p38 mitogen-activated protein (MAP) kinase but not p44/p42 MAP kinase is involved in prostaglandin E₁-induced vascular endothelial growth factor synthesis in osteoblasts

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Abstract

We investigated the mechanism underlying vascular endothelial growth factor (VEGF) synthesis stimulated by prostaglandin E₁ (PGE₁) in osteoblast-like MC3T3-E1 cells. PGE₁ induced the phosphorylation of both p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase. SB203580, a specific inhibitor of p38 MAP kinase, inhibited the PGE₁-stimulated VEGF synthesis as well as PGE₁-induced phosphorylation of p38 MAP kinase. PD98059, an inhibitor of the upstream kinase that activates p44/p42 MAP kinase, which reduced the PGE₁-induced phosphorylation of p44/p42 MAP kinase, had little effect on the VEGF synthesis stimulated by PGE₁. AH-6809, an antagonist of the subtypes of the PGE receptor, EP₁ and EP₂, or SC-19220, an antagonist of EP₁ receptor, did not inhibit the PGE₁-induced VEGF synthesis. H-89, an inhibitor of cAMP-dependent protein

kinase, and SQ22536, an inhibitor of adenylate cyclase, reduced the VEGF synthesis induced by PGE₁. Cholera toxin, an activator of G_s, and forskolin, an activator of adenylate cyclase, induced VEGF synthesis. SB203580 and PD169316, another specific inhibitor of p38 MAP kinase, reduced the cholera toxin-, forskolin- or 8bromo-cAMP-stimulated VEGF synthesis. However, PD98059 failed to affect the VEGF synthesis stimulated by cholera toxin, forskolin or 8-bromoadenosine-3',5'-cyclic monophosphate (8bromo-cAMP). SB203580 reduced the phosphorylation of p38 MAP kinase induced by forskolin or 8bromo-cAMP. These results strongly suggest that p44/p42 MAP kinase activation is not involved in the PGE₁-stimulated VEGF synthesis in osteoblasts but that p38 MAP kinase activation is involved.

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Introduction

It is well recognized that prostaglandins (PGs) act as autocrine/paracrine modulators of osteoblasts, and play important roles in cell function (Nijweide *et al.* 1986, Smith 1986). Among them, PGE₁ is known to stimulate cyclic AMP (cAMP) production and to induce alkaline phosphatase activity (Pilbeam *et al.* 1996), a marker of osteoblast phenotype (Robinson *et al.* 1973), in osteoblasts. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts (Nijweide *et al.* 1986), the former being responsible for bone formation and the latter for bone resorption. Accumulating evidence indicates that osteoblasts possess receptors for bone resorptive agents such as parathyroid hormone and 1,25-(OH)₂ vitamin D₃ (Nijweide *et al.* 1986), suggesting that osteoblasts also play pivotal roles in bone resorption. During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which proliferate

locally and differentiate into osteoblasts, migrate into the resorption lacuna. Thus, it is currently recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely co-ordinated via humoral factors as well as by direct cell-to-cell contact, and these cells co-operatively regulate bone metabolism (Erlebacher *et al.* 1995).

Vascular endothelial growth factor (VEGF) is known as a specific growth factor of vascular endothelium (Ferrara & Davis-Smyth 1997). VEGF binds to its respective tyrosine kinase receptor Flt-1 (VEGFR1) and Flk-1/kinase domain region (KDR) (VEGFR2), both of which are expressed on endothelial cells, and induces mitogenic and chemotactic actions on endothelial cells (Ferrara & Davis-Smyth 1997). As for bone metabolism, it has recently been reported that inactivation of VEGF causes complete suppression of blood vessel invasion, concomitant with impaired trabecular bone formation and expansion of the hypertrophic chondrocyte zone in the mouse tibial epiphyseal growth

plate (Gerber *et al.* 1999). Evidence is accumulating that VEGF is produced and secreted by osteoblasts in response to various humoral factors (Goad *et al.* 1996, Wang *et al.* 1996, Ferrara & Davis-Smyth 1997, Schallaeppi *et al.* 1997). It has been shown that PGE₁ increases the levels of mRNA for VEGF and produces VEGF in primary cultured rat calvarial cells and RCT-3 osteoblast-like cells, and that cAMP mediates the synthesis of VEGF (Harada *et al.* 1994). However, the mechanism behind VEGF synthesis induced by PGE₁ in osteoblasts has not yet been precisely clarified.

It is well known that mitogen-activated protein (MAP) kinases play important roles in the intracellular signaling of a variety of agonists (Nishida & Gotoh 1993, Widmann *et al.* 1999). In osteoblasts, MAP kinases are reportedly activated by several agonists such as basic fibroblast growth factor (bFGF) and estrogen (Hurley *et al.* 1996, Endoh *et al.* 1997). We have recently reported that the interleukin-6 (IL-6) synthesis induced by PGF_{2α} or endothelin-1 is mediated by protein kinase C-dependent activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells (Kawamura *et al.* 1999). In addition, we have demonstrated that bFGF-induced IL-6 synthesis is mediated through p38 MAP kinase activation, and that IL-1-induced IL-6 synthesis is dependent upon the activation of both p44/p42 MAP kinase and p38 MAP kinase in these cells (Kozawa *et al.* 1999, Miwa *et al.* 1999). However, the roles of MAP kinase in osteoblast cell function remain to be clarified. Thus, we have hypothesized that PGE₁ activation of VEGF synthesis is mediated by p44/p42 MAP kinase and/or p38 MAP kinase in osteoblasts.

In the present study, we have investigated the involvement of MAP kinases in the PGE₁-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that PGE₁ activates both p44/p42 MAP kinase and p38 MAP kinase in these cells, and that p44/p42 MAP kinase is not involved in PGE₁-stimulated VEGF synthesis but that p38 MAP kinase is involved.

Materials and Methods

Materials

PGE₁, cholera toxin, forskolin and 8-bromoadenosine-3',5'-cyclic monophosphate (8bromo-cAMP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2'-Amino-3'-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), 1-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316) and 9-(tetrahydro-2'-furyl)adenine (SQ22536) were obtained from Calbiochem Novabiochem Co. (La Jolla, CA, USA). Phospho-specific p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified),

phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) and p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) were purchased from New England BioLabs, Inc. (Beverly, MA, USA). *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2 HCl (H-89) was obtained from Seikagaku Kogyo Inc. (Tokyo, Japan). 6-Isopropoxy-9-xanthone-2-carboxylic acid (AH-6809) was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). 8-Chloro-dibenz[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide (SC-19220) was from Cayman Chemical (Ann Arbor, MI, USA). A mouse VEGF enzyme immunoassay kit was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). An ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PGE₁ was dissolved in ethanol. SB203580, PD98059, H-89, SQ22536, AH-6809, SC-19220 and PD169316 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

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 bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The cells were seeded into 35 mm diameter dishes or 90 mm diameter dishes in α -MEM containing 10% FBS. After 5 days, the medium was exchanged for α -MEM containing 0.3% FBS. The cells were used for experiments after 48 h.

Assay for VEGF

The cultured cells were stimulated by PGE₁ (10 μ M), cholera toxin (1 μ g/ml), forskolin (50 μ M) or 8bromo-cAMP (0.3 mM) in 1 ml α -MEM containing 0.3% FBS for the periods indicated. When indicated, the cells were pretreated with SB203580 (0.3–30 μ M), PD98059 (0.3–50 μ M), H-89 (1–10 μ M), SQ22536 (200 μ M), AH-6809 (1 μ M), SC-19220 (1 μ M) or PD169316 (0.3–30 μ M) for 60 min. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of MAP kinases

The cultured cells were stimulated by PGE₁ (10 μ M), forskolin (50 μ M) or 8bromo-cAMP (1 mM) in α -MEM containing 0.3% FBS for the periods indicated. The cells were washed twice with phosphate-buffered saline and

Figure 1 Effects of PGE₁ on the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 10 μ M PGE₁ for 1 min (lane 2), 3 min (lane 3), 5 min (lane 4), 10 min (lane 5), 20 min (lane 6), 30 min (lane 7) and 60 min (lane 8). The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase or p38 MAP kinase. Lane 1, control cells.

then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% 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-PAGE was performed according to Laemmli (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato *et al.* 1996) by using 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 nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pre-treated with SB203580 (30 μ M) or PD98059 (50 μ M) for 60 min.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with an EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparison between pairs, and *P*<0.05 was considered significant. All data are

presented as the mean $\pm$ S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effects of PGE₁ on the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase in MC3T3-E1 cells

In order to investigate whether PGE₁ activates p44/p42 MAP kinase and/or p38 MAP kinase, we examined the effects of PGE₁ on the phosphorylation of these MAP kinases. PGE₁ time-dependently induced the phosphorylation of both p44/p42 MAP kinase and p38 MAP kinase (Fig. 1). The maximum stimulatory effects of PGE₁ on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase was observed at 3 and 10 min respectively after the stimulation.

Effects of SB203580 or PD98059 on the PGE₁-induced VEGF synthesis in MC3T3-E1 cells

PGE₁ reportedly induces VEGF synthesis in osteoblasts (Harada *et al.* 1994). We confirmed that PGE₁ induces VEGF synthesis in a dose-dependent manner in the range between 10 nM and 10 μ M in MC3T3-E1 cells (data not shown). To investigate whether p38 MAP kinase or p44/p42 MAP kinase is involved in the PGE₁-induced VEGF synthesis, we examined the effects of SB203580, a

Figure 2 Effect of SB203580 on the PGE₁-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 for 60 min, and then stimulated by 10 μM PGE₁ (●) or vehicle (○) for 24 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 with the value of PGE₁ alone.

Figure 3 Effect of PD98059 on the PGE₁-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 for 60 min, and then stimulated by 10 μM PGE₁ (●) or vehicle (○) for 24 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

specific inhibitor of p38 MAP kinase (Cuenda *et al.* 1995), or PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi *et al.* 1995), on the VEGF synthesis induced by PGE₁. SB203580, which by itself had little effect on the VEGF synthesis, significantly reduced the PGE₁-stimulated VEGF synthesis in a dose-dependent manner between 0.3 and 30 μM (Fig. 2). On the other hand, PD98059, which alone did not affect the VEGF synthesis, had no effect on the PGE₁-stimulated VEGF synthesis (Fig. 3).

Effects of SB203580 and PD98059 on the PGE₁-induced phosphorylation of p38 MAP kinase and p44/p42 MAP kinase respectively in MC3T3-E1 cells

SB203580 (30 μM), which by itself had little effect on the phosphorylation of p38 MAP kinase, significantly attenuated the PGE₁-induced phosphorylation of p38 MAP kinase (Fig. 4A). In addition, we found that PD98059 (50 μM) markedly suppressed the PGE₁-induced phosphorylation of p44/p42 MAP kinase (Fig. 4B).

Effects of H-89 or SQ22536 on the PGE₁-induced VEGF synthesis in MC3T3-E1 cells

It has been reported that cAMP mediates the synthesis of VEGF induced by PGE₁ in osteoblasts (Harada *et al.* 1994). We examined the effects of H-89, an inhibitor of cAMP-dependent protein kinase (protein kinase A) (Chijiwa *et al.* 1990), and SQ22536, an inhibitor of adenylate cyclase (Goldsmith & Abrams 1991), on the PGE₁-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. H-89 which, by itself, had little effect on VEGF synthesis, dose-dependently reduced the PGE₁-induced VEGF synthesis in the range between 1 and 10 μM (Fig. 5). SQ22536 (200 μM) which, alone, hardly affected VEGF synthesis, also inhibited the VEGF synthesis induced by PGE₁ (Table 1).

Effects of AH-6809 or SC-19220 on the PGE₁-induced VEGF synthesis in MC3T3-E1 cells

The PGE receptor is classified into four subtypes, namely, EP₁, EP₂, EP₃ and EP₄ (Coleman *et al.* 1994). Among

Figure 4 Effects of SB203580 or PD98059 on the PGE₁-induced phosphorylation of p38 MAP kinase or p44/p42 MAP kinase in MC3T3-E1 cells. (A) The cultured cells were pretreated with 30 μ M SB203580 or vehicle for 60 min, and then stimulated by 10 μ M PGE₁ or vehicle for 10 min. (B) The cultured cells were pretreated with 50 μ M PD98059 or vehicle for 60 min, and then stimulated by 10 μ M PGE₁ or vehicle for 3 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against each MAP kinase.

them, EP₂ and EP₄ are coupled to the GTP-binding protein that mediates the stimulation of adenylate cyclase (G_s), which transduces stimulation from the receptor to adenylate cyclase (Gilman 1987). AH6809, an antagonist of EP₁ and EP₂ (Coleman *et al.* 1994, Kiriya *et al.* 1997) which, alone, had no effect on VEGF synthesis, did not reduce the PGE₁-induced VEGF synthesis (Table 1). SC19220, an antagonist of EP₁ (Coleman *et al.* 1994), did not inhibit the VEGF synthesis induced by PGE₁ (data not shown).

Effects of SB203580 or PD169316 on the cholera toxin-, forskolin- and 8bromo-cAMP-induced VEGF synthesis in MC3T3-E1 cells

To further clarify whether or not p38 MAP kinase is involved in the PGE₁-stimulated VEGF synthesis, we next examined the effect of SB203580 on the VEGF synthesis induced by cholera toxin, a direct activator of G_s (Gilman 1987), in MC3T3-E1 cells. SB203580 dose-dependently inhibited the cholera toxin (1 μ g/ml)-induced VEGF synthesis in the range between 0.3 and 30 μ M (Fig. 6A). PD169316, another specific inhibitor of p38 MAP kinase which is structurally different from SB203580 (Kummer *et al.* 1997), also reduced the VEGF synthesis stimulated by cholera toxin in a dose-dependent manner in the range between 0.3 and 30 μ M (Fig. 6B). In addition, we

Figure 5 Effect of H-89 on PGE₁-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of H-89 for 60 min, and then stimulated by 10 μ M PGE₁ (hatched bars) or vehicle (open bars) for 24 h. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. * $P < 0.05$, compared with the value of PGE₁ alone.

Table 1 Effects of SQ22536 or AH-6809 on the PGE₁-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 200 μ M SQ22536, 1 μ M AH-6809 or vehicle for 60 min, and then stimulated by 10 μ M PGE₁ for 24 h. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

	VEGF (pg/ml)
Control	12 $\pm$ 2
PGE ₁	264 $\pm$ 23
SQ22536	15 $\pm$ 3
SQ22536 + PGE ₁	111 $\pm$ 22*
AH-6809	16 $\pm$ 3
AH-6809 + PGE ₁	252 $\pm$ 16

* $P < 0.05$, compared with the value of PGE₁ alone.

examined the effects of SB203580 or PD169316 on the VEGF synthesis by forskolin, a direct activator of adenylate cyclase (Seamon & Daly 1981), in these cells. We found that forskolin (50 μ M) markedly induced VEGF synthesis. SB203580 or PD169316 inhibited the VEGF synthesis induced by forskolin in a dose-dependent manner in the

Figure 6 Effects of SB203580 or PD169316 on the cholera toxin-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 (A) or PD169316 (B) for 60 min, and then stimulated by 1 μg/ml cholera toxin (●) or vehicle (○) for 24 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 with the value of cholera toxin alone.

Figure 7 Effects of SB203580 or PD169316 on the forskolin-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 (A) or PD169316 (B) for 60 min, and then stimulated by 50 μM forskolin or vehicle for 24 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 with the value of forskolin alone.

range between 0.3 and 30 μM (Fig. 7). Furthermore, we tested the effects of SB203580 or PD169316 on the 8bromo-cAMP-induced VEGF synthesis in these cells. SB203580 or PD169316 significantly reduced the synthesis stimulated by 0.3 mM 8bromo-cAMP (Table 2).

Effects of PD98059 on the cholera toxin-, forskolin- and 8bromo-cAMP-induced VEGF synthesis in MC3T3-E1 cells

We next examined the effect of PD98059 on the cholera toxin-induced VEGF synthesis in these cells. PD98059 did not affect the VEGF induced by cholera toxin in the

Table 2 Effects of SB203580, PD169316 or PD98059 on the 8bromo-cAMP-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 30 μ M SB203580, 30 μ M PD169316, 50 μ M PD98059 or vehicle for 60 min, and then stimulated by 0.3 mM 8bromo-cAMP for 24 h. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations

	VEGF (pg/ml)
Control	12 $\pm$ 2
8bromo-cAMP	264 $\pm$ 23
SB203580	13 $\pm$ 2
B203580+8bromo-cAMP	124 $\pm$ 17*
PD169316	16 $\pm$ 3
PD169316+8bromo-cAMP	138 $\pm$ 16*
PD98059	15 $\pm$ 3
PD98059+8bromo-cAMP	282 $\pm$ 22

* $P < 0.05$, compared with the value of 8bromo-cAMP alone.

Figure 8 Effects of PD98059 on the VEGF synthesis induced by cholera toxin or forskolin in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 for 60 min, and then stimulated by 1 μ g/ml cholera toxin (●), 50 μ M forskolin (▲) or vehicle (○) and (△) for 24 h. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

range between 0.3 and 50 μ M (Fig. 8). In addition, PD98059 had little effect on the forskolin-induced VEGF synthesis (Fig. 8). The VEGF synthesis stimulated by

Figure 9 Effects of SB203580 on the phosphorylation of p38 MAP kinase induced by forskolin or 8bromo-cAMP in MC3T3-E1 cells. The cultured cells were pretreated with 50 μ M SB203580 or vehicle for 60 min, and then stimulated by 50 μ M forskolin (A), 1 mM 8bromo-cAMP (B) or vehicle for 45 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against each MAP kinase.

8bromo-cAMP, as well as that by cholera toxin or forskolin, was not affected by PD98059 (Table 2).

Effects of SB203580 on the forskolin- and 8bromo-cAMP-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells

To further clarify whether p38 MAP kinase mediates PGE₁-induced VEGF synthesis in MC3T3-E1 cells, we examined the effects of SB203580 on the phosphorylation of p38 MAP kinase induced by forskolin and 8bromo-cAMP. We found that forskolin (50 μ M) or 8bromo-cAMP (1 mM) significantly induced the p38 MAP kinase phosphorylation, and the maximum stimulatory effects of forskolin or 8bromo-cAMP on the phosphorylation of p38 MAP kinase were observed at 45 min after the stimulation (data not shown). SB203580 (50 μ M) markedly attenuated the phosphorylation of p38 MAP kinase induced by forskolin or 8bromo-cAMP (Fig. 9).

Discussion

In the present study, we have demonstrated that PGE₁ induced the phosphorylation of both p44/p42 MAP kinase

and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase (Raigneaud *et al.* 1995, Widmann *et al.* 1999). Therefore, our findings suggest that PGE₁ activates both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. As far as we know this is probably the first report showing that PGE₁ activates MAP kinases in osteoblasts.

The MAP kinase superfamily is known to play important roles in the intracellular signaling of a variety of agonists (Nishida & Gotoh 1993, Widmann *et al.* 1999). These major MAP kinases such as p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase are recognized to transduce signals in mammalian cells (Nishida & Gotoh 1993, Widmann *et al.* 1999). In osteoblast-like MC3T3-E1 cells, we previously reported that p44/p42 MAP kinase is involved in the IL-6 synthesis induced by PGF_{2α} or endothelin-1 (Kawamura *et al.* 1999, Tokuda *et al.* 1999), and that bFGF-induced IL-6 synthesis is dependent upon the activation of p38 MAP kinase (Kozawa *et al.* 1999). We have also demonstrated that IL-1-induced IL-6 synthesis is mediated by both p44/p42 MAP kinase and p38 MAP kinase in these cells (Miwa *et al.* 1999). Thus, we decided to investigate whether p38 MAP kinase or p44/p42 MAP kinase is involved in the VEGF synthesis induced by PGE₁ in MC3T3-E1 cells. We have shown here that SB203580 significantly reduced the PGE₁-stimulated VEGF synthesis, while PD98059 had little effect on the synthesis. We found that SB203580 and PD98059 actually suppressed the PGE₁-induced phosphorylation of p38 MAP kinase and p44/p42 MAP kinase respectively. It is therefore probable that the activation of p38 MAP kinase but not p44/p42 MAP kinase is involved in the PGE₁-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. It has been reported that PD98059 inhibits, but that SB203580 enhances the expression of tissue inhibitor of metalloproteinase-1 induced by a combination of all-trans retinoic acid and bFGF in human skin fibroblasts, and possibly in human osteoblasts (Bigg *et al.* 1999). It is likely that the roles of p44/p42 MAP kinase and p38 MAP kinase are quite different in each stimulus and cell species. In addition, the phosphorylation of p44 MAP kinase induced by PGE₁ shown here was weaker than that of p42 MAP kinase, suggesting that activation of p44 MAP kinase is greater than that of p42 MAP kinase in MC3T3-E1 cells. As for the activation of p44/p42 MAP kinase, also known as extracellular signal-regulated kinase (ERK) 1 and ERK2 respectively, the activation of ERK2 to several growth factors including bFGF is reportedly indicated to be more important than that of ERK1 in human osteoblastic cells and bone marrow cells (Chaudhary & Avioli 1998). On the other hand, it has been reported that ERK1 activation is greater than that of ERK2 in rat osteosarcoma ROS 17/2.8 cells, and that ERK2 is prominently activated in

MC3T3-E1 cells (Chaudhary & Avioli 1998). Our present finding does not conflict with this report. Thus, it is probable that there are interspecies differences in the activation and role of each MAP kinase. Further investigation would be required to clarify the details.

PGE₁ reportedly increases mRNA for VEGF and the synthesis through cAMP production in primary cultured rat calvarial cells and RCT-3 osteoblast-like cells (Harada *et al.* 1994). Among PGE receptor subtypes, EP₁, EP₂ and EP₄ are reportedly expressed in MC3T3-E1 cells (Suda *et al.* 1996), and EP₂ and EP₄ are known to be coupled to increase cAMP levels (Coleman *et al.* 1994). From our present results showing that neither AH-6809 nor SC-19220 affected the VEGF synthesis induced by PGE₁, it is unlikely that PGE₁-induced VEGF synthesis is mediated via EP₁ or EP₂ in MC3T3-E1 cells. The increase of adenylate cyclase activity elicits the formation of cAMP from ATP, resulting in the activation of protein kinase A. As shown here, H-89 and SQ22536 reduced the PGE₁-induced VEGF synthesis in MC3T3-E1 cells. It is most likely that the activation of adenylate cyclase and subsequent protein kinase A activation mediates the VEGF synthesis induced by PGE₁ in these cells. Furthermore, we have shown here that both SB203580 and PD169316 inhibited the cholera toxin-, forskolin- or 8bromo-cAMP-induced VEGF synthesis in MC3T3-E1 cells. It is well known that cholera toxin is a direct activator of G_s (Gilman 1987), and forskolin is a direct activator of adenylate cyclase (Seamon & Daly 1981). In addition, forskolin or 8bromo-cAMP actually elicited the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. Thus, it is probable that protein kinase A acts at a point upstream from p38 MAP kinase. We have shown here that SB203580 and PD169316 suppressed the phosphorylation of p38 MAP kinase induced by forskolin or 8bromo-cAMP. These findings therefore suggest that the inhibition by SB203580 or PD169316 of the VEGF synthesis induced by cholera toxin, forskolin or 8bromo-cAMP results from the attenuation of p38 MAP kinase activity. In the present study, SB203580 or PD169316 more weakly reduced the cholera toxin-, forskolin- or 8bromo-cAMP-induced VEGF synthesis and the forskolin- or 8bromo-cAMP-induced p38 MAP kinase phosphorylation than those induced by PGE₁. PGE₁ affects through its binding to the specific receptor, and the activation of adenylate cyclase through G_s is physiological. On the other hand, the effects of cholera toxin, forskolin and 8bromo-cAMP are irreversible and not physiological. Thus, the discrepancies in the inhibitions by SB203580 or PD169316 between the effects of PGE₁ and those of cholera toxin etc. shown here are probably due to the differences in their affecting mechanisms, namely, physiological actions or not. Based on our results, it is most likely that p38 MAP kinase is involved in PGE₁-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. On the contrary, PD98059 had little effect on cholera toxin-, forskolin- or

Figure 10 Diagram of the potential mechanism of PGE₁-induced VEGF synthesis in MC3T3-E1 cells. AC, adenylate cyclase; PKA, cAMP-dependent protein kinase.

8bromo-cAMP-induced VEGF synthesis as well as that by PGE₁ in these cells. Thus, it seems unlikely that p44/p42 MAP kinase is involved in the VEGF synthesis induced by the activation of protein kinase A. The transcriptional factors binding to cAMP response element (CRE), AP-1, nerve factor (NF)-IL6 and NF-κB, have been reported to be increased in MC3T3-E1 cells expressing the activating mutant of G_{sα} (Motomura *et al.* 1998). It has also been demonstrated that protein kinase A is the enzyme responsible for phosphorylating CRE-binding protein (Tyson *et al.* 1999), which requires c-fos expression in osteoblasts including MC3T3-E1 cells (Evans *et al.* 1996, Tyson *et al.* 1999, Fitzgerald *et al.* 2000). The pathway(s) other than p38 MAP kinase might also be involved in the VEGF synthesis induced by PGE₁ in osteoblasts. The potential mechanism of PGE₁-induced VEGF synthesis in osteoblasts shown here is summarized in Fig. 10.

PGs are known to be autocrine/paracrine modulators of osteoblasts (Nijweide *et al.* 1986, Smith 1986). As VEGF

is a specific mitogen of vascular endothelial cells (Ferrara & Davis-Smyth 1997), the synthesis by osteoblasts is recognized to be an important intercellular mediator between the osteoblasts and the vascular endothelial cells. In addition, VEGF is reportedly involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate (Gerber *et al.* 1999). The expansion of the capillary network providing microvasculature is thought to be essential for the promotion of bone remodeling. It is therefore possible that PGE₁-synthesized VEGF by osteoblasts through an autocrine/paracrine fashion promotes the migration of vascular endothelial cells in the micro-environment, resulting in the modulation of bone remodeling. Further investigation is required to clarify the details.

In conclusion, our present results strongly suggest that p38 MAP kinase takes part in the PGE₁-induced VEGF synthesis in osteoblasts but that p44/p42 MAP kinase does not.

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