Unique, Highly Proliferative Growth Phenotype Expressed by Embryonic and Neointimal Smooth Muscle Cells Is Driven by Constitutive Akt, mTOR, and p70S6K Signaling and Is Actively Repressed by PTEN

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Background—At distinct times during embryonic development and after vascular injury, smooth muscle cells (SMCs) exhibit a highly proliferative, serum-independent growth phenotype. The aim of the present study was to evaluate the functional role of S6 ribosomal protein (S6RP) and upstream positive and negative regulators in the control of SMC serum-independent growth.

Methods and Results—We previously reported increased expression of S6RP mRNA was associated with this unique growth phenotype. Using immunohistochemistry and Western blot analysis, we report high levels of total and phospho-S6RP and increased levels of Akt and p70S6K phosphorylation, upstream positive regulators of S6RP, in rat embryonic aortas and adult balloon-injured carotid arteries compared with quiescent adult aortas and uninjured carotid arteries. Western blot analysis demonstrated that cultured embryonic and neointimal SMCs that exhibited serum-independent growth capabilities expressed high levels of S6RP and constitutively active Akt, mTOR, and p70S6K. Pharmacological and molecular inhibition of phosphatidylinositol 3-kinase (PI3K) signaling pathways, using PI3K inhibitors, rapamycin, or dominant-negative Akt adenovirus, suppressed embryonic and neointimal SMC serum-independent growth. Finally, decreased activity of PTEN, an endogenous negative regulator of PI3K signaling, was associated with high in vivo SMC growth rates, and morpholino-mediated loss of endogenous PTEN induced a serum-independent growth phenotype in cultured serum-dependent SMCs.

Conclusions—The possibility exists that cells that exhibit a distinct embryonic-like growth phenotype different from traditional SMCs are major contributors to intimal thickening. Growth of SMCs that exhibit this phenotype is dependent on constitutive Akt and mTOR/p70S6K signaling and is actively inhibited through the timed acquisition of the endogenously produced growth suppressor PTEN. (Circulation. 2004;109:1299-1306.)

Key Words: cells ■ restenosis ■ muscle, smooth ■ aorta ■ carotid arteries

Vascular smooth muscle cell (SMC) proliferation plays a prominent role in normal vessel development and in many vascular pathologies, including restenosis after angioplasty and stent placement and atherosclerosis. The mechanisms that regulate SMC proliferation during vascular development and in response to vascular injury in the adult blood vessel, however, have yet to be fully elucidated. During development, aortic SMCs undergo a phase of rapid proliferation, during which the vessel wall acquires its complement of SMCs. Replication rates decrease as the animal matures, with SMCs eventually reaching a quiescent state in the adult animal. However, after injury to an adult artery, transient increases in SMC replication to levels similar to those exhibited during embryonic life are observed in the neointima.

Corresponding to high in vivo growth rates, SMCs cultured from embryonic aortas exhibit a distinct growth phenotype characterized by rapid serum-stimulated growth and the ability to replicate in a mitogen- or serum-independent manner. This growth phenotype is lost by fetal life, with fetal-, neonatal-, and adult-derived SMCs exhibiting slower growth rates in response to serum stimulation and loss of serum-independent growth. SMCs derived at specific times from injured adult vessels, however, exhibit a similar growth phenotype at a time correlating to peak in vivo neointimal SMC growth. Thus, SMCs are capable of transiently reexpressing an embryonic-like, serum-independent growth phenotype. Additionally, we reported adult-derived serum-dependent SMCs possess a “suppressor activity” that continually and actively controls SMC replication by extinguishing serum-independent growth potential. This suppressor activity is first detected in the fetal period of development.

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when the potential for SMC serum-independent growth is no longer detected and is only lost or inactivated after vascular injury corresponding to reexpression of a serum-independent growth phenotype. These observations are consistent with the possibility that mechanisms that contribute to vascular injury repair are a partial reiteration of early vascular developmental processes.

Our previous work used subtractive hybridization analysis to identify genes associated with both high rates of in vivo SMC growth rates. A, Embryonic (Emb) and adult (Ad) aortic and sham-injured and 7-day postinjury carotid artery (7d inj CA) tissue sections were immunostained for S6RP and phospho-S6RP (P-S6RP); positive reaction color is reddish-brown. Rabbit anti-human rotavirus was used as negative control (injured carotid artery shown). Lumen is to top in each photomicrograph; arrowheads indicate internal elastic laminae of carotid artery sections. RI indicates SMC replication indices (medial cells for aortic and sham-injured carotid artery; neointimal cells for injured carotid artery). B, Whole-cell lysates from embryonic and adult aortas (Ao) and sham-injured and 7-day-injured carotid arteries were examined by Western blot analysis for expression of total and phospho (P)-S6RP, phospho-Akt, and phospho-p70S6K with specific antibodies. Western blots were scored for relative densitometry normalized against β-actin (data shown in graph).

Results

High Levels of Total S6RP, Phospho-Akt, Phospho-p70S6K, and Phospho-S6RP Correlate to High In Vivo SMC Growth Rates

Our previous data showed S6RP mRNA was upregulated in embryonic-derived, serum-independent SMCs compared with adult-derived, serum-dependent SMCs. Using immunohistochemistry on tissue sections, we found higher total S6RP in embryonic aortas and in the neointima of 7-day postinjury carotid arteries compared with adult aortic and sham-injured carotid arterial tissues (Figure 1A). S6RP phosphorylation increases cell cycle progression. In addition to an increase in total protein, we also found increased levels of phospho-S6RP in embryonic and injured arterial sections compared with adult aortic and sham-injured carotid artery sections.
Figure 1A). Western blot analysis of vessel protein expressions confirmed the immunohistochemistry data (Figure 1B).

S6RP has a known role in translation initiation and cell cycle progression and is a downstream effector of PI3K/Akt/p70S6K signaling pathways.\(^5,\)\(^6\) We therefore hypothesized increased activity of this signaling pathway in embryonic and postinjury vessels may be associated with high growth rates. Using Western blot analysis on arterial tissues, we found increased levels of Akt and p70S6K phosphorylation in embryonic and postinjury vessels compared with adult aortic and sham-injured carotid artery vessels (Figure 1B).

Cultured, Serum-Independent Embryonic and Neointimal SMCs Express High Levels of S6RP and Constitutively Active Akt, mTOR, and p70S6K

PI3K/Akt and mTOR/p70S6K signaling plays a pivotal role in essential cellular functions such as cell growth and survival.\(^5\)\(^–\)\(^7\) PI3K controls translation initiation and cell cycle progression through the activation of its downstream effector, Akt/PKB. Akt can signal to p70S6K via mTOR activation, which results in phosphorylation of S6RP and increased translation of cell cycle–associated genes, such as cyclins and cyclin-dependent kinases.\(^6\)\(^–\)\(^8\) We therefore next determined whether embryonic aortic and carotid artery neointimal SMCs, which exhibit high rates of DNA synthesis when cultured under serum-free conditions, exhibit constitutively active Akt-mediated signaling, thus contributing to serum-independent growth. Using Western blot analysis, we found increased levels of total S6RP and phospho-S6RP and constitutively phosphorylated Akt, mTOR, and p70S6K in serum-deprived but growing embryonic and neointimal SMCs (Figure 2). In contrast, growth-arrested adult SMCs showed significantly decreased total S6RP and decreased phosphorylation of all signaling molecules, which suggests that rapid and serum-independent growth of embryonic and neointimal SMCs is driven, at least in part, by increased activity of Akt- and mTOR/p70S6K-dependent signaling.

Pharmacological and Molecular Inhibition of PI3K Signaling Blocks Embryonic and Neointimal SMC Serum-Independent Growth

To examine the possibility that PI3K-mediated signaling plays a functional role in conferring this highly proliferative growth phenotype, we used a systematic pharmacological and molecular approach to inhibit several levels of this pathway. The effects of rapamycin, a specific mTOR inhibitor, an adenoviral construct expressing an inactive form of Akt (dnAkt), and the PI3K inhibitors LY294002 and wortmannin on SMC serum-independent growth were tested. Control (untreated), vehicle-treated, and green-fluorescing protein-transduced embryonic and neointimal SMCs showed high rates of DNA synthesis under serum-deprived conditions, whereas adult SMCs were effectively growth arrested under serum-deprived conditions (Figure 3A). Rapamycin decreased embryonic and neointimal SMC DNA synthesis by 72% and 68%, respectively. Likewise, embryonic and neointimal SMCs incubated with pAd-dnAkt or treated with LY294002 or wortmannin showed decreased rates of DNA synthesis under serum-deprived conditions (pAd-dnAkt 58% and 61%, respectively; LY294002 80% and 78%, respectively; Wortmannin 58% and 56%, respectively). Inhibitors and dnAkt virus had little effect on the already low DNA synthesis of quiescent adult SMCs (Figure 3A). Western blot analysis confirmed LY-294002 or wortmannin showed decreased rates of DNA synthesis under serum-deprived conditions (pAd-dnAkt 58% and 61%, respectively; LY294002 80% and 78%, respectively; Wortmannin 58% and 56%, respectively). Inhibitors and dnAkt virus had little effect on the already low DNA synthesis of quiescent adult SMCs (Figure 3A). 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tigated here, can induce a serum-independent growth phenotype. Serum-independent growth and Akt, mTOR, p70S6K, and S6RP phosphorylation of adult SMCs were induced when cells were transduced with an adenoviral vector expressing constitutively active Akt (MyrAkt; Figure 3A). Transduction of MyrAkt in embryonic and neointimal SMCs did not significantly enhance the proliferative responses or phosphorylation levels of downstream targets, which suggests that the contribution of Akt activity in these cells is at a maximal state. Collectively, these results suggest that Akt-dependent, mTOR/p70S6K-sensitive pathways are essential regulators of SMC serum-independent growth.

Decreased Activity of PTEN Is Associated With High In Vivo SMC Growth Rates, and Loss of PTEN Induces a Serum-Independent Growth Phenotype In Vitro

The above data strongly suggest constitutive PI3K/Akt-induced signaling mediates SMC serum-independent growth, at least in part. Control of this pathway, as observed in adult-derived SMCs, could occur via loss of upstream positive regulators or via the acquisition of negative regulators. Our previous work suggested serum-independent growth is actively controlled through the timed acquisition of a suppressor activity.3 We therefore focused our studies on the potential role of PTEN, an endogenous negative regulator of PI3K signaling, in controlling embryonic and neointimal SMC serum-independent growth. PTEN, a dual-specificity lipid and protein phosphatase, promotes cell cycle arrest by downregulating PI3K/Akt signaling9–12 and is inactive when phosphorylated.13,14 Except for a single report,15 little is known of the physiological role of PTEN in vascular SMCs. Western blot analysis showed equal amounts of total PTEN protein expressed by embryonic and adult aortas and sham-injured and 7-day-injured carotid arteries (Figure 4). In contrast, we found increased levels of phospho-PTEN (inactive) in embryonic and adult aortas and sham-injured and 7-day-injured carotid arteries (Figure 4), consistent with increased growth rates.

To determine whether PTEN inactivity is associated with serum-independent SMC growth, embryonic, neointimal, and adult SMCs were maintained under serum-free conditions for 72 hours and then analyzed by Western blot analysis for total versus phospho-PTEN. In agreement with our previous work,3 total PTEN protein was expressed at similar levels by all SMC cultures. In contrast, increased levels of phospho-PTEN (inactive) were observed in serum-deprived but growing embryonic and sham-injured carotid artery tissues (Figure 4), consistent with increased growth rates.
growth rates of embryonic and neointimal SMCs under serum-free conditions. We next examined whether loss of endogenous PTEN induces a serum-independent phenotype in adult SMCs by treating adult SMCs with PTEN-specific antisense morpholino oligonucleotides and analyzing their ability to replicate DNA under serum-deprived and serum-stimulated conditions. Western blot analysis showed endogenous PTEN levels were reduced significantly by antisense treatment (Figure 5B). Control (untreated) and inverse antisense-treated SMCs exhibited low replication rates under serum-deprived conditions but increased rates in response to serum stimulation (Figure 5C). In contrast, antisense-treated SMCs exhibited high rates of growth under serum-free conditions, with replication rates that approached those observed in serum-stimulated control SMCs (Figure 5C). Serum-stimulated growth of antisense-treated SMCs was also significantly higher than control SMCs. Collectively, these data suggest PTEN is a potent endogenous inhibitor of SMC serum-independent proliferation.

Discussion

At distinct times during development and after vascular injury, rat vascular SMCs undergo periods of rapid but transient proliferation and, in culture, exhibit high rates of replication under serum-deprived conditions. Embryonic and neointimal SMC serum-independent growth is independent of secreted growth factors known to stimulate growth of traditional adult-derived SMCs, such as platelet-derived growth factor or basic fibroblast growth factor, but rather is dependent on an intrinsically controlled mechanism. Likewise, control of SMC replication during intimal thickening has been shown to be independent of such growth factors, which suggests that SMCs that exhibit a unique growth phenotype distinct from traditional vascular SMCs preferentially contribute to vascular lesion formation. We report high SMC growth rates observed in embryonic and postinjury vessels are associated with increased Akt-mediated signaling and decreased PTEN activity. Serum-deprived but proliferating embryonic and neointimal SMCs exhibit constitutively activated Akt, mTOR, and p70S6K and increased expression of S6RP, consistent with increased cell cycle progression. Selective inhibition of this pathway by both pharmacological and molecular approaches blocks serum-independent SMC growth. Finally, loss of serum-independent growth potential, as observed in SMCs cultured from mature, uninjured vessels, occurs, at least in part, through the developmentally timed increase in PTEN activity.

The findings of the present study show rapid and serum-independent growth of embryonic and neointimal SMCs is dependent on constitutive activity of the translational machinery, and these findings are consistent with neointimal cells expressing a highly proliferative phenotype characteristic of embryonic SMCs rather than traditional adult-derived vascular SMCs. Others have noted transient increases in Akt, mTOR, and p70S6K activities correlating to rapid SMC replication in various vascular injury models. Because expression of this phenotype only occurs at very distinct times, it is likely that SMCs execute a cellular program in response to appropriate environmental cues, which results in the transient expression of a highly proliferative phenotype characterized by increased PI3K/Akt-mediated signaling. In culture, this would manifest as a stable cellular program characterized by the ability of these cells to replicate under serum-independent conditions. Therefore, we can speculate there is an underlying positive regulator controlling constitutive activity of the PI3K/Akt pathway to drive serum-independent growth, although we have yet to define this factor.

In addition, or alternatively as we previously speculated, the ability of SMCs to replicate under unstimulated condi-
tions could occur through loss or inactivation of previously unidentified negative growth regulators. In the present report, we show the timed acquisition of PTEN actively represses SMC serum-independent growth. Elevated levels of phosphatidylinositol 3,4,5-trisphosphate, the predominant PTEN substrate, are observed in PTEN-deficient cells and are sufficient to activate Akt to transduce proliferative signals in the absence of other stimuli.\textsuperscript{9–11} In our system, the ability of SMCs to replicate under unstimulated conditions via inactivation of PTEN could occur through gain of upstream negative regulators or through loss of upstream positive regulators of PTEN. Therefore, identification of modulators of PTEN activity will significantly advance our knowledge of the vascular developmental process and the SMC response to injury.

PTEN, first discovered as a potent tumor suppressor, is a dual-specificity lipid and protein phosphatase.\textsuperscript{9–12} As a direct negative regulator of PI3K/Akt- and FAK-mediated signaling, PTEN promotes cell cycle arrest, apoptosis, and decreased cell migration. Studies have shown PTEN inactivation results in early embryonic lethality and altered differentiation and organization of all 3 germ layers.\textsuperscript{19,20} The present data suggest PTEN is less active in developing SMCs, thus contributing to a highly proliferative phenotype. At distinct times during the developmental process, an upregulation of PTEN activity contributes to the loss of constitutive Akt signaling and SMC quiescence. However, vascular injury results in PTEN inactivation, constitutive Akt signaling, and the reexpression of a serum-independent growth phenotype. Figure 6 summarizes our proposed role of PTEN in the control of SMC serum-independent growth. Ongoing studies in our laboratory are addressing other issues, for instance, upstream regulators, associated with PTEN function.

Neointimal proliferation contributing to restenosis after balloon angioplasty and stent placement continues to be the most important limitation to the success of such vascular interventions, with the overall rate of restenosis remaining relatively high (\textsuperscript{\textasciitilde}30\%).\textsuperscript{21–23} Most approaches to limit the rate of restenosis, designed to target factors that stimulate growth of traditional adult-derived serum-dependent SMCs,\textsuperscript{16,17,21} have been largely unsuccessful. Recently, an increasing number of animal and human studies have shown significant reductions in intimal thickening associated with attenuated PI3K/Akt/mTOR/p70S6K signaling after rapamycin treatment.\textsuperscript{21–28} Two-year follow-up studies in humans have shown persistent inhibition of in-stent restenosis with few, if any, adverse effects, which demonstrates the effectiveness and safety of rapamycin.\textsuperscript{27,28} Our studies show the high proliferative capability, even under serum-deprived conditions, exhibited by both embryonic and neointimal SMCs is dependent on low PTEN activity resulting in constitutive Akt-dependent

Figure 5. Knockdown of endogenous PTEN induces serum-independent growth of adult-derived SMCs. A, Embryonic (Emb), neointimal (Neo), and adult (Ad) SMCs were serum deprived for 72 hours. Whole-cell lysates were collected and analyzed for phospho-PTEN (pPTEN) with specific antibodies. Western blots were scored for relative densitometry normalized against total PTEN (data shown in graph). B, Adult SMCs were transfected with FITC-labeled PTEN-specific morpholino antisense (AS) or inverse antisense oligonucleotides (Inv) and allowed to recover for 30 hours. Control SMCs (Ctrl) were treated with morpholino delivery solution only. Cell lysates were analyzed by Western blotting for total PTEN. Filters were stripped and reprobed for \(\beta\)-actin as control for protein loading. C, SMCs transfected as described were growth arrested in serum-free medium for 48 hours then stimulated with 10% CS (black bars) or kept in serum-free medium (gray bars) for an additional 24 hours in the presence of 10 mmol/L BrDu. SMCs were analyzed by immunocytochemistry for BrDu-positive cells. Percentage of BrDu-positive SMCs was determined by counting a minimum of 200 cells for each condition. Data are presented as mean \(\pm\)SE. *Different than control and inverse AS in SFM; \(P<0.01\). **Different than control and inverse AS in 10% CS; \(P<0.01\).
signaling. Loss of this phenotype is associated with increased PTEN activity and regulated, rather than constitutive, activity of Akt-mediated signaling. Pharmacological inhibition of serum-independent growth by PI3K and/or mTOR inhibitors essentially mimics the endogenous effects of PTEN. Therefore, given the recent success of rapamycin on in-stent restenosis combined with the present data (in particular, the rapamycin data), we propose the efficacy of rapamycin in inhibiting in-stent restenosis is the result of selective growth inhibition of cells that exhibit this unique growth phenotype.

It is currently unclear whether all SMCs in the vasculature are capable of reexpressing a highly proliferative, embryonic growth phenotype after vascular injury. It was originally thought new proliferative SMCs were derived solely from resident adventitial cells and circulating and/or bone marrow–derived precursor cells. Regardless of origin, a number of studies provide evidence that cells responding to injury undergo a developmental sequence of events during the injury-repair process. Our studies demonstrate the capacity of SMCs to transiently reexpress a serum-independent growth phenotype after injury that is associated with constitutive Akt/mTOR/p70S6K activities mediated via PTEN inactivation. Therefore, selectively targeting of PTEN and/or elucidation of upstream modulators of PTEN activity might hold promise for the development of antirestenotic and antiatherosclerotic therapies.

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