Caveolin-1 Can Regulate Vascular Smooth Muscle Cell Fate by Switching Platelet-Derived Growth Factor Signaling From a Proliferative to an Apoptotic Pathway

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Background—Caveolin-1 is a regulator of signaling events originating from plasma membrane microdomains termed caveolae. This study was performed to determine the regulatory role of caveolin-1 on the proliferative events induced by platelet-derived growth factor (PDGF) in vascular smooth muscle cells (VSMCs).

Methods and Results—Treatment of VSMCs with PDGF for 24 hours resulted in a loss of caveolin-1 protein expression and plasma membrane–associated caveolae, despite a 3-fold increase in caveolin-1 mRNA. Pretreatment of VSMCs with chloroquine, an inhibitor of lysosomal function, inhibited the PDGF-induced loss of caveolin-1. These studies demonstrated that caveolin-1 was a target of PDGF signaling events. Adenoviral overexpression of caveolin-1 was associated with a switch in PDGF-induced signaling events from a proliferative response to an apoptotic response. This overexpression inhibited PDGF-induced expression of cyclin D1 in the presence of unaffected mitogen-activated protein kinase activation.

Conclusions—Taken together, these studies suggest that caveolin-1 is an inhibitor of PDGF proliferative responses and might be capable of transforming PDGF-induced proliferative signals into death signals. (Arterioscler Thromb Vasc Biol. 2003;23:1521-1527.)

Key Words: caveolae ■ smooth muscle ■ proliferation ■ platelet-derived growth factor ■ apoptosis

Several studies have demonstrated that platelet-derived growth factor (PDGF) signaling events take place in specialized plasma membrane microdomains known as caveolae. Caveolae are 50- to 100-nm, flask-shaped invaginations on the surface of vascular cells that participate in several diverse cellular functions. Electron microscopy demonstrates large “aggregates or clusters” of caveolae in vascular endothelial and smooth muscle cells that function, in part, as endocytotic vesicles carrying extracellular ligands and solutes to their intracellular destinations.

Caveolae are formed by the oligomerization of a family of proteins termed caveolins (caveolin-1, -2, and -3, respectively) and by the sequestration of cholesterol and glycosphingolipids into membranous microdomains, resulting in a platform for the assembly of signaling complexes at the surface of the cell. Molecules such as endothelial nitric oxide synthase and c-Src undergo posttranslational modifications that allow them to be sequestered in these microdomains. Other mediators of signal transduction can be localized to these domains by binding to caveolin-1 via a 20–amino acid scaffolding motif located on the amino terminus of the molecule. The close proximity of caveolin-1 with growth factor receptors and downstream mediators suggests a functional role for caveolin-1 in the regulation of proliferative events. Several studies have shown that tumor-derived cells or transformed cells often lack caveolin-1, and restoration of caveolin-1 in these cells leads to phenotypic changes, including formation of caveolae and decreased proliferative capacity. These findings have been further substantiated by the generation of caveolin-1–null mice that show a hypercellular lung phenotype and an increased proliferative capacity of embryonic fibroblasts taken from these animals. In addition to modulating the proliferative signals of cells, caveolin-1 has also been shown to facilitate cellular signals leading to apoptosis.

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Vasculoproliferative diseases are associated with a combination of vascular smooth muscle cell (VSMC) proliferative and apoptotic phenotypes. The regulatory role of caveolae and caveolin-1 in VSMC signal transduction is still largely unknown. It has been shown that the transition of smooth muscle cells from a contractile to a synthetic phenotype is associated with a loss of plasma membrane caveolae.
suggesting that the reduction of caveolae might play a mechanistic role in VSMC phenotypic changes. Other studies have shown that caveolin-1 is critical for angiotensin II signaling as well as focal adhesion complex formation in VSMCs.\textsuperscript{19,21} We have previously demonstrated that serum stimulation of human VSMCs resulted in VSMC proliferation associated with the reduction of caveolin-1 protein.\textsuperscript{22} These studies have prompted us to further investigate the role of caveolin-1 in the regulation of growth factor signaling in VSMCs. For these studies, PDGF, an important mediator of VSMC proliferation in vitro as well as in vivo, was used. These studies indicate that caveolin-1 protein expression is negatively regulated by PDGF in human VSMCs and caveolin-1 overexpression results in a switch from a proliferative to an apoptotic phenotype after PDGF stimulation.

Methods

Materials

Anti–caveolin-1, anti–caveolin-2 and anti–cycillin D1 monoclonal antibodies were purchased from Transduction Labs. Anti–Myc polyclonal antibody was from Clontech Laboratories Inc. and anti–cleaved poly–ADP ribose polymerase (PARP) monoclonal antibody and cleaved caspase-9 were purchased from Cell Signaling Technology. Anti–muscle actin monoclonal antibody (HHF35) was purchased from Dako Co. Anti–Erk-2 and anti–phospho–Erk 1/2 polyclonal antibodies were purchased from Santa Cruz. PDGF–BB was purchased from R&D Systems, Inc. All other buffer reagents were purchased from Sigma, unless otherwise noted.

Cell Culture and Western Blotting

Human coronary VSMCs were obtained from Clonetics Inc. To characterize changes in caveolin protein levels in whole-cell lysates, 20 μg total protein was size-fractionated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot analysis was performed as previously described.\textsuperscript{22}

Rabbit Injury Model

Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. New Zealand White rabbits (Harlan Laboratories, Indianapolis, Ind; n = 3) were sedated with ketamine (35 mg/kg IM) and xylazine (5 mg/kg IM). Surgical exposure and arteriotomy of the right femoral artery was performed, and a 3F Fogarty balloon catheter (Baxter Healthcare Corp) was passed into the common iliac artery. The balloon was then inflated in the femoral artery distal to the arteriotomy. Immediately after the animals were killed, injured rabbit iliac arteries were ligated distally, and the incision was closed. After surgery, the animals were administered ketamine (35 mg/kg IM) and xylazine (5 mg/kg IM). Surgical exposure and arteriotomy of the right femoral artery was performed, and a 3F Fogarty balloon catheter (Baxter Healthcare Corp) was passed into the common iliac artery. The balloon was then inflated in the right iliac artery and withdrawn 3 times. The right femoral artery was ligated distally, and the incision was closed. After surgery, the rabbits were fed a high-fat diet consisting of 0.5% cholesterol and 2.3% peanut oil until they were humanely killed at 21 days. Animals were anesthetized by using ketamine (50 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg) cocktail delivered intramuscularly and were then euthanized by using pentobarbital (100 to 125 mg/kg) by intravenous delivery.

Immunohistochemical Analysis of Injured Arteries

Immediately after the animals were killed, injured rabbit iliac arteries were embedded in OCT (Sakura) and flash-frozen in an LN\textsubscript{2}-cooled isopentane bath. Sections (5 μm) were fixed in acetone for 10 minutes at −20°C, blocked with 10% normal goat serum, and incubated for 1 hour at room temperature with 5 μg/mL of either anti–caveolin-1 or anti–caveolin-2 monoclonal antibodies or 0.5 μg/mL for human muscle actin. Control sections were incubated with mouse immunoglobulin G at the same concentration. Sections were then washed and incubated with a goat anti-mouse biotinylated antibody (Amersham) for 30 minutes. Caveolin-1 and caveolin-2 were detected by incubation with streptavidin–alkaline phosphatase (Vector Laboratories) for 45 minutes and visualized with Vector Blue (Vector Laboratories). Actin was detected by incubation with streptavidin–horseradish peroxidase (Vector Laboratories) for 45 minutes and visualized with Sigma Fast diaminobenzidine (Sigma).

Quantification of Caveolin-1 mRNA by Real-Time RT-PCR

Total RNA was isolated from VSMCs with Trizol reagent (Life Technologies). cDNA was prepared with an oligo(dT) primer and Moloney leukemia virus reverse transcriptase. After reverse transcription (RT), the cDNA product was amplified by polymerase chain reaction (PCR) with Taq DNA polymerase and standard protocols. Primers for human caveolin-1 used in this experiment were as follows: forward, 5′-GGGGCAAATACGTAGACTCG-3′ and reverse, 5′-CAGCAAGCGTGAAACCCAGT-3′. The amplified product was separated on a 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination. For the quantification of caveolin-1 mRNA, real-time PCR was performed with the Roche LightCycler (Roche Molecular Biochemicals) with SYBR green as the fluorophore (Molecular Probes). After electrophoresis in 1% agarose gel, the expected PCR product was cut out and eluted into Tris-HCl with use of a DNA elution kit (Qiagen). The caveolin-1 standards were prepared at concentrations of 10\textsuperscript{7}, 10\textsuperscript{6}, 10\textsuperscript{5}, and 10\textsuperscript{4} copies/μL. The linear relation between copy number and cycle number was then determined. The standard curve was used to calculate the copy number in the experimental sample.

Quantification of Caveolae by Electron Microscopy

VSMCs were plated onto tissue-culture-chamber slides (Laboratory-Tek, Nunc) and serum-deprived for 24 hours before treatment with 20 ng/mL PDGF for 24 hours. Both serum-free and PDGF-stimulated VSMCs were fixed overnight in 4% formaldehyde plus 1% glutaraldehyde in phosphate buffer. Specimens were rinsed in phosphate buffer, postfixed in 1% Os\textsubscript{4}O\textsubscript{4} and stained en bloc in 2% aqueous uranyl acetate. VSMCs were then dehydrated in a series of ethanol to 100%, infiltrated, and embedded in Spurr resin.

Immunofluorescence Imaging

VSMCs were plated onto tissue-culture-chamber slides (Laboratory-Tek, Nunc) and serum-deprived for 24 hours before treatment with 20 ng/mL PDGF or 20 ng/mL PDGF + 100 μmol/L chloroquine (Sigma). After treatment, cells were loaded with lysotracker Red (Molecular Probes) for 1 hour and fixed with 2% paraformaldehyde for 30 minutes. Cells were then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 2 minutes and blocked with 10% normal goat serum for 30 minutes. Cells were then incubated for 1 hour with mouse anti–caveolin-1 antibody at a dilution of 1:200 and for 1 hour with a goat anti–mouse fluorescein isothiocyanate–labeled antibody at a dilution of 1:1000. Cells were then incubated for 5 minutes with 10 μg/mL Hoechst 33258 (Sigma) to stain for nuclei. After being labeled, coverslips were mounted on the slides with Prolong mounting medium (Molecular Probes) and visualized by confocal microscopy.

Preparation and Infection of VSMCs With Adenoviral Constructs

First-generation recombinant adenoviruses were prepared as previously described.\textsuperscript{23} In brief, the cDNA encoding Myc-tagged canine caveolin-1 was inserted into a shuttle plasmid and recombined in 293 cells with E1- and E3-deleted adenoviral (Ad5) DNA. An adenovirus with no insert was used as a viral control (Ad-ΔE1).

Cell Proliferation Assays

Two different approaches were used to quantify cell proliferation of virally infected human VSMCs. In the first set of experiments, human VSMCs were infected with either Ad-Cav1 or Ad-ΔE1 at a multiplicity of infection (MOI) of 10 to 30 for 48 hours under serum-free conditions and then trypsinized and plated in triplicate into 60-mm dishes at a cell density of 10\textsuperscript{5} cells per dish. Cells were then fed normal growth medium and counted every 2 days with use of a hemocytometer. Alternatively, human VSMCs were plated into
96-well plates and infected with either Ad-Cav1 or Ad-ΔE1 at an MOI of 30 for 48 hours under serum-free conditions and then fed with medium containing 20 ng/mL PDGF for the determination of bromodeoxyuridine (BrdU) incorporation. BrdU incorporation into human VSMCs was assessed with a BrdU cell proliferation kit from Boehringer Mannheim.

**Apoptosis Assays**

Apoptosis was analyzed by terminal dUTP nick end-labeling (TUNEL) and Western blot analysis of cleaved caspase-9 and cleaved-PARP. Before each assay, human VSMCs were infected with either Ad-Cav1 or Ad-ΔE1 at an MOI of 30 for 48 hours under serum-free conditions and treated with 20 ng/mL PDGF for 1 to 3 days. TUNEL was performed with use of an apoptosis detection kit (Promega). In brief, VSMCs were plated and infected in Laboratory-Tek II chamber slides (Nunc) and stimulated with 20 ng/mL PDGF for 24 hours. Cells were fixed and labeled with fluorescein-12-dUTP to assay for DNA strand breaks. VSMCs were then stained with propidium iodide to visualize all cells. Apoptotic cells were determined by the appearance of green fluorescence on a red background (propidium iodide). Western blotting for both cleaved caspase-9 and cleaved-PARP was performed on nonadherent cells 1, 2, and 5 days after PDGF stimulation. Media were collected from Ad-Cav1- or Ad-ΔE1-infected cells and centrifuged at 3000 rpm for 10 minutes. Cell pellets were lysed, and equal amounts (20 μg) of protein were loaded in each lane and processed as described earlier.

**Statistical Analysis**

All studies were performed in triplicate unless otherwise stated in the figure legends. Results were normalized to the control cells in each experiment to account for variability in cell preparations. Normal distribution was tested with the Shapiro-Wilk statistic, and transformations were performed when appropriate. Caveolin-1 protein expression data were analyzed with 1-way ANOVA. The influence of the viral vector (ΔE1 or Cav1) and the time on cell proliferation was tested with 2-way ANOVA. All multiple pairwise comparisons were performed with Scheffe’s test. A value of P<0.05 was considered significant. Results are reported as mean±SEM.

**Results**

**PDGF Stimulation Decreases Caveolin-1 Expression in VSMCs In Vitro and In Vivo**

Previous studies have suggested that caveolin-1 might be an important regulator of VSMC proliferative potential and has prompted an investigation of the molecular fate of caveolin-1 in response to PDGF, a known effector of VSMC proliferative potential and has previously been shown to play an important role in regulating VSMC proliferation. To determine whether caveolin-1 protein expression is regulated by PDGF stimulation, serum-deprived human VSMCs were stimulated with 0 to 200 ng/mL PDGF-BB for 24 hours, followed by immunoblot analysis for caveolin-1 and caveolin-2. Immunoblot analysis revealed a dose-dependent decrease in caveolin-1 expression after PDGF stimulation (Figure 1A). Densitometric analysis of 5 experiments demonstrated that a dose of 20 ng/mL PDGF was sufficient to reduce caveolin-1 levels by 60% (P<0.05). Immunoblot analysis of caveolin-2 showed only moderate decreases in protein expression with higher doses of PDGF (data not shown), suggesting that the down-regulation of the caveolin-1 isoform might be a distinct target of PDGF signaling. Time-course studies showed caveolin-1 protein downregulation to occur 12 hours after PDGF stimulation (Figure 1B). Studies were performed to verify that the observed downregulation of caveolin-1 protein levels was not due to a decrease in mRNA levels. Quantitative analysis of caveolin-1 mRNA levels by real-time RT-PCR demonstrated a time-dependent increase in caveolin-1 mRNA, which was increased by 3-fold 24 hours after PDGF stimulation (Figure 1C). These data demonstrate that although PDGF stimulation results in an increase of steady-state levels of caveolin-1 mRNA, there is a significant reduction in caveolin-1 protein levels.

Because caveolae are important mediators of intracellular signaling complexes and caveolin-1 is required for caveolae formation, we assessed changes in caveolae number in VSMCs under serum-free conditions and after 24 hours of PDGF stimulation by electron microscopy. Caveolae number was evaluated by counting cell-surface, flask-shaped invaginations on multiple cells that encompassed a distance of 100 μm. These studies showed that PDGF stimulation caused a 75% reduction in overall caveolae number when compared with untreated VSMCs (Figure 1D), consistent with our observation that caveolin-1 levels are reduced under the same conditions.

Many studies have demonstrated that PDGF plays a significant role in VSMC proliferation and migration after balloon injury in vivo. To determine whether caveolin-1 expression was similarly decreased in VSMCs in vivo, a rabbit arterial injury model was studied. Three weeks after balloon-injury, injured rabbit iliac arteries were harvested and stained for muscle actin to confirm that the neointima was composed primarily of VSMCs (Figure 2A and 2E) and with hematoxylin/eosin to confirm the formation of a hypercellular neointima (Figure 2B and 2F). Immunostaining for caveolin-1 showed intense staining throughout the media; however, less caveolin-1 immunoreactivity within the neointima was clearly evident when compared with the media, suggesting an attenuation of caveolin-1 expression from these phenotypically altered intimal cells (Figure 2C and 2G). In distinction, immunoreactivity for caveolin-2 in the neointima suggests an active phenotype change in these cells.
was similar to that of the media (Figure 2D and 2H). These studies confirm that growth factor stimulation causes a significant reduction of VSMC caveolin-1 expression both in vitro and in vivo.

**PDGF-Induced Downregulation of Caveolin-1 Is Mediated by Lysosomal Degradation**

To further investigate whether PDGF stimulated the degradation of caveolin-1 via the proteasome or lysosomal pathway, inhibitors of these pathways were used before stimulation with PDGF for 24 hours. Initial studies with MG-132, an inhibitor of proteasomal degradation, showed little effect on the PDGF-induced downregulation of caveolin-1 (data not shown). In contrast, pretreatment of VSMCs with chloroquine, an inhibitor of the lysosomal degradative pathway, significantly inhibited caveolin-1 loss after PDGF stimulation (Figure 3A). To further investigate caveolin-1 distribution under serum-free conditions and after PDGF stimulation in the presence and absence of chloroquine, VSMCs were loaded with LysoTracker Red to detect acidic endosomal/lysosome compartments and analyzed by confocal microscopy (Figure 3B). Microscopic analysis showed that under serum-free conditions, caveolin-1 was localized to the plasma membrane and other intracellular compartments; however, there was little colocalization of caveolin-1 with LysoTracker Red. In contrast, treatment of VSMCs with PDGF for 24 hours resulted in the formation of multiple intracellular, punctate structures that stained for caveolin-1 and showed strong colocalization with LysoTracker Red. Treatment of VSMCs with chloroquine inhibited the apparent internalization of caveolin-1 caused by PDGF stimulation and resulted in an accumulation of LysoTracker Red–positive vesicles with a distinct lack of caveolin-1 colocalization to these regions. These data suggest that caveolin-1 is targeted for degradation via the lysosomal pathway after PDGF stimulation.

### Overexpression of Caveolin-1 Inhibits Proliferation and Induces Apoptosis in PDGF-Stimulated VSMCs

Because VSMC proliferation is associated with decreased caveolin-1 levels, the effects of overexpression of caveolin-1 were studied in human VSMCs. For these studies, cells were infected with an adenovirus expressing caveolin-1 (Ad-Cav1) or a similar vector without cDNA insert (Ad-ΔE1) for 48 hours in serum-free media, followed by growth factor stimulation. Western blot analysis demonstrated a dose-dependent increase of Myc-tagged caveolin-1 after adenoviral infection (Figure 4A). There was no apparent alteration in VSMC phenotype after delivery of either viral vector before growth factor stimulation; therefore, this infection protocol was used in all of the overexpression studies. To define changes in the proliferative capacity of virally infected VSMCs, a standard procedure was used to determine the incorporation of BrdU into the DNA of proliferating VSMCs. BrdU incorporation into Ad-Cav1–infected VSMCs was completely inhibited compared with Ad-ΔE1–infected cells 24 hours after stimulation with 20 ng/mL PDGF (Figure 4B), demonstrating that caveolin-1 overexpression was sufficient to inhibit VSMC proliferation. Additional experiments were performed to determine the growth rate of Ad-Cav1–infected cells. For these experiments, VSMCs were infected with either Ad-Cav1 or Ad-ΔE1 at an MOI of 30, and overall cell numbers were counted over the course of 7 days. Cell counts demonstrated an initial decrease in Ad-Cav1 cells at day 3, followed by a reduced growth rate in the remaining Ad-Cav1–infected cells. Control Ad-ΔE1–infected cells showed normal growth kinetics (Figure 4C).
20% of the adherent Ad-Cav1 and assayed for TUNEL staining. These studies revealed that MOI of 30, stimulated with 20 ng/mL PDGF for 24 hours, adherent Ad-Cav1 cells demonstrated significant reduction in cell number compared with Ad-ΔE1-infected cells. Because detachment from the plate might be indicative of cell death in these cells, it was hypothesized that PDGF stimulation might result in the induction of apoptosis in Ad-Cav1 cells. As an initial test for apoptosis, VSMCs infected with Ad-Cav1 or Ad-ΔE1 as described in Methods, and assayed for TUNEL staining. These studies revealed that ~20% of the adherent Ad-Cav1-infected cells demonstrated TUNEL staining 24 hours after PDGF stimulation, whereas Ad-ΔE1 cells displayed negligible staining (Figure 5A). Additionally, adherent cells were significantly fewer after Ad-Cav1 infection compared with Ad-ΔE1–infected cells, confirming a lower growth rate and a higher death rate in caveolin-1–overexpressing cells. Western blot analysis of nonadherent Ad-Cav1–infected cells demonstrated that PDGF stimulation resulted in the activation of caspase-9 and cleavage of PARP, a substrate of caspase-3 and caspase-7, with none detectable in the few nonadherent Ad-ΔE1–infected cells (Figure 5B). PDGF stimulation of both Ad-ΔE1– and Ad-Cav1–infected cells showed no evidence of apoptosis in unstimulated Ad-Cav1–infected cells or in Ad-ΔE1–infected cells stimulated with PDGF. These studies reveal that the proliferative potential of VSMCs is closely correlated with caveolin-1 protein levels. Adenoviral overexpression of caveolin-1 dose-dependently inhibits VSMC proliferation and can induce cells to undergo apoptosis in a setting of growth factor stimulation when cells have higher levels of caveolin-1.

During the course of these experiments, it was noted that although both Ad-Cav1– and Ad-ΔE1–infected cells were phenotypically normal while maintained under serum-free conditions. PDGF stimulation resulted in phenotypic alterations of several Ad-Cav1–infected cells when infected with an MOI of 30. Ad-Cav1–infected cells tended to “round up” and detach from the plate, whereas Ad-ΔE1–infected cells displayed a “streamline” phenotype similar to that of noninfected control cells. Because detachment from the plate might be indicative of cell death in these cells, it was hypothesized that PDGF stimulation might result in the induction of apoptosis in Ad-Cav1 cells. As an initial test for apoptosis, VSMCs were infected with either Ad-ΔE1 or Ad-Cav1 at an MOI of 30, stimulated with 20 ng/mL PDGF for 24 hours, and assayed for TUNEL staining. These studies revealed that ~20% of the adherent Ad-Cav1–infected cells demonstrated TUNEL staining 24 hours after PDGF stimulation, whereas Ad-ΔE1 cells displayed negligible staining (Figure 5A). Additionally, adherent cells were significantly fewer after Ad-Cav1 infection compared with Ad-ΔE1–infected cells, confirming a lower growth rate and a higher death rate in caveolin-1–overexpressing cells. Western blot analysis of nonadherent Ad-Cav1–infected cells demonstrated that PDGF stimulation resulted in the activation of caspase-9 and cleavage of PARP, a substrate of caspase-3 and caspase-7, with none detectable in the few nonadherent Ad-ΔE1–infected cells (Figure 5B). PDGF stimulation of both Ad-ΔE1– and Ad-Cav1–infected cells showed no evidence of apoptosis in unstimulated Ad-Cav1–infected cells or in Ad-ΔE1–infected cells stimulated with PDGF. These studies reveal that the proliferative potential of VSMCs is closely correlated with caveolin-1 protein levels. Adenoviral overexpression of caveolin-1 dose-dependently inhibits VSMC proliferation and can induce cells to undergo apoptosis in a setting of growth factor stimulation when cells have higher levels of caveolin-1.

To further understand the mechanisms involved in the inhibition of VSMC proliferation by caveolin-1 and to verify that the observed induction of apoptosis was not an artifact of gross adenoviral overexpression, additional studies were performed to determine the effects of caveolin-1 overexpression on the early signaling events leading to VSMC proliferation. For these studies, VSMCs were infected with Ad-Cav1 or Ad-ΔE1 at an MOI of 30 and stimulated with 20 ng/mL PDGF for 0, 5, 10, or 30 minutes, and Western blotting for the phosphorylated form of Erk 1/2 was performed (Figure 6A). Western blot analysis demonstrated that Erk 1/2 activation in both Ad-ΔE1– and Ad-Cav1–infected cells was similar, demonstrating that initial signaling events leading to cell proliferation were unchanged in Ad-Cav1–infected cells.
significant finding that growth factor stimulation of VSMCs results in a loss of caveolae and its coat protein caveolin-1. The mechanism by which caveolin-1 is degraded in response to PDGF stimulation appears to involve the internalization of cell surface caveolae, followed by the merger with the acidic endosomal/lysosomal compartments, because neutralization of the acidic environment in these compartments with chloroquine inhibited the PDGF-induced degradation of caveolin-1. Our studies with the proteasome inhibitor MG-132 suggest that the proteasome does not significantly participate in the degradation of caveolin-1 under conditions of growth factor stimulation. However, we cannot rule out that parts of the proteasome process are not important for the eventual loss of caveolin-1. The observation that caveolin-1 degradation occurs relatively late after PDGF stimulation suggests that caveolin-1 might be participating in other cellular functions, such as focal adhesion complex formation, before being targeted for degradation. We have observed caveolin-1 phosphorylation and association with focal adhesion complexes after PDGF stimulation in our cells, as previously reported by others. However, treatment of VSMCs with chloroquine did not inhibit the phosphorylation of caveolin-1 in response to PDGF authors’ unpublished observation), yet it was able to inhibit caveolin-1 degradation, suggesting that phosphorylation of caveolin-1 was not a requirement for caveolin-1 degradation.

In this study, we found that adenoviral overexpression of caveolin-1 caused the inhibition of VSMC proliferation associated with an induction of apoptosis in cells containing relatively high levels of caveolin-1 after addition of PDGF. The use of adenoviral constructs was necessary for efficient overexpression of caveolin-1 in these primary cells. When VSMCs were infected with an MOI of 30, there was an increase in the number of apoptotic cells after PDGF stimulation. Because inhibition of proliferation coincided with apoptosis, it is difficult to determine absolutely that our observed inhibition of proliferative indices was not due to apoptosis alone. However, biochemical analysis of early signaling events suggest that VSMCs infected with Ad-Cav1 at an MOI of 30 were able to phosphorylate Erk 1/2 to the same extent as VSMCs infected with control virus at the same MOI. Furthermore, there was an inhibition of cyclin D1 activity in Ad-Cav1 cells, suggesting that caveolin-1 overexpression resulted in the inhibition of cell-cycle progression. We therefore believe that in the presence of these discordant signaling events, inhibition of cell cycle progression was the trigger for apoptosis in these cells.

The mechanism by which caveolin-1 was able to facilitate and/or induce apoptosis in our cells is unknown. Apoptosis can be initiated in VSMCs through several pathways. The detection of activated caspase-9 in our current study suggests that apoptosis might be mediated by the release of cytochrome c from mitochondria, leading to the activation of caspase-9 and other effector caspsases, which ultimately results in the cleavage of PARP. However, further studies are required to define the pathways involved leading to cell death in our Ad-Cav1--infected cells when compared with viral controls.

In summary, we report that PDGF stimulation causes the upregulation of caveolin-1 mRNA, yet results in decreased

Discussion

PDGF has been shown to activate a number of signaling molecules in VSMCs, many of which participate in cell proliferation and cell survival. The results of this study suggest that the key regulator of caveolae formation and function, caveolin-1, might be a target and a modulator of PDGF-induced proliferative events. We demonstrate that although caveolin-1 mRNA expression is increased in primary VSMCs after PDGF stimulation, caveolin-1 protein levels and overall caveolae numbers are dramatically reduced. Furthermore, in vivo studies of injured rabbit arteries also demonstrate a decrease in caveolin-1 expression in the phenotypically altered neointimal compartment. Finally, overexpression of caveolin-1 was associated with inhibition of proliferation and growth factor induction of apoptosis in primary VSMCs.

Many studies have now shown that caveolae are important regulators of normal vascular hemostasis. It is therefore a significant finding that growth factor stimulation of VSMCs

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Adenoviral overexpression of caveolin-1 does not inhibit early signaling events leading to the activation of mitogen-activated protein kinase but does inhibit cyclin D expression. A, Western blot (WB) analysis of VSMC lysates demonstrates that early signaling events leading to the activation of mitogen-activated protein kinase (Erk 1/2) are intact in both Ad-Cav1- and Ad-ΔE1-infected cells (MOI of 30) after stimulation with 20 ng/mL PDGF for 0, 5, 10, and 30 minutes. B, Western blot (WB) analysis of VSMC lysates demonstrates that cyclin D1 expression by PDGF is inhibited in Ad-cav1- overexpressing cells (MOI of 30) compared with control Ad-ΔE1–infected cells (MOI of 30).
caveolin-1 protein owing to enhanced lysosomal degradation in VSMCs. This downregulation of caveolin-1 protein might be necessary for VSMC cell-cycle progression, because overexpression of caveolin-1 was shown to inhibit VSMC cyclin D1 activation. Finally, caveolin-1 overexpression resulted in the induction of apoptosis after growth factor stimulation in primary VSMCs, suggesting that caveolin-1 is an integral component in the regulation of VSMC proliferative responses.

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