Role of JNK, p38, and ERK in Platelet-Derived Growth Factor–Induced Vascular Proliferation, Migration, and Gene Expression

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Objective—We investigated the comparative roles of mitogen-activated protein (MAP) kinases, including c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38, in vascular smooth muscle cell (VSMC) proliferation, migration, and gene expression.

Methods and Results—VSMCs were infected with recombinant adenovirus containing dominant-negative mutants of ERK, p38, and JNK (Ad-DN-ERK, Ad-DN-p38, and Ad-DN-JNK, respectively) to specifically inhibit the respective MAP kinases and then stimulated with platelet-derived growth factor (PDGF)-BB. Ad-DN-ERK attenuated PDGF-BB–induced VSMC proliferation more potently than Ad-DN-p38 or Ad-DN-JNK, indicating the dominant role of ERK in VSMC proliferation. Ad-DN-ERK, Ad-DN-p38, and Ad-DN-JNK similarly inhibited PDGF-induced VSMC migration. Ad-DN-ERK and Ad-DN-JNK suppressed PDGF-BB–induced downregulation of cyclin-dependent kinase inhibitor p27Kip1, whereas Ad-DN-p38 decreased PDGF-BB–induced upregulation of p21Cip1. Ad-DN-ERK inhibited PDGF-BB–induced plasminogen activator inhibitor type-1 (PAI-1), monocyte chemotactic protein-1, and transforming growth factor-β (TGF-β) expression but not PAI-1, whereas Ad-DN-JNK suppressed only PAI-1 expression. Moreover, in vivo gene transfer of Ad-DN-p38 to rat carotid artery caused the inhibition of intimal hyperplasia by balloon injury, indicating the involvement of p38 in vascular remodeling in vivo.

Conclusions—We propose that these 3 MAP kinases participate in vascular diseases via differential molecular mechanisms and are new therapeutic targets for treatment of vascular diseases. (Arterioscler Thromb Vasc Biol. 2003;23:795-801.)

Key Words: platelet-derived growth factor ■ gene transfer ■ vascular smooth muscle cell ■ proliferation ■ gene expression
notion that MAP kinases may be involved in vascular remodeling or diseases. The important role of ERK and p38 in PDGF-induced vascular SMC responses is proposed. However, the detailed role of MAP kinases is poorly understood. In particular, there is no available information on the role of JNK in PDGF-induced vascular SMC responses.

In this study, by using gene transfer technique with adenoviral vector, we examined the comparative role of 3 MAP kinases, including JNK, p38, and ERK, in PDGF-BB–induced proliferation, migration, and gene expression of vascular SMCs. We obtained evidence that all 3 MAP kinases participate in PDGF-induced vascular SMC proliferation, migration, and gene expressions in differential manners.

Methods

Aortic Vascular Smooth Muscle Cells

Rat aortic SMCs were prepared from thoracic aortas of male Sprague-Dawley rats by using the collagenase digestion method and cultured, as described. For all experiments, rat aortic SMCs from passages 4 to 7 were used. SMCs were grown to 70% to 80% confluence and then made quiescent by incubation with DMEM containing 0.1% FBS for 48 hours before addition of PDGF-BB (10 ng/mL).

Construction of Recombinant Adenovirus Containing Dominant-Negative Mutant of ERK, p38, and JNK

Dominant-negative mutants of p44ERK cDNA (DN-ERK) and of p46JNK cDNA (DN-JNK) were produced by polymerase chain reaction (PCR) using primers as described. Dominant-negative mutant of p38α cDNA (DN-p38) was produced by PCR using primers to produce a mutant in the site of dual-activating phosphorylation through substitution of TGY (threonine180-glycine-tyrosine182) with AGF (alanine180-glycine-phenylalanine182). As negative control, recombinant adenoviruses containing bacterial β-galactosidase gene (Ad-LacZ) were also constructed in the same way. The titer of the virus was determined by limiting dilution in 293 cells and expressed as plaque-forming units.

In Vivo Gene Transfer and Balloon Injury

All procedures were in accordance with institutional guidelines for animal research. Sprague-Dawley rats (Clea Japan, Tokyo, Japan) were anesthetized with sodium pentobarbital (40 mg/kg, IP). In vivo gene transfer to carotid artery was performed, as described in detail. The adenovirus containing DN-p38 or LacZ as control (each 2×10⁹ plaque-forming units) was infused into the closed luminal segment of common carotid. At 2 days after gene transfer, the endothelial denudation of the left common carotid artery was carried out, and at 14 days after balloon injury, intimal/medial area ratio of carotid artery in each rat was estimated as described in detail. Furthermore, we measured the percentages of BrdU-positive cells at 7 days after injury, as previously reported. BrdU immunohistochemistry was performed with a mouse anti-BrdU monoclonal antibody (Amersham) and LSAB2 kit (DAKO JAPAN Co, Ltd).

Results

Effects of Dominant-Negative Mutants on Activation of MAP Kinases by PDGF-BB

Stimulation of aortic SMCs with PDGF-BB activated ERK, p38, and JNK with the peak at 5, 5, and 15 minutes, respectively (online Figure IA, available at http://atvb.ahajournals.org). Ad-DN-ERK, Ad-DN-p38, and Ad-DN-JNK at 100 MOI blocked PDGF-BB–induced activation of ERK, p38, and JNK, respectively, in a specific manner and did not significantly affect other MAP kinase activities (online Figure IA, available at http://atvb.ahajournals.org).

Effects on PDGF-BB–Induced Aortic SMC Proliferation

As shown in Figure 1, treatment of aortic SMCs with PDGF-BB significantly increased the rate of DNA synthesis and cell number. Compared with Ad-LacZ, infection with Ad-DN-ERK, Ad-DN-p38, and Ad-DN-JNK inhibited PDGF-BB–induced increase in [³H]thymidine incorporation by 98%, 28%, and 32%, respectively (Figure 1A) and suppressed the increase in cell number by 82%, 20%, and 36%, respectively (Figure 1B). Ad-LacZ infection did not significantly affect PDGF-BB–induced cell proliferation. Without PDGF-BB stimulation, infection of SMCs with Ad-LacZ, Ad-DN-ERK, Ad-DN-p38, or Ad-DN-JNK did not significantly affect [³H]thymidine incorporation and cell number.

Treatment of SMCs with PD98059 (50 µmol/L), SB202190 (10 µmol/L), and JNK inhibitor I (1 µmol/L) for 1 hour inhibited PDGF-BB–induced increase in [³H]thymidine incorporation by 78%, 39%, and 33% (n=6, P<0.01), respectively, and suppressed the increase in cell number by...
88%, 20%, and 41% (n=6, P<0.01), respectively. On the other hand, SB202474 (10 μmol/L), negative control of p38 inhibitor, or JNK inhibitor I negative control (1 μmol/L) did not affect PDGF-BB–induced cell proliferation.

Effects on Cell Cycle Progression
As shown in online Figure II, infection of rat aortic SMCs with Ad-DN-ERK, Ad-DN-p38, or Ad-DN-JNK significantly attenuated PDGF-BB (10 ng/mL for 20 hours)-induced increases in S entry of cell cycle, resulting in G1 arrest, whereas Ad-LacZ did not inhibit it. However, without PDGF-BB stimulation, infection of SMCs with these dominant-negative mutants did not affect cell cycle progression.

Treatment of SMCs with PD98059 (50 μmol/L) and SB202129 (10 μmol/L) produced the inhibition of PDGF-BB–induced increases in S entry of cell cycle to similar extent to Ad-DN-ERK and Ad-DN-p38, respectively (data not shown).

Effects on Cell Cycle Progression
As shown in online Figure II, infection of rat aortic SMCs with Ad-DN-ERK, Ad-DN-p38, or Ad-DN-JNK significantly attenuated PDGF-BB (10 ng/mL for 20 hours)-induced increases in S entry of cell cycle, resulting in G1 arrest, whereas Ad-LacZ did not inhibit it. However, without PDGF-BB stimulation, infection of SMCs with these dominant-negative mutants did not affect cell cycle progression.

Effects on p27kip1, p21Cip1, and p53
As shown in Figure 2, stimulation of vascular SMCs with PDGF-BB induced the significant downregulation of p27kip1, being consistent with previous reports.11,28 Ad-DN-ERK or Ad-DN-JNK completely prevented PDGF-BB–induced downregulation of p27kip1 (Figures 2A and 2B). On the other hand, Ad-DN-p38 infection did not alter PDGF-BB–induced downregulation of p27kip1. In contrast to p27kip1, PDGF-BB significantly increased p21Cip1, being in good agreement with previous findings.11,29 Neither Ad-DN-ERK nor Ad-DN-JNK altered PDGF-BB–induced upregulation of p21Cip1 (Figures 2A and 2C). However, Ad-DN-p38 infection significantly inhibited PDGF-BB–induced increase in p21Cip1. PDGF-BB did not significantly change p53 levels, as previously reported by us.11 Ad-DN-ERK, Ad-DN-p38, or Ad-DN-JNK did not significantly alter p53 levels, with or without PDGF-BB treatment (Figure 2A).

Effects on PDGF-BB–Induced AP-1 DNA Binding Activity
EMSA in online Figure III showed that treatment of aortic SMCs with PDGF-BB increased AP-1 DNA binding activity. Infection with Ad-DN-ERK and Ad-DN-JNK attenuated PDGF-BB–induced increase in AP-1 DNA binding activity by 45% and 25%, respectively (online Figure IIIA). However, Ad-DN-p38 did not alter AP-1 DNA binding activity induced by PDGF-BB.

As shown in online Figure IIIB, PD98059, but not SB202190, significantly inhibited PDGF-BB–induced elevation of AP-1 DNA binding activity, being consistent with the above results on DN-ERK and DN-p38.

Effects on PDGF-BB–Induced Cell Migration
As shown in online Figure IV, PDGF-BB stimulation significantly induced vascular SMC migration by 2.3-fold. Infection of SMCs with Ad-DN-ERK, Ad-DN-p38, or Ad-DN-JNK suppressed PDGF-BB–induced SMC migration by 91%, 81%, and 77%, respectively (online Figure IV). Ad-LacZ did not significantly affect PDGF-BB–induced cell migration.

Treatment of SMCs with PD98059 (50 μmol/L), SB202129 (10 μmol/L), and JNK inhibitor I (1 μmol/L) for 1 hour suppressed PDGF-BB–induced SMC migration by 73%, 69%, and 52%, respectively (n=6, P<0.01). On the other hand, SB202474 (10 μmol/L), negative control of p38 inhibitor, or JNK inhibitor I negative control (1 μmol/L) did not affect PDGF-BB–induced cell migration.
Effects on PDGF-BB–Induced Gene Expression of PAI-1, TGF-β1, and MCP-1

Treatment of aortic SMCs with PDGF-BB increased mRNA levels for PAI-1, TGF-β1, and MCP-1 with the peak at 3 hours, 18 to 24 hours, and 3 hours, respectively, and the increase in these mRNA levels by PDGF-BB was completely blocked by AG1295 (50 μmol/L), an inhibitor of PDGF receptor kinase (online Figure V).

As shown in Figure 3A, infection of aortic SMCs with Ad-DN-ERK significantly inhibited PDGF-BB–induced increases in mRNAs for PAI-1, TGF-β1, and MCP-1 by 80%, 57%, and 55%, respectively. Ad-DN-p38 significantly inhibited the increases in TGF-β1 and MCP-1 mRNAs by 30% and 37%, respectively, but did not affect PAI-1 expression. On the other hand, Ad-DN-JNK infection significantly prevented the increase in PAI-1 expression but did not inhibit TGF-β1 or MCP-1 expression. Ad-LacZ did not significantly affect PAI-1, TGF-β1, or MCP-1 mRNAs.

As shown in Figure 3B, PD98059 (50 μmol/L) and SB202190 (10 μmol/L) produced similar effects on PAI-1, TGF-β1, and MCP-1 mRNA levels to Ad-DN-ERK and Ad-DN-p38, respectively. SB203580 (10 μmol/L), another p38 inhibitor, showed similar effects to SB202190. SB202474 (10 μmol/L), the negative control of SB202190 or SB203580, did not affect these mRNA levels.

Effect of Ad-DN-p38 Gene Transfer on Neointimal Formation After Rat Balloon Injury

As shown in Figure 4A, p38 was significantly activated with the peak at 5 minutes after balloon injury. Ad-DN-p38 gene transfer produced the significant amount of the transgene expression all over the media (Figure 4B). The peaked activity of p38 in arterial wall at 5 minutes after balloon injury (6.2-fold increase compared with control) was prevented by Ad-DN-p38 gene transfer by 69% (P<0.01, n=3).
whereas there was no significant difference in the peaked activity of ERK or JNK between Ad-DN-p38 and Ad-LacZ (Figure 4C). As shown in Figure 4D, compared with Ad-LacZ, Ad-DN-p38 gene transfer significantly reduced the ratio of intimal to medial area at 14 days after balloon injury. Medial area at 14 days after balloon injury was not different between Ad-LacZ and Ad-DN-p38 gene transfer (Data not shown). Furthermore, compared with Ad-LacZ, Ad-DN-p38 gene transfer significantly reduced intimal BrDU index in balloon-injured artery at 7 days (Figure 4E).

**Discussion**

PDGF, particularly PDGF-BB, is known to play the central role in pathogenesis of various vascular disorders.\(^2\)\(^-\)\(^5\)\(^,\)\(^30\) Both proliferation and migration of SMCs is the essential process for the formation of intimal hyperplasia in vascular disorders.\(^1\)\(^6\) However, the molecular mechanisms underlying proliferation and migration of vascular SMCs by PDGF-BB remain to be well defined. Furthermore, the mechanisms responsible for the PDGF-induced induction of a variety of gene expressions, including PAI-1, MCP-1, and TGF-\(\beta\),\(^13\)\(^-\)\(^15\)\(^,\)\(^31\) which are proposed to be involved in the onset and development of vascular remodeling,\(^16\)\(^17\)\(^,\)\(^19\) remain to be determined. Therefore, in this study, by using recombinant adenoviruses containing DN-JNK, DN-P38, and DN-ERK for specific inhibition of each endogenous MAP kinase, we examined the comparative role of JNK, p38, and ERK in PDGF-BB-induced proliferation, migration, and gene expressions of vascular SMCs.

Accumulating evidence indicates that either JNK or p38 can be proapoptotic, have no effect, or even be apoptotic, depending on the cellular context. Our present work showed that either Ad-DN-JNK or Ad-DN-p38 slightly but significantly inhibited PDGF-BB-induced increase in \(^3\)H-thymidine incorporation, cell number, and entry to S phase from G1 in vascular SMCs, being consistent with the effect of a chemical JNK inhibitor I and a chemical p38 inhibitor SB202190 (Figure 1 and online Figure II). These results show the partial contribution of JNK and p38 to vascular SMC growth. However, in contrast to the slight involvement of JNK and p38 in vascular SMC proliferation, ERK inhibition with Ad-DN-ERK or PD98059 led to a greater inhibition of PDGF-induced vascular SMC proliferation. These observations provided the evidence that among the 3 MAP kinases, ERK plays a dominant role in mediating growth of vascular SMCs in response to PDGF.

p27\(^{\text{kip1}}\) is one of the cyclin-dependent kinase inhibitors (CKIs) that regulate activation of cyclin-dependent kinases required for cell cycle progression.\(^2\) Servant et al\(^2\) have
reported that the downregulation of \( p27^{kip1} \) participates in PDGF-BB–induced vascular SMC proliferation. Furthermore, very recently, using rabbit vascular SMCs, Castro et al\(^{33} \) reported that pharmacological inhibition of ERK leads to the inhibition of PDGF-BB–induced downregulation of \( p27^{kip1} \). Our present work showed that Ad-DN-ERK prevented PDGF-induced downregulation of \( p27^{kip1} \), without affecting CKI \( p21^{cip1} \) or \( p53 \), confirming that ERK pathway participates in PDGF-induced downregulation of \( p27^{kip1} \). Of note are the observations that Ad-DN-JNK had similar effects on \( p27^{kip1} \), \( p21^{cip1} \), and \( p53 \) to Ad-DN-ERK. Therefore, \( p27^{kip1} \) may be involved in the partial suppression of vascular SMC migration by JNK inhibition. Moreover, in a variety of cells, JNK and ERK are known to transduce signaling through activation of activator protein-1 (AP-1), which is composed of c-Jun and c-Fos families.\(^{34} \) By transfer of vascular SMCs with Ad-DN-ERK and Ad-DN-JNK, our results showed that PDGF-mediated AP-1 activation in SMCs is partially attributable to ERK or JNK but not \( p53 \). Taken together with our previous findings on the critical role of AP-1 in PDGF-induced vascular SMC proliferation,\(^{11} \) our observations suggest that the mechanism underlying either JNK- or ERK-mediated SMC proliferation under PDGF-BB stimulation may be partially attributable to AP-1 activation. However, in our present study, because the involvement of ERK in vascular SMC proliferation by PDGF was greater than that of JNK, other molecular mechanisms than \( p27^{kip1} \) and AP-1 are proposed to be more implicated in ERK-mediated vascular SMC proliferation, and additional study is needed to elucidate this point.

Notably, in this work, unlike the case of Ad-DN-JNK and Ad-DN-ERK, infection of SMCs with Ad-DN-p38 significantly inhibited PDGF-BB–induced upregulation of \( p21^{cip1} \) but did not affect \( p27^{kip1} \) or \( p53 \). At present, it is unclear whether the induction of \( p21 \) is responsible for cell proliferation induced by PDGF. However, using several lines of vascular smooth muscle cells, Weiss et al\(^{29} \) recently found that transfection with antisense oligodeoxynucleotide specific to \( p21^{cip1} \) is associated with decreased cyclin D1/cyclin-dependent kinases 4, unexpectedly, resulting in dose-dependent inhibition of PDGF-BB-stimulated DNA synthesis and cell proliferation. Taken together with the recent findings, our present results suggest the possibility that the upregulation of \( p21^{cip1} \) by PDGF-BB plays some role in p38-mediated SMC proliferation by PDGF-BB, although it is unknown whether the findings on cell lines can be applicable for the primary culture of vascular SMCs. It must await additional study to elucidate the difference in the underlying molecular mechanism among the 3 MAP kinases.

Migration of vascular SMCs is regarded as the essential step leading to neointimal hyperplasia,\(^{6} \) and PDGF is the most potent chemoattractant of vascular SMCs.\(^{4} \) To our knowledge, there is no report on the role of JNK in migration of vascular SMCs. Our present work provided the first evidence that JNK is involved in PDGF-BB–induced migration of vascular SMCs. Previous reports indicated that MEK inhibitor PD98059\(^{15} \) and p38 inhibitor SB 203580\(^{26,36,37} \) significantly prevent PDGF-induced vascular SMC migration. Our present study, using Ad-DN-p38 and Ad-DN-ERK, confirmed the importance of p38 and ERK in PDGF-induced migration of vascular SMCs.

In vascular SMCs, PDGF-BB is well-known to induce gene expression of PAI-1, MCP-1, and TGF-\( \beta_2 \),\(^{13-15,31} \) which play a pivotal role in vascular remodeling or diseases.\(^{16,17,19} \) However, whether JNK, p38, or ERK contribute to these gene expressions by PDGF-BB is still unclear. In this study, we obtained the evidence that ERK is responsible for PAI-1, MCP-1, and TGF-\( \beta_2 \) gene expressions, p38 is responsible for MCP-1 and TGF-\( \beta_2 \) expression but not PAI-1, whereas JNK is involved in only PAI-1 expression. Thus, these 3 MAP kinases play differential roles in these gene expressions in vascular SMCs in vitro.

Previously, we have reported that JNK or ERK activation is involved in intimal hyperplasia induced by balloon injury.\(^{24} \) Our present work, using in vivo gene transfer technique of Ad-DN-p38, demonstrated that p38 activation also contributes to intimal SMC proliferation and the subsequent intimal thickening induced by balloon injury, indicating the important role of p38 in vascular remodeling in vivo. Interestingly, despite the smaller inhibition of vascular SMC proliferation in vitro by DN-p38 than DN-ERK, the inhibitory effect of DN-p38 on intimal hyperplasia in vivo was as potent as that of DN-ERK.\(^{24} \) The present work did not allow us to explain the reason for the difference between the in vivo and in vitro effects of DN-p38. However, it is possible that the significant inhibition of intimal hyperplasia by DN-p38 in vivo is only partially mediated by the inhibition of vascular SMC growth, because either vascular SMC migration\(^{6} \) or vascular remodeling-related gene expressions, such as TGF-\( \beta_2 \),\(^{20} \) and MCP-1,\(^{38} \) is reported to participate in intimal hyperplasia in vivo as well as vascular SMC proliferation, and DN-p38 inhibited vascular SMC migration and TGF-\( \beta_2 \) and MCP-1 induction in vitro to a comparable extent to DN-ERK. Alternatively, it is also possible that proliferative signaling pathway in vivo may be significantly different from that induced by PDGF in vitro, because the underlying mechanism of intimal hyperplasia in vivo is well-known to be very complex, and a variety of other factors as well as PDGF participate in the pathophysiology of intimal hyperplasia.\(^{1,6,30} \) Additional study is needed to elucidate the reason for this difference.

**Study Limitations**

Our present in vitro study demonstrates the differential role among the 3 MAP kinases in the regulation of CKIs and PAI-1, MCP-1, and TGF-\( \beta_2 \) gene expressions. However, it is an open question whether our present data obtained by in vitro experiments can apply to the mechanism of MAP kinase–induced vascular hyperplasia in vivo.

In summary, in PDGF-BB–stimulated vascular SMCs, ERK plays a dominant role in vascular SMC growth, whereas ERK, JNK, and p38 are comparably involved in vascular SMC migration. Furthermore, these 3 MAP kinases play differential roles in PAI-1, TGF-\( \beta_2 \), and MCP-1 gene expressions. In conclusion, JNK, p38, and ERK are proposed to be involved in vascular diseases via differential molecular mechanisms and the new therapeutic targets for treatment of vascular diseases.
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References

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Expanded Materials and Methods

Reagents
Human recombinant PDGF-BB was purchased from Sigma Chemical Company. PD98059 was purchased from New England Biolabs, Inc. SB202190, SB202474, JNK Inhibitor I \(^1\) and JNK Inhibitor I negative control were purchased from Calbiochem.

Adenovirus-mediated gene transfer to SMCs

In vitro gene transfer to aortic SMCs was carried out by incubation with Ad-DN-p38, Ad-DN-ERK, Ad-DN-JNK, or Ad-Lac Z at multiplicity of infection (MOI) of 10, 50 or 100 in D-MEM containing 0.1% FBS for 1 hour at 37°C, 5% CO\(_2\) and 95% air. Then, SMCs were made quiescent for 48 hours before being assessed for the expression and the effect of the transferred gene.

ERK, p38 and JNK kinase assays

Kinase assays of ERK, p38 and JNK were performed using each commercial kit (Cell Signaling Technology, Inc.), according to the manufacturer’s instruction. Briefly, 200 µl cell lysate containing 200 µg total protein was incubated with 20 µl of immobilized phospho-p44/p42 MAP kinase (Thr202/Tyr204) monoclonal antibody (for ERK kinase assay) or immobilized phospho-p38 MAP kinase (Thr180/Tyr182) monoclonal antibody (for p38 kinase assay). Then the mixtures were incubated at 4°C overnight with gentle rocking and centrifuged to obtain the pellet. The pellet was suspended with 50 µl kinase reaction buffer containing 100 µM ATP (kinase reaction buffer), and 2 µg Elk-1 fusion protein for ERK kinase assay or 2 µg ATF-2 fusion protein for p38 kinase assay. For JNK kinase assay, 200 µl cell lysate containing 200 µg total protein was added to 20 µl of c-Jun fusion protein beads, incubated at 4°C overnight with gentle rocking, centrifuged and then the resulting pellet was suspended with 50 µl kinase reaction buffer. The reaction was performed at 30°C for 30 min and terminated by addition of SDS sample buffer. Phosphorylated substrates were analyzed with western blotting using phospo-Elk-1 antibody (1:1000) for ERK kinase assay, phospo-ATF-2 antibody (1:1000) for p38 kinase assay.
assay and phospho-c-Jun antibody (1:1000) for JNK kinase assay.

**Measurement of DNA synthesis and cell growth**

SMCs in 6-well plates were stimulated by PDGF-BB (10 ng/ml) for 15 hours and pulsed with 1 μCi/ml $[^3]$H]thymidine for 5 hours. Then cells were washed twice with PBS, incubated for 5 min in 5% trichloroacetic acid, washed by methanol, and dissolved in 99% formic acid. The incorporation of $[^3]$H]thymidine into trichloroacetic acid-insoluble material was measured by liquid scintillation spectrophotometer.

For the assay of cell growth, SMCs in 60 mm plates were stimulated by PDGF-BB (10 ng/ml, 72 hr), and cell number was counted by Coulter counter (Beckman).

**RNA preparation and northern blot analysis**

Total RNA preparation and northern blot analysis was performed, as described in detail. PAI-1, TGF-β 1, MCP-1 and acidic ribosomal phosphoprotein PO (36B4) cDNA were used for hybridization.

**Western blot analysis**

Western blot analysis was carried out, as described. Antibodies against p44/42ERK (#9102; New England Biolabs), phospho- p44/42ERK (#9101S; New England Biolabs), p38 (sc-535; Santa Cruz ), phospho-p38 (#9211S; Cell Signaling Technology ), p27$^{kip1}$ (sc-528; Santa Cruz) (1:200), p21$^{cip1}$ (sc-471; Santa Cruz) (1:200), p53 (sc-99; Santa Cruz) (1:2000) or α-tubulin (CP06-100UG; Calbiochem) (1:1000) were used.

**Electrophoretic mobility shift assay (EMSA)**

For DNA-protein-binding reaction, electrophoretic mobility shift assay was performed as described. The sequence of double-stranded consensus oligonucleotide of AP-1 was 5’-CGCTTTGATGACTCAGCCCGGAA-3’.

**Cell migration assay**

Cell migration was measured with 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD) as described. Briefly, SMCs were washed three times with PBS, harvested with trypsin, and then resuspended at 1×10^6 cell/ml in 0.25% BSA DMEM medium. A polycarbonate membrane
(polyvinylpyrrolidone-free, pore size, 8.0 \mu m) was coated with 100 \mu g/ml type I collagen (Vitrogen 100, Collagen Biomaterials, Palo Alto, CA) before use. The bottom wells of 48-well chamber were filled with 0.25 % BSA DMEM medium containing PDGF-BB (10 ng/ml) or without PDGF-BB as control. The cell suspension (50 \mu l) was placed in the upper chamber. The assembled chamber was incubated 4 hours. Cells that migrated to the lower face of the membrane were fixed and stained with Diff-Quik (International Reagents Corp., JAPAN) and was counted in three fields under 40×magnification. Experiments were performed in triplicated and were repeated three times.

**Flow cytometric analysis**

Cells were trypsinized, washed twice with PBS, and fixed in 70% ethanol at -20°C. Before measurement, fixed cells were centrifuged at 3000 rpm for 5 min and resuspended in dye combination containing 50 \mu g/ml RNase and 25 \mu g/ml propidium iodide (PI) (Sigma) for DNA staining. Cellular DNA content was assessed by FACS flow cytometer (Becton Dickinson). The DNA histogram analysis of cell distribution was measured by using ModFitLT software (Verity software house, Inc.).

**Statistic analysis**

The data are presented as mean±SEM. Statistical significance was determined with one-way analysis of variance (ANOVA), followed by Dunnett test using SuperANOVA (Abacus Concepts, Inc., Berkeley, U.S.A.) when more than two groups were compared. Statistical significance was determined with an unpaired Student’s t-test when two groups were compared. Differences were considered statistically significant at a value of P<0.05.
Online References


Online Figure legends

Online Figure I. Time course of activation of ERK, p38 and JNK in aortic SMCs with PDGF-BB (A) and effects of dominant negative mutants on ERK, p38 and JNK activity in aortic SMCs

(A) Activation of ERK and p38 in aortic SMC stimulated with PDGF-BB (10 ng/ml) was estimated by western blot analysis with anti-phospho-ERK antibody and anti-phospho-p38 antibody, respectively. Activation of JNK in aortic SMC stimulated with PDGF-BB (10 ng/ml) was estimated by JNK kinase assay kit, as described in Materials and Methods. (B). Aortic SMCs were infected with adenovirus containing each dominant negative mutant or not infected with adenovirus (Ad(-)). SMCs, infected with Ad-DN-ERK, Ad-DN-p38 and Ad-DN-JNK, were subjected to kinase assay of ERK, p38 and JNK. The experiments were repeated three times and similar results were obtained.

Online Figure II. Effects of Ad-DN-ERK, Ad-DN-p38 and Ad-DN-JNK on PDGF-BB-induced cell cycle progression of SMCs.

(A) Representative DNA histogram of PI fluorescence in SMCs, assessed by FACS flow cytometer. (B) Percentage of cells in S phase of cell cycle, measured by FACS flow cytometer. Each value represents mean±SEM (n=5). * p < 0.01 vs. PDGF (-). The experiments were repeated three times and similar results were obtained. PDGF(+) and PDGF(-) indicate stimulation of SMCs with PDGF or not, respectively. Ad(-), no adenoviral infection.

Online Figure III. Effects of blockade of ERK, p38 and JNK on PDGF-BB-induced AP-1 DNA binding activity in SMCs.

(A) Aortic SMCs, infected with Ad-DN-ERK, Ad-DN-p38, Ad-DN-JNK or Ad-Lac Z for 48 hours or not infected with adenovirus (Ad(-)), were stimulated with PDGF-BB (10 ng/ml, 2 hours). Each value represents mean±SEM. * p < 0.01 vs Ad(-). (B) Aortic SMCs were treated with PD98059 (50 µM), SB202190 (10 µM), SB203580 (10 µM), SB202474
(10 µM) or vehicle (DMSO) for 1 hour before PDGF-BB (10 ng/ml, 2 hours) stimulation. Each value represents mean±SEM. *p < 0.01 vs control.

Nuclear extracts were prepared from SMCs and electrophoretic mobility shift assay was performed with double-stranded AP-1 consensus oligonucleotide. Super-shift assay was performed with rabbit polyclonal anti-c-Jun IgG (sc-822X) (6 µg). Open arrow in (B) indicates supershifted band of AP-1 by anti-c-Jun IgG (sc-822X) (c-jun Ab(+)). The experiments were repeated three times and similar results were obtained.

**Online Figure IV.** Effects of Ad-DN-ERK, Ad-DN-p38 and Ad-DN-JNK on PDGF-BB-induced migration of aortic SMCs.

Each value represents mean±SEM. * p < 0.01 vs. PDGF(-). Experiments were performed in tetraplicated and were repeated three times.

**Online Figure V.** Induction of PAI-1, TGF-beta1 and MCP-1 mRNA in aortic SMC by PDGF-BB (A) and the inhibition by AG1295 (B)

(A) indicates time course of PAI-1, TGF-beta1 and MCP-1 mRNA levels in aortic SMCs stimulated with PDGF-BB (10 ng/ml). Each value represents mean±SEM. † p < 0.05, * p < 0.01 vs 0 time  (B) Aortic SMCs were treated with or without AG1295 (50 µM) for 1 hour before PDGF-BB (10 ng/ml, 3 hours) stimulation, and northern blot analysis was carried out. The 36B4 (acidic ribosomal phosphoprotein) was used to correct RNA loading and membrane transfer. Each value represents mean±SEM. * p < 0.01 vs. PDGF(+). The experiments were repeated three times.
**Figure I**

A

![Graph showing time (min) vs. various phospho-proteins](image)

B

![Diagram showing PDGF and Ad-DN proteins](image)
Figure II

A

B

Percentage of cells in S phase

Cell number

PDGF (-) Ad (-)

PDGF (+) Ad-Lac Z

PDGF (+) Ad-DN-ERK

PDGF (+) Ad-DN-p38

PDGF (+) Ad-DN-JNK

PI fluorescence

p < 0.01

p < 0.01

p < 0.01

p < 0.01

p < 0.01

p < 0.01

*
Figure III
Figure IV

![Graph showing relative cell number with PDGF (-) and PDGF (+) conditions.]

- Ad (-)
- Ad-Lac Z
- Ad-DN-ERK
- Ad-DN-p38
- Ad-DN-JNK

* p < 0.01

Legend:
- PDGF (-)
- PDGF (+)
Figure V

A

B

Time (hours)

Relative density of mRNA

PAI-1
TGF-beta 1
MCP-1
36B4

Relative density of mRNA

PDGF (-) PDGF (+) PDGF (+) + AG1295

PAI-1
TGF-beta 1
MCP-1
36B4

PDGF (-) PDGF (+) AG1295 + PDGF(+)

PAI-1 TGF-beta 1 MCP-1