p38 MAP kinase regulates BMP-4-stimulated VEGF synthesis via p70 S6 kinase in osteoblasts

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VASCULAR ENDOThelial GROWTH FACTOR (VEGF) has been characterized as a heparin-binding angiogenic factor that displays high specificity for endothelial cells (26). VEGF, which induces endothelial cell proliferation, angiogenesis, and capillary permeability, is produced and secreted from many cell types (26). It is well known that bone metabolism is regulated by osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively (27). The formation of bone structures and bone remodeling result from the coupling process, bone resorption by osteoclasts, and subsequent deposition of new matrix by osteoblasts. During bone remodeling, capillary endothelial cells provide the microvasculature. It is currently recognized that the activity of osteoblasts, osteoclasts, and vascular endothelial cells are closely coordinated with one another to promote the bone-remodeling process (4). As for osteoblasts, it has been reported that prostaglandin (PG)E2, PGE1, and insulin-like growth factor I stimulate the synthesis of VEGF in osteoblasts (8, 9). Accumulating evidence suggests that VEGF secreted from osteoblasts plays important roles in bone remodeling. It has been reported that VEGF is an essential coordinator of extracellular matrix remodeling, angiogenesis, and bone formation in the growth plate (7). However, the exact mechanism underlying VEGF production in osteoblasts has not yet been precisely clarified.

Bone-morphogenetic proteins (BMPs) are multifunctional cytokines that were originally identified by their ability to form ectopic bone (14, 29). BMPs belong to the transforming growth factor-β (TGF-β) superfamily (14, 29). BMPs are recognized as crucial regulatory factors in the early development of vertebrates (10). Osteoblasts reportedly synthesize BMP-2 and BMP-4, which stimulate alkaline phosphatase activity and the expression of osteocalcin, markers of mature osteoblast phenotype (2, 35, 36). The intracellular signaling of BMPs is mediated by Smad proteins such as Smad 1 and Smad 5, similar to TGF-β (12, 14, 24). In addition to the Smad-signaling pathway, other signaling pathways have recently been shown to mediate TGF-β superfamily signaling (13). We have previously reported (16) that...
BMP-4 stimulates the synthesis of VEGF in osteoblast-like MC3T3-E1 cells and that p70 S6 kinase is involved in the synthesis. Additionally, it has recently been shown that BMP-2 stimulates the phosphatidylinositol (PI) 3-kinase/p70 S6 kinase and p38 mitogen-activated protein (MAP) kinase cascades, which have a negative role in osteoblast differentiation (32). The MAP kinase superfamily plays a crucial role in the intracellular signaling of a variety of agonists (34). The three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and stress-activated protein kinase/c-Jun NH2-terminal kinase, are known as central elements used by mammalian cells to transduce the diverse messages (34). It has been reported that p44/p42 MAP kinase is involved in BMP-2-induced osteoblast differentiation (23). We have recently shown (15) that BMP-4-stimulated osteocalcin synthesis is regulated by p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. In the present study, we investigated the possible involvement of the MAP kinases in BMP-4-stimulated VEGF synthesis in these cells.

MATERIALS AND METHODS

Materials. BMP-4 and mouse VEGF ELISA kits were purchased from R&D Systems (Tokyo, Japan). PD-98059, U-0126, SB-203580, PD-169316, or SB-202474 for 60 min as previously described (15). The cells were pretreated with PD-98059, U-0126, SB-203580, PD-169316, or SB-202474 for 60 min as previously described (15). The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF ELISA kit.

Western blot analysis. The cultured cells were stimulated by BMP-4 or anisomycin in α-MEM for the indicated periods. The cells were washed twice with phosphate-buffered saline (PBS) 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 the supernatant after centrifugation at 125,000 g for 10 min at 4°C. The soluble fraction containing 20 μg of protein was loaded, and SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (20) in 10% polyacrylamide gel. Western blot analysis was performed as previously described (25) by using phosphospecific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phosphospecific p70 S6 kinase antibodies, p70 S6 kinase antibodies, phosphospecific PDK-1 antibodies, PDK-1 antibodies, phosphospecific Akt antibodies, or phosphospecific mTOR antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Protein determination. Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with BSA as the standard protein. The absorbance of ELISA samples was measured at 450 nm with SLT-Lab instruments EAR 340 AT. Absorbance was correlated with concentration through a standard curve. The concentration of VEGF obtained (pg/ml) was adjusted against cell number at the end of incubation and presented as VEGF synthesis (pg/1 × 106 cells). A densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories).

Statistical analysis. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and P < 0.05 was considered significant. All data are presented as means ± SD of triplicate determinations. Each experiment was repeated three times with similar results.

RESULTS

Effects of PD-98059 or U-0126 on BMP-4-induced VEGF synthesis in MC3T3-E1 cells. We previously reported (15) that BMP-4 activates p44/p42 MAP kinase, resulting in negatively regulating the BMP-4-induced osteocalcin synthesis in MC3T3-E1 cells. To clarify whether p44/p42 MAP kinase is involved in the BMP-4-stimulated VEGF synthesis in these cells, we examined the effect of PD-98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (5), on the synthesis of VEGF. We have previously demonstrated (15) that PD-98059 (50 μM) significantly reduces the phosphorylation of p44/p42 MAP kinase induced by BMP-4 in MC3T3-E1 cells. In this study, PD-98059 at 30 μM markedly reduced the BMP-4-induced phosphorylation of p44/p42 MAP kinase (Fig. 1A). However, PD-98059 had little effect on BMP-4-induced VEGF synthesis in the range between 1 and 30 μM (Fig. 1B). We further examined the effect of U-0126, another specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (5), on the VEGF synthesis induced by BMP-4. U-0126 (3 μM) definitely reduced the BMP-4-induced phosphorylation
of p44/p42 MAP kinase (Fig. 2A). However, U-0126 (3 μM), which by itself hardly affected VEGF synthesis, had little effect on the BMP-4-induced VEGF synthesis in these cells (Fig. 2B). We confirmed that the cell number changed little before and after these treatments (data not shown).

Effects of SB-203580 or PD-169316 on BMP-4-induced VEGF synthesis in MC3T3-E1 cells. We have shown (15) that BMP-4-activated p38 MAP kinase positively regulates the BMP-4-induced osteocalcin synthesis in MC3T3-E1 cells. To investigate the involvement of p38 MAP kinase in the synthesis of VEGF stimulated by BMP-4 in these cells, we next examined the effect of SB-203580, an inhibitor of p38 MAP kinase (3) on VEGF synthesis. SB-203580, which alone had little effect on the level of VEGF, significantly inhibited the VEGF synthesis stimulated by BMP-4 (Fig. 3A). The inhibitory effect of SB-203580 was dose dependent in the range between 1 and 30 μM. We confirmed that the cell number changed little by the treatment (8.1 ± 0.2 × 10⁵ cells before incubation; 8.0 ± 0.3 × 10⁵ cells after incubation with 30 μM SB-203580 and 30 ng/ml BMP-4; 7.9 ± 0.3 × 10⁵ cells after incubation with 30 μM SB-203580 alone). In addition, PD-169316, another inhibitor of p38 MAP kinase (18), which by itself had little effect on VEGF level, markedly reduced the BMP-4-stimulated VEGF synthesis (Fig. 3B). The inhibitory effect of PD-169316 was dose dependent between 0.1 and 30 μM. The cell number was not affected by the treatment (data not shown).

Effect of SB-202474 on BMP-4-induced VEGF synthesis in MC3T3-E1 cells. To clarify the involvement of p38 MAP kinase in the BMP-4-induced VEGF synthesis in MC3T3-E1 cells, we further examined the effect of SB-202474, a negative control for p38 MAP kinase inhibitor (22), on VEGF synthesis. SB-202474 (10 μM) failed to affect the VEGF synthesis induced by BMP-4, whereas SB-203580 (10 μM) significantly inhibited VEGF synthesis (Fig. 4). The cell number was not affected by the treatment (data not shown).

Effects of rapamycin or LY-294002 on BMP-4-induced p38 MAP kinase phosphorylation in MC3T3-E1 cells. In a previous study (16), we reported that p70 S6 kinase and PI 3-kinase are involved in BMP-4-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. To clarify whether p70 S6 kinase affects the BMP-4-stimulated p38 MAP kinase activation in these cells, we examined the effect of rapa-

![Fig. 1. Effects of PD-98059 on bone-morphogenetic protein (BMP)-
4-induced p44/p42 MAP kinase phosphorylation and vascular endo-
thelial growth factor (VEGF) synthesis in MC3T3-E1 cells. A: cul-
tured cells were pretreated with 30 μM PD-98059 or vehicle for 60 
min and then stimulated by 30 ng/ml BMP-4 or vehicle for 90 min. 
Extracts of cells were subjected to SDS-PAGE against phosphospe-
cific p44/p42 MAP kinase or p44/p42 MAP kinase antibodies. Histo-
gram shows quantitative representations of BMP-4-induced phos-
phorylation of p44/p42 MAP kinase obtained from laser densitomet-
ric analysis after normalization to the control of 3 different sample 
sets. B: cultured cells were pretreated with various doses of PD-
98059 for 60 min and then stimulated by 30 ng/ml BMP-4 (●) or 
vehicle (○) for 48 h. PD-98059 was present throughout the incuba-
tion. Each value represents the mean ± SD of triplicate determina-
tions. Similar results were obtained with 2 additional and different 
cell preparations. *P < 0.05 compared with the value of BMP-4 
alone.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00131.2001)
mycin, a specific inhibitor of p70 S6 kinase (19, 28), on the phosphorylation of p38 MAP kinase induced by BMP-4. We previously demonstrated (16) that 30 ng/ml rapamycin significantly reduced the phosphorylation of p70 S6 kinase in these cells. However, rapamycin did not affect the BMP-4-induced p38 MAP kinase phosphorylation (Fig. 5). We next examined the effect of LY-294002, an inhibitor of PI 3-kinase (33), on the phosphorylation of p38 MAP kinase induced by BMP-4 in these cells. LY-294002 (10–50 μM) failed to suppress the BMP-4-induced phosphorylation of p38 MAP kinase (data not shown).

Effect of SB-203580 on BMP-4-induced p70 S6 kinase phosphorylation in MC3T3-E1 cells. To investigate whether p38 MAP kinase affects the phosphorylation of p70 S6 kinase stimulated by BMP-4 in MC3T3-E1 cells, we examined the effect of SB-203580 on p70 S6 kinase phosphorylation. SB-203580 markedly reduced the BMP-4-induced p70 S6 kinase phosphorylation (Fig. 6). According to the densitometric analysis, SB-203580 caused an almost complete reduction in the BMP-4 effect.

Effect of anisomycin on phosphorylation of p70 S6 kinase in MC3T3-E1 cells. To clarify the relationship between p38 MAP kinase and p70 S6 kinase in MC3T3-E1 cells, we examined the effect of anisomycin, an activator of p38 MAP kinase (11), on the phosphorylation of p70 S6 kinase. We confirmed that anisomycin phosphorylates p38 MAP kinase in these cells (Fig. 7). The maximum effect on the p70 S6 kinase phosphorylation was observed at 60 min after the stimulation.

Effect of SB-203580 on anisomycin-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. To elucidate the relationship between p38 MAP kinase and p70 S6 kinase in MC3T3-E1 cells, we further examined the effect of SB-203580 on the anisomycin-induced phosphorylation of p70 S6 kinase. SB-203580 significantly reduced the anisomycin-stimulated phosphorylation of p70 S6 kinase (Fig. 8).

Effects of BMP-4 or anisomycin on phosphorylation of PDK-1 in MC3T3-E1 cells. It is generally recognized that PDK-1 and mTOR are involved in the activation of p70 S6 kinase (31). We examined the effects of anisomycin or BMP-4 on the phosphorylation of PDK-1 in MC3T3-E1 cells. Anisomycin (0.1 μM) induced the phosphorylation of PDK-1 in parallel with that of p38 MAP kinase (Fig. 9). However,
BMP-4 (30 ng/ml) had little effect on the phosphorylation of PDK-1 (Fig. 9). Furthermore, anisomycin affected neither mTOR nor Akt in these cells (data not shown).

**DISCUSSION**

In a previous study (16), we showed that BMP-4 stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells. We recently reported (15) that p44/p42 MAP kinase acts as a negative regulator in the BMP-4-stimulated synthesis of osteocalcin, whereas p38 MAP kinase takes part in the osteocalcin synthesis as a positive regulator in these cells. In the present study, we investigated whether there is a causal relationship between these MAP kinases and the BMP-4-stimulated VEGF synthesis in MC3T3-E1 cells. PD-98059, a specific inhibitor of mitogen-activated and extracellular signal-regulated kinase (1), failed to affect the BMP-4-induced VEGF synthesis. Therefore, it seems unlikely that p44/p42 MAP kinase is involved in the synthesis of VEGF stimulated by BMP-4 in osteoblast-like MC3T3-E1 cells. Next, we showed herein that SB-203580, an inhibitor of p38 MAP kinase (3), suppressed the BMP-4-induced VEGF synthesis. Because SB-203580 reportedly blocks PDK activity and Akt activation in addition to p38 MAP kinase (18), on VEGF synthesis. We found that PD-169316 reduced the synthesis of VEGF induced by BMP-4. Thus our findings suggest
that p38 MAP kinase is involved in the BMP-4-stimulated VEGF synthesis in MC3T3-E1 cells. Furthermore, SB-202474, a negative control of p38 MAP kinase inhibitor (22), had little effect on VEGF synthesis. Taking these results into account, it is most likely that p38 MAP kinase takes part in BMP-4-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells.

We have reported (16) that p70 S6 kinase participates in BMP-4-stimulated VEGF synthesis as a positive regulator in osteoblast-like MC3T3-E1 cells. We have demonstrated (19, 28) that rapamycin did not affect the BMP-4-induced p38 MAP kinase phosphorylation. Thus it seems unlikely that p70 S6 kinase functions at a point upstream from p38 MAP kinase. On the other hand, SB-203580 inhibited the BMP-4-stimulated phosphorylation of p70 S6 kinase. Therefore, our findings suggest that p38 MAP kinase affects the p70 S6 kinase phosphorylation induced by BMP-4 in MC3T3-E1 cells. It has been shown that BMP-2 stimulates the PI 3-kinase/p70 S6 kinase and p38 MAP kinase cascades in C2C12 cells (32). We have reported (16) that wortmannin or LY-294002, inhibitors of PI 3-kinase, significantly reduced BMP-4-induced VEGF synthesis in MC3T3-E1 cells, suggesting that PI 3-kinase is involved in BMP-4-induced VEGF synthesis. However, LY-294002 failed...
to suppress the phosphorylation of p38 MAP kinase induced by BMP-4. Next, we compared the time course of phosphorylation of p38 MAP kinase with that of phosphorylation of p70 S6 kinase. The time course of the phosphorylation of p38 MAP kinase stimulated by anisomycin, an activator of p38 MAP kinase (11), was faster than that of p70 S6 kinase in osteoblast-like MC3T3-E1 cells. We previously reported (15) that BMP-4 phosphorylates p38 MAP kinase and that the maximum effect of BMP-4 is observed at 90 min after the stimulation. On the other hand, p70 S6 kinase phosphorylation induced by BMP-4 is observed at these cells (16). Thus these results suggest that p38 MAP kinase acts at a point upstream from p70 S6 kinase in BMP-4-stimulated VEGF synthesis without involvement of PDK-1, Akt, or mTOR in osteoblast-like MC3T3-E1 cells.

VEGF is well known to be an angiogenic growth factor displaying high specificity for endothelial cells and promoting angiogenesis, providing the microvasculature (26) that is essential for bone remodeling. In our recent study (15), we reported that p38 MAP kinase is a positive regulator of the BMP-4-induced synthesis of osteocalcin, an osteoblast-specific phenotype marker. It is generally recognized that osteocalcin normally functions to limit, without impairing bone resorption and mineralization. With these findings taken into account, it is probable that BMP-4-activated p38 MAP kinase regulates bone remodeling via VEGF and osteocalcin synthesis. On the other hand, BMP-2-mediated p38 MAP kinase reportedly acts as a negative regulator of osteoblast differentiation in C2C12 cells (32). C2C12 cells are murine myoblasts, whereas MC3T3-E1 cells are murine preosteoblastic cells. Therefore, the discrepancy in the role of p38 MAP kinase between C2C12 cells and osteoblasts-like MC3T3-E1 cells might be due to the difference of cell differentiation stage.

In conclusion, our present findings strongly suggest that p38 MAP kinase regulates BMP-4-stimulated VEGF synthesis via p70 S6 kinase in osteoblasts.
REFERENCES


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