Macrophage-Specific p53 Expression Plays a Crucial Role in Atherosclerosis Development and Plaque Remodeling

Aksam J. Merched, Elizabeth Williams, Lawrence Chan

Objective—We first showed that absence of p53 accelerates atherosclerosis development in apoE-deficient mice. In this study, we investigated how macrophage-specific loss of p53 function might modulate atherosclerosis development in LDL receptor-deficient mice, a model for familial hypercholesterolemia.

Methods and Results—We transferred bone marrow cells isolated from p53<sup>+/−</sup> and p53<sup>−/−</sup> mice to lethally irradiated LDL receptor<sup>−/−</sup> mice and evaluated the aortic atherosclerotic lesion areas in the recipients at different times afterward. At 15 weeks and again at 20 weeks, we found larger aortic lesion size in mice receiving p53<sup>−/−</sup> cells compared with those that received p53<sup>+/−</sup> cells. By measuring the rate of bromodeoxyuridine incorporation, we found that the absence of p53 in macrophages stimulates cellular proliferation. In contrast, the rate of apoptosis in the atheromatous lesion was similar in the two groups of mice. Furthermore, we found that the absence of macrophage-specific p53 expression was associated with vulnerable-appearing lesions marked by increased tissue necrosis and reduced collagen deposition.

Conclusions—p53 plays a crucial role in atherosclerosis lesion development and remodeling, and macrophage-specific p53 deficiency stimulates cellular proliferation leading to a vulnerable-appearing phenotype of lesions in a mouse model of familial hypercholesterolemia. (Arterioscler Thromb Vasc Biol. 2003;23:1608-1614.)

Key Words: atherosclerosis ■ apoptosis ■ proliferation ■ necrosis ■ macrophage

Macrophages are a major cellular component of atheromatus plaques, where they perform key functions in modulating atherosclerosis development. In these plaques, macrophages take up native and modified lipoproteins and are transformed into foam cells in the process. Furthermore, they are an important source of bioactive inflammatory cytokines that influence lesion progression and plaque remodeling. Macrophages within the plaque undergo proliferation as well as apoptosis, processes that are tightly regulated by the tumor suppressor protein p53.

The in vivo role of p53 in atherosclerosis has been examined by two groups. Our laboratory first reported the effect of p53 inactivation on atherosclerosis development in apoE-deficient mice. We found that, despite a similar degree of hypercholesterolemia, p53<sup>−/−</sup>/apoE<sup>−/−</sup> mice developed aortic atherosclerosis much faster than p53<sup>+/−</sup>/apoE<sup>−/−</sup> littermates. Interestingly, the increased cellularity of the atheromas in the absence of p53 was the result of increased cellular proliferation and not decreased apoptosis. In another study, van Vlijmen et al<sup>8</sup> examined apoE<sup>−/−</sup> transgenic mice that had received bone marrow transplantation from p53<sup>−/−</sup> or p53<sup>+/−</sup> donors and found that mice reconstituted with p53<sup>−/−</sup> bone marrow developed atherosclerosis at a faster pace than those that received p53<sup>+/−</sup> cells. Although this result corroborated that of Guevara et al<sup>7</sup> on apoE<sup>−/−</sup> mice, there was apparently a difference in the mechanism that led to the increase in lesion size in the two mouse models. In contradiction to the observation of Guevara et al<sup>7</sup> on p53<sup>−/−</sup>/apoE<sup>−/−</sup> mice, van Vlijmen et al<sup>8</sup> found an insignificant trend toward increased apoptosis but no difference in cellular proliferation as measured by BrdU labeling in the apoE<sup>−/−</sup>/Leiden mice that received p53<sup>−/−</sup> marrow.

The difference in the two reports could be explained at least partly by the different animal models used. Guevara et al<sup>7</sup> used apoE-deficient mice to examine the effect of global p53 deficiency whereas van Vlijmen et al<sup>8</sup> used apoE<sup>−/−</sup>/Leiden transgenic mice to investigate the effect of p53 deficiency involving macrophages only. The two mutant apoE mouse models develop accelerated atherosclerosis because of the massive accumulation of remnant lipoproteins, a highly unusual cause of atherosclerosis in humans. In an accompanying editorial, Tabas<sup>9</sup> opined that, to determine the antiatherogenic role of p53 in humans, observations should be made in animal models with high plasma LDL, the common form of atherogenic hyperlipoproteinemia in humans.

In this communication, we have studied the effect of macrophage-specific p53 expression on atherosclerosis development in LDL receptor (LDL-R)<sup>−/−</sup> mice. In this model the atherosclerotic lesion development is not itself affected by the provision of normal leukocyte-specific LDL-Rs by bone marrow transplantation. We found that p53 expression in the atherosclerotic...
lesion was generally coupled to that of p21, a downstream effector of p53. Not only did we observe retardation of atherosclerotic lesion progression in mice transplanted with p53−/− macrophages, we also found that p53 expression in macrophages promoted the remodeling of the atherosclerotic lesion from a vulnerable-appearing phenotype (in the absence of p53) to a more stable-looking phenotype (in its presence). Macrophage-specific p53 expression thus appears to play an important role both in lesion progression as well as in atheroma remodeling.

Materials and Methods

Animals and Bone Marrow Transplantation

LDL-R knockout and p53 knockout mice backcrossed onto the C57BL/6j background were purchased from Jackson Laboratories. Six- to 7-week-old female LDL-R knockout mice (n=36) were subjected to 1000 rads total body irradiation to eliminate endogenous bone marrow stem cells and most of the bone marrow-derived cells. Bone marrow cells for repopulation were prepared from either wild-type (p53+/+) mice or p53−/− C57BL/6j mice as described.10 Five hours after irradiation the LDL-R−/− mice were injected intravenously with 1×10⁷ bone marrow cells from donor mice. After bone marrow transplantation, mice were fed a diet containing 0.2% (wt/wt) cholesterol and 10% (vol/wt) coconut oil, which maintained the plasma cholesterol level at 9.05 to 14.22 mmol/L. Groups of mice were sacrificed after 15 weeks (11 mice) and 20 weeks (25 mice), and blood was collected by cardiac puncture into tubes containing EDTA. To control the repopulation of the bone marrow into the recipient, gene expression of LDL-R of donor origin was verified by using the reverse transcription polymerase chain reaction technique (RT-PCR).10 Total RNA was isolated by using Trizol LS method (Gibco) from the liver, spleen, lung, and peripheral blood mononuclear cells of control and treated mice 20 weeks after bone marrow transplantation. All procedures were approved by the animal protocol review committee of our institution.

Plasma Cholesterol and Lipoprotein Analysis

Total cholesterol and triglyceride concentrations in plasma were measured at the end of diet feeding by using enzymatic procedures (Sigma). Fast-protein liquid chromatography separation of lipoprotein particles were achieved as described earlier.11 The cholesterol content of the fast-protein liquid chromatography fractions was measured enzymatically.

Quantitative Morphometry of Atherosclerotic Lesion Area.

Cross-sectional analysis of the aortic sinus was conducted after 15 weeks of bone marrow transplantation. En face study of atherosclerotic lesion area was performed on the 20-weeks time point by using complete aortas isolated from the root of the aorta to the iliac bifurcation. For the histological study, we used a modification of the method of Paigen et al12 Briefly, the heart was excised with a small portion of the ascending aorta remaining. Sectioning started within the heart and worked in the direction of the aorta. Once the aortic valves were identified, 7-µm cryostat sections were taken and mounted on poly-L-lysine–coated slides. In total, 70 sections were cut and divided over 10 slides so that every slide contained 10 sections at 490-µm intervals. One slide was stained with oil red O for fat and the remaining slides for immunohistochemistry. The extent of atherosclerosis was visually assessed by analyzing 4 sections stained with Oil Red O and counterstained with hematoxylin and examining under a light microscope.

After being fed a high-fat diet for 20 weeks, mice were killed and their aortas were isolated for en face study as described previously.7

Immunohistochemistry and Histologic Evaluation

Cryostat sections of the aortic root and aorta were fixed in acetone for 10 minutes and air-dried for at least 30 minutes. After blocking the endogenous peroxidase activity and washing in PBS (pH 7.4), sections were incubated for 30 minutes with the various monoclonal antibodies followed by a peroxidase or alkaline phosphatase–conjugated secondary antibody for 30 minutes. Smooth muscle cells were stained by using with anti-α-actin (Sigma, 1:800) and the M.O.M. Basic Kit (Vector) was used for detection. Macrophages were detected by immunostaining with MOMA-2 (1:25) by using a rat-specific kit (Vector). The peroxidase and alkaline phosphatase activities were demonstrated with diaminobenzidine and fast red (Sigma), respectively. Histology was performed by using standard techniques for Oil Red–O, hematoxylin and trichrome. Aortic en face lesions were evaluated by quantitative morphometry as previously described.

The collagen was stained by using Masson’s trichrome (Dako). Collagen content in the lesion area was assessed in 4 serial 7-µm sections at 49-µm intervals. After capturing images of the aortic sections, we determined collagen-positive area by computer-assisted color gated measurement on the total intimal surface area (SigmaScanPro5) and expressed the data as a percentage of total positive area per section.

Detection of Proliferating Cells and Cell Immunostaining

Mice that had been fed a high-fat diet for 15 and 20 weeks received an intraperitoneal injection of bromodeoxyuridine (BrdU) Labeling Reagent (Amerham), 1 mL per 100 g of body weight, 22 hours before being killed. For detection of S-phase cells in the plaques, we used a BrdU staining kit (Zymed Laboratories) combining biotinylated mouse monoclonal antibodies against BrdU and horseradish peroxidase–conjugated streptavidin Complex. The substrate for horseradish peroxidase was diaminobenzidine, which produces a brown color in positive nuclei. Hematoxylin was used as a counterstain. Cells were counted manually under microscope taken at magnification of 200.

Cell type–specific primary antibodies were mouse antibody against β-actin (Dako) and rabbit antibody against mouse macrophages (Accurate). Double immunostaining was made by using Dako EnVision Doublestain System combining BrdU staining and cell type–specific staining.

Detection of Apoptotic Cells and Necrotic Area

Apoptotic cells were detected by using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–digoxigenin nick end labeling (TUNEL; Roche) technique as described by the manufacturer. We included only those TUNEL-positive nuclei that displayed morphological features of apoptosis, including cell shrinkage, aggregation of chromatin into dense masses, and nuclear fragmentation. Cells were counted manually on photographs taken with a magnification of 200. The percent of positively stained cells was determined by using 3 to 4 serial sections of 7 µm at 49-µm intervals from each mouse.

Analysis of necrosis was performed in the aorta valve region. Images of the aorta for computer analysis were captured and necrotic areas were selected and quantified by using SigmaPro5 morphometric tools. Entire necrotic areas were included encompassing cells with shrunken and pyknotic nuclei, necrotic debris and large cell-free necrotic cores. Necrosis was estimated in 3 to 4 serial sections of 7 µm at 49-µm intervals and expressed as a percentage of total lesion area.

Statistical Analysis.

The t test was routinely used except when value distribution failed the normality test of ANOVA, in which case the Mann–Whitney rank sum test was used as specified (SigmaStat, Jandel Scientific, San Rafael, California). Values are expressed as means ± SD. The level of P equal or less than 0.05 was set to be significant.
Results

Bone Marrow Transplantation in LDL-R<sup>−/−</sup> Mice

LDL-R<sup>−/−</sup> mice were lethally irradiated and were transplanted with bone marrow from p53<sup>+/+</sup> or p53<sup>−/−</sup> mice. The transplantation rescued the mice, which were put on a high-cholesterol (0.2% wt/wt) high-fat (10% vol/wt coconut oil; HF/HC) diet 4 weeks later. Repopulation of the bone marrow cells (mainly as T cells and macrophages) was confirmed by detection of LDL-R gene of donor origin by RT-PCR. As shown in Figure 1, LDL-R mRNA was detected in the tissues and downstream of the disrupted region in exon 4 of LDL-R gene. Only wild-type LDL-R mRNA produced a 275-bp RT-PCR amplification product (lower band). Beta-actin RT-PCR reactions were used as internal control (upper band). Lg, lung; Lv, liver; Sp, spleen; BM, bone marrow.

We measured blood leukocyte counts in wild-type and p53<sup>+/+</sup> mice were as follows: monocytes, 3.30 ± 0.44%; lymphocytes, 79.20 ± 1.90% and those in and p53<sup>−/−</sup> mice were as follows: monocytes, 2.92 ± 1.18%; lymphocytes, 81.7 ± 11.20%.

Effect of Macrophage-Specific p53 Expression on Atherosclerosis

Mice that received bone marrow transplantation with p53<sup>+/+</sup> or p53<sup>−/−</sup> cells had similar levels of plasma cholesterol (443.56 ± 58.60 mg/dL in p53<sup>+/+</sup> vs 473.46 ± 56.90 mg/dL in p53<sup>−/−</sup>) and triglycerides (104.29 ± 43.20 mg/dL vs 101.72 ± 30.10 mg/dL). There was also no difference in the plasma lipoprotein profile between the two types of mice; both showed intermediate- and low-density lipoprotein elevation as the major class of lipoprotein accounting for the hypercholesterolemia (data not shown).

We quantified the extent of aortic atherosclerosis at two different time points by assessment of histological cross-sections of the aortic valve area as well as by en face staining of the aorta cut open longitudinally. At 15 weeks after HF/HC diet feeding, atherosclerotic lesion area measured by quantitative morphometry of multiple cross-sections of the aortic root (as described in Materials and Methods) was significantly larger in mice that received p53<sup>−/−</sup> mice (180%) compared with those that received p53<sup>+/+</sup> bone marrow cells (102,730 ± 37,730 μm<sup>2</sup> vs 184,960 ± 82,540 μm<sup>2</sup>, P = 0.05, Figure 2). At 20 weeks of HF/HC diet feeding, we compared the degree of atherosclerosis involvement by quantitative morphometric measurement of en face aorta preparations. We observed that the p53<sup>−/−</sup> aortas displayed a proportionately even greater (233%) lesion area compared with the p53<sup>+/+</sup> samples (1.20 ± 0.50 mm<sup>2</sup> vs 2.82 ± 1.84 mm<sup>2</sup>, P = 0.006, Figure 3). Therefore, lack of p53 expression in macrophages resulted in accelerated atherosclerosis development at two different time points as quantified by two different techniques.

Effect of Macrophage-Specific p53 Expression on Cellular Proliferation and Apoptosis in the Atheromatous Lesion

The rate of accumulation of the different cellular elements that make up the atheromatous plaque is determined by the rates of cell proliferation and cell death as well rate of cells that migrate to and from the lesion. Because p53 is a key regulator of cell proliferation and apoptosis, we compared these rates in LDL-R<sup>−/−</sup> mice that received bone marrow transplantation from p53<sup>+/+</sup> donors with the rates in LDL-R<sup>−/−</sup> mice that received marrow from p53<sup>−/−</sup> donors. The rate of cell proliferation was estimated by the rate of incorporation
of BrdU into cells in the atherosclerotic lesion after a timed injection of the label. By this method, we observed a 330% increase in the rate of cellular proliferation in the lesions of mice that harbored p53

\[ p53^{-/-} \]

macrophages compared with mice reconstituted with p53

\[ p53^{+/+} \]

macrophages (8.95 ± 0.70% vs 2.70 ± 0.60%, \( P < 0.01 \), Figure 4A). Although the proliferating cells in the lesion involved all types of cells in both kinds of mice, by double immunostaining we found that macrophages accounted for a larger proportion of proliferating cells in the lesions of p53

\[ p53^{-/-} \] mice than in those of the p53

\[ p53^{+/+} \] group (78% in p53

\[ p53^{-/-} \] vs 68% in p53

\[ p53^{+/+} \], \( P < 0.005 \)). We further compared smooth muscle staining with BrdU/MOMA-2 double-staining in serial sections. We found that in mice receiving p53

\[ p53^{+/+} \] bone marrow cells, 33% of the proliferating cells were macrophages, whereas in those receiving p53-deficient bone marrow cells, 80% of these cells were macrophages (Figure 4C and 4D).

We determined the frequency and location of apoptotic cells in atherosclerotic lesions 15 weeks after bone marrow transplantation. As determined by the technique of in situ DNA end extension (see Materials and Methods), we found that apoptotic cells appeared to be randomly distributed (Fig. 4B, 4E, and 4F). The frequency of apoptotic cells in mice that received p53

\[ p53^{+/+} \] bone marrow was 0.87 ± 0.06%, which was not significantly different (\( P = 0.73 \)) from that (0.92 ± 0.20%) found in mice that received p53

\[ p53^{-/-} \] marrow. Thus, the presence or absence of p53 expression in bone marrow-derived macrophages in the atherosclerotic lesions had no effect on the rate of programmed cell death in the lesions. The accelerated lesion growth in mice harboring p53

\[ p53^{-/-} \] macro-
p53 and its Downstream Effector p21 in the Lesion

p53 exerts much of its growth arrest function via p21, an inhibitor of cdks or GADD45, which suppress critical enzymes in DNA synthesis, resulting in inhibition of E2F-mediated transactivation of genes needed for cells to enter S phase. To examine the putative effector role of p21, we compared the expression of p53 and p21 in atheromatous plaques by immunolocalizing the expression of p53 (Figure 5A and 5C) and p21 (Figure 5B and 5D) in serial sections of atherosclerotic lesions. Lesions from mice transplanted with p53$^{-/-}$ bone marrow were packed with p53-positive–staining cells (Figure 5A). In contrast, very rare p53-positive cells were identified in lesions of mice transplanted with p53$^{-/-}$ marrow (Figure 5C). In serial sections from the same animal, we found that most of p21 immunostaining (Figure 5B and 5D) coincided with that of p53 (compare Figure 5A and 5B). However, we also detected p21 in some p53-negative cells (Figure 5C and 5D). This cellular distribution of p21 strongly suggests a p53-independent expression of p21, whose significance in atherosclerosis development is yet to be determined.

Effect of Macrophage-Specific p53 Expression on the Phenotype of Atheromatous Plaques in LDL-R$^{-/-}$ Mice

The cellular composition and other phenotypic features of an atheromatous plaque determine its stability. We compared the histopathology of the atherosclerotic lesion in LDL-R$^{-/-}$ mice that received p53$^{-/-}$ bone marrow cells with that in LDL-R$^{-/-}$ mice that received p53$^{-/-}$ cells. Because so-called “necrotic centers” have been postulated to often precede plaque rupture, we first measured the areas of necrosis contained within the lesion by quantitative morphometry (Figure 6, upper right histogram). We found that the presence of p53 in the transplanted cells protected against necrosis because the relative area involved in necrosis in mice that received p53$^{+/+}$ bone marrow cells was only 41% that in mice that received p53$^{-/-}$ cells. Atherosclerotic lesions are thought to be more stable if they have higher collagen content. We found that the collagen-positive area was 158% higher in lesions of mice that received p53$^{+/+}$ cells compared with those that received p53$^{-/-}$ cells (49±1.0% in p53$^{+/+}$ vs 31±5.0% in p53$^{-/-}$, P<0.05, Figure 6A and 6B and upper left histogram). Therefore, the presence in the lesion of p53 expression in the bone marrow–derived macrophages confers histopathologic features of stability to atheromatous lesions. Conversely, absence of p53 function renders them more vulnerable looking.

Discussion

This investigation extends two previous reports on the role of p53 in atherosclerosis.$^{7,8}$ All three studies found that the absence of p53 promotes atherosclerosis in mice. The two previous reports involved mice with absent or mutant apoE expression. These mutant mice developed a massively elevated serum cholesterol concentration that is about 500% (for the apoE$^{-/-}$ study$^{7}$) to 1000% (for the apoE$^{*3}$-Leiden study$^{8}$) the normal human level. This degree of hypercholesterolemia is rarely encountered clinically. Furthermore, the lipoprotein fraction that accumulates in the absence of apoE function consisted of mainly remnant particles, an unusual form of dyslipidemia in humans. Thus, the observations on cell proliferation and apoptosis made in the extremely aggressive
atherosclerosis in the apoE models may have limited applicability to the situation in human patients who develop mainly LDL-dependent atherosclerosis.9

It is reassuring, therefore, to find that absence of p53 also leads to accelerated atherosclerosis in a mouse model of LDL-R deficiency that displays a serum cholesterol level (about 2 to 3 times normal) very similar to that seen clinically. Observations in this model suggest that p53 expression may be relevant to clinical atherosclerosis. We note that a recent report showed that marked overexpression of p53 in smooth muscle cells was associated with atherosclerotic plaque destabilization in apoE−/− mice.14 This apparent divergent effect of p53 may be related to the different cell types involved. Alternatively, it is probable that adenovirus-induced massive overexpression of p53 could produce consequences that are very different from those resulting from physiological levels of expression.

In this study, we examined macrophage-specific instead of global p53 expression as was done in our previous report.7 Thus, the absence of p53 expression confined to the bone marrow–derived macrophages is sufficient to have a profound effect on atherosclerosis development in the presence of moderate hypercholesterolemia. Moreover, we found that the accelerated atherosclerosis is accompanied by an increased rate of cellular proliferation and not by any detectable change in the rate of apoptosis of the cells in the lesion. These findings parallel those made by our laboratory on apoE-deficient mice. They differ from those of van Vlijmen et al, who found no significant alteration in either cellular proliferation or apoptosis (with the latter parameter showing an insignificant trend toward a decrease). The reason for the difference is unclear, as the same two techniques were used to investigate these cellular processes in both studies. We speculate that the extreme hypercholesterolemia, or the presence of a dominant-negative mutant apoE (apoE*3-Leiden), in the previous study might have produced to a cellular proliferative and/or apoptotic response that is different from that in models with moderate hypercholesterolemia and normal apoE function.

p53 effects on cell cycle progression are mediated by a number of pathways.15,16 We found that for the most part p53 and p21 are coexpressed by the same cells in the atheroma, an observation that supports the intermediary role of p21 in the downstream action of p53. However, some p21 expression was not associated with that of p53. p21 is known to be involved in cell cycle progression and it is plausible that the antiproliferative effect of p53 in the LDL-R−/− mice was largely mediated by p21. However, p21 has been shown to be regulated in many situations independently of p53 and display functions that are entirely independent of p53.17-19 Future experiments using p21-inactivated mice will be informative in the elucidation of its role in atherogenesis.

Compared with p53−/− macrophages, p53+/− macrophages might contribute to reduced collagen deposition via different pathways, rendering the lesions less stable and more prone to inflammation and necrosis. Smooth muscle cells may be a major source of collagen; however, it is known that macrophages are also capable of producing type VIII collagen.20 Furthermore, the production of interstitial collagens by smooth muscle cells is regulated by cytokines produced by macrophages and lymphocytes.21 Finally, p53 itself has been reported to be directly involved in the expression of some of the matrix metalloproteinases because it downregulates MMP1.22 Thus, not only did we observe that p53 expression plays a protective role in slowing down atherosclerosis development, it also seems to promote remodeling of the lesion from a vulnerable phenotype to a stable-looking phenotype (Figure 6). Vulnerable lesions are recognized by a number of histological characteristics, including the absence of a fibrous cap and the presence of necrotic centers, which could develop into so-called “lipid cores.”23 Plaque composition rather than plaque size determines its vulnerability. The vulnerable lesion is much more likely to rupture (or fissure) and produce an acute coronary event.24,25 The fact that the presence of p53 reduced the amount of necrosis but increased the amount of collagen in the lesion suggests that macrophage-specific p53 expression or some of its downstream effector molecules may be novel targets for therapeutic intervention in coronary artery disease.

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