Distinct Regulation of Mitogen-activated Protein Kinases and p27Kip1 in Smooth Muscle Cells from Different Vascular Beds

A POTENTIAL ROLE IN ESTABLISHING REGIONAL PHENOTYPIC VARIANCE*

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Excessive proliferation and migration of vascular smooth muscle cells (SMCs) participate in atherosclerotic plaque growth. In this study, we investigated whether SMCs from vessels with different atherogenicity exhibit distinct growth and migratory potential and investigated the underlying mechanisms. In fat-fed rabbits, we found increased cell proliferation and atheroma formation in the aortic arch versus the femoral artery. When examined in culture, SMCs isolated from the aortic arch (ASMCs) displayed a greater capacity for inducible proliferation and migration than paired cultures of femoral artery SMCs. Two lines of evidence suggested that distinct regulation of the growth suppressor p27Kip1 (p27) contributes to establishing these phenotypic dissimilarities. First, p27 expression was comparably lower in ASMCs, which exhibited a higher fraction of p27 phosphorylated on Thr-187 and ubiquitinated. Second, forced p27 overexpression in ASMCs impaired their proliferative and migratory potential, a finding corroborated by forced MAPK activation diminished p27 expression and attenuated ASMC proliferation and migration. We propose that intrinsic differences in the regulation of MAPKs and p27 play an important role in creating variance in the proliferative and migratory capacity of vascular SMCs, which might in turn contribute to establishing regional variability in atherogenicity.

Atherosclerotic cardiovascular disease is the leading cause of mortality and morbidity in developed countries. Although percutaneous transluminal angioplasty has become a well-established technique for revascularization of patients with arterial occlusive disease, the occurrence of restenosis at the site of angioplasty remains the major limitation despite the successful procedure. The molecular basis of atherosclerosis and restenosis involves dedifferentiation of vascular smooth muscle cells (SMCs) to a so-called “synthetic state” characterized by abundant production of matrix components and excessive proliferative and migratory activities (1–3). Therefore, a better understanding of the molecular mechanisms underlying these processes should help develop novel therapeutic approaches for the treatment of cardiovascular disease.

Cellular proliferation is regulated by the balance between multiple cyclin-dependent kinases (CDKs), MAPK, and members of the Cip/Kip and INK4 families of CDK inhibitors (4, 5). Active CDK/cyclin complexes promote cell cycle progression by phosphorylating the retinoblastoma gene product, pRb, and the related pocket proteins p107 and p130 from the activity of CDK/cyclin holoenzymes. Studies arguing for a role of the Cip/Kip protein p27Kip1 (p27) in the pathophysiology of the cardiovascular system include the following. 1) p27 may contribute to the reestablishment of the quiescent phenotype after the initial proliferative response to balloon angioplasty in rat and porcine arteries, and adenosinemediated overexpression of p27 inhibited neointimal growth in these experimental models (6–8). 2) p27 may function as a molecular switch that regulates the phenotypic response of vascular SMCs to both hyperplastic and hypertrophic stimuli (9, 10). 3) p27 is a negative regulator of endothelial cell proliferation and migration in vitro, and adenosine-mediated overexpression of p27 inhibited angiogenesis in vivo (11, 12). 4) p27 may contribute to integrin-mediated control of vascular SMC proliferation (13). 5) p27 may limit cardiomyocyte proliferation during early postnatal development and after injury in adult mice (14, 15). 6) Changes in p27 expression might regulate human vascular cell proliferation within atherosclerotic lesions (7, 16), and a causal link between reduced p27 expression and atherosclerosis has been established in apolipoprotein E-deficient mice (17). It has been established that the expression of p27 is regulated mainly

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¹ The abbreviations used are: SMC, smooth muscle cells; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; p27, p27Kip1; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated ERK; BrdUrd, bromodeoxyuridine; ASMCs, SMCs isolated from the aortic arch; CSMCs, carotid artery SMCs; FSCMs, femoral artery SMCs; FBS, fetal bovine serum; ITC, insulin-transferrin-selenium medium with 250 μmol/liter ascorbic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PBS, phosphate-buffered saline; FGF, fibroblast growth factor; Rev, retroviral vector; SMα-actin, smooth muscle α-actin.
at the level of translation and protein turnover (18).
Multiple growth factors and cytokines interact with specific receptors located in the cytoplasmic membrane of vascular cells in response to a variety of pathological stimuli, thus triggering a complex signal transduction cascade, which culminates in changes in gene expression that execute a proliferative and migratory response (2, 3). The activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway is thought to play an important role during cardiovascular disease (19–22).

It has been well established that different segments of the arterial tree display significant differences in their susceptibility to atherosclerosis, both in animal models and humans. In this regard, it is notable that vascular SMCs display regional phenotypic variance both when comparing cells obtained from different compartments of the same vessel or cells isolated from vessels from different vascular beds (24–30). The findings of this study demonstrate that p27 and MAPKs are critical regulators of vascular SMC proliferation and migration. Our results suggest that intrinsic differences in the regulation of p27 and MAPKs may contribute to the establishment of regional variance in the proliferative and migratory capacity of SMCs from distinct regions of the vascular system.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were purchased from Santa Cruz Biotechnology: cyclin D1 (sc-450), cyclin A (sc-751), cyclin E (sc-198), p27 (sc-1641), α-tubulin (sc-8035), CDK2 (sc-163-G), PDGF receptor isoform β (PDGFR-β) (sc-432), P-ERK1/2 (sc-7383), reactive with Tyr-202-phosphorylated ERK1 and ERK2, and ERK2 (sc-154, reactive with ERK2 and, to a lesser extent, ERK1). Other antibodies were purchased from Calbiochem (anti-p27 phospho-specific Thr-187 (catalog number 506128) and anti-ubiquitin (catalog number 662099)), Dako (anti-5-bromodeoxyuridine (BrdUrd)), and Master Diagnostica (anti-5′-bromo2′-deoxyuridine (BrdUrd)), and Merck (anti-ubiquitin (clone I4091)).

Rabbit Studies—Male white New Zealand rabbits (4–5-month-old) were fed either control chow (n = 5) or received a high fat diet (n = 10) for 2 months. To assess arterial cell proliferation, the photomicrographs showed representative examples of BrdUrd immunoreactivity in sections of the aortic arch (A) and femoral arteries (B) of fat-fed rabbits. Specimens were counterstained with eosin. Two different magnifications are shown for each specimen as indicated in each photomicrograph. Arrows in the ×200 photomicrograph of the femoral artery indicate two BrdUrd-positive cells within the intimal lesion. White arrowheads point to the internal elastic lamina.

FIG. 1. Arterial cell proliferation and atherogenesis in the aortic arch and femoral artery of hypercholesterolemic rabbits. Rabbits received either control chow (n = 5) or a high fat diet (n = 10) for 2 months. Prior to sacrifice, animals were injected with BrdUrd to assess arterial cell proliferation. The photomicrographs show representative examples of BrdUrd immunoreactivity in sections of the aortic arch (A) and femoral arteries (B) of fat-fed rabbits. Specimens were counterstained with eosin. Two different magnifications are shown for each section as indicated in each photomicrograph. Arrows in the ×200 photomicrograph of the femoral artery indicate two BrdUrd-positive cells within the intimal lesion. White arrowheads point to the internal elastic lamina.

(Continued) Infected cells were selected in the presence of puromycin (2.5 μg/ml, Sigma).

Immunofluorescence Labeling of Vascular SMC Differentiation Markers and TUNEL Assay—Cells were plated onto glass coverslips. To examine the expression of differentiation markers, cells were grown until reaching confluence and then were maintained in mitogen-free insulin-transferrin-selenium (Invitrogen) supplemented with 25 μmol/liter ascorbic acid (ITC, Sigma) medium (33) for 2 days. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 1 h and permeabilized with 0.1% Triton X-100/PBS. Cells were blocked with 1% bovine serum albumin/PBS and expression of smooth muscle α-actin (SMA) and desmin was examined by indirect immunofluorescence. Microscopic images were digitally recorded on an Axioscope II microscope (Zeiss).

For TUNEL assays, cells were grown to ~60% confluence and were maintained in mitogen-free ITC medium for 2 days. For UV light irradiation, cell culture medium was removed and the cells were washed twice with PBS. The cultures then were placed in the tissue culture hood and exposed to UV light for 45 min (UV G-30-watt lamp, Sylvania, Japan). Control (not irradiated) and UV-irradiated cells were fixed and permeabilized as indicated above, and TUNEL assay was performed using an in situ cell death detection kit as suggested by the manufacturer (Roche Molecular Biochemicals).

Migration Assays—Migration of cultured cells labeled with the fluorescence dye calcin-AM (Molecular Probes) was assessed with the FALCON HTS FluoroBlock system as suggested by the manufacturer (BD Biosciences). Labeled cells were placed in the inserts (8.0-μm pore size, 5 × 104 cells/insert) in serum-free media. The lower chamber contained

PDGF-BB) (10 ng/ml) to induce cell cycle reentry, and cells were pulsed with 1 μCi/106 [3H]thymidine (Amersham Biosciences) during the last 4 h of incubation. After washes with cold PBS, DNA was precipitated with 15% trichloroacetic acid and solubilized with 0.2 mol/liter NaOH. Radioactivity incorporated into DNA was measured in a scintillation counter (Wallac).

Migration Assays—Migration of cultured cells labeled with the fluorescent dye calcin-AM (Molecular Probes) was assessed with the FALCON HTS FluoroBlock system as suggested by the manufacturer (BD Biosciences). Labeled cells were placed in the inserts (8.0-μm pore size, 5 × 104 cells/insert) in serum-free media. The lower chamber contained

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either serum-free media (unstimulated cells) or the chemotactic agent (10% FBS or 10 ng/ml PDGF-BB) (induced cells). Serum-free medium was supplemented with 0.1% bovine serum albumin. Chemotaxis at different times after plating the cells was assessed by detecting the fluorescence in the lower chamber at the indicated time points after plating the cells. Results represent the average fluorescence of induced cells. Positive controls for chemotaxis were performed using polystyrene beads. Chemotaxis was assessed by detecting the fluorescence of cells migrating to the lower chamber at the indicated time points after plating the cells. Results represent the average fluorescence of induced cells.

Western Blot Analysis, Immunoprecipitation, and CDK Assays—Cell lysates were prepared with either ice-cold lysis buffer A or buffer B supplemented with protease inhibitor Complete™ Mini-mixture (Roche Molecular Biochemicals). Buffer A contained 50 mmol/liter Hepes (pH 7.5), 1% Triton X-100, 150 mmol/liter NaCl, 1 mmol/liter diethio- reitol, 0.1 mM orthovanadate, 10 mM β-glycerophosphate, and 10 mM sodium fluoride. Buffer B contained 20 mmol/liter Tris-HCl (pH 7.5), 0.5% Triton X-100, 0.5% deoxycholate, 150 mmol/liter NaCl, 10 mmol/liter EDTA, and 1 mmol/liter diethio- reitol. 50 µg of protein was electro- phoresed on 12% SDS-PAGE to perform Western blot analysis as described previously (6). Antibody dilutions were 1:100 (cyclin D1, cyclin A, cyclin E, P-ERK1/2, and pS7), 1:200 (α-tubulin and CDK2), and 1:250 (PDGFR-β). 1:500 (anti-p27 phospho-specific Thr-187), and 1:700 (ERK2). For immunoprecipitation/Western blot assays, cell lysates were incubated with anti-ubiquitin antibody (0.5 µg) and protein A/G Plus-agarose (Santa Cruz Biotechnologies) for 4 h at 4°C under rotation. The immune complexes were extensively washed and subjected to Western blot analysis using anti-p27 antibody.

CDK activity in cell lysates (100 µg of protein) was determined as described previously (6) with the exception that CDK/cyclin holoenzymes were immunoprecipitated with 0.2 µg of each of the anti-cyclin E and anti-cyclin A antibodies.

Statistical Analysis—Results are reported as the mean ± S.E. Differences were evaluated using either two-tail unequal Student’s t test or ANOVA and Fisher’s post hoc test (Statview, SAS Institute).

RESULTS

Arterial Cell Proliferation and Atherogenesis in Different Vascular Beds of Hypercholesterolemic Rabbits—We investigated atherogenesis in fat-fed New Zealand White rabbits, which rapidly develop atheromas in response to dietary manipulation (34). To examine arterial cell proliferation, animals received four injections of BrdUrd prior to sacrifice. Although aortic atherosclerosis and BrdUrd immunoreactivity were essentially undetectable in rabbits fed with control chow (n = 5, data not shown), all of the fat-fed rabbits included in our study displayed atheromatous lesions in the aortic arch and exhibited abundant BrdUrd immunoreactivity in both intimal and me-
dial cells \( n = 10 \), Fig. 1A). In marked contrast, only 3 of 10 fat-fed rabbits displayed small atherosclerotic lesions in the femoral artery (Fig. 1B). Moreover, the number of BrdUrd-positive cells in femoral arteries was negligible in the media and was lower within the lesions as compared with the aortic arch (Fig. 1B). These findings are consistent with previous rabbit studies demonstrating that the aortic arch is highly susceptible to diet-induced atherosclerosis (34–37).

ASMCs and FSMSCs Display Dissimilar Migratory and Proliferative Activity in Vitro—Having demonstrated distinct proliferative response and atherogenicity in the aortic arch and femoral artery, we isolated SMCs from these vessels (ASMCs and FSMSCs, respectively) to ascertain whether their phenotypic dissimilarities were maintained in vitro. In primary cultures grown to confluence in serum-free media, ASMCs exhibited an epithelioid shape (Fig. 2A), whereas FSMSCs disclosed a bipolar, spindle-shaped morphology (Fig. 2B). We next performed indirect immunofluorescence experiments in passage 2 cultures to examine the expression of SMC differentiation markers. Both ASMCs and FSMSCs revealed abundant SM \( \alpha \)-actin immunoreactivity in a prominent stress fiber pattern (Fig. 2, C and D). In contrast, desmin expression appeared more abundant in FSMSCs (Fig. 2, E and F). These phenotypes were stable at least up to passage 8 (data not shown).

We next compared the migratory and proliferative capacity of cultured ASMCs and FSMSCs. Although FSMSCs did not migrate in response to PDGF-BB or FBS, both agents elicited a robust migratory response in paired

![Graph A](image)

**Fig. 3.** ASMCs and CSMCs display similar migratory and proliferative capacity. Statistical analysis was performed using ANOVA and Fisher's post hoc test. A, migration was assayed as described in Fig. 2G using 10% FBS as the chemotactic agent. *, \( p < 0.0001 \) versus \( t = 0 \). B, [\( ^3H \)]thymidine incorporation was assayed as indicated in Fig. 2H \( (n = 4 \text{ each time point}) \). Comparisons between ASMC and CSMC at each time point: \( \dagger, p < 0.001; \) comparisons versus corresponding \( t = 0 \); *, \( p < 0.0001 \).

![Graph B](image)

**Fig. 4.** ASMCs and FSMSCs display dissimilar cell cycle regulatory protein expression and CDK activation. Confluent cultures were maintained for 72 h in mitogen-free ITC medium and then exposed to 10 ng/ml PDGF-BB as indicated. Cell extracts were prepared in lysis buffer A containing phosphatase inhibitors \((A, B, D, \text{ and } E)\) or buffer B \((C)\), which did not contain phosphatase inhibitors. The analysis of lysates included cyclin A/cyclin E-associated CDK activity using histone H1 and \( [\gamma-\text{32P}] \)ATP substrates \((A)\), Western blot with the indicated antibodies \((B–D)\), and immunoprecipitation with an anti-ubiquitin antibody followed by Western blot of the immunoprecipitated material using anti-p27 antibodies \((E)\). A, kinase reactions were analyzed by SDS-PAGE and autoradiography. Relative activity was estimated after densitometric analysis (0 h is set as 1 for each cell type). B, densitometric analysis was performed to estimate the relative level of cyclin D1 and A. Each cyclin value was divided by its corresponding CDK2-loading control. Shown below is the PVDF membrane stained with Ponceau prior to incubation with antibodies. C, densitometric analysis was performed to estimate the relative p27 level. Each p27 value was divided by its corresponding tubulin-loading control (ASMC at 0 h = 1; nd, not detected). D and E, the phospho-specific anti-p27 antibody only recognizes p27 phosphorylated on Thr-187. Open and closed arrowheads point to the slow and faster migrating p27-immunoreactive band, respectively. Note that the slow migrating band that undergoes phosphorylation on Thr-187 and ubiquitination prevailed in ASMCs. By contrast, the faster migrating p27 band that does not contain protein phosphorylated on Thr-187 and does not undergo ubiquitination predominated in FSMSCs.
cultures of ASMCs (Fig. 2G). Likewise, \(^{[3}\text{H}]/\text{thymidine incorporation in starvation-synchronized cultures restimulated with PDGF-BB was lower in FSMSCs than in ASMCs (data not shown). In contrast, as determined by the TUNEL assay, apoptosis was similar in ASMCs and FSMSCs both under control conditions and after UV irradiation (Fig. 2I).}

Lineage analysis experiments have suggested that neural crest-derived (ectoderm) SMCs prevail in arterial segments proximal to the heart (i.e. aortic arch and great vessels of the head and neck), whereas arteries located more distally to the heart contain mainly mesoderm-derived SMCs (i.e. abdominal aorta and hind limb arteries) (1, 27, 38). Thus, dissimilar behavior and morphology of ASMCs and FSMSCs raised the possibility that adult SMC phenotypic properties are related, at least in part, to their primary embryonic lineage. Consistent with this notion, we found that CSMSCs (also of neural crest origin) behaved in a similar fashion as the ASMCs in proliferation and migration assays (Fig. 3).

Role of p27 in the Establishment of Phenotypic Variance between ASMCs and FSMSCs—Differences in proliferation and migration between ASMCs and FSMSCs prompted us to investigate the underlying molecular mechanisms. Consistent with the results of Fig. 2H showing greater PDGF-BB-dependent proliferation in ASMCs than in FSMSCs, CDK activity was higher in PDGF-BB-stimulated ASMCs (Fig. 4A). Likewise, the up-regulation of the positive cell cycle regulators cyclin D1 and cyclin A, whose expression is induced as starvation-synchronized cells resume progression through G1 and S-phase upon mitogen restimulation (4, 5), occurred earlier and was more prominent in PDGF-BB-stimulated ASMCs versus FSMSCs (Fig. 4B). The expression of the PDGFR-\(\beta\) was similar in ASMCs and FSMSCs, both under mitogen-free conditions and upon PDGF-BB stimulation (Fig. 4C), suggesting that dissimilar PDGF-BB-dependent proliferation and migration in ASMCs and FSMSCs were not a consequence of distinct regulation of PDGFR-\(\beta\) expression. Down-regulation of PDGFR-\(\beta\) 9 h after PDGF-BB stimulation is consistent with the notion that binding of PDGF to its receptor leads to internalization and degradation of the ligand-receptor complex in endosomes (39).

We next investigated the expression of the growth suppressor p27 in the same confluent cultures of ASMC and FSMSC used for the PDGFR-\(\beta\) immunoblot. Of note, the lysis buffer used in these assays did not contain phosphatase inhibitors (buffer B). Both under mitogen-free conditions and at different time points after PDGF-BB stimulation, p27 was detected as a single band that was more abundant in confluent cultures of FSMSCs versus ASMCs (Fig. 4C). For example, whereas p27 was not detected in ASMC after 9 h of stimulation, FSMSCs expressed more p27 at this time point than did unstimulated ASMCs. An analysis of subconfluent cultures also disclosed higher level of p27 expression in FSMSCs (data not shown). We next examined cell lysates prepared in the presence of phosphatase inhibitors (buffer A), which also disclosed higher p27 expression in FSMSCs versus ASMC (Fig. 4D, top blot). Notably, these experiments demonstrated the presence of two p27-immunoreactive bands of different electrophoretic mobility and

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maintained for 72 h in ITC media and then were exposed to 10 ng/ml PDGF-BB. When indicated, mitogen-depleted ASMCs were pretreated with antibodies. Treatment with 50 ng/ml PDGF-BB for short (A or long (B) periods of time (8 h). Cell lysates were prepared in lysis buffer A to perform immunoblot analysis with the indicated antibodies. Treatment with 50 μM PD98059 was initiated 1 h before the addition of PDGF-BB. P-ERK1/2 and ERK1/2 indicate phosphorylated (active) and total ERK1/2, respectively. Densitometric analysis was performed to estimate the relative level of P-ERK1/2 and p27. Each P-ERK or p27 value was divided by its corresponding loading control (total ERK or tubulin, respectively; nd, not detected). For p27, results are shown relative to control (set as 1).

C. asynchronously growing ASMCs were treated for 1 h in mitogen-free ITC medium supplemented with PD98059 or vehicle, and then cells were incubated for 24 h with 10 ng/ml PDGF-BB. Cultures were pulsed with [3H]thymidine during the last 4 h periods of time (8 h). Cell lysates were prepared in lysis buffer A to perform immunoblot analysis with the indicated antibodies. Treatment with 50 μM PD98059 was initiated 1 h before the addition of PDGF-BB. P-ERK1/2 and ERK1/2 indicate phosphorylated (active) and total ERK1/2, respectively. Densitometric analysis was performed to estimate the relative level of P-ERK1/2 and p27. Each P-ERK or p27 value was divided by its corresponding loading control (total ERK or tubulin, respectively; nd, not detected). For p27, results are shown relative to control (set as 1).

D. Mitogen-depleted or PDGF-BB-stimulated ASMCs were treated with 50 μM PD98059 for 1 h prior to PDGF-BB stimulation. Cells were pulsed with [3H]thymidine (n = 4; *, p < 0.0001 versus mitogen-depleted cells; †, p < 0.0001 versus 50 μM PD98059).

**Discussion**

We next investigated the effect of p27 overexpression on ASMC proliferation and migration by infecting these cells with retroviral vectors encoding for p27 (Rev-p27). Rev-p27-infected ASMCs disclosed a 3-fold increase in p27 expression, which caused a reduction in [3H]thymidine incorporation (Fig. 5A) and migration (Fig. 5B) as compared with control cultures infected with Rev-LacZ. These findings demonstrate that increased p27 expression is sufficient to attenuate the growth and migratory capacity of ASMCs. Thus, distinct regulation of p27 expression might contribute to establishing differences in the proliferative and migratory capacity of ASMCs and FSCMs.

**Differential Regulation of MAPKs in ASMCs and FSCMs and Role in the Regulation of Vascular SMC Proliferation and Migration**—Because the MAPK pathway plays a pivotal role in transducing environmental signals required for both cellular proliferation and migration (40), we examined the kinetics of expression and activation of individual MAPKs in ASMCs and FSCMs. Western blot analysis using an antibody specific for the phosphorylated (active) form of the MAPK isoforms of 44 and 42 kDa (dubbed ERK1 and ERK2, respectively) revealed a rapid activation of these proteins upon PDGF-BB stimulation of mitogen-depleted ASMCs and FSCMs (Fig. 6, top blot). However, the maximum level of ERK1/2 activation was higher in ASMCs than in FSCMs. Moreover, ERK1/2 activation was more prolonged in ASMCs. These differences occurred despite similar level of total ERK1/2 in ASMCs and FSCMs (Fig. 6, bottom blot).

To determine whether dissimilar MAPK regulation might contribute to phenotypic differences between ASMCs and FSCMs, we performed loss- and gain-of-function experiments. Treatment of ASMCs with PD98059, a selective inhibitor of MEK, impaired PDGF-BB-dependent ERK1/2 activation (Fig. 7A) and up-regulated p27 expression (Fig. 7B). Importantly, the exposure of asynchronously growing ASMCs to PD98059 inhibited [3H]thymidine incorporation in a dose-dependent manner (Fig. 7C), and preincubation of starvation-synchronized ASMCs with PD98059 blocked de novo DNA synthesis upon mitogen stimulation (Fig. 7D). Moreover, the exposure of ASMCs to PD98059 inhibited migration (Fig. 8A).

We also examined the effect of forced ERK1/2 activation on
FSMC proliferation and migration by infecting cultures with a retroviral vector encoding for a constitutively active MEK1 mutant (Rev-MEKE). As compared with control cultures, Rev-MEKE-infected FSMCs disclosed constitutive activation of ERK1/2 (Fig. 9A), which markedly reduced p27 expression (Fig. 9B), increased $[^{3}H]$thymidine incorporation (Fig. 9C), and augmented cell migration (Fig. 8B). Collectively, the above studies suggest that differential regulation of ERK1/2 in ASMCs and FSMCs plays an important role in the establishment of intrinsic differences in the proliferative and migratory potential of these cells.

**DISCUSSION**

Vascular SMCs undergo dedifferentiation and excessive proliferation and migration during atherosclerosis and restenosis post-angioplasty (1–3). Up-regulation of the growth suppressor p27 in the arterial wall might limit SMC proliferation at late time points after balloon angioplasty in rat and porcine arteries (6, 7), and adenovirus-mediated overexpression of p27 inhibited neointimal thickening in these animal models (8, 41). Regarding the role of p27 on atherosclerosis, genetic disruption of p27 increased arterial cell proliferation and accelerated atheroma formation in hypercholesterolemic apolipoprotein E-deficient mice (17). Moreover, p27 might mediate transforming growth factor-β-dependent inhibition of cell growth in human atheromas (16), and proliferating cells within human coronary atheromas appear to express a low level of p27 (7). Consistent with the observation that p27 overexpression attenuated human vascular endothelial cell migration in vitro (12) and that p27 inactivation reduced rapamycin-dependent inhibition of vascular SMC migration (42), we found that retrovirus-mediated overexpression of p27 inhibited vascular SMC migration. Thus, p27 might control neointimal thickening through the regulation of both cell proliferation and migration.

Our studies with fat-fed rabbits showed that aortic arch tissue displays increased cell proliferation and atherogenicity as compared with femoral artery. We found that primary cultures of ASMCs and FSMCs maintained marked differences in their growth and migratory potential, which might be related, at least in part, to their distinct primary embryonic lineage (neural crest and mesoderm, respectively) (1, 27, 38). Indeed, ASMCs and CSMCs, which are thought to derive from neural crest ectoderm, behaved similarly in our proliferation and migration assays. We chose to examine ASMCs and FSMCs as an in vitro model to elucidate molecular mechanisms involved in the establishment of dissimilar atherogenicity in distinct vessel segments. Greater ASMC proliferation and migration corre-
lated with the lower expression of p27 when compared with FSMCs, and retrovirus-mediated overexpression of p27 attenuated the growth and migratory potential of ASMCs. Previous studies also support the notion that distinct regulation of p27 expression plays an important role in establishing differences in the phenotypic response of vascular SMCs toward a variety of stimuli. First, Yang et al. (29) reported reduced proliferation of human internal mammary artery compared with saphenous vein SMCs. Importantly, PDGF-BB markedly down-regulated p27 protein level in saphenous vein, but this response was much less pronounced in internal mammary artery. Thus, sustained p27 expression despite growth stimuli may contribute to the resistance to growth of SMCs from internal mammary artery and to the longer patency of arterial versus venous grafts. Second, p27 may regulate the proliferative response of vascular SMCs toward fibroblast growth factor 2 (FGF2 or basic FGF). Whereas FGF2 plays a critical role in the induction of medial SMC proliferation after balloon angioplasty (30, 43, 44), neutralizing antibodies to FGF2 failed to inhibit neointimal SMC proliferation in balloon-injured arteries (45). Moreover, only a small increase in growth was observed when arteries with existing neointimal lesions were exposed to FGF2 (30, 43). Attenuated FGF2-dependent proliferation of neointimal SMCs occurred despite a robust induction of positive cell cycle regulators (30). Interestingly, neointimal SMCs expressed high levels of p27 compared with medial SMCs, and FGF2 infusion did not reduce the level of this inhibitor in arteries with established neointimal lesions.

Protein turnover is thought to play a major role in the regulation of p27 expression. Phosphorylation of p27 on Thr-187 triggers its ubiquitination and rapid turnover in the proteasome (18). Our Western blot assays demonstrate that the majority (90%) of p27 in ASMCs corresponds to a slow migrating form that undergoes phosphorylation on Thr-187 and ubiquitination (46). In marked contrast, ~96% of p27 in FSMCs corresponded to a faster migrating p27 band that was not recognized by the phospho-specific antibody and did not contain ubiquitinated protein. Thus, the relative amount of p27 phosphorylated on Thr-187 and ubiquitinated appears higher in ASMCs compared with FSMCs, which might account for the lower level of p27 detected in ASMCs. Of note, ubiquitinated p27 in the faster migrating band that does not contain phosphorylated Thr-187 was also detected in ASMCs (cf. Fig. 4E). This finding is in agreement with recent studies demonstrating an additional pathway for p27 ubiquitination and proteolysis independent of phosphorylation of p27 on Thr-187 (46, 47).

We investigated additional regulatory networks involved in the establishment of vascular SMC-phenotypic variance. A wealth of evidence implicates the rapid activation of the MAPK signal transduction pathway during the pathogenesis of cardiovascular disease (19, 21). For example, it has been suggested that persistent activation and hyperexpression of ERK1/2 are critical elements to initiate and perpetuate cell proliferation during diet-induced atherogenesis in the rabbit (48). Moreover, ERK1/2 activation occurs rapidly after angioplasty of porcine and rat arteries (20, 22), and all three MAPKs are activated in ERK1/2 activation occurs rapidly after angioplasty of porcine coronary arteries with established neointimal lesions. Second, p27 expression is under the control of MAPKs and p27 expression is under the control of MAPKs as demonstrated by the work of Philipp-Staheli et al. (32). This group demonstrated that MAPK-independent mechanisms of p27 regulation might be attributable, at least in part, to their distinct primary embryonic origin. Further clarification of the molecular networks underlying vascular SMC-phenotypic variance should shed significant insight into the mechanisms leading to regional variability in the susceptibility to intimal lesion development.

In conclusion, we propose that intrinsic differences in MAPK-dependent signaling and p27 expression in rabbit ASMCs and FSMCs contribute to establishing variance in their proliferative and migratory potential. These dissimilarities might be attributable, at least in part, to their distinct primary embryonic origin. Further clarification of the molecular networks underlying vascular SMC-phenotypic variance should shed significant insight into the mechanisms leading to regional variability in the susceptibility to intimal lesion development.

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