proliferation and G_1 cell-cycle progression [15]. As for osteoblasts, it has been shown that fluoroaluminate induces an increase in p70 S6 kinase phosphorylation [16]. In our previous study [17], we have reported that p70 S6 kinase plays as a positive regulator in bone morphogenetic protein 4–stimulated synthesis of vascular endothelial growth factor in osteoblast-like MC3T3-E1 cells. In addition, we recently demonstrated that p38 mitogen-activated protein (MAP) kinase, a member of the MAP kinase superfamily, functions at a point upstream from p70 S6 kinase in the synthesis of vascular endothelial growth factor in these cells [18]. However, the exact role of p70 S6 kinase in osteoblasts has not yet been fully clarified.

In the present study, we investigated the mechanism behind PDGF-BB–stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that PDGF-BB stimulates IL-6 synthesis through activation of 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), in these cells, and that p70 S6 kinase concomitantly activated by PDGF-BB has an inhibitory role in the IL-6 synthesis.

2. Materials and methods

2.1. Materials

Platelet-derived growth factor–BB and mouse IL-6 and osteocalcin enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN). Indomethacin was purchased from Sigma Chemical (St Louis, MO). PD98059, SB203580, SP600125, and rapamycin were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p70 S6 kinase antibodies (Thr389), and p70 S6 kinase antibodies were purchased from Cell Signaling (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. PD98059, SB203580, SP600125, or rapamycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for IL-6 or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [19] were maintained as previously described [20]. 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 hours.

2.3. Interleukin 6 ELISA

The cultured cells were stimulated by various doses of PDGF-BB in 1 mL of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated

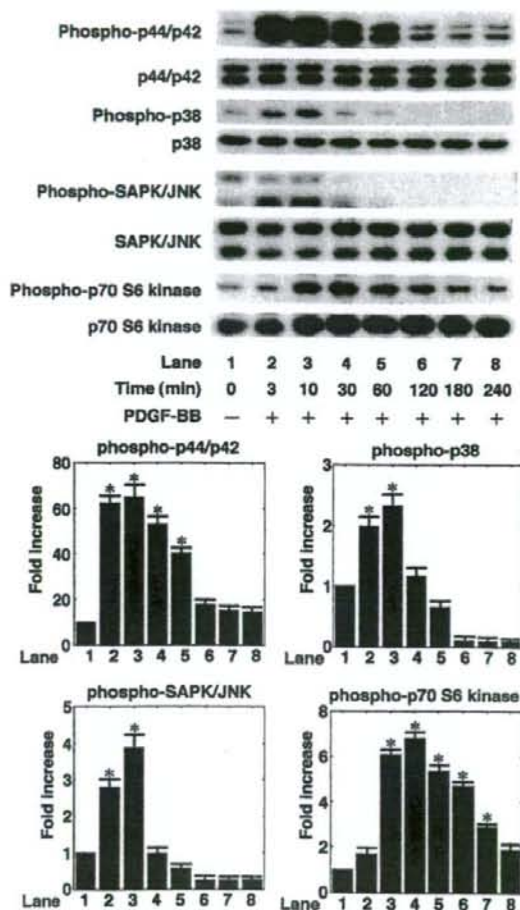

Fig. 1. Effects of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK, or p70 S6 kinase in MC3T3-E1 cells. The cultured cells were stimulated by 50 ng/mL PDGF-BB for the indicated periods. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p70 S6 kinase, or p70 S6 kinase. Similar results were obtained with 2 additional and different cell preparations. The histogram shows quantitative representations of the levels of PDGF-BB–induced phosphorylation obtained from laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of control.

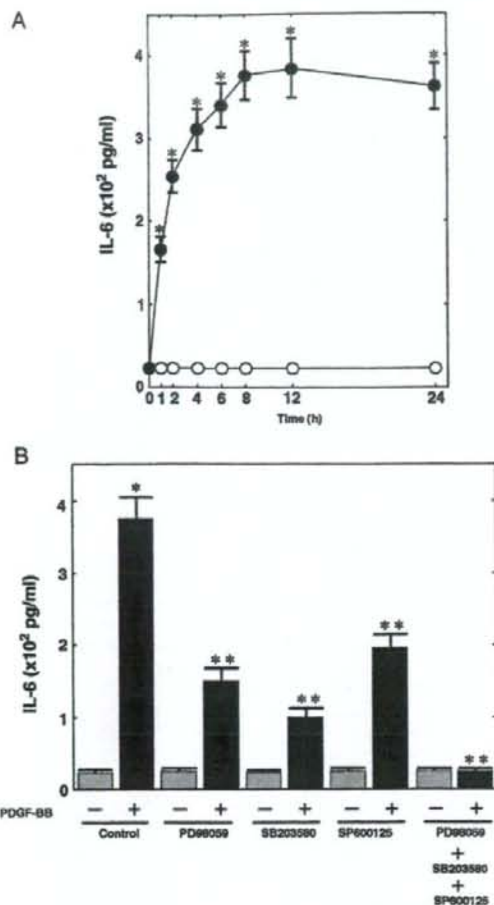

Fig. 2. Time course of PDGF-BB–induced IL-6 synthesis from MC3T3-E1 cells and effects of PD98059, SB203580, or SP600125 on the IL-6 synthesis by PDGF-BB in MC3T3-E1 cells. A, The cultured cells were stimulated by 50 ng/mL PDGF-BB (●) or vehicle (○) for the indicated periods. B, The cultured cells were pretreated with 3 μmol/L PD98059, 3 μmol/L SB203580, 3 μmol/L SP600125, or vehicle for 60 minutes and then stimulated by vehicle (gray bar) or 50 ng/mL PDGF-BB (black bar) for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of control. **$P < .05$ compared with the value of PDGF-BB alone.

with PD98059, SB203580, SP600125, indomathacin, or rapamycin for 60 minutes. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration was measured by ELISA kit.

2.4. Osteocalcin ELISA

The cultured cells were pretreated with various doses of rapamycin for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 24 hours. The conditioned medium was collected at the end of the incubation, and the osteocalcin concentration was measured by ELISA kit.

2.5. Western blot analysis

The cultured cells were stimulated by PDGF-BB in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer (pH 6.8) containing 62.5 mmol/L Tris/HCl, 2% sodium dodecyl sulfate, 50 mmol/L dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125 000g for 10 minutes at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by Laemmli [21] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [22] by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p70 S6 kinase antibodies, or p70 S6 kinase antibodies, with peroxidase-labeled antibodies raised in goat-against-rabbit immunoglobulin G being used as second antibodies. Peroxidase activity on the polyvinylidene difluoride (PVDF) sheet was visualized on x-ray film by means of the ECL Western blotting detection system.

2.6. Determination

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

2.7. Statistical analysis

The data were analyzed by analysis of variance followed by the Bonferroni method for multiple comparisons between pairs, and $P < .05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated 3 times, with similar results.

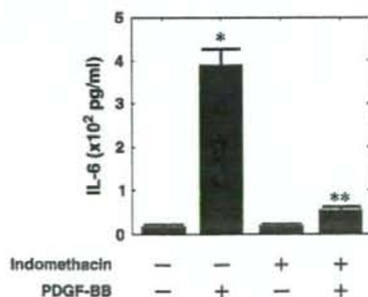

Fig. 3. Effect of indomethacin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 10 μmol/L indomethacin or vehicle for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the control. **$P < .05$ compared with the value of PDGF-BB alone.

3. Results

3.1. Effects of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells

It is well recognized that 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce the various messages of a variety of agonists [23]. To investigate whether PDGF-BB activates MAP kinases in osteoblast-like MC3T3-E1 cells, we examined the effect of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK. Platelet-derived growth factor–BB time-dependently induced the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK. The effect of PDGF-BB on the p44/p42 MAP kinase phosphorylation reached its peak at 10 minutes and continued to 60 minutes after the stimulation of PDGF-BB (Fig. 1). On the other hand, the effect on the phosphorylation of p38 MAP kinase reached its peak at 10 minutes and diminished within 30 minutes after the stimulation of PDGF-BB (Fig. 1). In addition, the maximum effect on the SAPK/JNK phosphorylation was observed at 10 minutes and diminished within 30 minutes after the stimulation of PDGF-BB (Fig. 1).

3.2. Effects of PD98059, SB203580, or SP600125 on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells

It has been reported that PDGF-BB induces IL-6 transcription in osteoblasts from fetal rat calvariae [14]. We found that PDGF-BB time-dependently stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells (Fig. 2A). To

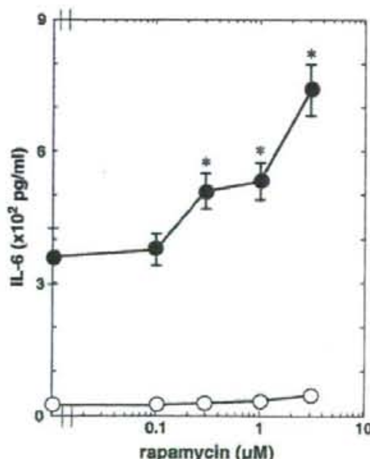

Fig. 4. Effect of rapamycin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of rapamycin for 60 minutes and then stimulated by 50 ng/mL PDGF-BB (●) or vehicle (○) for 24 hours. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of PDGF-BB alone.

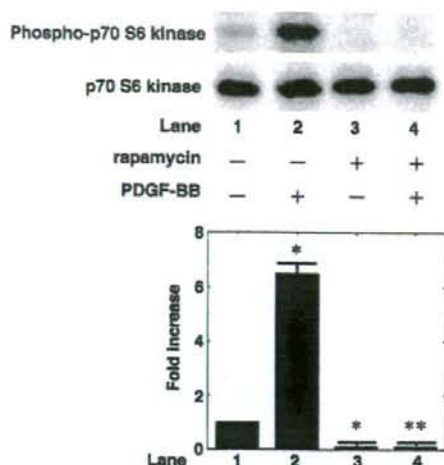

Fig. 5. Effect of rapamycin on the PDGF-BB–induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μmol/L rapamycin for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 30 minutes. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representations of the levels of PDGF-BB–induced phosphorylation obtained from laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations. *$P < .05$ compared with the value of control. **$P < .05$ compared with the value of PDGF-BB alone.

clarify the involvement of the MAP kinase pathway in the PDGF-BB–stimulated IL-6 synthesis in these cells, we first examined the effect of PD98059, a specific inhibitor of MAP kinase/extracellular signal-regulated kinase kinase (MEK, an upstream kinase that activates p44/p42 MAP kinase) [24], on the IL-6 synthesis. PD98059, which by itself had little effect on the IL-6 levels, significantly suppressed the PDGF-BB–stimulated synthesis of IL-6 (Fig. 2B). Similarly, the IL-6 synthesis stimulated by PDGF-BB was markedly reduced by SB203580, a specific inhibitor of p38 MAP kinase [25], or SP600125, a specific SAPK/JNK inhibitor [26] (Fig. 2B). In addition, a combination of PD98059, SB203580, and SP600125 completely suppressed the PDGF-BB–stimulated synthesis of IL-6 (Fig. 2B). To determine whether these inhibitors themselves could affect cell survival, or cell number, the cell viability had been assessed by trypan blue dye exclusion test. We confirmed that the viability of the cells incubated at 37°C for 24 hours in the presence of 3 μmol/L PD980590, 3 μmol/L SB203580, or 3 μmol/L SP600125 was more than 90% compared with that of the control cells.

3.3. Effect of indomethacin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells

Because we have previously reported that prostaglandins (PGs) increase IL-6 synthesis in MC3T3-E1 cells [27-30], to address whether endogenous PGs are involved in the

PDGF-BB–induced IL-6 synthesis in MC3T3-E1 cells, we examined the effect of indomethacin, an inhibitor of cyclooxygenase [31], on the IL-6 synthesis. Indomethacin, which by itself had no effect on the IL-6 levels, significantly reduced the PDGF-BB–induced synthesis of IL-6 (Fig. 3). These findings suggest that PGs mediate the stimulatory effect of PDGF-BB on IL-6 synthesis in these cells.

3.4. Effect of PDGF-BB on the phosphorylation of p70 S6 kinase in MC3T3-E1 cells

To clarify whether PDGF-BB activates p70 S6 kinase in MC3T3-E1 cells, we next examined the effect of PDGF-BB on the phosphorylation of p70 S6 kinase. p70 S6 kinase was time-dependently phosphorylated by PDGF-BB (Fig. 1). The maximum effect on the p70 S6 kinase phosphorylation was observed at 30 minutes after the stimulation of PDGF-BB, and the PDGF-BB effect continued 180 minutes after the stimulation.

3.5. Effect of rapamycin on the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells

To investigate whether p70 S6 kinase is involved in the PDGF-BB–induced synthesis of IL-6 in MC3T3-E1 cells, we examined the effect of rapamycin, a specific inhibitor of p70 S6 kinase [32,33], on the synthesis of IL-6 induced by PDGF-BB. Rapamycin, which alone failed to affect the IL-6 levels, significantly enhanced the PDGF-BB–induced synthesis of IL-6 (Fig. 4). The amplifying effect of rapamycin was dose-dependent in the range between 0.1 and 3 μmol/L. Rapamycin at 3 μmol/L caused approximately 110% enhancement in the PDGF-BB effect.

3.6. Effect of rapamycin on the PDGF-BB–induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells

We examined the effect of rapamycin on the PDGF-BB–induced phosphorylation of p70 S6 kinase. Rapamycin, which itself significantly suppressed the phosphorylation of p70 S6 kinase in itself, truly suppressed the PDGF-BB–induced phosphorylation of p70 S6 kinase (Fig. 5).

3.7. Effect of rapamycin on the proliferation or the differentiation of MC3T3-E1 cells

To determine whether rapamycin could affect cell survival, or cell number, the cell viability had been assessed by trypan blue dye exclusion test. We confirmed that the viability of the cells incubated at 37°C for 24 hours in the

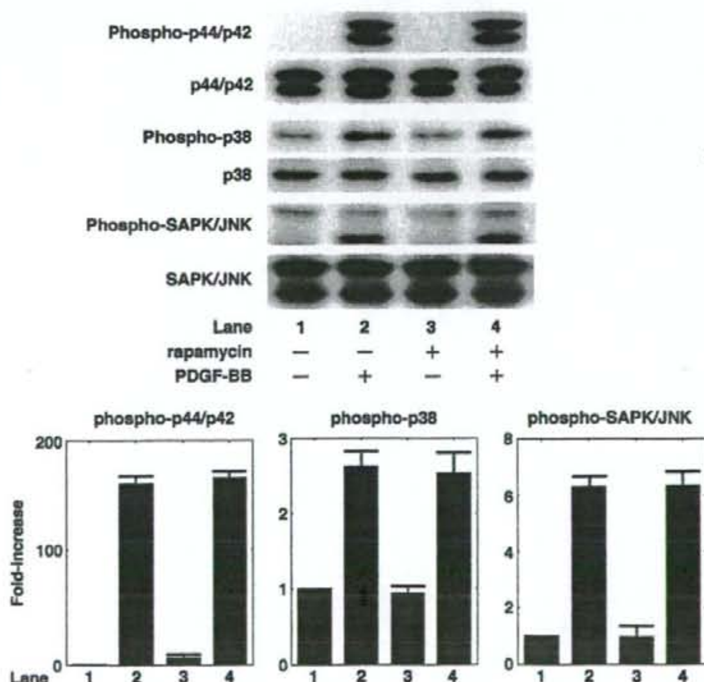

Fig. 6. Effects of rapamycin on the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μmol/L rapamycin or vehicle for 60 minutes and then stimulated by 50 ng/mL PDGF-BB or vehicle for 10 minutes. The extracts of cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, or SAPK/JNK. The histogram shows quantitative representations of the levels of PDGF-BB–induced phosphorylation obtained from laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with 2 additional and different cell preparations.

presence of 3 μmol/L rapamycin was more than 90% compared with that of the control cells. To determine whether rapamycin could affect the cell proliferation, we counted the cell number before and after the 24-hour incubation with rapamycin. We confirmed that rapamycin did not affect the cell number at a dose of 3 μmol/L ($9.7 \pm 1.1 \times 10^5$ cells/mL for control; $15.6 \pm 1.6 \times 10^5$ cells/mL for 50 ng/mL PDGF-BB alone; $10.1 \pm 1.4 \times 10^5$ cells/mL for 3 μmol/L rapamycin alone; and $14.7 \pm 1.6 \times 10^5$ cells/mL for 50 ng/mL PDGF-BB with 3 μmol/L rapamycin, as measured during the stimulation for 24 hours).

Next, to determine whether rapamycin affects the differentiation of these cells, we examined the effect of rapamycin on the production of osteocalcin, a mature osteoblast phenotype [34], in MC3T3-E1 cells. Platelet-derived growth factor–BB or rapamycin did not induce osteocalcin production in MC3T3-E1 cells (2.7 ± 0.3 ng/mL for control; 2.6 ± 0.3 ng/mL for 50 ng/mL PDGF-BB alone; 2.5 ± 0.4 ng/mL for 3 μmol/L rapamycin alone; and 2.5 ± 0.3 ng/mL for 50 ng/mL PDGF-BB with 3 μmol/L rapamycin, as measured during the stimulation for 24 hours). These findings as a whole suggest that rapamycin hardly affects the proliferation and the differentiation of osteoblast-like MC3T3-E1 cells within 24 hours.

3.8. Effects of rapamycin on the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells

To investigate whether rapamycin's effect on the PDGF-BB–stimulated IL-6 synthesis is dependent on the activation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK, we next examined the effect of rapamycin on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK induced by PDGF-BB in these cells. However, rapamycin failed to affect the PDGF-BB–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK (Fig. 6).

4. Discussion

In the present study, we found that PDGF-BB time-dependently induced the phosphorylation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells, using phospho-specific p70 S6 kinase (Thr389) antibodies. It is generally recognized that the activity of p70 S6 kinase is regulated by multiple phosphorylation events [15]. It has been shown that phosphorylation at Thr389 most strongly correlates with p70 S6 kinase activity [15]. Taking these results into account, it is most likely that PDGF-BB activates p70 S6 kinase in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, this is probably the first report showing the PDGF-BB–induced p70 S6 kinase activation in osteoblasts.

We next demonstrated that PDGF-BB induces the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK in these cells. 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 [23]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce diverse messages [23]. It has been shown that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual-specificity MAP kinases [23]. Therefore, our findings strongly suggest that PDGF-BB activates 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, in osteoblast-like MC3T3-E1 cells. In addition, we showed that the PDGF-BB–stimulated IL-6 synthesis was suppressed by a MEK inhibitor, PD98059 [24]; a specific p38 MAP kinase inhibitor, SB203580 [25]; or a specific SAPK/JNK inhibitor, SP600125 [26], in these cells. Thus, it is probable that PDGF-BB stimulates the synthesis of IL-6 via the 3 MAP kinases in osteoblast-like MC3T3-E1 cells. We have previously reported that PGs increase IL-6 synthesis in MC3T3-E1 cells [27-30]. In the present study, we found that indomethacin significantly reduced the PDGF-BB–induced synthesis of IL-6. These results suggest that PDGF-BB–induced IL-6 production is mediated, at least in part, by PDGF-BB–stimulated PG production in osteoblast-like MC3T3-E1 cells. In addition, we have previously shown that PGE_2, a major product of eicosanoids in osteoblasts, significantly stimulates IL-6 synthesis after 3 hours in MC3T3-E1 cells [30]. On the contrary, PDGF-BB significantly stimulated the IL-6 production within 3 hours. Taking our findings into account, it is quite likely that there will be PG-dependent and PG-independent effects of PDGF-BB–stimulated IL-6 synthesis, as has been demonstrated for so many growth factors and cytokines in bone cells, and it would be important to be define these. Therefore, experiments using PGE_2 itself instead of PDGF-BB are required.

We investigated whether p70 S6 kinase functions in the PDGF-BB–stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. The PDGF-BB–stimulated synthesis of IL-6 was significantly amplified by rapamycin, a specific inhibitor of p70 S6 kinase [31,32]. We confirmed that rapamycin truly suppressed the PDGF-BB–induced phosphorylation of p70 S6 kinase. It seems that the activated p70 S6 kinase plays an inhibitory role in the IL-6 synthesis by PDGF-BB in osteoblast-like MC3T3-E1 cells. Therefore, taking our results into account, it is most likely that PDGF-BB activates p70 S6 kinase, resulting in down-regulation of IL-6 synthesis. It is probable that the p70 S6 kinase signaling pathway activated by PDGF-BB limits the PDGF-BB–stimulated IL-6 synthesis. As far as we know, our present finding is probably the first report to show that the activation of p70 S6 kinase leads to the negative-feedback regulation of IL-6 synthesis in osteoblasts.

We investigated the relationship between p70 S6 kinase and 3 MAP kinases in the PDGF-BB–stimulated IL-6 synthesis in MC3T3-E1 cells. However, rapamycin failed to enhance the PDGF-BB–induced phosphorylation levels of

p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK. Therefore, it seems unlikely that p70 S6 kinase signaling pathway affects the PDGF-BB–stimulated synthesis of IL-6 through the amplification of activities of 3 MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, in osteoblast-like MC3T3-E1 cells.

The p70 S6 kinase pathway is recognized to play a crucial role in various cellular functions, especially cell-cycle progression [15]. Our present results indicate that the p70 S6 kinase pathway 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 [3,4]. The mitogenic activities of PDGF-BB and its release by platelets suggest an important role in wound healing and fracture repair [35]. It is also possible that PDGF-BB plays a role in acute bone repair after inflammation because the mitogenic actions of PDGF-BB are enhanced in the presence of cytokines [35]. Therefore, our present findings lead us to speculate that PDGF-BB–activated p70 S6 kinase acts as a negative regulator of bone resorption through the fine tuning of the local cytokine network. Thus, the p70 S6 kinase pathway in osteoblasts might be considered to be a new candidate as a molecular target of bone resorption concurrent with various bone diseases. On the contrary, we have previously shown that p70 S6 kinase acts as a positive regulator in bone morphogenetic protein-4–stimulated synthesis of vascular endothelial growth factor in MC3T3-E1 cells [17]. The physiologic significance of regulatory mechanism by p70 S6 kinase in osteoblasts still remains unclear. Further investigation is required to clarify the exact role of p70 S6 kinase in osteoblasts.

In conclusion, our results strongly suggest that p70 S6 kinase plays an important role in the regulation of PDGF-BB–stimulated, MAP kinase–mediated IL-6 synthesis in osteoblasts and may serve as a negative feedback mechanism to prevent from oversynthesizing IL-6 in these cells.

Acknowledgments

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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-3β, 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 μM, while it suppressed the FGF-2-induced phosphorylation of GSK-3β. 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; Schalaeppi 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; Debiais 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-3β antibodies, GSK-3β 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 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 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 (25×10^4 cells) in α-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 α-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 α-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® 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 caliblator diluent provided with the kit, and re-assayed.

2.4. Western blot analysis

The cultured cells were stimulated by FGF-2 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 $125,000 \times g$ 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-3β antibodies, GSK-3β 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 ± 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.

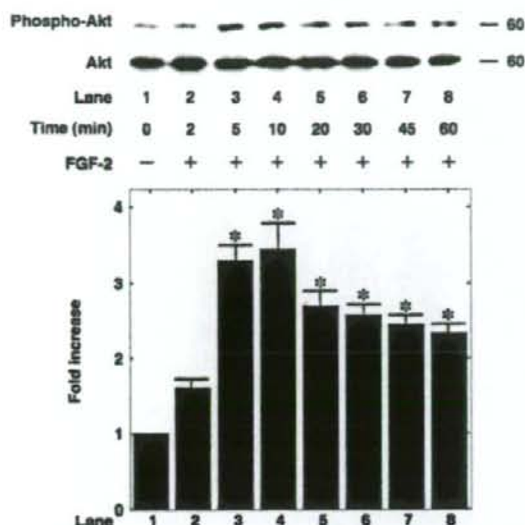

Fig. 1. Effect of FGF-2 on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The 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 ± 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 μM (Fig. 2A). The Akt inhibitor at 70 μM 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 μM (Fig. 2C). The maximum effect of the Akt inhibitor (30 μM) caused about 60% enhancement in the FGF-2-effect.

3.4. Effect of Akt inhibitor on the phosphorylation of Akt and GSK-3β 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-3α at Ser21 and GSK-3β at Ser9 (Cross et al., 1995; Srivastava and Pandey, 1998). We found that FGF-2 truly induced the Akt mediated phosphorylation of GSK-3β time-dependent manner in MC3T3-E1 cells (Fig. 3A). Then, we examined the effect of Akt inhibitor on the phosphorylation of GSK-3β induced by FGF-2 in these cells. Akt inhibitor markedly suppressed the FGF-2-induced phosphorylation of GSK-3β (Fig. 3B). The inhibitor (50 μM) 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 μM (Fig. 4A). The LY294002 at 30 μM 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-3β 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).

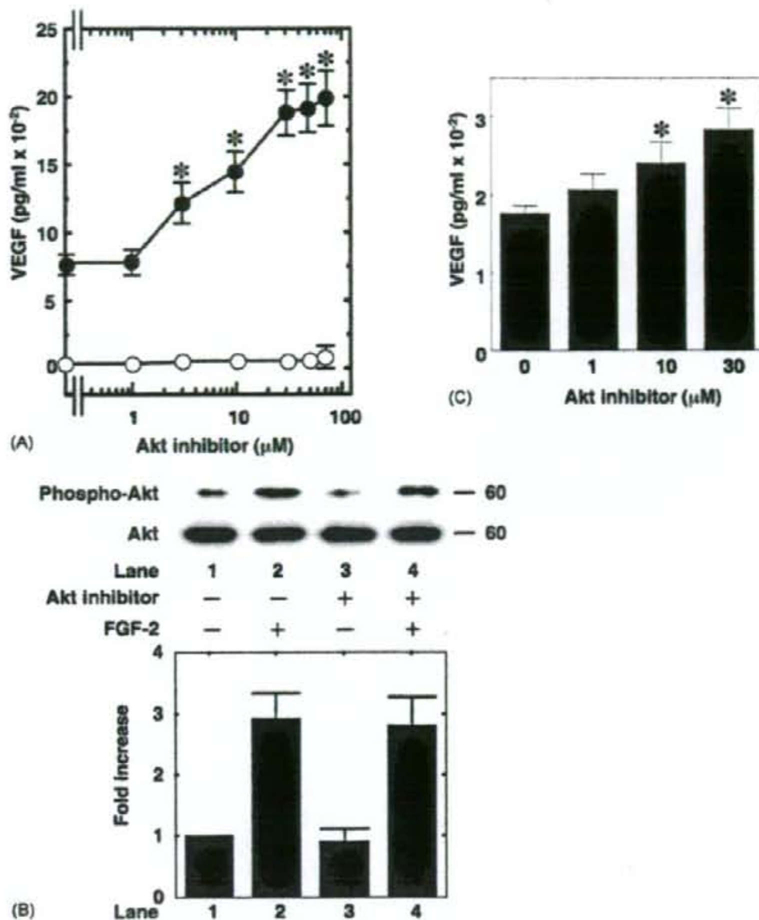

Fig. 2. Effects of Akt inhibitor on the VEGF release by FGF-2 or the FGF-2-induced phosphorylation of Akt. (A) Osteoblast-like MC3T3-E1 cells were pretreated with various doses of Akt inhibitor for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean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

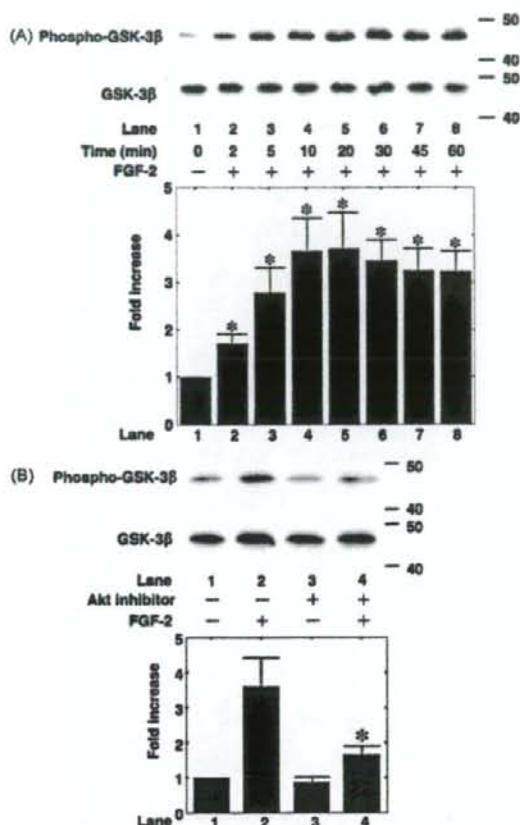

Fig. 3. Effect of FGF-2 on the phosphorylation of GSK-3β, and effect of Akt inhibitor on the FGF-2-induced phosphorylation of GSK-3β in MC3T3-E1 cells. (A) The cultured cells were stimulated by 30 ng/ml FGF-2 for the indicated periods. $^{*}p<0.05$, compared to the value of control. (B) The cultured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

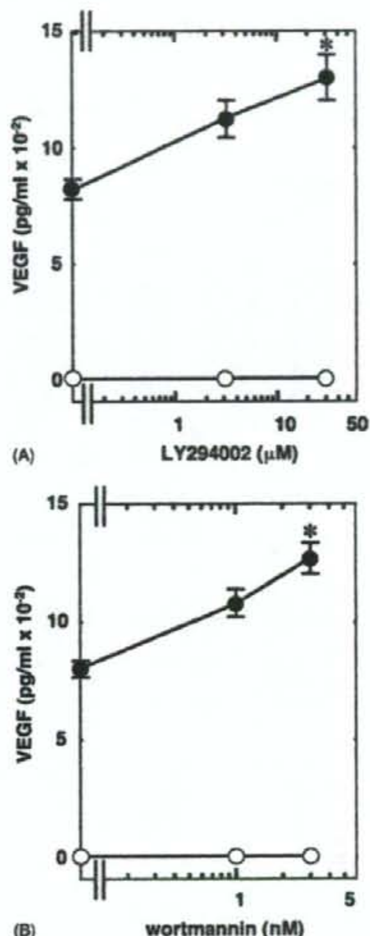

Fig. 4. Effects of wortmannin or LY294002 on the FGF-2-induced VEGF release in MC3T3-E1 cells. Osteoblast-like MC3T3-E1 cells were pretreated with various doses of LY294002 (A) or wortmannin (B) for 60 min, and then stimulated by 30 ng/ml FGF-2 (●) or vehicle (○) for 48 h. Each value represents the mean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

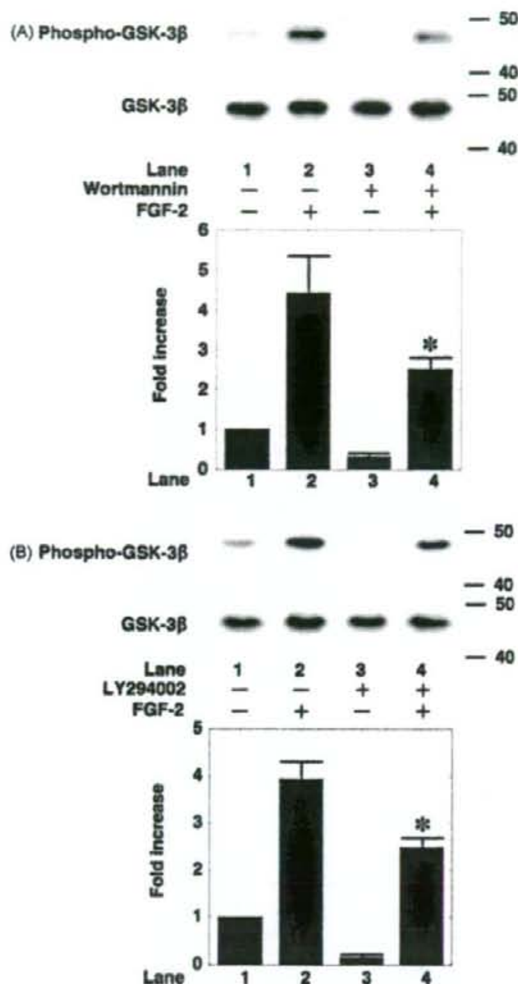

Fig. 5. Effects of wortmannin or LY294002 on the FGF-2-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM of wortmannin (A), 30 μM of LY294002 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells 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.

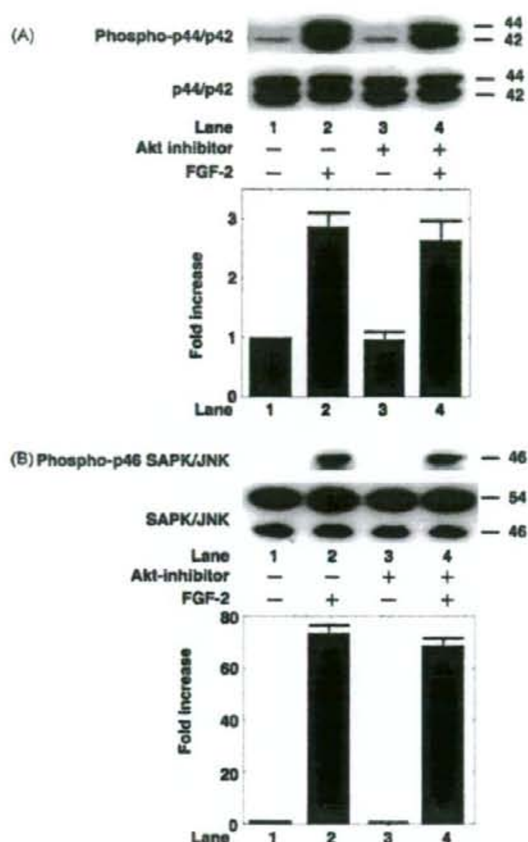

Fig. 6. Effects of Akt inhibitor on the FGF-2-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected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

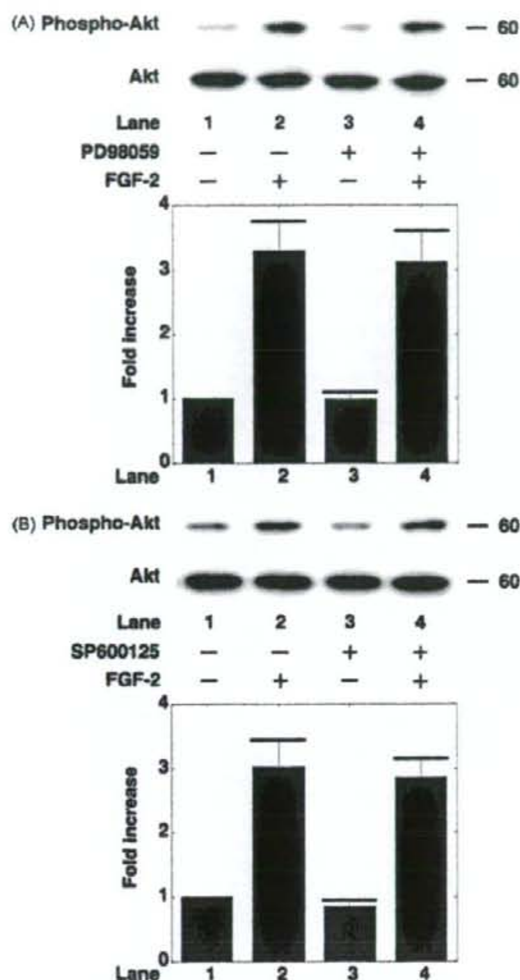

Fig. 7. Effects of PD98059 or SP600125 on the FGF-2-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM of PD98059 (A), 30 μM of SP600125 (B) or vehicle for 60 min, and then stimulated by 30 ng/ml FGF-2 or vehicle for 10 min. The extracts of cells were subjected 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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(−)-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.

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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, 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.

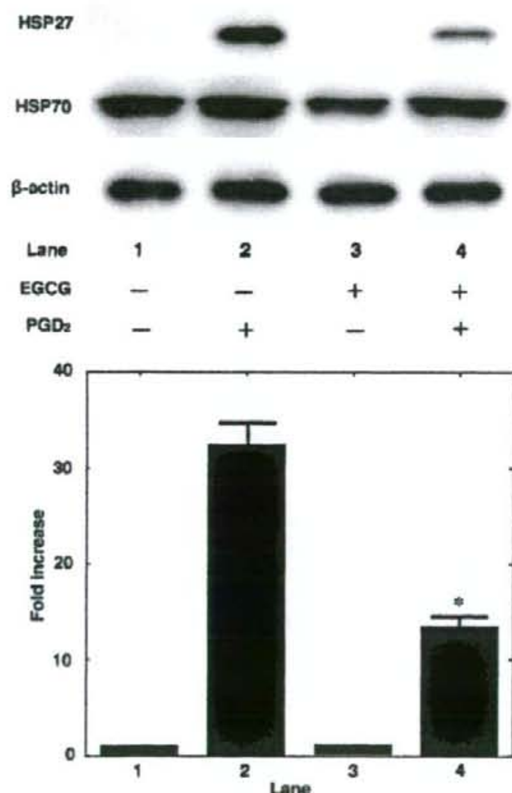

Fig. 1. Effect of EGCG on the PGD_2-stimulated HSP27 induction in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM EGCG for 60 min, and then stimulated with 10 μM PGD_2 for 9 h. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against 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

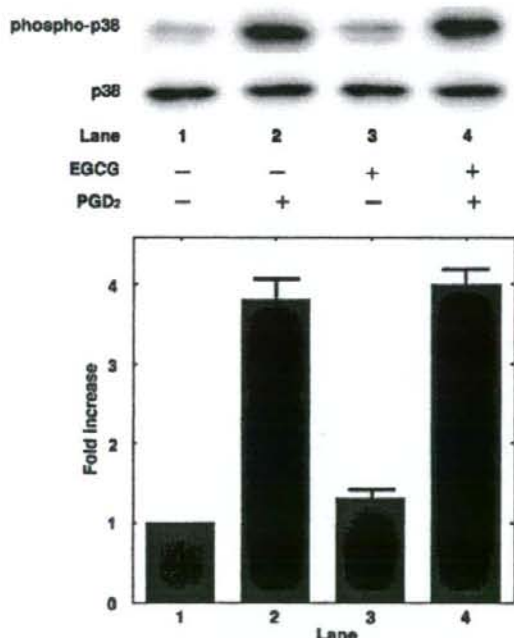

Fig. 2. Effect of EGCG on the PGD_2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting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

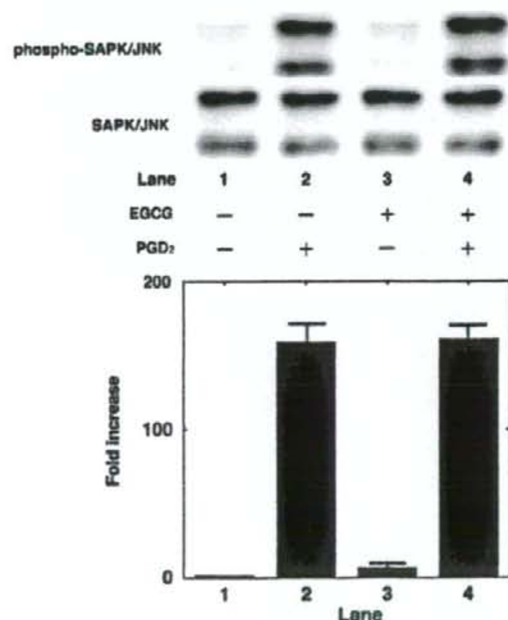

Fig. 3. Effect of EGCG on the PGD_2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis 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.

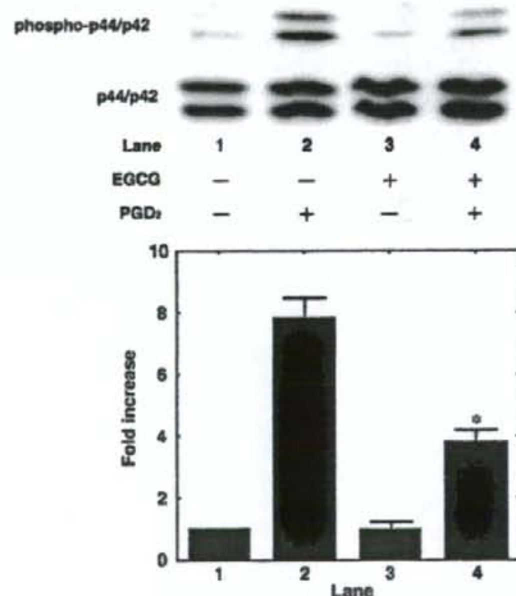

Fig. 4. Effect of EGCG on the PGD_2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM EGCG for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 15 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting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