Increased p53 gene dosage reduces neointimal thickening induced by mechanical injury but has no effect on native atherosclerosis

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Objective: The tumor suppressor p53 regulates cell proliferation and apoptosis, two key processes in the pathogenesis of occlusive vascular disease. Here, we examined the consequences of heightening p53 function on neointimal lesion formation in the setting of atherosclerosis and mechanical injury.

Methods: For this study we employed immunohistopathological characterization of neointimal lesions in atherosclerosis-prone apolipoprotein E-null mice with normal p53 gene dosage (apoE-KO) and carrying a p53 transgene (Super-p53/apoE-KO). We also carried out molecular studies in macrophages and smooth muscle cells (SMCs) obtained from these mice.

Results: The p53 transgene conferred p53 gain-of-function in cultured cells and mice. In vitro, survival of irradiated Super-p53 macrophages and femoral SMCs was reduced, but only Super-p53 SMCs exhibited attenuated proliferation. In vivo, whereas the size of spontaneously formed and diet-induced aortic atheromas was indistinguishable in apoE-KO and Super-p53/apoE-KO mice, the latter exhibited attenuated neointimal thickening in mechanically injured femoral artery. In both models, neither apoptosis nor cell proliferation were affected by additional p53 gene dosage when examined in established neointimal lesions. However, at 2 days after mechanical injury when neointimal lesions were not yet formed, cell proliferation was significantly attenuated within medial SMCs of Super-p53/apoE-KO mice.

Conclusion: Heightening p53 function has differential effects on in vitro proliferation of macrophages (unaffected) versus SMCs (reduced), and on native atherosclerosis (unaffected) versus mechanically induced neointimal thickening (reduced) in apoE-KO mice. The protective effect of p53 in mechanically injured femoral artery coincided with limited medial SMC proliferation at early time points preceding neointima formation, but neither medial nor neointimal cell proliferation was affected in vessels with established occlusive lesions. These findings corroborate p53 gain-of-function as a promising therapeutic strategy to limit post-angioplasty restenosis but not native atherosclerosis.

Keywords: Atherosclerosis; Restenosis; p53; Genetically modified mice

1. Introduction

Atherosclerosis and restenosis are multifactorial processes involving complex interactions among diverse cell types [1–4]. In both diseases, excessive proliferation of vascular...
SMCs, macrophages and adventitial myofibroblasts contributes to neointimal lesion development and diminished vascular patency [5–7]. Vascular cell loss by apoptotic death, which has been documented in animal and human atherosclerosis and restenosis, also appears to be a major determinant of the size and stability of neointimal lesions [8]. Therefore, unraveling the molecular mechanisms that control neointimal cell growth and apoptosis is of utmost importance to identify therapeutic targets to limit neointimal thickening and prevent plaque rupture.

The tumor suppressor gene p53 is expressed ubiquitously in all cell types as an inactive, latent, transcription factor which undergoes activation in response to a variety of cellular insults, namely DNA damage (signaled through the ATM and Chk kinases) and oncogenic stress (signaled through the p53-stabilizing protein ARF) [9]. Transcriptionally active p53 participates in the expression of pro-apoptotic (e.g., Bax, Fas, PUMA) and anti-proliferative (e.g., p21(CIP1), GADD45) genes, and in the repression of anti-apoptotic (e.g., bcl-2) and pro-proliferative (e.g., IGF-II) genes. Whether p53 activation provokes apoptosis or growth arrest, either reversible or permanent (replicative senescence), depends on the context and cellular type. Mounting evidence has implicated p53 as a critical regulator of pathological vascular remodeling. Human vascular SMCs in restenotic lesions exhibit augmented responses to p53 [10], and arterial p53 inactivation after human cytomegalovirus infection might contribute to coronary restenosis [11,12]. Moreover, p53 is overexpressed, but not mutated, in human atherosclerotic tissue [13], and expression studies in human endarterectomy specimens suggested a role for p53 as a negative regulator of neointimal thickening [14,15]. In line with this notion, gain- and loss-of-function studies have causally linked p53 and fibro-proliferative vascular disease. First, both global and hematopoietic cell-specific p53 deficiency aggravates atheroma progression in murine models of diet-induced atherosclerosis [16–19]. Likewise, p53-null mice exhibit accelerated neointimal thickening induced by vein grafting [20], external vascular cuff placement [21], and mechanical denudation [22]. Second, intraluminal antisense p53 oligodeoxynucleotide transfection into intact rat carotid artery promotes vascular SMC growth [23], and p53 gene transfer attenuates neointimal thickening in balloon-injured rat and rabbit carotid artery [24,25], porcine interposition saphenous vein grafts [26], and organ cultures of human saphenous vein [27]. Notably, adenosine-mediated p53 transfer to pre-established atheromas induced by implantation of a perivascular collar promoted vulnerability to plaque rupture [28].

While the mentioned studies have firmly established that endogenous and ectopically expressed p53 limit neointimal thickening, the role of p53 on neointimal cell proliferation and apoptosis is controversial (see Discussion). Moreover, none of the studies examined the consequences of heightening p53 function on diet-induced atherosclerosis. We have previously engineered a p53 transgene that behaves as a functional replica of the endogenous p53 gene when expressed in p53-null mice [29]. Compared with controls, Super-p53 transgenic mice exhibited an enhanced DNA damage response and protection from cancer. Remarkably, in contrast to the early ageing phenotype observed in transgenic mice overexpressing deregulated p53 [30] or its naturally occurring N-terminally truncated p44 variant [31], Super-p53 mice age normally [29]. Thus, cancer resistance can be enhanced by a simple genetic modification and in the absence of undesirable effects. In this study, we crossed atherosclerosis-prone apoE-KO [32] and Super-p53 [29] mice to generate doubly mutant Super-p53/apoE-KO mice and apoE-KO littermates. We have examined these animals to investigate the effects of heightening p53 function in the setting of atherosclerosis and mechanical injury of the vessel wall.

2. Materials and methods

2.1. Mice and diets

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. apoE-KO (C57BL6/J, Charles River) and Super-p53 (C57BL6/J, tg/tg carrying two extra copies of the p53 transgene) [29] mice were mated. The resulting heterozygous F1 was crossed with apoE-KO mice to obtain apoE-KO mice with or without one extra p53 allele (Super-p53/apoE-KO and apoE-KO, respectively).

Mice for spontaneous atherosclerosis and mechanical injury were kept on standard chow (catalog number 2014, Harlan Interfauna, Spain). For diet-induced atherosclerosis, mice received standard chow for 2 months and then were switched to high-fat diet (12.8% fat, 1.25% cholesterol, 0.5% sodium cholate, catalog #S4892-S010, Ssniff, Germany). Blood was withdrawn from the retroorbital plexus to measure plasma cholesterol (Kit 1: Infinity Cholesterol liquid stable reagent, Iberdiagnóstica, Spain; Kit 2: Cholesterol MR, Linear Chemicals, Spain). Mice were euthanized with ether.

2.2. Immunohistopathological characterization of neointimal lesions

For atherosclerosis studies, aortas were washed in situ with PBS and fixed with freshly prepared 4% paraformaldehyde/PBS. The heart, pulmonary artery and aorta were extracted, and fixation continued for 22–26 h at 4 °C. Characterization of neointimal lesions was carried out by an investigator who was blinded to genotype. The extent of aortic atherosclerosis was determined as the intima-to-media ratio (I/M) in hematoxylin/eosin-stained 3-μm cross-sections (obtained from three different zones of the ascending aorta separated ~30 μm, starting at the end of the aortic valve), or by en face Oil Red O staining (0.2% in 80% MeOH) (SIGMA), essentially as described before [33]. Images were captured with an Olympus CAMEDIA C5060 digital camera.
mounted on a Zeiss Axiolab stereomicroscope. No differences in atheroma size were observed between males and females of the same genotype.

For the mechanical injury model, mice were anesthetized with Forane and underwent bilateral endoluminal injury to the common femoral artery by passing 3 times a 0.25 mm-diameter angioplasty guidewire as described previously [34]. Buprex was administered subcutaneously for post-operative analgesia. Seams, ulcers and scars suggesting limb ischemia were controlled daily. One apoE-KO mouse was sacrificed during the post-operative period because it developed severe foot ischemia. At 2 days and 4 weeks post-injury, mice were killed and perfused in situ with 5 mL of PBS followed by 10 mL of freshly prepared 4% paraformaldehyde/PBS using a peristaltic pump at approximately 1 mL/min. Both hind limbs and pelvis were isolated in block and fixation continued for 24–28 h. Specimens were decalcified for 24 h at room temperature with mild shaking in Osteodec (Bio-Optica). After washes with PBS, transverse segments (approximately 2-mm thick) were cut at the level of the injury, embedded in paraffin, and 5-μm cross-sections were obtained throughout the injured segment. Specimens were hydrated and images captured with a Leica TCS/SP2 confocal microscope using the 488 nm argon laser to visualize internal and external elastic lamina autofluorescence. Only sections showing both the external and internal elastic lamina were used to quantify intimal, medial and luminal areas (Leica LCS Lite software). Results for each artery represent the average of all the measurements obtained from 3–5 different injured regions.

Immunohistopathological examination of neointimal lesions included quantification of lesion cellularity (hematoxylin/eosin staining), cell proliferation (Ki67 and PCNA immunoreactivity), apoptosis (Apoptag kit, caspase 3 immunoreactivity), and macrophage content (Mac3 immunoreactivity). Details on these studies can be found in Supplemental material.

2.3. Cell culture studies

Murine femoral SMCs (FSMCs) and bone marrow-derived macrophages (BMD/macrophages) were obtained essentially as described elsewhere [35,36] (see Supplementary material).

Fig. 1. In vitro studies of p53 functionality. BMD/macrophages and FSMCs were obtained from wild-type and Super-p53 mice. (A) BMD/macrophages were untreated or subjected to UV light (20 J/m²). Results represent the percentage of sub-G0/G1 cells relative to its non-irradiated control (=100%). (B) Western blot analysis of tubulin and p21 in primary FSMCs. Cells were maintained in 20% FBS/DMEM/Fungizone. Blots are representative of two independent experiments. The numbers below the blots correspond to the p21/tubulin ratio estimated by densitometric analysis (relative to wild-type). (C) Percentage of surviving FSMCs 24 h after UV-irradiation (120 J/m²). (D) FSMCs were serum-starved and restimulated for 24 h with 10 ng/mL PDGF-BB in the presence of 50 μM BrdU.
To evaluate cellular responses to UV light, BMD/macrophages and FSMCs were irradiated at 20 J/m² and 120 J/m², respectively. BMD/macrophages were stained with propidium iodide to determine the percentage of sub-G0/G1 cells (FACSCanto flow cytometer, Becton Dickinson), and FSMCs were counted 24 h after irradiation to quantify cell survival.

5′-bromodeoxyuridine (BrdU) incorporation was used for cell proliferation studies. Serum-starved FSMCs (0.5% FBS/DMEM/Fungizone, 3 days) were restimulated for 24 h with 10 ng/mL PDGF-BB in the presence of 50 μM BrdU. BMD/macrophages differentiated on DMEM containing 10% FBS/10% L929 cell-conditioned medium [36] were switched for 6 h to 10% FBS with or without 0.5 μg/mL LPS in the presence of 50 μM BrdU. Cells were fixed with 4% paraformaldehyde/PBS, washed with PBS and incubated for 30 min with 0.5% Triton X100/2N HCl. Cells were washed first with PBS and then with 122 mM Na₂B₄O₇/32 mM KH₂PO₄. After 30 min incubation with 3% H₂O₂/10% methanol to inactivate endogenous peroxidase, cells were blocked with 5% horse serum and BrdU incorporation was visualized using anti-BrdU-biotinylated antibody (Zymed, 1/50, 1 h, room temperature) and diaminobenzidine substrate kit (Serotec).

2.4. Western blot analysis

FSMC whole lysates were prepared in ice-cold 50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 10 mM β-glycerophosphate, 10% glycerol, 0.1% Tween 20, 0.1 mM NaVO₃, 1 mM DTT, supplemented with protease inhibitor Complete Mini cocktail (Roche Diagnostics). Lysates were centrifuged at 14,000 rpm for 20 min (4 °C) and protein concentration in the supernatants was determined using the Bradford assay (BioRad Laboratories). Proteins (50 μg) were separated onto 15% SDS-polyacrylamide gels and Western blot analysis was carried out using anti-p21 (1/200, WA-1 clone, ref. 11-338, EXBIO) and anti-tubulin (1/200, sc-8035, Santa Cruz) antibodies. Relative protein level was determined by densitometry using the MetaMorph software (Universal Imaging Corporation).

2.5. Statistical analysis

Results are reported as mean±SEM. In experiments with two groups, differences were evaluated using a 2-tail, unpaired t-test. Otherwise, differences were evaluated using ANOVA and Fisher’s post-hoc test (Statview, SAS institute, Fig. 2).
3. Results

3.1. p53 transgene functionality

We first examined the functionality of the p53 transgene in BMD/macrophages and FMSCs in culture. Both when examined at 8 and 16 h post-UV irradiation, Super-p53 BMD/macrophages elicited a stronger apoptotic response than control cells from apoE-KO mice (Fig. 1A). Consistent with the notion that p21 is a direct transcriptional target of p53, its expression was increased by ∼2-fold in Super-p53 FSMC cultures compared with wild-type controls (Fig. 1B). Moreover, both cell survival after UV irradiation (Fig. 1C) and PDGF-BB-dependent proliferation were significantly reduced in Super-p53 FSMCs (Fig. 1D). Thus, in line with previous studies [29], Super-p53 macrophages and FMSCs harbouring one extra p53 allele exhibit p53 gain-of-function.

3.2. Heightening p53 function has no effect on the extent of diet-induced and spontaneous atherosclerosis

The apoE-KO mouse spontaneously develops hypercholesterolemia and complex atherosclerotic lesions and these processes can be accelerated by a high-fat cholesterol-rich diet [32]. We first compared atherosclerotic lesion size in apoE-KO and Super-p53/apoE-KO mice fed an atherogenic diet for 7 and 5 weeks (Figs. 2 and 3, respectively). The severity of hypercholesterolemia induced by fat feeding was not affected by the p53 transgene (Figs. 2A, 3A). Likewise, body weight was indistinguishable in apoE-KO and Super-p53/apoE-KO mice under all conditions studied (Figs. 2B, 3B). As shown in Fig. 2C, quantification of the I/M in cross-sections from three different zones (I, II, III) of the ascending aorta of mice fed the fat diet for 7 weeks revealed diminished atherosclerosis as specimens were farther from the aortic valve (I/M_I > I/M_II > I/M_III). However, no statistically significant differences were observed in none of the regions under study when comparing apoE-KO and Super-p53/apoE-KO mice. Examination of the pulmonary artery in the same

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Fig. 3. Gain of p53 function does not affect the size of diet-induced and spontaneously formed atheromas within the aortic arch and thoracic aorta. (A–C) Two-month-old mice received the atherogenic diet for 5 weeks. (A) Plasma cholesterol level was measured using kit 2 in 3 females/3 males of each genotype. *, p<0.0001 versus pre-diet same genotype. Differences between genotypes were not significant. (B) Body weight was not significantly affected by dietary regimen or genotype (apoE-KO: 8 females/4 males; Super-p53/apoE-KO: 11 females/9 males). (C) Atherosclerosis was quantified in whole mounted aorta stained with Oil Red O as the ratio of total lesion area (red staining) versus total area. (D) Spontaneous atherosclerosis in 6-month-old mice was quantified as in C. A representative aorta from each gender and genotype is shown in C and D. The discontinuous black lines across photomicrographs mark the separation between aortic arch and thoracic aorta. The discontinuous black lines in the graphs indicate the mean relative lesion size for each group.
specimens also revealed similar I/M in both groups of mice (not shown). Likewise, Oil Red O staining of whole-mounted aorta revealed no statistically significant differences in atheroma extent within the aortic arch and thoracic regions of apoE-KO and Super-p53/apoE-KO mice fed the high-fat diet for 5 weeks (Fig. 3C).

We also analyzed the development of spontaneous atherosclerosis in a group of 6-month-old mice that had been always fed control chow. In agreement with our results in fat-fed mice, plasma cholesterol (not shown) and the size of atheromas within the aortic arch and thoracic aorta were indistinguishable between apoE-KO and Super-p53/apoE-

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**Fig. 4.** Gain of p53 function reduces neointimal thickening in mechanically injured femoral arteries. Four five-month-old female mice underwent bilateral endoluminal injury to the common femoral artery and were sacrificed 4 weeks post-surgery. Images of femoral artery cross-sections were captured with a confocal microscope to determine the I/M ratio and percentage of luminal stenosis. Only sections showing both the external (arrow) and internal (arrowhead) elastic lamina were analyzed. The photomicrographs show representative specimens.

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**Fig. 5.** Gain of p53 function does not affect proliferation, apoptosis, cellularity, and macrophage content in aortic atheromas induced by high-fat feeding. Mice received the atherogenic diet for 7 weeks. Studies were carried out in cross-sections from region I of the ascending aorta (see Fig. 2C). (A) Cellular proliferation, estimated as Ki67 immunoreactivity. (B) Apoptosis, determined with the Apoptag kit. (C) Lesion cellularity determined in hematoxylin/eosin-stained cross-sections. (D) Macrophage content, determined as Mac-3 immunoreactivity.
KO mice (Fig. 3D). Likewise, analysis of 5-month-old mice revealed no differences in spontaneously formed aortic atheromas (not shown). Collectively, these studies demonstrate that increasing p53 function does not attenuate neither diet-induced (up to 7 weeks of fat feeding) nor spontaneously formed atheromas in apoE-KO mice.

3.3. Neointimal thickening is reduced in mechanically injured femoral artery of Super-p53/apoE-KO mice

Using a femoral artery model of endoluminal mechanical injury [34], we found that both the I/M and the percentage of luminal stenosis were significantly attenuated in Super-p53/apoE-KO compared to apoE-KO mice when these parameters were examined 4 weeks post-injury (Fig. 4).

3.4. Immunohistopathological characterization of neointimal lesions

Neointimal cell proliferation and apoptotic cell death are a hallmark of atherosclerosis and restenosis [8]. The net balance between these antagonistic processes is a major determinant of cell number within the neointimal lesion, thus influencing its size, composition and stability. Since p53 is a key regulator of cell proliferation and apoptosis, we sought to examine these processes in the arterial wall of apoE-KO and Super-p53/apoE-KO mice.

For diet-induced atherosclerosis, cross-sections of the ascending aorta from mice fed the high-fat diet for 7 weeks were examined for Ki67 immunoreactivity and the Apoptag kit to estimate cell proliferation and apoptosis, respectively. Neointimal proliferation (Fig. 5A) and apoptosis (Fig. 5B) were unaffected by the p53 transgene. Comparatively, Ki67 immunoreactivity and apoptosis were very scant within the media (not shown). Neointimal cellularity (Fig. 5C) and macrophage content (Fig. 5D) were also similar when comparing atheromas from fat-fed apoE-KO and Super-p53/apoE-KO mice.

Cellular proliferation in mechanically injured femoral arteries of apoE-KO and Super-p53/apoE-KO mice was examined by PCNA-immunostaining because the decalcification protocol employed for tissue sectioning drastically hindered Ki67 immunoreactivity. We examined post-injury time points preceding neointimal thickening (2 days, not shown) and when neointimal lesions were prominent (4 weeks, confer Fig. 4). At 2 days, the media consisted mostly of smooth muscle α-actin-immunoreactive cells in both groups of mice and macrophage-specific Mac-3 protein was undetectable (not shown). Whereas medial PCNA immunoreactivity was significantly reduced in Super-p53/apoE-KO mice, the percentage of luminal stenosis was similar in both groups (Fig. 4).

Fig. 6. Effect of extra p53 dosage on arterial cell proliferation and apoptosis in mechanically injured femoral artery. Mice were subjected to femoral artery denudation and cross-sections were analyzed 2 days and 4 weeks post-injury. Proliferation was assessed by PCNA immunostaining (A, C) and apoptosis by caspase-3 immunostaining (B) or Apoptag kit (D).
apoE-KO mice (Fig. 6A), no significant differences were seen in the number of apoptotic cells (Fig. 6B). At 4 weeks post-injury, medial and neointimal proliferation (Fig. 6C) and apoptosis (Fig. 6D) were unaffected by the p53 transgene.

4. Discussion

Studies in p53-null mice have demonstrated that endogenous p53 expression limits neointimal thickening induced by high-fat feeding [16–19], vein grafting [20], perivascular cuff placement [21], and mechanical denudation [22]. In line with these findings, transient p53 gene transfer attenuated neointimal lesion formation in balloon-injured rat and rabbit carotid artery [24,25], in porcine interposition vein grafts [26], and in human saphenous vein organ cultures [27]. Taking advantage of the availability of atherosclerosis-prone apoE-KO mice [32] and Super-p53 transgenic mice carrying one additional p53 allele that reproduces the normal expression and regulation of the endogenous p53 gene [29], here we assessed whether heightening p53 function can reduce atherosclerosis and mechanically induced neointimal thickening. We observed increased p53 function in Super-p53 cells in culture. First, apoptosis was augmented in UV-irradiated macrophages harboring the p53 transgene (Fig. 1A). Second, primary cultures of Super-p53 FS-MCs exhibited increased expression of p21 (Fig. 1B), a direct target of p53, diminished cell survival after UV-irradiation (Fig. 1C), and reduced PDGF-BB-induced proliferation (Fig. 1D). However, heightening p53 function did not attenuate atherosclerosis within the ascending aorta, aortic arch, thoracic aorta and pulmonary artery of mice fed the high-fat diet for 5 and 7 weeks (Figs. 2C, 3C, and data not shown). Likewise, spontaneously formed atherosclerotic lesions exhibited similar size in apoE-KO and Super-p53/apoE-KO mice fed standard chow (Fig. 3D). In contrast, neointimal thickening was attenuated in mechanically injured femoral arteries of Super-p53/apoE-KO compared with apoE-KO mice (Fig. 4). Likewise, Super-p53 mice are significantly protected from cancer [29]. Thus, p53 gain-of-function attenuates the progression of highly proliferative disorders (e.g., cancer and mechanically induced neointimal hyperplasia), yet it is ineffective against atherosclerosis, a disease characterized by lower proliferative activity compared with restenosis. Of note in this regard, other significant differences exist in the mechanisms underlying neointimal formation in the setting of mechanically induced vascular injury and native atherosclerosis. For instance, it would be interesting to assess whether p53 gain-of-function affects differentially the production of cytokines and growth factors by de-differentiated SMCs in atherosclerotic lesions and mechanically injured vessels.

Unbalanced cell proliferation and/or apoptosis may underlie the consequences of altering p53 homeostasis on mechanically induced neointimal thickening. Adenovirus-mediated p53 gene transfer to the rat carotid artery promotes medial cell apoptosis 2 days post-angioplasty before neointimal lesions are formed [37]. On the other hand, p53-null mice display increased proliferation and decreased apoptosis in both medial and neointimal femoral artery cells at 2 weeks post-injury, but rapid (4 h post-injury) and late (4 weeks post-injury) apoptosis were unaffected as compared to wild-type controls [22]. We found significantly reduced medial cell proliferation in femoral arteries of Super-p53-apoE-KO mice compared with apoE-KO controls at 2 days post-injury (Fig. 6A); however, the number of medial apoptotic cells was similar in both groups (Fig. 6B). At 4 weeks post-injury, we found no differences in neointimal and medial cell proliferation and apoptosis when comparing apoE-KO and Super-p53/apoE-KO mice (Fig. 6C, D). Thus, inhibition of medial cell proliferation at early time points after mechanical injury may contribute to reduced neointimal thickening in the femoral artery of Super-p53/apoE-KO mice.

Studies in apoE-KO, low-density lipoprotein receptor (LDLR)-KO and apoE*3-Leiden transgenic mice have shown that global and macrophage-specific p53 deficiency accelerates diet-induced aortic atherosclerosis [16–18]. Subsequently, Mercer et al. [19] found that p53 genetic disruption aggravates atherosclerosis in the aorta but not in the brachiocephalic artery of apoE-KO mice; however, transplant of p53 wild-type/apoE-KO bone marrow to p53/AIDS doubly deficient mice reduced aortic plaque formation in brachiocephalic plaques [19]. Collectively, these studies demonstrate site and cell-type specificity in the effects of p53 on atheroma progression. We found no statistically significant differences in the size of diet-induced (5 and 7 weeks of high-fat feeding) and spontaneously formed aortic atheromas in Super-p53/apoE-KO compared with apoE-KO mice (Figs. 2C, 3C, D). This lack of atheroprotection coincided with similar proliferative and apoptotic activity, cellularity and macrophage content in established atheromas of fat-fed mice of both genotypes (Fig. 5). Given that macrophages are the predominant cell type in atherosclerotic lesions of apoE-KO mice, it is noteworthy that de novo DNA synthesis was undistinguishable in cultures of mitogen-induced wild-type and Super-p53 BMD/macrophages (Fig. S1A). On the other hand, uptake of modified LDLs by p53-null [19] and Super-p53 (Fig. S1B) peritoneal macrophages was unaffected compared to wild-type controls. Thus, two key events during atherosclerosis in apoE-KO mice (e.g., macrophage proliferation and LDL uptake) seem to be independent of p53 expression. It remains to be determined whether p53 plays any role on additional cellular processes which modulate atherosclerosis development (e.g., leukocyte recruitment, extracellular matrix formation, production of pro- and anti-inflammatory cytokines/chemokines). The role of p53 in plaque stability also remains uncertain. On the one hand, transplantation of p53-deficient bone marrow into apoE3-Leiden [17] or LDLR-KO [18] mice led to higher aortic necrotic index and diminished aortic neointimal collagen content, respectively, suggesting that macrophage-specific p53 disruption promotes plaque instability. However, in
apoE-KO mice, transluminal adenovirus-mediated p53 gene transfer into carotid artery atheromas induced by perivascular collar placement also led to more vulnerable plaques, as revealed by lower collagen content [28]. We found no statistically significant differences in collagen content when comparing aortic atheromas and mechanically injured femoral arteries of apoE-KO and Super-p53/apoE-KO mice (Fig. S2). Additional studies are thus warranted to elucidate the mechanisms by which p53 loss- and gain-of-function affect differently the stability of neointimal lesions in various vascular beds subjected to distinct forms of damage (e.g., hypercholesterolemia, perivascular collar deployment, mechanical denudation).

The role of endogenous p53 in the control of neointimal proliferation and apoptosis in the context of murine atherosclerosis has hitherto remained subject to controversy. Two studies suggest that p53 deficiency leads to increased neointimal proliferation without significantly affecting apoptosis (although a tendency towards more apoptosis was seen in one study) [16,18], and a third study disclosed a tendency towards reduced apoptosis but unaffected cell proliferation [17]. None of these studies identified the lineage of the growing/dying cells. Subsequently, Mercer et al. [19] reported that p53 disruption in brachiocephalic plaques of apoE-KO mice significantly increases neointimal cell proliferation and reduces apoptosis. Although most neointimal proliferating cells were monocyte/macrophages (92–97%), neointimal apoptotic cells were both macrophages (75%) and SMCs (25%). In tissue culture experiments, these authors observed that endogenous p53 promotes peritoneal macrophage apoptosis but attenuates apoptotic death of aortic SMCs and bone marrow stromal cell, which can transdifferentiate into cells expressing SMC-specific makers [38]. We found reduced viability of in vitro UV-irradiated BMD/macrophages (Fig. 1A) and FSMCs (Fig. 1C) carrying the p53 transgene. However, p53 gain-of-function appears to have cell-type specific effects on proliferation, since BrdU incorporation was reduced in Super-p53 FMSCs (Fig. 1D) but not in Super-p53 BMD/macrophages (Fig. S1A). Thus, the consequences of both p53 disruption and gain-of-function on neointimal cell proliferation and cell death seem to depend upon the cell type and vascular bed under study.

In summary, we have demonstrated that, in the absence of undesirable effects, increased p53 gene dosage in apoE-KO mice reduces neointimal thickening induced by mechanical vessel denudation. In contrast, this genetic manipulation did not affect either diet-induced or spontaneous atherosclerosis in several vascular beds (e.g., ascending aorta, aortic arch, thoracic aorta, and pulmonary artery). It remains to be determined whether heightening p53 function beyond that achieved in Super-p53/apoE-KO would protect from atherosclerosis. This could be achieved by generating apoE-KO mice carrying ≥2 additional p53 alleles, or inhibiting the Mdm2/p53 interaction with “AP peptide”, nutlins, RITA, or inhibitors of the E3 ubiquitin ligase activity of Mdm2, a negative regulator of p53 that can both prevent effective p53-dependent transcriptional activation and target it for proteasomal degradation [9,39]. However, the potential benefits of these strategies may be hindered by noxious effects. First, transgenic mice with hyperactivated p53, or its naturally occurring N-terminal truncated isoform p44, exhibit symptoms of premature ageing (e.g., reduced longevity, osteoporosis, generalized organ atrophy and a diminished stress tolerance) [30,31], perhaps because p53 in these mice has an altered transcriptional program rather than an overall increase in activity [9,39]. Second, indiscriminate p53 activation in normal cells upon drug-based Mdm2 inactivation may lead to fatal pathologies, as shown in Mdm2-null mice [40]. Whilst these potential side effects might preclude the use of prophylactic measures based on p53 hyperactivation for the treatment of atherosclerosis, a chronic disease that requires long-term treatment, restenosis might be amenable for these strategies because treatment might be limited to the first few months after revascularization when neointimal hyperplasia typically occurs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.05.002.

References


