Effects of Dominant-Negative c-Jun on Platelet-Derived Growth Factor–Induced Vascular Smooth Muscle Cell Proliferation

Yumei Zhan, Shokei Kim, Hideo Yasumoto, Masashi Namba, Hitoshi Miyazaki, Hiroshi Iwao

Abstract—Although platelet-derived growth factor (PDGF)-BB is thought to participate in vascular disorders, the mechanism of PDGF-induced vascular smooth muscle cell (SMC) proliferation is not fully understood. This study was undertaken to examine the role of c-Jun in PDGF-BB–induced proliferation of rat aortic SMCs. PDGF-BB (10 ng/mL) significantly increased activator protein (AP)-1 DNA binding activity in SMCs, followed by the increase in [3H]thymidine incorporation and cell number. SMCs were infected with recombinant adenovirus containing TAM67, a dominant-negative c-Jun lacking the transactivation domain of wild c-Jun (Ad-DN-c-Jun), to inhibit endogenous AP-1.

Ad-DN-c-Jun, which specifically blocked AP-1 transcriptional activity, significantly inhibited PDGF-BB–induced increases in [3H]thymidine incorporation or cell number. As shown by flow cytometric analysis, Ad-DN-c-Jun inhibited PDGF-BB–induced entrance of SMCs into S phase, leading to a G1 arrest. Ad-DN-c-Jun attenuated PDGF-BB–induced downregulation of p27Kip1, as shown by Western blot analysis, and the prevented PDGF-BB–induced decrease in cyclin E/cyclin-dependent kinase 2 complex–associated p27Kip1, as shown by immunoprecipitation study. Furthermore, protein kinase assay showed that Ad-DN-c-Jun blocked PDGF-BB–induced activation of cyclin-dependent kinase 2. Our results provide the first evidence that dominant-negative c-Jun inhibits PDGF-BB–induced vascular SMC proliferation by preventing the downregulation of p27Kip1, thereby supporting the important role of c-Jun in vascular SMC proliferation.

(Arterioscler Thromb Vasc Biol. 2002;22:82-88.)

Key Words: platelet-derived growth factor ▪ smooth muscle cells ▪ c-Jun ▪ gene transfer ▪ proliferation

Platelet-derived growth factor (PDGF)-BB is known as a multifunctional growth factor capable of stimulating various quiescent cells to proliferate. Accumulating evidence indicates that PDGF-BB is involved in various vascular diseases by stimulating vascular smooth muscle cell (SMC) proliferation, as reviewed.1 However, the mechanism underlying PDGF-induced vascular SMC proliferation is not fully understood. In vascular proliferative diseases, entry into the progression of vascular cells through the cell cycle is thought to be a key event.2 and PDGF-BB, through activation of the β-PDGFR receptor, is a critical mediator of the lesion formation after vascular injury.3 Therefore, detailed investigation into the molecular mechanism underlying PDGF-BB–induced vascular SMC proliferation is essential not only to elucidate the mechanism of vascular diseases but also to develop a new therapeutic strategy for vascular diseases.

The mitogenic action of PDGF-BB is initiated by its interaction with PDGF-BB receptor. Receptor-mediated relocation of cytoplasmic proteins to the inner surface of the plasma membrane, accompanied by subsequent tyrosine phosphorylation, results in the stimulation of multiple signal-
by PDGF-BB, we constructed a recombinant adenovirus containing the dominant-negative mutant of c-Jun (Ad-DN-c-Jun) and infected rat aortic SMCs with this recombinant adenovirus to inhibit endogenous c-Jun. We obtained the first evidence that c-Jun participates in PDGF-BB–induced vascular SMC proliferation and found a novel mechanism: the downregulation of p27Kip1 by c-Jun is involved in PDGF-BB–induced vascular SMCs proliferation.

Methods

Reagents and Cells

Human recombinant PDGF-BB was purchased from Sigma Chemical Co. Rat aortic SMCs were prepared from thoracic aortas of male Sprague-Dawley rats (Clea Japan, Tokyo, Japan) 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 the addition of PDGF-BB.

Construction of Ad-DN-c-Jun

The dominant-negative mutant of c-Jun, called TAM67, was generated by removal of the transactivational domain of amino acids 3 to 50, 100, 250, or 500 in DMEM containing 0.1% FBS for 1 hour at 37 °C before being assessed for the expression and the effect of the transferred gene.

Adenovirus-Mediated Gene Transfer to SMCs

In vitro gene transfer to aortic SMCs was carried out by incubation with the adenoviral vector with a multiplicity of infection (MOI) of 50, 100, 250, or 500 in DMEM containing 0.1% FBS for 1 hour at 37 °C and 5% CO2/95% air. Then, SMCs were made quiescent for 48 hours before being assessed for the expression and the effect of the transferred gene.

An expanded Methods section is available online at http://atvb.ahajournals.org.
more, we examined the effects of Ad-DN-c-Jun on PDGF-BB–induced aortic SMC proliferation at the above-mentioned MOI and found that Ad-DN-c-Jun at 250 MOI significantly inhibited aortic SMC proliferation, whereas Ad-LacZ at the same MOI did not inhibit proliferation at all. Therefore, in all experiments, we compared the effects of Ad-DN-c-Jun with those of Ad-LacZ at 250 MOI. Aortic SMCs infected with Ad-DN-c-Jun at 250 or 500 MOI expressed significant amounts of DN-c-Jun mRNA (see online Figure II-A, which can be accessed at http://atvb.ahajournals.org) and protein (see online Figure II-B), indicating successful DN-c-Jun gene transfer with adenoviruses. As indicated by gel mobility shift analysis in Figure 1A, Ad-DN-c-Jun infection increased AP-1 DNA binding activity. However, the position of the AP-1 band that was due to Ad-DN-c-Jun was higher than that of the PDGF-BB–induced endogenous AP-1 band. Ad-LacZ did not affect AP-1 DNA binding activity with or without PDGF-BB. As shown by supershift analysis in Figure 1B, PDGF-BB–induced AP-1 was supershifted with the anti-c-Jun antibody recognizing the transactivation domain of wild-type c-Jun (sc-822X), the anti-c-Jun antibody recognizing the conserved DNA binding domain (sc-44X), or the anti-c-Fos antibody. On the other hand, the Ad-DN-c-Jun–induced AP-1 band was supershifted only with the anti-c-Jun antibody recognizing the conserved DNA binding domain, ie, sc-44X, but not with the anti-c-Jun antibody sc-822X or anti-c-Fos antibody (Figure 1B). To confirm the specificity of this increased AP-1 activity that was due to Ad-DN-c-Jun, we examined the serum response element (SRE), nuclear factor (NF)-κB, and stimulatory protein-1 (Sp1) DNA binding activity. Figure 2 shows that Ad-DN-c-Jun infection did not affect the SRE, NF-κB, and Sp1 DNA binding activity. Moreover, as shown by dual luciferase assay in Figure 3, PDGF-BB increased AP-1 transcriptional activity in aortic SMCs, and this increase was significantly inhibited by Ad-DN-c-Jun but not by Ad-LacZ. Furthermore, Ad-DN-c-Jun infection did not change SRE and NF-κB transcriptional activity, indicating specific blockade of AP-1 transcriptional activity by Ad-DN-c-Jun.

Ad-DN-c-Jun Attenuates PDGF-BB–Induced Proliferation Through G1 Arrest

Infection of aortic SMCs with Ad-DN-c-Jun at 250 MOI diminished the PDGF-BB–induced increase in [3H]thymidine incorporation by 42%, whereas Ad-LacZ had no effect (Figure 4A). Ad-DN-c-Jun or Ad-LacZ did not affect [3H]thymidine incorporation in SMCs without PDGF-BB stimulation. Ad-DN-c-Jun also inhibited the PDGF-BB–induced increase in cell number by 38% (Figure 4B), whereas this increase was not affected by Ad-LacZ, even at 500 MOI (Figure 4B). Neither adenoviral infection affected the SMC number without PDGF-BB stimulation (data not shown).
Flow cytometric analysis showed that Ad-DN-c-Jun significantly inhibited PDGF-BB–induced S-phase entrance of SMCs and increased the percentage of SMCs in G0/G1 phase, whereas Ad-LacZ did not significantly affect it (Figure 5).

**Effects of DN-c-Jun on p27Kip1, p21Cip1, p53, and Cyclin-Dependent Kinase 2 Activity**

As shown in Figure 6A, PDGF-BB exposure of aortic SMCs downregulated p27Kip1 in a time-dependent manner, which is consistent with the findings by Servant et al. On the other hand, PDGF-BB increased p21Cip1 in a time-dependent manner and did not change p53 levels.

As shown in Figure 6B, this downregulation of p27Kip1 in SMCs exposed to PDGF-BB (16 hours) was prevented by Ad-DN-c-Jun (250 MOI) by 62% but not by Ad-LacZ. In SMCs not subjected to PDGF-BB stimulation, neither Ad-LacZ nor Ad-DN-c-Jun significantly affected p27Kip1.

p27Kip1 is known to inhibit the cyclin-regulatory subunit and catalytic cyclin-dependent kinase (Cdk) complex activity, including cyclin E/Cdk2 activity. Cyclin E/Cdk2 activity is essential for entry into S phase. To further elucidate the molecular mechanism of PDGF-BB–induced SMC proliferation, an immunoprecipitation study with anti–cyclin E antibody or anti-p27Kip1 antibody was performed to determine cyclin E–associated p27Kip1 levels. As shown in Figure 6C, the addition of PDGF-BB (10 ng/mL, 16 hours) significantly decreased cyclin E–associated p27Kip1 levels, which is in good agreement with the results of Servant et al. Compared with Ad-LacZ, Ad-DN-c-Jun infection significantly prevented the PDGF-BB–induced decrease in cyclin E–associated p27Kip1.

Furthermore, Cdk2 activity was measured by the immune complex kinase assay by using histone H1 as a substrate. As shown in Figure 6D, treatment of aortic SMCs with PDGF-BB (10 ng/mL, 16 hours) activated Cdk2, and this activation was significantly blocked by Ad-DN-c-Jun but not by Ad-Lac Z. Neither adenoviral infection affected the Cdk2 activity in aortic SMCs not subjected to PDGF-BB treatment.

As shown in Figure 6E, Ad-DN-c-Jun did not affect p21Cip1 or p53 levels in SMCs with or without PDGF-BB (16 hours). In addition, we examined the effects of Ad-DN-c-Jun on the protein expression of cyclin D1, cyclin E, Cdk2, and Cdk4. Compared with Ad-LacZ, Ad-DN-c-Jun did not prevent an increase in cyclin D1, cyclin E, Cdk2, or Cdk4 proteins by PDGF-BB (data not shown).

**Discussion**

Accumulating evidence supports the notion that PDGF is involved in a variety of vascular diseases by stimulating vascular SMC proliferation, as reviewed. We and other groups of investigators have shown that tyrosine phosphorylation of the PDGF receptor, particularly the PDGF β-receptor activated by PDGF-BB, is enhanced in balloon-injured arteries, hypertensive aortas, and other vascular lesions. Vascular SMC proliferation by PDGF in vitro is mediated by multiple intracellular signaling molecules, such as Ras, protein kinase C, Src, or phosphatidylinositol 3-kinase, as reviewed. In the present study, using an adenoviral expression vector containing TAM67 (a dominant-negative c-Jun gene), we investigated the novel mechanism responsible for PDGF-BB–induced vascular SMC proliferation and obtained the first evidence that c-Jun partially but significantly contributes to PDGF-BB–induced vascular SMC proliferation through the downregulation of p27Kip1, the activation of Cdk2, and the subsequent induction of G0/S transition.

As recently reviewed, c-Jun exerts diverse biological functions, including cell proliferation, transformation, differentiation, and apoptosis, depending on the cell type and the context of other regulatory influences that the cell is receiving. c-Jun plays a critical role in the proliferation of cultured fibroblasts and hepatoblasts, whereas c-Jun overexpression is shown to induce apoptosis in 3T3 fibroblasts and trigger apoptosis in vascular endothelial cells. However, the biological role of c-Jun in vascular SMCs remains to be determined. We have previously reported that AP-1 binding activity, composed of c-Jun, is significantly enhanced in injured rat artery by balloon angioplasty and angiotensin II–mediated hypertension, suggesting that c-Jun may play...
some role in various vascular diseases. These findings encouraged us to elucidate the role of c-Jun in PDGF-BB–induced vascular SMC proliferation. The use of adenoviruses containing the dominant-negative c-Jun gene allowed us to specifically suppress endogenous c-Jun. To our knowledge, the present work provides the first evidence that c-Jun participates in PDGF-BB–induced vascular SMC proliferation. Furthermore, our recent work, 23 in which we used a dominant-negative mutant of extracellular signal–regulated kinase (ERK) or c-Jun amino-terminal kinase (JNK), indicates that ERK and JNK are involved in vascular SMC proliferation. Taken together with the fact that these kinases can activate AP-1,24 these findings suggest that c-Jun may participate in ERK- or JNK-induced SMC proliferation.

The progression that exit cells from G1 phase into S phase is regulated by the activation of multiple holoenzymes composed of cyclin-regulatory subunit and catalytic Cdk.2 Particularly, cyclin E/Cdk2 activity is essential for entry into S phase.17,18,25 Cyclin-dependent kinase inhibitor (CKI) p27Kip1 has been initially characterized as an inhibitor of cyclin E/Cdk2 activation that is responsible for retinoblastoma gene product phosphorylation.26,27 Ectopic expression of p27Kip1 causes cell cycle arrest in G1 phase,26 and conversely, antisense inhibition of p27Kip1 expression suppresses quiescence in fibroblasts.28,29 The expression of p27Kip1 is increased in serum-starved or density-arrested cells and in cells exposed to antiproliferative signals, such as transforming growth factor-β1.30–32 In contrast, p27Kip1 levels decline in response to mitogenic factors,26,31,33 and overexpression of p27Kip1 cDNA in vascular SMCs inhibits mitogen-stimulated [3H]thymidine incorporation.34 Importantly, downregulation of p27Kip1 during late G1 phase plays an important role in cell cycle progression from G1 to S phase.28,32 Recently, Servant et al16 reported that PDGF-BB downregulates p27Kip1 and then enhances Cdk2 activity in the late G1 phase of mitogenic response in vascular SMCs, whereas in angiotensin II–induced hypertrophic response, neither enhanced Cdk2 activity nor downregulation of p27Kip1 is found, showing that the downregulation of p27Kip1 is important in vascular SMC proliferation. However, the mechanism responsible for the downregulation of p27Kip1 by PDGF-BB remains to be determined. Therefore, in the present study, we investigated the role of c-Jun in PDGF-BB–induced downregulation of p27Kip1 in vascular SMCs. Our present results showed that Ad-DN-
c-Jun infection of vascular SMCs prevented PDGF-BB–induced p27Kip1 downregulation (Figure 6B) and inhibited a PDGF-BB–induced decrease in cyclin-E/Cdk2 complex–associated p27Kip1 (Figure 6C), leading to the blockade of Cdk2 activation (Figure 6D) and the subsequent cell cycle arrest in G1 phase (Figure 5). Thus, the present work provided the first evidence that there is a link between c-Jun and p27Kip1 in the regulation of mitogenic cell progression and that c-Jun–dependent p27Kip1 downregulation participates in PDGF-induced proliferation of vascular SMCs.

Although the Kip/Cip family of CKIs, including p27Kip1 and p21Cip1, are thought to be negative regulators of the cyclin/Cdk complex, there have been some reports indicating that the CKIs positively regulate cyclin/Cdk activation.31,32,35,36 Recently, CKI p21Cip1 has been reported to exhibit permissive effects on PDGF-BB–induced vascular SMC cycle progression, as shown by the fact that transfection of several lines of vascular SMCs with p21Cip1 antisense decreased the association of cyclin D1/Cdk4 but not cyclin E/Cdk2 and resulted in the inhibition of PDGF-BB–induced proliferation.37 In the present study, we found that PDGF-BB increased p21Cip1 in a time-dependent manner within 24 hours, which is in good agreement with the previous report.37 However, Ad-DN-c-Jun infection of aortic SMCs did not significantly affect the PDGF-BB–induced increase in p21Cip1 (Figure 6D), thereby providing no evidence for the involvement of p21Cip1 in c-Jun–dependent vascular SMC proliferation by PDGF-BB.

Previous reports have shown that c-Jun–mediated cell proliferation is associated with p53 in mouse embryo fibroblasts38 and with cyclin D1 in NIH 3T3 mouse fibroblasts.39 Nevertheless, in vascular SMCs, the present study showed no effect of PDGF-BB on p53 protein levels (Figure 6A) and no alteration of p53 levels by Ad-DN-c-Jun (Figure 6D), providing no evidence for the important role of p53 in c-Jun–dependent SMC proliferation by PDGF-BB. In the present study, we also examined whether the regulation of cyclin D1, cyclin E, Cdk2, and Cdk4 protein levels is involved in this c-Jun–mediated proliferation and found no contribution of c-Jun to the PDGF-BB–induced increase in these protein levels. Thus, in vascular SMCs, p27Kip1 seems to specifically play a pivotal role in c-Jun–dependent G1/S transition and proliferation induced by PDGF-BB.

The present study did not allow us to elucidate the inhibitory mechanism of PDGF-induced p27Kip1 downregulation by DN-c-Jun. Generally, p27Kip1 levels are controlled by various mechanisms, including (1) posttranscriptional mechanisms, such as the ubiquitin-proteasome pathway and the ubiquitin-independent processing pathway,40 (2) the transcriptional mechanism,16 and (3) the phosphorylation of p27Kip1 by activated cyclin E/Cdk2.41 However, further study is needed to elucidate which mechanism(s) participates in the inhibition of PDGF-induced p27Kip1 downregulation by DN-c-Jun.

In conclusion, c-Jun plays a pivotal role in PDGF-BB–induced vascular SMC proliferation, which is mediated by CKI p27Kip1. Our work provides not only a new insight into the molecular mechanism underlying PDGF-induced vascular SMC proliferation but also a new therapeutic approach for targeting the cell cycle in proliferative vascular diseases.

**Acknowledgments**

This study was supported in part by the Sasagawa Medical Scholarship of the Japan-Sino Medical Association, the Soroptimist Scholarship of Japan Chuao Region of Soroptimist International of the Americas, Inc, and the Hoh-ansha Foundation.

**References**


---

**Zhan et al**

**c-Jun–Mediated SMC Proliferation by PDGF-BB**

---

**Downloaded from** [http://atvb.ahajournals.org/](http://atvb.ahajournals.org/) by guest on October 15, 2017


Effects of Dominant-Negative c-Jun on Platelet-Derived Growth Factor–Induced Vascular Smooth Muscle Cell Proliferation
Yumei Zhan, Shokei Kim, Hideo Yasumoto, Masashi Namba, Hitoshi Miyazaki and Hiroshi Iwao

Arterioscler Thromb Vasc Biol. 2002;22:82-88
doi: 10.1161/hq0102.101821

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/22/1/82

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2002/01/10/22.1.82.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Online figure II

**A**

- PDGF (+)
- PDGF (-)
- Ad (-)
- Ad/Lac-Z
- Ad/DN-c-Jun

- c-Jun mRNA
- DN-c-Jun mRNA
- 36B4 mRNA

**B**

- Ad (-)
- Ad/Lac-Z
- Ad/DN-c-Jun

- DN-c-Jun protein
- tubulin
Online Figure I

A

B