Human Vascular Smooth Muscle Cells From Restenosis or In-Stent Stenosis Sites Demonstrate Enhanced Responses to p53 Implications for Brachytherapy and Drug Treatment for Restenosis

Stephen Scott, Michael O’Sullivan, Sassan Hafizi, Leonard M. Shapiro, Martin R. Bennett

Abstract—The p53 tumor suppressor gene regulates growth arrest and apoptosis after DNA damage. Recent studies suggest that p53 is inactive in vascular smooth muscle cells (VSMCs) in human angioplasty restenosis, promoting VSMC accumulation and vessel stenosis. In contrast, the success of irradiation (brachytherapy) for in-stent restenosis argues that DNA-damage p53 responses are intact. We examined p53 expression and function in human VSMCs from normal vessels (n-VSMCs) and angioplasty/in-stent restenosis sites (r-VSMCs). p53 expression was uniformly low in all VSMCs and was induced by DNA damage. However, p53 induced profoundly different biological effects in r-VSMCs versus n-VSMCs, causing growth arrest and apoptosis in r-VSMCs only. In addition, dominant-negative p53 promoted cell proliferation and apoptosis in r-VSMCs but not n-VSMCs. Cytotoxic drug- or irradiation-induced growth arrest and apoptosis in both cell types was mediated only partly by p53. In contrast, cyclin D degradation in response to DNA damage, a critical early mediator of growth arrest, was impaired in r-VSMCs, an effect that required p53. We conclude that p53 expression and function are normal or increased in r-VSMCs and may underlie the success of brachytherapy. We also identify a restenosis VSMC-specific defect in cyclin D degradation induced by DNA damage.

Key Words: atherosclerosis ■ apoptosis ■ restenosis ■ intravascular stenting ■ brachytherapy

The tumor suppressor gene p53, whose loss/inactivation is the commonest single lesion in human neoplasia,1 exerts its tumor suppressor effect via transcription of target genes that control cell-cycle checkpoints2 or induce apoptosis. Growth arrest and apoptosis after DNA damage restrict propagation of damaged DNA, protecting the integrity of the genome. These complementary actions of p53 occur via distinct but overlapping pathways (reviewed in Bennett3).

Recent studies suggest that p53 is a critical regulator of human vascular smooth muscle cell (VSMC) proliferation in atherosclerosis. Thus, atherosclerotic plaques exhibit increased expression of both p53 and p53 targets such as the cyclin-dependent kinase (cdk) inhibitor p21 compared with normal vessels.4 p53 expression correlates with low levels of cell proliferation,5 suggesting that p53 limits VSMC proliferation in vivo. In contrast, loss of p53 activity is implicated in restenosis after angioplasty,6,7 possibly via interaction with a cytomegalovirus (CMV) gene product IE84. IE84 induces aberrant proliferation and inhibits apoptosis in VSMCs, directly promoting VSMC accumulation.6,7 Although exogenous administration of p53 limits neointimal formation after arterial injury,6–10 the status and role of p53 in human VSMCs in restenosis are unknown.

The role of p53 in restenosis after angioplasty or stenting has received new interest following successful clinical trials of brachytherapy.11–14 Brachytherapy reduces both angioplasty and in-stent stenosis (ISS), by inhibiting both neointimal accumulation and constrictive remodeling. Ionizing or UV irradiation induces growth arrest and apoptosis in part by p53 induction through DNA damage–sensing mechanisms. Thus, an intact p53 response may be critical to the success of brachytherapy, and virus protein inhibition of p53 in restenosis VSMCs (r-VSMCs) would significantly impair its therapeutic efficacy.

The present study examined the expression and function of p53 in human r-VSMCs and its contribution to responses to irradiation or cytotoxic drugs. We demonstrate that p53 in r-VSMCs is functional, regulating cell proliferation and apoptosis and that r-VSMCs show increased sensitivity to ectopic p53. Contrary to predictions, p53 inactivation increases the sensitivity of r-VSMCs to DNA damage. We also identify a novel p53-dependent mechanism of growth arrest that is absent in r-VSMCs.

Materials and Methods

Cell Isolation and Culture

VSMCs from human angioplasty restenosis or ISS sites were obtained by directional coronary atherectomy (DCA) and normal medial VSMCs from coronary arteries of patients undergoing cardiac transplantation for nonischemic cardiomyopathy (details can be found in the online data supplement). VSMC isolation followed standard methods.15 Four cultures of restenosis-derived or 3 of...
normal VSMCs between passages 3 to 8 were used for baseline experiments or gene transfer. Cultures from individual patients were kept separate and not pooled.

**Generation of Human VSMCs Expressing Ectopic p53, Dominant-Negative p53, or Human Papillomavirus E6**

Human VSMCs from restenosis sites or normal coronary arteries expressing exogenous genes were generated by retrovirus-mediated gene transfer as previously described.16

**Time-Lapse Videomicroscopy**

Cells were prepared for videomicroscopy as previously described.16

**Western Blotting/Immunohistochemistry**

Western blots and immunohistochemistry were performed as previously described,16 using a mouse monoclonal anti-human p53 antibody (14211A, Pharmingen) or cyclin D1, antibody (1481A, Pharmingen).

**Flow Cytometry**

Cell lines growing in 10% FCS for at least 24 hours or 24 hours after addition of 5 μmOL/L etoposide or after irradiation (1000 J/cm2) were prepared for flow cytometry as previously described.16 Cells demonstrating less than the diploid DNA content were excluded from measurements of cell-cycle phase.

**Statistical Analyses**

Analysis between 2 groups, between restenosis and normal VSMCs, or between treatments was performed using Student’s t test (unequal variances) for means of cell divisions/deaths.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

To analyze p53 function in human VSMCs, we cultured cells from both normal coronary artery media (n-VSMCs) and coronary angioplasty restenosis or ISS sites (r-VSMCs). Retrovirus vectors were used to suppress p53 activity via a dominant-negative p53 minigene (DN-p53) or overexpress p53. DN-p53 contains the p53 oligomerization domain but lacks a DNA-binding domain; the protein produced oligomerizes with native p53 thus preventing DNA binding17 and inhibiting p53-mediated apoptosis.18,19 p53ER™, a fusion protein of human p53 with a transactivation-defective form of the mouse estrogen receptor (ER),20 was used to overexpress p53. This protein is activated by 4-hydroxytamoxifen (4OHT)21 and increases p53 transcriptional activity, inducing apoptosis and growth arrest in primary plaque VSMCs.16 Resistant pools of r-VSMCs and n-VSMCs expressing these constructs or the empty vector were designated —DN-p53, —p53-ER™ or —vector. After all infections, expression of the inserted gene was confirmed by Northern and Southern blotting (not shown).

**p53 Protein Expression by Normal or Restenosis VSMCs**

The presence of mutant p53 protein or p53 interaction with virus proteins can elevate p53 protein levels 20- to 50-fold, attributable to a markedly increased half-life. We therefore examined p53 content of r-VSMCs and n-VSMCs by Western blot (Figure 1). p53 expression was at normal (low) levels in primary normal, plaque, or r-VSMCs, with no consistent difference between VSMC types. In particular, r-VSMCs did not show the high p53 expression seen when cells are infected with viruses such as simian virus large T antigen (SV40) (Figure 1) or CMV, which stabilize p53, or p53 degradation, seen when cells express human papillomavirus (HPV) E6. No difference was found in p53 expression between angioplasty and ISS VSMCs. Mutant p53 was not detected in any cell type (not shown). DN-p53 expression reduced wild-type p53 protein levels and p53ER™ expression increased p53 expression both of the wild-type protein...
and the high-molecular-weight p53ER™ protein (Figure 1). DN-p53 or p53ER™ expressing versions of normal and r-VSMCs showed similar p53 expression or transcriptional activity (not shown).

p53 Induction Induces Growth Arrest in Restenosis VSMCs

To examine whether p53 in r-VSMCs was functional, we studied cell proliferation and apoptosis in cells expressing DN-p53 or p53ER™ using time-lapse videomicroscopy. No significant differences in cell proliferation or apoptotic rates were found between cells from angioplasty or ISS sites (not shown), and cells were therefore classified as “restenosis” VSMCs. Proliferation of r-VSMCs or n-VSMCs was not affected by expression of the ER™ retrovirus vector alone (not shown), although r-VSMCs and r-vector VSMCs demonstrated increased apoptosis in low-serum conditions compared with normal VSMCs. Abrogation of p53 by the DN minigene had no effect on cell proliferation or apoptosis in n-VSMCs but markedly increased cell proliferation and apoptosis in r-VSMCs. p53ER™ activation also had a differential effect on r-VSMCs versus normal VSMCs, slowing proliferation and inducing apoptosis in r-VSMCs, but not normal medial VSMCs (Figure 2). 4OHT (100 nmol/L) did not affect either cell proliferation or apoptosis of cells with ER™ vector alone (not shown).

### Table 1. Growth Arrest After Etoposide or Irradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G(0)/G1</th>
<th>S</th>
<th>G/M</th>
<th>Deaths</th>
<th>Divisions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal VSMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% FCS</td>
<td>69.0</td>
<td>11.3</td>
<td>19.7</td>
<td>1.1 (0.6)</td>
<td>96.8 (6.7)</td>
</tr>
<tr>
<td>10% FCS + etoposide</td>
<td>62.9</td>
<td>12.2</td>
<td>24.9</td>
<td>2.6 (1.1)</td>
<td>8.5 (3.2)*</td>
</tr>
<tr>
<td>10% FCS + UV</td>
<td>68.0</td>
<td>15.9</td>
<td>16.1</td>
<td>3.8 (1.4)</td>
<td>0 (0)†</td>
</tr>
<tr>
<td>Restenosis VSMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% FCS</td>
<td>69.5</td>
<td>15.5</td>
<td>15.0</td>
<td>0 (0)</td>
<td>88.6 (8.6)</td>
</tr>
<tr>
<td>10% FCS + etoposide</td>
<td>38.1</td>
<td>11.5</td>
<td>50.4</td>
<td>5.3 (0.9)</td>
<td>9.4 (4.5)†</td>
</tr>
<tr>
<td>10% FCS + UV</td>
<td>53.9</td>
<td>28.6</td>
<td>17.6</td>
<td>3.6 (0.8)</td>
<td>0 (0)†</td>
</tr>
<tr>
<td>Restenosis DN-p53 VSMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% FCS</td>
<td>61.0</td>
<td>14.5</td>
<td>24.5</td>
<td>6.8 (5.5)</td>
<td>173.2 (2.3)</td>
</tr>
<tr>
<td>10% FCS + etoposide</td>
<td>49.0</td>
<td>6.7</td>
<td>44.3</td>
<td>17.4 (4.9)</td>
<td>43.7 (9.1)*</td>
</tr>
<tr>
<td>10% FCS + UV</td>
<td>51.0</td>
<td>21.7</td>
<td>27.3</td>
<td>68.2 (4.6)</td>
<td>2.6 (1.3)†</td>
</tr>
</tbody>
</table>

Flow cytometric analysis, apoptotic rates, and cell divisions (/100 cells/24 hours) of human normal, restenosis, or restenosis DN-p53 cells in culture either alone (10% FCS) or after etoposide treatment or irradiation. Cell-cycle profiles were analyzed 24 hours after treatment. Data are mean±SEM. *P<0.05, †P<0.01 vs 10% FCS for each cell type.

Effect of Irradiation on Restenosis VSMCs

Brachytherapy using either γ or β sources can successfully prevent restenosis after angioplasty or stenting.12–14 In addition, cytotoxic or antiproliferative drugs are in development for prevention of both angioplasty and ISS. Although irradiation and cytotoxic drugs inhibit cell proliferation and induce apoptosis in normal VSMCs, their effects on human restenosis VSMCs or the requirement for p53 is unknown. Etoposide is a topoisomerase II inhibitor that induces DNA damage, and the p53-dependent growth arrest after etoposide treatment is a well-characterized assay for functional p53.22,23 Similarly, UV irradiation induces p53 and a subsequent p53-dependent growth arrest in many cells.24,25

Etoposide and UV irradiation induced p53 protein expression within 24 hours in both n-VSMCs and r-VSMCs to a similar extent (Figure 3). Cell proliferation was analyzed over 24 hours after etoposide or UV irradiation and by flow cytometry 24 hours after treatment. Etoposide induced growth arrest in n-VSMCs and r-VSMCs (Table 1); growth arrest occurred in the G1 phase of the cell cycle in r-VSMCs as previously demonstrated in rat VSMCs.26 (Figure 1, Table 4A), although this effect was less prominent in n-VSMCs. In contrast, irradiation induced growth arrest throughout the cell cycle. Cells expressing DN-p53 showed slight reduction in the ability of etoposide (but not irradiation) to induce growth arrest (89% versus 75% reduction in proliferation) in r-VSMCs, but similar cell-cycle profiles to r-VSMCs were seen. Both etoposide and irradiation induced minimal apoptosis in both cell types in culture in the presence of serum. Importantly, abrogation of p53 actually promoted DNA damage–induced apoptosis in r-VSMCs (Table 1), particularly after UV irradiation.

When brachytherapy or cytotoxic drugs are delivered after intervention, most VSMCs in the vessel wall are in the quiescent (G0) state. We therefore examined the ability of etoposide or irradiation to inhibit cell-cycle entry of VSMCs. Cells were arrested in G0/G1 by incubation in low serum for 72 hours and then stimulated to enter the cell cycle using serum, either alone, with etoposide, or after irradiation. Cell-cycle analysis was performed at 24 hours, or cumulative cell divisions assessed over the subsequent 48 hours. Serum withdrawal resulted in G0/G1 arrest in all VSMCs (Table 2, Figure 4B). Serum addition resulted in rapid cell-cycle entry in all cell types with an increase in percentage of S or G2 at 24 hours and evidence of cell division at 48 hours. r-VSMCs showed faster cell-cycle entry than n-VSMCs, with a higher percentage of cells reaching G2 by 24 hours (Table 2, Figure 4B). Etoposide slowed cell-cycle entry in n-VSMCs and r-VSMCs with arrest in G0/G1 in n-VSMCs and S/G2 in r-VSMCs. The S phase but not the G2 arrest was abrogated by inhibition of p53 in r-VSMCs. Irradiation completely blocked cell division after serum stimulation, but different cell types proceeded to different points in the cell cycle. n-VSMCs and r-VSMCs entered the cell cycle but were arrested in S phase or G2/M, respectively. In contrast, r-DN-p53 VSMCs did not enter the cell cycle at all after irradiation.
D 1 protein. 27 This causes a rapid inhibition of G 1 -S transition product p105RB. We therefore examined cyclin expression in due to impaired phosphorylation of the retinoblastoma gene.

Table 2. Cell-Cycle Entry After Etoposide or Irradiation

<table>
<thead>
<tr>
<th></th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
<th>Deaths</th>
<th>Divisions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal VSMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% FCS alone</td>
<td>87.1</td>
<td>8.1</td>
<td>4.5</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>+10% FCS</td>
<td>61.6</td>
<td>30.4</td>
<td>8.0</td>
<td>0 (0)</td>
<td>92.1 (8.2)</td>
</tr>
<tr>
<td>+10% FCS + etoposide</td>
<td>81.7</td>
<td>14.6</td>
<td>3.7</td>
<td>1.1 (0.6)</td>
<td>20.2*</td>
</tr>
<tr>
<td>+10% FCS + UV</td>
<td>71.5</td>
<td>21.9</td>
<td>6.6</td>
<td>2.1 (0.8)</td>
<td>0†</td>
</tr>
<tr>
<td>Restenosis VSMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% FCS alone</td>
<td>76.7</td>
<td>12.6</td>
<td>10.7</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>+10% FCS</td>
<td>44.5</td>
<td>6.3</td>
<td>49.2</td>
<td>0 (0)</td>
<td>98.2 (10.4)</td>
</tr>
<tr>
<td>+10% FCS + etoposide</td>
<td>44.5</td>
<td>26.3</td>
<td>29.2</td>
<td>5.9 (5.9)</td>
<td>19.3 (19.3)*</td>
</tr>
<tr>
<td>+10% FCS + UV</td>
<td>63.4</td>
<td>4.2</td>
<td>32.4</td>
<td>0 (0)</td>
<td>0 (0)†</td>
</tr>
<tr>
<td>Restenosis DN-p53 VSMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% FCS alone</td>
<td>77.3</td>
<td>12.9</td>
<td>9.8</td>
<td>...</td>
<td></td>
</tr>
<tr>
<td>+10% FCS</td>
<td>65.9</td>
<td>12.5</td>
<td>21.6</td>
<td>0 (0)</td>
<td>120.4</td>
</tr>
<tr>
<td>+10% FCS + etoposide</td>
<td>59.3</td>
<td>12.5</td>
<td>28.2</td>
<td>1.4 (1.1)</td>
<td>0†</td>
</tr>
<tr>
<td>+10% FCS + UV</td>
<td>84.7</td>
<td>5.7</td>
<td>9.6</td>
<td>14.2 (0.5)*</td>
<td>0†</td>
</tr>
</tbody>
</table>

Flow cytometric analysis, apoptotic rates, and cell divisions (/100 cells/24 hours) of human normal, restenosis, or restenosis DN-p53 cells in culture after serum withdrawal for 72 hours (0% FCS) or after addition of serum-containing medium, either alone (10% FCS) or with etoposide treatment or irradiation. Cell-cycle profiles were analyzed at 24 hours and cell division over 48 hours after serum addition and treatment. Data are mean ± SEM.

The ability of DNA-damaging agents to cause growth arrest and apoptosis in cells with deficient p53 suggests that these agents also work via p53-independent pathways. Recently, it has been shown that DNA damage causes a p53-independent degradation of cyclin D1, via a destruction box motif in cyclin D1 protein.27 This causes a rapid inhibition of G1-S transition due to impaired phosphorylation of the retinoblastoma gene product p105RB. We therefore examined cyclin expression in VSMCs after DNA damage. In contrast to earlier studies, DNA damage in n-VSMCs caused a marked reduction in expression of all 3 D-type cyclins, but no consistent effect on cyclins E or A (Figure 5A). r-VSMCs were resistant to DNA damage-induced cyclin D degradation, an effect that required p53, as this was lost in r-VSMCs expressing DN-p53 cells or HPV E6. The effect of DN-p53 was due to recovery of degradation after irradiation rather than blocking resynthesis of cyclins D1−3 (Figure 5B), although resynthesis of cyclin D was seen at 48 hours after irradiation, and could be blocked by cycloheximide (Figure 5C). p21 expression was slightly increased after irradiation rather than blocking resynthesis of cyclin D1−3 in vivo at restenosis sites, we examined normal coronary arteries or ISS atherectomy specimens by immunohistochemistry. Figure 6 demonstrates that p53 and cyclins D1−3 were not detectable in normal vessels. In contrast, although p53 was not detectable, VSMCs positive for cyclins D1−3 were found in ISS specimens.

**Discussion**

The role of p53 in regulating VSMC proliferation and apoptosis in atherosclerosis or restenosis after angioplasty/stenting is highly controversial. Some studies have identified increased wild-type p53 expression in VSMCs in atherosclerosis5,23; the coincident increase in p53 transcriptional target expression suggests that this p53 is functional.5,28 Suppression of p53 also promotes VSMC accumulation after arterial injury in animals,8–10 suggesting that basal p53 activity limits the response to injury after human angioplasty. Consistent with this idea, some studies have found an association between positive CMV serology and restenosis; CMV
immediate-early gene expression may inhibit p53 function and thus would be predicted to promote restenosis. However, the demonstration that brachytherapy, a modality based in part on p53 activation after DNA damage, is a highly effective treatment for restenosis after stenting argues that the growth arrest/apoptosis response of restenosis VSMCs is intact. We therefore examined p53 function in VSMCs derived from human angioplasty or ISS sites.

We find that r-VSMCs show normal expression of wild-type p53 protein in vitro and in vivo and respond to irradiation or DNA damage induced by drugs by induction of p53. Thus, all of the upstream DNA damage–sensing machinery required to stabilize and activate p53 is functional in r-VSMCs. We find that upon p53 activation, growth arrest and apoptosis occur very efficiently in r-VSMCs; indeed r-VSMCs are far more sensitive to p53 induction than n-VSMCs. We therefore find no evidence of a functional defect in p53 in restenosis VSMCs. Furthermore, in r-VSMCs, irradiation and etoposide potently induce growth arrest and apoptosis that is minimally p53-dependent. Thus, r-VSMCs lacking transcriptionally active p53 undergo growth arrest and apoptosis in response to DNA damage. However, r-VSMCs expressing DN-p53 have increased cell proliferation and apoptosis compared with vector-expressing cells, an effect not seen in VSMCs from normal vessel media. Although it is possible that this is a direct effect of the DN-p53 minigene and not a result of suppression of p53 per se, HPV E6 suppression of p53 also increases apoptosis and proliferation in human VSMCs. This further argues that basal p53 expression limits the response to injury in r-VSMCs. To our knowledge, this is the first published study to demonstrate that cell-cycle regulation in human coronary r-VSMCs differs from that in n-VSMCs.

Our data do not support the hypothesis that p53 inactivation predisposes some patients to restenosis. Inactivation of p53 has been suggested to lead to deregulated cell proliferation and/or reduced apoptosis in r-VSMCs, promoting development of restenosis. Virus inactivation of p53 may occur in one of two ways. Virus proteins such as SV40 bind and inactivate p53, preventing p53-induced transcription. Binding also stabilizes p53 protein, resulting in high levels of (inactive) protein (see Figure 1). In contrast, HPV E6 binds p53 and targets p53 for ubiquitin-mediated degradation, manifest as little or no detectable p53. Most mutant p53 proteins have a longer half-life (and thus more detectable expression), but with reduced activity. In contrast to earlier studies, p53 expression in our cultured r-VSMCs did not differ from normal VSMCs. Although this may reflect changes in p53 in culture, immunohistochemistry of ISS sites did not demonstrate increased levels of p53 expression consistent with a mutant or stabilized protein.

However, a note of caution in interpretation of our studies is warranted. Earlier studies have identified high p53 expression correlating with CMV expression in 38% of restenosis lesions. It remains possible that the smaller number of samples obtained by DCA studied in long-term culture missed a subgroup of samples having high p53 expression. Indeed, none of our VSMC cultures expressed CMV proteins IE72 or IE84 by RT-PCR (not shown). In contrast, our studies examined stable expression of p53 in pure VSMC cultures, passaged to the point where expression of cellular proteins should no longer be affected by mode of isolation or type of tissue from which cells were isolated. Studies examining immediate-early gene expression may inhibit p53 function and thus would be predicted to promote restenosis. However, the demonstration that brachytherapy, a modality based in part on p53 activation after DNA damage, is a highly effective treatment for restenosis after stenting argues that the growth arrest/apoptosis response of restenosis VSMCs is intact. We therefore examined p53 function in VSMCs derived from human angioplasty or ISS sites.

We find that r-VSMCs show normal expression of wild-type p53 protein in vitro and in vivo and respond to irradiation or DNA damage induced by drugs by induction of p53. Thus, all of the upstream DNA damage–sensing machinery required to stabilize and activate p53 is functional in r-VSMCs. We find that upon p53 activation, growth arrest and apoptosis occur very efficiently in r-VSMCs; indeed r-VSMCs are far more sensitive to p53 induction than n-VSMCs. We therefore find no evidence of a functional defect in p53 in restenosis VSMCs. Furthermore, in r-VSMCs, irradiation and etoposide potently induce growth arrest and apoptosis that is minimally p53-dependent. Thus, r-VSMCs lacking transcriptionally active p53 undergo growth arrest and apoptosis in response to DNA damage. However, r-VSMCs expressing DN-p53 have increased cell proliferation and apoptosis compared with vector-expressing cells, an effect not seen in VSMCs from normal vessel media. Although it is possible that this is a direct effect of the DN-p53 minigene and not a result of suppression of p53 per se, HPV E6 suppression of p53 also increases apoptosis and proliferation in human VSMCs. This further argues that basal p53 expression limits the response to injury in r-VSMCs. To our knowledge, this is the first published study to demonstrate that cell-cycle regulation in human coronary r-VSMCs differs from that in n-VSMCs.

Our data do not support the hypothesis that p53 inactivation predisposes some patients to restenosis. Inactivation of p53 has been suggested to lead to deregulated cell proliferation and/or reduced apoptosis in r-VSMCs, promoting development of restenosis. Virus inactivation of p53 may occur in one of two ways. Virus proteins such as SV40 bind and inactivate p53, preventing p53-induced transcription. Binding also stabilizes p53 protein, resulting in high levels of (inactive) protein (see Figure 1). In contrast, HPV E6 binds p53 and targets p53 for ubiquitin-mediated degradation, manifest as little or no detectable p53. Most mutant p53 proteins have a longer half-life (and thus more detectable expression), but with reduced activity. In contrast to earlier studies, p53 expression in our cultured r-VSMCs did not differ from normal VSMCs. Although this may reflect changes in p53 in culture, immunohistochemistry of ISS sites did not demonstrate increased levels of p53 expression consistent with a mutant or stabilized protein.

However, a note of caution in interpretation of our studies is warranted. Earlier studies have identified high p53 expression correlating with CMV expression in 38% of restenosis lesions. It remains possible that the smaller number of samples obtained by DCA studied in long-term culture missed a subgroup of samples having high p53 expression. Indeed, none of our VSMC cultures expressed CMV proteins IE72 or IE84 by RT-PCR (not shown). In contrast, our studies examined stable expression of p53 in pure VSMC cultures, passaged to the point where expression of cellular proteins should no longer be affected by mode of isolation or type of tissue from which cells were isolated. Studies examining

Figure 5. Effect of irradiation on cyclin and cdk inhibitor expression in VSMCs. A, Normal (2 separate cultures), restenosis, or r-DN-p53 VSMCs were irradiated (+) or left untreated (−). Cyclin or cdk inhibitor expression was analyzed 24 hours after treatment. Cyclin D degradation was also examined in r-HPV E6 VSMCs (R-E6) after irradiation. B, Time course of cyclin D expression in r-VSMCs after irradiation. C, Resynthesis of cyclin D at 48 hours can be blocked by cycloheximide (+). D, Cyclin D degradation is also seen in 2 cultures of primary carotid plaque VSMCs after irradiation (+).

Figure 6. Expression of p53 and cyclins D₁–₃ in human ISS lesions or normal arteries. Positive control tissue (A and D), normal human coronary artery sections (B and E), or ISS specimens (C and F) were examined by immunohistochemistry for expression of p53 (A through C) or cyclin D₁–₃ (D through F). Positive control tissues were lung (A) or colonic (D) carcinomas.
freshly isolated cells from restenosis lesions risk examining non-VSMC types, which may have different p53 expression or virus infection, or still be subject to local microenvironmental or systemic stimuli.

We next examined the hypothesis that p53 function is reduced in r-VSMCs. p53 induces growth arrest in both G1 and G2,29–31 The G1 arrest is due mostly to transcriptional activation of the cdk inhibitor p21.32–34 In contrast, G2 arrest is due to both p21 and other genes such as GADD4535 and 14.3.3r, a protein that sequesters cyclin B1-cdc2 complexes.36–38 p53 also induces p53R2, a homologue of ribonucleotide reductase39 that controls G2 transit, and is required for p53-induced G2 arrest, protecting cells with DNA damage from apoptosis. p53-induced apoptosis is also part transcriptionally dependent, with target genes including Bax, Fas, DR-3, and a number of redox-related genes.40 Indeed, apoptosis in response to genotoxic stress is impaired in the absence of p53.41–42

We find that, as with primary plaque VSMCs, ectopic p53 induction potently causes both growth arrest and apoptosis in r-VSMCs, although not in n-VSMCs. These latter findings are in contrast to earlier studies,43 which demonstrated that infection of normal human VSMCs with a p53-encoding adenovirus induced apoptosis. The differing results may be due to the expression levels of ectopic p53. The retrovirus construct we use causes a 2- to 3-fold increase in p53 expression in human VSMCs, similar to that seen after irradiation or cytotoxic drugs. In contrast, adenoviruses encoding p53 cause much higher expression of the protein, which may be nonphysiological. In addition, adenovirus infection of cells can result in significant transgene-independent elevations of both p53 and other cell-cycle proteins, leading to both apoptosis and G1/M retardation.44

Ionizing radiation via either γ or β sources induces DNA damage, causing double-stranded DNA breaks (DsBs). DsBs activate a number of DNA repair enzymes, including ATM (mutated in ataxia telangiectasia) and ATR (ATM-related kinase), which may mediate the response to types of DNA damage other than DsBs. ATM and ATR directly phosphorylate and activate specific checkpoint kinases, such as chk2 and hCDS1, which in turn phosphorylate p53,45,46 Phosphorylation of p53 stabilizes p53 protein, increasing transcriptional activity of p53, resulting in both p53-induced growth arrest and apoptosis. Thus, DNA damage leads directly to p53 stabilization and activation, and the growth arrest and apoptosis response of cells after irradiation depend at least in part on p53.

We find that DNA damage caused by cytotoxic drugs or irradiation in dividing cells induces both p53 and growth arrest in n-VSMCs and r-VSMCs. The S/G2 arrest of etoposide is a predicted feature based on the mode of action of the drug, whereas irradiation causes growth arrest in G1, S, and G2 in both cell types. Etoposide reduces and irradiation completely inhibits cell proliferation from quiescence in both n-VSMCs and r-VSMCs. However, the cell-cycle arrest is different in n-VSMCs compared with r-VSMCs. In particular, after irradiation, n-VSMCs proceed to S phase whereas r-VSMCs transit S phase and accumulate in G2. This difference is not due to p53, as DN-p53 VSMCs are sensitive to growth arrest after DNA damage, and do not enter the cell cycle. These experiments demonstrate that DNA damage induced by cytotoxic drugs or irradiation induces growth arrest via both p53-dependent and -independent mechanisms.

Although ectopic p53 is functional in r-VSMCs, we identify a novel p53-dependent mechanism for rapid growth arrest, which is lost in r-VSMCs. Previous studies have shown that DNA damage causes an immediate p53-independent growth arrest via proteolysis of cyclin D1 via a destruction box motif.27 This releases p21 from cdk4 to bind cdk2 and induce growth arrest. Prevention of cyclin D1 degradation blocks arrest and makes the cells more susceptible to apoptosis. We show that all 3 cyclin D isoforms are reduced in n-VSMCs after irradiation, an effect that is lost in r-VSMCs. This may account for the ability of r-VSMCs to bypass S-phase arrest seen on cell-cycle entry after irradiation. The resistance of r-VSMCs to DNA damage-induced cyclin D degradation is dependent on p53, as it is lost in DN-p53 cells. The inability to degrade cyclin D may also partly explain the increased sensitivity of r-VSMCs to apoptosis induced by p53. The effect of DN-p53 on cyclin D degradation in the absence of irradiation is unclear. Because DN-p53 does not reduce basal expression of cyclins D1,3 and irradiation suppresses cyclins D1,3, to barely detectable levels in n-VSMCs, any additional effect of DN-p53 on cyclin D1,3 degradation could not be elucidated.

Our data have important implications for the development of novel therapies for restenosis, including brachytherapy. Successful brachytherapy requires growth arrest and (probably) apoptosis of VSMCs comprising the restenosis lesion, which requires an intact DNA-damage response and functional p53 protein. Intact DNA-damage sensing and responses may also restrict propagation of damaged DNA after brachytherapy. Additionally, extensive medial or adventitial damage may cause exaggerated constrictive remodeling and failure of medial dissections to heal, promoting vessel stenosis or lesion instability. Our finding that p53 in restenosis VSMCs is functional and that DNA-damage responses (whether p53-dependent or -independent) are, if anything, more efficient than in normal medial VSMCs predicts a therapeutic dose of irradiation that could induce apoptosis and growth arrest of neointimal but not medial VSMCs, inhibiting restenosis without impairing medial healing. Our data also suggest that both normal medial and intimal r-VSMCs may enter the cell cycle after injury and irradiation; however, neither cell type will complete mitosis, being arrested in S phase or G1/M, respectively.

In conclusion, we have shown that human restenosis VSMCs exhibit similar p53 expression to normal medial VSMCs. p53 is induced after DNA damage and is functional to induce both growth arrest and apoptosis in restenosis VSMCs. Restenosis VSMCs entering the cell cycle after irradiation demonstrate novel cell-cycle regulation, being able to bypass the S-phase block induced by irradiation, an effect that may be due to an inability to degrade cyclin D after DNA damage. The growth arrest and apoptotic responses after DNA damage, in brachytherapy, for example, require both p53-dependent and -independent pathways.

Acknowledgments
This work was supported by British Heart Foundation Grants FS/97024, PG/97199, PG/98045, and RG/98009. Michael O’Sullivan is a Wellcome Trust Training Fellow.
References

Human Vascular Smooth Muscle Cells From Restenosis or In-Stent Stenosis Sites Demonstrate Enhanced Responses to p53: Implications for Brachytherapy and Drug Treatment for Restenosis

Stephen Scott, Michael O’Sullivan, Sassan Hafizi, Leonard M. Shapiro and Martin R. Bennett

Circ Res. 2002;90:398-404; originally published online January 31, 2002;
doi: 10.1161/hh0402.105900

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/90/4/398

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2002/03/03/90.4.398.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Human vascular smooth muscle cells from restenosis or in stent stenosis sites demonstrate enhanced responses to p53: implications for brachytherapy and drug treatment for restenosis

1 Unit of Cardiovascular Medicine, Addenbrooke’s Centre for Clinical Investigation, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK

2 Heart Sciences Centre, Harefield Hospital, Middlesex UB9 6JH

3 Cardiac Unit, Papworth Hospital, Papworth Everard Cambridge

Address correspondence to Professor M R Bennett

Phone/ Fax 01223 331504/5, E-mail mrb@mole.bio.cam.ac.uk

Running title - Role of p53 in restenosis
ONLINE SUPPLEMENT

Methods

Cell isolation and culture

The use of material from DCAs or recipient hearts was approved by Hospital Local Ethics Committees, and informed consent was obtained from patients. Briefly, cultures were obtained from specimens from individual patients of both sexes including a range of ages between 34 and 62 years (mean age of patients was 49.8 (restenosis) and 47.8 years (Normal coronary artery). Restenosis cultures were obtained from patients with stable angina; times from original intervention to sampling were 4-9m. Cells from both atherectomy specimens and normal vessels were cultured in DMEM medium containing 10% foetal calf serum, penicillin, amphotericin, glutamate and streptomycin, equilibrated with 95% air and 5% CO₂.

Characterisation of VSMCs as intimal from atherectomy tissues, and medial, from normal vessels was performed by H&E histology of the atherectomies or arteries removed. DCA or medial specimens in which an elastic lamina was identified, indicating contamination with media or intima/adventitia respectively were not used for culture. Cells were characterised as VSMCs by immunocytochemical staining at passage 3 (α-actin positive, vimentin positive, VWF negative, desmin negative and smooth muscle myosin negative). In isolates from atherosclerotic plaques, both macrophages and T-lymphocytes were present; however, by passage 3, the only cells identifiable were VSMCs.

Generation of human VSMCs expressing introduced p53 or dominant negative p53.
Briefly, full length cDNAs encoding human p53ER™, a dominant negative p53 minigene encoding amino acids 302-390 \(^1\) or Human Papilloma Virus Type 16 E6 protein were cloned into the retrovirus vector pBabe (neo/puro) using conventional techniques, and transfected into PA317 packaging cells. Amphotropic virus was harvested and used to infect human VSMCs at passage 3 as previously described \(^2\). Resistant human VSMCs were selected in 2.5 µg/ml of puromycin after at least 4 weeks in antibiotic-containing medium. Pooled populations of both plaque-derived and normal human VSMCs were used for experiments rather than clones of antibiotic-resistant cells. All experiments were performed on passages 5-8 cells.

**Time-lapse videomicroscopy**

Briefly, cells were maintained in medium containing 10% FCS, washed 3 times in PBS and then cultured in 0% FCS medium. For experiments involving etoposide (5µM) or irradiation, cells were maintained in 10% FCS medium and filmed for 24 hours after treatment. Flasks were gassed with 95% air and 5% CO\(_2\) every 24 hours and sealed. An Olympus OM-70 microscope was enclosed in a plastic environment chamber and maintained at 37°C by an external heater. The time-lapse equipment consisted of a Sony 92D CCD camera with a Panasonic 6730 time-lapse video recorder. Films were analysed for morphology of apoptosis and cell death rates as previously described \(^2\) using an observer blind to cell type and treatment conditions. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and fragmented, an interval of typically 60-90 minutes. Cell division was scored at the time at which septa appeared between two daughter cells. Individual cell cultures were analysed in duplicate as a minimum.
Western blotting

Western blots were prepared by lysis of cells cultured in medium containing 10% FCS. Protein isolation, electrophoresis and blotting were as previously described, using a mouse monoclonal anti human p53 antibody (14211A, Pharmingen) or cyclin D1,3 antibody (1481A, Pharmingen). Protein concentrations were assessed by modified Bradford assay (Biorad) prior to loading.

Drug treatment / irradiation of vascular smooth muscle cells

Cells grown on petri dishes were drained of medium, washed three times in PBS, and exposed to 1000μJ/m² of UV irradiation in a Stratalinker. Cells were then washed once in PBS, the medium replaced, and cells returned to the incubator. Growth arrest was analysed by time-lapse videomicroscopy or flow cytometry 24-48 hours after either drug treatment or irradiation. p53 protein levels were analysed 24 hours after either treatment.

Immunohistochemistry

p53 or cyclins D1,3 expression was examined in both normal coronary arteries or in stent stenosis sites by immunohistochemistry as previously described using the same antibodies to p53 or cyclin D1,3 as described for Western blotting.
Online references


