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Involvement of p38 MAP Kinase in TGF-β-Stimulated VEGF Synthesis in Aortic Smooth Muscle Cells

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Abstract Although it is known that transforming growth factor (TGF)-β induces vascular endothelial growth factor (VEGF) synthesis in vascular smooth muscle cells, the underlying mechanisms are still poorly understood. In the present study, we examined whether the mitogen-activated protein (MAP) kinase superfamily is involved in TGF-β-stimulated VEGF synthesis in aortic smooth muscle A10 cells. TGF-β stimulated the phosphorylation of p42/p44 MAP kinase and p38 MAP kinase, but not that of SAPK (stress-activated protein kinase)/JNK (c-Jun N-terminal kinase). The VEGF synthesis induced by TGF-β was not affected by PD98059 or U0126, specific inhibitors of the upstream kinase that activates p42/p44 MAP kinase. We confirmed that PD98059 or U0126 did actually suppress the phosphorylation of p42/p44 MAP kinase by TGF-β in our preparations. PD169316 and SB203580, specific inhibitors of p38 MAP kinase, significantly reduced the TGF-β-stimulated synthesis of VEGF (each in a dose-dependent manner). PD169316 or SB203580 attenuated the TGF-β-induced phosphorylation of p38 MAP kinase. These results strongly suggest that p38 MAP kinase plays a part in the pathway by which TGF-β stimulates the synthesis of VEGF in aortic smooth muscle cells. J. Cell. Biochem. 82: 591–598, 2001. © 2001 Wiley-Liss, Inc.

Key words: MAP kinase; TGF-β; VEGF; vascular smooth muscle cells

VEGF has been characterized as a heparin-binding angiogenic growth factor, displaying high specificity for endothelial cells [Keck et al., 1989; Neufeld et al., 1999]. VEGF, which increases capillary permeability and endothelial cell proliferation [Keck et al., 1989], is produced and secreted from a variety of cell types. Within the vascular system, vascular smooth muscle cells, which play a crucial role in the pathogenesis of atherosclerosis and hypertension, are reportedly the main source of VEGF [Tischer et al., 1991; Stavri et al., 1995]. Interestingly, hypoxia and hypoglycemia are known to stimulate VEGF expression in these cells [Shweiki et al., 1992; Natarajan et al., 1997]. It has been shown that the hypoxia-induced transcription of VEGF mRNA is mediated by the binding of hypoxia-inducible factor 1 (HIF-1) to an HIF-1 binding site located in the VEGF promoter [Levy et al., 1995]. In addition, evidence is accumulating to indicate that many humoral factors—basic fibroblast growth factor, platelet-derived growth factor, tumor necrosis factor-α, adenosine, hepatocyte growth factor, endothelin, interleukin-1β, interleukin-6, and TGF-β—induce VEGF production in vascular smooth muscle cells [Brogi et al., 1994; Li et al., 1995; Stavri et al., 1995a; Cohen et al., 1996; Pedram et al., 1997; Belle et al., 1998; Pueyo et al., 1998; Lynch et al., 1999]. As for the intracellular signaling leading to VEGF synthesis in these cells, it has been shown in human umbilical vein smooth muscle cells [Pedram et al., 1997] that endothelin-1 induces VEGF synthesis through a stimulation of protein kinase C (PKC) and Raf-1, which is a substrate for PKC, leading to an activation of Erk. In addition, the enhancement of cAMP by adenosine reportedly contributes to an upregulation of VEGF mRNA expression in rat aortic smooth muscle cells [Pueyo et al., 1998]. However, the exact mechanisms behind VEGF

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synthesis in vascular smooth muscle cells remain unclear.

TGF-β is a multifunctional cytokine with effects on cellular growth, differentiation, morphogenesis, inflammatory responses, and extracellular matrix formation [Massague, 1998]. TGF-β binds directly to TGF-β type-II receptors which, in turn, phosphorylate the TGF-β type-I receptor (originally known as activin receptor-like kinase (ALK) 5), a member of the type-I TGF-β superfamily receptor with intrinsic serine/threonine kinase activity [Heldin et al., 1997]. It is well recognized that the subsequent phosphorylation of Smad2 or Smad3 by the activated type-I receptors results in a translocation of these complexes to the nucleus, where they can regulate gene transcription [Heldin et al., 1997]. Very recently, it has been reported that ALK1 mediates TGF-β-induced angiogenesis through the phosphorylation of Smad1 or Smad5 [Lux et al., 1999]. In addition, other signaling pathways (e.g., via mitogen-activated protein (MAP) kinases) have been shown to be involved in TGF-β signaling [Yamaguchi et al., 1995; Wang et al., 1997; Hannigan et al., 1998; Hanafusa et al., 1999; Hedges et al., 1999; Hocevar et al., 1999]. The MAP kinase superfamily plays an important role in transducing an extracellular signal into a cellular response [Waskiewicz and Cooper, 1995; Widmann et al., 1999], the specificity of the cellular response being determined by the activation of a particular MAP kinase pathway in response to given stimulus. Three major MAP kinases, p42/p44 MAP kinase, p38 MAP kinase, and SAPK (stress-activated protein kinase)/JNK (c-Jun N-terminal kinase) are generally recognized as being the central elements used by mammalian cells to transduce such diverse messages [Waskiewicz and Cooper, 1995; Widmann et al., 1999]. In vascular smooth muscle cells, MAP kinases have been implicated in the signaling pathways, mediating cell proliferation and migration [Marrero et al., 1997; HedgeHs et al., 1999], and it has recently been demonstrated that p42/p44 MAP kinase activation is one of the requirements for the TGF-β stimulation of fibronectin production [Kaiura et al., 1999]. However, the exact roles, played by the MAP kinase superfamily in TGF-β-signaling in these cells, have not yet been fully clarified.

In the present study, we investigated the involvement of the MAP kinase signaling pathway in the TGF-β-stimulation of VEGF synthesis in an aortic smooth muscle cell line, A10 cells. Our results demonstrate an involvement of p38 MAP kinase, but not of p42/p44 MAP kinase or SAPK/JNK.

MATERIALS AND METHODS

Materials

Mouse VEGF enzyme immunoassay (EIA) kits and TGF-β were purchased from R & D Systems (Tokyo, Japan). PD98059, PD169316, and, SB203580 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). U0126 was purchased from Promega Co. (Madison, WI). Antibodies against the following kinases, phospho-specific p42/p44 MAP kinase (rabbit polyclonal IgG, affinity purified), p42/p44 MAP kinase (rabbit polyclonal IgG, affinity purified), phospho-specific p38 MAP kinase (rabbit polyclonal IgG, affinity purified), p38 MAP kinase (rabbit polyclonal IgG, affinity purified), phospho-specific SAPK/JNK (rabbit polyclonal IgG, affinity purified), and SAPK/JNK (rabbit polyclonal IgG, affinity purified), were purchased from New England BioLabs, 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. PD98059, U0126, PD169316, and SB203580 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the measurements made in the VEGF immunoassay or the Western blot analysis.

Cell Culture

An aortic smooth muscle cell line, A10 cells, derived originally from the fetal rat aorta [Kimes and Brandt, 1976], was obtained from the American Type Culture Collection (Rockville, MD). The cells were seeded into 35-mm (1×10^5) or 90-mm (5×10^5) diameter dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. After 5 days, the medium was exchanged for serum-free DMEM. The cells were used for experiments 48 h later.

Assay for VEGF Synthesis

Cultured cells were stimulated by TGF-β in 1 ml of DMEM for the periods indicated in the

text and figures. The conditioned medium was collected, and the VEGF in the medium was measured using a VEGF EIA kit that recognizes rat VEGF (according to the manufacturer), as previously described [Seko et al., 1999]. As indicated, the cells were pretreated with PD98059, U0126, SB203580, or PD169316 for 60 min prior to stimulation with TGF-β. The absorbance of EIA samples was measured at 450 nm using an SLT-Labinstruments EAR 340AT. Absorbance was correlated with concentration by means of a standard curve.

The data were analyzed by a one-way ANOVA, followed by the Bonferroni correction for multiple comparisons between pairs. $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations from three independent experiments.

Western Blot Analysis of p42/p44 MAP Kinase, p38 MAP Kinase, and SAPK/JNK

Cultured cells were stimulated by TGF-β in DMEM for the periods indicated in the text and figures. The stimulated cells were rinsed twice with phosphate-buffered saline, then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mmol/l Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mmol/l dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000*g* for 10 min at 4°C. The supernatant was used for the analysis of each MAP kinase by Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [Laemmli, 1970] in 10% polyacrylamide gel. Western blotting analysis was, then, performed as previously described [Kozawa et al., 1999] using the antibodies listed above under "Materials", 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.

RESULTS

Effect of TGF-β on VEGF Synthesis

It has been reported that TGF-β induces VEGF synthesis in human vascular smooth muscle cells [Brogi et al., 1994]. We found that TGF-β stimulated VEGF synthesis time-dependently (up to 84 h) in A10 cells (Fig. 1A). The VEGF synthesis was significant for 12 h or more, after the TGF-β stimulation. The stimulatory effect of TGF-β on VEGF synthesis was dose-dependent over the range 0.1 to 50 ng/ml (Fig. 1B), the maximum effect being observed at 50 ng/ml.

Effects of TGF-β on the Phosphorylation of p42/p44 MAP Kinase, p38 MAP Kinase, and SAPK/JNK

To investigate the involvement of the MAP kinase superfamily in TGF-β-stimulated VEGF synthesis in A10 cells, we first examined the effects of TGF-β on the phosphorylation of p42/p44 MAP kinase, p38 MAP kinase, and SAPK/JNK. TGF-β induced a significant phosphorylation of both p42/p44 MAP kinase and p38 MAP kinase (Fig. 2), although the time courses of these two effects appeared to be quite different from each other. The phosphorylation of p42/p44 MAP kinase by TGF-β reached its peak at 20 min after the stimulation and then declined. On the other hand, the TGF-β-induced phosphorylation of p38 MAP kinase reached its peak at 45 min. In contrast, TGF-β had little effect on the phosphorylation of SAPK/JNK in A10 cells (Fig. 2).

Effects of PD98059 and U0126 on the TGF-β-Stimulated Synthesis of VEGF

The TGF-β-stimulated phosphorylation of p42/p44 MAP kinase was significantly reduced by PD98059, a specific inhibitor of the upstream kinase that activates p42/p44 MAP kinase [Dudley et al., 1995] (Fig. 3). However, PD98059 did not affect the TGF-β-stimulated synthesis of VEGF over the range 0.3 to 50 μmol/l (Fig. 4A). U0126, another inhibitor of the upstream kinase that activates p42/p44 MAP kinase [Favata et al., 1998], also had no effect on the TGF-β-stimulated synthesis of VEGF (over the range 0.1 to 30 μmol/l)(Fig. 4B).

Effects of PD169316 and SB203580 on the TGF-β-Stimulated Synthesis of VEGF

The TGF-β-stimulated synthesis of VEGF was significantly reduced by PD169316, a specific inhibitor of p38 MAP kinase [Kummer et al., 1997] (Fig. 5A). This inhibitory effect was dose-dependent over the range 0.1 to 30 μmol/l, the maximum reduction (about 70%) being observed at 10 μmol/l. SB203580, another inhibitor of p38 MAP kinase [Cuenda et al.,

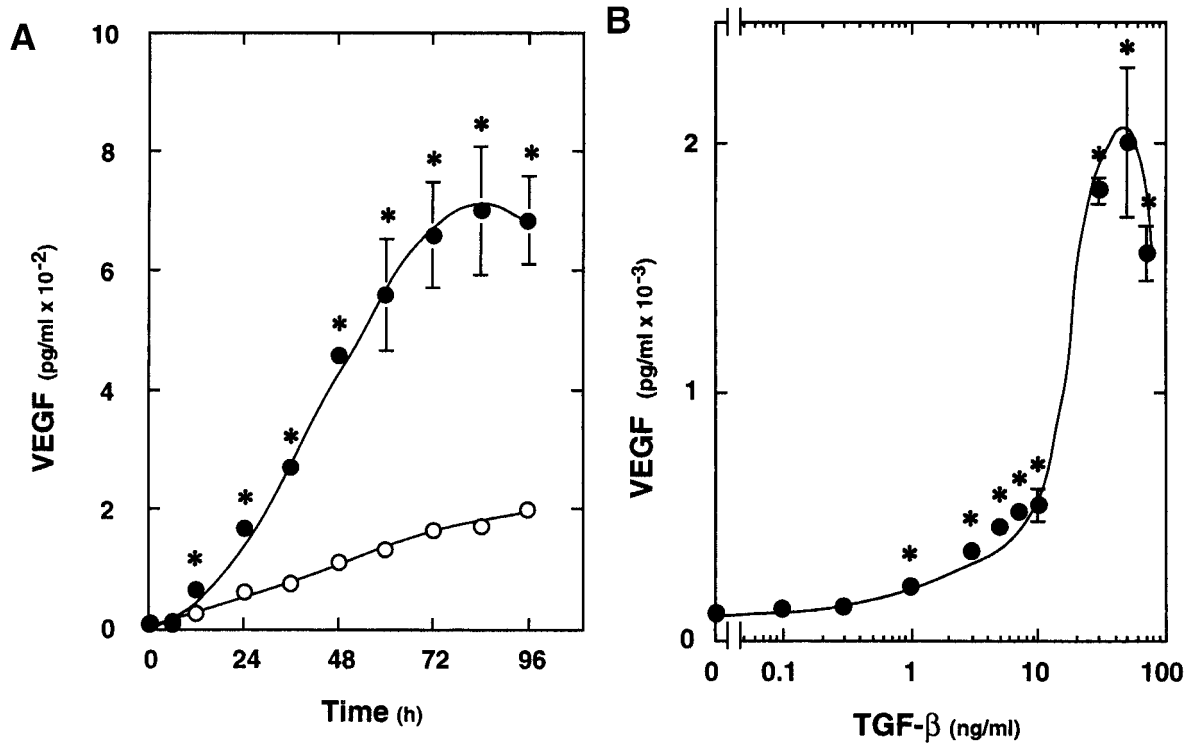

Fig. 1. **A:** Effect of TGF-β on VEGF synthesis in A10 cells. Cultured cells were exposed to 5 ng/ml of TGF-β (●) or vehicle (○) for the indicated periods. **B:** Dose-dependent effect of TGF-β on VEGF synthesis in A10 cells. Cultured cells were stimulated by various doses of TGF-β for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations. $*P < 0.05$ compared with control value.

1995], had little effect on the level of VEGF when given alone, but it too reduced the TGF-β-stimulated synthesis of VEGF, the effect being dose-dependent over the range 0.1 to 30 μmol/l (Fig. 5B). We confirmed that the TGF-β-stimulated phosphorylation of p38 MAP kinase was significantly reduced by PD169316 (Fig. 6). We found that U0126 had little effect

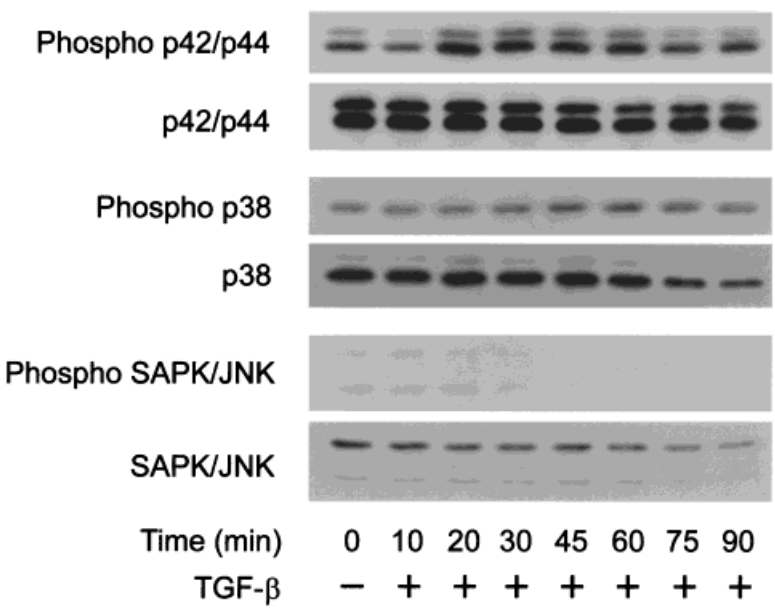

Fig. 2. Effects of TGF-β on the phosphorylation of p42/p44 MAP kinase, p38 MAP kinase, and SAPK/JNK in A10 cells. Cultured cells were stimulated by 5 ng/ml of TGF-β for the indicated periods. Cell lysates were subjected to SDS-PAGE followed by Western blot analysis using antibodies against phospho-specific p42/p44 MAP kinase, p42/p44 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, or SAPK/JNK.

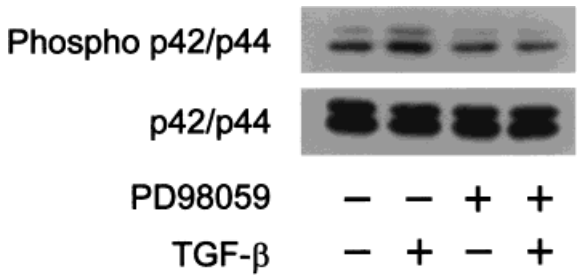

Fig. 3. Effect of PD98059 on TGF-β-induced p42/p44 MAP kinase phosphorylation in A10 cells. Cultured cells were pretreated with 10 μM of PD98059 for 60 min, then stimulated by 5 ng/ml of TGF-β for 20 min. Cell lysates were subjected to SDS-PAGE followed by Western blot analysis using antibodies against phospho-specific p42/p44 MAP kinase or p42/p44 MAP kinase.

on the TGF-β-stimulated phosphorylation of p38 MAP kinase.

DISCUSSION

In the present study, we have demonstrated that in aortic smooth muscle A10 cells, TGF-β stimulates the phosphorylation of both p42/p44 MAP kinase and p38 MAP kinase, but has little or no effect on the phosphorylation of SAPK/JNK. It is recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [Raingeaud et al., 1995]. Thus, our findings suggest that TGF-β activates both p42/p44 MAP kinase and p38 MAP kinase, but not SAPK/JNK, in A10 cells. We next investigated whether p42/p44 MAP kinase or p38 MAP kinase is involved in the pathway by which TGF-β stimulates the synthesis of VEGF. In fact, the TGF-β-induced synthesis of VEGF was not affected by either PD98059 or U0126, specific inhibitors of the upstream kinase that activates p42/p44 MAP kinase [Dudley et al., 1995; Favata et al., 1998]. In this study, we confirmed that PD98059 did truly suppress the phosphorylation of p42/p44 MAP kinase by TGF-β. To judge from our findings, it is unlikely that activation of p42/p44 MAP kinase is involved in the TGF-β-stimulated synthesis of VEGF in A10 cells. On the other hand, PD169316 and SB203580, specific inhibitors of p38 MAP kinase [Cuenda et al., 1995; Kummer

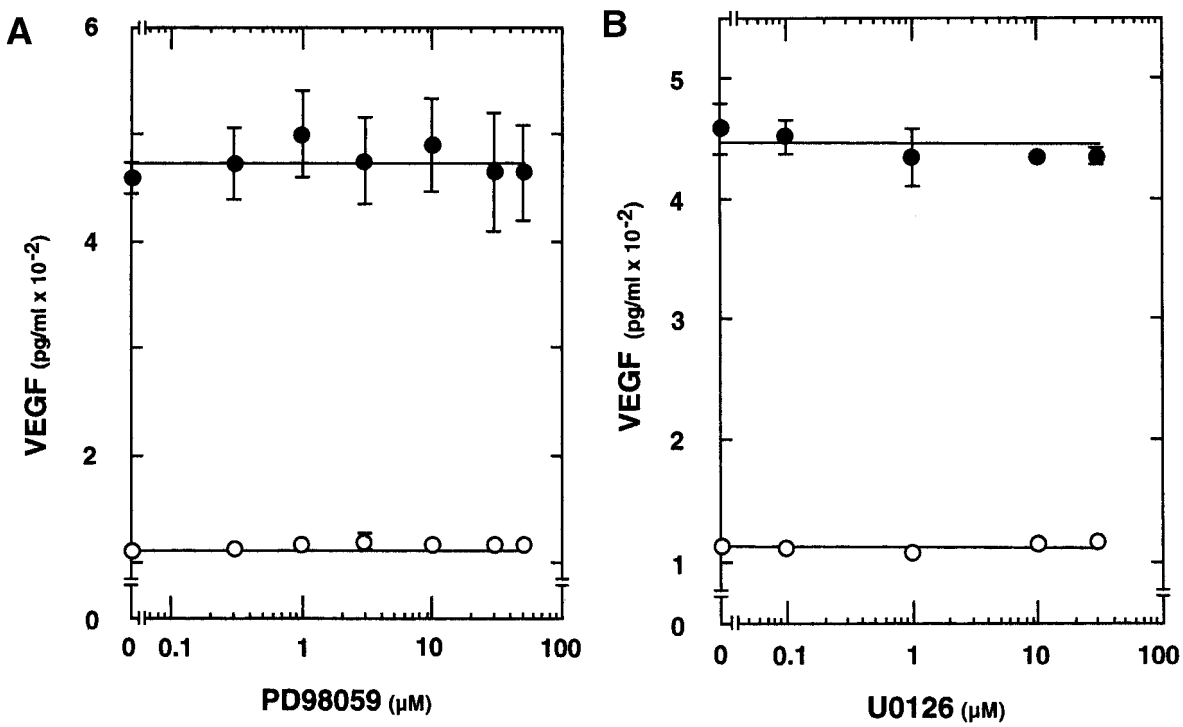

Fig. 4. **A:** Effect of PD98059 on TGF-β-induced synthesis of VEGF in A10 cells. Cultured cells were pretreated with various doses of PD98059 for 60 min, then exposed to 5 ng/ml of TGF-β (●) or vehicle (○) for 48 h. (**B**) Effect of U0126 on TGF-β-induced synthesis of VEGF in A10 cells. Cultured cells were pretreated with various doses of U0126 for 60 min, then exposed to 5 ng/ml of TGF-β (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations.

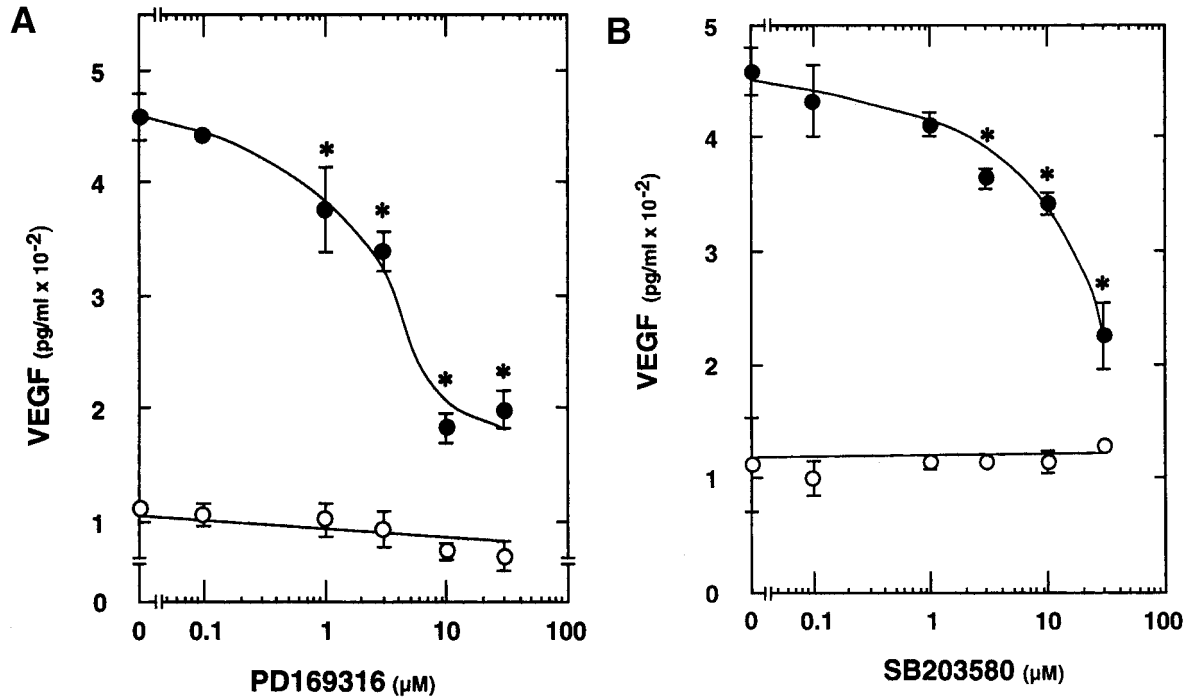

Fig. 5. **A:** Effect of PD169316 on TGF-β-induced synthesis of VEGF in A10 cells. **B:** Effect of SB203580 on TGF-β-induced synthesis of VEGF in A10 cells. Cultured cells were pretreated with various doses of PD169316, SB203580 or vehicle for 60 min, then exposed to 5 ng/ml of TGF-β (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations. *$P < 0.05$ compared with TGF-β alone.

et al., 1997], significantly reduced the TGF-β-stimulated synthesis of VEGF, suggesting that p38 MAP kinase is involved in this process in A10 cells. We confirmed that PD169316 did suppress the p38 MAP kinase phosphorylation induced by TGF-β. In addition, U0126 had little effect on the TGF-β-stimulated phosphorylation of p38 MAP kinase. Taking all our findings into account, it is most likely that activation of p38 MAP kinase is required for TGF-β-stimulated VEGF synthesis in A10 cells. Recent reports have demonstrated a link between TGF-β signal transduction and the activation of MAP kinases [Yamaguchi et al., 1995; Wang et al., 1997; Hannigan et al., 1998; Hanafusa et al., 1999; Hedges et al., 1999; Hocevar et al., 1999]. For instance, TAK-1, a member of the MAP kinase kinase kinase family, is reportedly activated by TGF-β, resulting in a stimulation of SAPK/JNK and p38 MAP kinase, in human embryonic kidney cells, mink lung epithelial cells, and osteoblast-like MC3T3-E1 cells [Yamaguchi et al., 1995; Hannigan et al., 1998]. It has also been shown that stimulation of human neutrophils with TGF-β induces a phosphorylation of p38 MAP kinase and its substrate MAPKAP (mitogen-activated protein kinase activated protein) kinase 2 [Hanafusa et al., 1999]. In addition, p38 MAP kinase activation by TGF-β has been reported to be required for cell migration in dog tracheal smooth muscle cells [Hedges et al., 1999]. To the best of our knowledge, the present study represents the first

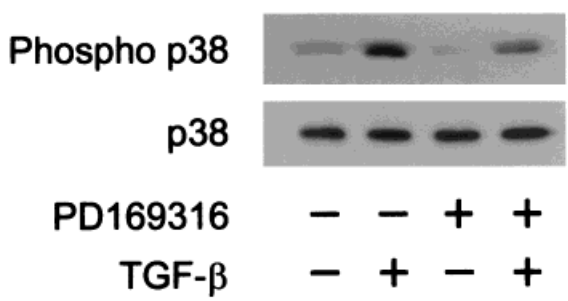

Fig. 6. Effect of PD169316 on TGF-β-induced p38 MAP kinase phosphorylation. Cultured cells were pretreated with 30 μmol / ml of PD169316 for 60 min, then exposed to 5 ng/ml of TGF-β or vehicle for 90 min. Cell lysates were subjected to SDS-PAGE followed by Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase.

report showing that p38 MAP kinase mediates the TGF-β-induced synthesis of VEGF in vascular smooth muscle cells (in this case, A10 cells).

TGF-β has a wide range of biological effects on a large variety of cell types. It is known to play a pivotal role in promoting alterations in vessel structure (on the basis of its pleiotropic properties as a modulator of cell growth, differentiation, migration, and matrix production) [Massague, 1998]. Furthermore, recent evidence suggests that alterations in the local abundance of TGF-β in the arterial wall promote vascular cell transdifferentiation, vascular wall remodeling, arterial lesion growth, and lesion regression associated with apoptosis [Schulick et al., 1998]. In these processes, it has been shown that TGF-β promotes VEGF synthesis in vascular smooth muscle cells and exerts angiogenic effects [Brogi et al., 1994; Stavri et al., 1995]. Indeed, evidence is accumulating to indicate that VEGF is a central regulator of angiogenesis [Plate et al., 1992; Shweiki et al., 1992]. These findings, together with ours, suggests to us that in vascular smooth muscle cells, p38 MAP kinase may play a crucial role in angiogenesis, although it should be admitted that our results were obtained in smooth muscle cells derived from the aorta, rather than from small blood vessels. A recent publication indicated that anisomycin, strong activator of the p38 MAP kinase and JNK, increases VEGF mRNA stabilization through the activation of p38 MAP kinase and JNK in hamster fibroblast's derivatives [Pages et al., 2000]. This action is presumably mediated through an AU-rich region of the 3′-untranslated region (UTR) of VEGF mRNA. But it remains unclear whether VEGF 3′-UTR-interacting proteins are direct targets for phosphorylation by p38 MAP kinase. Several diseases, including cancer, are characterized by abnormal angiogenesis, and it has been reported that in many cases these diseases are accompanied by an aberrant production of VEGF [Kim et al., 1993]. Thus, it is conceivable that an investigation inducing a modulation of VEGF function could contribute to a successful therapeutic treatment for these diseases, although this idea is speculative at present. At the moment, the exact mechanisms underlying VEGF production are not fully understood. Hence, a complete elucidation of the mechanisms involved in VEGF production may be necessary, before methods aimed at an appropriate modulation of VEGF-induced angiogenesis can be developed.

In conclusion, our results strongly suggest that p38 MAP kinase is required for TGF-β - stimulated VEGF synthesis in rat aortic smooth muscle cells.

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