Paradoxical Upregulation of Tumor Suppressor Protein p53 in Serum-Stimulated Vascular Smooth Muscle Cells

A Novel Negative-Feedback Regulatory Mechanism

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Background—The proliferative response of vascular smooth muscle cells (VSMCs) to various growth stimuli is critical for atherosclerosis and postangioplasty restenosis. Although tumor suppressor protein p53 plays a critical role in the elimination of cancerous cells, recent genetic studies have indicated that it also protects against atherosclerosis and restenosis.

Methods and Results—We examined the levels of p53 protein in normal VSMCs before and after serum stimulation. The p53 protein levels increased robustly on stimulation. Upregulated p53 protein was capable of binding to the p53 consensus sequence, as shown by electrophoretic mobility shift assay. In addition, p53 upregulation was associated with increases in the transcript and protein levels of p21\(^{\text{WAF1/CIP1}}\) and Bax, as shown by real-time reverse transcriptase–polymerase chain reaction and Western blot analysis, respectively. Furthermore, the upregulation of p21\(^{\text{WAF1/CIP1}}\) and Bax was followed by cell-cycle arrest and apoptosis induction, as shown by 5-bromo-2’-dUTP incorporation assay and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling staining, respectively. Finally, double-staining analyses showed that the majority of p53-expressing cells also expressed p21\(^{\text{WAF1/CIP1}}\) and Bax proteins.

Conclusions—p53 protein expression in quiescent VSMCs is paradoxically increased by application of a growth stimulus. Through the mediation of p21\(^{\text{WAF1/CIP1}}\) and Bax, the induced p53 protein negatively regulates the growth of dividing VSMCs, thereby minimizing the inappropriate accumulation of VSMCs. Therefore, p53 may be a negative regulator of VSMC growth. (Circulation. 2003;108:464-471.)

Key Words: angioplasty ■ restenosis ■ atherosclerosis ■ growth substances ■ apoptosis

The tumor suppressor protein p53 is a tetrmeric nuclear phosphoprotein,\(^1\) the activities of which are mediated predominantly through the transcriptional activation of cell-cycle–regulatory genes\(^2\)–\(^4\) and proapoptotic genes.\(^5\)–\(^7\) In the clinical arena, p53 is best known as the “guardian of the genome” that prevents the propagation of cells that have come to harbor a genetic aberration.\(^8\)–\(^10\)

In recent years, p53 has also been shown to play an important role in proliferative cardiovascular disorders, namely atherosclerosis and postangioplasty restenosis. To study the role of p53 in atherosclerosis, Guevara and others cross-bred p53\(^{-/-}\) mice\(^11\) with apolipoprotein E (apoE)\(^{-/-}\) mice\(^12\) and produced p53\(^{+/+}\)/apoE\(^{-/-}\), p53\(^{-/-}\)/apoE\(^{+/+}\), p53\(^{-/-}\)/apoE\(^{+/+}\), or p53\(^{+/+}\)/apoE\(^{-/-}\) strains.\(^13\) In comparison with p53\(^{+/+}\)/apoE\(^{-/-}\) mice, p53\(^{-/-}\)/apoE\(^{-/-}\) mice developed accelerated aortic atherosclerosis in the presence of similarly elevated serum cholesterol, thus supporting the notion that p53 protects against atherosclerosis. Meanwhile, Spier et al\(^14\) and others\(^15\) demonstrated the frequent presence in restenotic plaques of IE84, a human cytomegalovirus protein that physically interacts with and blocks the transcriptional activity of p53, suggesting that the dysfunction of p53 caused by the presence of IE84 might lead to overgrowth of neointima and to postangioplasty restenosis.\(^14\)

Despite this increasing body of evidence that p53 plays an important role in the pathogenesis of proliferative vascular disorders and despite meticulous studies of growth-promoting genes\(^16\) in vascular smooth muscle cells (VSMCs), the regulatory role of p53 in VSMC proliferation has not been clearly addressed. Therefore, we investigated the expression of p53 using a defined system in which quiescent VSMCs were activated by serum stimulation. Here, we report that, in contradiction to our initial hypothesis that p53 expression would be downregulated in proliferating VSMCs, such expression was instead increased by serum stimulation. On the basis of the present observations, we propose that p53 represents a built-in “growthostat” in serum-stimulated VSMCs and that, once activated by growth stimuli, it can
subsequently prevent the inappropriate growth of VSMCs through its activation of cell-cycle-regulatory and apoptotic genes.

**Methods**

**Tissue Culture**

Adult human aortic VSMCs (HAVSMCs) were purchased from Cascade Biologics and maintained according to the manufacturer’s instructions in Medium 231 with 1% penicillin-streptomycin and serum (5%) supplements. All experiments were performed in cells of the seventh passage or less.

**Molecular Cloning**

Fragments of p21\(^{WAF1/CIP1}\), p53, and Bax cDNA were obtained by standard polymerase chain reaction (PCR) techniques using appropriate primer sets and then ligated in-frame into a pcDNA3.1 vector containing a T7 promoter site (Invitrogen).

**Cell Growth Proliferation Assay**

The growth pattern of HAVSMCs in response to serum stimulation was assessed in quadruplicate by use of the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer’s instructions. Growth index was calculated as follows:

\[
\frac{A_{\text{cells at indicated time}} \times A_{\text{media at indicated time}} - A_{\text{cells at time 0}} \times A_{\text{media at time 0}}}{100}
\]

**Real-Time Reverse Transcription–PCR**

Absolute copy numbers of Bax and p21\(^{WAF1/CIP1}\) transcripts were determined with quantitative real-time reverse transcription–PCR (RT-PCR), as described previously. The primers and a fluorogenic probe for p21\(^{WAF1/CIP1}\) and Bax had the following sequences: p21\(^{WAF1/CIP1}\), forward primer, 5'-CGCTAATGGCGGGCTT-3'; reverse primer, 5'-CATCCAGGAGGCCGGTGAGCC-3'; probe, 5'-CGCAGAGATATTGCCAA-3'; p53, forward primer, 5'-CGTGACAAAGTCGAAGTTCCAA-3'; reverse primer, 5'-GCCACTCGGAAAAGACCTCC-3'; probe, 5'-GCCACTCGGAAAAGACCTCC-3'. The sample RNAs were quantified and the results normalized to ascertain the constant amount of starting template with respect to the amplification of the 18S ribosomal RNA (Ambion, Inc). The quantitative real-time RT-PCRs were carried out in quadruplicate by use of the TaqMan RT-PCR kit (ABI) in the Applied Biosystems 7700 Sequence Detector system, according to the manufacturer’s instructions. Standard curves covering a range of 10\(^4\) to 10\(^9\) copies were constructed by use of in vitro synthesized transcripts of p21\(^{WAF1/CIP1}\) and Bax. For an assay to be considered successful, the correlation coefficient of the standard curve had to be >0.98.

**Western Blot Analysis of VSMCs**

In brief, ~1x10\(^6\) cells in 10-cm tissue culture dishes were made quiescent by maintenance for 48 hours in Medium 231 (Cascade Biologics) containing no serum. Cells were then exposed to Medium 231 containing serum and harvested at 0, 4, 8, 24, 48, and 72 hours after a 1-time medium change at time 0. Western blot and densitometric analysis of HAVSMCs was performed as described previously, with well-characterized antibodies against p53 (clone DO-1), p21\(^{WAF1/CIP1}\) (Clone 187), and Bax (N-20) (all from Santa Cruz Biotechnology). The densitometric signal intensities of p53, p21\(^{WAF1/CIP1}\), and Bax bands at the indicated time points were divided by the signal intensity of the \(\alpha\)-tubulin band at the same time points and expressed in terms of fold increase from the signals at time 0, after signal intensities at time 0 had been normalized to a value of 1. Experiments were repeated at least 3 times.

**ELISA of p53**

Cells were harvested at the indicated time points (Figure 1C) and subjected to ELISA of p53 according to the manufacturer’s instructions (Roche Diagnostics Corp).

**Immunostaining of p53 and Downstream Genes**

Cells were seeded in 2 glass chamber slides (Nalge Nunc International) in duplicate. After 48 hours of serum starvation, the first slide was harvested in 4% paraformaldehyde in PBS. Medium in the second slide was then exchanged for medium containing sera, after which the cells were incubated for 48 hours and then harvested as described above. For the single immunostaining of p53, cells were probed by anti-p53 antibody (clone pBP53-12, mouse IgG2a; Santa Cruz Biotechnology) and goat anti-mouse IgG2a-Alexa 568 (Molecular Probes) as described previously. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma Co). At least 500 cells were counted per well, and the p53 expression index was calculated as the number of p53-positive cells divided by the number of total cells counted and expressed as a percentage. For the
double immunostaining of p53 and p21<sup>WAF1/CIP1</sup>, cells were stained with anti-p53 antibody (clone pBP53-12) and anti-p21<sup>WAF1/CIP1</sup> antibody (clone 187, mouse IgG1; Santa Cruz Biotechnology).<sup>23,24</sup> Bound antibodies were detected by goat anti-mouse IgG2α–Alexa 568 and goat anti-rabbit IgG–Alexa 488, respectively (both from Molecular Probes). Nuclei were counterstained with DAPI. At least 275 cells were counted per staining experiment, and the number of cells with red nuclei divided by the total number of cells counted and expressed as a percentage, was then calculated. At least 500 cells were counted per chamber. The BrdU incorporation index, defined as the number of cells with red nuclei divided by the total number of cells counted and expressed as a percentage, was calculated.

**TUNEL Staining of VSMCs**

Terminal deoxynucleotidyl transferase (TdT)-deoxuryridine nick end-labeling (TUNEL) staining<sup>25</sup> was performed with a FragEL DNA Fragmentation Detection Kit (Oncogene Research Products) according to the manufacturer’s instructions. Briefly, ~1×10<sup>4</sup> HAVSMCs were seeded in duplicate in 8-well Laboratory-Teck chamber slides (Nalge Nunc International). Cells were serum-starved for 48 hours; pulsed with 50 μmol/L BrdU; and finally fixed in −20°C ethanol-glycine solution. BrdU staining was performed with a BrdU staining kit (Roche) according to the manufacturer’s instructions, with the following modifications. Bound mouse anti-BrdU antibody was detected by anti-mouse rhodamine Red X antibody (Jackson ImmunoResearch Laboratories). The nuclei were counterstained with DAPI. At least 500 cells were counted per chamber. The BrdU incorporation index, defined as the number of cells with red nuclei divided by the total number of cells counted and expressed as a percentage, was calculated.

The resulting DNA–protein complexes were resolved on a 4.5% native polyacrylamide gel, vacuum-dried, exposed overnight to a Kodak Phosphoscreen, and then analyzed for signals with a Bio-Rad Molecular Imager-FX laser scanner equipped with Quantity One image analysis software (Bio-Rad).
Statistical Analysis
For the comparison of 2 means, probability values were calculated by the 2-sample t test. To assess the statistical significance of the concordance between p53 expression and the expression of p21WAF1/CIP1 or Bax, the chi² test was used (Minitab Inc). In all cases, a probability value of P<0.05 was considered significant.

Results
Tumor Suppressor Protein p53 Levels Increase in Serum-Stimulated HAVSMCs
For the present study, we adopted a simple and well-defined experimental system of serum starvation and stimulation. As shown in Figure 1A, the proliferation of serum-stimulated HAVSMCs was evident as early as 2 hours after serum stimulation began. HAVSMCs then underwent robust proliferation over the next 70 hours (Figure 1A, closed circles); in comparison, control HAVSMCs, which were kept serum-starved, did not (Figure 1A, open circles).

We then determined the p53 expression levels by Western blot analysis, ELISA, and immunostaining. First, Western blot analysis using lysates prepared from serum-stimulated HAVSMCs showed a distinct increase in p53 protein level as early as 1 hour after serum stimulation began (Figure 1B). The modest increase was sustained until 24 hours, when it increased dramatically up to 8-fold (Figure 1B). Second, ELISA showed a significant moderate (≈2-fold) increase in p53 level at 8 hours, followed by a dramatic (≈7-fold) increase at 24 hours (Figure 1C). Finally, p53 immunostaining of HAVSMCs at time 0 and at 48 hours after serum stimulation began showed that the percentage of cells expressing p53 increased from 5.35±0.21% to 13.4±1.5% (P<0.05; Figure 1D). These data, taken together, suggest that p53 protein became upregulated (Figure 1, B–D) in serum-stimulated HAVSMCs.

Upregulated p53 Is Capable of Binding Its Consensus Sequence In Vitro
Next, we conducted EMSAs in which nuclear fractions were isolated from HAVSMCs and evaluated for the presence of p53 capable of binding to 32P-labeled double-stranded DNA comprising the p53 consensus sequence. This system was first validated by use of nuclear cell extracts from U2OS osteosarcoma cells transduced by adenovirus vectors encoding p53 (Figure 2A, nuclear extract [Ad-p53]). In the absence of p53, all of the 32P-labeled probes migrated rapidly to the bottom of the gel (ie, negative control; Figure 2, A and B, lanes 1, free probe). The presence of p53 capable of binding the target sequence was indicated by a shifted band containing probe–p53 complexes (ie, positive control; Figure 2, A and B, lanes 2, shift), whose migration was further slowed by the addition of anti-p53 antibody PAb1801 (Figure 2A, lane 3, super-shift). The addition of nonradioabeled double-stranded consensus p53 templates abolished the shift (Figure 2A, lane 4, competitor).

Using this system, we evaluated the status of activated p53 in serum-stimulated HAVSMCs. In quiescent HAVSMCs, shifted bands were only faintly visible, indicating that only a small amount of activated p53 was present and capable of binding the probes (Figure 2B, lanes 3 and 4; signal intensity [SI]=1±0.22 [arbitrary unit]). By 24 hours after the start of serum stimulation, the signal intensities of the shifted bands had increased dramatically, 2.9-fold (Figure 2B, lanes 5 and 6, SI=2.9±0.07, P<0.01 compared with time 0). At 48 and 72 hours, the shifted bands remained robust, albeit less so than at 24 hours (Figure 2B, lanes 7 to 10, SI=2.2±0.32, P<0.05 compared with time 0, and 1.3±0.66 for 48 and 72 hours, respectively). Together, these data suggest that upregulated p53 protein in serum-stimulated HAVSMCs was in fact capable of transcriptionally activating its target genes.

Both p21WAF1/CIP1 and BAX Are Transcriptionally Activated on Serum Stimulation
After the initiation of serum stimulation, p21WAF1/CIP1 transcript levels showed the largest increase during the first 8 hours, as measured by real-time quantitative RT-PCR (Figure 3A). The p21WAF1/CIP1 transcript levels then showed a small but steady increase up to 48 hours, when they peaked with a 1.7-fold increase (3.7±0.06×10⁶ at time 0 versus 6.47±0.06×10⁶ at 48 hours, P<0.001) (Figure 3A). Conversely, bax transcript levels increased minimally in the first 8 hours after serum stimulation but then increased drastically thereafter, peaking at 24 hours with a 3.9-fold increase over the level at time 0 (3.35±0.77×10⁶ versus 1.30±0.02×10⁷, P<0.05) (Figure 3A). Together, these data suggest that p53 protein, whose expression increased in response to serum stimulation and was detectable in the very early hours thereafter by Western blot analysis, in turn transcriptionally...
activated both cell-cycle–regulatory genes (eg, p21WAF1/CIP1) and proapoptotic genes (eg, Bax) in HAVSMCs.

**p21WAF1/CIP1 and Bax Protein Expression Levels Are Both Elevated on Serum Stimulation**

Next, we evaluated p21WAF1/CIP1 and Bax protein expression levels in HAVSMCs by Western blot analysis. As shown in Figure 3B, p21WAF1/CIP1 expression was almost undetectable at time 0, but by 2 hours after the initiation of serum stimulation, it had increased moderately (2- to 3-fold), and by 24 hours, it had increased robustly (6-fold). Bax, which was only modestly upregulated on serum stimulation during the first 8 hours, was drastically upregulated at 24 hours and remained so at 48 and 72 hours (Figure 4B). These data, taken together, are consistent with the notion that serum stimulation led in turn to an increase in intracellular levels of functionally active p53 protein (Figures 1 and 2), to the transcriptional activation of p21WAF1/CIP1 (Figure 3A) and Bax (Figure 4A), and to an increase in the levels of p21WAF1/CIP1 (Figure 3B) and Bax (Figure 4B) proteins.

**Increased p21WAF1/CIP1 Expression Is Temporally Associated With Reduction in S-Phase Cell Number After Serum Stimulation**

Next, we evaluated the BrdU incorporation indices of HAVSMCs at various time points after serum stimulation (Figure 5A). Essentially none of the serum-starved HAVSMCs took up BrdU at time 0 (0.18±0.02%) (Figure 5B), which suggests that they had achieved complete quiescence. On serum stimulation, however, the BrdU index increased significantly as early as 1 hour later (0.80±0.13%, P<0.05 in comparison with time 0) and increased drastically by 24 hours (24.9±1.98%, P<0.005 for the comparison between the index at 1 and 24 hours), indicating a robust increase in the number of S-phase cells. In this system, BrdU incorporation decreased at 48 and 72 hours after its peak at 24 hours (16.8±0.42% and 7.85±0.35% at 48 and 72 hours; P<0.005 and P<0.001, respectively, in comparison with the index at 24 hours) (Figure 5B). Cells continued to grow during this time period (Figure 1A). Because p21WAF1/CIP1 expression as determined by Western blot analysis showed a drastic increase at 24 hours (Figure 3B), p21WAF1/CIP1 was probably at least partially responsible for the decreasing BrdU incorporation after 24 hours of serum stimulation.

**Increased Bax Expression Is Temporally Associated With Increase in Apoptotic Cell Number After Serum Stimulation**

Next, we performed a standard TUNEL assay to assess the apoptosis of HAVSMCs at various time points after serum stimulation (Figure 6A). After serum starvation for 48 hours, the apoptotic index (ie, percentage of cells TUNEL-positive, or apoptotic) was 1.62±0.01% (Figure 6B). After the initiation of serum stimulation, however, the apoptotic index dropped to 0.66±0.11% by 24 hours, suggesting that cell attrition caused by serum starvation had ceased; then the index increased again to 1.13±0.08% by 48 hours (P<0.05 for the comparison between the index at 24 and 48 hours). Strikingly, at 72 hours, the apoptotic index was 3.03±0.11%, almost 5-fold that at 24 hours (P<0.005). This peak occurred ~48 hours after the peak of Bax protein at 24 hours (Figure 4B). Together, these data suggest that Bax contributed, at
least partially, to the increased apoptosis of HAVSMCs 48 to 96 hours after the initiation of serum stimulation.

There Is Causality Between p53 Expression and Expression of Its Downstream Genes

Finally, using double immunostaining techniques, we examined the status of the expression of genes downstream of p53 (ie, p21WAF1/CIP1 and Bax) in relation to p53 expression in serum-stimulated HAVSMCs. As shown in Figure 7A, there was a significant correlation between p53 and p21WAF1/CIP1 expression: 91.6±3.2% (152/[152+14], Figure 7A) of p53-positive cells were also positive for p21WAF1/CIP1, 96.6±0.4% (1085/[1085+38], Figure 7A) of p53-negative cells were also negative for p21WAF1/CIP1. χ² analysis showed this correlation to be highly statistically significant (P<0.001, **** in Figure 7A). There was also a significant correlation between p53 and Bax expression (Figure 7B): 76.1±2.9% of p53-positive cells (331/[331+103], Figure 7B) were also positive for Bax; 92.5±1.3% of p53-negative cells (1575/[1575+127], Figure 7B) were also negative for Bax (P<0.001, **** in Figure 7B). Together, these data suggest that there was a significant correlation between p53 expression and expression of its downstream genes, namely, p21WAF1/CIP1 and Bax.

Discussion

Although various growth factors have been studied meticulously as positive regulators of VSMC growth kinetics, the paradoxical upregulation of p53 in actively proliferating HAVSMCs has not been reported previously. Our present observations suggest the existence of a novel, negative feedback mechanism in proliferating VSMCs that operates even in the continued presence of growth factors (sera). In other words, at the same time that cells prepare themselves to proliferate in response to growth stimuli, they also appear to take measures to minimize the inappropriate cellular proliferation by increasing their p53 protein levels. This concept is in agreement with the work of Reich and Levine demonstrating the same paradoxical upregulation of p53 in mouse fibroblast cell lines on serum stimulation.

On its face, our observation seems to contradict previous reports that serum starvation upregulates p53. However, this apparent contradiction is not because of one observation being right and the other wrong but rather of differences in...
methods used. In the earlier reports, the investigators compared p53 expression in serum-fed VSMCs and in VSMCs serum-starved for 48 hours and found that p53 expression was upregulated in the serum-starved cells.34,35 In our experiments, we compared p53 expression in HAVSMCs that had been serum-starved for 48 hours and HAVSMCs that had been serum-stimulated after 48 hours of serum starvation. The upregulation of p53 was not a result of the waning serum effect, as shown by experiments in which we could reproduce our present data even when media were changed daily (Figure S1, Data Supplement).

The results of our present work also suggest that quiescent HAVSMCs may or may not induce p53 when activated by serum stimulation (Figure 1D). Those HAVSMCs that induce p53 would more likely induce p21WAF1/CIP1 and Bax proteins (Figure 7), and a fraction of those cells would eventually undergo cell-cycle arrest (Figure 5) and apoptosis (Figure 6). Conversely, those HAVSMCs that fail to induce p53 would go on to proliferate. If this is indeed the case, one would expect to see a mixed “mosaic” of proliferating, growth-arrested, and apoptotic cells in the growth-stimulated vascular wall. Intriguingly, Kearney et al36 reported this very mosaic nature in in-stent restenosis tissue, in which one third of cells (mostly VSMCs) showed signs of ongoing proliferation, another third showed signs of apoptosis, and the remaining third showed no evidence of either apoptosis or proliferation. Although this simultaneous presence of proliferating, nonproliferating, and apoptotic cells has long been considered a mystery, it is possible that all or part of the solution may lie in the variable response of VSMCs to growth stimuli in terms of p53 induction.

Further studies are needed to identify and characterize any “molecular switches” that might determine the ultimate fate of VSMCs on growth stimulation.37 These molecular switches would determine which cells would undergo p53 induction in response to growth stimuli and which of those cells would then undergo induction of p21WAF1/CIP1 (and subsequent cell-cycle arrest) or Bax (and subsequent apoptosis). VSMCs from p53−/− mice11 will be a useful tool for further validating the cause-and-effect relationship between p53 upregulation and Bax/p21WAF1/CIP1 expression and for investigating more exhaustively the molecular mechanisms of p53 signal transduction.

If p53 does in fact function as a negative growth regulator of VSMCs entering the proliferative phase, then the genetic difference in the degree of p53 induction in response to growth stimuli may determine the susceptibility of individuals to proliferative cardiovascular disorders (ie, primary atherosclerosis and restenosis). In other words, VSMCs from different individuals may induce p53 to quite different degrees in response to the same degree of proliferative stimuli in vivo. And if that is so, then it is possible that certain genetic traits that allow p53 to be upregulated more vigorously may be associated with lesser degrees of VSMC growth and more resistance to proliferative cardiovascular disorders. Furthermore, medical interventions to enhance the p53 response to growth stimuli would prove to be protective against proliferative cardiovascular disorders, the concept of which has been supported in part by the exogenous p53-mediated prevention of proliferative response in an animal model of vascular injury.38–40 Finally, although serum-starved, quiescent and serum-stimulated, proliferating VSMCs may be a reasonable in vitro representation of the normal and postinjury states of arteries, respectively, studies evaluating the expression status of p53, p21WAF1/CIP1, and Bax in injured animal arteries will be necessary to establish definitively the role of p53 as a negative regulator in vivo.

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