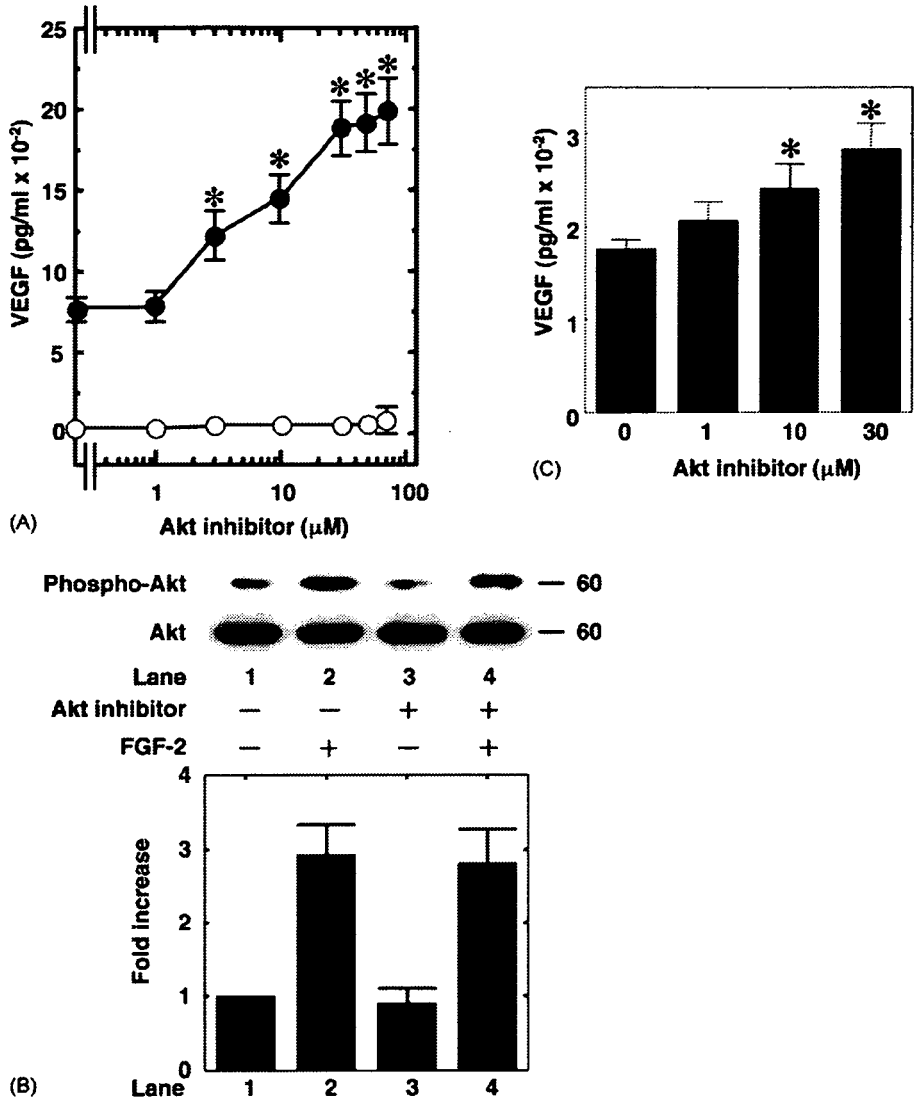

Fig. 2. Effects of Akt inhibitor on the VEGF release by FGF-2 or the FGF-2-induced phosphorylation of Akt. (A) Osteoblast-like MC3T3-E1 cells were pretreated with various doses of Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean ± 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 μM 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 ± 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

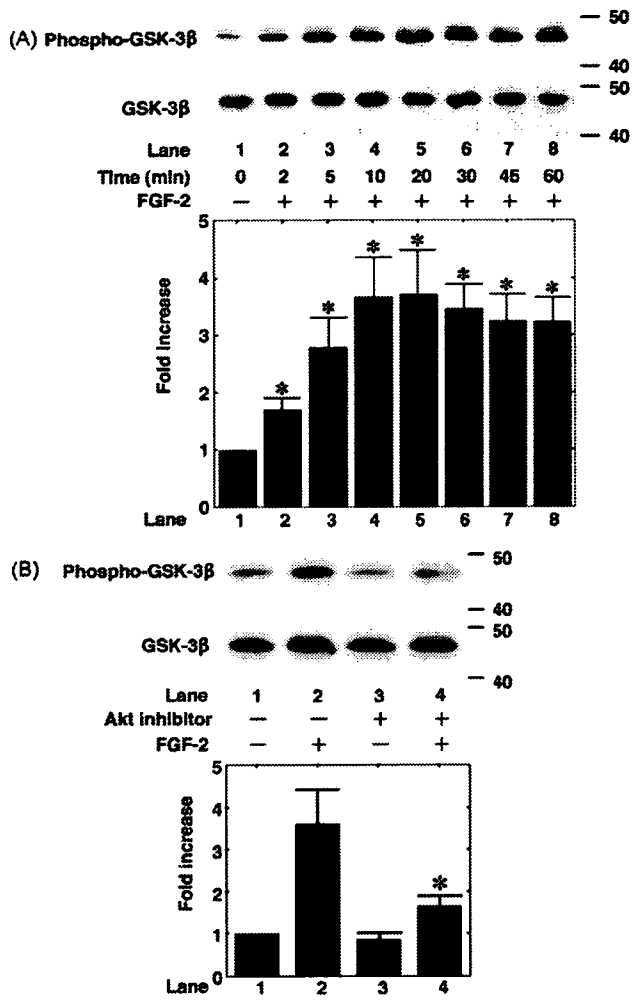

Fig. 3. Effect of FGF-2 on the phosphorylation of GSK-3β, and effect of Akt inhibitor on the FGF-2-induced phosphorylation of GSK-3β in MC3T3-E1 cells. (A) The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. $^{*}p < 0.05$, compared to the value of control. (B) The cultured cells were pretreated with 50 μM 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-3β or GSK-3β. 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 ± 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.

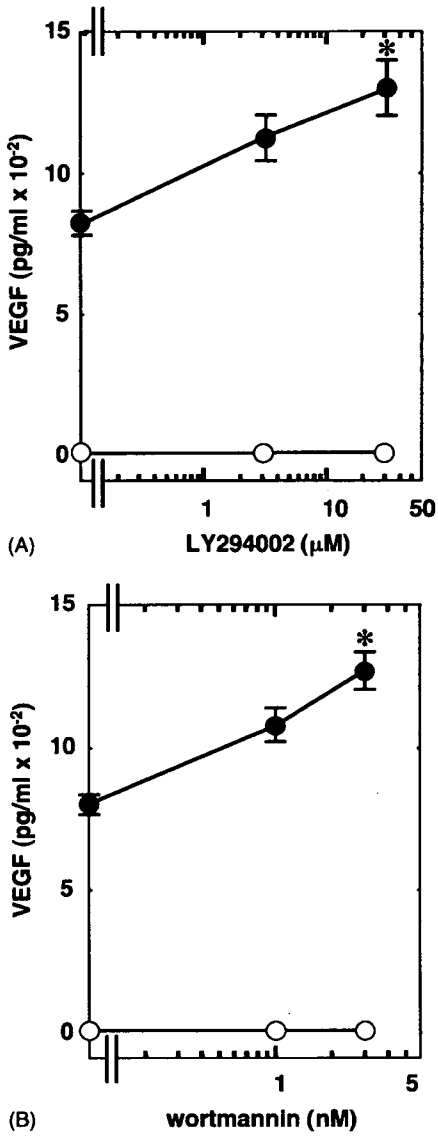

Fig. 4. Effects of wortmannin or LY294002 on the FGF-2-induced VEGF release in MC3T3-E1 cells. Osteoblast-like MC3T3-E1 cells were pretreated with various doses of LY294002 (A) or wortmannin (B) for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of FGF-2 alone.

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 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,

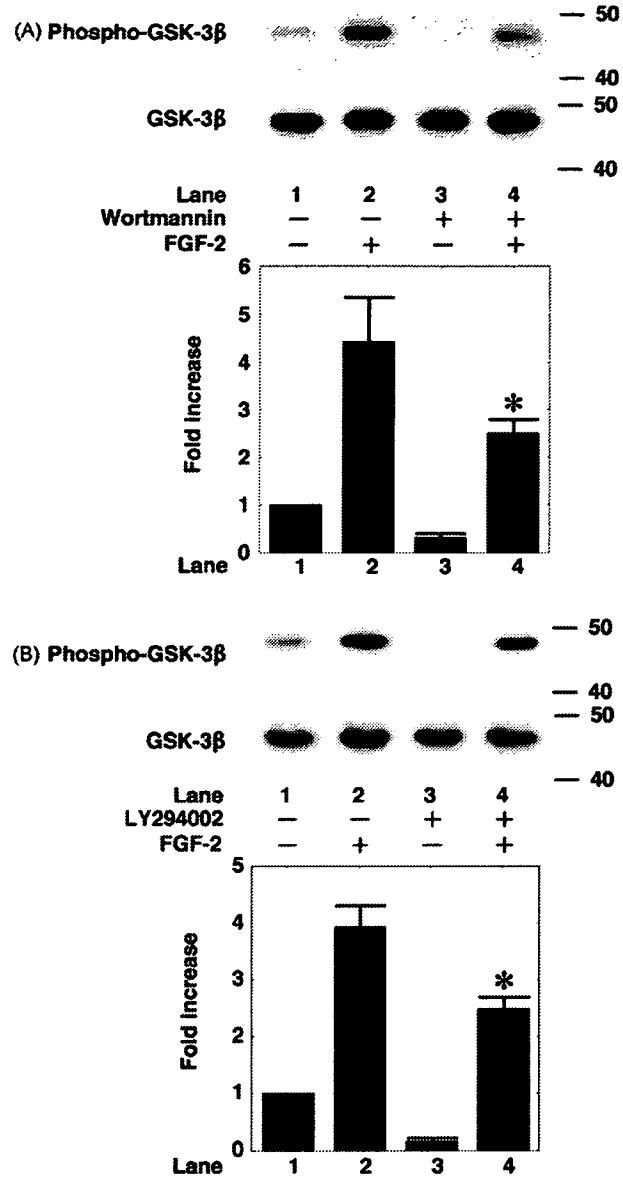

Fig. 5. Effects of wortmannin or LY294002 on the FGF-2-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM of wortmannin (A), 30 μM of LY294002 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. 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 ± 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.

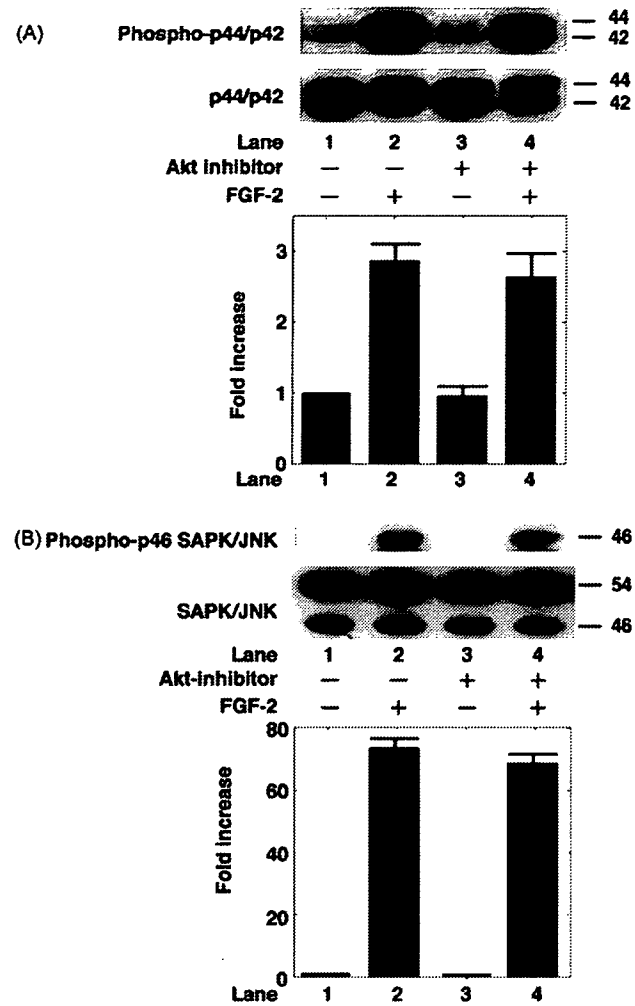

Fig. 6. Effects of Akt inhibitor on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected 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 ± 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.

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 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

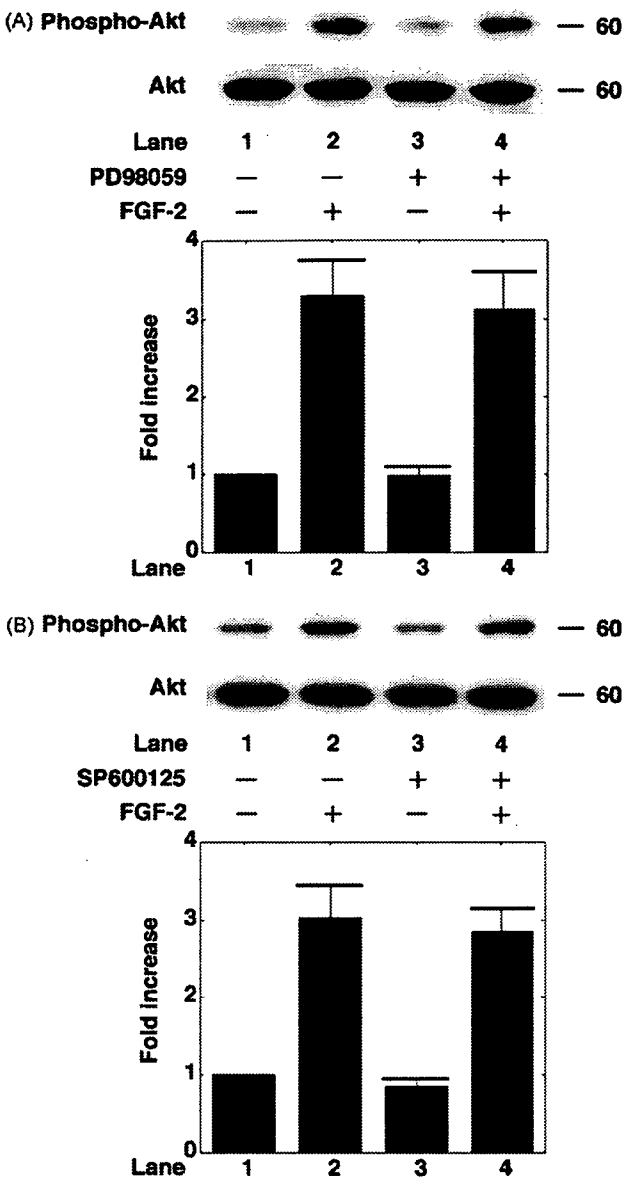

Fig. 7. Effects of PD98059 or SP600125 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM of PD98059 (A), 30 μM of SP600125 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected 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 ± 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-3β, which is well known as a downstream target of 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-3β 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 phosporylation. 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.

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 ± 2
–	–	+	819 ± 52
–	+	+	1560 ± 104
+	–	–	12 ± 2
+	–	+	411 ± 31*
+	+	+	1169 ± 68**

The cultured cells were pretreated with 10 ng/ml of actinomycin D, 10 μM 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 ± 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.

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 transducer 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.

Acknowledgements

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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.

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

It is well recognized that interleukin-6 (IL-6) is a multifunctional 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-α 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_A receptors and ET_B receptors [11]. We have shown that ET-1 activates phospholipase C and phospholipase D through ET_A 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 form 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 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- 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.

IL-6 assay. The cultured cells were stimulated by various doses of ET-1 in 1 ml of α-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 α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000*g* for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [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 ± 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 μM. Y27632 at 10 μM 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 μM. Fasudil (3 μM) 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

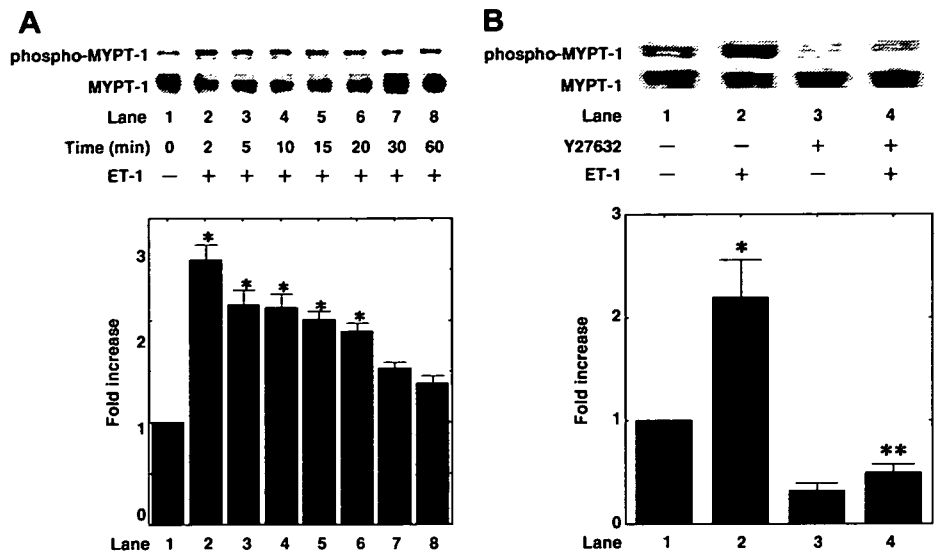

Fig. 1. Effect of ET-1 on the phosphorylation of MYPT-1 and effect of Y27632 on the ET-1-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. (A) The cultured cells were stimulated by 0.1 μM ET-1 for the indicated periods. (B) The cultured cells were pretreated with 10 μM Y27632 or vehicle for 60 min, and then stimulated by 0.1 μM 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 ± 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.

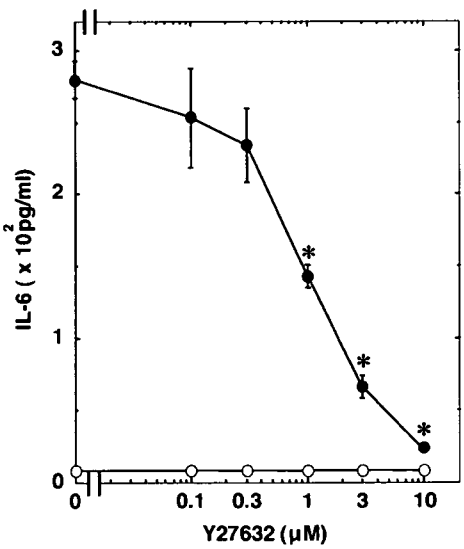

Fig. 2. Effect of Y27632 on the ET-1-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained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

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

Fasudil (μM)	ET-1	IL-6 (pg/ml)
0	–	<7.8
0	+	287 ± 16*
0.3	–	<7.8
0.3	+	261 ± 6
1	–	<7.8
1	+	171 ± 5**
3	–	<7.8
3	+	70 ± 5**

The cultured cells were pretreated with various doses of fasudil for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
* $p < 0.05$, compared to the control.
** $p < 0.05$, 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-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

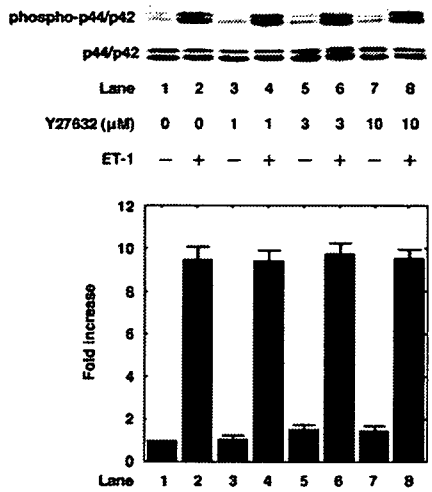

Fig. 3. Effect of Y27632 on the ET-1-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle for 5 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western 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 ± 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 μM. Y27632 (10 μM) 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 μM. Fasudil (10 μM) 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-

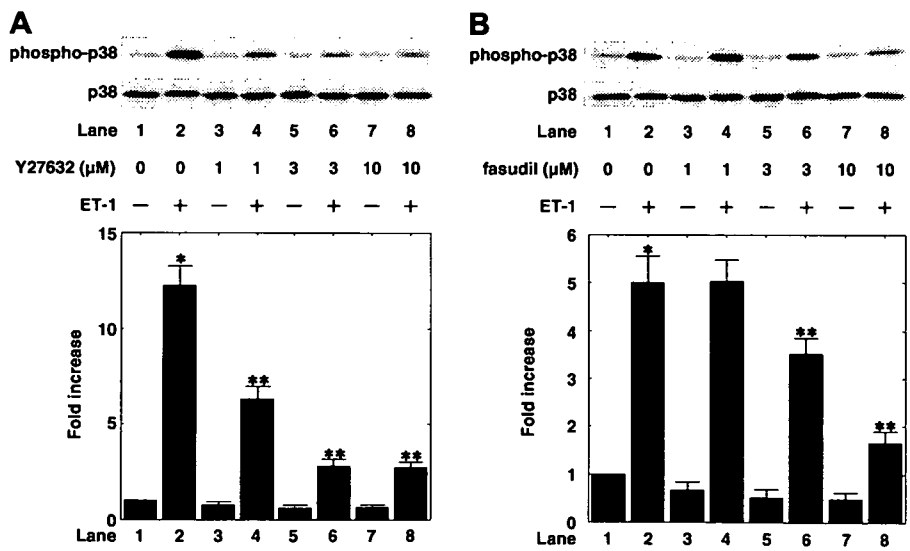

Fig. 4. Effects of Y27632 or fasudil on the ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle for 20 min. The extracts of cells were subjected to 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 ± 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

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(−)-Epigallocatechin gallate inhibits prostaglandin D_2-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_2 (PGD_2) 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_2 without affecting the levels of HSP70. The PGD_2-induced phosphorylation of p38 MAP kinase or SAPK/JNK was not affected by EGCG. On the contrary, EGCG markedly suppressed the PGD_2-induced phosphorylation of p44/p42 MAP kinase and MEK1/2. However, the PGD_2-induced phosphorylation of Raf-1 was not inhibited by EGCG. These results strongly suggest that EGCG suppresses the PGD_2-stimulated induction of HSP27 at the point between Raf-1 and MEK1/2 in osteoblasts.

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, αB-crystallin and HSP20 share high homology in amino acid sequences "α-crystallin domain" [2]. Though the functions 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_2 is generally known as a potent regulator of osteoblastic functions [11,12]. In our previous study [13], we have reported that PGD_2 stimulates the synthesis of interleukin-6 through calcium-dependent manner in osteoblast-like MC3T3-E1 cells. In addition, we showed that PGD_2 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_2 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_2.

In the present study, we investigated the effect of (−)-epigallocatechin gallate (EGCG), one of the major green tea flavonoids [17], on PGD_2-stimulated induction of HSP27 and the mechanism in osteoblast-like MC3T3-E1 cells. We here show that EGCG suppresses the PGD_2-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_2 and β-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_2 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 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 90-mm diameter dishes (25×10^4 per dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with EGCG.

2.3. Western blot analysis

The cultured cells were stimulated by PGD_2 in serum-free α-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, β-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±SEM of triplicate determinations.

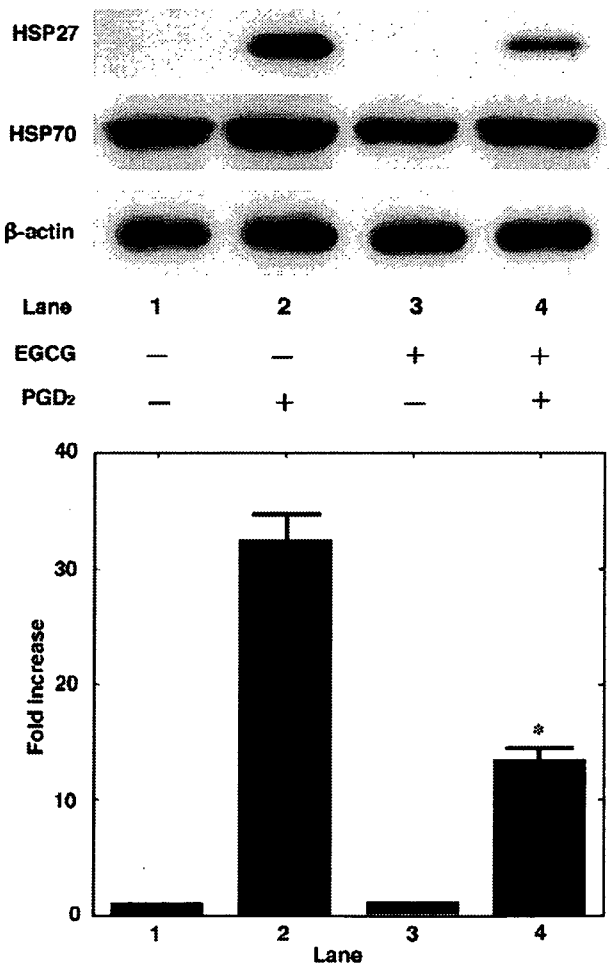

Fig. 1. Effect of EGCG on the PGD_2-stimulated HSP27 induction in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM EGCG for 60 min, and then stimulated with 10 μM PGD_2 for 9 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27, HSP70 or β-actin. The histogram shows quantitative representations of the levels of PGD_2-induced HSP27 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_2 alone.

3. Results

3.1. Effect of EGCG on the PGD_2-stimulated HSP27 induction in MC3T3-E1 cells

We examined the effect of EGCG on the PGD_2-stimulated induction of HSP27. EGCG significantly reduced the PGD_2-induced levels of HSP27 (Fig. 1). EGCG (30 μM) caused about 60% reduction in the PGD_2-effect. We have shown that PGD_2 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_2-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

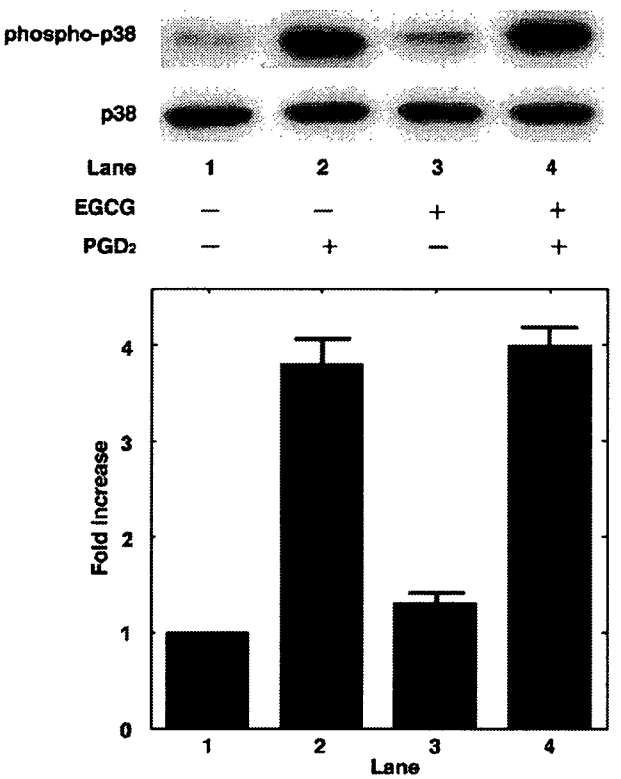

Fig. 2. Effect of EGCG on the PGD_2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of PGD_2-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.

p44/p42 MAP kinase mediate the PGD_2-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_2-stimulated phosphorylation of three MAP kinases. However, EGCG did not influence the PGD_2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (Fig. 2). In addition, the PGD_2-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_2 (Fig. 4). EGCG (30 μM) caused about 50% reduction in the PGD_2-effect.

3.3. *Effect of EGCG on the PGD_2-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 upstream kinase known as Raf-1 [25]. We found that both MEK1/2 and Raf-1 were time dependently phosphorylated by PGD_2 (data not shown). EGCG significantly suppressed the PGD_2-induced phosphorylation of MEK1/2 (Fig. 5). EGCG (30 μM) caused about 50% reduction in the PGD_2-effect. On the contrary, EGCG failed to attenuate the PGD_2-induced phosphorylation of Raf-1 (Fig. 6).

4. Discussion

In the present study, we first showed that EGCG markedly inhibited the PGD_2-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

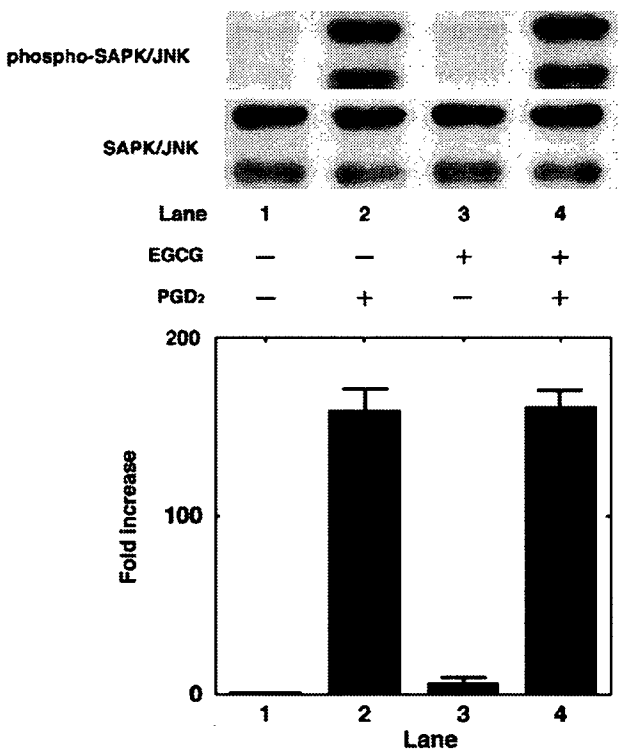

Fig. 3. Effect of EGCG on the PGD_2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of PGD_2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

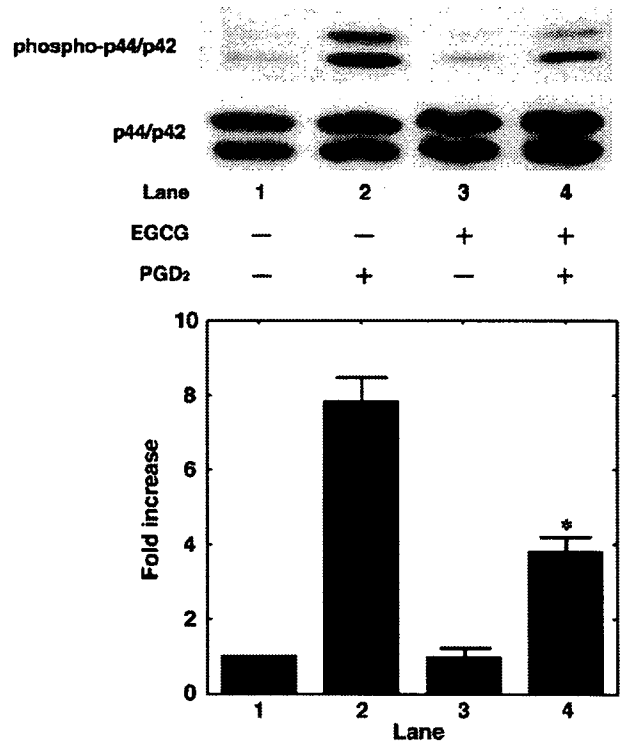

Fig. 4. Effect of EGCG on the PGD_2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 15 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting 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_2-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_2 alone.

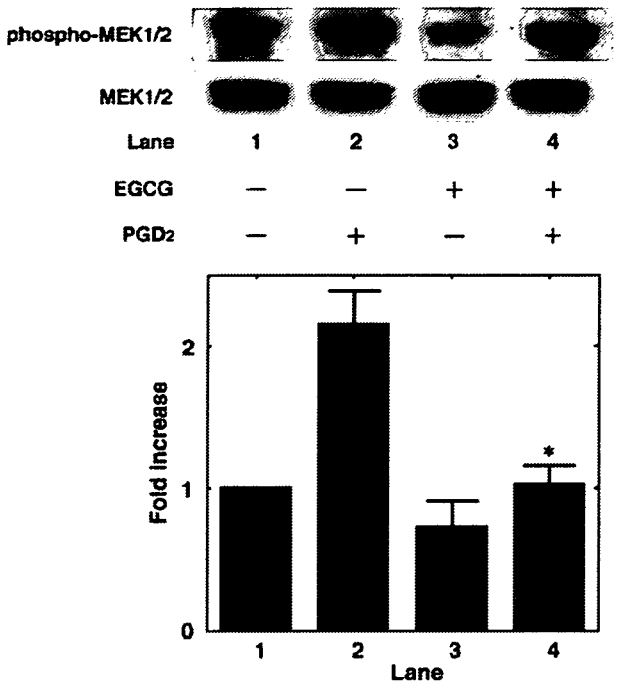

Fig. 5. Effect of EGCG on the PGD_2-induced phosphorylation of MEK1/2 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 15 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific MEK1/2 or MEK1/2. The histogram shows quantitative representations of the levels of PGD_2-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_2 alone.

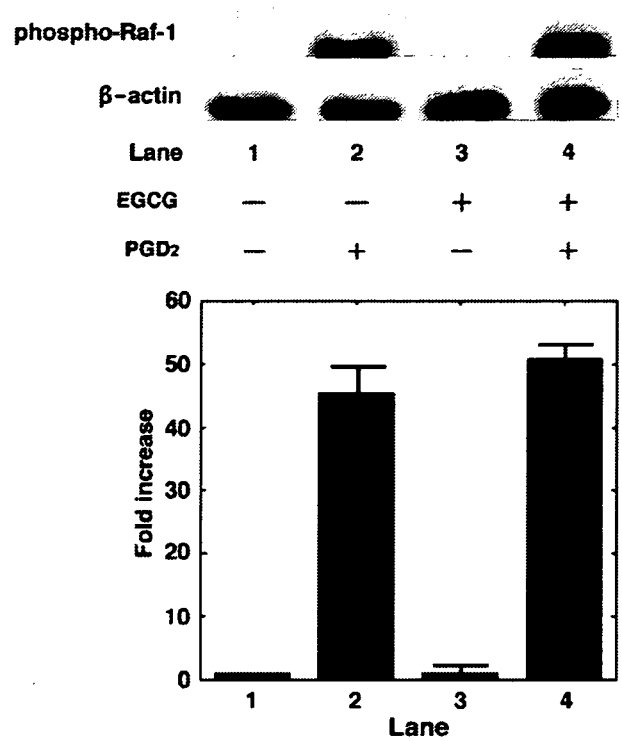

Fig. 6. Effect of EGCG on the PGD_2-induced phosphorylation of Raf-1 in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Raf-1 or β-actin. The histogram shows quantitative representations of the levels of PGD_2-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.

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 transducer the various messages. We have previously shown that the three MAP kinases function as positive regulators in the PGD_2-stimulated induction of HSP27 in osteoblast-like MC3T3-E1 cells [14,15]. In the present study, the PGD_2-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_2-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 MEK as MAP kinase kinase kinase [25]. In the present study, the PGD_2-stimulated phosphorylation of p44/p42 MAP kinase was significantly attenuated by EGCG. This result suggests that EGCG suppresses PGD_2-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_2-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_2-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_2-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

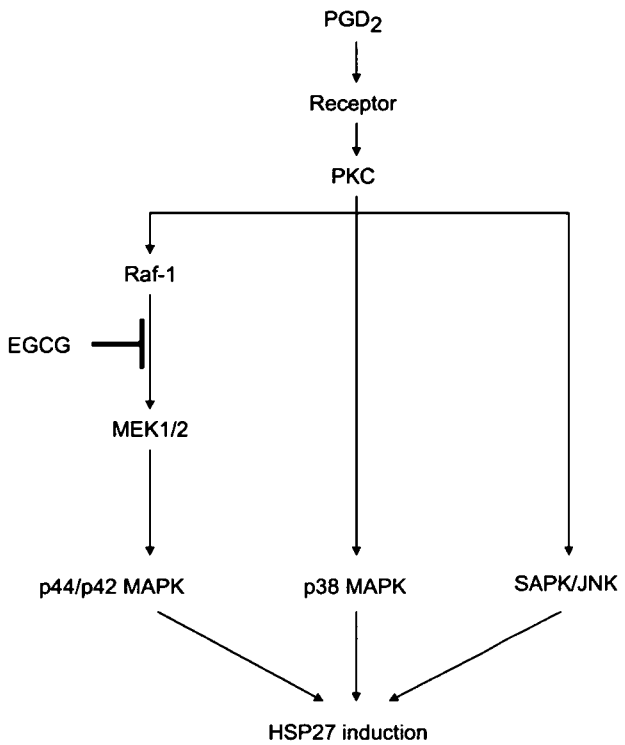

Fig. 7. Diagram of the mechanism of PGD_2-stimulated HSP27 induction via three MAP kinases and the inhibition by EGCG in osteoblasts. PGD_2, prostaglandin D_2; PKC, protein kinase C; EGCG, (−)-epigallocatechin gallate; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; HSP27, heat shock protein 27.

bone resorption [19]. Consistent with this, catechin reportedly induces apoptotic cell death of osteoclasts [26]. It has recently been reported that catechin increases cell viability and alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells and apoptosis of these cells is suppressed by catechin [20]. These evidences lead us to speculate that catechin promotes bone formation and reduces bone resorption, resulting in at least preventing bone loss. Therefore, it is possible that intake of catechin-containing beverage such as green tea could prevent the progression of postmenopausal osteoporosis. Although the physiological significance of HSP27 in osteoblasts has not yet been clarified, it is probable that EGCG-induced suppression of the p44/p42 MAP kinase cascade in osteoblasts contributes to the modulation of osteoblastic cell function toward bone formation at least in part via specifically down regulating HSP27 induction. Further investigation is required to clarify the exact role of catechin in bone metabolism.

In conclusion, these results strongly suggest that EGCG suppresses the PGD_2-stimulated induction of HSP27 via suppression of the p44/p42 MAP kinase pathway in osteoblasts.

Acknowledgments

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Platelet-derived growth factor-BB amplifies $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblasts: Function of phosphatidylinositol 3-kinase

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Abstract

We have reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates the synthesis of vascular endothelial growth factor (VEGF) via p44/p42 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells. In addition, we recently showed that phosphatidylinositol 3 (PI3)-kinase activated by platelet-derived growth factor-BB (PDGF-BB) negatively regulates the interleukin-6 synthesis in these cells. In the present study, we investigated the effect of PDGF-BB on the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells. PDGF-BB, which alone did not affect the levels of VEGF, significantly enhanced the $PGF_{2\alpha}$-stimulated VEGF synthesis. The amplifying effect of PDGF-BB was dose dependent in the range between 10 and 70 ng/ml. LY294002 or wortmannin, specific inhibitors of PI3-kinase, which by itself failed to affect the $PGF_{2\alpha}$-stimulated VEGF synthesis, significantly suppressed the amplification by PDGF-BB. PD98059, a specific inhibitor of MEK1/2, suppressed the amplification by PDGF-BB of the $PGF_{2\alpha}$-stimulated VEGF synthesis similar to the levels of $PGF_{2\alpha}$ with PD98059. PDGF-BB itself induced the phosphorylation of p44/p42 MAP kinase in these cells, and the effects of PDGF-BB and $PGF_{2\alpha}$ on the phosphorylation of p44/p42 MAP kinase were additive. Moreover, LY294002 had little effect on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ with PDGF-BB. These results strongly suggest that $PGF_{2\alpha}$-stimulated VEGF synthesis is amplified by PI3-kinase-mediating PDGF-BB signaling in osteoblasts, and that the effect is exerted at a point downstream from p44/p42 MAP kinase.

1. Introduction

Platelet-derived growth factor (PDGF) is a mitogenic polypeptide, which mainly acts on connective tissue cells [1,2]. PDGF occurs as five different isoforms [2]. PDGF isoforms were initially isolated from human platelets, but have been shown to be synthesized and released from a variety of cell types including osteosarcoma and osteoblasts [1,3,4]. It is well recognized that bone metabolism is strictly regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [5]. As for bone metabolism, PDGF-BB is a potent stimulator of osteoblast proliferation and induces bone resorption [4]. It is recognized that PDGF, released during platelet aggregation, plays a crucial role in fracture healing as a systemic factor and that PDGF also regulates bone remodeling as a local factor [4]. PDGF receptor has an intrinsic protein tyrosine kinase activity and associates with SH-2 domain-containing substrates such as phospholipase C-γ and phosphatidylinositol 3-kinase (PI3-kinase) [1]. We have previously reported that

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