Effect of $p53$ Deficiency on External Vascular Cuff-Induced Neointima Formation

Masao Moroi, MD; Taro Izumida, MD*; Toshisuke Morita, MD**; Junko Tatebe, BS***; Chikara Ishii, MD*; Tomihiko Imai, MD*; Shinji Yagi, MD*; Tetsu Yamaguchi, MD†; Shigehiro Katayama, MD*

The $p53$ tumor suppressor gene may act as an inhibitor of vascular neointima formation in response to injury and in the present study the effects of $p53$ deficiency on external vascular cuff-induced neointima formation were evaluated. Vascular neointima formation was induced by an external vascular cuff; a polyethylene tube placed around a 2 mm segment of the left femoral artery ensheathed the adventitia, but avoided direct intraluminal injury. Two weeks after cuff placement, the cuff-sheathed and contralateral control arteries without cuff from wild-type (n=10) and $p53$ deficient (n=8) mice were harvested and analyzed by quantitative morphometry. The areas of the lumen, intima, and media were measured in 10 cross-sections from one edge to the other of the cuffed portion, and in the corresponding 2 mm segment of the contralateral control artery. The volume ratio of the intima to media (I/M) was calculated. The contralateral control arteries without a cuff did not have intima in either wild-type or $p53$ deficient mice. In the cuff-sheathed arteries, neointima formation of $p53$ deficient mice with an I/M of 93% was significantly greater than that of wild-type mice with an I/M of 50% (P=0.001). The absence of $p53$ is associated with increased neointima formation in response to cuff injury. (Circ J 2003; 67: 149–153)

Key Words: Arteries; Atherosclerosis model; Genes; Inflammatory response; Mice

The $p53$ tumor suppressor gene is thought to negatively regulate the cell cycle in various types of cells, and loss of the wild-type $p53$ allows the abnormal proliferation that can lead to cancer. Recently, it was reported that inhibition of $p53$ accelerated the proliferation of cultured vascular smooth muscle cells (VSMC) into which an antisense $p53$ gene was transfected and resulted in increased neointima of a rat carotid artery into which antisense $p53$ was transfected. It has also been suggested that VSMC migration as well as proliferation may be controlled by $p53$. Furthermore, $p53$ expression is associated with VSMC apoptosis. Bennett et al demonstrated $p53$-induced VSMC apoptosis by enhancing the cell surface expression of the cell death ligand receptor Fas. Bennett et al also reported that there is a relatively low level of cell proliferation and a high level of apoptosis induced by $p53$ in cultured VSMCs from human atherosclerotic plaques. Mayr et al demonstrated that $p53$ deficiency accelerates neointima formation by facilitating VSMC proliferation as well as abrogating cell apoptosis in a mouse vein bypass graft model. Although there are not enough data to determine whether $p53$-induced apoptosis occurs in VSMC after their migration to the subendothelial space, $p53$ may partially regulate vascular intima formation through the apoptosis of VSMC that migrate to the site. In addition to the studies on the effects of $p53$ on VSMC migration, proliferation, and apoptosis, one study examined the interaction between atherosclerosis and $p53$ in mice deficient in both $p53$ and apoE. In that model, the absence of $p53$ exacerbated atherosclerotic lesion expansion caused by increased VSMC proliferation. In contrast to the absence of $p53$, forced overexpression of $p53$ inhibited neointima formation in a rabbit carotid balloon-injury model. These data indicate that a deficiency of the wild-type $p53$ may lead to increased vascular intima formation in response to a balloon injury and in an atherosclerosis mouse model; however, it is unknown what its effects are on endothelial cells in vivo, because endothelial cells are removed in the balloon model and were dysfunctional in the atherosclerosis mouse model.

Restenosis after percutaneous coronary interventions (PCI) is thought to be strongly associated with medial smooth muscle cell proliferation; there have been some clinical studies that suggested an interaction among $p53$, cytomegalovirus (CMV) infection and restenosis after PCI. In approximately one-third of patients with restenosis, CMV DNA sequences can be found in the lesions, and the VSMCs from such lesions obtained by coronary atherectomy express IE2, which is one of the immediate early proteins of CMV and which binds to $p53$ and inhibits its function. Thus, restenosis in humans after PCI may be associated with inactivation of the $p53$ protein by CMV.

We previously described an external vascular cuff-induced neointima formation mouse model by which we can quantitatively and reproducibly analyze vascular neointima formation. The model avoids direct intraluminal injury such as induced by the filament model and consequently enables study of the effect of endothelial factors. In this regard, the cuff model is completely different from other...
rodent models for studying vascular intima formation and we can study the effect of p53 on vascular intima formation without directly injuring endothelial cells. In addition, previously reported methods, such as gene transfer and gene blockade by antisense, may be suboptimal for studying the action of p53 because there is incomplete blocking of the actions of p53. Therefore, we used the external vascular cuff-induced intima formation model in mice deficient in p53 and analyzed the effects of p53 on neointima formation in response to an external vascular cuff injury.

Methods

Animals
We used p53 deficient mice, generated by Jacks et al,7 that have the combined genetic background of the SV129 and C57BL/6J strains. The p53 homozygous mutant mice are viable, fertile, and indistinguishable from wild-type mice in appearance until 12 weeks of age, after which they are highly predisposed to malignancy and 50% of homozygotes die by 20 weeks of age. In comparison, the wild-type mice typically live for more than 90 weeks. In the present experiment, we used 8–10-week-old males that weighed 19–22 g, because the homozygotes at that age have a low cancer risk. Furthermore, litter mates were used for comparison between wild-type and p53 deficient mice on vascular neointima formation. All mice received humane care in accordance with the Guide for Animal Experiments of Saitama Medical School.

Identification of p53 Deficient Mice
The tails of the offspring were biopsied at 4 weeks of age and the genomic DNA was isolated using proteinase K and then analyzed with polymerase chain reaction. Mice with the wild-type p53 and mutant p53 were identified by Southern blotting with the following probes: wild-type p53, p53-036:5′-ACAGCGTGGTGGTACCTTAT or p53-040:3′-TATACTCAGAGCCGGCTT; mutant p53, p53-038:5′-CTATACAGACATAGCGTTTG or p53-040:3′-GCCCCCATGCAGGAGCTAT.

Femoral Artery Cuff Placement
The external vascular cuff-induced intima formation mouse model has been previously described by Moroi et al.15 After mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg), the left femoral artery was exposed and isolated from the surrounding tissue. Next, a nonocclusive, flexible polyethylene cuff (length, 2 mm; inner diameter, 0.56 mm; outer diameter, 0.965 mm; Becton Dickinson, Mountain View, CA, USA) was placed around it. The right femoral artery was dissected from the surrounding tissues, but a cuff was not placed (sham-operated). The wounds were sutured. After recovery from anesthesia, the mice were fed a standard diet and water ad libitum.

Tissue Harvesting and Histologic Staining
Two weeks after cuff placement, the mice were anesthetized and killed. The vessels were fixed in situ by constant pressure fixation at 100 mmHg with 10% formalin through a 22-gauge butterfly angiocatheter placed in the left ventricle of the heart. Both the right and left femoral arteries were harvested. Each artery was embedded in paraffin, and cross-sections (10 μm) were continuously cut from one edge to the other of the cuffed portion, and in the corresponding segment of the contralateral control artery. Each section was mounted in order on 5 series of slides. Parallel sections were stained with hematoxylin and eosin (H&E) or elastica van Gieson, or underwent immunohistochemical staining.

Morphometry
Morphometric analyses were performed on the H&E-stained tissue. For each mouse, 10 cross-sections from the cuffed left femoral artery and the control right femoral artery were saved as digital images. For the area/volume calculations, 4 measurements for each artery section were made using an image analysis computer program (Scion Image; Scion Corporation, Frederick, MD, USA): luminal circumference, luminal area, area inside the inner elastic lamina, and area inside the outer elastic lamina. The mean vascular diameter was calculated as the luminal circumference/π. The intima was defined as the area between the lumen and the internal elastic lamina. The media was defined as the area between the internal and external elastic lamina. The volumes of the intima and media were calculated by integrating the areas over the length of the cuffed region. Thickness of the intima and the media were calculated from each volume. The raters were unaware of the genotype of the mice.

Immunohistochemistry
Immunohistochemical stains were performed using the following antisera and dilutions: anti–mouse smooth muscle actin (1:500 dilution, monoclonal mouse; Sigma Chemical Co, St Louis, MO, USA), anti-von Willebrand factor (1:3200 dilution, polyclonal rabbit; DAKO Corp, Carpinteria, CA, USA). The secondary antibodies (biotinylated anti-mouse or anti-rabbit) were applied to the avidin-peroxidase complexes (Peroxidase Vectastain Elite ABC kit, Vector Laboratories, Inc, Burlingame, CA, USA). The reaction was visualized with 3,3‘-diaminobenzidine.

Counts of the number of new vessels identified by von Willebrand factor (vWF)-positive cells in the adventitia per 0.5 mm2 were performed on 5 sections per cuffed artery equally spaced, and then averaged. The adventitia was defined as the area outside the external elastic lamina and inside the cuff.

Statistical Analysis
All values are expressed as the mean±SEM. The luminal diameter, intimal and medial thicknesses, and the ratios of the intimal to medial volumes of the 2 groups of mice studied 14 days after cuff placement were first tested by analysis of variance (ANOVA). When ANOVA demonstrated significant differences, Bonferroni/Dunn’s analysis was used posthoc to compare the groups. The luminal diameters and medial thickness of the 2 groups (cuffed arteries and contralateral control arteries) were compared using the paired Student’s t test. For all statistical analyses, a p value less than 0.05 was considered significant.

Results
Vessel Growth in p53-Deficient Mice
At the time of removal of the vessels (10–12-week-old mice), as shown in Table 1, there was no significant difference in the body weights between the wild-type and p53-deficient mice, which is consistent with previous studies in which young adult p53-deficient mice exhibited normal growth.17,18 In addition, the luminal diameter of the control femoral artery of young adult p53-deficient mice was the
same as that of the wild-type mice (Table 1). Furthermore, the luminal diameter with and without the cuff 2 weeks after cuff placement was not different between p53-deficient and wild-type mice. The intima was not present in the contralateral control femoral arteries of either the wild-type or p53-deficient mice, nor was there any difference between the 2 groups in the medial thickness. These results suggest that vessel growth is normal in p53-deficient mice up to the age of 10–12 weeks.

**Vessel Response to External Vascular Cuff Placement**

Neointima formation was present in wild-type and p53-deficient mice at 2 weeks after external vascular cuff placement and the degree was expressed as the intimal/medial volume ratio (I/M). Two weeks after cuff placement, the p53-deficient mice had greater neointima formation with an I/M of 92.9% compared with the wild-type mice with an I/M of 49.6% (p=0.001, Table 1). Cuffed intima thickness of the p53-deficient mice was greater than that of wild-type mice, but cuffed medial thickness was not significantly different between the 2 groups. Neointima formation in p53 heterozygous mice was intermediate between the wild-type and homozygous mutant mice (data not shown). Similar to the wild-type mice, p53-deficient mice did not have any visible intima at baseline, nor did their sham-operated vessels show any intima proliferation. Fig 1 shows representative sections of sham-operated and cuff-injured vessels.

**Histologic Characterization of Intimal Response**

Fig 2 shows sections of cuff-injured and sham-operated

---

**Table 1  Effect of p53 Disruption on Response to Vessel Injury**

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>p53&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>24.6±1.2</td>
<td>24.6±1.2</td>
<td>0.95</td>
</tr>
<tr>
<td>Cuff placement</td>
<td>25±1.1</td>
<td>25±1.1</td>
<td>0.67</td>
</tr>
<tr>
<td>Luminal diameter before cuff placement (µm)</td>
<td>169±5.2</td>
<td>166±2.4</td>
<td>0.62</td>
</tr>
<tr>
<td>Control luminal diameter (µm)</td>
<td>184±7.1</td>
<td>181±8.4</td>
<td>0.72</td>
</tr>
<tr>
<td>Control intimal thickness (µm)</td>
<td>5.6</td>
<td>0.5</td>
<td>0.72</td>
</tr>
<tr>
<td>Cuffed luminal diameter (µm)</td>
<td>22.0±0.6</td>
<td>21.2±1.9</td>
<td>0.72</td>
</tr>
<tr>
<td>Cuffed intimal thickness (µm)</td>
<td>182±5.3</td>
<td>177±6.3</td>
<td>0.56</td>
</tr>
<tr>
<td>Cuffed medial thickness (µm)</td>
<td>13.9±1.8</td>
<td>20.3±1.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Cuffed IM volume ratio (%)</td>
<td>19.2±0.9</td>
<td>18.8±1.8</td>
<td>0.81</td>
</tr>
</tbody>
</table>

I/M, intima to media volume ratio. Wild-type, wild-type mice; p53<sup>−/−</sup>, p53 homozygous mutant mice. Data are the mean±SEM.
control arteries from a p53-deficient male mouse. The smooth muscle cells in the media normally stain for α-smooth muscle actin (Fig 2F) and in the corresponding cuffed artery (Fig 2E), not only do cells in the media (outside the internal elastic lamina and inside the external elastic lamina), but also the majority of cells in the neointima (inside the internal elastic lamina) show this staining. On the other hand, staining for vWF occurred only in the endothelial layer in both the cuff-injured and sham-operated vessels. In the cuff-injured vessels, the vWF staining is separated from the internal elastic lamina by the neointima (Fig 2G), whereas on the sham-operated side, the vWF-stained cells directly appose the internal elastic lamina because there is no intima (Fig 2H). The endothelial layer is preserved in both the sham-operated and the cuff-injured vessels.

Adventitial Neovascularization in p53-Deficient Mice

The number of new vessels detected by vWF-positive cells in the adventitia per 0.5 mm² was 4±2 and 4±1 in wild-type and p53-deficient mice, respectively. There was no significant difference in neovascularization in the adventitia between the wild-type and p53-deficient mice (Table 2).

Discussion

The external vascular cuff-induced neointima formation model used in the present study differs from other rodent models, such as the balloon model and the filament model in several important ways. Endothelial cells are not directly injured, but the primary endpoint is neointima formation. In the cuff model, the primary endpoint is neointima formation. In the filament model, intima formation occurs to varying degrees, but the primary endpoint is an increase in medial thickness. The mechanism of intima formation after cuff injury is not well known. Based on our observations, the adventitia of cuff-injured vessels is infiltrated with inflammatory cells and this adventitial inflammation may induce neointimal formation. Three potential origins for cells in the neointima have been postulated. First, recent studies have found that adventitial passive (static) fibroblasts can become active (mobile) myofibroblasts under certain conditions such as adventitial inflammation. The myofibroblasts in the adventitia may migrate to the subendothelial space and make up the neointima in the cuff model. To our knowledge, there is no known association between myofibroblasts and p53. A second possible source for neointima cells is based upon transplanted heart studies in which vascular intima formation in the transplanted heart was found to consist mainly of cells that originated from bone marrow. Because inflammation-associated cells, such as leukocytes and macrophages, generally originate from bone marrow, it is reasonable to propose that cells in the intima in the presence of adventitial inflammation may at least partially originate from the bone marrow. In fact, the absence of p53 in macrophages leads to enhanced atherosclerosis. Finally, there is a classical hypothesis that proposes that medial smooth muscle cells change their phenotype and migrate to the subendothelial space where they proliferate, thus generating the neointima. Because VSMC migration and proliferation occur after the down-regulation of its p53, the absence of p53 could lead to the accumulation of medial smooth muscle cells in the subendothelial space. Whatever the mechanism after injury, our data indicate that a deficiency of p53 is associated with increased intima formation in response to cuff injury.

Because the cuff model preserves the endothelial cells, at least immediately after cuff placement, the initial effect of p53 on them can be examined in vivo. In humans, it has been reported that the CMV immediate early proteins upregulate endothelial p53 function and that the upregulated p53 promotes endothelial apoptosis. Another study showed that inhibition of endothelial p53 induces angiogenesis. These previous data suggest that a deficiency of p53 promotes endothelial proliferation and migration, such as in angiogenesis. In our experiment, we compared the angiogenesis in the adventitia (an area outside the external elastic lamina but inside the cuff) between wild-type and p53-deficient mice at 2 weeks after cuff placement. There was no significant difference in angiogenesis in the adventitia between the 2 types of mice. One possible explanation is that the 10–12-week-old mice are too young for studying differences in angiogenesis, because the p53-deficient mice of this age do not yet have any malignancy. The pathophysiology of endothelial p53 function is unknown except for its role in angiogenesis; however, using the cuff model without direct injury of the endothelial cells, we have for the first time demonstrated an increased neointima in association with deficiency of p53.

In summary, we have developed a cuff model of vessel injury in mice, which avoids direct injury of endothelial cells, and results in the reproducible formation of neointima in 2 weeks. Mice in which p53 is disrupted show substantially increased vascular neointima formation in response to cuff injury. This suggests that the absence of p53 in vivo results in increased vascular intima even if the endothelial cells are not directly injured. Endothelial cells lacking p53 cannot prevent increased neointima formation in response to cuff injury.

Acknowledgements

We thank Ryuichi Taki for excellent technical assistance. This work was partly supported by a grant (No.10877117) from the Japanese Ministry of Education, Science and Culture and by Project Research Grant No.12-7 of Toho University School of Medicine.

References


Table 2  p53 Disruption and Neovascularization in the Adventitia

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>p53&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>No. of vessels in the adventitia</td>
<td>4±2</td>
<td>4±1</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Counts of the number of vessels in the adventitia per 0.5 mm² were performed on sections stained by von Willebrand factor per cuffed artery equally spaced, which were then averaged. The adventitia was defined as the area outside the external elastic lamina and inside the cuff. Wild-type: wild-type mice, p53<sup>−/−</sup>: p53 homozygous mutant mice. Data are the mean±SEM.
Vascular Neointima and p53 Deficiency

153

70b: 523–526.


