Kawamura and Seiko Sakakibara for their skillful technical assistance. This investigation 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 Grant on Proteomics and Research Grant on Longevity Sciences from the Ministry of Health, Labour and Welfare of Japan.

#### References

- [1] S. Akira, T. Taga, T. Kishimoto, Interleukin-6 in biology and medicine, *Adv. Immunol.* 54 (1993) 1–78.
- [2] D. Heymann, A.V. Rouselle, gp130 Cytokine family and bone cells, *Cytokine* 12 (2000) 1455–1468.
- [3] S. Kwan Tat, M. Padrines, S. Theoleyre, D. Heymann, Y. Fortun, IL-6 is produced by osteoblasts and induces bone resorption, *Cytokine Growth Factor Rev.* 15 (2004) 49–60.
- [4] P.J. Nijweide, E.H. Burger, J.H.M. Feyen, Cells of bone: proliferation, differentiation, and hormonal regulation, *Physiol. Rev.* 86 (1986) 855–886.
- [5] Y. Ishimi, C. Miyaura, C.H. Jin, T. Akatsu, F. Abe, Y. Nakamura, Y. Yamaguchi, S. Yoshiki, T. Matsuda, T. Hirano, T. Kishimoto, T. Suda, IL-6 is produced by osteoblasts and induces bone resorption, *J. Immunol.* 145 (1990) 3297–3303.
- [6] G.D. Roodman, Interleukin-6: an osteotropic factor? *J. Bone Miner. Res.* 7 (1992) 475–478.
- [7] M. Helle, J.P.J. Brakenhoff, E.R. DeGroot, L.A. Aarden, Interleukin 6 is involved in interleukin 1-induced activities, *Eur. J. Immunol.* 18 (1998) 957–959.
- [8] A.J. Littlewood, J. Russil, G.R. Harvey, D.E. Hughes, R.G.G. Russel, M. Gowen, The modulation of the expression of IL-6 and its receptor in human osteoblasts in vitro, *Endocrinology* 129 (1991) 1513–1520.
- [9] T. Miyauchi, T. Masaki, Pathophysiology of endothelin in the cardiovascular system, *Annu. Rev. Physiol.* 61 (1999) 391–415.
- [10] P.H. Stern, A. Tatrai, D.E. Semler, S.K. Lee, P. Lakatos, P.J. Strieman, G. Tarjan, J.L. Sanders, Endothelin receptors, second messengers, and actions in bone, *J. Nutr.* 125 (1995) 2028S–2032S.
- [11] T.A. Guise, J.J. Yin, K.S. Mohammad, Role of endothelin-1 in osteoblastic bone metastases, *Cancer* 97 (2003) 779–784.
- [12] A. Suzuki, J. Shinoda, Y. Watanabe-Tomita, N. Ozaki, Y. Oiso, O. Kozawa, ET<sub>A</sub> receptor mediates the signalling of endothelin-1 in osteoblast-like cells, *Bone* 21 (1997) 143–146.
- [13] H. Kawamura, T. Otsuka, H. Tokuda, H. Matsuno, N. Niwa, N. Matsui, T. Uematsu, O. Kozawa, Involvement of p42/p44 MAP kinase in endothelin-1-induced interleukin-6 synthesis in osteoblast-like cells, *Bone* 24 (1999) 315–320.
- [14] H. Tokuda, S. Takai, Y. Hanai, R. Matsushima-Nishiwaki, T. Hosoi, A. Harada, T. Ohta, O. Kozawa, (–)-Epigallocatechin gallate suppresses endothelin-1-induced interleukin-6 synthesis in osteoblasts: inhibition of p44/p42 MAP kinase activation, *FEBS Lett.* 581 (2007) 1311–1316.

- [15] Y. Fukata, M. Amano, K. Kaibuchi, Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells, *Trends Pharmacol. Sci.* 22 (2001) 32–39.
- [16] H. Shimokawa, M. Rashid, Development of Rho-kinase inhibitors for cardiovascular medicine, *Trends Pharmacol. Sci.* 28 (2007) 296–302.
- [17] K. Riento, A.J. Ridley, Rocks: multifunctional kinases in cell behaviour, *Nat. Rev. Mol. Cell Biol.* 4 (2003) 446–456.
- [18] W. Windischhofer, D. Zach, G. Fauler, G. Raspotnig, H. Kofeler, H.J. Leis, Involvement of Rho and p38 MAPK in endothelin-1-induced expression of PGHS-2 mRNA in osteoblast-like cells, *J. Bone Miner. Res.* 17 (2002) 1774–1784.
- [19] D. Harmey, G. Stenbeck, C.D. Nobes, A.J. Lax, A.E. Grigoriadis, Regulation of osteoblast differentiation by *Pasteurella multocida* toxin (PMT): a role for Rho GTPase in bone formation, *J. Bone Miner. Res.* 19 (2004) 661–670.
- [20] H. Sudo, H. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, *J. Cell Biol.* 96 (1983) 191–198.
- [21] O. Kozawa, H. Tokuda, M. Miwa, J. Kotoyori, Y. Oiso, Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E<sub>2</sub> in osteoblast-like cells, *Exp. Cell Res.* 198 (1992) 130–134.
- [22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [23] K. Kato, H. Ito, K. Hasegawa, Y. Inaguma, O. Kozawa, T. Asano, Modulation of the stress-induced synthesis of hsp27 and alpha B-crystallin by cyclic AMP in C6 rat glioma cells, *J. Neurochem.* 66 (1996) 946–950.
- [24] M. Ito, T. Nakano, F. Erdodi, D.J. Hartshorne, Myosin phosphatase: structure, regulation and function, *Mol. Cell. Biochem.* 259 (2004) 197–209.
- [25] C. Widmann, S. Gibson, M.B. Jarpe, G.L. Johnson, Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human, *Physiol. Rev.* 79 (1999) 143–180.
- [26] H. Kawamura, T. Otsuka, H. Matsuno, M. Niwa, N. Matsui, K. Kato, T. Uematsu, O. Kozawa, Endothelin-1 stimulates heat shock protein 27 induction in osteoblasts: involvement of p38 MAP kinase, *Am. J. Physiol.* 277 (1999) E1046–E1054.



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## (–)-Epigallocatechin gallate inhibits prostaglandin D<sub>2</sub>-stimulated HSP27 induction via suppression of the p44/p42 MAP kinase pathway in osteoblasts

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### Abstract

We previously reported that prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) stimulates heat shock protein 27 (HSP27) induction through p38 mitogen-activated protein (MAP) kinase, stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether (–)-epigallocatechin gallate (EGCG), the major polyphenol found in green tea, affects the induction of HSP27 in these cells and the mechanism. EGCG significantly reduced the HSP27 induction stimulated by PGD<sub>2</sub> without affecting the levels of HSP70. The PGD<sub>2</sub>-induced phosphorylation of p38 MAP kinase or SAPK/JNK was not affected by EGCG. On the contrary, EGCG markedly suppressed the PGD<sub>2</sub>-induced phosphorylation of p44/p42 MAP kinase and MEK1/2. However, the PGD<sub>2</sub>-induced phosphorylation of Raf-1 was not inhibited by EGCG. These results strongly suggest that EGCG suppresses the PGD<sub>2</sub>-stimulated induction of HSP27 at the point between Raf-1 and MEK1/2 in osteoblasts.

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### 1. Introduction

Heat shock proteins (HSP) are induced in cells in response to the biological stress such as heat stress and chemical stress [1]. HSPs are classified into high-molecular-weight HSPs such as HSP90 and HSP70, and low-molecular-weight HSPs based on apparent molecular sizes. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27,  $\alpha$ B-crystallin and HSP20 share high homology in amino acid sequences “ $\alpha$ -crystallin domain” [2]. Though the func-

tions of the low-molecular-weight HSPs are known less than those of the high-molecular-weight HSPs, it is generally believed that they may have chaperoning functions like the high-molecular-weight HSPs [2]. It is well recognized that HSP27 activity is regulated by post-translational modification such as phosphorylation [3,4]. Under unstimulated conditions, HSP27 exists as a high-molecular-weight aggregated form. It is rapidly dissociated as a result of phosphorylation [5,6]. The phosphorylation-induced dissociation from the aggregated form correlates with the loss of molecular chaperone activity [5,6]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [7]. The formation of bone structures and bone remodeling

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results from the coupling process, bone resorption by activated osteoblasts with subsequent deposition of new matrix by osteoblasts. In osteoblasts, it has been shown that down-regulation of proliferation is accompanied by a transient increase of the HSP27 mRNA expression [8]. In addition, heat-stimulated induction of HSP27 is reportedly facilitated by estrogen [9]. However, the exact role of HSP27 in osteoblasts remains to be clarified.

Prostaglandins (PGs) act as autocrine/paracrine modulators in bone metabolism and play important roles in the regulation [7,10]. Among PGs, PGD<sub>2</sub> is generally known as a potent regulator of osteoblastic functions [11,12]. In our previous study [13], we have reported that PGD<sub>2</sub> stimulates the synthesis of interleukin-6 through calcium-dependent manner in osteoblast-like MC3T3-E1 cells. In addition, we showed that PGD<sub>2</sub> stimulates the induction of HSP27 via three mitogen-activated protein (MAP) kinases, p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in these cells [14,15]. However, the mechanism by which PGD<sub>2</sub> modulates osteoblast functions is not fully clarified.

It is well known that compounds in foods such as vegetables and fruits have beneficial properties to human beings. Among them, flavonoids reportedly show antioxidative, antiproliferative and proapoptotic effects [16,17]. Osteoporosis is one of major problems in health of elderly persons in the advanced countries. It is recognized that tea drinkers appear to have low risk in osteoporosis [18]. Catechins are one of the major flavonoids contained in various species of plants including tea [17]. In bone metabolism, it has been reported that catechin suppresses bone resorption [19]. As for osteoblasts, it has been shown that catechin stimulates alkaline phosphatase activity, a mature osteoblast phenotype, and reduces bone-resorptive cytokine production in osteoblast-like MC3T3-E1 cells [20]. These evidences lead us to speculate that catechin could affect osteoblast function through the modulation of HSP27 induction stimulated by the local factors such as PGD<sub>2</sub>.

In the present study, we investigated the effect of (–)-epigallocatechin gallate (EGCG), one of the major green tea flavonoids [17], on PGD<sub>2</sub>-stimulated induction of HSP27 and the mechanism in osteoblast-like MC3T3-E1 cells. We here show that EGCG suppresses the PGD<sub>2</sub>-stimulated induction of HSP27 via inhibition of p44/p42 MAP kinase but not p38 MAP kinase or SAPK/JNK in these cells.

## 2. Materials and methods

### 2.1. Materials

PGD<sub>2</sub> and  $\beta$ -actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). EGCG was

obtained from Calbiochem-Novabiochem (La Jolla, CA). HSP27 antibodies were obtained from R&D Systems Inc. (Minneapolis, MN). HSP70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific MEK1/2 antibodies, MEK1/2 antibodies and phospho-specific Raf-1 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PGD<sub>2</sub> was dissolved in ethanol. The maximum concentration of ethanol was 0.1%, which did not affect Western blot analysis.

### 2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [21] were maintained as previously described [22]. Briefly, the cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cells were seeded into 90-mm diameter dishes (25 × 10<sup>4</sup> per dish) in  $\alpha$ -MEM containing 10% FCS. After 5 days, the medium was exchanged for  $\alpha$ -MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with EGCG.

### 2.3. Western blot analysis

The cultured cells were stimulated by PGD<sub>2</sub> in serum-free  $\alpha$ -MEM for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized, sonicated and immediately boiled in a lysis buffer (pH 6.8) containing 62.5 mM Tris/Cl, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The sample was used for the analysis by Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [23] in 10% polyacrylamide gel. Western blot analysis was performed as described previously [24], using HSP27 antibodies, HSP70 antibodies,  $\beta$ -actin antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific MEK1/2 antibodies, MEK1/2 antibodies or phospho-specific Raf-1 antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on polyvinylidene

difluoride (PVDF) membranes was visualized on X-ray film by means of the ECL Western blotting detection system and was quantitated using NIH image software. All of Western blot analyses were repeated at least three times in independent experiments.

#### 2.4. Statistical analysis

The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a  $p < 0.05$  was considered significant. All data are presented as the mean  $\pm$  SEM of triplicate determinations.

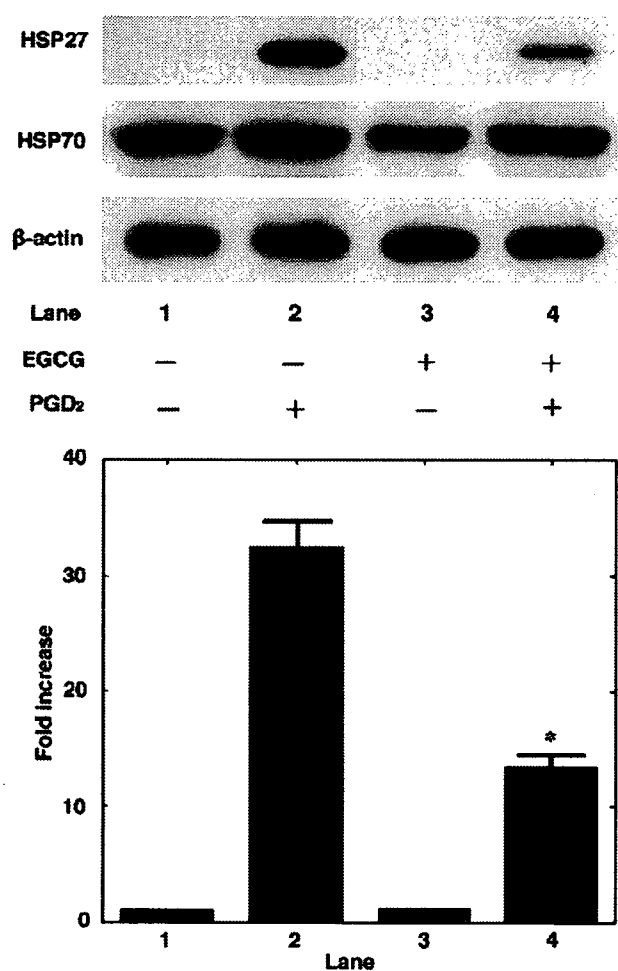


Fig. 1. Effect of EGCG on the PGD<sub>2</sub>-stimulated HSP27 induction in MC3T3-E1 cells. The cultured cells were pretreated with 30  $\mu$ M EGCG for 60 min, and then stimulated with 10  $\mu$ M PGD<sub>2</sub> for 9 h. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against HSP27, HSP70 or  $\beta$ -actin. The histogram shows quantitative representations of the levels of PGD<sub>2</sub>-induced HSP27 obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. \* $p < 0.05$ , compared to the value of PGD<sub>2</sub> alone.

### 3. Results

#### 3.1. Effect of EGCG on the PGD<sub>2</sub>-stimulated HSP27 induction in MC3T3-E1 cells

We examined the effect of EGCG on the PGD<sub>2</sub>-stimulated induction of HSP27. EGCG significantly reduced the PGD<sub>2</sub>-induced levels of HSP27 (Fig. 1). EGCG (30  $\mu$ M) caused about 60% reduction in the PGD<sub>2</sub>-effect. We have shown that PGD<sub>2</sub> does not affect the levels of HSP70, a high-molecular-weight HSP, in osteoblast-like MC3T3-E1 cells [14]. EGCG had little effect on the levels of HSP70 (Fig. 1).

#### 3.2. Effects of EGCG on the PGD<sub>2</sub>-stimulated phosphorylation of p38 MAP kinase, SAPK/JNK or p44/p42 MAP kinase in MC3T3-E1 cells

In our previous studies [14,15], we have shown that the activations of p38 MAP kinase, SAPK/JNK and

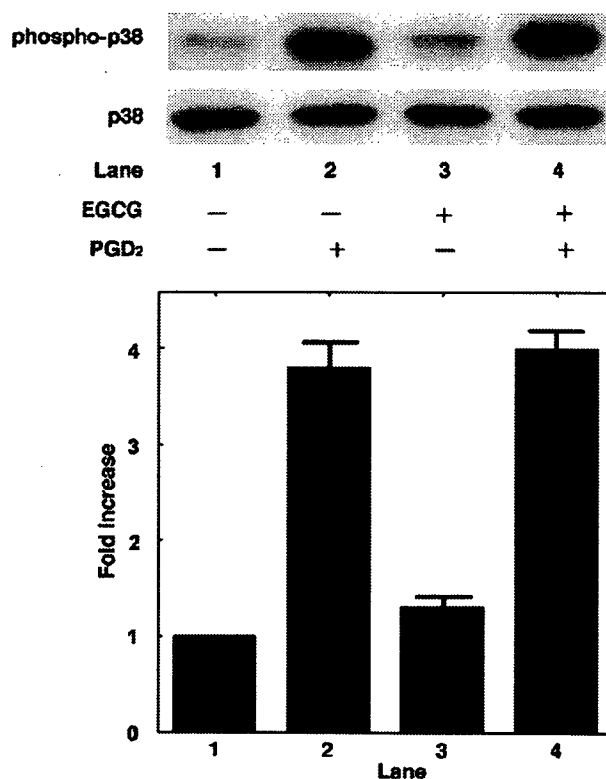


Fig. 2. Effect of EGCG on the PGD<sub>2</sub>-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 100  $\mu$ M EGCG for 60 min, and then stimulated by 10  $\mu$ M PGD<sub>2</sub> or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with 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 PGD<sub>2</sub>-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean  $\pm$  SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

p44/p42 MAP kinase mediate the PGD<sub>2</sub>-stimulated induction of HSP27 in osteoblast-like MC3T3-E1 cells. In order to clarify what kind of kinase among three MAP kinases is involved in the EGCG-induced suppression of HSP27 induction in MC3T3-E1 cells, we next examined the effect of EGCG on the PGD<sub>2</sub>-stimulated phosphorylation of three MAP kinases. However, EGCG did not influence the PGD<sub>2</sub>-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (Fig. 2). In addition, the PGD<sub>2</sub>-induced phosphorylation of SAPK/JNK was not affected by EGCG (Fig. 3).

On the contrary, EGCG markedly reduced the p44/p42 MAP kinase phosphorylation by PGD<sub>2</sub> (Fig. 4). EGCG (30 μM) caused about 50% reduction in the PGD<sub>2</sub>-effect.

### 3.3. Effect of EGCG on the PGD<sub>2</sub>-induced phosphorylation of MEK1/2 and Raf-1 in MC3T3-E1 cells

It is generally known that p44/p42 MAP kinase is activated by MEK1/2, which is regulated by the

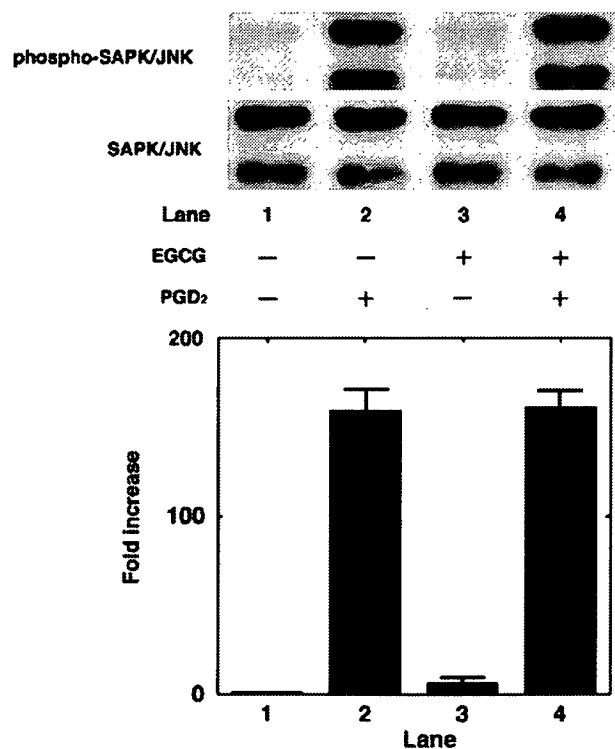


Fig. 3. Effect of EGCG on the PGD<sub>2</sub>-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD<sub>2</sub> or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of PGD<sub>2</sub>-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.

upstream kinase known as Raf-1 [25]. We found that both MEK1/2 and Raf-1 were time dependently phosphorylated by PGD<sub>2</sub> (data not shown). EGCG significantly suppressed the PGD<sub>2</sub>-induced phosphorylation of MEK1/2 (Fig. 5). EGCG (30 μM) caused about 50% reduction in the PGD<sub>2</sub>-effect. On the contrary, EGCG failed to attenuate the PGD<sub>2</sub>-induced phosphorylation of Raf-1 (Fig. 6).

## 4. Discussion

In the present study, we first showed that EGCG markedly inhibited the PGD<sub>2</sub>-stimulated induction of HSP27, a low-molecular-weight HSP, while EGCG failed to affect the levels of HSP70, a high-molecular-weight HSP in osteoblast-like MC3T3-E1 cells. We next investigated the mechanism of EGCG behind the suppressive effect on the HSP27 induction. The MAP kinase superfamily plays a pivotal role in cellular

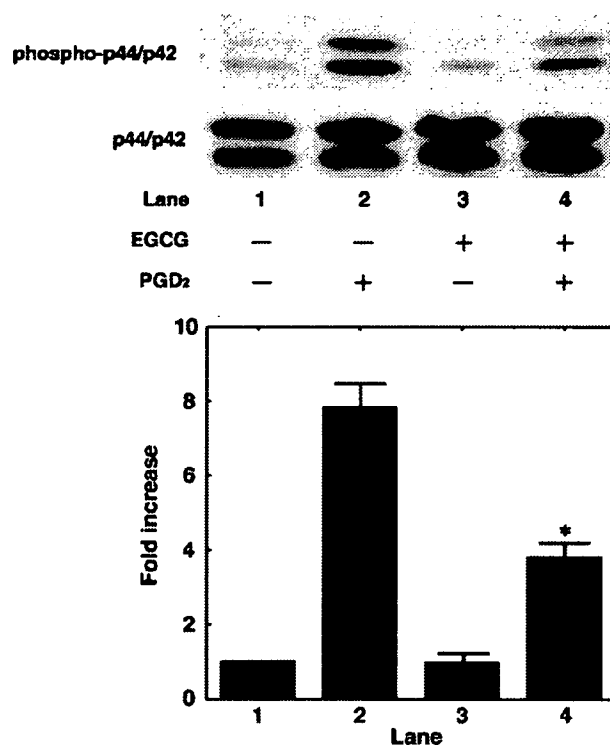


Fig. 4. Effect of EGCG on the PGD<sub>2</sub>-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD<sub>2</sub> or vehicle for 15 min. The extracts of cells were subjected to SDS-PAGE with subsequent 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 PGD<sub>2</sub>-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. \**p* < 0.05, compared to the value of PGD<sub>2</sub> alone.

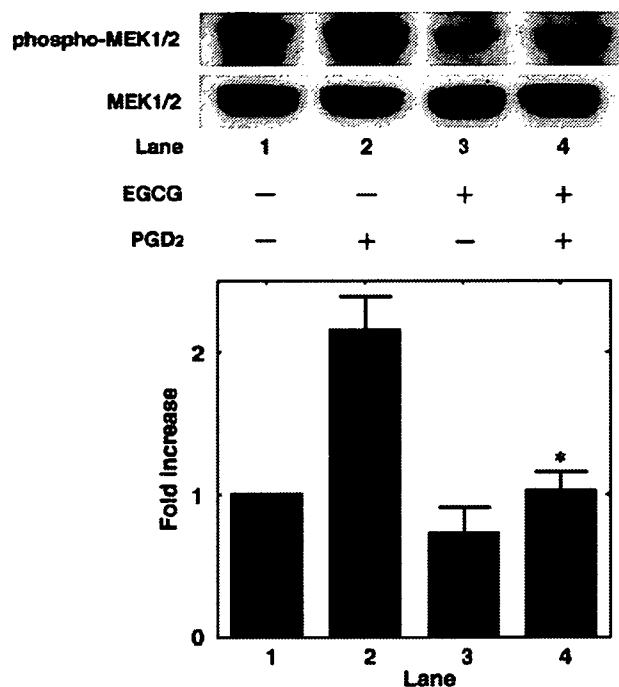


Fig. 5. Effect of EGCG on the PGD<sub>2</sub>-induced phosphorylation of MEK1/2 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD<sub>2</sub> or vehicle for 15 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific MEK1/2 or MEK1/2. The histogram shows quantitative representations of the levels of PGD<sub>2</sub>-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. \**p* < 0.05, compared to the value of PGD<sub>2</sub> alone.

functions including proliferation, differentiation, and apoptosis in a variety of cells [25]. It is generally known that three major MAP kinases such as p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are known as central elements used by mammalian cells to transduce the various messages. We have previously shown that the three MAP kinases function as positive regulators in the PGD<sub>2</sub>-stimulated induction of HSP27 in osteoblast-like MC3T3-E1 cells [14,15]. In the present study, the PGD<sub>2</sub>-induced phosphorylation of p38 MAP kinase or SAPK/JNK was not affected by EGCG. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [25]. Therefore, it seems unlikely that the EGCG-induced suppression of PGD<sub>2</sub>-stimulated induction of HSP27 is due to the inhibition of p38 MAP kinase pathway or SAPK/JNK pathway in osteoblast-like MC3T3-E1 cells.

p44/p42 MAP kinase (extracellular signal-regulated kinase) is activated by MEK (mitogen extracellular kinase) as MAP kinase kinase and that Raf-1 regulates

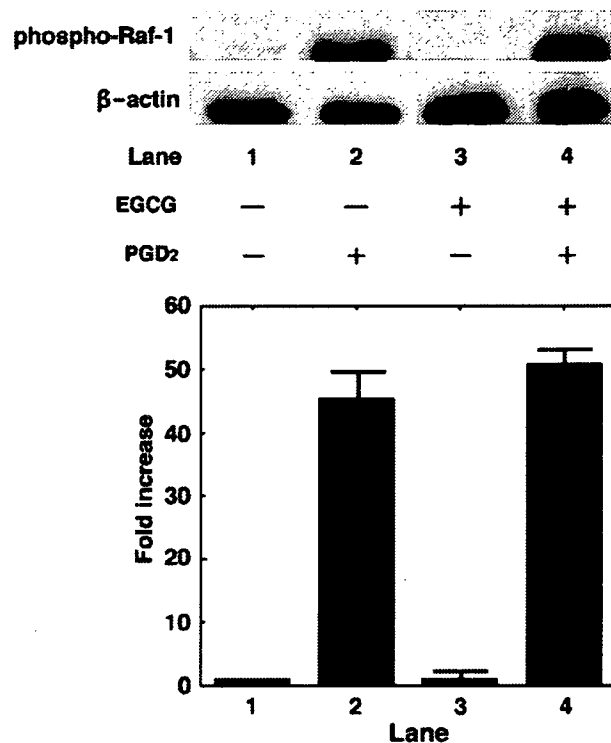


Fig. 6. Effect of EGCG on the PGD<sub>2</sub>-induced phosphorylation of Raf-1 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD<sub>2</sub> or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Raf-1 or β-actin. The histogram shows quantitative representations of the levels of PGD<sub>2</sub>-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.

MEK as MAP kinase kinase [25]. In the present study, the PGD<sub>2</sub>-stimulated phosphorylation of p44/p42 MAP kinase was significantly attenuated by EGCG. This result suggests that EGCG suppresses PGD<sub>2</sub>-stimulated HSP27 induction via inhibiting the p44/p42 MAP kinase cascade in osteoblast-like MC3T3-E1 cells. In addition, we showed that EGCG attenuated the levels of the PGD<sub>2</sub>-induced phosphorylation of MEK1/2 without affecting the levels of Raf-1 phosphorylation. Taking our findings into account, it is most likely that EGCG inhibits the PGD<sub>2</sub>-stimulated induction of HSP27 at the point between Raf-1 and MEK1/2 in osteoblast-like MC3T3-E1 cells. The potential mechanism of EGCG in PGD<sub>2</sub>-stimulated HSP27 induction in osteoblasts shown here is summarized in Fig. 7. Further investigations are necessary to elucidate the exact mechanism of catechin underlying the down-regulation of HSP27 induction in osteoblasts.

EGCG is one of the major polyphenolic flavonoids present in a green tea [17]. As for bone metabolism, it has been shown that catechin has an inhibitory effect on