Role of Pim-1 in Smooth Muscle Cell Proliferation*

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Ad, adenovirus; DN, dominant negative; FCS, fetal calf serum; GFP, green fluorescent protein; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; X-gal, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside.

The proliferation of vascular smooth muscle cells (VSMCs) and alterations of their phenotype are implicated in the pathogenesis of atherosclerosis. Arterial wall injury induces the expression of proto-oncogenes, leading to the proliferation of VSMCs. In particular, c-Myc and c-Myb play a central role in cell cycle progression and are essential for VSMC replication. The proto-oncogene Pim-1 cooperates with c-Myc and enhances the transcriptional activity of c-Myb in hematopoietic cells, suggesting that Pim-1 is involved in cell cycle regulation. The aim of this study was to examine the possible involvement of Pim-1 in VSMC proliferation. Pim-1 was substantially induced in neointimal VSMCs of balloon-injured rat carotid arteries, and in vivo infection with a dominant negative Pim-1-expressing adenovirus (Ad-DN-Pim-1) markedly suppressed neointima formation and cell cycle progression in the balloon-injured arteries. In cultured VSMCs, treatment with serum or H₂O₂ induced Pim-1 expression, and H₂O₂-stimulated cell cycle progression and DNA synthesis were almost completely inhibited by DN-Pim-1 overexpression. Furthermore, we performed immunohistochemical staining for Pim-1 in human thoracic aortas and coronary arteries obtained from six individuals at autopsy and found that Pim-1-positive cells are observed predominantly in the thickened intima of the aortas and coronary arteries. To the best of our knowledge, this is the first report showing Pim-1 expression in rodent and human arterial walls. To summarize, Pim-1 expression was observed in the neointima of balloon-injured rat carotid arteries and in human thoracic aortas and coronary arteries showing intimal thickening, and the specific inhibition of Pim-1 function markedly suppressed neointima formation after balloon injury and the proliferation of cultured VSMCs, suggesting that Pim-1 plays a role in VSMC proliferation.

The proliferation of vascular smooth muscle cells (VSMCs) and alterations of their phenotype are implicated in the pathogenesis of atherosclerosis and restenosis after the angioplasty of vascular lesions (1–3). Injury of the arterial wall initiates the synthesis and release of numerous mitogens and the rapid induction of proto-oncogenes, leading to the proliferation of VSMCs. In particular, c-Myc and c-Myb have been found to play a central role in cell cycle progression and to be essential for VSMC replication, which has been demonstrated by various in vitro and in vivo studies (4–7). Furthermore, the down-regulation of these molecules has been reported to interfere with VSMC proliferation (8–12). Cyclin/cyclin-dependent kinases are also important regulators of cell proliferation; it has been shown that the cyclin-dependent kinase inhibitor p21 blocks VSMC proliferation and inhibits neointima formation after injury (13, 14).

Pim-1 is a proto-oncogene that encodes a serine/threonine kinase whose expression is associated with the survival and proliferation of hematopoietic cells (15, 16). Its expression is induced by a variety of cytokines, growth factors, and mitogens (17–19), suggesting that Pim-1 may be an important intermediate in signal transduction. In hematopoietic cells, Pim-1 cooperates with c-Myc (20, 21) and enhances the transcription activity of c-Myb (22, 23). It has also been shown that Pim-1 activates the cyclin-dependent kinase inhibitor p21 (24) and that the antiapoptotic factor Bcl-2 is regulated downstream of Pim-1 (25), suggesting that Pim-1 is involved in cell cycle regulation and apoptosis in hematopoietic cells. In addition, functional analysis by RNA interference in embryonic stem cells has recently demonstrated that Pim-1 is required for their differentiation into endothelial cells and VSMCs (26). In this study, we show that Pim-1 expression is observed in balloon-injured rat carotid arteries and human coronary arteries and that dominant negative Pim-1 markedly suppresses VSMC proliferation in the balloon-injured model and in a cell culture system.

**MATERIALS AND METHODS**

Preparation of Recombinant Adenovirus—An adenovirus was designed to express a dominant negative Pim-1 in which lysine 67 in the ATP binding domain was replaced by methionine, resulting in inactivation of its kinase domain (7, 24). Adenovirus-expressing dominant negative Pim-1 (Ad-DN-Pim-1) was prepared using the Adeno-X Expression System (Clontech) according to the manufacturer’s protocol. Briefly, 6× c-Myc-tagged DN-Pim-1 cDNA was cloned into a shuttle vector, and then the resultant plasmid was cloned into the PI-SceI/I-Dig site of the adenoviral vector. After transfection of the adenovirus plasmids into human embryonic kidney 293 cells, the adenovirus particles were propagated in the cells and purified on a cesium green fluorescent protein; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; X-gal, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside.

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Pim-1 is a proto-oncogene that encodes a serine/threonine kinase whose expression is associated with the survival and proliferation of hematopoietic cells (15, 16). Its expression is induced by a variety of cytokines, growth factors, and mitogens (17–19), suggesting that Pim-1 may be an important intermediate in signal transduction. In hematopoietic cells, Pim-1 cooperates with c-Myc (20, 21) and enhances the transcription activity of c-Myb (22, 23). It has also been shown that Pim-1 activates the cyclin-dependent kinase inhibitor p21 (24) and that the antiapoptotic factor Bcl-2 is regulated downstream of Pim-1 (25), suggesting that Pim-1 is involved in cell cycle regulation and apoptosis in hematopoietic cells. In addition, functional analysis by RNA interference in embryonic stem cells has recently demonstrated that Pim-1 is required for their differentiation into endothelial cells and VSMCs (26). In this study, we show that Pim-1 expression is observed in balloon-injured rat carotid arteries and human coronary arteries and that dominant negative Pim-1 markedly suppresses VSMC proliferation in the balloon-injured model and in a cell culture system.

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FIG. 1. Pim-1 expression in the neointima of balloon-injured rat carotid arteries. Fourteen days after balloon injury, neointima formation is clearly visible, and Pim-1 expression is substantially induced in the neointima of injured arteries (right) but not in the control unjured artery (left). IEL, internal elastic lamina.

FIG. 2. Adenovirus-mediated expression of GFP and DN-Pim-1 in balloon-injured rat carotid arteries. Ad-DN-Pim-1 and Ad-GFP were prepared, and 50 μl of each adenovirus (1 × 10^9 plaque-forming units/ml) was delivered to balloon-injured rat carotid arteries. A, top, representative balloon-injured rat carotid artery 14 days after Ad-GFP infection. As revealed by the GFP signal in this fluorescence micrograph, many neointimal cells are infected after exposure to the adenovirus. Bottom, immunostaining for DN-Pim-1 in balloon-injured rat carotid arteries infected with or without Ad-DN-Pim-1. DN-Pim-1 expression is observed in neointimal smooth muscle cells after exposure to Ad-DN-Pim-1. A, Bottom, DN-Pim-1 protein expression after Ad-DN-Pim-1 infection. Fourteen days after balloon injury, total protein was obtained from uninjected, Ad-GFP injected, or Ad-DN-Pim-1 infected carotid arteries, and Western blot analysis was performed with an antibody for Pim-1.

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chloride gradient followed by extensive dialysis. The titer of the virus stock was assessed by a plaque formation assay using 293 cells and expressed as plaque-forming units. We also prepared two control adenoviruses, namely Ad-LacZ, expressing bacterial β-galactosidase, and Ad-GFP, expressing green fluorescent protein.

Rat Carotid Artery Balloon Injury Model—Male Sprague-Dawley rats (weighing 300–350 g at 9 to 10 weeks of age; Clea Japan, Tokyo, Japan) anesthetized with pentobarbital (50 mg/kg intraperitoneal) were prepared, and 50 μl of each adenovirus (1 × 10^9 plaque-forming units/ml) was delivered to balloon-injured rat carotid arteries. A, top, representative balloon-injured rat carotid artery 14 days after Ad-GFP infection. As revealed by the GFP signal in this fluorescence micrograph, many neointimal cells are infected after exposure to the adenovirus. Bottom, immunostaining for DN-Pim-1 in balloon-injured rat carotid arteries infected with or without Ad-DN-Pim-1. DN-Pim-1 expression is observed in neointimal smooth muscle cells after exposure to Ad-DN-Pim-1. B, DN-Pim-1 protein expression after Ad-DN-Pim-1 infection. Fourteen days after balloon injury, total protein was obtained from uninjected, Ad-GFP injected, or Ad-DN-Pim-1 infected carotid arteries, and Western blot analysis was performed with an antibody for Pim-1.

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Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End-labeling (TUNEL) Assay—Perfusion-fixed, paraffin-embedded tissues were stained for apoptotic nuclei with an in situ apoptosis detection kit (Takara). In brief, after treatment with proteinase K, slides were incubated with a TUNEL reaction mixture, treated with converter-peroxidase solution, and exposed to diaminobenzidine black nickel chromogen. Positive signals were visualized using nickel enhancement and analyzed by a light microscopy for total and TUNEL-positive cells.

Culture and Stimulation of VSMCs—VSMCs were grown from explants of 4-week-old male Sprague-Dawley rat aortic arteries and maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin, streptomycin, glutamine, nonessential amino acids, and 20% fetal bovine serum (FCS). The VSMCs were used for experiments at passages <5. The identity of the smooth muscle cells was confirmed by immunocytochemistry for smooth muscle cell α-actin. Synchronous populations of quiescent cells were obtained by placing cultures in media that contained 0.5% FCS for 48 h. Cells were then infected with adenovirus (Ad-DN-Pim-1 or Ad-LacZ) diluted in starvation medium at a multiplicity of infection of 100 for 1 h. Cells were then stimulated with the addition of serum mitogens (10% FCS in Dulbecco’s modified Eagle’s medium) or H2O2 (5 or 10 μM).

Immunocytochemistry—VSMCs were cultured onto Lab-Tek tissue culture chamber slides (Invitrogen), fixed in ice-cold methanol, quenched using bovine serum albumin, and incubated with a specific primary antibody (anti-Pim-1 (C-20) or anti-c-Myc (9E10 epitope)) for 2 h. After three washes in phosphate-buffered saline, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody for 60 min. After three washes in phosphate-buffered saline, stained cells were examined using fluorescence microscopy.

Northern Blot Analysis—Total RNA was isolated from VSMCs before and after treatment with 200 μM H2O2. Twenty micrograms of the total RNA was size-fractionated by 1.2% agarose gel electrophoresis and transferred to a nylon membrane. The probes used were 5′-labeled Pim-1 cDNA (1 kb; EcoRI-Xhol fragment). After hybridization with the labeled probes at 42°C, the membrane was washed twice with 2× stock was assessed by a plaque formation assay using 293 cells and expressed as plaque-forming units. We also prepared two control adenoviruses, namely Ad-LacZ, expressing bacterial β-galactosidase, and Ad-GFP, expressing green fluorescent protein.

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sodium chloride–sodium citrate (1× SSC, 15 mM sodium citrate and 150 mM NaCl, pH 7.5), which contained 0.1% sodium dodecyl sulfate at 50 °C for 60 min and was then washed with 0.2× SSC and 0.1% SDS at 50 °C for 30 min. Autoradiography was performed using an intensifying screen at 80 °C, and the exposure time was varied so that the band intensity was kept within the linear range.

Determination of DNA Synthesis—Relative rates of DNA synthesis were assessed by the determination of [3H]thymidine incorporation into trichloroacetic acid-precipitable material. Rat VSMCs (5×10^3 cells) were plated per well of a 24-well plate in growth medium. Quiescent cells were then stimulated with serum or H_2O_2. The cells were exposed to [3H]thymidine (Amersham Biosciences) at a concentration of 1 μCi/ml for the last 4 h of the 24-hour stimulation period. The cells were then incubated with 5% trichloroacetic acid at 4 °C for 20 min, dissolved in 1 N NaOH at 37 °C for 20 min and then neutralized. Radioactivity was determined by liquid scintillation counting. Each experiment was performed in triplicate and repeated on VSMCs isolated from several separate donors.

Flow Cytometric Analysis of Cell Cycle Stage—After detachment with 0.25% trypsin, VSMCs were resuspended, fixed in 70% ethanol, stained in a solution of phosphate-buffered saline, propidium iodide (50 μg/ml), and RNase A (500 μg/ml), and measured with a FACScan apparatus (BD Biosciences). The data were analyzed for cell cycle distribution using the ModFitLT program.

Thoracic Aortas and Coronary Arteries from Individuals at Autopsy—Thoracic aortas were obtained at autopsy from three individuals aged 73 (case 1), 45 (case 2), and 35 years (case 3) whose causes of death were acute myocardial infarction (case 1 and case 2) and dilated cardiomyopathy (case 3). Coronary arteries were obtained at autopsy from three individuals aged 62 (case 4), 83 (case 5), and 32 years (case 6) whose causes of death were acute myocardial infarction, aortic aneurysm, and primary pulmonary hypertension, respectively. The study protocol was approved by the hospital ethics committee, and informed consent was obtained from each patient’s bereaved relative.

Statistical Analysis—All values are expressed as mean ± S.D. Data between two groups were compared by a two-tailed unpaired Student’s t test, and data between more than two groups were compared by a one-way analysis of variance followed by Scheffe’s test. A value of p < 0.05 was considered to be statistically significant.

RESULTS

Pim-1 Expression in Balloon-injured Rat Carotid Arteries—To examine the possible involvement of Pim-1 in the progression of atherosclerosis, immunohistochemical staining for Pim-1 was performed in balloon-injured rat carotid arteries. We first examined whether the expression of Pim-1 is induced in the arterial wall. As shown in Fig. 1, 14 days after balloon injury neointima formation was clearly visible, and Pim-1 expression was substantially induced in the neointima of injured arteries but not in the control uninjured artery. To the best of our knowledge, this is the first report on the expression of Pim-1 in the vascular wall.

Inhibition of Pim-1 Activity Attenuates Neointima Formation—To examine the possible role of Pim-1 in smooth muscle cell proliferation, we evaluated the effect of the suppression of Pim-1 activity on neointima formation after balloon injury. For this purpose, we prepared adenovirus-expressing dominant negative Pim-1 (Ad-DN-Pim-1) and control adenovirus-expressing GFP (Ad-GFP) and delivered 50 μl of each adenovirus...
(1 x 10^9 plaque-forming units/ml) to balloon-injured rat carotid arteries. Fig. 2A, top, shows a representative balloon-injured rat carotid artery 14 days after Ad-GFP infection. As revealed by the GFP signal in this fluorescence micrograph, many neointimal cells were infected after exposure to the adenovirus. Immunostaining of balloon-injured arteries after Ad-DN-Pim-1 infection revealed that DN-Pim-1 expression was induced in neointimal smooth muscle cells (Fig. 2A, bottom). Expression of the DN-Pim-1 protein was also confirmed by Western blotting (Fig. 2B). Fig. 3A shows a representative rat carotid artery 14 days after balloon injury. In the injured artery exposed to saline or Ad-GFP, a significant concentric neointima was observed with the medium clearly defined by the internal and external elastic laminae (neointimal cross-sectional area for saline treated was 0.12 ± 0.05 mm^2, and for Ad-GFP treated it was 0.12 ± 0.06 mm^2) (n = 6). Overexpression of DN-Pim-1 resulted in marked suppression of the neointimal cross-sectional area (0.04 ± 0.02 mm^2) (n = 6). The intima/medium ratio in arteries exposed to Ad-DN-Pim-1 (0.29 ± 0.10) was also significantly less than in arteries exposed to either saline (0.88 ± 0.37) or Ad-GFP (0.85 ± 0.26). There was no significant difference in the medial area (Fig. 3B). In addition, to elucidate the mechanism for the suppression of neointima formation in Ad-DN-Pim-1-treated arteries we examined the effect of DN-Pim-1 overexpression on the cell proliferation rate. Many PCNA-positive cells were observed in Ad-GFP-treated arteries but not in Ad-DN-Pim-1-treated arteries (Fig. 4). In addition, because it has recently reported that Pim-1 could regulate apoptosis (27), we performed a TUNEL assay to examine whether the effects of DN-Pim-1 in our present study relate to an increase of apoptosis. In contrast to the decrease in the number of PCNA-positive cells, the number of apoptotic cells was very low in both GFP-treated and DN-Pim-1-treated balloon-injured rat carotid arteries; the number of apoptotic cells was not increased at all even after treatment with DN-Pim-1 (data not shown). Thus, although practically it was very difficult to perform quantitative analysis because of the very low frequency of apoptosis, it is likely that the suppression of neointima formation by DN-Pim-1 is due to the reduction of cell proliferation rather than increased apoptosis.

**FIG. 5.** Induction of Pim-1 mRNA and protein expression by oxidative stress in rat aortic VSMCs. A, VSMCs isolated from Sprague-Dawley rat aortic arteries were treated with H_2O_2 (0–10 μM) for 6 h followed by Western blot analysis (top) and fluorescent immunocytochemistry (bottom). B, VSMCs isolated from Sprague-Dawley rat aortic arteries were treated with H_2O_2 (200 μM) for 0–4 h followed by Northern blot analysis.

**FIG. 6.** Adenovirus-mediated expression of β-galactosidase (β-Gal) and DN-Pim-1 in rat aortic VSMCs. Isolated rat VSMCs were infected with Ad-DN-Pim-1 or Ad-LacZ (multiplicity of infection of 100). Forty eight hours after exposure to Ad-DN-Pim-1 or Ad-LacZ, Western blot analysis (A), immunostaining (B, top row), and X-gal staining (B, bottom row) were performed to confirm the induction of DN-Pim-1 and β-galactosidase expression. More than 95% of cells were positive for DN-Pim-1 upon infection with Ad-DN-Pim-1 but negative upon infection with Ad-LacZ or without infection. In contrast, >95% of cells are X-gal-positive upon infection with Ad-LacZ but negative upon infection with Ad-DN-Pim-1 under the same conditions.
Pim-1 mRNA level is induced by oxidative stress, we performed Northern blot analysis after H2O2 treatment. As shown in Fig. 5B, induction of Pim-1 mRNA was also observed after H2O2 treatment. Similarly, Pim-1 expression was induced by serum (10% FCS) stimulation (data not shown).

Inhibition of Pim-1 Activity Attenuates Cultured VSMC Proliferation—To further elucidate the role of Pim-1 in VSMC proliferation, we infected isolated rat VSMCs with Ad-DN-Pim-1 (multiplicity of infection of 100). As shown in Fig. 6A, 48 h after exposure to Ad-DN-Pim-1, induction of DN-Pim-1
expression was observed by Western blot analysis. Similarly, after exposure to Ad-LacZ (control virus coding β-galactosidase), β-galactosidase expression was also confirmed. Immunocytochemistry for DN-Pim-1 showed that >95% of cells were positive upon infection with Ad-DN-Pim-1 but negative upon infection with Ad-LacZ or without transfection. In contrast, >95% of cells were X-gal-positive upon infection with Ad-LacZ but negative upon infection with Ad-DN-Pim-1 under the same conditions (Fig. 6B). No toxicity was observed in cell culture by infection of the adenovirus at a multiplicity of infection of 100. We examined whether the induction of DN-Pim-1 affects VSMC proliferation by directly counting cell number. As shown in Fig. 7, DN-Pim-1 expression suppressed serum- or H2O2-stimulated cell proliferation, although no such effect was observed after exposure to Ad-LacZ. As shown in Fig. 8, serum-induced [3H]thymidine incorporation was completely suppressed in Ad-DN-Pim-1-treated VSMCs (p < 0.05) but not in Ad-LacZ-treated VSMCs. Similarly, 5 and 10 μM H2O2 induced an increase in [3H]thymidine incorporation, which was also significantly inhibited by the infection of Ad-DN-Pim-1 or treatment with the antioxidant N-acetyl-l-cysteine but not by infection of Ad-LacZ. Furthermore, we performed florescence-activated cell sorter analysis to test whether Ad-DN-Pim-1 affected the cell cycle. Whereas serum and H2O2 treatment produced a significantly higher percentage of cells in the S phase and G2-M phase, DN-Pim-1 expression counterbalanced this phenomenon; Ad-DN-Pim-1 infection resulted in a higher percentage of cells in the G0-G1 phase and a considerably lower percentage of cells in the S phase (Fig. 9). These results suggest that growth inhibition by DN-Pim-1 overexpression is mainly due to the suppression of cell cycle progression.

Pim-1 Expression in the Thickened Intima of Human Thoracic Aortas and Coronary Arteries Obtained from Individuals at Autopsy—Although the characteristics of human and rodent arterial walls is thought to be similar, we cannot exclude the possibility that Pim-1 is induced only in rodents. Thus, to determine whether Pim-1 is also induced in humans, we performed immunohistochemical staining for Pim-1 using human thoracic aortas and coronary arteries obtained from individuals at autopsy. As shown in Fig. 10, Pim-1-positive cells were observed in the thickened intima of thoracic aortas obtained from three individuals at autopsy aged 73 (case 1), 45 (case 2), and 35 years (case 3) whose causes of death were acute myocardial infarction (case 1 and case 2) or dilated cardiomyopathy (case 3). Pim-1-positive cells (brown) are observed predominantly in the thickened intima of the thoracic aortas obtained from all three individuals. Counter-staining for the nucleus (blue) was carried out by Mayer’s hematoxylin. Top row, original magnification ×100; bottom row, original magnification ×400.

DISCUSSION

Injury of the arterial wall initiates the synthesis and release of numerous mitogens and the rapid induction of proto-oncogenes, leading to the proliferation of VSMC and neointima formation (1, 2). In the present study, we identified Pim-1 as one of the candidate molecules involved in this process; the immunoreactivity of Pim-1 was detected in thickened intima induced by balloon injury of rat carotid arteries and in the thickened human aortas and coronary arteries, including atherosclerotic lesions. To the best of our knowledge, this is the first report showing Pim-1 expression in the neointima of balloon-injured rat carotid arteries and in human arteries showing intimal thickening. To evaluate the role of Pim-1, we prepared an adenovirus expressing a dominant negative form...
of the Pim-1 protein and found that the suppression of Pim-1 activity markedly attenuated neointima formation and reduced the number of PCNA-positive VSMCs in the balloon-injured rat carotid artery, indicating that DN-Pim-1 exerted potent anti-proliferative effects. Furthermore, results from the cell culture experiments showed that reduction of cell proliferation by DN-Pim-1 overexpression is mainly due to the suppression of cell cycle progression, implying that this is the mechanism underlying the reduction of neointima formation in balloon-injured carotid arteries infected with Ad-DN-Pim-1. These findings suggest that Pim-1 plays a critical role in VSMC proliferation under certain conditions. Because functional analysis by RNA interference in embryonic stem cells has recently demonstrated that Pim-1 is required for their differentiation into VSMCs (26), it is likely that Pim-1 plays at least two roles in VSMCs; one is a physiological role in differentiation into VSMCs, and another is a role in VSMC proliferation under certain conditions.

It is well known that reactive oxygen species stimulate VSMC growth and expression of various proto-oncogenes (28). Although we have not studied the precise molecular mechanism of the regulation of Pim-1 expression in VSMCs, we demonstrated that Pim-1 expression in cultured VSMCs was markedly induced by oxidative stress (Fig. 5) and that the oxidative stress-mediated increases in cell number, thymidine incorporation, and cell cycle progression were clearly suppressed by DN-Pim-1 overexpression (Figs. 7–9). These results indicate that oxidative stress-mediated Pim-1 expression plays an important role in VSMC proliferation, at least under the in vitro conditions used. Thus, although not examined in this study, it would be important to clarify the mechanism as to how Pim-1 is induced by oxidative stress in VSMCs. Several signal transduction pathways including c-Jun N-terminal kinase, IκB kinase, and p38 mitogen-activated protein kinase are known to be activated by oxidative stress in several cell types. Among the various kinases, inactivation of the c-Jun N-terminal kinase pathway has clearly been shown to suppress VSMC proliferation in a balloon injury model (29), and, thus, we assume that activation of the c-Jun N-terminal kinase pathway at least in part explains the oxidative stress-mediated induction of Pim-1.

Pim-1 is a serine/threonine kinase that is able to phosphorylate and regulate different proteins. Several substrates of Pim-1 have been identified in hematopoietic cells, some of which are potentially involved in cell cycle progression in VSMCs, although the substrate that mediates the proliferative effect of Pim-1 in VSMCs remains unknown. For example, it was reported that Pim-1 phosphorylates and activates Cdc25A (30), a phosphatase that promotes cell cycle progression, and c-Myb (22, 23), a transcription factor that is essential for VSMC replication (7, 8). These factors are likely to be involved in cell cycle progression in VSMCs and, thus, may explain the molecular mechanism for Pim-1-mediated VSMC proliferation. It was also reported that Pim-1 inactivates the cyclin-dependent kinase inhibitor p21 by phosphorylation (24), which could lead to cell cycle progression in VSMCs. Pim-1 may also phosphorylate yet unknown targets involved in the amplification of VSMCs. In addition, Pim-1 cooperates with c-Myc (20, 21), which is well known to play an important role in VSMC pro-
liferation and thereby may contribute to cell cycle progression in VSMCs. Furthermore, although not examined in this study, DN-Pim-1 may also have some other beneficial effects in addition to its anti-proliferative effect. For example, there is the possibility that DN-Pim-1 inhibits the migration of VSMCs and/or the production of the extracellular matrix and, thus, contributes to the suppression of neointima formation.

As shown in this study, Pim-1 is likely to play a crucial role in VSMC proliferation, but how we can apply the present results to clinical medicine (e.g. treatment for occlusive vascular diseases) remains to be elucidated. Although adenovirus experiments have been useful for clarifying the mechanisms underlying various diseases, it would obviously be very difficult to use adenovirus treatment itself in clinical medicine because of its side effect. Thus, it will be very important in the future to investigate how we can apply the various significant data obtained to date with different kinds of adenovirus into clinical medicine. One possibility would be to develop a specific inhibitor of the target molecule. Considering all of our present data, small Pim-1-specific kinase inhibitor molecules could be useful for suppressing VSMC proliferation (neointimal thickening), which eventually leads to occlusive vascular diseases. Another possibility would be to establish new adenovirus systems that do not exert a deleterious side effect.

Taken together, Pim-1 expression was observed in the neointima of balloon-injured rat carotid arteries and in human aortas and coronary arteries showing intimal thickening, and specific inhibition of the Pim-1 function markedly suppressed neointima formation after balloon injury and the proliferation of cultured VSMCs, suggesting that Pim-1 plays a role in VSMC proliferation.

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