PTEN reduces cuff-induced neointima formation and proinflammatory cytokines

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Koide S, Okazaki M, Tamura M, Ozumi K, Takatsu H, Kamezaki F, Tanimoto A, Tasaki H, Sasaguri Y, Nakashima Y, Otsuji Y. PTEN reduces cuff-induced neointima formation and proinflammatory cytokines. Am J Physiol Heart Circ Physiol 292: H2824–H2831, 2007. First published February 2, 2007; doi:10.1152/ajpheart.01221.2006.—An inflammatory response followed by vascular injury plays an important role in neointima formation and development of atherosclerotic lesions, which are in part mediated by proinflammatory cytokines. Using a cuff injury model, we examined the effects of adenovirus-mediated overexpression of phosphatase and tensin homology deleted on chromosome 10 (PTEN) on neointima formation and the proinflammatory response. A cuff was placed around the femoral artery, and adenovirus expressing human PTEN type 1 (AdPTEN) or Escherichia coli β-galactosidase (AdLacZ) was injected between the cuff and the adventitia. After 14 days, the arteries were examined histopathologically and by Western blotting. The significant reduction of neointima formation by AdPTEN compared with AdLacZ was accompanied by reduced cell proliferation and increased adventitial cell apoptosis. AdPTEN also reduced expression of phosphorylated IκB-α, but not nonphosphorylated IκB-α. Western blotting revealed that AdPTEN reduced the cuff injury-induced expression levels of monocyte chemoattractant protein-1, TNF-α, and IL-1β and their expression in all layers of the arterial wall. In contrast, cuff-induced macrophage invasion, which was also inhibited by AdPTEN, was detected only at the intimal surface and in the adventitia. In cultured vascular smooth muscle cells, PTEN directly inhibited ANG II-induced monocyte chemoattractant protein-1 expression as quantified by real-time PCR and Western blotting. Our results suggest that overexpression of PTEN reduces neointima formation, possibly in part through inhibition of the inflammatory response by macrophage invasion and proinflammatory cytokine expression.

vascular inflammation; monocyte chemoattractant protein-1; gene transfer

PHOSPHATASE AND TENSIN homology deleted on chromosome 10 (PTEN) is a multifunctional lipid phosphatase that was originally identified as a tumor suppressor gene (20, 22). In addition to its tumor suppression capability, it is also known to modulate several cell functions, including migration, proliferation, and apoptosis (36). These actions of PTEN are mediated by antagonism of the phosphatidylinositol 3-kinase (PI3K)-mediated signaling pathway (22). Moreover, PTEN also has protein tyrosine phosphatase activity, which downmodulates signaling pathways involving focal adhesion kinase (FAK) or Shc (34, 36). Modulation of FAK/Shc activity by PTEN affects cell migration and adhesion activated by integrins and other tyrosine kinase receptors (34, 36). PTEN has recently been found to play an essential role in cells of the cardiovascular system (25). PTEN is expressed endogenously and regulates many cell functions in cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells (VSMCs), including proliferation, migration, survival/apoptosis, hypertrophy, contractility, metabolism, and mechanotransduction (13, 25, 31). Recently, an inhibitory effect of PTEN on neointima formation was demonstrated in a carotid arterial balloon injury model (14) and in aortocoronary saphenous vein grafts (9), but the detailed mechanism remains unclear, especially against vascular injury-mediated inflammation.

Proliferation and migration of VSMCs during neointima formation induced by arterial injury represent a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic lesions (4). Perivascular cuff placement results in a concentric neointima formation (1), and this animal model would represent the early events of atherosclerosis (11), because the histological changes include proliferation of VSMCs, but not other features of atherosclerosis, such as foam cell formation (11). Nevertheless, the exact mechanism of neointima formation in such injury remains to be elucidated. Recent studies have indicated that the inflammatory response in the adventitia plays an important role in neointima formation (15). Stimulation by the proinflammation process in the vessel wall induces the production of monocyte chemoattractant protein-1 (MCP-1), a potent chemoattractant for monocytes by vascular endothelial cells, VSMCs, and monocytic cells (17). Egashira et al. (8) demonstrated that mutant MCP-1, which acts as a dominant-negative inhibitor of MCP-1, attenuated vascular inflammation induced by perivascular cuff placement due to the suppression of monocyte infiltration and activation and downregulation of inflammatory cytokines, including MCP-1. The expression of these inflammatory cytokines is regulated by transcription factors, such as NF-κB (32). In addition, blockade of the ANG II type 1 receptor, which mediates MCP-1 expression (21), also attenuated cuff-induced perivascular inflammation and neointima formation (21). These results suggest that ANG II-induced MCP-1 expression plays an important role in neointima formation.

The present study was designed to determine the effect of PTEN on cuff-induced neointima formation. Specifically, we...
examined whether adenovirus-mediated overexpression of PTEN can inhibit the cuff-induced inflammatory response, including neointima formation and cytokine expression and ANG II-induced MCP-1 expression in VSMCs.

MATERIALS AND METHODS

Animals. Male Wistar rats (8 wk old) were housed under conditions of constant temperature (22°C) and a 12:12-h dark-light cycle and given a standard diet and water ad libitum. The study was reviewed and approved by the Ethics Committee on Animal Care and Experimentation of the University of Occupational and Environmental Health. The study was carried out in accordance with the Institutional Guidelines for Animal Experiments and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Adenovirus-mediated gene transfer into cuffed arteries. Recombinant E1- and E3-deleted adenovirus expressing human PTEN type 1 (AdPTEN) under transcriptional control of the CAG promoter was kindly provided by Prof. Hirofumi Hamada (Sapporo Medical University, Sapporo, Japan). A control adenovirus, AdLacZ, containing the Escherichia coli LacZ gene under the CAG promoter was constructed by our group, as described previously (33). Adenoviruses were purified by ultracentrifugation on a CsCl density gradient. The titers of the adenoviruses were determined by plaque assays using HEK-293 cells.

Surgery was performed essentially as reported previously (26). Briefly, rats were anesthetized with pentobarbital sodium (45 mg/kg ip), and the femoral artery was isolated from the surrounding tissues. A polyethylene (PE-160) tube (5 mm long, 1.14 mm ID, 1.57 mm OD; Becton Dickinson, Franklin Lakes, NJ) was loosely placed around the artery. A 30-μl aliquot of virus fluid containing AdPTEN or AdLacZ was delivered into the space between the cuff and the artery for viral infection. This virus fluid was dissolved with polyoxyethylene-polyoxypropylene block copolymer F127 (also known as Pluronic or poloxamer F127 or P407; BASF, Ludwigshafen, Germany). After the poloxamer was dissolved in solution, the mixture became liquid at cold temperatures and gelatinous at body temperature (16). Therefore, this virus solution showed a marked increase in gene transfer. The final concentration of the virus fluid was 2 × 10^9 plaque-forming units/ml in 20% poloxamer. Cuffs were placed on arteries of the cuff-treated control rats, but they were not infected with virus. Sham-operated arteries were dissected from the surrounding tissue, but no cuffs were placed around them, nor were they infected with virus.

Measurement of intimal and medial areas. Morphometric analysis was performed as described previously with minor modification (26). Briefly, 2 wk after cuff placement, the rats were killed, and the relevant portions of the femoral arteries were removed, fixed in 10% formalin-neutral buffer solution, and embedded in paraffin. Serial cross sections (5 μm thick, at 250-μm intervals) were prepared and then stained with hematoxylin and eosin. The cross-sectional intimal and medial areas of each lesion in a given photomicrograph were measured using NIH Image software. The ratio of intimal area to medial area was calculated for all sections from each artery, and the average of the ratios from the three sections showing the most stenotic lumen was calculated as the intima-to-media ratio for each artery.

Immunostaining and apoptosis. At 2 wk after cuff placement, the rats were killed and the femoral arteries were harvested, fixed, and cut into sections. They were incubated with the primary antibodies: mouse anti-PTEN antibody (1:50 dilution; Cell Signaling Technology, Beverly, MA), mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:50 dilution; clone PC 10, Dako Cytomation, Glostrup, Denmark), goat anti-TNF-α/TNFSF1A antibody (1:100 dilution; R & D Systems, Minneapolis, MN), goat anti-IL-1β antibody (1:100 dilution; R & D Systems), goat anti-MCP-1 antibody (1:100 dilution; R & D Systems), mouse anti-PTEN antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-PTEN antibody (1:50 dilution; CD68, Chemicon International, Temecula, CA). The sections were incubated with the secondary antibody, visualized by NovaRed (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin. At 3 days after cuff placement, apoptosis was observed by TdT-mediated dUTP nick end labeling (TUNEL). Sections were stained with VasoTACS In Situ Apoptosis Detection Kit (R & D Systems) as instructed by the manufacturer. The stain areas were determined by three investigators, including a pathologist, who were blinded to the treatment.

Cell culture. The procedure described by Okazaki et al. (24) for enzymatic dissociation was used to isolate VSMCs from the thoracic aorta of 8-wk-old male Wistar rats. Primary culture and subculture were carried out in DMEM supplemented with 10% FBS and antibiotics (GIBCO BRL, Paisley, UK) at 37°C in a CO2 incubator. Cells were used at passages 3–9.

Western blotting. At 3 days or 2 wk after cuff placement, the rats were killed and the femoral arteries were harvested and then homogenized with radioimmune precipitation buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany)]. After transfection, cultured VSMCs were stimulated with rat recombinant ANG II (Wako Pure Chemical Industries, Osaka, Japan). Then VSMCs were harvested with radioimmune precipitation buffer at 4°C. Protein concentrations were determined using the Micro BCA Protein Assay kit (Pierce Biotechnology, Boston, MA). Extracts were separated by gel electrophoresis and transferred to membranes, which were incubated with the primary antibodies: mouse anti-phosphory-

Fig. 1. Overexpression of phosphatase and tensin homology deleted on chromosome 10 (PTEN) in the AdPTEN-transfected site of rat femoral arteries 2 wk after cuff placement and transfection. Photomicrographs are representative of AdLacZ-transfected (A) and AdPTEN-transfected (B) arteries stained immunohistochemically for PTEN. Arrow, internal elastic lamina; arrowhead: external elastic lamina. Original magnification ×100. Scale bar, 100 μm.
lated IκB-α antibody (1:1,000 dilution; Cell Signaling Technology), rabbit anti-IκB-α antibody (1:1,000 dilution; Cell Signaling Technology), rabbit anti-MCP-1 antibody (1:5,000 dilution; Chemicon), goat anti-TNF-α/TNFSF1A antibody (1:1,000 dilution; R & D Systems), goat anti-IL-1β antibody (1:1,000 dilution; R & D Systems), and rabbit anti-α-tubulin (1:10,000 dilution; Abcam, Cambridge, UK). The membranes were then incubated with the secondary antibody and detected by the ECL Western Blotting Detection Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). The band intensity was quantified using NIH Image software.

Fig. 2. Effect of PTEN overexpression on neointima formation. A: representative photomicrographs of sections of sham-operated (sham) and cuff-treated control (control), AdLacZ-transfected (LacZ), and AdPTEN-transfected (PTEN) arteries stained with hematoxylin and eosin. Original magnification ×200. Scale bar, 50 μm. B: ratio of intima to media (I/M) area was significantly smaller in AdPTEN-transfected arteries than in cuff-treated control and AdLacZ-transfected arteries. Values are means ± SE of 4 rats in each group. *P < 0.05 vs. sham. †P < 0.05 vs. control and AdLacZ. C: representative photomicrographs of sections stained immunohistochemically for proliferating cell nuclear antigen (PCNA, red). Original magnification ×400. Scale bar, 50 μm. D: PCNA-positive nuclei as percentage of whole nuclei in the intima and media determined by counting the number of nuclei of both types of cells. Percentage of PCNA-positive cells was significantly smaller in AdPTEN-transfected arteries than in cuff-treated control and AdLacZ-transfected arteries. Values are means ± SE of 5–6 rats in each group. *P < 0.05 vs. sham. †P < 0.05 vs. control and AdLacZ. E: representative photomicrographs showing TUNEL staining (blue). Original magnification ×200. Scale bar, 50 μm. Arrow, internal elastic lamina; arrowhead, external elastic lamina.
PTEN REDUCES NEOINTIMA FORMATION AND CYTOKINES

H2827

Statistical analysis. Values are means ± SE. ANOVA with Tukey-Kramer post hoc analysis was used to analyze differences between experimental groups. \( P < 0.05 \) was considered statistically significant.

RESULTS

Overexpression of PTEN in AdPTEN-transfected arteries. We assessed PTEN expression in the cuff-injured arteries by immunohistochemistry to confirm the efficiency of adenoviral gene transfer resulting in PTEN protein production at the transfected arteries. At 2 wk after cuff placement, PTEN protein was homogenously expressed in all layers of the AdLacZ-transfected arteries (Fig. 1A). In contrast, in AdPTEN-transfected arteries, PTEN was also detected in all the arterial layers, but PTEN-positive cells were predominantly localized in the adventitia and outer layer of the media (Fig. 1B), suggesting that PTEN was overexpressed mainly in these areas.

Effect of PTEN transfection on neointima formation, proliferation, and apoptosis. There were no differences in body weight among all groups (data not shown). We examined the effects of adenoviral gene transfer of PTEN or LacZ on neointima formation by estimating the ratio of the intima to media area (Fig. 2, A and B). At 2 wk after cuff placement, the intima-to-media area ratio was significantly lower in AdPTEN-transfected (0.121 ± 0.009, \( n = 4 \)) than in AdLacZ-transfected arteries (0.369 ± 0.077, \( n = 4 \), \( P < 0.05 \) vs. AdPTEN) or cuff-treated control arteries (0.412 ± 0.060, \( n = 4 \), \( P < 0.05 \) vs. AdPTEN). On the other hand, neointima formation in AdLacZ-transfected arteries was comparable to that in cuff-treated control arteries. There were no differences in the area of the media layer among all groups: 0.761 ± 0.091, 0.708 ± 0.112, 0.691 ± 0.105, and 0.605 ± 0.103 mm² in sham-operated and cuff-treated control, AdLacZ-transfected, and AdPTEN-transfected arteries, respectively (\( P = \) not significant by ANOVA).

Immunostaining for PCNA was simultaneously compared between all vessels (Fig. 2C). The vessels showed scarce PCNA-positive nuclei in the neointima and media layers of

Real-time PCR. After VSMCs reached subconfluence, they were infected with AdPTEN or AdLacZ at 5 plaque-forming units/ml in 10% FBS-DMEM for 24 h. The virus solution was removed, and the cells were washed and incubated in serum-free DMEM for starvation. After 24 h, the medium was changed to DMEM containing 10⁻⁷ M ANG II and incubated for 3 h. On the basis of the protocol provided by the manufacturer, mRNA was extracted with the QuickPrep mRNA Purification Kit (GE Healthcare), and the first-strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit (GE Healthcare). Quantitative real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). GAPDH mRNA levels were used as an internal control. The fold change in mRNA levels for MCP-1 was calculated using the \( 2^{-\Delta\Delta CT} \) method, as recommended by the manufacturer (Applied Biosystems). Several samples were run on agarose gels by electrophoresis, and all showed a single unique band at the expected size for each amplicon (data not shown).

**A**

![MCP-1, TNF-α, IL-1β, α-tubulin](image)

**B**

![MCP-1 protein expression level](image)

**C**

![TNF-α protein expression level](image)

**D**

![IL-1β protein expression level](image)

Fig. 3. Effects of PTEN overexpression on IkB-α phosphorylation. Cuffed and sham-operated arteries were harvested 3 days after operation. Equal amounts of tissue extracts (50 μg of protein) were subjected to electrophoresis, and IkB-α phosphorylation levels were normalized to α-tubulin levels. Values are means ± SE (\( n = 4 \)). *\( P < 0.05 \) vs. sham. †\( P < 0.05 \) vs. control and AdLacZ.

Fig. 4. Effects of PTEN overexpression on inflammation. Cuffed and sham-operated arteries were harvested 2 wk after operation. A: vessel homogenates were subjected to Western blotting with antibodies. Equal protein loading (50 μg) was confirmed by blotting with an antibody against α-tubulin. Band intensity of monocyte chemoattractant protein-1 (MCP-1; \( B, n = 6 \)), TNF-α (\( C, n = 4 \), each), and IL-1β (\( D, n = 6 \), each) was quantified by NIH Image. Values are means ± SE. *\( P < 0.05 \) vs. sham. †\( P < 0.05 \) vs. control and AdLacZ.
AdPTEN-transfected arteries: the percentage of total nuclei that stained positive for PCNA was 12.12 ± 5.07% in the neointima and 15.47 ± 3.73% in the media (n = 5; Fig. 2D). On the other hand, PCNA-positive nuclei were readily recognized in the neointima and media of AdLacZ-transfected (40.78 ± 2.31% and 30.72 ± 3.42%, respectively, P < 0.05 vs. AdPTEN, n = 5) and cuff-treated control (34.94 ± 3.30% and 27.47 ± 1.64%, respectively, P < 0.05 vs. AdPTEN, n = 6) groups.

Next, we used the TUNEL method to evaluate the extent of apoptosis. At 3 days after cuff placement, TUNEL-positive nuclei were easily detected in the adventitia in AdPTEN-transfected arteries compared with other arteries (Fig. 2E). However, 2 wk after cuff placement, TUNEL-positive nuclei were scarce in all vessels (data not shown).

Overexpression of PTEN inhibits IkB-α phosphorylation. To investigate whether overexpression of PTEN reduces activation of NF-κB, we examined phosphorylated IkB-α and non-phosphorylated IkB-α expression. Phosphorylated IkB-α expression was increased significantly in cuff-treated control and AdLacZ-transfected arteries compared with sham-operated arteries, suggesting that active NF-κB was increased (Fig. 3). On the other hand, phosphorylated IkB-α expression was markedly reduced in AdPTEN-transfected arteries compared with AdLacZ-transfected arteries (Fig. 3). However, IkB-α expression was comparable among the groups.

Overexpression of PTEN reduces cuff-induced expression of proinflammatory chemokines and cytokines. Using Western blotting, we investigated whether overexpression of the PTEN gene affected the expression of proinflammatory chemokines and cytokines in cuff-injured arteries. MCP-1, TNF-α, and IL-1β expression levels were significantly increased in cuff-treated control and AdLacZ-transfected arteries compared with sham-operated arteries (Fig. 4). In contrast, MCP-1 expression was markedly reduced in AdPTEN-transfected arteries vs. AdLacZ-transfected arteries (Fig. 4A). Similarly, TNF-α and IL-1β expression levels were significantly suppressed in AdPTEN-transfected arteries (Fig. 4, B and C).

Fig. 5. Effect of PTEN overexpression on inflammation of the arterial wall. Cuffed and sham-operated arteries were harvested 2 wk after operation. Representative photomicrographs of sham-operated and cuff-treated control, AdLacZ-transfected, and AdPTEN-transfected arteries were stained immunohistochemically for CD68 (monocytes and macrophages), MCP-1, TNF-α, and IL-1β. Arrow, internal elastic lamina; arrowhead, external elastic lamina. Original magnification ×400. Scale bar, 50 μm.
Macrophage, proinflammatory chemokine, and cytokine localization in cuff-injured arteries. To examine the recruitment of macrophages in the cuffed lesion, we stained the harvested arteries with anti-CD68 antibody. We found many CD68-positive cells recruited to the endothelial surface and adventitia of the AdLacZ-transfected and cuff-treated control arteries (Fig. 5). In the AdPTEN-transfected arteries, we found some CD68-positive cells in the adventitia. Many MCP-1-positive areas were identified in the neointima, media, and adventitia (Fig. 5), although CD68-positive-stained nuclei were found only on the endothelial surface and in the adventitia. Similarly, many TNF-α- and IL-1β-positive areas were detected in the neointima, media, and adventitia (Fig. 5). Furthermore, in agreement with the results of Western blotting, we also found that the chemokine- and cytokine-stained areas were reduced in AdPTEN-transfected arteries compared with AdLacZ-transfected or cuff-treated control vessels (Fig. 5).

Overexpression of PTEN inhibits MCP-1 mRNA and protein expression in cultured VSMCs. Next, we examined whether MCP-1 expression was suppressed by PTEN in vitro. First, we investigated the time of peak MCP-1 mRNA expression as quantified by real-time PCR. VSMCs were stimulated with ANG II for 0, 1, 3, and 6 h. Peak MCP-1 mRNA expression was observed 3 h after stimulation with ANG II (data not shown). Therefore, in the following experiments, VSMCs were stimulated with ANG II for 3 h. MCP-1 mRNA expression was suppressed in AdPTEN-transfected VSMCs compared with AdLacZ-transfected VSMCs (Fig. 6A).

To explore the time of peak MCP-1 protein expression by Western blotting, cultured VSMCs were stimulated with ANG II for 0, 1, 3, 6, 12, and 24 h. Peak MCP-1 protein expression was observed 6 h after stimulation with ANG II (data not shown). After stimulation of VSMCs with ANG II for 6 h, MCP-1 protein expression was suppressed in AdPTEN-transfected VSMCs compared with AdLacZ-transfected VSMCs (Fig. 6B).

DISCUSSION

In the present study, we demonstrated in rats that adenovirus-mediated overexpression of PTEN in the adventitia suppressed neointima formation induced by placement of a cuff around the femoral artery and enhanced accumulation of CD68-positive macrophages in the adventitia. Moreover, cuff-induced neointima formation was associated with increased MCP-1, TNF-α, and IL-1β expression in all layers of the vessel wall; such expression was inhibited in AdPTEN-transfected arteries. The results suggest that PTEN inhibits neointima formation through suppression of proinflammatory chemokine and cytokine expression, in addition to its antiproliferative and proapoptotic properties. In our in vitro study, we also demonstrated that PTEN directly inhibited ANG II-induced MCP-1 expression in VSMCs, which possibly plays an important role in cuff-induced neointima formation.

By histological examination, Kockx et al. (18) demonstrated that neointima formation consisted of aggregation of extracellular matrix and α-smooth muscle actin-positive cells. We reported previously that neointimal cells double-stained with anti-muscle actin antibody (HHF-35) and anti-PCNA antibody (PC 10) (26). Moreover, Tanaka et al. (35) revealed that, in the cuff injury model, neointimal cells were not bone marrow-derived cells, whereas, in wire-mediated endovascular injury and in a model of ligation of the common carotid artery, they were. These results strongly suggest that, in the present study, neointimal cells were composed of proliferating smooth muscle cells migrating largely from medial smooth muscles.

Accumulating evidence suggests that PTEN inhibits cell migration, invasion, and growth by inhibiting the PI3K pathway, the downstream effector molecules of which are PDK1, Akt/PKB, p70S6K, and mammalian target of rapamycin (19). It is well appreciated that PTEN affects a variety of cellular processes, including cell proliferation, survival, and metabolism, cytoskeletal reorganization, and membrane trafficking (19). PTEN also dephosphorylates FAK and Shc, which regulate cell migration (36). These mechanisms consist of two components: 1) a directionally persistent migratory component promoted by the FAK-FAK-p130cas signaling pathway and 2) a random-motility component promoted by the Shc-MAPKK-ERK...
signaling pathway (36). It is likely that, in our in vivo model, PTEN, which was mainly overexpressed in the adventitia and outer layer of the media, reduced cuff-induced neointima formation and also simultaneously reduced cell migration through inhibition of signaling pathways, including the PI3K, FAK-FAK-p130*Sk, and Shc-MAPK-ERK signaling pathways. Other mechanisms through which PTEN reduced cuff-induced neointima formation include inhibition of cell proliferation and proapoptosis. In this study, we demonstrated decreased PCNA expression in the neointima and media layers and increased apoptosis in the adventitia of AdPTEN-transfected arteries.

In the development of atherosclerosis, inflammatory cytokines and chemokines play many important roles, including cell proliferation and migration (3, 28). On the other hand, clinical reports indicated a tendency to restenosis after percutaneous coronary intervention in patients with high expression of inflammatory factors (29). Furthermore, molecular biology studies have indicated that the mRNA expression levels of proinflammatory cytokines and chemokines such as IFN-γ, IL-1β, IL-6, TNF-α, MCP-1, and RANTES (regulated upon activation, normal T cell expressed and presumably secreted) were markedly elevated in cuff-injured arteries compared with control intact arteries (1, 8). Similarly, using Western blotting, we demonstrated that IL-1β, TNF-α, and MCP-1 protein levels were significantly increased in cuff-treated control and Ad-LacZ-transfected arteries compared with sham-operated arteries. Moreover, elevations of cytokine protein levels were completely suppressed in AdPTEN-transfected arteries. The results of immunohistochemistry, which were consistent with those of Western blotting, suggest that inhibition of proinflammatory cytokine and chemokine expression by PTEN involves at least in part neointima formation in the cuff injury model. One possible mechanism of the cuff-induced inflammatory response is the perivascular cuff placement acting as a foreign body, initiating immunologic reactions and inflammation. Several reports have demonstrated infiltration of inflammatory cells in the adventitia of a cuff-injured artery (30). This is compatible with our results, i.e., recruitment of many CD68-positive cells to the adventitia of the cuff-injured arteries. We also found recruitment of some CD68-positive cells to the endothelial surface of the cuff-injured arteries, suggesting that the cuff-induced vascular injury prompted a monocyte-endothelium interaction. In this study, adenovirus-mediated overexpression of PTEN in the adventitia reduced intima formation in cuff-injured arteries and reduced infiltration of CD68-positive cells in the adventitia.

Infiltration of CD68-positive cells into the vessel wall, a key initial step in the process of vascular inflammation and neointima formation, is mediated in part by MCP-1 (7). Egashira et al. (8) demonstrated that the dominant-negative inhibitor of MCP-1 suppressed monocyte infiltration and activation and downregulated expression of inflammatory cytokines, including MCP-1, after arterial injury induced by perivascular cuff placement, resulting in marked inhibition of neointima formation. Therefore, vascular inflammation mediated by MCP-1 seems to play a central role in the development of experimental restenosis and atherosclerosis (8). MCP-1 is best known for its ability to recruit monocytes (7). However, few studies have reported that MCP-1 also directly induces VSMC proliferation in vitro (27). We obtained similar results of MCP-1-induced VSMC growth (data not shown). Furthermore, in our study, the MCP-1-stained area was dissociated from CD68-positive cell localization. Thus it seems that MCP-1 not only regulates macrophage chemotaxis, but it also has other roles, such as cell proliferation.

On the other hand, previous studies have indicated that ANG II stimulates MCP-1 expression via the MAPK-dependent signaling pathway and NF-κB, resulting in vascular inflammation (5, 10). ANG II is a potent mediator of oxidized stress and stimulates the release of cytokines, chemokines, growth factors, and adhesion molecules that mediate vascular wall inflammation and vascular remodeling (6). In the cuff injury model, blockade of angiotensin-converting enzyme and angiotensin type 1 receptor antagonist inhibited neointima formation (2, 21), suggesting that the angiotensin type 1 receptor plays an important role in neointima formation. In VSMCs, we directly demonstrated that ANG II-induced MCP-1 mRNA and protein expressions were completely suppressed by overexpression of PTEN. ANG II-induced MCP-1 expression is mediated by ERK and by NF-κB, which is downstream of Akt/PKB (5, 10). It is likely that the inhibitory effect of PTEN on MCP-1 expression is mediated through inhibition of PI3K. These results are compatible with a previous report indicating that PTEN inhibits ERK and NF-κB (23, 36). In this context, we also showed that overexpression of PTEN suppressed phosphorylation of IkB-α in cuff-treated arteries, suggesting that the effect of overexpression of PTEN on cuff-induced neointima formation is partially mediated by inhibition of NF-κB activation.

In summary, the present study demonstrated that overexpression of PTEN reduced cuff-induced neointima formation, in association with inhibition of macrophage invasion, cell proliferation, and expression of proinflammatory cytokines in the rat cuff injury model. Moreover, overexpression of PTEN directly inhibited ANG II-induced MCP-1 expression in cultured VSMCs. These results support the hypothesis that PTEN not only regulates inflammation and vascular remodeling but also has other roles, such as cell proliferation and apoptosis.

These results support the hypothesis that PTEN acts as a strong anti-inflammatory protein, resulting in inhibition of neointima formation. Taken together with our results and recent experience with rapamycin-coated stents (12), PTEN might be useful in drug-eluting stents.

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PTEN REDUCES NEOINTIMA FORMATION AND CYTOKINES

H2831


