Loss of p53 Accelerates Neointimal Lesions of Vein Bypass Grafts in Mice

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Abstract—The transcription factor p53 is essentially involved in regulation of cell death and proliferation. Recently, we have established a mouse model for vein graft arteriosclerosis by grafting autologous jugular veins or vena cava to carotid arteries. Using this model, we studied the role of p53 in the development of vein graft arteriosclerosis in p53−/− mice. Four weeks after grafting, neointimal hyperplasia of vein grafts in p53−/− mice was increased 2-fold compared with that of wild-type controls. Cell component analysis revealed that neointimal lesions in p53−/− mice consisted mainly of α-actin positive smooth muscle cells (SMCs), whereas the majority of cells in wild-type mice were MAC-1 (CD11b/18)–positive at 4 weeks. Importantly, SMC apoptosis as determined by TUNEL assay was significantly reduced in p53−/− vein grafts. TUNEL positive cells in wild-type vein grafts markedly increased from 0.5% to 6.4% of total cells 4 weeks postoperatively, but remained virtually unchanged in p53−/− grafts (0.8%). Immunofluorescence analysis revealed that increased p53 expression in neointimal SMCs of wild-type, but not p53−/−, mice coincided with oxidative DNA damage in vein grafts. Interestingly, SMCs of p53−/− mice showed increased apoptosis in response to TNFα and decreased apoptosis in response to sodium nitroprusside. Additionally, p53-deficient SMCs showed a higher rate of proliferation and migration and expressed higher levels of matrix metalloproteinases. Thus, p53 deficiency accelerates neointima formation by facilitating SMC proliferation as well as abrogating cell apoptosis. (Circ Res. 2002;90:197-204.)

Key Words: animal models ■ vein grafts ■ apoptosis ■ p53 ■ neointima hyperplasia

Autologous vein grafts are a common procedure for vascular reconstruction, but their patency rate is limited due to neointimal hyperplasia that develops rapidly in veins subject to arterial blood pressure.1 The principal cause of graft failure is obliterative stenosis of the vessel because of proliferative thickening of the intima. A hallmark of neointimal lesions is smooth muscle cell (SMC) migration/proliferation and extracellular matrix deposition.2 Recently, we established a new mouse model of vein graft arteriosclerosis, which resembles human venous bypass graft disease in many respects.3 We demonstrated that one of the initial events after grafting veins to arteries was SMC apoptosis followed by mononuclear cell infiltration and SMC proliferation.4 Thus, the balance between SMC proliferation and apoptosis could be crucial in determining lesion development in vein grafts.

The tumor suppressor gene p53 is an important transcription factor regulating cell death and proliferation. High levels of p53 cause cells either to undergo apoptosis or prolonged cell-cycle arrest. Hypoxia, serum deprivation, and genotoxic stress are known activators of p53.5 Key to the function of p53 is its ability to activate the transcription of its target genes including the cyclin-dependent kinase inhibitor p21WAF and Bax, a proapoptotic member of the Bcl-2 family.6 Colocalization of p53 with p21WAF and Bax8 in atherosclerotic plaques indicates an involvement of p53 in the control of vascular proliferation and apoptosis, respectively. Additionally, there is evidence that p53 activation upregulates the surface expression of death receptors in SMCs.9 Loss of activity of p53 has been postulated in the pathogenesis of atherosclerosis and restenosis.10,11 Similarly, apoE/p53 double knockout mice had a significant increase in cell proliferation and atherosclerotic lesions induced by hyperlipidemia.12 However, no information is available on the role of p53 in the development of vein graft disease. In the present study, we performed vein bypass grafts in p53 knockout and wild-type mice and compared neointima lesions 4 weeks after surgery. We also investigated whether accelerated proliferation or a decrease in apoptosis contributed to neointima formation in vivo. To determine the molecular mechanism of the role of p53 in SMCs, we cultivated aortic SMCs from both p53−/− and p53+/+ mice, and assessed cell proliferation/migration and apoptosis in vitro.
Materials and Methods

Mice
All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. p53−/− mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and used as breeders. The mice were maintained on a light/dark (12/12 hours) cycle at 22°C, receiving food and water ad libitum.

Polymerase Chain Reaction
Three genotypes of p53−/−, +/−, and +/+ mice were identified using Jackson Laboratory’s polymerase chain reaction protocol with a slight modification. Briefly, genomic DNA was isolated from tail tissues by enzyme digestion and precipitation. DNA (50 ng) in reaction buffer was thermocycled with 0.4 μmol/L of each primer (oIMR013 5'-CTTGGGTGGAAGGCTATTC-3', oIMR014 5'- AGGTGGAGATGACAGGAGATC-3', oIMR336 5'-ATAG- GTCCGGCGGTTCAT-3', and oIMR337 5'-CCCGAGTATCT-GGAAGACAG-3') at optimal temperature. After reaction, the mixture was electrophoresed on agarose gel. p53−/− mice have a 280-bp band, p53+/− a 600-bp band, and p53+/+ mice have both bands.

Vein Graft Procedure
The vein grafts were performed using animals of the same genotype as donors and recipients. The procedure used for vein grafts was similar to that described previously. Briefly, 3-month-old mice were anesthetized with pentobarbital sodium (50 mg/kg body weight, IP). The vena cava was harvested. The right common carotid artery was mobilized free from the bifurcation at the distal end toward the proximal, cut in the middle, and a cuff placed at the end. The cuff was made of an autoclavable nylon tube 0.63 mm in diameter outside and 0.5 mm inside (Portex Ltd). The artery was turned inside out and ligated. The vein segment wasgrafted between the 2 ends of the carotid artery by sleevving the ends of the vein over the artery-cuff and ligating them together with the 8 to 0 suture.

Histology and Lesion Quantification
For histological analysis, in vivo perfusion with 4% phosphate-buffered formaldehyde was performed, as described previously. Vein grafts were harvested by cutting the implanted segments from the native vessels at the cuff end. The grafts were dehydrated in graded ethanol baths, cleared in xylol, embedded in paraffin, and sectioned. Neointimal lesions were defined as the region between the lumen and media. For lesional area measurement, sections were reviewed using a BX60 microscope (Olympus Optical Co Ltd) equipped with a Sony 3CCD camera and television monitor. Images were first scanned, saved, and then overlaid by different staining techniques to trace the lumen and media. The lesion area was determined by subtracting the area of the lumen from the area enclosed by the line inside of the media. Cell counts in the intima and media were performed on 2 regions of each section and expressed as the number of nuclei per 100 μm of the vessel wall.

Immunofluorescent Staining
For frozen section preparation, vein grafts were harvested without perfusion and immediately frozen in liquid nitrogen. The procedure used for immunofluorescent staining was similar to that described previously. Briefly, serial 5-μm thick frozen sections were labeled with a rat monoclonal antibody against mouse MAC-1 (CD11b/18) leukocytes (PharminGen), a mouse monoclonal antibody against α-actin conjugated with Cy3 (Sigma), or a rabbit antibody against p53 (Santa Cruz Biotech). The sections were visualized with swine anti-rat or anti-rabbit Ig conjugated with FITC (Dakopatts). Semi-quantitative evaluation was performed at 400× magnification. Positive stained cells in the neointima were counted on 2 regions of each section and expressed as the range of cell number or the percentage of total nuclei.

Measurement of Oxidative DNA Damage
A specific fluorescent probe for 8-oxoguanine (DNA damage assay, Biotin) was used for direct detection of oxidized DNA. Tests were performed according to manufacturer’s instructions. Briefly, cells were fixed with 4% paraformaldehyde, then blocked and incubated with the FITC-conjugated probe for 8-oxoguanine for 1 hour at 37°C. Additionally, tissue sections were stained with dihydroethidium to detect reactive oxygen species. After washing, samples were analyzed by confocal microscopy.

TUNEL Assay
Accumulated intermolecular DNA fragments (apoptosis) were detected using an in situ apoptosis detection kit (Boehringer Mannheim, Roche) as described previously. Percentages of positive stained cells were determined by counting the numbers of labeled and total cells. Positive cells of 2 regions of each section of 4-week grafts were counted.

Annexin V/Propidium Iodide Double Staining and FACS Analysis
Annexin staining was performed according to the manufacturer’s instructions (PharMingen) and as described previously. The cellular fluorescence signal was recorded on the FL1 and FL2 channel of a FACS scan flow cytometer (Program Cell Quest, Becton Dickinson) and expressed on a logarithmic scale. After appropriate markings for negative and positive populations, the percentage of annexin V+/propidium iodide (PI)+ or V+/PI− cells was determined.

SMC Proliferation Assay
Vascular SMCs from p53−/− and p53+/+ mice were cultured from their aortas, as described elsewhere. Experiments were conducted on SMCs that had just achieved confluence. For proliferation assays, SMCs (2×10^5) cultured in 96-well plates in medium containing 10% fetal calf serum at 37°C for 24 hours were serum-starved for 2 days. Then, 5% fetal calf serum was added for 24 hours. A solution from a proliferation kit (Promega) was added 4 hours before measurement. The optical density at 490 nm was determined with a photometer.

Western Blot Analysis
The cells were washed twice with prechilled (4°C) PBS and harvested on ice in buffer A as described previously. Total SMC protein (50 to 100 μg) was separated by electrophoresis through a 7% or 15% SDS-polyacrylamide gel. The membranes were probed with antibodies to pan- or phosphorylated-ERK1/2, -JNK1/2, p53, and MMP-2 (Santa Cruz Biotech). Specific antibody-antigen complexes were detected using the ECL Western Blot Detection Kit (Amersham).

Migration Assays
Migration of SMCs was assayed by scraping off the cells on the chamber slide bottom using a rubber policeman (0.8 mm). The bottles were incubated at 37°C for 24 and 72 hours. Migration activity was represented by the closure of the wound. For migration assays using Boyden chamber, RPMI1640 medium containing 100 ng/mL of platelet-derived growth factor (PDGF)-BB was added to wells of the chamber (NeuroProbe). SMCs (2×10^5/well) in 50 μL of RPMI 1640 medium were added to the upper chambers, and incubated at 37°C for 6 hours in a cell culture incubator. SMCs on the upper side of the filter were removed and the filter was stained with Diff-Quick Staining Solution (Baxter). Migrated SMCs were then counted under the microscope.

Zymography
Zymograms from gelatinolytic activity were determined by gelatin zymography, as described previously. Ratelike tissue samples were incubated for 2 days in 2% bovine type I collagen gel. After incubation, gels were stained with Coomassie blue for 16 hours.
followed by destaining for 4 to 8 hours. Bands of lysis representing gelatinase activity were visualized against a blue background.

In Situ Zymography
MMP activity was determined by in situ zymography as described previously. Briefly, 8-μm frozen sections of 4-week vein grafts from p53+/− and p53+/+ mice on glass slides were coated with LM-1 photographic emulsion diluted 1:2 with incubation medium (50 mmol/L Tris, 50 mmol/L NaCl, 10 mmol/L CaCl2, 0.05% Brij 35, pH 7.6) and incubated overnight at 37°C. Sections were developed in the light with Kodak D-19 developer and fixed. Gelatinolytic activity was identified as white holes of lysis on a black background.

Statistical Analysis
Statistical analyses were performed on Macintosh Computer using the Mann-Whitney U-test and ANOVA, respectively. A value of P<0.05 was considered significant.

Results

Accelerated Lesions in p53-Deficient Mice
Vein grafts of wild-type mice showed neointimal hyperplasia, i.e., thickening of the vessel wall of more than 10 layers of cells 4 weeks after grafting (Figures 1a and 1b). Interestingly, a marked increase in lesion size as well as in cell density was observed in neointima of vein grafts in p53+/− mice (Figures 1c and 1d). Figure 1e summarizes data of neointima thickness as measured microscopically. The lesion area of vein grafts derived from p53+/− mice was approximately doubled compared with wild-type controls. Statistical data summarized in Figure 1e showed a significant increase in lesional areas of vein grafts in p53+/− mice. Furthermore, acellular extracellular matrix deposits were observed in neointima of wild-type (Figure 1b), but not in p53+/− mice. When cell nuclei in the lesions of grafted vessels were counted in 100-μm length, total cell numbers in 4-week grafts of p53+/− mice were markedly higher than those of wild-type animals (Figure 1f). Because p53+/− mice are susceptible to tumor development, leukemia was excluded in all animals used for the present experiments.

Cell Composition in p53+/− Vein Grafts
Figure 2a shows intensive immunostaining for MAC-1+ macrophages especially in the surface area of vein grafts from p53+/− mice, whereas α-actin+ SMCs were found in the deeper layers of the neointima. In contrast, α-actin positive SMCs were abundant in lesions of p53+/− mice (Figure 2b). Significant differences in numbers of α-actin+ SMCs were observed between p53+/− and p53+/+ mice (Figure 2c). MAC-1+ cells were the predominant cell type in wild-type lesions (55%), whereas α-actin positive SMCs constituted the majority of cells in lesions of p53+/− mice (50%). In comparison, only 20% of cells were MAC-1+ cells in p53+/− mice, and 10% were α-actin positive in wild-type mice. However, the absolute number of MAC-1+ cells in lesions of p53+/− and +/+ was at a similar level (Figure 2c, lower panel), indicating that increased SMCs in p53+/− grafts are responsible for exacerbated neointimal lesions.

p53 Expression in Vein Grafts
p53 has been shown to be highly expressed in atherosclerotic lesions, but its presence in vein grafts remains to be determined. No staining was seen in freshly harvested veins or in vein segments stained with normal rat serum as a negative control (Figure 3a). Significantly increased immunostaining was observed in neointimal SMCs 4 weeks after grafting (Figure 3b). As expected, no positive staining for p53 was detectable in the vein grafts of p53+/− mice (Figure 3c).

Because reactive oxygen species (ROS) are implicated in causing both reversible and irreversible DNA damage that is a known activator of p53, we determined the presence of ROS and DNA damage in vein graft lesions. Data shown in Figures 3e and 3f (red) demonstrated higher levels of ROS compared with the normal vessel (Figure 3d). Because the nucleotide guanine is highly susceptible to oxidative modifications, a marker of free radical-induced DNA damage, 8-oxoguanine,
was detectable in neointimal lesions of 4-week vein grafts (green; Figure 3e), but rare at 8 weeks after surgery (Figure 3f). Additionally, no staining was observed in freshly-isolated veins (Figure 3d). Thus, oxidative DNA damage was present, which may be responsible, at least in part, for p53 expression in vein grafts.

**Apoptosis in Vein Grafts**

Because p53 is a critical determinant in the regulation of cell death, we measured the rate of apoptosis in vein grafts by TUNEL staining. Similar to our previous studies, a marked increase in apoptosis was found in the neointima of wild-type mice 4 weeks after grafting (Figures 4a and 4b). Notably, neointimal lesions of p53$^{-/-}$ mice showed a lower rate of apoptosis compared with the wild-type controls, indicating p53-dependent SMC apoptosis and providing a possible explanation for the increased cell density in p53$^{-/-}$ grafts.

**Susceptibility to Cell Death in p53$^{-/-}$ and p53$^{+/+}$ SMCs**

Because of the predominance of SMCs and decreased cell death in p53$^{-/-}$ grafts, we compared the susceptibility of p53$^{-/-}$ and p53$^{+/+}$ SMCs to various cytotoxic effects, including relevant cofactors in atherogenesis, such as nitric oxide (NO) and death receptor ligand TNFα. FACS analysis of SMCs double stained with annexin and propidium iodide indicated that the main population of dying SMCs underwent apoptosis (annexin+/PI+) (Figure 4c). Surprisingly, we found a resistance of p53-deficient SMCs to NO donor sodium nitroprusside, but not death receptor ligand TNFα (Figure 4c). Because Jun kinase (JNK) can phosphorylate p53 on...
residue Ser 37 of human and Ser 35 of mouse p53, and mediate apoptosis induced by TNFα and H2O2. JNK phosphorylation or activation was monitored by Western blot analysis. No difference in JNK activation between p53+/+ and p53−/− SMCs was found after treatment with TNFα, indicating that p53 deficiency did not abrogate TNFα-induced upstream signaling in SMCs (Figure 4d). Pan-JNK proteins showed no significant changes in p53+/+ and p53−/− SMCs. These results indicate that different signal pathways are involved in TNFα- and NO-induced cell death.

**Proliferation in p53−/− SMCs**

In response to serum stimulation, SMCs from both types of mice replicated markedly (Figure 5a). However, cell numbers of p53−/− SMCs 24 hours after serum starvation were significantly higher compared with p53+/+ mice. Because ERK-mediated signal pathways are crucial in mediating cell proliferation, we measured ERK1/2 phosphorylation in both cell types. The activated (phosphorylated) forms of p42 and p44 were identified in Western blots (Figure 5b). p53−/− SMCs showed higher ERK activity under serum free conditions and enhanced ERK activation in response to PDGF and serum stimulation.

**p53-Dependent SMC Migration**

Mitogen-induced suppression of p53 precedes SMC outgrowth of the tunica media. To explore the role of p53 in SMC migration, cells were disrupted by scraping, and migration was evaluated after 1 and 3 days. p53 deficiency markedly enhanced SMC migration, resulting in faster closure of the wound (Figure 6a). Similar results were observed when both p53−/− and p53+/+ SMCs were assayed using a Boyden Chamber, a well-established method to test cell migration (Figure 6b; Reference 18). As mentioned above, a large amount of extracellular matrix deposit was observed in neointima lesions of wild-type (Figure 1b), but not in p53−/− mice. Degradation of extracellular matrix involves a family of zinc-dependent proteolytic enzymes, namely matrix metalloproteinases (MMP). To study the mechanisms by which p53 is involved in SMC migration, proteins and activity of

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**Figure 4.** Apoptosis in p53+/+ and p53−/− SMCs. a and b, TUNEL positive cells in vein grafts. Sections of 4-week grafts were stained with TUNEL as described in Materials and Methods. TUNEL positive cells and total cell nuclei were enumerated under the microscope. The percentage of TUNEL positive nuclei in 4 week grafts was compared with control veins. **Significant difference from other groups, P < 0.01 (n=8). The absolute numbers of apoptotic cells in each vein graft were shown in panel b expressed as numbers per 20× field. c, Comparison of apoptosis between p53+/+ and p53−/− SMCs. SMCs were incubated with sodium nitroprusside (100 μmol/L) or TNFα (100 ng/mL) at 37°C for 24 hours. Annexin and propidium iodide staining was performed to detect apoptosis by FACS analysis. Cellular fluorescence was recorded on a FACS scan flow cytometer and expressed on a logarithmic scale. Note the resistance for apoptosis to sodium nitroprusside in p53−/− cells, but not to TNFα. d, JNK activity in p53+/+ and p53−/− SMCs. SMCs were stimulated with TNFα (100 ng/mL) or UV (10 J/m²). The upper panel demonstrates results of Western blot analysis for JNK1/2 phosphorylation. Pan-JNK1 protein is shown in the lower panel.
MMP-2, a predominant gelatinase in freshly isolated veins, were determined. Western Blot analysis revealed a 3-fold increase in protein levels of MMP-2 in p53-deficient SMCs (Figures 6c and 6d). Increased protein expression in p53−/− SMCs was paralleled by enhanced MMP-2 activity as demonstrated by zymography in gel (Figure 6e). These results suggest that p53 regulates SMC migration, at least partially, via the regulation of MMP-2 production.

To determine MMP activities in vivo, in situ zymography was performed using frozen sections of murine vein grafts. Moderate levels of MMP activities were detected in vein grafts of wild-type animals at 4 weeks (Figure 7b), and elevated MMP activities were found in vein grafts of p53−/− mice (Figure 7d). MMP activities of both vein grafts were abolished by addition of EDTA in the reactive solution (Figures 7a and 7c), indicating a calcium dependent process.

Discussion

Previously, we reported that one of the earliest cellular events in the neointimal formation of vein bypass grafts is cell death4 that evokes inflammatory responses28 followed by SMC proliferation. In this article, by using our murine vein graft model, we demonstrate that p53 deficiency accelerates neointima lesions by facilitating migration and proliferation as well as abrogating apoptosis of SMCs. This conclusion is based on 3 observations: (1) a decrease in apoptosis may contribute to neointima formation by prolonging the life span of intimal cells. Indeed, inverse correlations were observed between the density of SMCs and the frequency of apoptotic cell death. Resistance to apoptosis may therefore, at least partially, account for differences in lesion size between vein grafts of p53−/− and p53+/+ mice. (2) Abnormal proliferation/accumulation of vascular SMCs lacking p53 activity was observed, indicating that p53 is an essential factor for controlling cell growth.29 Supporting our observations are reports that increased SMC proliferation can be achieved by antisense treatment against either p53 or Rb.11 However, cell numbers only increase if cells are transfected with antisense p53 because increased proliferation alone, as achieved by Rb inactivation, results in a simultaneous upregulation of p53 followed by Bax-mediated apoptosis.11,30 (3) p53-deficiency enhances expression and activity of MMP-2 in SMCs and their migration rate. This finding provides a possible explanation why p53 downregulation is required for SMC migration (b). A polycarbonate filter with 8-μm pores was placed between the lower and upper chambers. SMCs (2×10⁴/well) in 50 μL of RPMI 1640 medium were added to the upper chambers, PDGF-BB in the lower chamber, and incubated at 37°C for 6 hours. SMCs on the lower side of the filter were stained with a quick stain kit and counted under the microscope. Protein extracts of both cell types were analyzed for p53 and MMP-2 expression by Western blot (c). MMP-2 expression was quantified by densitometry (d). Data represent means of optical density (±SD). *Significant difference from p53−/− cells, P<0.05. MMP-2 activity was assayed in p53−/− and p53+/+ SMCs by zymography (e). Bands of lysis represent gelatinase activity. M indicates purified MMP-2 as a positive control.

Studies concerning the involvement of p53 in the vessel wall are focusing on native or hyperlipidemia-induced ath-
SMCs as well as neointimal lesions of vein grafts in p53−/− mice. Gene transfer of tissue inhibitor of MMP (TIMP-2) significantly inhibits MMP activities of vein grafts resulting in reduced lesions.22 Similarly, we found that inhibition of MMP activities of both p53−/− and p53+/+ SMCs by TIMP-2 gene transfer inhibits cell migration in vitro (C. Li, Y. Hu, Q. Xu, unpublished data, 2001). Interestingly, overexpression of wild-type p53 in cultured vein segments inhibits migration of SMCs leading to reduced intimal thickening although no effect on SMC proliferation was found.36 These results suggest that p53-regulated MMP gene expression could contribute to SMC migration during the development of neointimal lesions in vein grafts.

In summary, p53 is crucial in determining lesion development in vein bypass grafts by inducing apoptosis, limiting proliferation and migration, and enhancing matrix protein accumulation. Because cell death is an early event after vein grafting, it could be beneficial to inhibit p53 expression by gene transfer or drug treatment at this stage. Alternatively, overexpression of p53 in advanced lesions might inhibit lesion progress and increase the lesion stability via enhanced matrix protein production. To prove this hypothesis, further experiments are being carried out in our laboratory focusing on therapy for vein graft atherosclerosis.37

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