PDGF-BB regulates p27 expression through ERK-dependent RNA turn-over in vascular smooth muscle cells

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Summary

Cyclin-dependent kinase inhibitor p27, a critical determinant for cell cycle progression, is an important regulation target of mitogenic signals during arterial injury. In this study, we showed in rat aortic smooth muscle cells that PDGF-BB downregulated p27 protein and mRNA in an ERK-dependent mechanism. Inhibition of ERK, but not other subtypes of the MAP kinase family, prevented the reduction of p27 protein and mRNA. Conversely, direct activation of ERK via adenovirus-mediated expression of a constitutively active form of MEK led to a reduction of p27 protein and mRNA, further supporting the central role of ERK in regulation of p27 expression. Rapamycin, which potently inhibited PDGF-induced activation of p70 S6 Kinase as well as proliferation of smooth muscle cells, did not alter the expression of p27. To delineate the molecular mechanism underlying the p27 downregulation, we examined the effect of PDGF-BB on p27 promoter activity as well as mRNA stability. Stimulation with PDGF-BB significantly shortened the half-life of p27 mRNA without affecting its promoter activity. To further understand the PDGF-stimulated p27 mRNA turn-over, we inserted the 5’ and/or 3’ untranslational regions of p27 cDNA into a non-PDG-sensitive gene luciferase. Only those chimeric genes that contained the 3’ untranslational region responded to PDGF-BB with reduced expression. Moreover, inhibition of ERK completely prevented the effect of PDGF on the chimera expression. In summary, our data suggest p27 is downregulated by PDGF-BB in vascular smooth muscle cells through an ERK-dependent posttranscriptional mechanism.

Introduction

Vascular smooth muscle cells (SMCs), the major type of cells composed of the medial layer of blood vessels, are normally quiescent and growth arrested at the G0/G1 phase of the cell cycle. At the time of vascular injury, vascular SMCs change from a quiescent or “contractile” phenotype to a hyperproliferative phenotype (1). The mitogenic response of SMCs during injury is characterized in part by activation of cyclin-dependent kinases (CDKs), hyperphosphorylation of Rb, and DNA synthesis. Many growth factors and cytokines released at the site of vascular injury contribute to the activation of SMCs, and among these factors PDGF-BB possesses the most potent mitogenic effect.

The G1 to S phase progression, which requires a coordinated activation of CDKs, is the most critical point of cell cycle regulation. In addition to their dependence on various cyclins, CDK activities are also subjected to regulation by a group of proteins called CDK inhibitors (2). Through association with distinct cyclin/CDK complexes, CDK inhibitors suppress CDK activities and therefore inhibit cell cycle progression. CDK inhibitor proteins can be classified into two families based on their structure and CDK targets: The Cip/Kip and INK4 families (2). The Cip/Kip family, which includes p21, p27, and p57, inhibits CDK4, CDK6 and CDK2 activities. The INK family, which includes p16,
p15, p18, and p19, specifically inhibits CDK4 and CDK6 activities.

Normal quiescent cells express high level of p27, which could be viewed as a high threshold that prevents cells from entering the S phase (3). Target deletion of the p27 gene in mice leads to enhanced growth and hyperplasia in multiple organs (4-6). In addition to its role in controlling cell cycle progression during normal development, p27 appears to function as a tumor suppressor, and reduced expression of p27 has been associated with the poor survival of cancer patients (3).

The importance of p27 in vascular homeostasis has been indicated by several in vivo findings. Constitutively expressed in normal arteries, p27 expression is altered by vascular injury in a temporal pattern that is inversely correlated with vascular SMC proliferation. Following balloon injury of rat carotid artery, the level of p27 is downregulated immediately and becomes upregulated during the later phases of arterial repair (7). Overexpression of exogenous p27 in vascular SMC antagonizes the mitogenic effect of growth factors and results in reduction of proliferation and thus prevents the formation of intimal hyperplasia, a high cellular lesion resulted from SMC proliferation as well as migration (8,9). Furthermore, a recent study by Boehm et al indicates p27 also contributes to vascular repair through regulation of bone marrow-derived immune cells and their infiltration to the injured vessel wall (10).

As an important intracellular cell cycle regulator, the expression of p27 is subjected to regulation by growth factors in vascular SMC. Upon stimulation of PDGF-BB, the level of p27 protein in vascular SMCs is dramatically reduced (11,12). Despite the critical role of p27 and PDGF-BB in vascular SMC proliferation as well as vascular repair, the molecular mechanism underlying p27 downregulation in vascular SMCs is not fully understood.

The mitogen-activated protein kinase (MAPK) family consists of three major subfamilies with multiple members: the extra-cellular regulated (ERK), c-jun N-terminal kinase (JNK) and the p38 MAPK (p38). Each MAPK is activated, in response to diverse extracellular stimuli including PDGF-BB, through concomitant Tyr and Thr phosphorylation within a conserved Thr-X-Tyr motif in the activation loop of the kinase (13). The critical role of ERK in PDGF-BB signaling, vascular SMC proliferation and arterial injury has been well established (14-16). Recently, inhibition of JNK or p38, with either pharmacological inhibitors or dominant negative mutants, was also found to attenuate SMC proliferation, however, the effect is much less profound compared to that caused by inhibition of ERK (17,18).

p70 S6 kinase is a mitogen-activated Ser/Thr kinase that controls translation of a unique family of mRNAs, presumably by inducing multiple phosphorylations of the 40 S ribosomal protein S6 (19). Selective inhibition of p70 S6 kinase by rapamycin impedes PDGF-stimulated SMC proliferation, (20) suggesting the mammalian target of rapamycin (mTOR) / p70 S6 kinase pathway plays an essential role in SMC proliferation.

In the current study, we investigated the role of MAPK and p70 S6 kinase in p27 downregulation in arterial SMCs. Using both pharmacological and molecular approaches, we showed that the ERK subfamily of MAPK, is both necessary and sufficient in the regulation of p27. Subsequently, we demonstrated that PDGF-BB inhibits the expression of p27 by affecting the stability of its message through a cis-element located within the 3’ untranslated region (UTR).

Materials and Methods

Materials
Human recombinant PDGF-BB was obtained from Upstate (Lake Placid, N.Y.). Lactacystin, β-lactone, PD98059, SB203580, SP600125, and rapamycin were purchased from Calbiochem (San Diego, CA). Actinomycin D was obtained from Biomol (Plymouth Meeting, PA). All other reagents if not specified were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture
Rat aortic A10 SMCs, obtained from American Tissue Culture Collection (ATCC), were grown as recommended at 37°C in 5% CO₂. The cells were maintained in Dulbecco’s minimal essential medium (DMEM) modified to contain 4 mM L-
glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate supplemented with 10% FBS (Gemini, Woodland, CA) and antibiotics.

Immunoblot Analysis
SMCs (70% confluent) were made quiescent by incubation in medium containing 0.5% FBS for 48 h and then stimulated with PDGF-BB. A10 cells were lysed in radioimmunoprecipitation (RIPA) buffer (25 mM Tris-HCl pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 0.5 mM EGTA, 1.0 % Triton X-100, 1 mM PMSF, and 10 mM Okadaic acid) and subjected to SDS-PAGE and transfer as described previously (21). The membrane was incubated with rabbit polyclonal antibodies to p27, p21 (Santa Cluz Biotechnology, Santa Cruz, CA) or β-actin (Sigma-Aldrich, St. Louis, MO), followed by biotinylated goat anti-rabbit or anti-mouse IgG (BioRad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (Perkin Elmer, Boston, MA).

Northern Blot Analysis
Total RNA was extracted using a RNAqueous kit (Ambion, Austin, TX). Equal amounts of total RNA (10–20 μg) were resolved, transferred to Hybond-N membranes and hybridized as previously describe (22). Restriction enzyme fragments of human p27 cDNA or GAPDH cDNA (Sigma Chemical Co., St. Louis, MO) were labeled with 32P using a Prime-a-Gene Labeling System from Promega (Madison, WI). Hybridization as well as washing were carried out as previously described (22).

Real-time RT-PCR
Total RNA was isolated as above. A Taqman quantitative real-time RT-reverse-transcription polymerase chain reaction (RT-PCR) analysis was carried out by means of the standard protocol provided by Applied Biosystems (Foster City, CA). Briefly, samples were reverse transcribed into cDNA with random hexamer primers, and real-time PCR was performed using ABI Prism 7700 Sequence Detector (Perkin Elmer, Boston, MA). On the basis of the published sequences, (23) the forward and reverse primers for p27 were designed as follows: 5’-GCTTGGATGTCCAGCGGA-3’ and 5’-TCAGAGTTTGCTGAGACCCA-3’, and Taqman probe as 5’-CGCCAGCGGTTGCCTCAA-3’. The primer pair and probe for the housing keeping gene GAPDH were purchased from Applied Biosystems. The expression of p27 of each sample was standardized to the level of GAPDH.

Adenoviral infection
The recombinant adenovirus vector Ad-MEKca, expressing the catalytically active form of MEK, was constructed from a replication-deficient adenovirus type 5 as described by Foshi (24). An empty adenovirus vector (Ad-Null) and a recombinant adenovirus vector Ad-GFP were obtained from The Gene Therapy Core Facility at Weill Medical College Cornell University. Adenoviruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation as described by He et al. (25) A10 cells were incubated with various dosages of adenoviruses, 5000 – 30000 particle unit (pu)/cell, in media containing 2% FBS. After 4 h of infection, cells were changed to fresh media containing 10% FBS and incubated overnight. The efficiency of viral infection of A10 cells was evaluated using Ad-GFP. Under the condition used in this experiment, over 80% of cells were infected and became GFP positive (data not shown).

MAP kinase assay
Activation of JNK and the p38 was measured by dual phosphorylation of Thr and Tyr using anti-phospho-JNK (Thr183/Tyr185) and phospho-p38 (Thr180/Tyr182) antibodies (Cell Signaling Technology, Inc., Beverly, MA), respectively. The ERK activity was measured using a MAPK IP-Kinase Assay Kit from Upstate (Lake Placid, NY). Cells were lysed with buffer containing 1% Nonidet P-40 provided by the kit. Lysates were immunoprecipitated with an anti-ERK1/2 antibody immobilized with agarose. After four rounds of vigorous wash, the immunoprecipitate complexes were subjected to the phosphorylation assay using myelin basic protein (MBP) as a substrate and the phosphorylated MBP was analyzed by immunoblotting using an antibody specific for phosphorylated MBP.
Transient transfection and luciferase assay
Luciferase constructs that contain the human p27 UTR were described elsewhere (26). A p27 promoter reporter was constructed by inserting the -1751 to +455 region of the mouse p27 gene into the pGL2 basic vector (Promega). The transcription start site (+1) is based on the published data (27). Transient transfection and luciferase assay were carried out as previously described (22). Briefly, plasmid DNA was introduced into A10 SMCs by using SuperFect (Qiagen, Valencia, CA). Cells, at 70% confluence, were incubated with DNA/lipid mixture for 3 h in the presence of 10% FBS. After transfection, cells were incubated in media containing 0.5% FBS with or without PDGF-BB (25 ng/ml) for 48 h. In all experiments, 50 ng of the renilla luciferase construct (pRL-CMV) was co-transfected as an internal control. Cell lysates were prepared using a passive lysis buffer provided by Promega and luciferase activities were measured using a Dual-luciferase detection system. The reporter gene activity (firefly luciferase) was normalized to the co-transfected renilla activity. Triplicates were used in each experiment.

Statistical analysis
Values were expressed as mean ± standard error. Unpaired Student’s t test was used to evaluate the statistical differences between control and treated groups. Values of p<0.05 were considered significant. All experiments were repeated at least three times.

Results
PDGF reduced the level of p27 protein in vascular SMCs
We used A10, a rat aortic SMC line, as an in vitro model to study the mechanism underlying p27 downregulation. When treated with PDGF-BB (25 ng/ml), A10 cells responded with a 263.7 ± 5.6 % increase in DNA synthesis, which is a typical mitogenic response for vascular SMCs. To determine the expression of p27, we stimulated A10 SMCs with 5, 25 or 50 ng/ml of PDGF-BB for 24 h. Cell lysates were analyzed by Western blotting using a p27 specific antibody. PDGF decreased the level of p27 in a dose-dependent manner (Fig. 1A). Next, we determined the time-course of the p27 downregulation. The reduction of p27 was evident at 17 h of PDGF-treatment, but more prominent at 24 h (Fig 1B). Since p21, a related CDK inhibitor, is also expressed by vascular SMCs and known to be involved in regulation of proliferation, we tested whether the level of p21 was also altered. Treatment of A10 cells with PDGF-BB (25 ng/ml) did not affect the intracellular level of p21 at any time point studied (Fig. 1B). These results suggest that the effect of PDGF on CDK inhibitors is specific to p27 in vascular SMCs.

Downregulation of p27 is mediated by the ERK MAPK
The results described above indicate that p27 might be an important target of the PDGF signal that contributes to SMC proliferation. We have previously demonstrated that MAPK is essential for PDGF-induced proliferation of vascular SMCs, (15,28) thereby, we wish to examine whether the MAPK activity is also required for p27 downregulation. To this end, we explored specific inhibitors to ERK, JNK or p38, the three major types of MAPK. PD98059, a selective cell permeable inhibitor of MAP ERK kinase (MEK), is widely used to inhibit ERK activation and has been shown to inhibit SMC proliferation in response to PDGF (15,17). Pretreatment of A10 SMCs with PD98059 (50 μM) completely eliminated the effect of PDGF on p27 protein (Fig. 2A). In contrast, inhibition of p38 with SB203580 (20 μM) or JNK with SP600125 (20 μM) had no effect on p27 downregulation. The efficacy of the latter two inhibitors was confirmed by their ability to abolish PDGF-induced phosphorylation of p38 or JNK, a commonly used measurement for MAPK activation (Fig. 2B). Thus, our data suggest that the ERK family of MAPK is necessary for mediating downregulation of p27 by PDGF in vascular SMCs.

The p70 S6 kinase pathway is not necessary for the p27 downregulation
We next tested whether the p70 S6 kinase pathway, which is also activated by PDGF in vascular SMCs, plays a role in the PDGF-induced p27 downregulation. Rapamycin, which inhibits growth factor-induced activation of p70 S6 kinase through mammalian target of rapamycin (mTOR), potently inhibits SMC proliferation (20). To our
surprise, pretreatment of A10 cells with rapamycin (10 ng/ml) for 24 h did not prevent p27 downregulation (Fig. 3A). Subsequent analyses confirmed that the same concentration of rapamycin completely blocked the activation of p70 S6 kinase as well as cell proliferation in response to PDGF-BB (Fig. 3B and C). Therefore, it appears that the mTOR/p70 S6 kinase pathway, although important for proliferation, is not involved in p27 regulation in A10 SMCs.

**ERK activation is sufficient to induce p27 downregulation**

To address whether activation of ERK alone is sufficient to downregulate p27, we used a recombinant adenoviral vector (Ad-MEKca) (24), kindly provided by Dr. Sorokin, to express a constitutively active form of MEK, a MAP kinase kinase upstream of ERK. We confirmed the overexpression of MEK by infecting A10 SMC cells with increasing dosages of Ad-MEKca virus. A dose- and time-dependent increase in MEK was found to be associated with Ad-MEKca infection (Fig. 4A). Next using an immuno-kinase assay we confirmed that Ad-MEKca produced a dose- and time-dependent induction in ERK activity (Fig. 4B). To test whether activation of ERK alone can alter p27 expression, we infected A10 cells with 30,000 particle/cell of Ad-MEKca or control adenoviruses (Ad-Null). Cells were lysed 36 or 48 h after infection and cell lysates were immunoblotted with an anti- p27 antibody. Compared to Ad-Null infected cells, levels of p27 in Ad-MEKca infected cells were significantly reduced in both time points (Fig. 4C). Actually, the fold reduction of p27 induced by Ad-MEKca was equivalent to that produced by 25 ng/ml of PDGF-BB (76.2% versus 80.2%). Similar to PDGF-BB, Ad-MEKca induced p27 downregulation was insensitive to rapamycin (Fig. 4D). Taken together, these data further support the notion that PDGF-BB regulates p27 expression in vascular SMCs through ERK.

**The PDGF-induced p27 downregulation is not mediated by proteasome**

The ubiquitin-proteasome dependent protein degradation is an important mechanism that regulates the intracellular level of p27. Inhibiting the proteasome pathway by specific inhibitors lactacystin or β-lactone has been shown to block p27 downregulation induced by matrix proteins in Chinese hamster ovarian cells (CHO) or by BCR/ABL in a pre-B cell line (29,30). To test whether the PDGF-induced p27 downregulation is due to an accelerated degradation through the ubiquitin-proteasome pathway, we pretreated A10 SMCs with lactacystin (2 μM) or β-lactone (2 μM). Inhibition of proteasome activity by either inhibitor failed to prevent the downregulation of p27 in response to PDGF (Fig. 5A). To confirm that these inhibitors, at the concentration used here, were sufficient to inhibit the ubiquitin-proteasome pathway in vascular SMCs, we examined the level of the tumor suppressor p53, which is known to be modulated by the ubiquitin-proteasome pathway, in A10 SMCs treated with the proteasome inhibitors. As shown in Fig. 5B, lactacystin (2 μM) or β-lactone (2 μM) increased the accumulation of p53, confirmed that both inhibitors are sufficient to block proteasome-dependent protein degradation.

**PDGF reduces the accumulation of cellular p27 mRNA**

We next examined the effect of PDGF on the level of p27 mRNA. Quiescent A10 SMCs were treated with PDGF-BB (25 ng/ml) for various periods of time. Total cellular RNA was isolated and reverse transcribed into cDNA. The Taqman real-time PCR was then performed using primers designed for rat p27 as well as for the house-keeping gene GAPDH. The relative abundant of p27 in each sample was normalized to its corresponding GAPDH. At all tested time points, PDGF-BB (25 ng/ml) induced an over 50% reduction of p27 mRNA (Fig. 6A). Similar results were obtained from Northern blotting (data not shown). Furthermore, this PDGF-induced reduction in p27 mRNA was completely abolished by the MEK inhibitor PD 98059 (Fig. 6B). Next we addressed whether direct activation of ERK by Ad-MEKca was sufficient to regulate p27 mRNA. Total RNA was isolated 48 h following adenoviral infection and analyzed by real-time RT-PCR. As shown in Fig. 6C, Ad-MEKca reduced the accumulation of p27 message in a similar fashion similar to the PDGF-stimulation.
The p27 promoter activity is not affected by PDGF
To test whether PDGF reduced the abundance of p27 mRNA by inhibiting its promoter activity, we transfected A10 SMCs with a luciferase construct that contains the human p27 promoter (-1751 to +455 region). Following PDGF-BB treatment, luciferase activities in cell lysates were determined. There was no significant difference in luciferase activities of PDGF-treated cells compared to that of control cells (Fig. 7A), suggesting PDGF does not regulate the p27 promoter in vascular SMCs. In contrast, serum, which also downregulates p27, inhibited the p27 promoter reporter activity by ~50% (Fig. 7B).

PDGF shortens the half-life of p27 mRNA
Next, we evaluated the effect of PDGF on p27 mRNA stability, which could also affect the steady state level of p27 mRNA. A10 SMCs were treated with PDGF-BB in the presence of actinomycin D (5 μg/ml), a transcription inhibitor. Total cellular RNA was isolated at various times (0, 2, and 4 h) following the addition of actinomycin D from control or PDGF-treated cells. As a result of RNA decay in the presence of actinomycin D, the level of p27 mRNA (along with other cellular mRNAs) declined in both control and PDGF-treated cells. However, the rate of decline was significantly faster in cells that were treated with PDGF-BB compared to the vehicle-treated control cells (Fig. 8), with the half-life of p27 mRNA of 1.46 h in the PDGF-treated cells versus 4.15 h in control cells. These results suggest that PDGF regulates p27 expression by stimulating its mRNA degradation.

PDGF regulates the p27 mRNA turn-over through its 3’UTR
Regulation of mRNA decay is achieved by interactions between structural (cis-) elements within a mRNA and specific protein factors. The cis-elements include the 5’-cap structure, 5’UTR, the protein coding region, 3’UTR and the 3’-polyadenylate (poly A) tail. The human p27 mRNA contains a short 5’UTR (152 bp), a long 3’UTR (1341 bp) with a relative short poly A tail (26). To determine which of these elements mediates the effect of PDGF, we used luciferase constructs in which various non-coding sequences of human p27 cDNA, 5’UTR, 3’UTR or both, were inserted upstream or downstream of the coding region of the luciferase gene (Fig. 9A) (26). A luciferase construct that does not contain any p27 sequence (No UTR) was used as a control. These chimeric genes were transiently transfected into A10 SMCs as described in the method. The basal luciferase activity varied among these chimeras. Compared to the “No UTR” control, the insertion of the p27 5’UTR increased the luciferase activity by ~2 fold, whereas the 3’UTR inhibited it by ~80% (Fig. 9B). These effects of p27 UTRs on basal reporter activity is identical to what was observed previously in endothelial cells (26). Next, we examined the effect of PDGF-BB on the chimera expression. After transfection, A10 SMCs were stimulated with PDGF-BB (25 ng/ml) for 48 h. While PDGF-BB did not significantly affect the activity of “No UTR” or “5’ UTR”, it reduced the luciferase activity of “3’UTR” as well as “5’+3’ UTR” (Fig. 9C), suggesting that the PDGF-responsive element resides in the 3’ UTR of p27 mRNA.

Since our earlier results suggest that PDGF-BB may regulate p27 expression through ERK, we next tested whether the regulation of p27 UTR-luciferase chimera expression is also mediated by ERK. Following transfection with “5’+3’ UTR” or “No UTR” constructs, A10 SMCs were pretreated with the MEK inhibitor PD 98059 (50 μM) for 1 h then treated with PDGF-BB (25 ng/ml) as above. PD 98059 completely abolished the effect of PDGF-BB on “5’+3’ UTR” activity (Fig. 9C). This result confirmed that regulation of p27 is mediated by its UTR through an ERK mediated mechanism.

DISCUSSION
PDGF-BB is a potent mitogen for vascular SMCs and plays a central role in the development of hyperplastic lesion following vascular injury. Upon binding to its membrane receptors, PDGF-BB elicits multiple intracellular signaling pathways that lead to cell proliferation. One important downstream target of the PDGF signaling is the CDK inhibitor p27. By using both pharmacological and molecular approaches, we demonstrated that PDGF-BB reduced the accumulation of both p27 protein and mRNA through an ERK-dependent post-transcriptional mechanism. The activation ERK is not only
necessary but also sufficient to suppress the expression of p27.

The inhibitory effect of PDGF on p27 expression has been previously demonstrated in rat aortic SMCs (11) as well as human saphenous vein SMCs (12). Similar downregulation of p27 was also induced by serum, but not by angiotensin (12,31). The inhibitory effect of PDGF as well as serum on p27 is consistent with their mitogenic effects in vascular SMC. Furthermore, it is found that lower level of p27 is associated with the hyper-proliferative state of SMCs following vascular injury, (7) and gene transfer of p27 inhibits the mitogenic effects of growth factors and attenuate the hyperplastic response caused by vascular injury (8,9).

Mitogen-induced activation of ERK is essential for proliferation of vascular SMCs and contributes to the formation of hyperplastic lesions during vascular injury (14-16). We have previously shown that ERK is rapidly activated in vascular SMCs upon PDGF stimulation (28). The present study provides evidence that ERK promotes SMC proliferation, at least in part, by suppressing the expression of p27. Pharmacological inhibition of ERK blocked the effect PDGF on p27 protein, mRNA as well as UTR-luciferase chimera expression. Conversely, activation of ERK through a molecular manipulation led to reduction of p27 protein and mRNA. The importance of ERK in the p27 regulation as well as SMC proliferation is further supported by a recent study of Zhan et al. in which a dominant negative mutant of ERK suppressed PDGF-induced downregulation of p27 as well as proliferation of rat aortic SMC (18).

Using pharmacological inhibitors, we showed that the MAPK JNK and p38 were not critically involved in the regulation of p27. The lack of involvement of p38 is consistent with the finding by Zhan et al who used a dominant negative p38 mutant, (18) however, the results regarding JNK diverges. Zhan et al. found that a dominant negative mutant of JNK suppressed the downregulation of p27 (18). To ensure that the dosage of JNK inhibitor SP600125 (20 μM) used in this study was sufficient to inhibit JNK activation as well as a JNK-dependent cellular process in SMC, we evaluated the effect of SP600125 on fibronectin production by SMCs, a process that is reported to be mediated by JNK (32). Indeed, we found that SP600125 at a dosage of 20 μM inhibited the amount of fibronectin secreted by A10 SMCs (data not shown). Thus, our data suggest that in A10 SMCs JNK appears to be unnecessary for the regulation of p27 expression.

The involvement of ERK in p27 expression has also been reported in other cell types. Inhibition of ERK by PD98059 causes an accumulation of p27 as well as the S-phase entry in fibroblasts and epithelial cells (33), suggesting the ERK cascade is required for both mitogen-induced p27 downregulation and proliferation. Furthermore, genetic activation of ERK decreased the expression of p27 in NIH 3T3 cells (34).

The p70 S6K is essential for mitogen-stimulated SMC proliferation. Rapamycin, which inhibits S6K through its intracellular target TOR or mTOR, inhibits proliferation of many cell types including vascular SMCs (35). Downregulation of p27 by interleukin-2 in T lymphocytes was prevented by rapamycin (36). The involvement of p27 in the antimitogenic responses of rapamycin was suggested by a study using a rapamycin-resistant murine cell line as well as fibroblasts and T lymphocytes derived from p27 deficient mice (37). However, we did not observe any effect of rapamycin on p27, despite the fact that rapamycin, at the same concentration, inhibited A10 SMC proliferation. These data reiterate the complexity of proliferation regulation, in which distinct signaling pathways could be employed depended on cell types as well as the nature of mitogenic stimuli.

The PDGF-induced p27 downregulation appears not to be mediated by the proteasome-dependent protein degradation in A10 SMCs. Instead, PDGF caused a reduction in the accumulation of p27 mRNA during a time frame that proceeded the reduction of its protein. We speculate that the reduction of p27 mRNA by PDGF is the major mechanism contributing to the downregulation of its protein. Our finding that both reduction of p27 mRNA and protein required activation of ERK further supports this hypothesis. However, we cannot rule out the possibility that p27 protein is degraded in SMCs by proteases other than proteasome. Similarly, our data do not exclude any potential mechanism involving protein translation. But, our subsequent UTR analyses suggest that a post-transcriptional other than a translational
mechanism underlies the regulation of p27 by PDGF.

Using a p27 promoter reporter, we showed that the p27 promoter (-1751 to +455) was not regulated by the PDGF signal. In contrast, the same reporter responded to serum stimulation with a 50% reduction. It appears that PDGF and serum, although both stimulate SMC proliferation, regulate the p27 expression through different mechanisms.

RNA processing is increasingly recognized as a major regulation point of gene expression in mammalian cells. An alteration in the rate of mRNA degradation affects the steady state level of the mRNA and thus the protein. It is believed that the stability of a particular mRNA is controlled by specific interactions between its structural elements and RNA-binding proteins that can be general or mRNA-specific. The best described RNA element involved in stability of short lived messages is the AU-rich element (AREs) within the 3’UTR of mRNA (38). We demonstrated the importance of 3’UTR in p27 regulation by using UTR-luciferase chimeras. The insertion of 3’UTR but not 5’UTR of p27 cDNA conveyed the PDGF-response to the non-mammalian gene luciferase which is otherwise not regulated by PDGF. Furthermore, the p27 UTR-luciferase chimera responded to PDGF in a similar ERK dependent manner as the native p27 gene. This ERK-dependent regulation of p27 expression is different from the Rho-dependent translational regulation observed in MDA468 cells. The latter is mediated by the 5’UTR as well as the last 300 bp of 3’UTR of p27 (39). In addition, we found that inhibition of Rho by C3 exoenzyme did not affect the ability of PDGF-BB to inhibit p27 expression (data not shown).

Several regions throughout 3’UTR of human p27 mRNA exhibit high homology to the ARE core sequence. Future studies will be designed to test which of these putative AREs controls the decay of p27 mRNA. Beyond identifying the trans-acting factors that regulate p27 mRNA, it is important to understand how ERK influences the function of these protein factors and their interaction with 3’UTR.

In summary, we showed in this report that PDGF-BB negatively regulated the CDK inhibitor p27 in vascular SMCs. This PDGF-induced downregulation, mediated by ERK, occurred, at least in part, at the post-transcriptional level. The PDGF-induced activation of ERK, through a mechanism involving the 3’UTR, shortened the half-life of p27 mRNA and thus led to a reduction in the expression of its protein.

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References
Fig. 1 PDGF-BB decreases the level of p27 protein in vascular SMCs.
Western blots of cell lysates isolated from A10 cells stimulated with 5, 25 or 50 ng/ml of PDGF-BB for 24 h (A) or with 25 ng/ml of PDGF-BB for indicated durations (B).

Fig. 2 Inhibition of ERK prevents the effect of PDGF-BB on p27.
(A) A10 cells were pre-incubated with PD98059 (50 µM), SB203580 (20 µM), SP600125 (20 µM) for 1 h then stimulated with PDGF-BB (25 ng/ml) for 24 h. The level of p27 expression was evaluated with Western blotting. (B) Cells were treated with SB20380 or SP600125 and PDGF-BB as above. Activation of JNK or p38 was evaluated using antibodies specific for phosphorylated JNK (Thr183/Tyr185) or p38 (Thr180/Tyr182).

Fig. 3 Downregulation of p27 is insensitive to rapamycin.
Prior to the PDGF-stimulation, A10 cells were pre-incubated with rapamycin (10 nM) for 24 h. The expression of p27 (A), activation of S6 kinase (B) and cell proliferation (C) were evaluated as described in Methods. (n=3, * p<0.05)

Fig.4 Molecular stimulation of ERK mimics the effect of PDGF on p27.
(A) A10 SMCs were infected with Ad-Null (30000 pu/cell) or Ad-MEKca (5000, 10000, or 30000 pu/cell). 36 or 48 h after infection, cells were lysed and immuno-blotted using an anti-MEK antibody. (B) A10 cells were infected and lysed as above. Cell lysates were immunoprecipitated with an anti-ERK antibody and ERK activity was measured as described in the Methods. Uninfected quiescent A10 cells, treated with PDGF-BB (25 ng/ml) for 15 min, was used as a positive control. (C) A10 SMCs were infected with Ad-Null (30000 pu/cell) or Ad-MEKca (30000 pu/cell). Thirty-six or forty-eight h after infection, cells were lysed. The level of p27 was evaluated by immunoblotting. (D) Cells were pretreated with or without rapamycin (10 nM) for 24 h and then were infected with Ad-Null (30000 pu/cell) or Ad-MEKca (30000 pu/cell). Forty-eight h after infection, cell lysates were blotted for p27.

Fig. 5 Inhibition of proteasome has no effect on p27 downregulation.
(A) A10 cells were pre-incubated with lactacystin (2 µM) or β-lactone (2 µM) for 2 h then stimulated for 24 h with PDGF-BB (25 ng/ml). (B) A10 cells, grown in 10% serum to 70% confluent, were treated with lactacystin (2 µM) or β-lactone (2 µM) for 24 h. Cell lysates were blotted for p53.

Fig. 6 PDGF-BB decreases the abundance of p27 mRNA through an ERK-dependent mechanism.
(A) A10 cells were stimulated with PDGF-BB (25 ng/ml) for 0 – 12 h. Total cellular RNA was isolated and analyzed by real-time RT-PCR. (n=3, * p<0.05) (B) A10 cells were pre-incubated with or without PD98059 (50µM) for 1 h then stimulated with PDGF-BB (25 ng/ml) for 8 h. The level of p27 mRNA was determined by using the real-time RT-PCR analysis. (n=3, * p<0.05, compared to non-PDGF-BB, non-PD98059 treated control) (C) A10 cells were infected with Ad-MEKca or Ad-Null (30000 pu/cell). Cells were lysed 48 h post infection and the abundance of p27 mRNA was determined by real-time RT-PCR. (n=3, * p<0.05, compared to Ad-Null)

Fig. 7 The activity of p27 promoter is not responsive to PDGF-BB.
A10 cells were transfected with a p27/luc reporter. Following transfection, cells were stimulated for 48 h with 25 ng/ml of PDGF-BB (A) or 10% serum (B). Reporter activities were expressed as ratios of firefly luciferase to renilla luciferase. (n=6, * p<0.05, compared to 0.5% FBS treated control)
Fig. 8 PDGF-BB decreases the stability of p27 mRNA.
A10 SMCs, pretreated with 25 ng/ml of PDGF-BB or solvent for 6 h, were incubated with actinomycin D (5 ng/ml) for the indicated time. The level of p27 mRNA was determined with Northern blotting (A) or real time RT-PCR (B). Data, representative of three experiments, is expressed as percentage relative to the amount of p27 mRNA at time zero. The solid lines represent the least-squares fit of the data obtained by linear regression analysis.

Fig. 9 PDGF-BB deregulates the expression of p27 UTR/luc.
(A) Schematics of p27 UTR/luc. (B) A10 cells were transfected with a p27 UTR/luc construct as indicated. Following transfection, cells were stimulated with 25 ng/ml of PDGF-BB for 48 h. Luciferase activities were measured as described in the methods. (n=4, * p<0.05, compared to the non-PDGF-BB treated control) (C) Cells were transfected with No UTR or 5’+3’ luciferase constructs. The MEK inhibitor PD98059 (50 µM) was administrated 1 h prior to the PDGF-BB (25 ng/ml) stimulation. (n=3, * p<0.05, compared to the corresponding control)
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