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An essential role of PDCD4 in vascular smooth muscle cell apoptosis and proliferation: implications for vascular disease

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Liu X, Cheng Y, Yang J, Krall TJ, Huo Y, Zhang C. An essential role of PDCD4 in vascular smooth muscle cell apoptosis and proliferation: implications for vascular disease. *Am J Physiol Cell Physiol* 298: C1481–C1488, 2010. First published March 31, 2010; doi:10.1152/ajpcell.00413.2009.—It is well established that vascular smooth muscle cell (VSMC) apoptosis and proliferation are critical cellular events in a variety of human vascular diseases. However, the molecular mechanisms involved in controlling VSMC apoptosis and proliferation are still unclear. In the current study, we have found that programmed cell death 4 (PDCD4) is significantly downregulated in balloon-injured rat carotid arteries in vivo and in platelet-derived growth factor-stimulated VSMCs in vitro. Overexpression of PDCD4 via adenovirus (Ad-PDCD4) increases VSMC apoptosis in an apoptotic model induced by serum deprivation. In contrast, VSMC apoptosis is significantly decreased by knockdown of PDCD4 via its small interfering RNA. In the rat carotid arteries in vivo, VSMC apoptosis is increased by Ad-PDCD4. We have further identified that activator protein 1 is a downstream signaling molecule of PDCD4 that is associated with PDCD4-mediated effects on VSMC apoptosis. In addition, VSMC proliferation was inhibited by overexpression of PDCD4. The current study has identified, for the first time, that PDCD4 is an essential regulator of VSMC apoptosis and proliferation. The downregulation of PDCD4 expression in diseased vascular walls may be responsible for the imbalance of VSMC proliferation and apoptosis. The results indicate that PDCD4 may be a new therapeutic target in proliferative vascular diseases.

programmed cell death 4; activator protein 1

APOPTOSIS (PROGRAMMED CELL DEATH) is an important cellular event in the pathogenesis of atherosclerosis and restenosis, although the significance of apoptosis in atherosclerosis depends on the stage of the plaque, localization, and the cell types involved (5, 9). Indeed, apoptosis of vascular smooth muscle cells (VSMCs) has recently been identified as an important process in a variety of human vascular diseases, including atherosclerosis, arterial injury, and restenosis after angioplasty (3, 10, 17). It is well known that various stimuli such as oxidized lipoproteins, hemodynamic stress, free radicals, and other vascular injury factors can induce VSMC apoptosis. However, the detailed molecular mechanisms within the VSMCs that are involved in this stimulus-mediated apoptosis are still unclear.

Programmed cell death 4 (PDCD4) was initially discovered in blood and tumor cells, where it was upregulated during induced apoptosis and downregulated in many cancer cells (11, 12, 22, 24). The human *PDCD4* gene is localized to chromosome 10q24. Analysis of the deduced protein sequence re-

vealed that PDCD4 comprises 469 amino acids, with two basic domains at the NH₂ terminus and COOH terminus and two conserved α -helical MA-3 domains. Recently, the biological functions of PDCD4 and their potential molecular mechanisms have started to be investigated (11, 12). It was found that PDCD4 could regulate molecules such as p21Waf1/Cip1, Cdk4, ornithine decarboxylase, carbonic anhydrase II, JNK/c-Jun/activator protein 1 (AP-1), urokinase-receptor (u-PAR), and Sp1/Sp3 (11). Accordingly, PDCD4 had a putative role in regulating tumor cell proliferation, apoptosis, and invasion (11, 12). In general, PDCD4 is a potent tumor suppressor (12). However, the biological functions may be cell type specific (11). For example, overexpression of PDCD4 produced elevated levels of p21Waf1/Cip1 in the neuroendocrine cell line Bon-1, but had no effect or a slight decrease in HCT116 cells. Similarly, reduced dUTPase levels associated with high PDCD4 levels might contribute to the tumor suppressor function of PDCD4 in Bon-1 cells, but could not be observed in HCT116 cells (12).

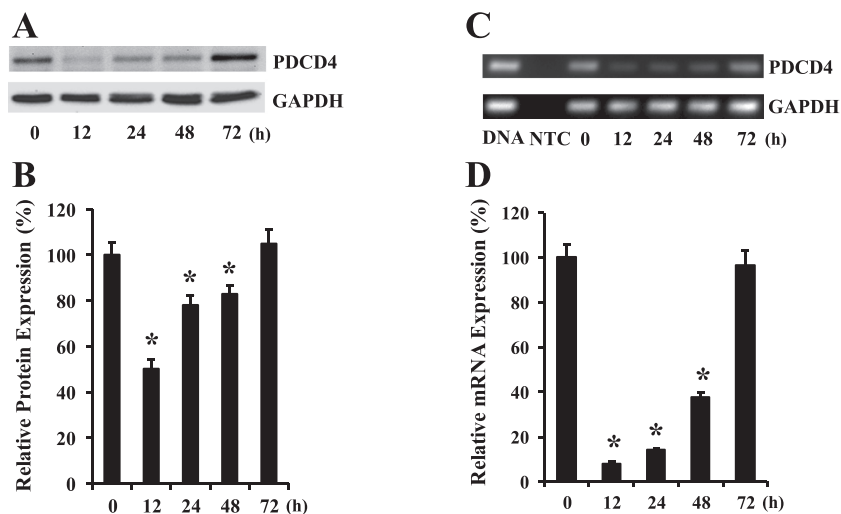
The biological roles of PDCD4 in cardiovascular cell biology and cardiovascular diseases are largely unknown. In our recent studies, we have found that PDCD4 is expressed in both cardiac cells and VSMCs (2, 15). We have further identified that PDCD4 is a target gene of microRNA-21 (miR-21) and is related to miR-21-mediated protective effects against hydrogen peroxide-mediated cell injuries (2, 15). These new findings encouraged us to test the following hypothesis in the current study: PDCD4 may be a critical regulator of VSMC apoptosis in vascular walls. We have found that PDCD4 is significantly downregulated in platelet-derived growth factor (PDGF)-stimulated cells in vitro and in balloon-injured rat carotid arteries in vivo. Our results suggest that PDCD4 is indeed a critical regulator of VSMC apoptosis, one which acts via its downstream signal AP-1.

EXPERIMENTAL PROCEDURES

Rat carotid artery balloon injury model. Carotid artery balloon injury was induced in male Sprague-Dawley rats (250 to 300 g) as described in our previous studies (2, 16). Briefly, rats were anesthetized with ketamine (80 mg/kg)-xylazine (5 mg/kg). Under a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision. A 2F Fogarty catheter (Baxter Edwards) was introduced via an arteriotomy in the external carotid artery, and the catheter was then advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, we inflated the balloon with saline and withdrew it three times from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After injury, the external carotid artery was permanently ligated with

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Fig. 1. The effect of PDGF on the expression of programmed cell death 4 (PDCD4) in cultured vascular smooth muscle cells (VSMCs). Cultured rat VSMCs were treated with PDGF (20 ng/ml). The proteins and RNAs in VSMCs were isolated before treatment and at 12, 24, 48, or 72 h after PDGF stimulation. *A*: representative blots of PDCD4 protein. *B*: quantification of PDCD4 protein levels determined by Western blot. *C*: gel electrophoresis of PCR products of experimental samples (0–72 h), genomic DNA (DNA), or no template control (NTC) after 25 cycles of amplification. *D*: quantification of PDCD4 mRNA levels determined by quantitative (q)RT-PCR. * $P < 0.05$ compared with 0 h group; $n = 6$.



a 6-0 silk suture, and blood flow in the common carotid artery was restored. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey and were consistent with the *Guide for the Care and Use of Laboratory Animals*, published by National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Cell culture. VSMCs were obtained from the aortic media of male Sprague-Dawley rats (5 wk old) using an enzymatic dissociation method as described (14). VSMCs were cultured with DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cultures were maintained at 37°C in a humidified 95% air and 5% CO₂ atmosphere. Cells between *passage 3* and *6* were applied for the experiments.

Construction of the adenovirus expressing PDCD4 and control virus expressing green fluorescent protein. The adenovirus expressing PDCD4 and control virus expressing green fluorescent protein (Ad-GFP) were generated using the Ad-

eno-X Expression Systems 2 kit (Clontech) according to the manufacturer's protocols. Briefly, a 1,410-bp fragment of the full-length coding sequence was amplified with primers tgaatcatgtagtagaaaacgagcagata and taagcttcagtagctctcagggttaagacga using RT-PCR and was inserted into pDNR-CMV donor vector (Clontech) at *EcoR* I and *Hind* III sites. This vector was named pDNR-CMV-PDCD4. The construct was sequenced to confirm the DNA sequence. The PDCD4 fragment was then excised from the pDNR-CMV-PDCD4 and was inserted into the pLP-Adeno-X-CMV vector using Cre recombinase, which was then termed pLP-Adeno-X-CMV-PDCD4. The pLP-Adeno-X-CMV-PDCD4 plasmid, digested by *Pac* I, was used to transfect low-passage human embryonic kidney (HEK) 293 cells to produce recombinant adenovirus with Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen). Adenovirus expressing GFP was generated as described (16). The GFP DNA fragment was excised from pGFP-N3 (Clontech) by digestion of the plasmid with *Sal*I and *Not*I and subcloned into an entry vector, pENTR3C (Invitrogen), producing pENTR3C-

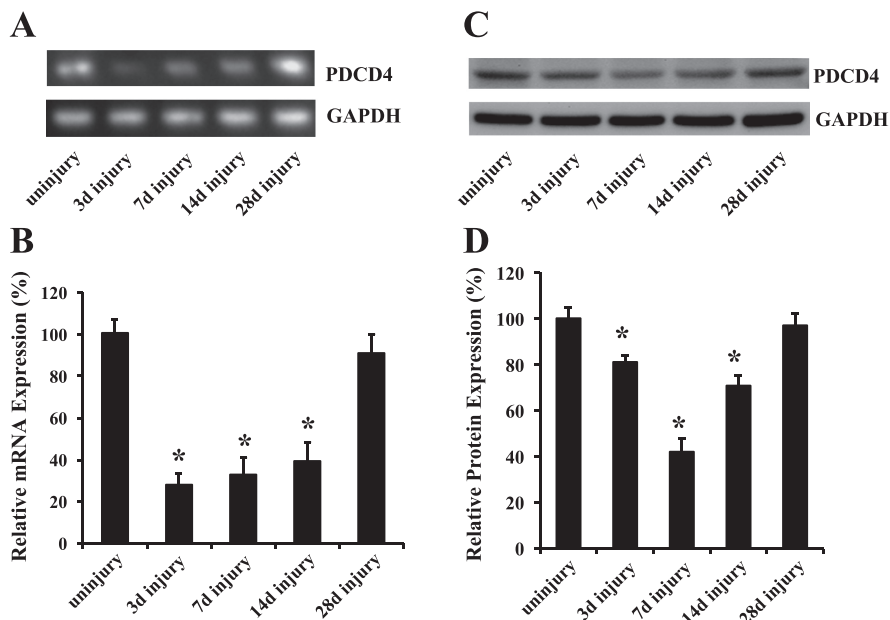


Fig. 2. PDCD4 expression is downregulated in balloon-injured rat carotid arteries. The rat right carotid arteries were isolated at 3, 7, 14, and 28 days (d) after angioplasty for PDCD4 measurement by Western blot and qRT-PCR. The uninjured left carotid arteries were used as the uninjured control group (uninjury). *A*: gel electrophoresis of PCR products of experimental samples after 25 cycles of amplification. *B*: quantification of PDCD4 mRNA levels determined by qRT-PCR. *C*: representative blots of PDCD4 protein. *D*: quantification of PDCD4 protein levels determined by Western blot. * $P < 0.05$ compared with that in uninjured control; $n = 6$.

GFP. pENTR3C-GFP was transformed into *Escherichia coli* DH5 α , and the plasmids were amplified. These plasmids were recombined with pAd/CMV/V5-DEST as described by the manufacturer (Invitrogen), producing pAd-GFP plasmids, which were verified by DNA sequencing. The pAd-enhanced GFP with *Pac I* was transfected into HEK 293A cells. The resulting adenoviruses (Ad-PDCD4 and Ad-GFP) were further amplified by infection of HEK293A cells and purified by cesium chloride gradient ultracentrifugation. The Ad-PDCD4 and Ad-GFP were titrated using a standard plaque assay.

Modulation of PDCD4 expression in cultured VSMCs. PDCD4 expression in cultured VSMCs was upregulated via Ad-PDCD4. The cells were treated with vehicle, control adenovirus Ad-GFP [50 multiplicity of infection (MOI)], or Ad-PDCD4 (10 or 50 MOI). For PDCD4 downregulation, small interfering RNA of PDCD4 (siRNA PDCD4) was applied. The cells were treated with vehicle, control oligo (scramble, 100 nM), or siRNA PDCD4 (100 nM). Forty-eight hours later, the cell protein and RNA were isolated to measure the expression of PDCD4.

Overexpression of PDCD4 in rat carotid arteries via Ad-PDCD4-mediated gene transfer. To upregulate PDCD4 expression in the injured vascular walls, Ad-PDCD4 was trans-

ferred into the balloon-injured rat carotid artery segments using a method established in our lab as described (1, 7, 16). Briefly, immediately after balloon injury, 100- μ l solutions of Ad-PDCD4 or Ad-GFP (5×10^9 plaque-forming units/ml) were infused into the ligated segment of the common carotid artery for 30 min. Then, the external carotid artery was permanently ligated with a 6-0 silk suture, and blood flow in the common carotid artery was restored. Animals with thrombosis after the balloon injury and adenovirus treatments ($\approx 20\%$) were excluded from the study.

Determination of PDCD4 expression by quantitative RT-PCR and Western blot analysis. Total RNA in cultured rat VSMCs and carotid arteries was extracted with TRIzol (Invitrogen) according to manufacturer's protocol. RT-PCR was performed according to RETROscript First Strand Synthesis Kit manual (Ambion). Primers (PDCD4 forward, tgagcagagatcgaacga; PDCD4 reverse, gctaaggacactgccaacacg; GAPDH forward, aagctcactggcatgacctt; and GAPDH reverse, cgcatgtcagatccacaac) were used to amplify *PDCD4* and *GAPDH*, respectively. Diluted RT-PCR solution containing 20 ng RNA per sample was added to a 20- μ l real-time PCR mixture containing 10 μ l SYBR Green I Master (Roche) and 500 nM forward and reverse primer, respectively. Real-time

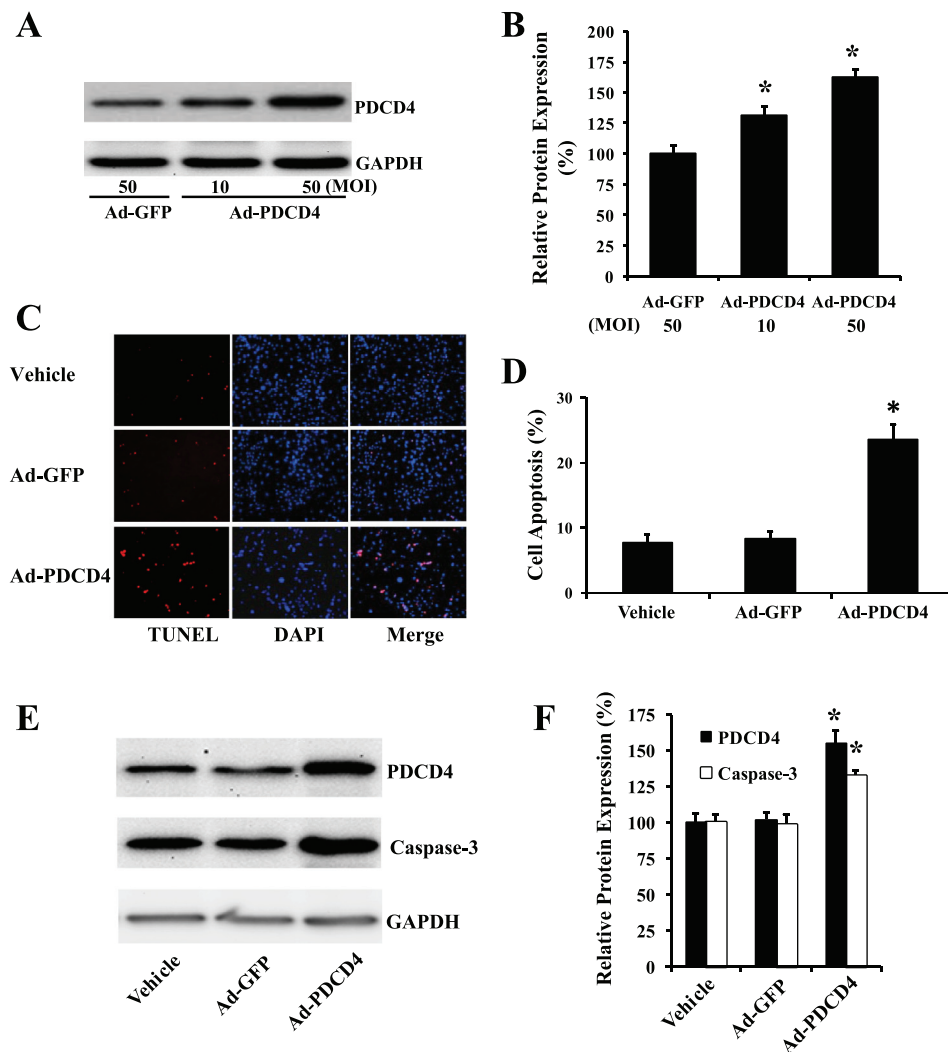


Fig. 3. Overexpression of PDCD4 increases VSMC apoptosis in cultured VSMCs. PDCD4 expression in cultured VSMCs was upregulated by adenovirus expressing PDCD4 (Ad-PDCD4). In this experiment, the cells were treated with vehicle, control adenovirus expressing green fluorescent protein [Ad-GFP; 50 multiplicity of infection (MOI)], or Ad-PDCD4 (10 or 50 MOI). Forty-eight hours later, the cell proteins were isolated to measure the expression of PDCD4. *A*: representative blots of PDCD4 protein. *B*: quantification of PDCD4 protein levels determined by Western blot. * $P < 0.05$ compared with adenovirus control (Ad-GFP) group; $n = 6$. The effect of PDCD4 upregulation on VSMC apoptosis was then determined in an apoptosis model induced by 48 h of serum deprivation. *C*: representative TUNEL-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from VSMCs treated with vehicle, Ad-GFP, or Ad-PDCD4. *D*: quantification of apoptotic cells. * $P < 0.05$ compared with Ad-GFP group; $n = 6$. *E*: representative blots of caspase-3 protein from VSMCs treated with vehicle, Ad-GFP, or Ad-PDCD4. *F*: quantification of caspase-3 protein determined by Western blot. * $P < 0.05$ compared with Ad-GFP group; $n = 6$.

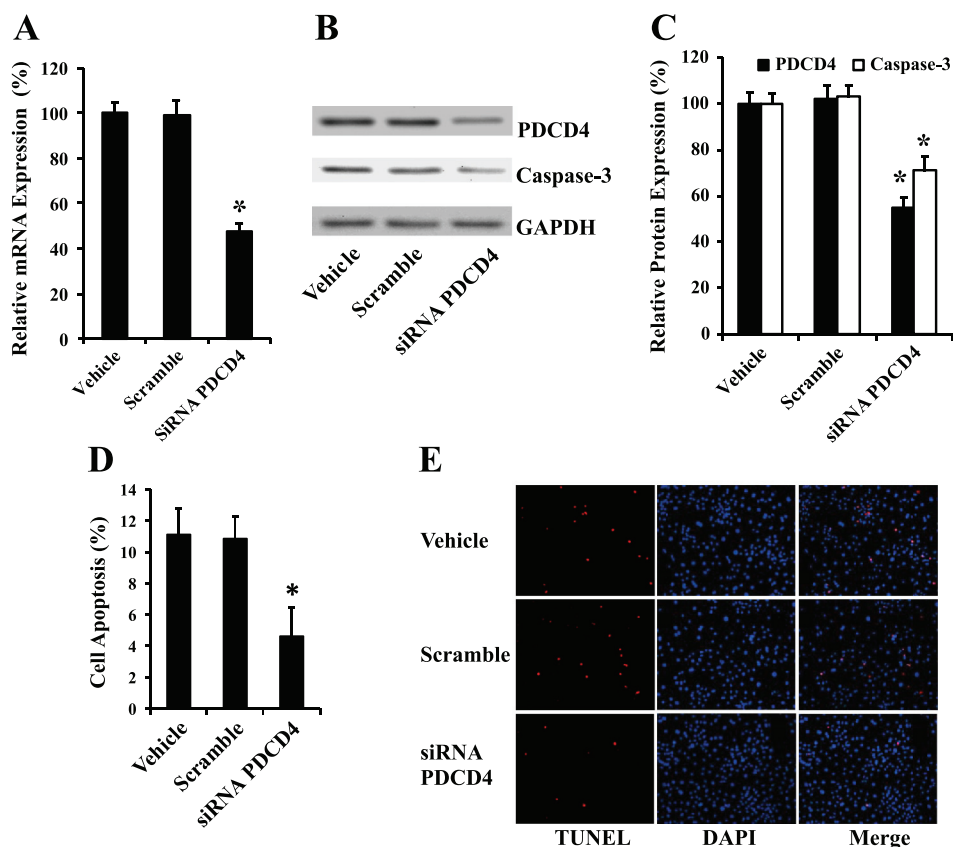
PCR was performed on a Roche LightCycler 480 Real-time PCR System. *GAPDH* was used as the internal reference gene. A rat genomic DNA control and a no template control (NTC) containing DNaseI-treated RNA but no reverse transcriptase were used to further control the quality of the quantitative (q)RT-PCR reactions. After 25 cycles of amplification of experimental samples, genomic DNA, or NTC control, gel electrophoresis of these PCR products was performed. Proteins isolated from cultured rat VSMCs and rat carotid arteries were determined by Western blot analysis. Equal amounts of protein were subjected to SDS-PAGE. A standard Western blot analysis was conducted using PDCD4 antibody (Cell Signaling). *GADPH* antibody (1:5,000 dilution; Cell Signaling) was used as the loading control.

Measurement of the effects of PDGF and vascular injury on PDCD4 expression in cultured VSMCs and in rat carotid arteries. In this experiment, VSMCs at 30–40% confluence were cultured in 0.1% serum for 24 h to reach growth arrest and synchronization. Then, the cells were treated with PDGF (20 ng/ml) or vehicle. The protein was isolated before treatment and at 12, 24, 48, and 72 h after PDGF stimulation for PDCD4 measurement. For the *in vivo* study, the rat carotid arteries were isolated at 3, 7, 14, and 28 days after balloon injury for PDCD4 measurement. Injured carotid artery segments or control uninjured carotid arteries were rinsed with cold phosphate-buffered saline (PBS), frozen immediately in liquid nitrogen, and ground into powder. Homogenization buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μ g/ml phenylmethylsulfonyl fluoride, 100 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM sodium or-

thovanadate) was added to the tissue powder followed by homogenization with Tissue Tearor (Biospect Products) and ultrasonic homogenizer Omni-Ruptor 250. Then the samples were lysed for 20 min on ice. Tissue extracts were transferred to 1.5-ml Eppendorf tubes and cleared by centrifugation at 12,000 rpm for 20 min at 4°C. MicroBCA Protein Assay Kit (Pierce) was used to measure sample protein concentration. RNAs isolated from these cells and arteries were used to determine the PDCD4 expression by qRT-PCR.

VSMC apoptosis. To determine the role of PDCD4 in VSMC apoptosis, we applied a VSMC apoptosis model in which apoptosis was measured after 48 h in serum-free culture (7, 14). In brief, the cultured VSMCs were pretreated with vehicle, Ad-PDCD4 (50 MOI), or Ad-GFP (50 MOI) for 48 h. Then, the cells were cultured in serum-free medium (0% serum) for 48 h to induce apoptosis. VSMC apoptosis in cultured cells was measured by TUNEL analysis and caspase-3 expression measurement as described (2, 7, 15). The VSMCs cultured on coverslips in 24-well plates were fixed in 4% paraformaldehyde. TUNEL staining was done using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's protocol. A VSMC apoptosis index was calculated using the following formula: [(the number of TUNEL-positive cells)/(total cells) \times 100]. VSMC apoptosis in vascular walls *in vivo* was induced by balloon injury of the rat carotid arteries (7). The balloon-injured rat carotid arteries were treated with Ad-GFP or ad-PDCD4 to modulate PDCD4 levels as described above. At 2 wk after angioplasty, the balloon-injured rat carotid arteries were isolated for apoptosis assay. Detection of apoptotic cells *in vivo* was performed using the TUNEL method by

Fig. 4. Knockdown of PDCD4 decreases VSMC apoptosis in cultured VSMCs. PDCD4 expression in cultured VSMCs was upregulated by adenovirus expressing its small interfering (si)RNA (siRNA PDCD4). In this experiment, the cells were treated with vehicle, control oligo (scramble, 100 nM), or siRNA PDCD4 (100 nM). Forty-eight hours later, the cell proteins and RNAs were isolated to measure the expression of PDCD4. **A:** PDCD4 mRNA determined by qRT-PCR. **B:** representative blots of PDCD4 and caspase-3. **C:** quantification of PDCD4 and caspase-3 protein levels determined by Western blot. **D:** quantification of apoptotic cells. **E:** representative TUNEL-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from VSMCs treated with vehicle, scramble, or siRNA PDCD4. * $P < 0.05$ compared with scramble control group; $n = 6$.



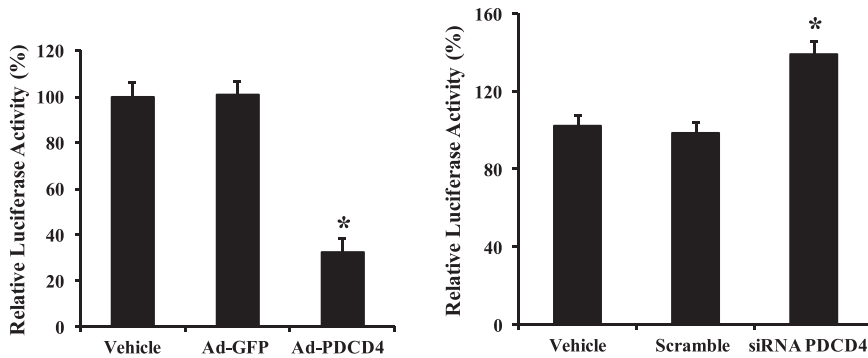


Fig. 5. The effect of PDCD4 on AP-1 activity in cultured VSMCs. Activator protein 1 (AP-1) was measured using luciferase assay. VSMCs pretreated with vehicle, control oligos (scramble, 100 nM), siRNA PDCD4 (100 nM), Ad-GFP (50 MOI), or Ad-PDCD4 (50 MOI) for 4 h were transfected with adenoviral vector (Ad-AP1-Luc) containing a *Photinus pyralis* (firefly) luciferase gene controlled by a synthetic promoter with direct repeats of the transcription recognition sequences for AP-1 for 5 h with 10 plaque-forming units/cell. Luciferase activity was measured after 24 h. *A*: luciferase activity was decreased by Ad-PDCD4. *B*: luciferase activity was increased by siRNA PDCD4. **P* < 0.05 compared with vehicle control; *n* = 5.

immunohistochemistry (7). Apoptotic cells were quantified by counting the percentage of TUNEL-positive cells against total nucleated cells in the tissue section. The average of the six sections was used as the measurement for one animal. TUNEL-positive cells were quantified by automated counting software (NIS-Elements BR 3.0) in six random ×100 fields of a fluorescence microscope. Caspase-3 expression both in cultured VSMCs and in rat carotid arteries was measured by Western blot.

VSMC proliferation. Rat VSMCs in 12-well plates were treated with vehicle, Ad-PDCD4 (50 MOI), or Ad-GFP (50 MOI) for 24 h followed by 0.1% serum culture medium for 24 h to reach synchronization. Then, the cells were cultured with medium containing 5% serum (FBS) for 48 h for the proliferation assay. VSMC proliferation was determined by cell counting and the Click-iT EdU incorporation assay kit (Invitrogen). For cell counting, the cells were detached by trypsinization and resuspended in PBS. The cells were then counted under a

microscope. For the Click-iT EdU incorporation assay, 20 μM EdU was added to the culture medium for incorporation into the replicating cells. After 1 h of incubation, the cells were fixed, and Click-iT reaction cocktail (Click-iT EdU assay) was added to each well for 30 min. The number of EdU-positive cells was measured by automated counting software (NIS-Elements BR 3.0) in six random ×100 fields of a fluorescence microscope.

Measurement of AP-1 activity. AP-1 activity was measured using a luciferase assay as previously described (2). Briefly, adenoviral vector (Ad-AP1-Luc) containing a *Photinus pyralis* (firefly) luciferase gene controlled by a synthetic promoter with direct repeats of the transcription recognition sequences for AP-1 was purchased from Vector Biolabs. Luciferase expression was measured on a scintillation counter by using a dual luciferase reporter system (Promega).

Statistical analysis. All data are presented as means ± SE. For relative gene expression, the mean value of the vehicle

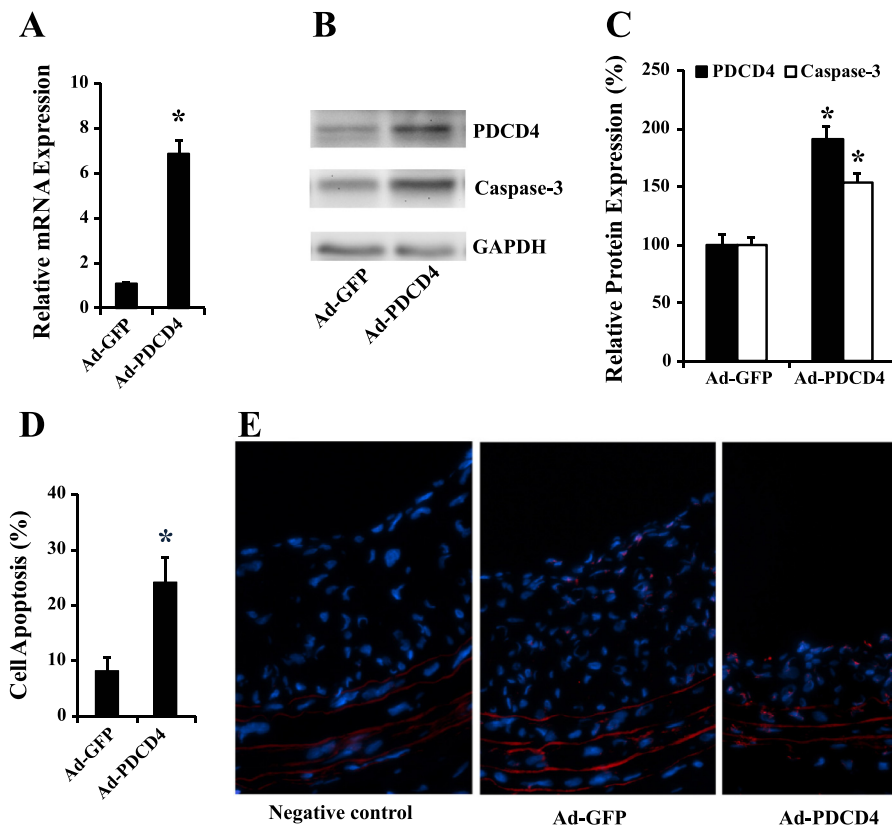


Fig. 6. The effect of PDCD4 restoration on cell apoptosis in rat carotid arteries after angioplasty. The balloon-injured rat carotid arteries were treated with Ad-GFP or Ad-PDCD4 to modulate PDCD4 levels, as described in EXPERIMENTAL PROCEDURES. At 2 wk after angioplasty, the balloon-injured rat carotid arteries were isolated for apoptosis assay. *A*: Ad-PDCD4 increases PDCD4 mRNA expression in balloon-injured rat carotid arteries. *B*: representative Western blots of PDCD4 and caspase-3 from rat carotid arteries treated with Ad-GFP or Ad-PDCD4. *C*: quantification of caspase-3 and PDCD4 proteins determined by Western blot. *D*: quantification of apoptotic cells in rat carotid arteries treated with Ad-GFP or Ad-PDCD4. *E*: representative TUNEL-stained photomicrographs of vessel sections from rat carotid arteries treated with Ad-GFP or Ad-PDCD4. The red color represents the apoptotic cells. **P* < 0.05 compared with Ad-GFP control; *n* = 6.

control group is defined as 100% or 1. Two-tailed unpaired Student's *t*-tests and ANOVA were used for statistical evaluation of the data. SigmaStat statistical analysis program was used for data analysis. $P < 0.05$ was considered significant.

RESULTS

PDCD4 is expressed in vascular walls and in cultured VSMCs, and its expression is significantly downregulated in PDGF-stimulated, proliferating VSMCs and in balloon-injured rat carotid arteries. As shown in Fig. 1, PDCD4 was expressed in cultured VSMCs. Its expression was quickly downregulated after PDGF stimulation at both protein (Fig. 1, *A* and *B*) and mRNA levels (Fig. 1, *C* and *D*). Similarly, PDCD4 expression was also downregulated in balloon-injured rat carotid arteries at 3, 7, and 14 days after angioplasty. At 28 days after injury, its expression was recovered (Fig. 2).

Overexpression of PDCD4 increases VSMC apoptosis. As shown in Fig. 3, *A* and *B*, the expression of PDCD4 was increased by Ad-PDCD4. To determine the potential involvement of PDCD4 in VSMC apoptosis, the effect of PDCD4 overexpression on VSMC apoptosis was determined in a VSMC apoptosis model elicited by 48 h of serum deprivation. The results demonstrated that overexpression of PDCD4 increased the number of apoptotic VSMC cells (Fig. 3, *C* and *D*) and the expression of apoptotic protein caspase-3 (Fig. 3, *E* and *F*). Representative TUNEL-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from VSMCs treated with vehicle, Ad-GFP, and Ad-PDCD4 are displayed in Fig. 3*C*.

Knockdown of PDCD4 decreases VSMC apoptosis. PDCD4 expression at both mRNA (Fig. 4*A*) and protein (Fig. 4, *B* and *C*) levels was downregulated by its siRNA (siRNA PDCD4), compared with vehicle or scrambled control oligo (scramble)-treated cells. Accordingly, caspase-3 levels (Fig. 4, *B* and *C*) and apoptotic cell numbers (Fig. 4, *D* and *E*) were significantly decreased in PDCD4 siRNA-treated VSMCs. Representative TUNEL-stained cell photomicrographs, corresponding total cell photomicrographs (DAPI), and merged photomicrographs (merge) from VSMCs treated with vehicle, scramble, or siRNA PDCD4 are displayed in Fig. 4*E*.

AP-1 is a downstream signaling molecule of PDCD4 that is associated with PDCD4-mediated effects on VSMC apoptosis. As shown in Fig. 5*A*, overexpression of PDCD4 by Ad-PDCD4 inhibited AP-1 activity. In contrast, decreasing PDCD4 expression via siRNA PDCD4 resulted in an increase in AP-1 activity (Fig. 5*B*). The results suggested that AP-1 might be a downstream signaling molecule of PDCD4 that was associated with PDCD4-mediated effects on VSMC apoptosis.

Restoration of PDCD4 expression in balloon-injured rat carotid arteries increases cell apoptosis in vivo. On the basis of the expression change of PDCD4 in injured vascular walls and the cellular effect of PDCD4 identified in cultured VSMCs, we hypothesized that restoration of the downregulated PDCD4 in balloon-injured arteries should increase cell apoptosis in vivo. To test it, PDCD4 expression was increased by Ad-PDCD4 as shown in Fig. 6, *A–C*. Interestingly, caspase-3 levels (Fig. 6, *B* and *C*) and apoptotic cells (Fig. 6, *D* and *E*) in balloon-injured rat carotid arteries were significantly increased compared with those in Ad-GFP-treated vessels. Representa-

tive TUNEL-stained cell photomicrographs from negative control, Ad-GFP, and Ad-PDCD4-treated vessel sections are displayed in Fig. 6*E*.

Overexpression of PDCD4 inhibits VSMC proliferation stimulated by FBS. As shown in Fig. 7, *A* and *B*, the cell proliferation of cultured VSMCs was inhibited by Ad-PDCD4, as demonstrated by the decreased cell number and EdU incor-

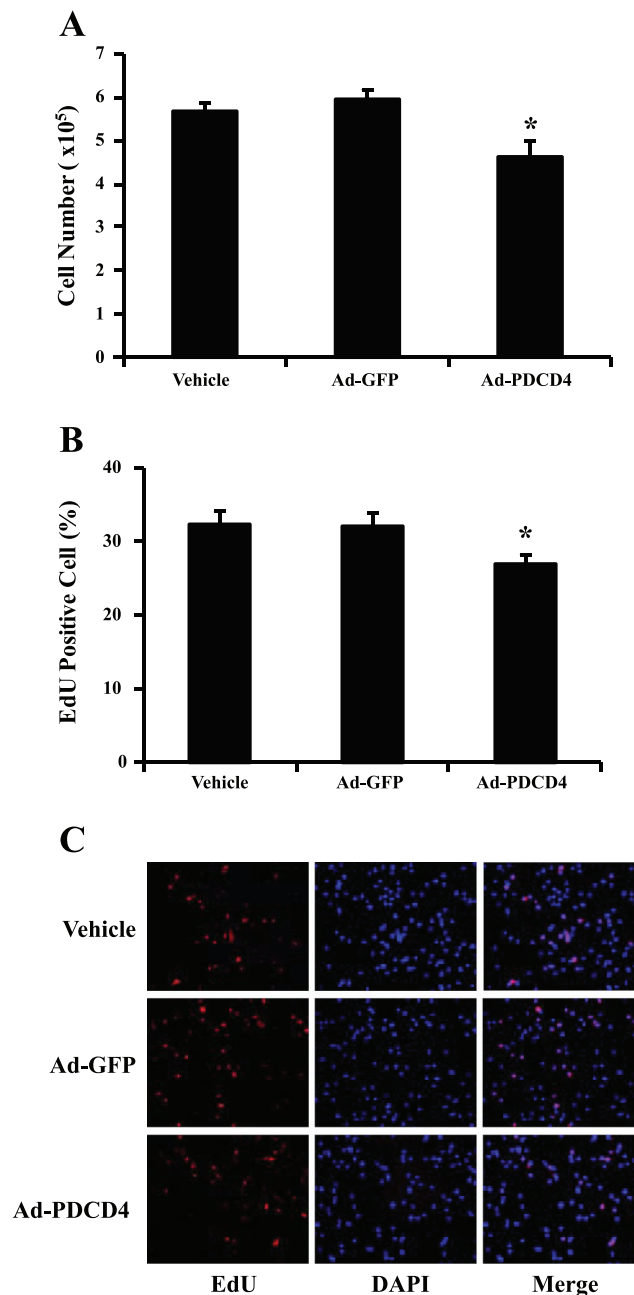


Fig. 7. Upregulation of PDCD4 inhibits VSMC proliferation in cultured VSMCs. In this experiment, VSMCs were treated with vehicle, control adenovirus Ad-GFP (50 MOI), or Ad-PDCD4 (10 or 50 MOI). Forty-eight hours later, cell proliferation was determined by cell counting and Click-iT EdU incorporation assay. *A*: cell proliferation determined by cell counting. *B*: quantification of EdU-positive cells. *C*: representative EdU-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from VSMCs treated with vehicle, Ad-GFP, or Ad-PDCD4. * $P < 0.05$ compared with Ad-GFP group; $n = 6$.

poration. In contrast, the control adenovirus, Ad-GFP, had no effect on VSMC proliferation under our experimental conditions. Representative EdU-positive cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from VSMCs treated with vehicle, Ad-GFP, and Ad-PDCD4 are displayed in Fig. 7C.

DISCUSSION

Atherosclerotic intimal lesion formation and neointimal lesion formation after angioplasty are complex processes in which multiple cellular events are involved: for example, white blood cell activation and infiltration; endothelial cell injury, activation, apoptosis, deendothelialization, and reendothelialization; platelet activation; and VSMC apoptosis, proliferation, migration, and production of extracellular matrix (18–20). Obviously, VSMC apoptosis and proliferation are two important cellular events both in atherosclerosis and restenosis.

Recent studies in cancer cells have revealed that PDCD4 has a strong proapoptotic effect and that its expression is downregulated in many tumors. However, the expression of PDCD4 in VSMCs and the potential expression changes in diseased vascular walls are unclear. In the current study, we have found that PDCD4 is expressed in VSMCs and that its expression is significantly downregulated in balloon-injured vascular walls and in PDGF-stimulated proliferative VSMCs. The negative relationship between VSMC cell number and the expression of PDCD4 indicates that PDCD4 may also have a proapoptotic effect on VSMCs.

In cultured VSMCs, we identified that overexpression of PDCD4 increased VSMC apoptosis. In contrast, VSMC apoptosis is inhibited by downregulation of PDCD4. The proapoptotic effect of PDCD4 on VSMCs was further confirmed in vivo in rat carotid arteries after balloon injury, in which the increased cell apoptosis was demonstrated by restoration of PDCD4 expression. Thus, the relative decrease in VSMC apoptosis induced by downregulation of PDCD4 may be an important and novel mechanism involved in PDGF- and vascular injury-mediated increase in VSMC number.

It is well established that AP-1 is a signaling molecule that plays a key role in determining life or death cell fates in response to extracellular stimuli such as reactive oxygen species, although its final outcome on cell apoptosis is cell specific (8, 21). Several recent studies suggest that AP-1 may be a downstream signaling molecule of PDCD4 in certain types of cells (6, 23). To determine the effect of PDCD4 on AP-1 in VSMCs, PDCD4 was downregulated by siRNA PDCD4 and was upregulated by Ad-PDCD4. The results demonstrated that overexpression of PDCD4 inhibits AP-1 activity. In contrast, AP-1 activity is increased by siRNA PDCD4. The results indicate that AP-1 may be a downstream signal of PDCD4 associated with PDCD4-mediated effects on VSMC apoptosis. However, it is still unclear whether the effect of PDCD4 on AP-1 activity is a direct inhibitory effect or an indirect effect via other signaling molecules in VSMCs.

PDCD4 has a strong negative effect on growth in cancer cells (4). In the current study, the effect of PDCD4 on VSMC growth was investigated. We found that upregulation of

PDCD4 expression in cultured VSMCs inhibited cell proliferation. However, unlike the effect on apoptosis and the antiproliferative on cancer cells (4), the inhibitory effect of PDCD4 on VSMC proliferation was mild and was much weaker than that on cancer cell proliferation. The molecular mechanisms responsible for the weaker antiproliferative effect of PDCD4 in VSMCs compared with that in cancer cells are still unclear. However, a recent report suggests that the action of PDCD4 may be cell type specific (13). We think that the cell type-specific effects of PDCD4 could explain its relative weaker antiproliferative effect on VSMCs.

VSMC apoptosis and proliferation are two cellular events involved in the pathogenesis of vascular neointimal lesion formation. In the current study, we have found that PDCD4 expression in the vascular walls is significantly downregulated after angioplasty. An investigation into the effect of Ad-PDCD4 on neointimal growth in diseased vascular walls is warranted in future studies.

In summary, the current study reveals that PDCD4 is an essential regulator for VSMC apoptosis and proliferation. The downregulated PDCD4 expression in diseased vascular walls may be involved in VSMC proliferation and apoptosis. PDCD4 may be a new therapeutic target in proliferative vascular diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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