Extracellular signal-regulated kinases and AP-1 mediate the up-regulation of vascular endothelial growth factor by PDGF in human vascular smooth muscle cells

HEE J. CHANG, JUNG S. PARK, MI H. KIM, MIN H. HONG, KI M. KIM, SEOK M. KIM, BOO A. SHIN, BONG W. AHN and YOUNG D. JUNG

Chonnam University Research Institute of Medical Sciences, Chonnam National University Medical School, Kwangju 501-190, Korea

Received August 16, 2005; Accepted September 30, 2005

Abstract. Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) have been shown to communicate with each other via cytokine signaling during neovascularization. In this study, we investigated the effect of platelet-derived growth factor (PDGF), a cytokine released from tumors and ECs, on vascular endothelial growth factor (VEGF) expression in human VSMCs and underlying signal transduction pathways. PDGF induced VEGF expression in a time- and concentration-dependent manner. PDGF induced the activation of extracellular signal-regulated kinase-1/2 (ERK-1/2), but not the activation of c-jun amino terminal kinase (JNK) and P38 mitogen-activated protein kinase (MAPK). Specific inhibitor of mitogen-activated protein kinase kinase (MEK)-1 was found to suppress VEGF expression and promoter activity. The expression of vectors encoding a mutated-type MEK-1 decreased the VEGF promoter activity. Electrophoretic mobility shift assay revealed that PDGF dose-dependently increased the DNA binding activity of AP-1. Transient transfection studies using an AP-1 decoy oligonucleotide confirmed that the activation of AP-1 is involved in PDGF-induced VEGF upregulation. Conditioned media from the human VSMCs pretreated with PDGF could remarkably stimulate the *in vitro* growth of human umbilical vein endothelial cells and this effect was partially abrogated by VEGF neutralizing antibodies. The above results suggest that ERK-1/2 and AP-1 signaling pathways are involved in the PDGF-induced VEGF expression in human VSMCs and that these paracrine signaling pathways induce endothelial cell proliferation.

Introduction

Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are the major cellular components of small tumor vessels. The interactions between these cells play significant roles in the homeostasis of the structure and function of the blood vessel (1). Angiogenesis, the growth of novel capillaries from pre-existing vessels, is an essential step in tumor growth and metastasis (2). The formation of new blood vessels is mediated by angiogenic growth factors (3). The growth factors not only bind their receptors on ECs and stimulate the cell proliferation initiating new blood vessel formation but also bind receptors on accessory cells, such as VSMCs, that maintain vessel integrity (4). One of the most potent angiogenic factors is vascular endothelial growth factor (VEGF), which stimulates capillary formation and has specific mitogenic and chemotactic activities for vascular ECs (5). VEGF is produced by various cell types, including ECs, VSMCs, fibroblasts, epithelial cells, mesenchymal cells, and macrophage (5-7).

A variety of cytokine and growth factors, including IL-1ß, IL-6, PDGF-BB, TGF-ß, basic fibroblast growth factor, and hepatocyte growth factor have been shown to induce VEGF expression in malignant and non-malignant cell lines (5). The binding of cytokine to specific cell surface receptors should initiate intracellular signaling that ultimately induces VEGF. However, the specific cytokine-induced signaling pathways that contribute to VEGF upregulation have not been fully elucidated. Hypoxia is an another well characterized inducer of VEGF expression (8-10). Hypoxia leads to a rapid increase in VEGF expression in numerous cells by both increasing the transcription of the gene and prolonging the mRNA half-life (11).

Two specific signal transduction pathways are known to mediate the upregulation of VEGF: the PI-3 kinase/Akt (9) and mitogen-activated protein kinase (MAPK) pathways (12). The located upstreams of these pathways are activated Src and Ras, which have also been associated with increased VEGF production and angiogenesis in *in vitro* models (10,13). Analysis of gene structure revealed that the VEGF gene has several potential binding sites for tanscription factors, AP-1, AP-2 and SP-1 (14), and hypoxic stress induced the activation of AP-1, NF-κB and HIF-1 in various cell types (15,16).

We have investigated the cytokine paracrine signalings in perivascular cells which induce VEGF expression, thereby enhancing the proliferation of ECs. In the present study, we demonstrate that PDGF upregulates VEGF in human VSMCs (hVSMCs) via activation of the ERK-1/2 and AP-1 pathways.

Correspondence to: Dr Young D. Jung, Department of Biochemistry, Chonnam National University Medical School, 5 Hakdong, Kwangju 501-190, Korea
E-mail: ydjung@chonnam.ac.kr

Key words: AP-1, vascular endothelial growth factor, platelet-derived growth factor, smooth muscle cells

We also show that the condition medium (CM) from hVSMCs treated with PDGF can enhance the proliferation of ECs.

Materials and methods

Cell culture and culture conditions. hVSMCs were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in Hank's modified Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 U/ml penicillin-streptomycin at 37˚C in 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection and cultured in DMEM supplemented with 15% FBS and 5 ng/ml basic fibroblast growth factor (bFGF). To determine the effects of PDGF (PDGF-BB, R&D Systems, Inc., McKinley Place, NE) on Erk-1/2, JNK and P38 MAPK activation, cells were harvested at various intervals and phosphorylated and total protein levels were determined by Western blot analysis. To examine the role of specific signaling pathways in VEGF induction by PDGF, hVSMCs were pretreated with 25 μM PD98059 (a MEK inhibitor, New England Biolabs Inc., Beverly, MA), 20 μM SP600125 (a JNK inhibitor, Calbiochem, San Diego, CA) and 10 μM SB203580 (a specific P38 MAPK inhibitor; Calbiochem) for 1 h prior to exposure to PDGF, and the level of VEGF mRNA was measured by Northern blot analysis.

ELISA for VEGF concentration. The concentration of VEGF in the culture supernatants was measured by ELISA using a commercially available kit (Immunoassay Kit Human VEGF; BioSource International Camarillo, CA). Briefly, samples were incubated in microtiter plates precoated with a monoclonal antibody specific for VEGF. After incubation at room temperature for 2 h and washing, a substrate solution was added. Color development was stopped after 30 min at room temperature, and the color intensity was read at 450 nm within 30 min.

Western blot analysis. Protein extraction and Western blot analysis were performed as previously described (5). The primary antibody preparations used in this study were 1:1000 dilutions of rabbit polyclonal anti-phosphospecific ERK-1/2, anti-phosphospecific JNK, and anti-phosphospecific P38 MAPK antibodies (New England Biolabs Inc.). The secondary antibody was horseradish peroxidase-labeled anti-rabbit immunoglobulins from donkey (Amersham Corp., Arlington Heights, IL) used at a 1:3000 dilution. Protein bands were visualized using a commercially available chemiluminescence kit (Amersham Corp.). Total protein levels were assayed by washing the blotted membrane with stripping solution [100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl (pH 6.7)] for 30 min at 50˚C and then reprobing the membrane with rabbit polyclonal anti-p44/42, anti-JNK, and anti-P38 antibodies diluted at 1:1000.

Northern blot analysis. Total RNA extraction and Northern blot hybridization were performed as previously described (5). The cDNA probe for VEGF was generated by PCR using sense (5'-TCCAGGAGTACCCTGATGAG-3') and antisense (5'-ATT CACATTTGTTGTGCTGT-3') primers (17). The glyceraldehyde-phosphate dehydrogenase (GAPDH) probe was purchased from American Type Culture Collection. Each cDNA probe was radiolabeled with [α-^{32}P]deoxyribonucleoside triphosphate by the random-priming technique using the Rediprime labeling system (Amersham Corp.). The probed nylon membranes were exposed to radiographic films (Life Technologies Inc., Grand Island, NY).

Measurement of VEGF promoter activity. A 2951-bp fragment containing the 5' flanking region and transcriptional start site of the VEGF gene (-2362 to +589 bp) was prepared by PCR with genomic DNA of VEGF as a template. The specific primers contained KpnI and SmaI linker sites (18). This PCR product was cloned into the pGL3-Basic vector (Promega, Madison, WI) to make a VEGF promoter-luciferase reporter construct (pGL3-VEGF). hVSMCs (5×10^5) were seeded and grown to 60-70% confluence and then pRLTK (an internal control plasmid containing the herpes simplex thymidine kinase promoter linked to the constitutively active Renilla luciferase reporter gene) and pGL3-VEGF were cotransfected into cells using FuGENE (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. pRLTK and pGL3 were cotransfected as a negative control. Cells were incubated in the transfection medium for 20 h and treated with PDGF for 8 h. The effects of signaling inhibitors on VEGF promoter activity were determined by pretreating cells with the inhibitors for 1 h prior to addition of PDGF. Cotransfection studies were performed in the presence or absence of AP-1 decoy oligodeoxynucleotides (ODNs) and dominant-negative mutants of MEK-1. Phosphorothioate double-stranded ODNs with sequences against the AP-1 binding site (5'-CAG CTC AGA AGT CAC TTC-3', 3'-GAA GTG ACT TCT GAG CTG-5') were prepared (Genotech Corp., Yusung, Korea) and annealed (AP-1 decoy ODNs). The expression vector encoding for the inactive MEK-1 (pMCL-K97M) was a gift from Dr N.G. Ahn (University of Colorado, Boulder, CO) (19). Cells were harvested using passive lysis buffer (Dual-Luciferase Reporter Assay System; Promega), and luciferase activity was determined using a single sample luminometer according to the manufacturer's protocol.

Transient transfection of AP-1-reporter. The AP-1-luciferase (Luc) reporter construct was purchased from Clontech (Palo Alto, CA). At 80-90% confluency, cells were washed and incubated with Hank's modified Dulbecco's minimal essential medium without serum and antibiotics for 5 h. The cells were then transfected with 1 μg AP-1-reporter containing pGL3 vector using FuGENE6 (Boehringer Mannheim) for 24 h. To determine the effect of PDGF on AP-1 activation, the transfected cells were incubated with 0-10 ng/ml PDGF for 8 h. After incubation, cells were lysed and luciferase activity was measured using a luminometer.

Extraction of nuclear proteins. Eighty to ninety percent confluent hVSMCs were incubated overnight in medium containing 1% FBS and treated with 0-10 ng/ml PDGF for 8 h. The cells were then resuspended in 500 μl cold buffer A [50 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 3% (v/v) glycerol, and 1.5 mM $MgCl_2$]. After the cells were

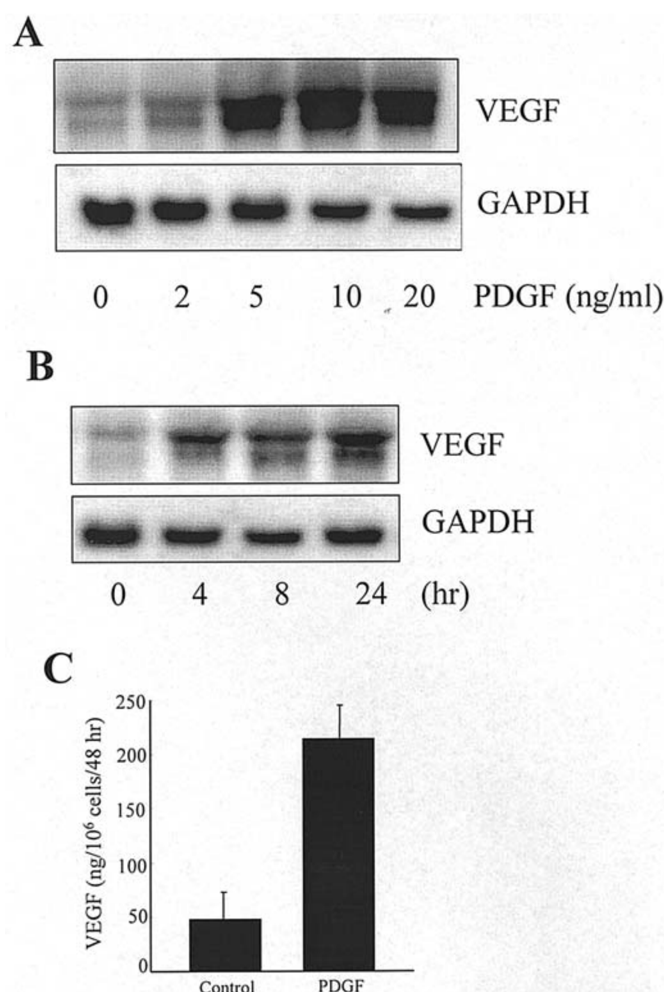

Figure 1. Induction of VEGF by PDGF in hVSMCs. Northern blot analysis was performed to determine the effect of PDGF on VEGF mRNA expression in hVSMCs. The cells were incubated with 0-20 ng/ml PDGF for 8 h (A), and with 10 ng/ml PDGF for the indicated times (B). ELISA was performed to determine the VEGF protein level in the culture medium derived from cells treated with or without 10 ng/ml PDGF for 48 h. Data represent the means ± SD from triplicate measurements (C).

allowed to swell for 5 min on ice, they were lysed with 500 μl buffer B [identical to buffer A except containing 0.05% Nonidet P-40 (Sigma)]. The homogenate was gently layered onto an equal volume cushion of buffer C [10 mM Tris (pH 7.4), 25% (v/v) glycerol, and 1.5 mM $MgCl_2$] and centrifuged for 5 min at 200 x g. The white nuclear pellet was resuspended in 75 μl cold high-salt lysis buffer (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). This suspension was agitated for 30 min at 4˚C and then microcentrifuged for 15 min at 4˚C. The resulting supernatant was stored in aliquots at -80˚C. Protein was quantitated spectrophotometrically using the BCA assay (Pierce) with bovine serum albumin as a standard.

Electrophoretic mobility shift assay (EMSA). EMSA was performed using the Gel Shift assay system (Promega). Briefly, oligonucleotides containing the consensus sequence for AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') were end-labeled with [α-^{32}P]adenosine triphosphate (3000 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase and then purified in a Microspin G-25 column (Sigma) and used as a probe for EMSA. Nuclear extract proteins (6 μg) were pre-incubated with binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% (v/v) glycerol, and 0.05 mg/ml poly (deoxyinosine-deoxycytosine)] for 5 min and then incubated with the labeled probe for 15 min at 37˚C. Each sample was electrophoresed in a 5% non-denaturing polyacrylamide gel in 0.5X Tris borate-EDTA buffer at 150 V for 4 h. The gel was dried and subjected to autoradiography

Determination of the effect of hVSMC-derived conditioned medium (CM) on proliferation of HUVEC. CM derived from hVSMCs was prepared as follows. Cells were grown to 95-100% confluence and incubated for 48 h in DMEM with 1% FBS and 10 ng/ml PDGF. After incubation, the supernatant (conditioned medium, CM) was centrifuged, filtered, and stored at -20˚C. To determine the effect of CM on endothelial cell proliferation, HUVECs (5x10^3) were seeded on 96-well plates (Falcon Laboratories, McLean, VA) and incubated for 24 h in DMEM containing 15% FBS and 10 ng/ml bFGF. The medium was replaced with CM, and the cells were incubated for 24 h. The neutralizing effect of anti-VEGF antibody on CM activity was determined by incubating the cells with CM after CM was treated for 1 h with 10 μg/ml anti-VEGF antibody or non-specific IgG (R&D Systems). Cell proliferation was determined by MTT assay.

Results

Effect of PDGF on VEGF mRNA and protein expression in hVSMCs. To determine the effect of PDGF on VEGF expression in hVSMCs, cells were incubated with 0-20 ng/ml PDGF for various periods and VEGF mRNA and protein were measured by Northern blotting and ELISA, respectively. As shown in Fig. 1A and B, PDGF treatment increased the level of VEGF mRNA in a time- and dose-dependent manner. Also, PDGF treatment increased the VEGF protein level. The VEGF protein level increased nearly four times after cells were incubated with 10 ng/ml PDGF for 48 h (Fig. 1C).

Involvement of Erk-1/2 in PDGF-induced VEGF expression. To study the signaling pathways involved in VEGF induction by PDGF, changes in the levels of phosphorylated and total ERK-1/2, JNK, and P38 MAPK were determined in hVSMCs after the cells were exposed to PDGF. As shown in Fig. 2A, PDGF treatment led to a remarkable increase in ERK-1/2 phosphorylation within 15 min and the increased level was maintained for 120 min. The level of total ERK-1/2 was not significantly altered after PDGF treatment. In contrast, however, PDGF did not induce the phosphorylation of JNK and P38 MAPK. To examine further the specific role of ERK-1/2 in PDGF-induced VEGF expression, hVSMCs were pretreated with 25 μM PD98059 (a MEK inhibitor) before PDGF treatment. The result showed that PD98059 remarkably blocked the PDGF-induced VEGF expression (Fig. 2B). However, 20 μM SP600125 (a JNK inhibitor) and 10 μM SB203580 (a specific P38 MAPK inhibitor) did not affect the PDGF-induced VEGF expression (Fig. 2B).

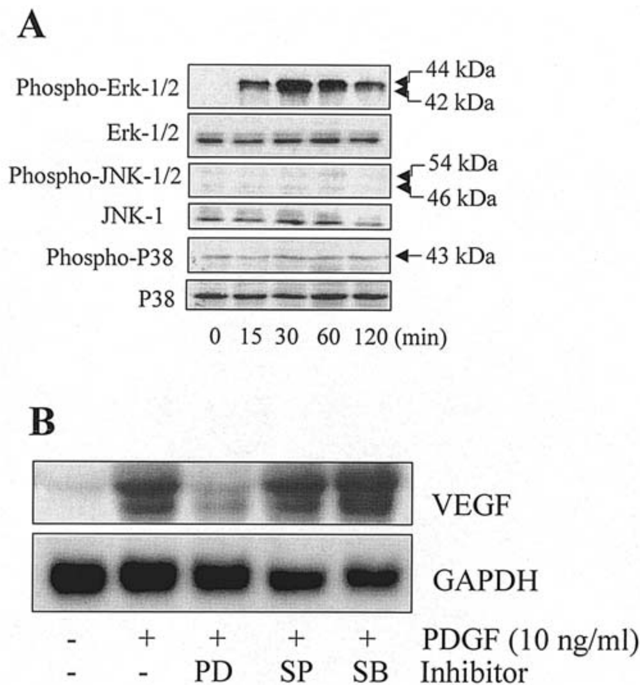

Figure 2. Activation of ERK-1/2 during PDGF-induced VEGF expression in hVSMCs. hVSMCs were incubated with 10 ng/ml PDGF for 0-120 min, and cell lysates were determined for the phosphorylated and total ERK-1/2, JNK-1/2 and P38 MAPK by Western blot analysis (A). hVSMCs pretreated with 25 μM PD98059 (PD), 20 μM SP600125 (SP), and 10 μM SB203580 (SB) for 1 h were incubated with 10 ng/ml PDGF for 8 h. After incubation, the cell lysates were determined for the VEGF mRNA by Northern blot analysis (B).

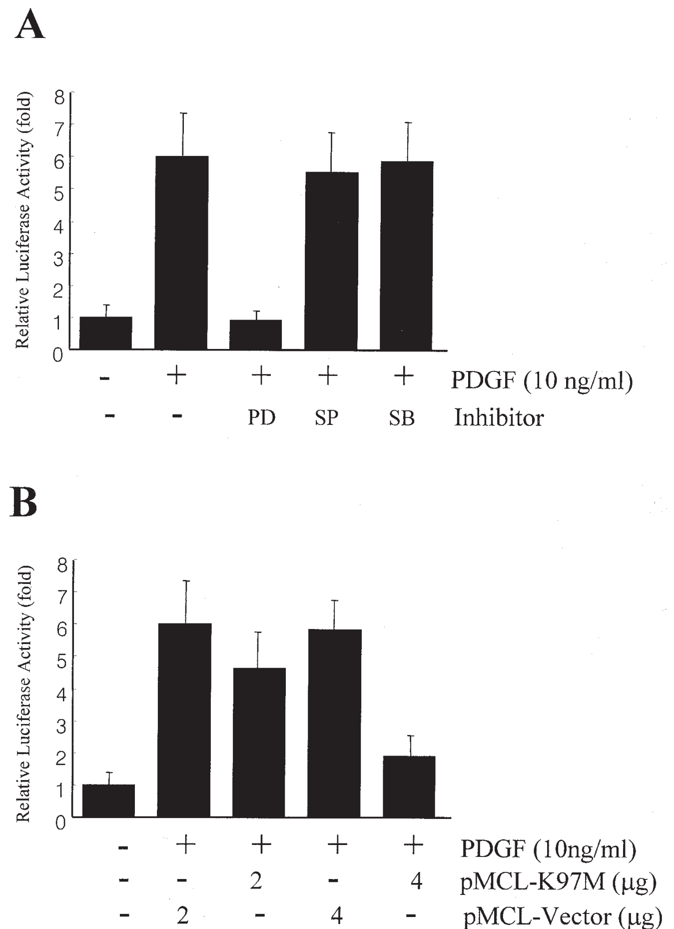

Figure 3. Effects of inhibitors of signaling pathways on VEGF promoter activity induced by PDGF. hVSMCs were transiently transfected with 1 μg pGL3-VEGF. The transfected cells, after being pretreated with 25 μM PD98059 (PD), 20 μM SP600125 (SP) and 10 μM SB203580 (SB), were incubated with 10 ng/ml PDGF for 8 h (A). Where indicated, different concentrations of a dominant negative mutant of MEK-1 (pMCL-K97M) were cotransfected with pGL3-VEGF into hVSMCs (B). After incubation, the cells were lysed and the luciferase activity was measured using a luminometer. Data represent the means ± SD from triplicate measurements.

Next, we sought to examine the effect of PDGF on the transcriptional regulation of the VEGF gene. To this end, hVSMCs were transiently transfected with the promoter-reporter construct (pGL3-VEGF) of the human VEGF gene fused to the luciferase gene. hVSMCs transfected with pGL3-VEGF showed a 6-fold increase in promoter activity after PDGF treatment (Fig. 3A). When the transfected cells were pretreated with PD98059 before PDGF treatment, the induction of VEGF promoter activity by PDGF was almost completely inhibited (Fig. 3A). Again, SP600125 and SB203580 did not affect significantly the VEGF promoter activity induced by PDGF. Also, when the mutant form of MEK-1 (K97M) was cotransfected with pGL3-VEGF into hVSMCs, the induction of VEGF promoter activity by PDGF was inhibited dose dependently (Fig. 3B). The above results strongly suggest that the ERK-1/2 signaling pathway is involved in the PDGF-induced activation of VEGF transcription.

Activation of transcription factor, AP-1, during PDGF-induced VEGF expression. Since earlier studies had suggested a potential role of the transcription factor, AP-1, in VEGF expression, we investigated the effect of PDGF on the activation of AP-1 in hVSMCs. As shown in Fig. 4A, PDGF treatment led to a remarkable increase in the amount of AP-1 which could form a complex with the radiolabeled oligonucleotide probe in EMSA. Consistent with the EMSA result, PDGF treatment increased the AP-1-dependent transcriptional activity, as revealed by transient transfection study using the pAP-1-Luc reporter construct (Fig. 4B). Also, when the hVSMCs were transiently transfected with an AP-1 decoy oligonucleotide, the PDGF-induced VEGF promoter activity was decreased dose dependently by the AP-1 decoy oligonucleotide (Fig. 5). These results suggest that the transcription factor, AP-1, is indeed involved in PDGF-induced VEGF expression in hVSMCs.

Effect of conditioned medium (CM) derived from PDGF-treated hVSMCs on proliferation of HUVECs. We investigated whether CM obtained from PDGF-treated hVSMCs could stimulate the growth of endothelial HUVECs. HUVECs were cultured in the presence or absence of the CM and cell proliferation was determined 24 h later by MTT assay. As shown in Fig. 6, the CM remarkably stimulated the *in vitro* growth of HUVECs. When the CM was pretreated with anti-VEGF antibody, its proliferation-stimulatory effect was significantly abolished. The non-specific IgG did not influence the proliferation-stimulatory effect of CM. These results suggest that VEGF induced by PDGF in hVSMCs may stimulate endothelial cell proliferation.

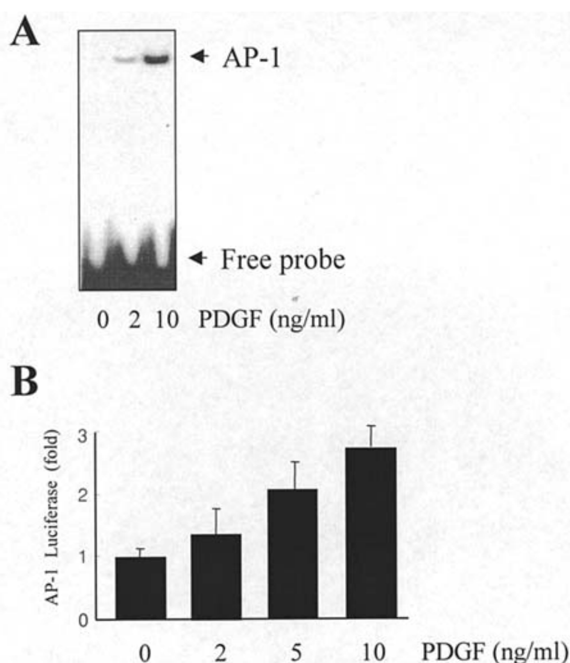

Figure 4. Activation of AP-1 during PDGF-induced VEGF expression in hVSMCs. hVSMCs were incubated with 0-10 ng/ml PDGF for 8 h, and nuclear extracts from the cells were analyzed by EMSA for activated AP-1 using a radiolabeled oligonuclotide probe (A). hVSMCs were transiently transfected with the pAP-1-Luc reporter construct. The transfected hVSMCs were exposed to 0-10 ng/ml PDGF for 8 h and the luciferase activity was determined using a luminometer. Data represent the means ± SD from triplicate measurements (B).

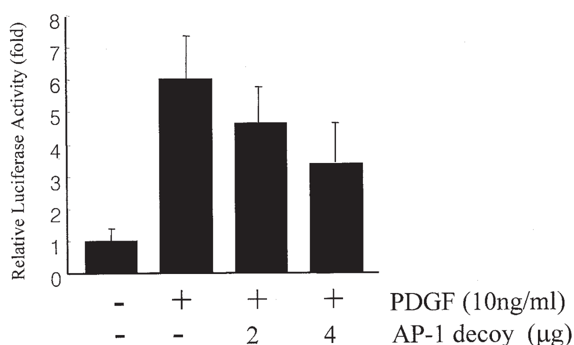

Figure 5. Effect of AP-1 decoy oligonucleotide on PDGF-induced VEGF promoter activity. AP-1 decoy oligonucleotide (AP-1 decoy, 0-4 μg) was cotransfected with 1 μg pGL3-VEGF into hVSMC cells. After incubation with 10 ng/ml PDGF for 8 h, the cells were lysed and the luciferase activity was measured using a luminometer. Data represent the means ± SD from triplicate measurements.

Discussion

PDGF is a potent mitogen and chemoattattractant for mesenchymal cells and fibroblasts (20). PDGF is composed of A, B, C, and D polypeptide chains that form the homodimers PDGF-AA, BB, CC and DD and the heterodimer PDGF-AB (21). Accumulated evidence has shown that PDGF-B plays an important role in angiogenesis. Genetic studies have demonstrated that PDGF-B is involved in vessel maturation through the recruitment of VSMCs and pericytes to growing vessels during embryonic development (20,22). Increased

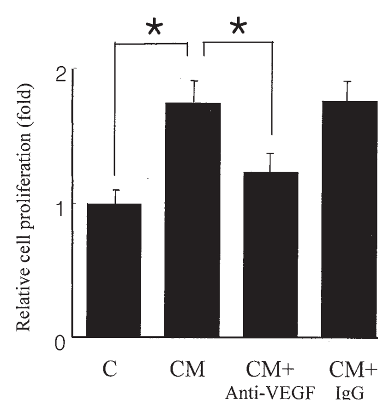

Figure 6. Effect of CM derived from PDGF-treated hVSMCs on HUVEC proliferation. HUVECs were incubated with CM for 24 h in the presence or absence of 10 μg/ml anti-VEGF antibody (anti-VEGF) and non-specific IgG (IgG), and the number of cells was counted using MTT. Data represent the means ± SD from triplicate measurements (*P<0.05).

PDGF and its receptor expression have also been implicated in the progression of human tumors (23).

Our results show that PDGF-BB induced VEGF mRNA and protein expression in hVSMCs and this induction of VEGF expression was caused, in part at least, by an increase in transcriptional activity. Recently, much effort has been made to define the signal transduction pathways induced by PDGF. Cellular responses to PDGF stimulation trigger a cascade of protein kinases that transmit signals from the cell surface to the nucleus and these signals ultimately upregulate the gene expression. In mammalian cells, three subgroups of MAPKs have been detected: ERK-1/2, JNK, and P38 MAPK (24). In our study, PDGF induced the activation of ERK-1/2 but not of JNK and P38 MAPK in hVSMCs. ERK-1/2 phosphorylation increased within 15 min after exposure of hVSMCs to PDGF and the increased levels were maintained for 120 min. These results suggest that the ERK-1/2 signaling pathway mediates the PDGF signal amplification and modulation that induces VEGF. This suggestion was further supported by observations that treatment with a specific inhibitor for MEK-1 (PD98059) and expression of vectors encoding a mutated-type MEK-1 (pMCL-K97M) resulted in a remarkable reduction in VEGF mRNA expression and promoter activity in hVSMCs.

It remains to be known how PDGF mediates ERK-1/2 activation in hVSMCs. Pukac *et al* (25) reported that the downregulation of PKC (which eliminates c/nPKC isoforms) partially blocked the PDGF-BB activation of ERK in rat aortic VSMCs, indicating that both c/nPKC and other signals mediated the PDGF-BB stimulation of ERK. According to Grammer and Blenis (26), both cPKC and PI-3 kinase pathways are lined to the PDGF-BB stimulation of ERK.

The following observations in our study suggest that AP-1 is also involved in the signaling pathway for VEGF induction by PDGF in hVSMCs: i) PDGF treatment increased the amount of AP-1 which could form a complex with the radiolabeled oligonucleotide probe for AP-1 in EMSA; ii) PDGF treatment caused an increase in AP-1-dependent transcriptional activity, as revealed by transient transfection study using the pAP-1-Luc reporter construct; and iii) inhibition of AP-1 by AP-1 decoy ODN reduced the VEGF promoter activity. Several *cis*-elements and trans-acing factors including AP-1, which

can regulate the transcription of the VEGF gene, have been identified (14). Also, it has been reported that MAPK cascades activate transcription factor AP-1 in various cells. AP-1 activity is regulated by different MAPKs depending on the kinds of cells and stimuli (27). AP-1 is a homo- or heterodimer composed of Fos and Jun. The transcription of c-fos and c-jun is upregulated by activated MAPK (28). Thus, our results suggesting that PDGF activates AP-1 through Erk-1/2 signaling in hVSMC cells are consistent with earlier observations.

Although VEGF is a well-characterized angiogenic factor, the role of VEGF secreted from hVSMCs is less defined. Reinmuth *et al* (4) suggested that VEGF secreted from pericytes or hVSMCs may enhance EC survival in a paracrine manner via specific intracellular signaling pathways. In our study, the CM collected from hVSMCs treated with PDGF increased EC proliferation and, when neutralizing antibodies to VEGF were added to the CM, this effect was significantly blocked, suggesting that VEGF secreted from hVSMCs enhances the proliferative activity of ECs.

The process of angiogenesis is essential for various physiological processes and for tumor growth and metastasis. In the search for a better understanding of the process of angiogenesis, it is necessary to appreciate that it is dependent not only upon the activity of endothelial cells but also upon the function and activity of other cells in the vascular microenvironment, such as VSMCs. In this study, our results suggest that ERK-1/2 and AP-1 signaling pathways are involved in the PDGF-induced VEGF expression in hVSMCs and that these paracrine signaling pathways induced EC proliferation. Further studies are needed to clarify the signaling pathways for the VEGF expression and paracrine interactions between ECs and VSMCs.

Acknowledgements

We are grateful to Dr Natalie Ahn (University of Colorado, USA) for the MEK-1 construct. M.H. Hong was financially supported by a Korea Research Foundation Grant (KRF-99-005-F00014). This work was supported by the Korea Science and Engineering Foundation through the Medical Research Center (R13-2002-013-00000-0) at Chonnam National University.

References

1. Antoine M, Wirz W, Tag CG, Mavituna M, Emans N, Korff T, Stoldt V, Gressner AM and Kiefer P: Expression pattern of fibroblast growth factors (FGFs), their receptors and antagonists in primary endothelial cells and vascular smooth muscle cells. Growth Factors 23: 87-95, 2005.
2. Hicklin DJ and Ellis LM: Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 23: 1011-1027, 2005.
3. Ferrara N: Molecular and biological properties of vascular endothelial growth factor. J Mol Med 77: 527-543, 1999.
4. Reinmuth N, Liu W, Jung YD, Ahmad SA, Shaheen RM, Fan F, Bucana CD, McMahon G, Gallick GE and Ellis LM: Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. FASEB J 15: 1239-1241, 2001.
5. Jung YD, Liu W, Reinmuth N, Ahmad SA, Fan F, Gallick GE and Ellis LM: Vascular endothelial growth factor is upregulated by interleukin-1 beta in human vascular smooth muscle cells via the P38 mitogen-activated protein kinase pathway. Angiogenesis 4: 155-162, 2001.
6. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O and Alitalo K: Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 269: 6271-6274, 1994.
7. McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM and Smith SK: Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 98: 482-489, 1996.
8. Shima DT, Deutsch U and D'Amore PA: Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. FEBS Lett 370: 203-208, 1995.
9. Mazure NM, Chen EY, Laderoute KR and Giaccia AJ: Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 90: 3322-3331, 1997.
10. Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS and Sukhatme VP: Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature 375: 577-581, 1995.
11. Levy NS, Goldberg MA and Levy AP: Sequencing of the human vascular endothelial growth factor (VEGF) 3' untranslated region (UTR): conservation of five hypoxia-inducible RNA-protein binding sites. Biochim Biophys Acta 1352: 167-173, 1997.
12. Jung YD, Nakano K, Liu W, Gallick GE and Ellis LM: Extracellular signal-regulated kinase activation is required for up-regulation of vascular endothelial growth factor by serum starvation in human colon carcinoma cells. Cancer Res 59: 4804-4807, 1999.
13. Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T and Kerbel RS: Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res 55: 4575-4580, 1995.
14. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC and Abraham JA: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266: 11947-11954, 1991.
15. Lukiw WJ, Ottlecz A, Lambrou G, Grueninger M, Finley J, Thompson HW and Bazan NG: Coordinate activation of HIF-1 and NF-kappaB DNA binding and COX-2 and VEGF expression in retinal cells by hypoxia. Invest Ophthalmol Vis Sci 44: 4163-4170, 2003.
16. Josko J and Mazurek M: Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. Med Sci Monit 10: 89-98, 2004.
17. Berse B, Brown LF, van de Water L, Dvorak HF and Senger DR: Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Mol Biol Cell 3: 211-220, 1992.
18. Akagi Y, Liu W, Zebrowski B, Xie K and Ellis LM: Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I. Cancer Res 58: 4008-4014, 1998.
19. Emrick MA, Hoofnagle AN, Miller AS, Ten Eyck LF and Ahn NG: Constitutive activation of extracellular signal-regulated kinase 2 by synergistic point mutations. J Biol Chem 276: 46469-46479, 2001.
20. Hellstrom M, Kalen M, Lindahl P, Abramsson A and Betsholtz C: Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126: 3047-3055, 1999.
21. Betsholtz C, Karlsson L and Lindahl P: Develpmental roles of platelet derived growth factors. Bioessays 23: 494-507, 2001.
22. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E and Betsholtz C: Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 8: 1875-1887, 1994.
23. Sun J, Wang DA, Jain RK, Carie A, Paquette S, Ennis E, Blaskovich MA, Baldini L, Coppola D, Hamilton AD and Sebti SM: Inhibiting angiogenesis and tumorigenesis by a synthetic molecule that blocks binding of both VEGF and PDGF to their receptors. Oncogene 24: 4701-4709, 2005.
24. Cobb MH and Goldsmith EJ. How MAP kinases are regulated. J Biol Chem 270: 14843-14846, 1995.

25. Pukac L, Huangpu J and Karnovsky MJ: Platelet-derived growth factor-BB, insulin-like growth factor-I, and phorbol ester activate different signaling pathways for stimulation of vascular smooth muscle cell migration. Exp Cell Res 242: 548-560, 1998.
26. Grammer TC and Blenis J: Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. Oncogene 14: 1635-1642, 1997.
27. Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, Iwao H and Yoshikawa J: Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. Cardiovasc Res 38: 116-124, 1998.
28. Whitmarsh AJ and Davis RJ: Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 74: 589-607, 1996.