Chapter 2

Macrophage p53 and atherosclerosis

Macrophage p53 controls foam cell death in atherosclerotic lesions of apolipoprotein E deficient mice

Lianne S.M. Boesten[1,5]; A. Susanne M. Zadelaar [2,5]; Anita van Nieuwkoop[1,5]; Lihui Hu[1]; Amina F.A.S. Teunisse[3]; Aart G. Jochemsen[3]; Bastiaan Evers[6]; Marion J.J. Gijbels[7,8]; Bart J.M. van Vlijmen[4]; Louis M. Havekes[1,2,5]; Menno P.J. de Winther[7]

[1]Dept. of General Internal Medicine, [2]Dept. of Cardiology, [3]Dept. of Molecular and Cell Biology, [4]Hemostasis and Thrombosis Research Center, Dept. of Hematology, Leiden University Medical Center, Leiden, The Netherlands, [5]TNO-Quality of Life/ Gaubius Laboratory, Leiden The Netherlands, [6]The Netherlands Cancer Institute, Amsterdam, The Netherlands, [7]Department of Molecular Genetics, Cardiovascular Research Institute Maastricht, [8]Department of Pathology, Maastricht University, Maastricht, The Netherlands

ABSTRACT

The cellular composition of an atherosclerotic lesion is determined by many factors including cell infiltration, proliferation and cell death, either by apoptosis or necrosis. The tumor suppressor gene p53 has been shown to regulate both cell proliferation and cell death in many cell types. To study the role of macrophage p53 in the development of atherosclerosis, we generated apoE-deficient mice with a macrophage-restricted deletion of p53 (LysMCre^{+} p53$^{loxP/loxP}$ apoE$^{-/-}$ mice) and control littermates (p53$^{loxP/loxP}$ apoE$^{-/-}$ mice) and analyzed early and advanced atherosclerosis development. Absence of macrophage p53 did not affect lesion area in both early and advanced atherosclerosis, neither in the aortic root nor in the aortic arch and thoracic aorta. In early atherosclerosis, absence of macrophage p53, resulted in reduced apoptosis (-59%), however without changes in lesion composition. In contrast, in advanced atherosclerosis, reduced apoptosis (-37%) upon absence of macrophage p53, coincided with increased necrotic death (+96%), increased foam cell content (+24%), and reduced lipid core formation (-41%). Proliferation was not affected by the absence of macrophage p53 in both early and advanced atherosclerosis. Hence, our data point towards an important role for macrophage p53 in induction of foam cell apoptosis and prevention of lesional necrosis.

Atherosclerosis is an inflammatory disease of the large vessels in which macrophages play a central role.[1,2] Accumulation of macrophage foam cells in the vessel wall results in the formation of fatty streaks. These lesions may be reversible and may not cause clinical symptoms. However, macrophage accumulation within the arterial intima sets the stage for progression of the atheroma and evolution into more complicated lesions that can cause clinical symptoms by eventual rupture or erosion of the plaque.[3] Changes in the cellular composition of an atherosclerotic lesion are important in modulating the risk of acute coronary syndromes. Cell proliferation and cell death are crucial processes in regulating cell numbers in the atherosclerotic lesion and may thereby directly influence lesion composition and stability.[4]

The p53 tumor suppressor protein is an essential gene in cell proliferation and cell death and plays a pivotal role in the cellular response to a range of environmental and intracellular stress signals.[5] Mutations in p53 occur in about half of the human cancers, resulting in loss of apoptotic function. P53 is a potent transcription factor, predominantly acting in the G1 phase of cell cycle progression, regulating multiple downstream genes implicated in cell cycle control, apoptosis, differentiation, and senescence.[6,7] In atherosclerosis, p53 was immunohistochemically visualized in human carotid atheromatous lesions in virtually all cell types (macrophages, smooth muscle cells, endothelial cells).[8] Recent mouse studies demonstrated that p53 plays an important role in the progression of atherosclerotic lesions. Whole body p53 inactivation in apolipoprotein E-deficient ($apoE^{-/-}$) mice accelerated atherosclerosis primarily by increased cellular proliferation.[9] In addition, using bone marrow transplantation in both apoE*3-Leiden[10] and LDL receptor deficient ($LDLR^{-/-}$)[11] mice it was shown that p53 of hematopoietic origin is, at least in part, responsible for the inhibition of atherogenesis in these models. Hence, these studies show an important role for both macrophage- and lymphocyte-derived p53 (or in addition: their interplay) in the development of atherosclerosis.

To distinguish the effects of p53 deficiency specifically in macrophages from processes affected by lymphocyte-p53 deficiency, we employed a conditional deletion approach using the Cre-loxP system. Macrophage specific p53 deletion was accomplished by combining mice carrying a p53 allele that was flanked by loxP sites[12] with LysMCre mice.[13] Expression of Cre in the myeloid lineage in the LysMCre mice will result in cell specific deletion of p53 and thereby give macrophages that lack p53. Using this approach, we found that absence of macrophage p53 did not affect lesion area in both early and advanced atherosclerosis, neither in the aortic root nor in the aortic arch and thoracic aorta. In early atherosclerosis, absence of macrophage p53 resulted in reduced apoptosis, however without changes in lesion composition. In contrast, in advanced atherosclerosis, reduced apoptosis due to absence of macrophage p53, coincided with increased necrotic death, increased foam cell content and reduced lipid core formation. These studies indicate that macrophage p53 is primarily involved in determining atherosclerotic lesion composition by controlling the balance of lesional apoptosis and necrosis.

METHODS

Mice and diet

The experimental animals were obtained by combining mice carrying the floxed p53 gene[12] with LysMCre mice[13] (a generous gift from Dr. B.E. Clausen, AMC, The Netherlands and Dr. I. Forster, University of Cologne, Germany), and apoE-deficient mice[14] resulting in mice that are homozygously floxed for p53 and deficient for apoE and that either express Cre in their macrophages (LysMCre^{+} p53$^{loxP/loxP}$ apo E$^{-/-}$ further referred to as p53^{del}) or do not express Cre and remain wildtype for p53 (p53$^{loxP/loxP}$ apoE$^{-/-}$ further referred to as p53^{fl}). Mice were genotyped by polymerase chain reaction (PCR) for LysMCre,[13] p53$^{loxP/loxP}$,[12] and apoE[14] status. For experiments, 8 week old male p53^{del} and littermate control p53^{fl} mice were used. Mice were fed a semi-synthetic cholesterol-rich diet composed essentially according to Nishina et al.[15] supplemented with cocoa butter (15%, by weight) and cholesterol (0.25%, by weight), without cholate (Hope Farms, Woerden, The Netherlands). The animals were fed the cholesterol-rich diet for either 7 weeks (early atherosclerosis development, n=18 and n=21 for p53^{del} and p53^{fl} mice, respectively) or 11 weeks (advanced atherosclerosis development, n=17 and n=15 for p53^{del} and p53^{fl} mice, respectively). Mice were given food and water ad libitum. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government.

Quantification of macrophage p53 by Western blotting

Peritoneal macrophages were obtained from p53^{del} and p53^{fl} mice four days after intraperitoneal injection of 1 ml thioglycollate broth (3% wt/vol.) by flushing the peritoneum with 10 ml ice-cold PBS. Macrophages were washed with RPMI 1640 containing 10% foetal calf serum and the cells of each mouse were subsequently divided over two 6-cm culture plates. After 2 hours the duplicate dishes were either mock-treated or incubated with a combination of etoposide (20 μM, Sigma Aldrich) and proteasome inhibitor MG132 (20 μM, Sigma Aldrich) for 2 hours. Subsequently, non-adherent cells were removed by washing twice with ice-cold PBS, and cells were harvested in Giordano buffer ((50 mM Tris HCl, pH 7.4; 250 mM NaCl; 0.1% Triton X-100; 5 mM EDTA), supplemented with protease inhibitors) and analyzed by Western blot as described previously.[16] Lysates from mouse embryo fibroblast derived from wild type mice or homozygous p53/mdm2-deficient double knock out (DKO) mice were used as positive and negative controls, respectively. Blots were incubated with anti-p53 (Ab-7; Merck Biochemicals) and after stripping with anti-αTubulin (CloneDM1A; Sigma-Aldrich) as loading control. Protein bands were detected by enhanced chemiluminescence with the use of the SuperSignal West-Dura kit (Pierce), visualized by autoradiography or by imaging with the ChemiGenius XE3 (Syngene, Cambridge, UK). Quantification of the p53 and tubulin protein levels was performed with use of the Syngene GeneTools software.

Blood sampling and analysis

Blood samples were collected, after 4 hours fasting, in EDTA-coated vials (Sarstedt,

Nümbrecht, Germany) by bleeding from the tail vein. Plasma cholesterol and triglyceride levels were measured enzymatically using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany). Total blood leukocyte ($CD45^+$), T-cell ($CD3^+$), B-cell ($CD19^+$) and myeloid ($CD11b^+$) numbers were determined by FACS analysis (FACSCalibur, BD Biosciences, California, USA) following standard protocol (TruCOUNT, BD Biosciences, California, USA), as described before.[17]

Atherosclerosis analysis

After either 7 or 11 weeks on a cholesterol-rich diet mice were sacrificed. Hearts were overnight fixed in formalin (pH 7.4) and embedded in paraffin. The aorta was snap-frozen and stored at -80°C. To quantify cross-sectional lesion area in the aortic root formalin-fixed hearts were processed as described before.[10,18] Sections of the aortic root were routinely stained with hematoxylin-phloxine-saffran (HPS) for morphometric analysis. Areas were determined using Leica Qwin image software (EIS, Asbury, NJ).

For *en face* analysis of lesion area in the aortic arch and thoracic aorta, the aorta was cleaned in situ from periadventitial tissue, dissected from the aortic arch down to the iliac bifurcation, opened longitudinally, pinned on a silicone basement and stained with Oil-Red-O. The percentage of surface area covered by atherosclerotic lesions (Oil-Red-O positive area) was quantified starting from the top of the aortic arch 1 cm down towards the thoracic aorta by computer-assisted analysis[19] (n=9 vs. n=10 for early atherosclerosis and n=9 vs. n=9 for advanced atherosclerosis for $p53^{del}$ and $p53^{fl}$ mice, respectively). All analyses were performed double blindly without prior knowledge of the genotype.

Lesion composition analysis

For compositional analysis of the lesions lipid core, necrosis and macrophage content were determined. Lipid core area was defined by the presence of cholesterol clefts, extracellular lipids and the complete absence of nuclei. In addition, necrosis was defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei.[20] Lipid core area and necrosis area were measured using morphometric analysis, as described above. Serial sections were stained for macrophages using a rabbit antibody to mouse macrophages (AIA-312040, 1/1500, Accurate Chemical and Scientific). AIA-312040-positive areas were quantified as described before.[17]

To label DNA-synthesizing cells the mice received 5'-Bromo-2'-Deoxyuridine (BrdU, Sigma; 60 mg/kg, intraperitoneally) for 3 consecutive days prior to sacrification. Sections were stained for proliferation using a monoclonal mouse anti-BrdU antibody (DAKO A/S Denmark) and for apoptosis using the TUNEL technique according to manufacturer's protocol (In situ cell detection kit POD, Roche Diagnostics GmbH, Mannheim, Germany). Numbers of BrdU- or TUNEL-positive nuclei were expressed per total atherosclerotic lesion area, corrected for lipid core area, as the lipid core is an acellular area of the atherosclerotic lesion without nuclei and therefore by definition does not contain any (BrdU- or TUNEL- positive) nuclei.

Statistical analysis

Statistical analyses were performed using Graphpad Prism 4.03. All data groups were

first tested for normality. If the data were distributed normally, groups were compared using Welch's corrected t-test. If data were not distributed normally, groups were compared using Mann-Whitney rank sum test. Data are expressed as mean±SD. P-value < 0.05 was regarded as significant.

RESULTS

General characteristics of apoE-deficient $p53^{del}$ mice

Littermate male $apoE^{-/-}$ mice either lacking p53 in their macrophages ($p53^{del}$) or wildtype for p53 ($p53^{fl}$) were fed a cholesterol-rich diet for 7 weeks (early atherosclerosis) or 11 weeks (advanced atherosclerosis). During the study, the mice appeared healthy and displayed no signs of abnormalities. As shown in Table 1, both after 7 and 11 weeks of a cholesterol-rich diet challenge, mean body weight was not different between $p53^{del}$ and $p53^{fl}$ mice. Plasma cholesterol, triglyceride and hematocrite levels (Table 1) and lipoprotein profiles (data not shown) did not differ between $p53^{del}$ and $p53^{fl}$ mice. Moreover, absence of macrophage p53 did not affect circulating T-cell, B-cell or myeloid cell concentrations (Table 1) as analyzed after 11 weeks feeding a cholesterol-rich diet.

Table 1. General characteristics of male $p53^{del}$ and $p53^{fl}$ mice after feeding a cholesterol-rich diet for 7 weeks (early atherosclerosis development) or 11 weeks (advanced atherosclerosis development)

		$p53^{fl}$	$p53^{del}$	$p53^{fl}$	$p53^{del}$
Atherosclerosis development		Early		Advanced	
Weight (g)		29.5±2.8	29.8±3.0	29.4±3.6	28.3±2.2
Plasma lipid levels (mmol/L)	Cholesterol	31.7±8.0	28.4±7.4	47.0±16.3	39.9±16.4
	Triglycerides	1.3±0.6	1.2±0.6	1.1±0.4	1.1±0.6
Hematocrite		0.47±0.02	0.47±0.02	0.47±0.02	0.47±0.04
Blood leukocytes (10^6 cells/mL)	T-cells ($CD3^+$)	n.d.	n.d.	1.8±0.4	1.7±0.5
	B-cells ($CD19^+$)	n.d.	n.d.	4.9±1.1	3.7±1.4
	Myeloid cells ($CD11b^+$)	n.d.	n.d.	3.6±0.5	3.7±1.3

n.d. = not determined

To confirm deletion of p53 in macrophages, we analyzed p53 protein levels in thioglycollate-elicited macrophages using Western blot analysis. Yields of thioglycollate-elicited peritoneal macrophages from $p53^{del}$ and $p53^{fl}$ mice were similar (data not shown). Western blot analysis showed that p53 was virtually absent in thioglycollate-elicited macrophages from $p53^{del}$ animals, while it could easily be detected in $p53^{fl}$ macrophages (Figure 1A). To further increase p53 signals in the macrophages, we treated the cells with the DNA damaging agent etoposide in the presence of the proteasome inhibitor MG132. Such treatments are known to increase the levels of the very unstable p53 protein.[21] As can be seen in figure 1A, this treatment strongly increased p53 levels in $p53^{fl}$ macrophages and resulted in the presence of

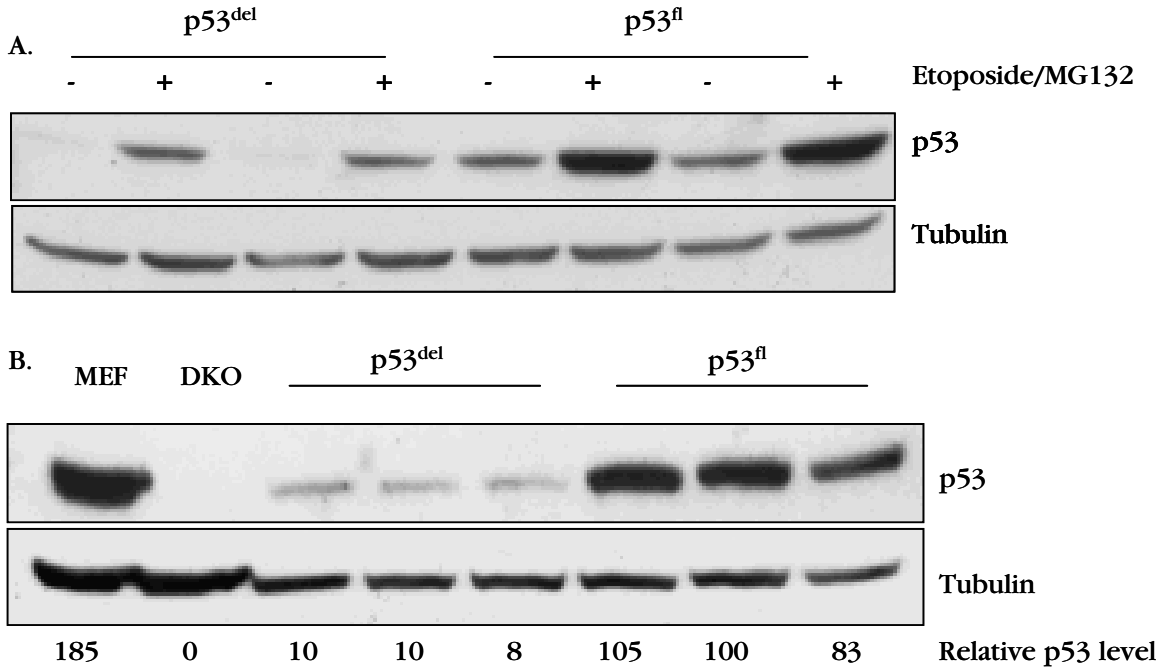

Figure 1. (A.) Protein extracts from $p53^{del}$ and $p53^{fl}$ macrophages, either mock-treated or treated with etoposide and proteasome inhibitor MG132, were analyzed for p53 and α-tubulin expression. (B.) p53 protein expression levels in lysates from representative $p53^{del}$ and $p53^{fl}$ macrophages treated with etoposide and MG132. P53 protein levels in $p53^{del}$ macrophages were correlated to the p53 expression levels in $p53^{fl}$ macrophages, corrected for the loading control α-tubulin. MEF: lysate from wild type mouse embryo fibroblasts (positive control); DKO: lysate from homozygous p53/mdm2 double knock out mouse embryo fibroblasts (negative control)

a p53 band in the $p53^{del}$ cells, indicating that some remaining p53 was left. Semi-quantitative PCR confirmed that some wild type allele was left in the $p53^{del}$ macrophages (data not shown). The relative difference in p53 levels between $p53^{del}$ and $p53^{fl}$ macrophages was quantified using Western blots of etoposide/MG132-treated cells and showed a reduction of approximately 90% in p53 protein levels in $p53^{del}$ macrophages as compared to $p53^{fl}$ mice (Figure 1B). Moreover, titration on Western blot using lysates from $p53^{fl}$ macrophages to obtain p53 protein levels similar to that of $p53^{del}$ macrophages also required over 10-fold dilution confirming the >90% reduction in p53 protein levels (data not shown). These data show that cell specific deletion of p53 results in a strong (>90%) reduction in p53 protein levels in macrophages.

Analysis of atherosclerotic lesion area

Mice fed the cholesterol-rich diet for 7 weeks (early atherosclerosis) or 11 weeks (advanced atherosclerosis) were sacrificed for collection of heart, aorta, and other organs. Morphometric analysis of the total atherosclerotic lesion area in the aortic root showed no difference between $p53^{del}$ and $p53^{fl}$ in early lesion size (P=0.40, Figure 2A and B) and in advanced lesion size (P=0.12, Figure 2A and B). In addition, *en face* analysis of Oil-Red-O stained aortas did also not reveal a difference in relative lesion area between $p53^{del}$ and $p53^{fl}$ mice on early (P=0.84, Figure 3 A and B) and advanced atherosclerosis development (P=0.84, Figure 3A and B). Hence,

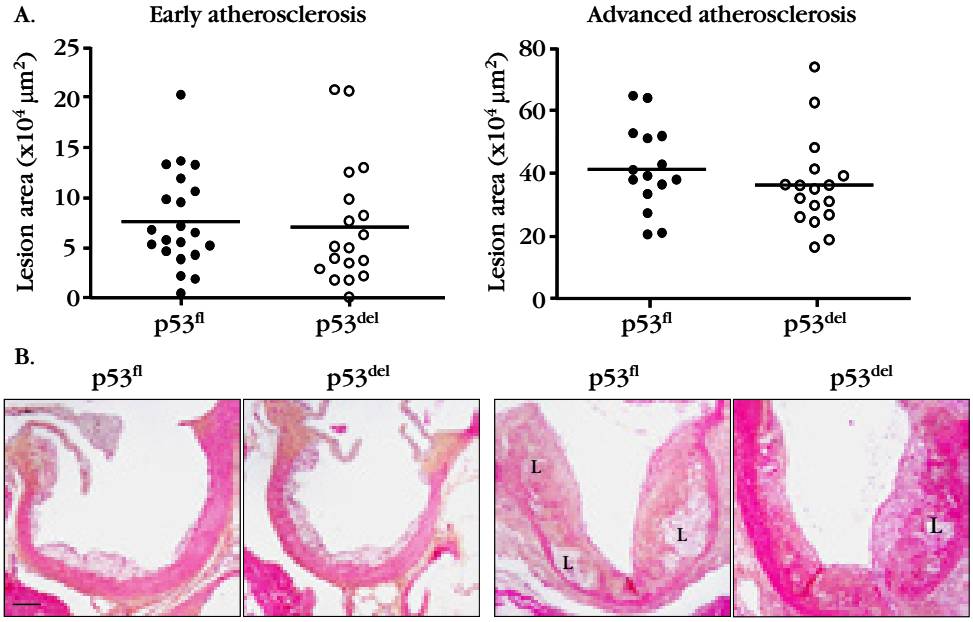

Figure 2. Aortic root atherosclerosis in $p53^{del}$ and $p53^{fl}$ mice. (A.) Early and advanced aortic root atherosclerosis in $p53^{fl}$ (closed circles) and $p53^{del}$ (open circles) mice. Symbols indicate individual mice. Line represents mean area for each group. (B.) Representative photomicrographs of atherosclerotic lesions in $p53^{fl}$ and $p53^{del}$ mice. Serial sections were stained with HPS. L: lipid core, magnification 50x, scale bar 100 µm.

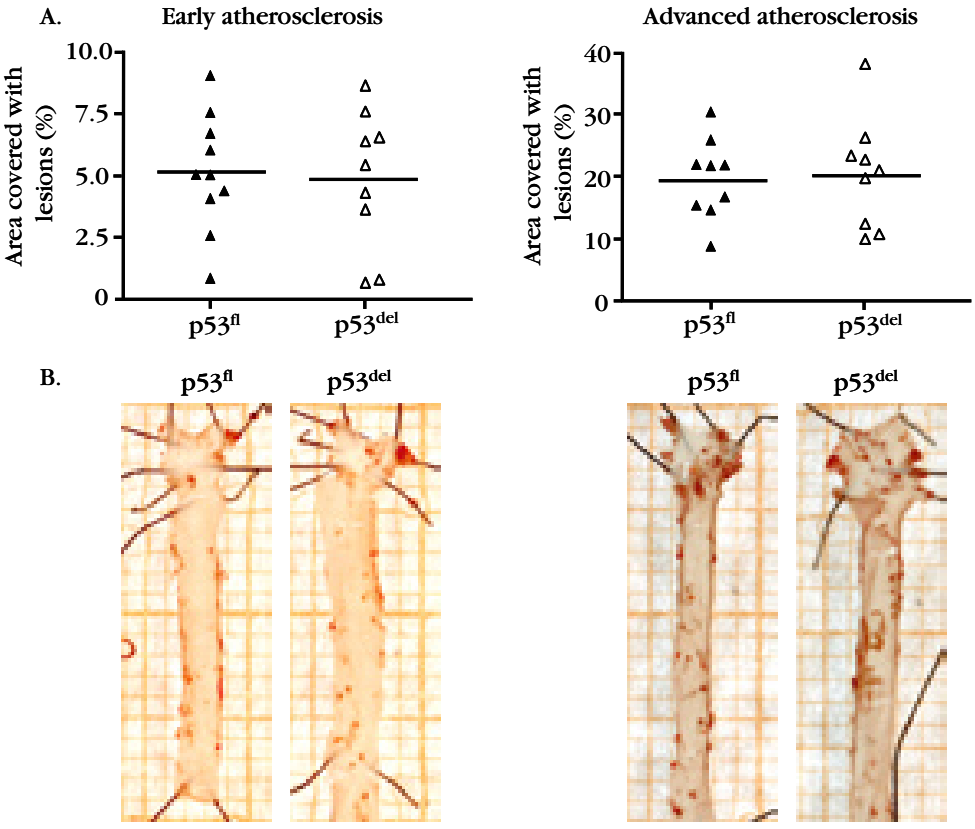

Figure 3. Aortic arch and thoracic aorta atherosclerosis in $p53^{del}$ and $p53^{fl}$ mice. (A.) Early and advanced atherosclerosis in $p53^{fl}$ (closed triangles) and $p53^{del}$ (open triangles) mice. Symbols indicate individual mice. Line represents mean area for each group. (B.) Representative Oil-Red-O stained aortas of $p53^{fl}$ and $p53^{del}$ mice in early and advanced atherosclerosis development.

absence of macrophage p53 did not affect total atherosclerotic lesion area, both in early and advanced atherosclerotic lesions at two different regions in the aortas of apoE-deficient mice.

Cell proliferation and cell death

To investigate whether macrophage p53 deficiency affects cell turnover in the lesions both cell proliferation and cell death were followed during atherosclerosis development. For analysis of cell proliferation mice were injected daily with BrdU for 3 days before the end of the experiment. In both the early and advanced atherosclerosis group the incidence of $BrdU^+$ cells did not differ between $p53^{del}$ and $p53^{fl}$ mice (Figure 4A), indicating that macrophage p53 deficiency did not affect lesional proliferation in both early and advanced atherosclerosis.

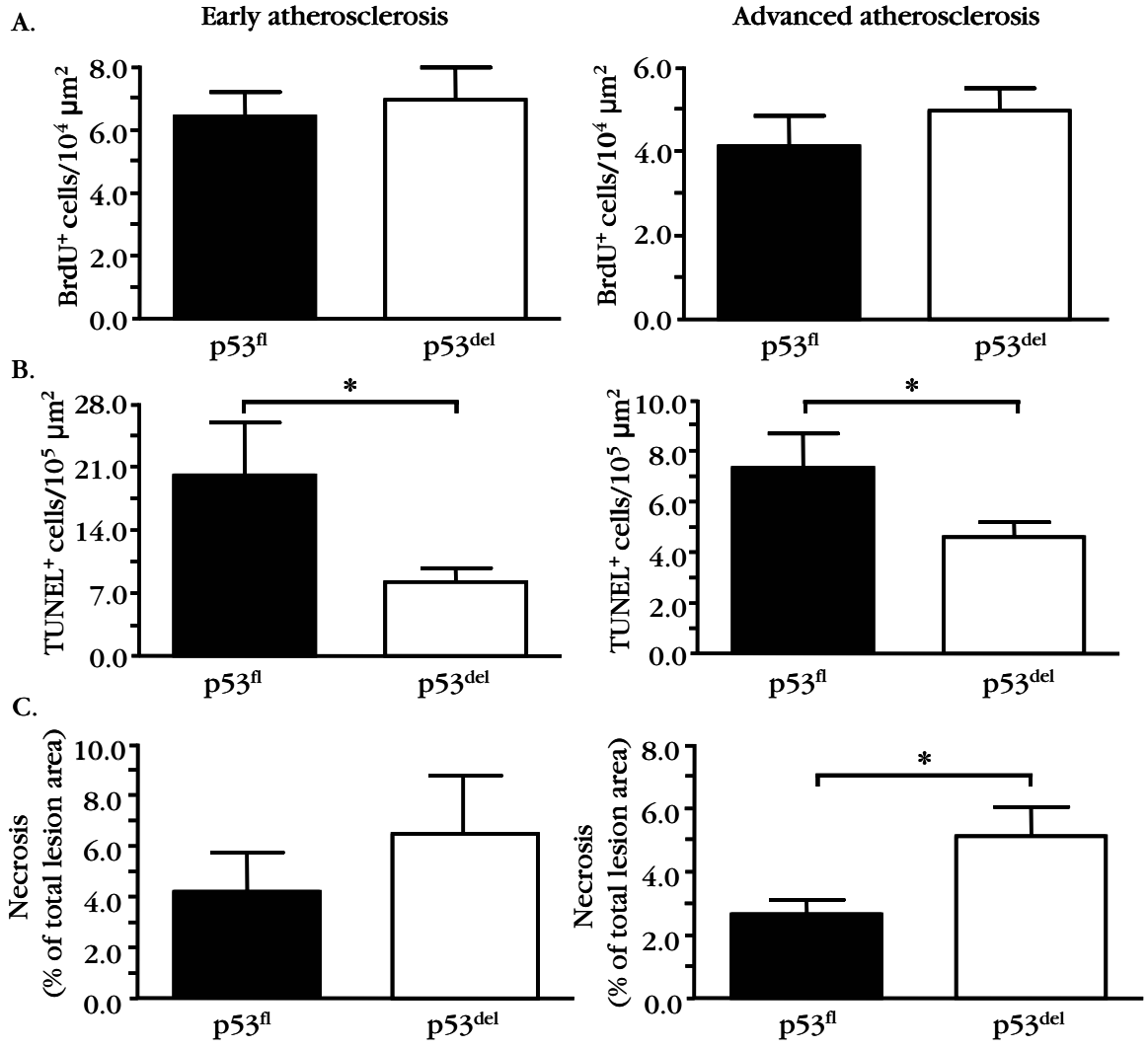

Figure 4. Proliferation, apoptosis and necrosis in $p53^{fl}$ and $p53^{del}$ mice. (A.) Proliferation was detected by BrdU-staining. Bars represent the number of $BrdU^+$ nuclei per lesion area. (B.) Apoptosis was detected by TUNEL staining. Bars represent the number of $TUNEL^+$ nuclei per lesion area. (C.) Necrosis, defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei, was analyzed on HPS sections. Bars represent necrotic area per lesion area. Black bars, $p53^{fl}$ mice; white bars $p53^{del}$ mice. Error bars indicate SEM. *$P<0.05$.

To investigate cell death in the atherosclerotic lesions apoptosis and necrosis were quantified. Apoptotic cells in the atherosclerotic lesions were identified as TUNEL-positive cells. Strikingly, the incidence of TUNEL-positive nuclei was strongly reduced in $p53^{del}$ mice in both early and advanced atherosclerosis development (-59% and -37%, respectively, $P<0.05$, Figure 4B). In addition to apoptosis, necrosis in the lesions was quantified. Necrosis in the advanced atherosclerosis group was 2-fold increased in the $p53^{del}$ group, as compared to the controls ($P<0.05$, Figure 4C). A similar trend was observed in early atherosclerosis although not significant (Figure 4C). These analyses show that macrophage p53 deletion prevents lesional macrophage apoptosis both in early and advanced atherosclerosis. In addition, prevention of macrophage apoptosis in advanced atherosclerosis switches the cell death pathway towards necrotic cell death.

Macrophages and lipid core

To analyze whether the changes in lesional cell death in $p53^{del}$ mice coincided with changes in atherosclerotic lesion composition we performed a more detailed phenotypic analysis of the lesions. Macrophage area was detected by immunostaining and lipid core area was defined by the presence of cholesterol clefts, extracellular lipids and complete absence of nuclei. In the early atherosclerosis group both macrophage area and lipid core content were not affected by macrophage restricted-p53 deletion (Table 2). However, quantification of macrophage area in the advanced atherosclerosis group revealed a 24% increase in $p53^{del}$ mice as compared to the $p53^{fl}$ control mice ($P<0.05$, Table 2). Interestingly, these changes coincided with a strong 41% reduction in the lipid core content of the lesions in $p53^{del}$ mice as compared to $p53^{fl}$ control mice ($P<0.05$, Table 2, Figure 2B, right panel). Hence, the decrease in lesional apoptosis in $p53^{del}$ mice coincides with a change in cellular lesion composition towards atherosclerotic lesions with an increased macrophage area and a decreased lipid core.

Table 2. Characteristics of early (7 weeks cholesterol-rich diet) and advanced (11 weeks cholesterol-rich diet) atherosclerotic lesions in $p53^{del}$ and $p53^{fl}$ mice

	$p53^{fl}$	$p53^{del}$	$p53^{fl}$	$p53^{del}$
Atherosclerosis development	Early		Advanced	
Lesion macrophage area (% of total lesion area)	66.4±10.5	63.6±11.5	42.2±8.2	52.5±9.8*
Lipid core (% of total lesion area)	3.1±3.6	2.5±3.3	11.1±4.2	6.5±3.7*

* $P<0.05$

DISCUSSION

In the present study, we investigated the role of macrophage p53 in the pathogenesis of atherosclerosis. Absence of macrophage p53 did not affect lesion area of early and advanced atherosclerosis analyzed both at the level of the aortic root and at the level of the aortic arch and thoracic aorta in ApoE-deficient mice. However, detailed analysis of atherosclerosis in the aortic root revealed that apoptosis of foam

cells was strongly reduced in $p53^{del}$ mice at both time points, while proliferation was unaffected. Moreover, the reduction in lesional apoptosis in $p53^{del}$ mice coincided with increased necrosis in the advanced atherosclerotic lesions. The changes in lesional cell death were accompanied by an increase in relative macrophage area and a decrease in relative lipid core area in the advanced atherosclerosis group. Hence, these studies demonstrate that macrophage p53 is a major mediator of foam cell apoptosis and inhibition of this pathway results in a shift of cell death towards necrotic death of lesional macrophages, thereby affecting lesion composition.

Previous bone marrow transplantation (BMT) studies demonstrated that p53 of hematopoietic origin clearly has atheroprotective properties.[10,11] Bone marrow harbors not only progenitors for the myeloid lineage including macrophages, but also the progenitors from which ultimately T-cells and B-cells originate. In atherosclerosis, T-cell derived cytokines as well as B-cell mediated antibody production clearly contribute to atherosclerosis progression.[1] Moreover, p53 is shown to be important in T- and B-cell turnover.[22,23] Hence, the atheroprotective effect of bone marrow derived p53 could be attributed in part to T and B-cell-specific p53. In this light it is of interest that hematopoietic p53 deficiency (i.e. combined lymphocyte and macrophage p53 deficiency) results in strong effects on the size of the lesions, while this study demonstrates that macrophage-restricted p53 deficiency results to more subtle effects confined to lesional macrophage turnover, eventually affecting lesion composition. Combining these data it can be suggested that T/B-cell p53 is involved in modulating size i.e. growth of the lesions. Future studies, using conditional approaches for lymphocyte-specific deletion of p53 may shine more light onto the role of lymphocyte-derived p53 in atherogenesis.

Additionally, the differences in findings on quantitative lesion area may also partly be attributed to the differences in choice of atherosclerotic background. Whereas the LDL-receptor deficient mouse model[11] and the APOE*3-Leiden mouse model[10] show mild atherosclerosis development, the $apoE^{-/-}$ mouse is a model of accelerated atherosclerosis development leading to complex atherosclerotic lesions.[24] Moreover, absence of ApoE inhibits cholesterol efflux from macrophages and thereby more progressively stimulates the formation of foam cells, our cells of interest.[25] Although the clearance of apoptotic cell remnants is attenuated in $apoE^{-/-}$ mice[26] the foam cell rich lesions enabled us to establish the role of macrophage p53 on macrophage turnover in the atherosclerotic lesions.

Based on the current study we hypothesize that in early lesions foam cells preferentially die quickly via a relatively clean apoptotic death which requires p53. However, eventually, the composition of the lesions may prevent complete proper phagocytosis of the apoptotic cells, resulting in secondary necrosis[27] and the formation of a lipid core. In contrast, upon impaired functioning of the p53 pathway in macrophages, lesional foam cells are no longer directed to die via the apoptotic pathway. This is in line with recent *in vitro* data of Mercer et al. showing that $p53^{-/-}$ peritoneal macrophages exhibit reduced apoptosis.[28] As a consequence of the inability to die via apoptosis, death of foam cells may be delayed, but eventually it results in increased death via necrosis, due to extensive lipid accumulation. Previously, it was also shown that inhibition of p53 in mouse embryonic fibroblasts results in a shift of NO-induced cell death towards relatively more necrosis and less apoptosis.[29]

The alternative necrotic death pathway of foam cells may be more slowly and is generally considered to be more detrimental since necrosis leads to the release of pro-inflammatory and pro-thrombotic substances. Hence, macrophage necrosis is detrimental to atherosclerotic lesion development, whereas macrophage apoptosis can be considered more beneficial in lesion development and plaque stability.Therefore, our data point towards an important role for macrophage p53 in controlling foam cell death by induction of apoptosis and prevention of lesional necrosis.

In conclusion, we demonstrate that macrophage p53 guarantees safe foam cell death via apoptosis and thereby prevents lesional necrosis, which directly affects lesion composition. Lesion composition rather than lesion size determines its vulnerability and thereby its clinical relevance.This implies that local targeting of processes that regulate p53 mediated apoptosis may be a powerful target for therapeutic intervention in coronary artery disease. Recently, the use of drug-eluting stents has emerged as a highly promising local approach to reduce in-stent restenosis.[30] The different drugs (i.e. rapamycin, flavopiridol) used in these drug-eluting stents target different apoptosis and proliferative genes, including p53.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. Jonkers for his contribution to the experiments leading to the manuscript.This work was supported by the Netherlands Heart Foundation (NHS 2000.051) and the Netherlands Organization for Scientific Research (NWO 902-26-242). B.J.M. van Vlijmen is a fellow of the Royal Netherlands Academy of Arts and Sciences, and M.P.J. de Winther is supported by NWO (906.02.075 and 917.66.329).

REFERENCES

1. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-1695.
2. Lusis AJ. Atherosclerosis. Nature 2000;407:233-241.
3. Libby P. Changing concepts of atherogenesis. J Intern Med 2000;247:349-358.
4. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature JID - 0410462 1993;362:801-809.
5. Meek DW. The p53 response to DNA damage. DNA Repair (Amst) 2004;3:1049-1056.
6. Klein C, Vassilev LT. Targeting the p53-MDM2 interaction to treat cancer. Br J Cancer 2004;91:1415-1419.
7. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594-604.
8. Ihling C, Menzel G, Wellens E, Monting JS, Schaefer HE, Zeiher AM. Topographical association between the cyclin-dependent kinases inhibitor P21, p53 accumulation, and cellular proliferation in human atherosclerotic tissue. Arterioscler Thromb Vasc Biol 1997;17:2218-2224.
9. Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nat Med 1999;5:335-339.
10. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 2001;88:780-786.

11. Merched AJ, Williams E, Chan L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. Arterioscler Thromb Vasc Biol 2003;23:1608-1614.
12. Jonkers J, Meuwissen R, van Der GH, Peterse H, van d, V, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001;29:418-425.
13. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-277.
14. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 1992;258:468-471.
15. Nishina PM, Verstuyft J, Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. J Lipid Res 1990;31:859-869.
16. Meulmeester E, Maurice MM, Boutell C, Teunisse AF, Ovaa H, Abraham TE, Dirks RW, Jochemsen AG. Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. Mol Cell 2005;18:565-576.
17. Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ. Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-leiden transgenic mice. Cardiovasc Res 2005;66:179-185.
18. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 1987;68:231-240.
19. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. J Lipid Res 1995;36:2320-2328.
20. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2003;112:1176-1185.
21. Damalas A, Ben Ze'Ev A, Simcha I, Shtutman M, Leal JF, Zhurinsky J, Geiger B, Oren M. Excess beta-catenin promotes accumulation of transcriptionally active p53. EMBO J 1999;18:3054-3063.
22. Moller MB, Gerdes AM, Skjodt K, Mortensen LS, Pedersen NT. Disrupted p53 function as predictor of treatment failure and poor prognosis in B- and T-cell non-Hodgkin's lymphoma. Clin Cancer Res 1999;5:1085-1091.
23. Ward JM, Tadesse-Heath L, Perkins SN, Chattopadhyay SK, Hursting SD, Morse HC, III. Splenic marginal zone B-cell and thymic T-cell lymphomas in p53-deficient mice. Lab Invest 1999;79:3-14.
24. Rosenfeld ME, Polinsky P, Virmani R, Kauser K, Rubanyi G, Schwartz SM. Advanced atherosclerotic lesions in the innominate artery of the ApoE knockout mouse. Arterioscler Thromb Vasc Biol 2000;20:2587-2592.
25. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 2004;24:1006-1014.
26. Grainger DJ, Reckless J, McKilligin E. Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. J Immunol 2004;173:6366-6375.
27. Tabas I. p53 and atherosclerosis. Circ Res 2001;88:747-749.
28. Mercer J, Figg N, Stoneman V, Braganza D, Bennett MR. Endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice. Circ Res 2005;96:667-674.
29. McLaughlin LM, Demple B. Nitric oxide-induced apoptosis in lymphoblastoid and fibroblast cells dependent on the phosphorylation and activation of p53. Cancer Res 2005;65:6097-6104.
30. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. Int J Cardiol 2005;99:9-17.