Role of Sp1 in the Induction of p27 Gene Expression in Vascular Smooth Muscle Cells In Vitro and After Balloon Angioplasty

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Abstract—The abnormal proliferation of vascular smooth muscle cells (VSMCs) plays an important role in atherosclerosis and restenosis. Although several studies have implicated the growth inhibitory protein p27Kip1 (p27) in the control of myocyte growth and hypertrophy, little is known about the molecular mechanisms that regulate p27 expression in the cardiovascular system. In the present study, we demonstrate the interaction of the transcription factor Sp1 with 2 GC-rich sequences within the p27 promoter in cultured VSMCs. Importantly, point mutations that disrupted Sp1 binding markedly reduced p27 promoter activity, demonstrating that Sp1 is required for efficient p27 gene transcription in cultured VSMCs. Because p27 expression is upregulated after balloon angioplasty, we investigated Sp1 expression and activity in control and balloon-injured rat carotid arteries to assess the role of Sp1 as a physiological regulator of p27 expression. Although immunohistochemical analysis disclosed Sp1 protein expression in both control and balloon-injured arteries, a high level of Sp1 DNA-binding activity was found only in response to balloon angioplasty. Collectively, these results demonstrate that Sp1 is essential for maximum p27 promoter activity in VSMCs and suggest that posttranslational induction of Sp1 DNA-binding activity contributes to the induction of p27 expression and VSMC growth arrest at late time points after balloon angioplasty. (Arterioscler Thromb Vasc Biol. 2001;21:342-347.)

Key Words: vascular smooth muscle cell ■ cell cycle ■ Sp1 ■ p27 ■ angioplasty

Excessive vascular smooth muscle cell (VSMC) growth is involved in the pathogenesis of vascular proliferative diseases, including atherosclerosis and restenosis (see reviews1–3). With several animal models of arterial injury, it has been shown that “activated” VSMCs resume a quiescent phenotype within 2 to 6 weeks after angioplasty.4–6 Therefore, a better understanding of the molecular mechanisms that limit VSMC proliferation during the remodeling process is of utmost importance to develop novel therapies for the treatment of vascular proliferative diseases.

Cell cycle progression is controlled by several cyclin-dependent protein kinases (CDKs), which can associate with activating subunits, the cyclins, and with CDK inhibitory proteins (CKIs).9,10 p27 and p21 (p21) are related CKIs that associate with CDK2-, CDK4-, CDK6-, and CDC2-containing complexes, thereby abrogating their catalytic activity leading to growth arrest.9,11 Recent studies have suggested a physiological role of p21 and p27 as regulators of VSMC growth during the pathogenesis of cardiovascular diseases (see review12). In vitro experiments have shown that VSMC responsiveness to growth signals is highly dependent on changes in specific components of the extracellular matrix through α1 integrin–dependent regulation of p27 and p21.13 Reduced CDK2 activity and the decline in VSMC proliferation that takes place at late time points after angioplasty correlated with a marked induction of p27 and p21.14–16 Moreover, overexpression of p27 efficiently blocked mitogen- and c-fos–dependent induction of cyclin A promoter activity in cultured VSMCs,15,17 and adenovirus-mediated overexpression of p2715 and p2114,18,19 limited neointimal thickening after balloon denudation. In contrast, recent studies with p27 knockout mice have demonstrated that the loss of p27 results in prolonged proliferation of cardiac myocytes.20 Moreover, p27 plays an important role in the regulation of cardiomyocyte hypertrophy20 and angiotensin II–stimulated VSMC hypertrophy.21,22

Although these studies identify p27 and p21 as important regulators of the phenotypic response of cardiac and vascular myocytes to mitogenic and hypertrophic stimuli, little is known about the molecular mechanisms involved in the regulation of CKI gene expression in the cardiovascular system. Therefore, we investigated the transcriptional mechanisms that control p27 gene expression and obtained results that demonstrate the trans–acting nuclear factor Sp1 is required for maximum p27 promoter activity in cultured VSMCs. Moreover, using the rat carotid model of balloon angioplasty.
Sp1-Dependent Regulation of p27 Expression in VSMCs

Methods

Cell Culture and Transient Transfection Assays

E19P cells (gift from C. Shanahan, University of Cambridge, Cambridge, UK) were obtained from explant cultures of embryonic day 19 aorta from Fischer rats. Through Northern blot analysis, these cells express SM22α, calponin, and smooth muscle α-actin mRNAs (C. Shanahan, personal communication). Cells were maintained in M199 supplemented with 10% FBS at 37°C (95% air and 5% CO2). Cells were seeded onto 6-well dishes, and on the next day (~60 to 80% confluence), they were transiently transfected with SuperFect reagent (Qiagen) and 2 μg of the indicated luciferase reporter plasmids driven by the murine p27 promoter fragments spanning from −170 to +54 relative to the transcriptional initiation site22 (gift from S. Lin, National University of Singapore). To correct for differences in transfection efficiency, luciferase activity was normalized relative to the alkaline phosphatase activity produced from S. Lin, National University of Singapore). To correct for differences in transfection efficiency, luciferase activity was normalized relative to the alkaline phosphatase activity produced from S. Lin, National University of Singapore). To correct for differences in transfection efficiency, luciferase activity was normalized relative to the alkaline phosphatase activity produced from S. Lin, National University of Singapore)

Balloon Angioplasty and Tissue Extraction

Male Sprague-Dawley rats (400 to 500 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital solution (45 mg/kg body wt) for performance of balloon denudation of the left common carotid artery in accordance with institutional guidelines as described previously.3,26 At the indicated times after angioplasty, rats were euthanized with sodium pentobarbital IP injection, 100 mg/kg body wt, and both injured and uninjured common carotid arteries were perfused with saline through a cannula inserted into the left ventricle. For immunohistochemistry, saline perfusion was followed by in situ fixation with 100% methanol; then, arteries were harvested, and the surrounding adventitia and fat tissue were carefully removed. Arteries for the preparation of protein extracts and cellular RNA were harvested without in situ fixation, cleaned of adventitia and fat tissue, snap-frozen in liquid nitrogen, and stored at −80°C until further use (see later).

Immunohistochemistry

Methanol-fixed arteries were embedded in paraffin and cut into 5-μm sections. Immunohistochemistry with rabbit polyclonal anti-p27 antibodies (sc-776, 1:20 dilution; Santa Cruz Biotechnology) and goat polyclonal anti-Sp1 antibody (sc-59-G; 1:50 dilution; Santa Cruz Biotechnology) was performed with a biotin/streptavidin peroxidase detection system according to the recommendations of the manufacturer (Signet Laboratories). Before incubation with primary antibodies, specimens were treated with an avidin/biotin blocking system (Signet Laboratories) to reduce nonspecific staining due to endogenous avidin or biotin.

Preparation of Protein Extracts and Electrophoresis Mobility Shift Assays

Cells and arteries were lysed in ice-cold buffer that contained 20 mmol/L HEPES buffer, pH 7.5, 10 mmol/L EGTA, 40 mmol/L β-glycerophosphate, 1% NP-40, 2.5 mmol/L MgCl2, 2 mmol/L orthovanadate, 1 mmol/L DTT, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Protein extracts were prepared from the pooled tissue from 7 to 9 animals at each time point. Lysates were cleared with centrifugation in a microfuge, and the supernatants were stored at −80°C. Western blot analysis with mouse monoclonal anti-p27 antibody (Ab-2, dilution 1:75; Oncogene) was performed with an ECL detection system (Amersham).

The following radiolabeled probes that span the murine p27 promoter regions from −140 to −109, −118 to −87, and −60 to −20 contained 2 GC-rich boxes (box I −133 to −117, box II −87 to −72) were used for electrophoresis mobility shift assays (EMSAs) (binding sites are underlined, and point mutations are given in lowercase letters): box I (wild-type), 5′-GGCGGAGAGGGCCCGGGCGCGCACCCGGGGGC-3′; box I (mutant), 5′-GGCGGAGACGGGaaGCGCGCACCCGGGGGC-3′; box II (wild-type), 5′-GAGCGCGCTCGAGGGGGAAGCGCGGCACCCGGGGGC-3′; and box II (mutant), 5′-GAGCGCGCTCGAGGGGGAAGCGCGGCACCCGGGGGC-3′.

Other probes used in these studies included the consensus Sp1 (5′-ATTGATCGGCGGCGGCGAC-3′) and AP1 (5′-CGCTTGAAGCTCCGGCGGCGAC-3′) binding sites. EMSA was performed as previously described.17 To demonstrate the presence of Sp1 in the retarded nucleoprotein complexes, cell extracts were preincubated for 20 minutes with 3 μg of mouse monoclonal anti-Sp1 antibody (sc-420X; Santa Cruz Biotechnology) before addition of the probe.

Northern Blot Analysis

Arterial total RNA from control and balloon-injured rats was isolated from the pooled tissue from 3 animals with Tri-Reagent according to the recommendations of the manufacturer (GIBCO BRL). Ten micrograms of RNA was electrophoresed on 1% agarose gels that contained 2.2 mmol/L formaldehyde, transferred to nylon membranes (Hybond-N; Amersham), and immobilized by short-wave UV irradiation (UV-Stratalinker 1800; Stratagene). Blots were hybridized with radiolabeled cDNA probes for p27 and GAPDH. Autoradiographs were scanned (Microtek Scan Maker II), and band intensity was determined after background subtraction with the densitometric program Sigma Gel (Jandel Scientific).

Results

Sp1 Interacts With Two GC-Rich Sequences

Within the p27 Gene Promoter That Are Required for Maximum Transcriptional Activity in VSMCs

Analysis of the proximal promoter region of the murine p27 gene revealed 2 GC-rich regions (box I −133 to −117, box II −87 to −72), which are putative binding sites for the transcription factor Sp1. As shown in the EMSA in Figure 1A, the incubation of E19P cell extracts with the box II probe leads to the formation of a slow migrating nucleoprotein complex (lane 2), which was disrupted when an excess of unlabeled wild-type box II oligonucleotide was added to the binding reaction (lane 3). A mutated box II oligonucleotide that disrupted the putative Sp1 binding site did not compete for binding (Figure 1A, lane 4), demonstrating the requirement of the GC-rich motif for nucleoprotein complex formation. Two additional faster migrating bands that could not be disrupted with either wild-type or mutated box II oligonucleotides corresponded to nonspecific interactions (Figure 1A, NS). Although less intense compared with autoradiographs with box II probe, a retarded band of the same electrophoretic mobility was seen with the box I probe (data not shown).

A series of experiments were then performed to demonstrate the interaction of Sp1 with these GC-rich motifs within the p27 proximal promoter region. First, the electrophoretic mobility of the specific complex detected with the wild-type box II probe was identical to that observed with a probe containing an Sp1 consensus site (Figure 1A, compare lanes 2 and 7). Similar results were obtained with the wild-type box I probe (data not shown). Moreover, the DNA-binding specificity of the factor bound by box I, box II, and the Sp1 consensus probes was indistinguishable on the basis of cross-competition experiments. Although both wild-type box I and box II efficiently competed for binding to the Sp1 consensus probe, their...
mutant versions did not affect binding under the same conditions (Figure 1B, lanes 6 to 8 and 12 to 14). Likewise, the Sp1 consensus oligonucleotide was very efficient at disrupting the nucleoprotein complex seen with box I (data not shown) and box II (Figure 1A, lane 5) probes. Finally, the addition of an anti-Sp1 antibody disrupted the binding activity detected with both the Sp1 consensus and box II probes (Figure 1C), demonstrating the presence of Sp1 in these nucleoprotein complexes.

Having demonstrated the binding of Sp1 to the p27 promoter in cultured VSMCs, we investigated the functional consequences of this interaction. To this end, E19P cells were transiently transfected with luciferase reporter genes driven by fragments of the murine p27 gene promoter spanning from +54 relative to the transcriptional initiation site. WT indicates wild-type promoter activity by 65% compared with the corresponding wild-type fragment. Collectively, these experiments demonstrated that Sp1 interacts with 2 GC-rich motifs within the p27 gene promoter that are required for maximum transcriptional activity in cultured VSMCs.

**Figure 1.** Sp1 interacts with 2 GC-rich sequences within the p27 gene promoter. E19P cells lysates (20 μg total protein) were incubated in binding reactions with radiolabeled probes containing a consensus Sp1 binding site (Sp1 cons) or the GC-rich box located at position −82 to −72 (box II) within the murine p27 promoter (see Methods). A, EMSA was performed with box II (lanes 1 to 5) and Sp1 cons (lanes 6 to 8) probes. Competition experiments were carried out with a 50-fold excess of unlabeled oligonucleotides. Lanes 1 and 6 show control reactions without cell lysate. Nonspecific bands that could not be disrupted with competitors are indicated (NS). B, EMSA was performed using the Sp1 consensus probe. A 50-fold excess of unlabeled Sp1 oligonucleotide competed for binding. Increasing amounts of wild-type box II and box I (position −133 to −117 within the murine p27 promoter) also disrupted binding. In contrast, mutated box I and box II did not compete under the same conditions. The amount of competitor was 150-fold (lanes 3, 6, 9, and 12), 250-fold (lanes 4, 7, 10, and 13), and 500-fold (lanes 5, 8, 11, and 14). C, Preincubation of cell extracts with monoclonal anti-Sp1 antibody inhibited binding to the box II and Sp1 consensus probes. Nonspecific bands are indicated (NS).

**Figure 2.** Sp1 binding sites are required for maximum p27 promoter activity in VSMCs. E19P cells were transiently transfected with luciferase reporter genes driven by fragments of the murine p27 gene promoter spanning from −170 to +54 activated reporter gene expression. Importantly, point mutations that disrupted the binding of Sp1 to box I and box II reduced promoter activity by 65% compared with the corresponding wild-type fragment. Collectively, these experiments demonstrated that Sp1 interacts with 2 GC-rich motifs within the p27 gene promoter that are required for maximum transcriptional activity in cultured VSMCs.

**Sp1 and p27 Spatial and Temporal Pattern of Expression After Balloon Angioplasty**

To assess the role of Sp1 as a physiological regulator of p27 expression in vivo, we investigated the spatial and temporal patterns of expression of these factors in control and balloon-injured rat carotid arteries. VSMC proliferation in this model of vascular injury peaks during the first week and then declines thereafter to return to baseline levels within 2 to 3 weeks.5,6,27 In agreement with previous studies that reported the induction of p27 in balloon-injured arteries,15,16 immunohistochemical analysis disclosed elevated p27 protein expression at 10 days after balloon angioplasty, which was maintained at 2 weeks after injury (Figure 3). Western blot analysis was more sensitive and revealed expression of p27 in uninjured vessels that was induced between 1 to 2 weeks after angioplasty (Figure 4A). Moreover, averaged over 2 independent experiments, Northern blot analysis disclosed a 3.5-fold induction of p27 steady-state mRNA level at 2 weeks after angioplasty compared with uninjured arteries (Figure 4B).

Immunohistochemical analysis revealed abundant expression of Sp1 in both control and balloon-injured carotid arteries (Figure 3). Importantly, lower p27 mRNA and protein expression in uninjured arteries and up to 2 days after angioplasty correlated with lower Sp1 DNA-binding activity (Figure 4C, lanes 2 to 4). By day 5 after angioplasty, Sp1 DNA-binding activity was markedly induced and remained elevated until day 18 (Figure 4C, lanes 5 to 7). The specificity of the nucleoprotein complex was demonstrated by the ability of unlabeled homologous oligonucleotide (lanes 8 to 13), but not unrelated AP1 oligonucleotide (lanes 14 and 15), to compete for binding.

Previous studies demonstrated a rapid induction in the expression and activity of AP1 transcription factors after balloon angioplasty, suggesting that early proto-oncogene expression contributes to injury-induced VSMC proliferation.17,28,29 Consistent with these findings, and in contrast to Sp1 DNA-binding activity, maximum...
AP1-DNA–binding activity was induced shortly after balloon angioplasty and then declined at later time points (Figure 4D, lanes 2 to 7). Unlabeled AP1 oligonucleotide (Figure 4D, lanes 8 to 13), but not unrelated Sp1 oligonucleotide (Figure 4D, lanes 14, 15), competed for binding to the AP1 consensus probe, demonstrating the specificity of the nucleoprotein complex. These results reveal striking differences between the temporal and spatial patterns of expression and activity of AP1 and Sp1 proteins in the arterial wall.

Discussion

High levels of the CKIs p21 and p27 have been found in VSMCs at later phases of arterial remodeling that correlated with reduced CDK2 activity and the decline in VSMC growth that takes place after the initial burst of proliferation.14–16 Thus, the upregulation of p21 and p27 may contribute to VSMC growth arrest at late time points after angioplasty. In agreement with this hypothesis, adenovirus-mediated overexpression of p2114,18,19 and p2715 attenuated neointimal thickening in balloon-injured arteries. Despite all of these findings that implicate p21 and p27 as inhibitors of VSMC proliferation, little is known about the molecular networks that regulate CKI expression during arterial remodeling in vivo.

The purpose of the present study was to elucidate regulatory mechanisms that control p27 gene expression in VSMCs in vitro and in the setting of balloon angioplasty. Our EMSAs demonstrated the interaction of the trans-acting factor Sp1 with 2 GC-rich regions within the p27 proximal promoter region. Moreover, maximum transcription from the p27 promoter was markedly reduced when VSMCs were transiently transfected with reporter genes harboring point mutations that disrupted both Sp1 binding sites. Although these results suggested that Sp1 is an important regulator of p27 gene expression in cultured VSMCs, the induction of p27 mRNA and protein expression after balloon angioplasty of the rat carotid artery did not correlate with changes in Sp1 protein expression. Importantly, a lower level of Sp1 DNA-binding activity was found in uninjured arteries and during the early time points after angioplasty, when p27 expression is lower and maximum VSMC proliferation is detected. Consistent with a role of Sp1 as a transcriptional activator of p27 in balloon-injured arteries, elevated p27 expression at later time points during vascular remodeling correlated with a
marked induction of Sp1 DNA-binding activity. Therefore, posttranslational induction of Sp1 in the injured vessel wall may contribute to transcriptional activation of p27 gene expression and the cessation of VSMC proliferation at later phases during arterial remodeling. However, we cannot rule out the possibility that increased mRNA stability plays an important role in the upregulation of steady-state p27 mRNA levels in the injured arterial wall. It is also noteworthy that induction of Sp1 DNA-binding activity at 5 days after injury preceded the upregulation of p27 gene expression, suggesting that another factor or factors independent of Sp1 are necessary for maximum p27 gene expression in vivo. In this regard, we have shown that activity of the box I motif in the proximal p27 gene regulatory region is required for p27 promoter activity in VSMCs and the molecular mechanisms that underlie this regulation.

Our immunohistochemical studies suggested distinct regulation of Sp1 expression in medial and neointimal VSMCs. Sp1 immunoreactivity was high within the neointima at all time points analyzed, but expression of Sp1 in medial VSMCs was low up to 10 days after angioplasty compared with uninjured vessels or 2 weeks after angioplasty. Thus, future studies should address whether Sp1 expression and DNA-binding activity are regulated during phenotypic modulation of VSMCs and the molecular mechanisms that underlie this regulation.

Our results with cultured VSMCs and balloon-injured arteries extend previous studies that demonstrate binding of Sp1 to both of the GC-rich motifs in the proximal p27 gene regulatory region is required for p27 promoter activity in cancerous human HeLa and U937 cells. Likewise, several studies have demonstrated that Sp1 binds specifically to oligonucleotides that correspond to Sp1-like motifs clustered within the proximal p21 promoter region and that this interaction is essential for constitutive p21 promoter activity in transiently transfected human hepatoma HepG2 cells. In vitro studies with different cell types showed that another factor or factors independent of Sp1 are necessary for maximum p27 gene transcription in VSMCs via its interaction with 2 GC-rich sequences within the p27 promoter. Using the rat carotid model of balloon angioplasty, we provide evidence that Sp1 may contribute to inducible p27 expression in vivo. Given that Sp1 is also an important transcriptional activator of p21, it is tempting to speculate that elevated Sp1 DNA-binding activity in the injured arterial wall contributes to p27 and p21 upregulation, which may in turn promote VSMC growth arrest at later phases during the vascular response to balloon denudation. The observation that Sp1 protein is expressed in uninjured arteries, which disclosed lower Sp1 DNA-binding activity, suggests that its induction after angioplasty is likely mediated by a posttranslational modification of Sp1. These results illustrate a link between the transcriptional and cell cycle machinery that may be relevant to the pathogenesis of vascular proliferative disorders.

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References