Rapid Communication

Expression of Cyclin-Dependent Kinase Inhibitors in Vascular Disease

Felix C. Tanner, Zhi-Yong Yang, Eric Duckers, David Gordon, Gary J. Nabel, Elizabeth G. Nabel

Abstract—Arterial lesions in cardiovascular diseases are characterized by proliferation and migration of smooth muscle cells as well as deposition of connective tissue matrix. Factors that stimulate vascular smooth muscle cell (VSMC) proliferation are well described; however, the role of proteins that limit intimal hyperplasia is not well understood. To examine the function of Kip/Cip and INK cyclin-dependent kinase inhibitors (CKIs) in vascular diseases, the expression of p27Kip1 and p16INK was examined in VSMCs in vitro and in porcine arteries and human atherosclerosis in vivo. Western blot and fluorescence activated cell-sorting analysis demonstrated that levels of p27Kip1, but not p16INK, increased during serum deprivation of primary VSMC cultures and caused G1 arrest. p27Kip1 inhibited Cdk2 activity, suggesting that Kip CKIs promote G1 arrest in VSMCs by binding cyclin E/Cdk2. In porcine arteries, p27Kip1, but not p16INK, was constitutively expressed at low levels. Immediately after balloon injury, cell proliferation increased as p27Kip1 levels declined. Three weeks after injury, p27Kip1 was strongly expressed in intimal VSMCs when VSMC proliferation was <2%, suggesting that p27Kip1 functions as an inhibitor of cell proliferation in injured arteries. In contrast, p16INK expression was detected only transiently after injury. CKI expression was examined in 35 human coronary arteries, ranging from normal to advanced atherosclerosis. p27Kip1 expression was abundant in nonproliferating VSMCs and macrophages within normal (7 of 8) and atherosclerotic (25 of 27) arteries. p21Cip1 levels were undetectable in normal arteries but were elevated in atherosclerotic (19 of 27) arteries. p16INK could not be detected in normal or atherosclerotic arteries (0 of 35). Thus, the Kip/Cip and INK CKIs have different temporal patterns of expression in VSMCs in vitro and in injured arteries and atherosclerotic lesions in vivo. In contrast to p16INK, p27Kip1 likely contributes to the remodeling process in vascular diseases by the arrest of VSMCs in the G1 phase of the cell cycle. (Circ Res. 1998;82:396-403.)

Key Words: cyclin-dependent kinase ■ cell cycle ■ vascular smooth muscle cell

Atherosclerotic plaques are complex lesions in which repair of tissue injury is associated with VSMC proliferation, connective tissue formation, and calcium deposition.1 Mitogen-dependent stimulation of VSMC division has been described; however, the mechanisms by which cell proliferation is inhibited during vascular remodeling are not well understood. In many cells, transit through G1 of the cell cycle and entry into the S phase require the binding and activation of cyclin/CDK complexes, predominantly cyclin D/Cdk4 or Cdk6 and cyclin E/Cdk2.2,3 The CKIs are naturally occurring gene products that inhibit cyclin/CDK activity and phosphorylation of Rb4,5 resulting in G1 arrest.4,5 CKIs directly implicated in CDK regulation are p21Cip1,6,7 p27Kip1,8–10 and p16/p15INK.11 Since cell proliferation is a prominent feature of cardiovascular diseases, including atherosclerosis, angiogenesis, and restenosis, we hypothesized that CKIs regulate G1 progression in VSMCs and function as endogenous inhibitors of VSMC proliferation.

Two families of CKIs have been identified: the Kip/Cip proteins (p21Cip1, p27Kip1, and p57Kip2) and the INK proteins (p15INKA, p16INKB, p18INKC, and p19INKD). The Kip/Cip proteins contain a conserved domain that is necessary and sufficient for cyclin-CDK interaction and inhibition in proliferating cells.12 In vitro experiments indicate that multiple molecules of Kip are required with a cyclin/CDK complex for inhibition of kinase activity.13 Amino acids 17 to 71 of p21Cip1 contain the cyclin/CDK binding and inactivation domain.14 Similar homologous domains are present in p27Kip1 with amino acids 26 to 88.14,15 We and others have found that p21Cip1 is upregulated in arteries after vascular injury and that overexpression of p21Cip1 in VSMCs results in G1 arrest and inhibition of cell growth.16,17 There are several properties of p27Kip1 suggesting that it might also have an important role regulating entry into and exit from the mitotic cycle. Ectopic expression of p27Kip1 cDNA is sufficient to induce G1 arrest, and there is a correlation between growth arrest and the amount of p27/Cdk2 complex under antimitogenic conditions.14,18 Given these properties of p27Kip1, we hypothesized that p27Kip1 might be expressed in VSMCs and function to establish an inhibitory threshold that G1 CDKs must surpass before activation and entry into the S phase. We speculated further that p27 would be upregulated in arterial tissues after vascular injury to inhibit

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VSMC proliferation and promote arterial repair. Finally, we sought to compare the expression and function of p27Kip1 with an INK protein, p16INK.

**Materials and Methods**

**Cell Culture and Cell Cycle Analysis**

Primary porcine aortic VSMCs were isolated by an explantation method and maintained in medium 199 containing 20% FCS. Twenty-four hours before cell cycle analysis, VSMCs were carried in medium 199 with 0.2% BSA or medium 199 with 20% FCS. For analysis of the cell cycle distribution, cells were harvested 24 hours later, washed with PBS twice, fixed in 2% paraformaldehyde for 60 minutes, and permeabilized in 0.2% Tween 20. The cells were treated with 1 U of DNase-free RNase in 1 mL of PBS for 30 minutes, and permeabilized in 0.2% Tween 20. The cells were treated with 1 U of DNase-free RNase in 1 mL of PBS for 30 minutes at 37°C, resuspended in 0.03 mg/mL propidium iodide, and analyzed by flow cytometry using a FACScan model (Becton Dickinson).

**Western Blot and Immunoprecipitation**

Porcine aortic VSMCs were cultured in medium 199 with 20% FCS. Cell lysates were prepared as described. Western blot analysis was performed on whole-cell lysates by incubating VSMCs in lysis buffer (50 mmol/L Tris, pH 7.5, 250 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, and 0.1% NP-40) supplemented with protease and phosphatase inhibitors (0.5 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L NaF, 0.1 mmol/L Na3VO4, and 1 mmol/L DTT) on a rocking platform for 30 minutes. Cellular debris was removed by centrifugation at 16,000g for 10 minutes, and protein concentration was determined by the Bradford assay. Eight micrograms of protein was loaded per lane, resolved by SDS-PAGE under reducing conditions, blotted on polyvinylidene fluoride membranes, and incubated with monoclonal antibodies to detect p27 (No. K25020, Transduction Laboratories) and p16 (No. 13251A, Pharmingen) with a horseradish peroxidase–coupled secondary antibody using SuperSignal (Pierce) as substrate.

Immunoprecipitations were performed by dilution of whole VSMC lysates in 1 mL of buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, and 0.1% NP-40) supplemented with protease and phosphatase inhibitors (0.5 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L NaF, 0.1 mmol/L Na3VO4, and 1 mmol/L DTT). Diluted lysates were precleared with an isotype antibody and then incubated with the CdK2 antibody for 3 hours on a rocking platform at 4°C. Immunoprecipitates were washed three times in 1 mL of buffer and then used for Western blot analysis or for kinase assays. For H1 kinase assays, immunoprecipitates were washed in 1 mL of kinase buffer (50 mmol/L Tris, pH 7.5, 10 mmol/L MgCl2, 20 μmol/L ATP, 1 mmol/L NaF, 0.1 mmol/L Na3VO4, and 1 mmol/L DTT) and resuspended in 25 μL of this buffer. The kinase reaction was performed for 30 minutes at 37°C in the presence of 10 μCi [γ-32P]ATP (ICN) with 1 μg histone H1 (Boehringer) as substrate. Labeled proteins were resolved on 15% SDS-PAGE and subjected to autoradiography.
centrifuged into pellets. The pellets were formalin-fixed and paraffin-embedded, and immunohistochemistry was performed.

Immunohistochemistry was also performed on formalin-fixed paraffin-embedded specimens with a mouse monoclonal anti-Ki67 antibody, 1:500 dilution (AMAC), using immunoperoxidase staining. Serial sections were placed onto poly-L-lysine–coated slides, deparaffinized in three changes of xylene, and rehydrated in 100%, 95%, and 75% ethanol. Slides were then blocked with 10% normal goat serum in PBS containing 1% BSA and incubated with the Ki67 antibody for 30 minutes at room temperature. A biotinylated horse anti-mouse secondary antibody was applied for 30 minutes, followed by an avidin-biotin-peroxidase conjugate (Vector Labs) for 30 minutes at room temperature. 3,3′-Diaminobenzidine with nickel chloride was added to yield a black reaction product, and methyl green was used as a nuclear counterstain.

Double-label immunohistochemistry was performed using previously described methods4 on formalin-fixed tissue using the following primary antibodies: a mouse monoclonal anti-mouse p27 antibody, 1:100 dilution (No. 13231A, Pharmingen); a rabbit polyclonal anti-human p21 antibody, 1:500 dilution (No. 756, Santa Cruz); a mouse monoclonal anti-smooth muscle α–actin antibody, 1:500 dilution (Boehringer-Mannheim); a mouse monoclonal anti-human CD68 antibody, 1:500 dilution (Dako); a monoclonal SP1.d8 aminopeptide–specific type I procollagen antibody, 1:500 dilution (Developmental Studies, Hybridoma Bank, University of Iowa); and a monoclonal mouse anti-BrdC antibody, 1:1000 dilution (Amersham Life Sciences).

To determine the percentage of intimal cells expressing procollagen, immunohistochemistry was performed with a procollagen antibody as described above. Total nuclei and procollagen-positive nuclei were counted in the intima of normal and injured arteries by a microscope-based video image analysis system (Image One Systems, Universal Imaging Corp).19 Eight high-power fields were counted in four sections from each artery. A percentage of positive procollagen cells was calculated as the ratio of labeled cells to the total number of cells. The data for each artery were averaged, and a mean and standard error were calculated for eight arteries at each time point. The data are expressed as mean±SEM.

Figure 1. Effect of growth factors on cell cycle distribution of VSMC and CKI protein levels. A, Primary VSMCs cultured in the presence of 0.2% BSA (left) were arrested in the G1 phase. In the presence of 20% FCS (right), VSMCs were stimulated to proliferate, and the number of G1 phase–arrested cells decreased substantially. B, Effect of growth factors on protein levels of p27Kip1 and p16INK4a is shown. p27Kip1 levels were higher in the presence of 0.2% BSA compared with 20% FCS, whereas p16INK4a levels did not vary. Western blot analysis was performed to detect p27Kip1 and p16INK4a expression in VSMCs using whole-cell lysates and monoclonal antibodies to detect p27Kip1 (No. K25020, Transduction Laboratories) and p16INK4a (No. 13251A, Pharmingen). C, Cdk2 kinase activity in quiescent and proliferating VSMCs and porcine arteries is shown. Western blot analysis demonstrated that proliferation of VSMCs in response to serum resulted in undetectable levels of p27Kip1 in proliferating cells (row 1), whereas the levels of Cdk2 did not change in proliferating compared with arrested cells (row 2). Immunoprecipitation of Cdk2 followed by Western blot analysis for p27Kip1 showed a decrease in Cdk2-associated p27Kip1 (row 3) and an increase in H1 kinase activity (row 4) in proliferating compared with arrested cells. p27Kip1 is present in cultured VSMCs as well as in normal arteries (row 1), whereas p16INK4a is detected only in cultured VSMCs but not in the arterial tissue (row 5). Western blot analysis, immunoprecipitations, and H1 kinase assays were performed on whole-cell lysates from arrested (BSA) or proliferating (FCS) VSMCs, and Western blot analysis was performed for p27Kip1 and p16INK4a expression using monoclonal antibodies to detect p27Kip1 (No. K25020, Transduction Laboratories) and p16 (No. 13251A, Pharmingen).
Results

CKI Expression in Arrested and Proliferating VSMCs

CKI expression in VSMCs was first analyzed in vitro in the absence or presence of serum. Cells incubated in media with 0.2% BSA for 24 hours displayed G1 arrest (82% in G1; Fig 1A, left) and showed high levels of p27Kip1 (Fig 1B, lane 1). After exposure to 20% FCS, proliferation resumed, with the proportion of G1 cells decreasing substantially (44% in G1; Fig 1A, right). At the same time, p27Kip1 levels decreased to undetectable levels as determined by Western blot analysis (Fig 1B, lane 2). Similarly, VSMCs synchronized in G1 by culture in BSA for 72 hours displayed high levels of p27Kip1 (Fig 1B, lane 3), which declined to undetectable levels within 24 hours after addition of FCS (Fig 1B, lanes 4 to 9). p21Cip1 showed a similar trend (data not shown). p16INK levels did not change in arrested compared with proliferating cells (Fig 1B, lanes 1 to 9).

Incubation of VSMCs in BSA for up to 72 days did not lead to cell death as determined by the sub-G1 peak of propidium iodide−stained cells (data not shown). Proliferation of VSMCs in response to serum resulted in decreased cellular p27Kip1 (Fig 1C, row 1) as well as Cdk2-associated p27Kip1 (Fig 1C, row 3), whereas the cellular level of Cdk2 did not change under these conditions (Fig 1C, row 2). Accordingly, the Cdk2-associated H1 kinase activity was increased in the proliferating cells, indicating that the low p27Kip1 levels in the presence of growth factors permitted proliferation to occur (Fig 1C, row 4). Western blot analysis of p27Kip1 and p16INK expression in cultured VSMCs and in the arterial wall demonstrated that p27Kip1 was present in both isolated cells and intact tissue, whereas p16INK could be detected in the cultured cells only (Fig 1C, rows 1 and 5). These studies suggest that p27Kip1, but not p16INK, is induced in VSMCs in association with G1 arrest in vitro and that this induction mediates cell cycle exit.

CKI Expression During Arterial Repair

Expression of CKI proteins was next characterized in porcine arteries, which are well studied as a model for human vascular disease. After balloon injury in this species, smooth muscle cell proliferation began in the intima within 1 to 2 days, peaked 7 days later with 15% to 18% of intimal cells undergoing proliferation, declined rapidly to <2% of intimal cells by day 14 (Fig 2J), and remained at this low level at days 21 through 60. In normal, uninjured arteries, p27Kip1 expression was detected in the intima, media, and adventitia (Fig 2A). After balloon injury to the artery, p27Kip1 expression decreased by day 4 (Fig 2B), and levels remained low through day 7 (Fig 2C). After balloon injury, there was an acellular region in the mid-medial region and an absence of staining in this region. Expression of p27Kip1 was observed in the developing neointima at day 14 (Fig 2D), and expression continued at day 21 (Fig 2E and 2F) and day 60 (Fig 2G). At these time points, p27Kip1 expression was observed throughout the intima (Fig 2F) but in many sections was seen primarily in the lower regions of the intima, adjacent to the internal elastic lamina (Fig 2E). p27Kip1 expression persisted in the artery even at day 60, when the response to injury was complete (Fig 2G).

To explore factors that might account for this pattern of p27Kip1 expression in the intima, immunohistochemical studies were performed with antibodies to procollagen and TGF-β. In the normal pig artery, ≈3% of cells in the intima and media were positive for procollagen, as might be expected for an artery not undergoing remodeling (Fig 3). After balloon injury,
procollagen expression in the intima increased to 6.8 ± 1.1% of cells at day 1 and remained at this approximate level until 7 days after injury (day 4, 6.3 ± 2.1%; day 7, 7.2 ± 2.6%). By 14 days, however, the percentage of positive procollagen cells in the intima increased further to 10.1 ± 0.8% and to 14.3 ± 1.4% at day 21. Procollagen immunostaining was observed throughout the intima (Fig 2H and 2I). The percent positive procollagen cells at 60 days was 5.0 ± 1.2%.

TGF-β expression was also present in the intima at 14 and 21 days after vascular injury. Two patterns of staining were observed. In some regions of the artery, TGF-β was present throughout the intima, whereas in other sections, expression was observed primarily in the lower regions of the intima, adjacent to the internal elastic lamina in a pattern similar to p27Kip1 expression (Fig 2L). This localization to the innermost regions of the intima was observed in previous studies of TGF-β overexpression in pig arteries after vascular gene transfer.20

In previous studies, in contrast to p27Kip1, p21Cip1 protein has not been detected in normal arteries; however, after injury, its levels were found to increase in the same region as p27Kip1 when VSMC proliferation decreased.16 Unlike p27Kip1, p21Cip1 was undetectable by 60 days when the response to injury was complete. Interestingly, the pattern of p16 expression differed from p27Kip1 and p21Cip1 and was detected only transiently before the peak onset of cell proliferation (day 4) (Fig 2N). This is consistent with the observation that p16 was not detectable in the uninjured artery by immunohistochemistry (Fig 2M).

These data suggest that p27Kip1 is normally expressed in VSMCs in pig arteries, downregulated after arterial injury, and upregulated in the intima when cell proliferation is decreasing and procollagen synthesis is present (Fig 4).

CKI Expression During Atherogenesis
To determine whether CKI expression was abnormal in human cardiovascular disease, we examined expression patterns in 35 coronary artery segments from cardiac transplant recipients classified as normal with DIT (n = 8 arteries), early atherosclerosis (n = 7 arteries), or advanced atherosclerosis (n = 20 arteries) (Table). p27Kip1 was expressed in intimal and medial VSMCs as well as in adventitial fibrocytes of nonatherosclerotic arteries (7 of 8 arteries) (Fig 5, top panel). p21Cip1 was detected only at low levels (1 of 8 arteries), was not expressed in the absence of p27Kip1, and was detected more prominently in the intima and media compared with the adventitia (Fig 5, top panel). p21Cip1 expression was often eccentric compared with p27Kip1 expression, suggesting that its presence may reflect a response to arterial injury or stress. p16INK was not detected in any artery with DIT (0 of 8 arteries).

In early atherosclerosis, p27Kip1 and p21Cip1 were present in similar regions of the intima and media (p27, 5 of 7 arteries; p21, 4 of 7 arteries), whereas p16INK was not detected in any artery with early atherosclerosis (Fig 5, middle panel). In advanced atherosclerotic lesions, p27Kip1 and p21Cip1 were both detected within plaques, particularly in regions with the greatest accumulation of matrix and the least cellularity (p27, 20 of 20 arteries; p21, 15 of 20 arteries) (Fig 5, bottom panel). Although p27Kip1 was more abundant than p21Cip1 in these lesions, the frequency of cells containing p21Cip1 was markedly increased with the increasing severity of atherosclerosis (Fig 5, bottom panel). A nearly 100-fold higher frequency was seen in advanced atherosclerosis compared with normal arteries or those with early atherosclerosis. In contrast, p16INK was not observed in atherosclerotic lesions. Cell proliferation was measured in these specimens by immunostaining for the proliferation marker Ki67.24 Cell proliferation indices in early and advanced atherosclerosis were low (< 2% of plaque cells), similar to previously reported findings.25 p27Kip1, p21Cip1, and p16INK expression was not observed within plaque cells...
undergoing proliferation, and CKI expression was more commonly observed in regions of the plaque not undergoing proliferation.

To identify the cells expressing these CKIs, costaining with antibodies to smooth muscle α-actin and the macrophage marker CD68 was performed. The majority of cells expressing p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} in early and advanced atherosclerosis were VSMCs (~87%) (Fig 6A and 6B), and the remainder were identified as macrophages (Fig 6E and 6F) by double-labeling techniques. Expression of p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} was variable in luminal endothelium of large arteries; however, there was abundant and uniform expression of p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} within plaque and adventitial microvessels in endothelium and smooth muscle cells (Fig 6C and 6D). Western blot analyses performed on extracts from coronary arteries confirmed the presence of the expected size proteins in these tissues (data not shown).

**Discussion**

Vascular proliferative diseases, such as atherosclerosis and restenosis, are characterized by intimal smooth muscle cell growth in response to vascular injury. The signaling pathways by which VSMCs proliferate in response to mitogenic signals are well described (reviewed in References 26, 27, and others). However, the role of cellular gene products that cause VSMCs to shift from a proliferative state to a quiescent state during the G\textsubscript{1} phase of the cell cycle are not well understood. The purpose of the present study was to examine the expression of the CKIs p27\textsuperscript{Kip1}, p21\textsuperscript{Cip1}, and p16\textsuperscript{INK} in normal and diseased arterial tissue. We found that p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} are expressed in distinct temporal patterns in normal, injured, and diseased arteries in vivo. p27\textsuperscript{Kip1}, but not p21\textsuperscript{Cip1}, is constitutively expressed in normal pig arteries. p27\textsuperscript{Kip1} is downregulated after arterial injury when cell proliferation increases. p27\textsuperscript{Kip1} is upregulated in later phases of arterial remodeling and is associated with a decline in cell proliferation and an increase in procollagen and TGF-β synthesis. Of interest, p16\textsuperscript{INK} was expressed only transiently in pig arteries after injury and was not observed in normal arteries or chronic lesions. In human lesions, p27\textsuperscript{Kip1} was expressed in atherosclerotic and nonatherosclerotic lesions in cells not undergoing proliferation. p21\textsuperscript{Cip1} expression increased as the severity of atherosclerotic lesions progressed and was not seen in the absence of p27\textsuperscript{Kip1}. p16\textsuperscript{INK} was not observed in any human lesion. These findings suggest that p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} are endogenous regulators of G\textsubscript{1} transit in VSMCs and function to inhibit cell proliferation during arterial repair. The CKI p16\textsuperscript{INK} probably plays little role in arterial remodeling. In addition, within a family of related CKIs, p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} may have different patterns of expression and regulation in normal and diseased arteries.

Recently, it has become evident that interactions between matrix and VSMCs may control CKI expression and that differential regulation of p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} CKIs can be achieved by engagement of integrin receptors by cross-linked collagen. This hypothesis is supported by recent work from Koyama et al.,\textsuperscript{26} who have demonstrated that...
fibrillar collagen regulates early integrin signaling, which induces p27Kip1 and p21Cip1 expression in VSMCs and downregulates Cdk2 expression. In the present study, we found that p27Kip1 and procollagen are expressed at low levels in the intima during the first 7 days after arterial injury. During this time period, smooth muscle cells are proliferating, and the neointima is forming. Between 7 and 14 days in this model, there is a decline in smooth muscle cell proliferation, an increase in p27Kip1 and p21Cip1 expression, and an increase in procollagen expression in the intima. There is also a correlation temporally and spatially between p27Kip1 and TGF-β expression. These data suggest that there is an association between p27Kip1, procollagen, and TGF-β expression and an inverse correlation between these factors and cell proliferation. These observations lend support to the report by Koyama et al that specific signaling through integrin receptors could contribute to differential expression of p21Cip1 and p27Kip1 in arterial lesions. In addition to signals mediated by matrix attachment, growth stimulatory signals also decline during this period after injury. This may contribute to increased CKI levels, especially in the case of p27Kip1, as suggested by the serum starvation experiments with cultured VSMCs. The decline in p27Kip1 expression during VSMC proliferation after arterial injury could also contribute to the increased Cdk2 activity.

We have previously reported that p21Cip1 expression is also increased in injured arterial lesions after 7 days. Although these findings are similar to those currently reported for p27Kip1, there are distinct differences in expression patterns between p21Cip1 and p27Kip1 in injured pig arteries and human atherosclerosis. p27Kip1 is constitutively expressed in normal arteries, whereas p21Cip1 is not, and p27Kip1 is found in all phases of atherosclerotic and nonatherosclerotic human coronary disease, whereas p21Cip1 is observed in advanced atherosclerosis. These findings suggest that p27Kip1 may function to maintain normal VSMCs in G1/G0 and to inhibit cell proliferation during the latter phases of arterial repair. p21Cip1 may function as a cofactor that is induced in the latter phases of arterial remodeling to inactivate CDK/cyclin complexes and cause G1 arrest.

These studies in pig arteries and human lesions suggest that p27Kip1 sets a balance between proliferating and nonproliferating cells in arterial lesions. These findings support evidence from previous studies in p27Kip1 knockout mice. Disruption of the CDK binding domain of the p27Kip1 gene in mice enhances growth.30–32 Growth results from an increase in cell number, due to increased cell proliferation, which is most obvious in tissues that ordinarily express p27Kip1 at the highest levels, such as thymus, pituitary, and ovaries. In the present study, we observed an inverse correlation between VSMC proliferation and p27Kip1 expression, again suggesting a role for p27Kip1 in setting a balance between proliferating and nonproliferating VSMCs. Although the gene disruption studies implicate p27Kip1 function in development, the expression studies reported here demonstrate a major role for p27Kip1 in repair and remodeling of arteries after vascular injury. As such, these studies lend support to the concept that CKIs regulate cell cycle progression in VSMCs and point to potential therapies for vascular proliferative diseases.

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