Involvement of SAPK/JNK in basic fibroblast growth factor-induced vascular endothelial growth factor release in osteoblasts

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Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates p44/p42 mitogen-activated protein (MAP) kinase resulting in the stimulation of vascular endothelial growth factor (VEGF) release in osteoblast-like MC3T3-E1 cells and that FGF-2-activated p38 MAP kinase negatively regulates VEGF release. In the present study, we investigated the involvement of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in FGF-2-induced VEGF release in these cells. FGF-2 markedly induced the phosphorylation of SAPK/JNK. SP600125, an inhibitor of SAPK/JNK, markedly reduced the FGF-2-induced VEGF release. SP600125 suppressed

the FGF-2-induced phosphorylation of SAPK/JNK without affecting the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase induced by FGF-2. PD98059, an inhibitor of upstream kinase of p44/p42 MAP kinase, or SB203580, an inhibitor of p38 MAP kinase, failed to affect the FGF-2-induced phosphorylation of SAPK/JNK. A combination of SP600125 and SB203580 suppressed the FGF-2-stimulated VEGF release in an additive manner. These results strongly suggest that FGF-2 activates SAPK/JNK in osteoblasts, and that SAPK/JNK plays a part in FGF-2-induced VEGF release.

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Introduction

Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara & Davis-Smyth 1997). It is well recognized that VEGF, which is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells (Ferrara & 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 the hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate (Gerber *et al.* 1999). Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors (Goad *et al.* 1996, Wang *et al.* 1996, Ferrara & Davis-Smyth 1997, Schallaeppi *et al.* 1997). Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption (Nijweide *et al.* 1986). During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption

lacuna. It is currently recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism (Erlebacher *et al.* 1995). Thus, there is no doubt that VEGF secreted from osteoblasts plays an important role in the regulation of bone metabolism. However, the mechanism behind VEGF synthesis in osteoblasts has not yet been fully clarified.

Basic fibroblast growth factor (FGF-2) is found 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). Thus, it is recognized that FGF-2 plays a crucial role in fracture healing and bone remodeling. There are four structurally related high affinity receptors (FGF receptors 1–4) that possess an intrinsic protein tyrosine kinase activity and induce tyrosine autophosphorylation of the receptors (Johnson & Williams 1993). We have shown that FGF receptors 1 and 2 are autophosphorylated by FGF-2 in osteoblast-like MC3T3-E1 cells (Suzuki *et al.* 1996). In addition, we have reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that p44/p42 mitogen-activated protein (MAP) kinase participates, at least partly, in VEGF release while p38 MAP kinase limits VEGF

Figure 1 Effect of FGF-2 on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated with 30 ng/ml FGF-2 (A) or 2 ng/ml FGF-2 (B) for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK (phospho-SAPK/JNK) or SAPK/JNK. The upper and lower bands indicate p54 and p46 SAPK/JNK respectively. Lane 1, control cells.

release (Tokuda *et al.* 2000). It has recently been reported that stress-activated protein kinase/*c-Jun* N-terminal kinase (SAPK/JNK), which belongs to the MAP kinase superfamily (Widmann *et al.* 1999), is activated by FGF-2 in mouse myocytes and breast cancer cells (Liu *et al.* 1999, Sheikh *et al.* 2001), leading us to speculate involvement of SAPK/JNK in FGF-2-induced VEGF release in osteoblasts. However, the involvement of JNK in FGF-2 signaling in osteoblasts has not yet been clarified.

In the present study, we investigated the involvement of SAPK/JNK in the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. Here we show that FGF-2 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in these cells, and that SAPK/JNK plays a role as a positive regulator in FGF-2-induced VEGF release.

Materials and Methods

Materials

FGF-2 and mouse VEGF enzyme immunoassay kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). SP600125, SB203580 and PD98059 were obtained from Calbiochem Novabiochem Co. (La Jolla, CA, USA). Phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific

Figure 2 Effect of SP600125 on the FGF-2-induced VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with 10 μ M SP600125 (closed symbols) or vehicle (open symbols) for 60 min, and then stimulated with 30 ng/ml FGF-2 (circles) or vehicle (triangles) for the indicated periods. 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 FGF-2 alone.

p38 MAP kinase antibodies and p38 MAP kinase antibodies were purchased from New England BioLabs, Inc. (Beverly, MA, USA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SP600125, SB203580 or PD98059 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of 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 calf serum (FCS) at 37 $^{\circ}$ 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% FCS.

Figure 3 Dose-dependent effect of SP600125 on the FGF-2-induced VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated with 30 ng/ml FGF-2 (solid circles) or vehicle (open circles) 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 FGF-2 alone.

After 5 days, the medium was exchanged for α -MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

VEGF assay

The cultured cells were stimulated with FGF-2 in 1 ml α -MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SB203580 or SP600125 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 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

Figure 4 Effect of SP600125 on the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 10 μ M SP600125 or vehicle for 60 min, and then stimulated with 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 phospho-specific SAPK/JNK (phospho-SAPK/JNK) or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. * $P < 0.05$ compared with the value of FGF-2 alone.

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 the method of Laemmli (1970) in a 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato *et al.* 1996) by using phospho-specific SAPK/JNK antibodies, SAPK/JNK 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 pretreated with SP600125 or SB203580 for 60 min.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with an EL 340 Bio Kinetic Reader

Figure 5 Effect of SP600125 on the FGF-2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 10 μ M SP600125 or vehicle for 60 min, and then stimulated with 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 phospho-specific p44/p42 MAP kinase (phospho-p44/p42) or p44/p42 MAP kinase (p44/p42). The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

Figure 6 Effect of SP600125 on the FGF-2-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 10 μ M SP600125 or vehicle for 60 min, and then stimulated with 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 p38 MAP kinase (phospho-p38) or p38 MAP kinase (p38). The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

(Bio-Tek Instruments, Inc., Winooski, VT, USA). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a value of $P<0.05$ 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

Effect of FGF-2 on the phosphorylation of SAPK/JNK in MC3T3-E1 cells

To clarify whether FGF-2 activates SAPK/JNK in osteoblast-like MC3T3-E1 cells, we examined the effect

of FGF-2 on the SAPK/JNK phosphorylation. FGF-2 (30 ng/ml) markedly induced phosphorylation of SAPK/JNK in a time-dependent manner up to 60 min (Fig. 1A). The FGF-2-induced phosphorylation of SAPK/JNK was also observed with 2 ng/ml, a more physiological concentration of FGF-2 (Esch *et al.* 1985) (Fig. 1B).

Effect of SP600125 on the FGF-2-induced VEGF release in MC3T3-E1 cells

We have previously shown that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells (Tokuda *et al.* 2000). To investigate whether SAPK/JNK is involved in the FGF-2-induced VEGF release in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of JNK, on the release (Bennett *et al.* 2001). SP600125, which alone hardly affected the basal level of VEGF, significantly reduced the FGF-2-induced VEGF release (Fig. 2). The inhibitory effect of SP600125 on the FGF-2-induced VEGF release was dose-dependent in the range between 1 and 10 μ M (Fig. 3). The maximum

Figure 7 Effect of PD98059 on the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 50 μ M PD98059 or vehicle for 60 min, and then stimulated with 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 phospho-specific SAPK/JNK (phospho-SAPK/JNK) or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

inhibitory effect of SP600125 was observed at 10 μ M, which caused about 70% reduction in the FGF-2 effect.

Effects of SP600125 on the phosphorylation of SAPK/JNK, p44/p42 MAP kinase and p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells

We found that SP600125 truly inhibited the phosphorylation of SAPK/JNK induced by FGF-2 (Fig. 4). According to the densitometric analysis, SP600125 caused about 50% reduction of the FGF-2 effect on the SAPK/JNK phosphorylation. On the other hand, SP600125 had little effect on the FGF-2-induced phosphorylation of p44/p42 MAP kinase (Fig. 5). In addition, p38 MAP kinase was not affected by SP600125 (Fig. 6).

Effect of PD98059 or SB203580 on the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells

PD98059, an inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi *et al.* 1995), failed to affect the FGF-2-induced phosphorylation of SAPK/JNK (Fig. 7). Additionally, SB203580, an inhibitor of p38 MAP

Figure 8 Effect of SB203580 on the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μ M SB203580 or vehicle for 60 min, and then stimulated with 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 phospho-specific SAPK/JNK (phospho-SAPK/JNK) or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean $\pm$ S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

kinase (Cuenda *et al.* 1995), had little effect on the FGF-2-induced phosphorylation of SAPK/JNK (Fig. 8).

Effect of a combination of SP600125 and PD98059 on the FGF-2-stimulated VEGF release in MC3T3-E1 cells

We further examined the effect of a combination of SP600125 and PD98059 on the FGF-2-stimulated VEGF release in these cells. A combination of SP600125 and PD98059 significantly reduced the FGF-2-stimulated VEGF release in an additive manner (Table 1).

Discussion

It is currently known that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and cell death in a variety of cells (Widmann *et al.* 1999). Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to

Table 1 Effect of a combination of SP600125 and SB203580 on the FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with 1 µM SP600125, 10 µM PD98059 or vehicle for 60 min, and then stimulated with 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

			VEGF (pg/ml)
SP600125	PD98059	FGF-2	
—	—	—	23 ± 3
—	—	+	518 ± 45
—	+	—	23 ± 3
—	+	+	326 ± 31*
+	—	—	21 ± 2
+	—	+	277 ± 26*
+	+	—	28 ± 5
+	+	+	128 ± 18**

*P<0.05 compared with the value of FGF-2 alone, **P<0.05 compared with the value of PD98059 and FGF-2.

transduce the diverse messages (Widmann *et al.* 1999). In the present study, we demonstrated that FGF-2 induces the phosphorylation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. We have previously shown that FGF-2 activates both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells (Tokuda *et al.* 2000). It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase (Raingeaud *et al.* 1995, Widmann *et al.* 1999). Therefore, these results strongly suggest that FGF-2 independently activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. Furthermore, 2 ng/ml FGF-2 in addition to 30 ng/ml FGF-2 was able to induce the phosphorylation of SAPK/JNK in these cells. It has been shown that the ED₅₀ of FGF-2 on most mesoderm-derived cell types is 60 pg/ml (Esch *et al.* 1985). Thus, it is probable that FGF-2 truly activates SAPK/JNK under physiological conditions. As far as we know this is the first report showing FGF-2-induced SAPK/JNK activation in osteoblasts.

Herein, we showed that SP600125, a specific inhibitor of SAPK/JNK (Bennett *et al.* 2001), significantly suppressed the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. In addition, we found that SP600125 truly attenuated the phosphorylation of SAPK/JNK induced by FGF-2 in these cells. We have previously reported that activation of p44/p42 MAP kinase acts as a positive regulator in the FGF-2-induced VEGF release in MC3T3-E1 cells (Tokuda *et al.* 2000). However, SP600125 did not affect the FGF-2-induced phosphorylation of p44/p42 MAP kinase. Thus, it is probable that the SP600125-induced suppression of FGF-2-stimulated VEGF release is due to the inhibition of SAPK/JNK activation. These results strongly suggest that the activation of JNK is involved in FGF-2-stimulated VEGF

synthesis in osteoblast-like MC3T3-E1 cells. In addition, we showed that PD98059, a specific inhibitor of mitogen-activated protein kinase (Alessi *et al.* 1995), had little effect on the phosphorylation of FGF-2-induced SAPK/JNK phosphorylation. Moreover, the FGF-2-stimulated VEGF release was suppressed additively by a combination of SP600125 and PD98059. Therefore, it seems that SAPK/JNK participates, at least partly, in the FGF-2-stimulated VEGF release independently of p44/p42 MAP kinase. Based on these findings, it is most likely that the VEGF release stimulated by FGF-2 is mediated through the activation of both p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells.

We have shown that the FGF-2-stimulated VEGF release is negatively regulated by FGF-2-activated p38 MAP kinase in osteoblast-like MC3T3-E1 cells (Tokuda *et al.* 2000). However, SP600125 failed to affect the FGF-2-induced phosphorylation of p38 MAP kinase. Furthermore, SB203580, a specific inhibitor of p38 MAP kinase (Cuenda *et al.* 1995), had little effect on the phosphorylation of FGF-2-induced SAPK/JNK phosphorylation. Taken together, it is probable that FGF-2-activated SAPK/JNK plays a part in VEGF release independently of both p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells.

The expansion of the capillary network providing microvasculature is an essential process of bone remodeling (Erlebacher *et al.* 1995). Since VEGF is a specific mitogen of vascular endothelial cells (Ferrara & Davis-Smyth 1997), it is probable that VEGF synthesized by osteoblasts acts as an important intercellular mediator between osteoblasts and vascular endothelial cells. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of the mouse (Gerber *et al.* 1999), supporting the importance of VEGF in bone metabolism. On the other hand, it is well recognized that FGF-2 is produced and secreted by osteoblasts, and is accumulated in the extracellular matrix of bone (Baylink *et al.* 1993, Hurley *et al.* 1993). During bone resorption, it is speculated that FGF-2 in extracellular bone matrix is released and affects osteoblasts existing in the neighborhood. Therefore, it is most likely that FGF-2-induced VEGF synthesis by osteoblasts plays a pivotal role in the process of bone remodeling through regulating the capillary endothelial cells proliferation.

In conclusion, our present results strongly suggest that FGF-2 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblasts, and that SAPK/JNK acts as a positive regulator, at least partly, in FGF-2-induced VEGF release.

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