Absence of p53 Leads to Accelerated Neointimal Hyperplasia After Vascular Injury

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Objective—It has been suggested that deregulated expression of the tumor suppressor protein p53 may play a role in the pathogenesis of occlusive vascular remodeling. However, the role of p53 in cell proliferation and apoptosis in vascular lesions has been controversial.

Methods and Results—We tested the potential involvement of p53-mediated molecular signaling in lesion formation using a mouse model of vascular injury that may resemble balloon angioplasty. A large wire was inserted into the femoral artery of p53+/+ and p53−/− mice. There was no significant difference in the occurrence of rapid-onset apoptosis, that is, 4 hours after injury. At 2 weeks, the number of proliferating cells in the lesion of p53−/− mice was significantly higher than that observed in p53+/+ mice. The frequency of apoptotic cells was significantly lower in p53−/− mice than in p53+/+ mice. At 4 weeks, the neointimal hyperplasia of p53−/− mice was greater than that of p53+/+ mice. There was no significant difference in the frequency of apoptosis in the lesions.

Conclusions—These results indicate a crucial role of p53 in pathological vascular remodeling after mechanical injury and provide the basis for the development of new therapies targeting p53 for a prophylactic treatment of vascular diseases.

Key Words: angioplasty ■ apoptosis ■ balloon injury ■ muscle, smooth ■ atherosclerosis

Percutaneous coronary intervention has been widely adopted as a treatment of atherosclerosis. However, in a significant number of patients, the procedure fails because of progressive vessel narrowing (post–percutaneous coronary intervention restenosis).1,2 The pathology of restenosis is heterogeneous, but abnormal hyperplasia of vascular smooth muscle cells (VSMCs) is a common feature of restenosis.3,4 Although much effort has been devoted to clarify the mechanisms that regulate VSMC accumulation, the pathogenesis remains unclear and no effective therapy has proved to limit restenosis.5

The tumor suppressor protein p53 has been postulated to play a critical role in both cell proliferation and apoptosis in many cell types.6 It was reported that p53 accumulates in VSMCs in human restenotic7 and atherosclerotic lesions.8 In vascular lesions, p53 was reported to be coexpressed with immediate-early gene products of human cytomegalovirus or MDM2, both of which are known to abolish p53 function.7–9 Thus, it was suggested that deregulated expression of p53 may play a role in the pathogenesis of human vascular diseases. This notion was supported by animal studies using local delivery of wild-type p53 gene10,11 or antisense p53 gene oligodeoxynucleotides12 into the arteries. However, untransfected bone marrow–derived cells may potentially contribute to lesion formation.13–15 Thus, the exact impact of p53 inactivation on vascular cell proliferation and apoptosis in vivo remains to be elucidated by genetic manipulation.

In this study, we used a new mouse model of acute vascular injury in p53+/+ and p53−/− mice. The endovascular injury induced marked enlargement of the artery with massive apoptosis of VSMCs, followed by luminal narrowing attributable to exuberant neointimal hyperplasia. There was no significant difference in the frequency of apoptosis. However, VSMC proliferation and the consequent neointimal hyperplasia were more prominent in p53−/− than in p53+/+ mice. Our findings provide genetic evidence that p53 may play an important role in controlling neointimal hyperplasia after mechanical vascular injury.

Methods

Animals
Eight-week-old male wild-type C57BL/6J mice were purchased from Japan SLC, Inc (Shizuoka, Japan). Eight-week-old male mice deficient for p53 (p53−/−, C57BL/6J background)16 were purchased from Jackson Laboratory (stock No. 002101, Bar Harbor, Maine). Mice were kept in microisolator cages on a 12-hour day/night cycle and fed regular chow. For all surgical procedures, the mice were anesthetized by intraperitoneal injection of 50 mg/kg Nembutal (Abbott Laboratories) diluted in 0.9% sodium chloride solution. All procedures involving experimental animals were performed in accordance with protocols approved by local institutional guidelines.
for animal care of the University of Tokyo and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

Mouse Femoral Injury Model
Surgery was carried out using a dissecting microscope (SMZ-800, Nikon). Transluminal mechanical injury of the femoral artery was induced by insertion of a large wire (0.38 mm in diameter, No. C-SF-15-15, COOK) as previously described.13,17,18 The mice were euthanized by intraperitoneal administration of an overdose of Nembutal at the time points indicated. At death, the mice were perfused with 0.9% NaCl solution followed by perfusion fixation with 4% paraformaldehyde in PBS (pH 7.4). The femoral artery was carefully excised, additionally fixed in 4% paraformaldehyde overnight at 4°C, and embedded in paraffin. Cross-sections (5 mm) were stained with H&E. Morphometric analysis was performed according to the instructions provided by the manufacturer (BrdU Staining Kit, ZYMED).

Transmission Electron Microscopy
At 2 hours after surgery, the injured and uninjured arteries were perfusion-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in PBS. The arteries were postfixed in 1% osmium tetroxide and embedded in epoxy resin (Epon 812). Thin sections were stained with 3% uranyl acetate and examined under a transmission electron microscope (H-7000, Hitachi).

Culture of Bone Marrow Cells From p53+/+ and p53−/− Mice
Total bone marrow cells were isolated from p53+/+ or p53−/− mice as described,13 and 4×10^6 cells were cultured in a well of 24-well dish in Humedia-SG media (KURABO, Osaka) supplemented with 50 ng/mL PDGF-BB. At 18 days, cells were fixed in 4% paraformaldehyde. After incubation in 0.5% NP40, cells were stained with an alkaline phosphatase–conjugated monoclonal antibody to α-smooth muscle actin (clone 1A4, Sigma) and Vector Red substrate (Vector Laboratories). The number of positive cells in a high-power field was counted.

Statistical Analysis
All data are presented as mean ± SEM. Statistical comparisons were made with ANOVA followed by the Student’s Newman-Keuls test. A value of P<0.05 was considered to be significant.

Results
Massive Apoptosis of Smooth Muscle Cells Induced by Wire Injury in p53+/+ and p53−/− Mice
We inserted a large wire into the femoral artery of p53+/+ and p53−/− mice. The wire was left in place for 1 minute to denude and dilate the artery. The injured artery was markedly enlarged with massive medial cells apoptosis (Figure 1A). As reported in balloon angioplasty models of rat carotid artery and rabbit femoral artery,19 VSMC apoptosis in the acute phase after mechanical injury was rapid and transient.17,18 At 17 hours after the injury, we seldom detected TUNEL-positive cells.17 There was no significant difference in the frequency of VSMC apoptosis between p53+/+ and p53−/−/H11001 mice.
mice (n=4 for each group) as determined by TUNEL staining on the sections harvested at 4 hours after injury (percent TUNEL-positive nuclei, 31.6±4.2% versus 29.7±6.6%). Because TUNEL staining is not specific for the detection of apoptosis, we also examined the morphology of VSMCs by electron microscopy. We did not observe any apoptotic cells in the uninjured arteries of p53−/− and p53+/− mice (Figure 1B). In the femoral arteries harvested 2 hours after the vascular injury, we readily detected typical apoptotic cells as reported in balloon-injured rat carotid artery.19 Apoptotic cells were characterized by chromatin condensation and nuclear fragmentation (Figure 1C). There was no detectable difference in the apoptotic morphology between p53−/− and p53+/− mice.

Accelerated Cellular Proliferation in p53−/− Mice
In this model, the dilated lumen gradually narrows because of neointimal hyperplasia.17 At 2 weeks after injury, proliferating cells were identified within the media and the small neointima formed on the luminal side of the internal elastic lamina as determined by BrdU incorporation into the DNA in S-phase (n=4 for each group) (Figure 2A). We found that the percentage of BrdU-positive cells in p53−/− lesions was significantly higher than that found in p53+/− lesions in neointima (55.7±10.0 versus 30.0±13.1%) and media (40.4±8.2 versus 23.0±6.4%). TUNEL staining revealed apoptotic cell death in neointima and media (Table). The number of TUNEL-positive nuclei found in p53−/− neointima was less than that observed in p53+/− lesion.

To investigate the mechanism by which the lack of p53 leads to increased cellular proliferation, we investigated the expression of cyclin-dependent kinase inhibitors, which play a pivotal role in the regulation of cellular proliferation. Immunostaining of the injured arteries harvested at 2 weeks revealed that p21WAF1 (p21) was expressed in the growing neointima. The number of p21-positive cells in p53−/− mice was significantly less than that in p53+/− mice in neointima (25.4±4.0% versus 8.3±2.4%) and media (23.6±5.2% versus 9.9±2.8%) (Figure 2B). We seldom detected p27KIP1 (p27) expression in neointimal cells of p53+/− mice.

Figure 3. Neointimal hyperplasia at 4 weeks after injury. A, Injured arteries were harvested at 4 weeks and stained with H&E. Bar=100 μm. Arrows indicate internal elastic lamina. B, Sections were stained for CD31 and α-smooth muscle actin to detect endothelial cells and smooth muscle cells, respectively. Bar=100 μm. Arrowheads indicate internal elastic lamina. C, Sections were stained with TUNEL (green) and Hoechst 33258 (blue) to detect apoptotic nuclei and all nuclei, respectively. Arrowheads indicate internal elastic lamina.
and p53−/− mice (Figure 2C), whereas p27 expression was readily detected in endothelial cells and adventitial inflammatory cells. These results suggest that p53 functions to inhibit vascular cell proliferation in the injured artery, at least in part, through induction of the p21 gene.

Enhanced Lesion Formation in p53−/− Mice
In this vascular injury model, the neointima continues to grow until 3 weeks, after when no significant change in the size of the neointima is observed.17 Concentric neointimal hyperplasia developed in the injured arteries of p53−/+ and p53−/− mice (Figure 3A). Morphological analysis revealed that the lesion of p53−/− mice was significantly larger than that of wild-type mice (intima to media ratio, 2.9±0.2 versus 1.8±0.2). In both p53−/+ and p53−/− mice, the neointima was exclusively composed of α-smooth muscle actin–positive cells (Figure 3B). The luminal side was almost completely reendothelialized, as determined by immunostaining for CD31. Consistent with our previous report17 and studies performed using clinical specimens,20,21 we detected occasional apoptosis of the smooth muscle cells in the neointima and media of the lesions (Figure 3C). There was no significant difference in the frequency of apoptosis in p53−/+ and p53−/− lesions in neointima (2.6±1.2% versus 2.0±0.9%) or in media (8.2±4.2% versus 9.8±4.4%).

Enhanced Differentiation of p53−/− Bone Marrow Cells Into α-Actin–Positive Cells
We recently reported that bone marrow–derived cells significantly contribute to neointimal formation in mouse model of vascular injury.13 To study the potential impact of genetic ablation of p53 on bone marrow–derived smooth muscle cells, we cultured bone marrow cells from p53−/+ or p53−/− mice. Bone marrow cells from p53−/− mice produced significantly more α-smooth muscle actin–positive cells than p53−/+ bone marrow cells did (23.0±4.1% versus 7.5±1.6%, P<0.05) (Figure 4).

Discussion
For a better understanding of the role of p53 in vascular diseases, p53-deficient mice have been analyzed in models of hyperlipidemia-induced atherosclerosis.22 Cross-breeding of the atherosclerosis-susceptible apolipoprotein E−/− mouse with a p53−/− mouse enhanced atherosclerotic lesion formation.23 Moreover, reconstitution of the bone marrow of APOE−/+ Leiden transgenic mice with that of p53−/− mice increased atherosclerosis.24 These results suggested that p53, particularly its expression in bone marrow–derived cells, plays an important role in regulating hyperlipidemia-induced atherosclerosis. However, the role of p53 in pathological vascular remodeling in response to mechanical injury remains to be clarified. Recently, we developed a mouse model of endovascular injury that may resemble balloon angioplasty.17 Like established models of balloon injury of rat and rabbit,19 this method induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia.17 In this study, we took advantage of this model to study the impact of genetic ablation of p53 on cellular proliferation and apoptosis, which have been implicated in pathological vascular remodeling. We found that targeted disruption of the p53 gene enhances neointimal hyperplasia. Accelerated cell proliferation in p53−/− mice was associated with impaired p21 induction and decreased occurrence of apoptotic cell death. These results suggest that p53 may play a crucial role in the pathogenesis of neointima formation after mechanical vascular injury.

The cyclin-dependent kinase inhibitor p21WAF1/CIP1 gene is transcriptionally activated by p53.6 Overexpression of p21 has been shown to inhibit VSMC proliferation in vitro and in vivo.25,26 In this study, the p53−/− mice displayed accelerated cellular proliferation 2 weeks after the vascular injury, which was correlated with reduced expression of p21. Thus, it is likely that p53 functions to inhibit cell proliferation, at least in part, through induction of the p21 gene.

Although it has been reported that VSMC apoptosis occurs in human vascular lesions20,21 and in animal models of angioplasty,19,27 the molecular mechanism of vascular cell apoptosis remains unknown28 and the role of smooth muscle cell apoptosis in vascular remodeling has been controversial.20,30 It was reported that immediate-early gene products of human cytomegalovirus inhibit VSMC apoptosis by antagonizing p53 function.9 p53 deficiency in bone marrow–derived cells decreased the percentage of TUNEL-positive cells in the lesions.24 Thus, it has been assumed that p53 functions to promote VSMC apoptosis and to inhibit their accumulation in vascular lesions.9,11,31 In this study, we found that the occurrence of apoptosis decreased in p53−/− lesions when neointima was growing. Our results suggest that p53 inhibits VSMC accumulation through proapoptotic
functions as well as through antiproliferative effects in our model of vascular injury.

We recently reported that bone marrow–derived cells significantly contribute to neointimal formation in mouse model of vascular injury. When we cultured bone marrow cells from p53+/− or p53−/− mice, we found that p53−/− bone marrow cells produced more α-smooth muscle actin–positive cells than p53+/− bone marrow cells did. It may be plausible that p53 may play a role in mobilization, differentiation, and proliferation of bone marrow–derived smooth muscle progenitor cells.

Conclusion

In conclusion, we found that loss of p53 function results in accelerated cell proliferation and enhanced lesion formation after mechanical vascular injury. Disruption of p53 may impart a proliferative advantage not only to tumor cells but also to untransformed smooth muscle cells in the vascular lesions. Our findings suggest a previously unappreciated role of p53 in the development of gene therapy or pharmacotherapeutics targeting p53 for a prophylactic treatment of postangioplasty restenosis.

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