Absence of p21Waf1/Cip1/Sdi1 Modulates Macrophage Differentiation and Inflammatory Response and Protects Against Atherosclerosis

Aksam J. Merched, PhD; Lawrence Chan, MBBS, DSc

Background—The tumor suppressor p53 protects against atherosclerosis progression in several different mouse models. A major target of p53 is p21, the cyclin-dependent kinase inhibitor that regulates entry into the cell cycle of different types of cells, including stem cells. p21 is also involved in the maturation and differentiation of monocytes into macrophages.

Methods and Results—We studied the effect of p21Waf1 inactivation on atherosclerosis development in apolipoprotein E–deficient mice (apoE−/−). Contrary to previous data suggesting a protective role for p21, we found that absence of p21, either globally or in bone marrow–derived cells, protects against atherosclerosis. Atherosclerotic lesions of p21−/−/apoE−/− mice exhibit a more stable phenotype, with increased apoptosis and reduced inflammatory vascular cell adhesion molecule-1 immunostaining but no difference in cellular proliferation compared with lesions of p21+/+/apoE−/− mice. Because bone marrow–derived cells mediate many of the effects of p21, we examined the expression profile of 23 genes in macrophages using real-time polymerase chain reaction. Compared with their p21+/+ counterparts, peritoneal macrophages of p21−/− mice express lower levels of proinflammatory markers, including macrophage inflammatory proteins 1 and 2 and interleukin-1α, and higher levels of putative protective genes, such as scavenger receptor type B-1 and LDL receptor–related protein. Furthermore, we found that, in comparison with p21+/+ macrophages, p21−/− macrophages displayed increased phagocytic activity toward fluorescent latex microspheres as well as apoptotic cells, thus uncovering a novel mechanism of the antiinflammatory activity of p21−/− macrophages.

Conclusions—Loss of p21 protects against atherosclerosis in apoE−/− mice. The data underscore the important role of p21 in macrophage function and inflammation and provide insight into the mechanism of the proatherogenic effect of p21.

(Circulation. 2004;110:3830-3841.)

Key Words: apoptosis • inflammation • atherosclerosis • phagocytosis • cell cycle

Atherosclerosis is an inflammatory disease1,2 in which macrophages play major roles in all stages of development, from initiation of the fatty streak to processes that lead to plaque rupture and acute coronary events. Macrophages interact with T cells to modulate the evolution of the inflammatory response; importantly, they also help contain the inflammatory process by engulfing and removing apoptotic cells before they disintegrate and release their toxic cellular contents.3

Atherosclerosis is an inflammatory disease1,2 in which macrophages play major roles in all stages of development, from initiation of the fatty streak to processes that lead to plaque rupture and acute coronary events. Macrophages interact with T cells to modulate the evolution of the inflammatory response; importantly, they also help contain the inflammatory process by engulfing and removing apoptotic cells before they disintegrate and release their toxic cellular contents.3

Interest in the contributions of apoptosis and cellular proliferation in atherosclerosis development prompted us to examine the role of p53 in the process. We first reported that p53 inactivation led to the development of accelerated atherosclerosis in apolipoprotein E–deficient (apoE−/−) mice.4 Interestingly, the increased cellularity of atheromas in the absence of p53 was caused primarily by increased cellular proliferation, because we observed no difference in the rate of apoptosis. Recently, we obtained similar data in LDL receptor–deficient (LDLR−/−) mice that received bone marrow transplantation from p53−/− donors, which support the crucial role of bone-marrow–derived cells in the process.5 A report on the proatherogenic effect of transplantation of p53−/− bone marrow to another mutant apoE, apoE*3-Leiden, mouse model further supports the importance of p53 in atherosclerosis development.6 In contrast, another study showed that induced smooth muscle cell (SMC)–specific overexpression of p53 using recombinant adenovirus leads to atherosclerotic plaque destabilization.7

The effects of p53 are mediated by different downstream effectors and targets.8,9 A Cdk inhibitor, p21Waf1/Cip1/Sdi1, is a major mediator of p53 action.10–12 p21 effects stress-induced p53-dependent and p53-independent cell proliferation arrest.
In addition, the protein possesses antiapoptotic capabilities, but it may also be involved in monocyte and dendritic cell differentiation. There is substantial evidence that p21 participates in the protective action of p53 on atherosclerosis. Adenoviral vector–mediated delivery of the p21 gene to the vessel wall protected porcine arteries against the development of intimal hyperplasia after balloon injury. Moreover, Condorelli et al showed that overexpression of a mutated form of p21 (that has increased biological activity) by adenovirus-mediated gene transfer protected against restenosis in apoE/− mice by reducing SMC proliferation and macrophage infiltration of the lesion. Thus, p21 is generally considered an antiatherogenic molecule and a potential therapeutic gene that can be used to treat or prevent atherosclerosis and postangioplasty restenosis.

To investigate the role of p21 in atherosclerosis, we evaluated aortic atherosclerotic lesion development in p21+/−/apoE−/− and p21+/+apoE−/− mice at different ages and under different diet conditions. The involvement of p21 in macrophages in the process was further explored by bone marrow transplantation experiments. To our surprise, not only does p21 fail to protect against atherosclerosis, but it also actually exhibits a potent proatherogenic effect on lesion development and progression. We examined the gene expression pattern of macrophages by quantifying 23 markers involved in inflammation, phagocytosis, and lipid metabolism. We undertook mechanistic experiments to elucidate the role of p21 in macrophage differentiation and activation. These experiments provide a plausible explanation for the unexpected proatherogenic properties of p21 and reveal an important function of this molecule on macrophage behavior that directly affects the development of atherosclerosis.

Methods

Animals and Diet

p21−/− mice were obtained from Dr Lawrence Donehower and Punmin Zhang at Baylor College of Medicine, and apoE−/− mice in C57BL/6J background were purchased from Jackson Laboratories, Bar Harbor, Me. p21+/− mice were backcrossed onto the C57BL/6J background for ≥8 generations. At 6 weeks of age, as specified below, we put some mice on a Western diet (Teklad adjusted-Western-type diet: 21% [wt/wt] fat, 0.15% [wt/wt] cholesterol, 19.5% [wt/wt] casein, without sodium cholate). We measured total cholesterol and triglyceride concentrations in plasma at the end of diet feeding by use of enzymatic procedures (Sigma). Fast performance liquid chromatography (FPLC) separation of lipoprotein particles was achieved as described previously. The cholesterol content of the FPLC fractions was measured by use of an enzymatic kit (Sigma). The animal protocol review committee of our institution approved all procedures.

Irradiation and Bone Marrow Transplantation

Fifteen-week-old female apoE−/− mice were subjected to 10 Gy 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 apoE−/−p21−/− or apoE−/− mice, and transplantation was performed as described previously. After bone marrow transplantation, mice were kept on a regular chow diet. They were killed after 19 weeks, and blood was collected by cardiac puncture into tubes containing EDTA.

Quantitative Morphometry and Immunohistochemistry

We performed cross-sectional analysis of the aortic sinus 19 weeks after bone marrow transplantation. En face study of the atherosclerotic lesion area was performed for all the other time points using complete aortas spanning from the root of the aorta to the iliac bifurcation. We prepared aortas for analysis as described previously. 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), we incubated the sections for 30 minutes with different monoclonal antibodies individually and exposed the sections to peroxidase– or alkaline phosphatase–conjugated secondary antibody for 30 minutes. Primary antibodies used include rat anti-mouse macrophages Mac3 (Santa Cruz), rat anti-mouse vascular cell adhesion molecule (VCAM)-1 (Santa Cruz), rat anti-mouse CD18 (Pharmingen), rabbit anti–SMC-specific epitope actin (Spring Bioscience), or rabbit anti-mouse scavenger receptor type B-1 (SR-BI) (Novus Biologicals). We detected proliferating and apoptotic cells using a bromodeoxyuridine (BrdU) staining kit (Zymed Laboratories) and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) (Roche), respectively, as described. We visualized collagen by use of trichrome staining and elastic fibers using Verhoeff’s–van Gieson staining.

Phagocytosis Activity of Isolated Peritoneal Macrophages

Peritoneal macrophages were activated with intraperitoneal injection of 1 mL of 3% aged Brewer’s thiglycollate (Difco Laboratories) 3 days before collection. We used 2-μm Nile red fluorescent latex FluoSpheres (F-8825, Molecular Probes) for the phagocytosis experiment. We washed the beads twice with Tris buffer, pH 9.0, and coated them with BSA by incubation with 5 mg/mL solution of BSA in the same Tris buffer for 2 hours at 37°C; we then washed them twice with PBS containing 0.1% gelatin and once with RPMI before resuspending them in RPMI containing 10% heat-inactivated FBS. For the phagocytosis experiment, we added the BSA-coated beads to the plated cell suspension at a 100:1 ratio of microspheres to cells and incubated the mixture at 37°C for 90 minutes. We then removed the free microspheres and other unattached cells by washing with RPMI, washed the cells with PBS, then fixed and processed them for immunohistochemistry.

To examine the macrophage-mediated phagocytosis of apoptotic thymocytes, we prepared mouse thymocytes as described by Chang et al. Briefly, we added 10×10⁶ CellTracker-labeled dexamethasone-treated thymocytes to the macrophage monolayers. We fixed them for 80 minutes at 37°C. Wells were washed 5 times with ice-cold PBS and treated with 0.25% trypsin/0.02% EDTA for 20 minutes at 22°C to remove uninternalized thymocytes. We fixed the cells with 4% paraformaldehyde in PBS and incubated the mixture at 37°C for 90 minutes. We then removed the free microspheres and other unattached cells by washing with RPMI, washed the cells with PBS, then fixed and processed them for immunohistochemistry.

Real-Time Quantitative RT-PCR and Western Blotting

Total RNA was extracted from macrophages by use of Absolutely RNA reverse transcription–polymerase chain reaction (RT-PCR) MiniPrep Kit (Stratagene) with an additional RNase-free DNase digest of genomic DNA. Total RNA was reverse-transcribed into cDNA by use of Superscript II reverse transcriptase (Invitrogen) and oligo (dT) primers in a total volume of 80 μL. Reaction proceeded at 42°C for 50 minutes and was terminated with a denaturation step at 70°C for 15 minutes. Reverse-transcribed RNA was primed with oligonucleotides specific for the following genes: SR-B1 (5′-CAGTAGTTCTGCCGTTGCTG-3′ and 5′-TGAATGGCCTCC- TTATCTG-3′), LDLR (5′-GAAAAGGCTACTGGCTTG-3′), ABCA1 (5′-TGTTCCAAAGAGCGCCATGG-3′ and 5′-TGCATGCGTACCAATATACAC-3′), MSR-A (5′-ATGAT-
CGCTGGGATATACGG-3' and 5'-ACCCACGATCTCTTGAATG-3', β-actin (5'-GAGGCCAGATCCTACATAT-3' and 5'-CTTTCGACCTTCGCAAG-3'), LDL receptor–related protein (LRP1) (5'-TTGTCTCAAGGCAAGG-3' and 5'-CCATCTGGTTGGTGCAAG-3'), PSR1 (5'-TCCTGCTCAAAGG-3' and 5'-TGAGCACCCTTGAGTCTC-3'), p16 (5'-CTCAACTCAAGGTGCAAGTCC-3' and 5'-TGGCAGGATGTC-3'), macrophage inflammatory protein (MIP)-1α (5'-CTGACTCTCGCTGAGTGGTC-3' and 5'-TGGGAGGAGGAGACGACTTAT-3'), tumor necrosis factor (TNF)-α (5'-CTGGCTAGCGCTGTCAATG-3' and 5'-AGTGAACTGCGCTGTCAATG-3'), transforming growth factor (TGF)-β (5'-TCGCGATACCTTCCCAGT-3' and 5'-CTCCTGTTGACCCAGAGCAT-3'), interleukin (IL)-1α (5'-TGCTGGGAGGAGACTTATCG-3' and 5'-CCACGCTACCTGCTGCTACT-3'), interleukin (IL)-1β (5'-TGGCAGCTGAGTGGTGAAAGA-3' and 5'-AGATGCCGAGAGTCCCTTCTC-3'), peroxisome proliferator–activated receptor-γ (5'-CTCTGTGACCCAGAGCAT-3' and 5'-AATGCGAGGTGTCCTCCCAT-3'), interleukin (IL)-2, IL-4 and 9, and females, P<0.05 set to be significant.

Results

p21 Inactivation Protects Against Atherosclerosis in apoE−/− Mice

We assessed the effect of p21 inactivation in apoE−/− mice maintained on a regular chow diet at an early stage (20 weeks of age) and then at an advanced stage (63 weeks, or ∼14 months) of atherosclerosis development. Measurement of lesions at this early time by use of computer-assisted histo-

![Figure 1. Presence of p21 promotes atherosclerosis in apoE−/− mice: quantification of aortic atherosclerotic lesion in p21+/−/apoE−/− and p21−/−/apoE−/− mice at different times and under different dietary conditions. A, Lesion area in male mice fed a normal chow diet for 20 weeks. Each data point represents total affected area at aortic valve and sinus in cross-sectional analysis (P<0.02) (see Methods). B, Aortic lesion area in female mice after 63 weeks (14 months) on normal chow by en face analysis, n=18, P<0.01. C, Quantification of aortic atherosclerotic lesion area by en face analysis in mice fed a Western diet for 15 weeks: again, p21−/−/apoE−/− mice had smaller lesion areas than did p21+/−/apoE−/− mice in both males P=0.05 (n=4 and 9) and females, P=0.07 (n=6 and 8). D, FPLC analysis of plasma lipoproteins in mice under normal chow diet. Vertical bars represent mean±SD.](http://circ.ahajournals.org/doi/abs/10.1161/CIRCULATIONAHA.104.158740)
logical cross-sectional analysis revealed that the absence of p21 reduced atherosclerotic lesion size by 53% (45 554±26 356 versus 97 386±30 798 µm², P<0.05, male mice, Figure 1A). We next evaluated atherosclerosis at a much more advanced stage at 14 months of age by use of an en face method, which computes the total lesion area in the entire aorta, and found again a highly significant protective effect of absence of p21; there was a 32% reduction in lesion size in p21+/−/apoE−/− compared with p21+/+/apoE−/− mice [(17.06±5.73)×10⁶ µm² versus (25.22±6.44)×10⁶ µm², P<0.01, female mice, Figure 1B]. To study the role of p21 in the setting of markedly accelerated atherosclerosis, we fed 6-week-old male and female p21+/−/apoE−/− and p21+/+/apoE−/− mice an atherogenic diet (western diet, see Methods) for 15 weeks and then compared their aortic lesion areas by quantitative en face analysis. Again, the presence of p21 expression was associated with a significant proatherogenic effect on the aggressive atherosclerosis occurring in the aorta of apoE−/− mice under these conditions (Figure 1C). Comparing male apoE−/− mice that expressed p21 with male apoE−/− mice lacking p21, we found that the lesion area was significantly larger (39%) (P<0.05); lesion area was also larger (31%) in female apoE−/− mice expressing p21 compared with female apoE−/− mice, although the difference did not reach statistical significance (P=0.07). The total blood counts (white blood cells, red blood cells, and platelets) were similar in p21+/−/apoE−/− and p21+/+/apoE−/− mice (data not shown), as were plasma cholesterol and triglyceride concentration and lipoprotein profiles, whether the mice were fed chow diet (Figure 1D) or western diet (data not shown).

Inspection of the luminal surface of the aorta revealed much larger lesions in p21+/−/apoE−/− (aorta on the left, Figure 2A) compared with those of p21+/+/apoE−/− mice (aorta on the right, Figure 2A). Analysis of trichrome-stained atherosclerotic lesion (Figure 2B) showed smaller lesions in p21+/−/apoE−/− mice (lefts) compared with p21+/+/apoE−/− mice (rights), amounting to a 52% and a 43% reduction in the valve (uppers) and sinus areas (middles), respectively. Examination at higher magnification of similar-sized lesions (Figure 2B, lowers) showed a thicker and better-formed fibrous cap (arrowhead, SMC stained red) in the p21+/−/apoE−/− mice (left) compared with apoE−/− mice (right), indicating the presence of more stable-looking lesions in mice lacking p21.

Lesions Associated With Global p21 Inactivation Display Increased Apoptosis, Reduced Vascular Cell Adhesion Molecule-1 Immunostaining, but No Change in Cellular Proliferation

Because p21 is a known regulator of cellular proliferation and apoptosis,17,20 we investigated how its absence affects these 2 processes in the atherosclerotic lesion. We determined the frequency and location of apoptotic cells in atherosclerotic lesions in 20-week-old p21+/−/apoE−/− versus p21+/+/apoE−/− mice. As determined by an in situ DNA end-extension technique, apoptotic cells appeared to be randomly distributed in both genotypes. The frequency of apoptotic cells was significantly (182%) higher in the lesions of apoE−/− mice lacking p21 compared with those expressing p21 (2.02±0.30% versus 1.11±0.19%, P<0.005, Figure 3A).

The increased rate of apoptosis in the absence of p21 corroborates its antiapoptotic activities reported by others.20 The cellular proliferation rate as measured by the rate of BrdU incorporation into cells in the atheromatous lesions was...
not different between p21+/+apoE−/− and p21+/−apoE−/− mice (3.46±0.22% versus 3.42±0.30%, P=0.84, Figure 3B). The content of leukocytes in the lesions was comparable between p21+/−apoE−/− mice (Figure 3C, left) and p21+/+/apoE−/− mice (right), 69.50±5.76% versus 73.33±4.4%, respectively. We further evaluated the expression of an adhesion molecule, VCAM-1, a marker of inflammation, by immunostaining. By this technique, there was lower VCAM-1 expression in the atherosclerotic lesion of p21+/+/apoE−/− mice compared with p21+/−apoE−/− mice (reduced by 55%, P<0.005; Figure 3D); the reduced inflammation in p21+/−apoE−/− mice may contribute to retardation of the atherosclerotic process in these animals.

**Transfer of p21+/−apoE−/− Bone Marrow Cells to p21+/+/apoE−/− Mice Protects Against Atherosclerosis and Increases Apoptosis of Macrophages In Situ**

We transferred bone marrow cells from p21+/−apoE−/− or p21+/+/apoE−/− mice to irradiated apoE−/− mice and found that the rate of successful rescue of lethally irradiated apoE−/− mice was correlated with the presence of p21 expression in the donor cells, because recipients of p21−/− cells started to die at 17 weeks after transplantation. At 19 weeks, whereas 100% (7/7) of the mice that received p21+/− bone marrow cells survived, only 62% (5/8) of those that received p21+/+ cells survived. The cause of death was not explored here, because it had been extensively investigated by Cheng et al., who showed elegantly that self-renewal of stem cells was impaired in serially transplanted bone marrow from p21+/− mice, which eventually died of hematopoietic failure. In the mice that survived 19 weeks after transplantation, we found that compared with mice that received p21+/− bone marrow, the absence of p21 expression in the donor cells was associated with a 32% reduction in atherosclerosis in the recipient apoE−/− mice (P<0.01) (Figure 4A and B). Cross-sectional analysis of oil red O–stained frozen sections showed significant reduction in lesion area involving the aortic valve region (Figure 4B, left, recipient of p21+/−/apoE−/− cells; right, recipient of p21+/+/apoE−/− cells). Plasma cholesterol and triglyceride levels were not different between the 2 groups of mice (Table 1). Leukocyte content of the lesions as assessed by CD18 specific immunostaining was not different between the 2 groups of apoE−/− mice with

---

**Figure 3.** Apoptotic and proliferating cells in atherosclerotic plaques of p21+/−/apoE−/− and p21+/+/apoE−/− mice fed a normal chow diet at 20 weeks of age. A, Apoptosis was detected by use of nonisotopic DNA end-extension in situ. Lesions of p21+/−/apoE−/− mice had higher content of apoptotic cells compared with those of p21+/+/apoE−/− mice (2.02±0.30% vs 1.11±0.19%, P<0.005). B, Proliferating cells, indicated by BrdU incorporation. Frequency of proliferating cells was not statistically different between p21+/−/apoE−/− and p21+/+/apoE−/− mice (3.46±0.22% vs 3.42±0.30%, respectively). C, Leukocyte-specific immunostaining by use of anti-CD18 (brown staining) in lesions from p21+/−/apoE−/− (right) and p21+/+/apoE−/− mice (left). D, Immunostaining of VCAM-1 in lesions from p21+/−/apoE−/− (right) and p21+/+/apoE−/− mice (left). Sections are shown before (top) and after hematoxylin counterstaining. VCAM-1 levels were significantly lower in p21+/−/apoE−/− mice, P<0.001 (right histogram). Error bars represent SD. Comparison included 3 to 5 mice in each group and 3 to 4 sections from each animal.
transplants (Figure 4C and Table 2). Immunostaining of β-actin revealed a more prominent fibrous cap in lesions from mice that had received p21−/− marrow (Figure 4D, left). Moreover, we observed the presence of buried fibrous caps in some lesions, indicative of previous plaque ruptures, a finding associated with accelerating lesion growth through repeated erosions. Quantification of buried caps among lesions revealed a lower frequency of these indicators of ruptured plaques in the absence of p21 (Figure 4E and Table 2). The difference in frequencies suggests that approximately

Figure 4. Bone marrow transplantation of apoE−/− mice with p21−/− cells protects against atherosclerosis. A, apoE−/− mice that received p21−/− bone marrow cells exhibited a 32% reduction in atherosclerosis (P<0.005) compared with those that received p21+/− marrow 19 weeks after transplantation. B, Cross-sectional analysis of oil red O–stained frozen sections showed smaller lesion involvement of aortic valves and sinus area in p21−/−/apoE−/− mice (left) compared with p21+/−/apoE−/− (right). C, CD18 immunostaining showed no differences in leukocyte content of lesions. D, Immunostaining of β-actin showed much more prominent fibrous cap in lesions from mice receiving p21−/− marrow (left). E, Verhoeff’s–van Gieson elastic fiber staining of lesions showed presence of a buried fibrous cap in a p21−/−/apoE−/− mouse (right, arrow) and a prominent fibrous cap shown in p21−/−/apoE−/− mice (elastic fibers stain in black). F, Characterization of apoptosis in vivo. By TUNEL technique, presence of FITC-positive cells inside lesions indicative of apoptotic cells (right). For cell typing, same slide was subject to an additional double immunostaining (left) for macrophages (Mac3, red) and SMCs (actin, brown). Arrows show 3 apoptotic macrophages (open arrows) and 1 apoptotic SMC (filled arrow). Lesions from mice receiving p21−/− marrow presented higher rate of apoptosis compared with those in control mice (left histogram). Cell typing of apoptotic cells reveals a significant difference involving macrophage (≈3 times higher, P<0.05, after transplantation of p21−/− bone marrow, right histogram) but no significant difference between apoptotic SMCs between groups receiving p21−/− or p21+/− bone marrow cells.
half of these events might have been averted in p21+/−/apoE−/− mice compared with p21+/−/apoE+/− (frequencies of 20% versus 43%, respectively, Table 2). We found an increased rate of apoptosis (Figure 4F) in the lesions of mice transplanted with p21+/−/apoE−/− bone marrow compared with those transplanted with p21+/+/apoE−/− marrow (Figure 4F, left histogram, and Table 2). Cell typing of apoptotic cells showed a 3-fold increase in apoptotic macrophages in lesions of mice that received p21+/−/apoE−/− bone marrow compared with those that received p21+/+/apoE−/− marrow (Figure 4F, right histogram, and Table 2). Although the majority of the Mac3-immunoreactive apoptotic cells appear to be apoptotic macrophages, we cannot exclude the possibility that some of the TUNEL+ material represents apoptotic fragments (from other cell types) engulfed by the macrophages. SMCs were not significantly affected by p21 status (Table 2). The absence of p21 and its associated antiapoptotic function in the lesional macrophages may account for their higher rate of apoptosis in mice transplanted with p21+/−/apoE−/− bone marrow.

**Macrophages Lacking p21 Display Increased Phagocytic Activity**

We studied the role of p21 in modulating the phagocytic avidity of isolated macrophages, a property that influences atherosclerosis development. Macrophages isolated from mice lacking p21 ingested approximately 3 times more latex microsphere beads when incubated with them in vitro (P<0.005) compared with macrophages from mice that expressed p21 (Figure 5A). To determine whether the increased phagocytic activity toward an artificial substrate such as latex beads is also seen with a more pathophysiologically relevant substrate, we evaluated the effect of p21 expression on the avidity with which the macrophages ingested apoptotic thymocytes, an antiinflammatory function. We found that the number of FITC-positive apoptotic cells internalized per macrophage per unit time was 2-fold higher in macrophages lacking p21 compared with those that expressed the protein (2.48±0.90 versus 1.27±0.25, P=0.01, Figure 5B).

**Gene Expression Profile of Thioglycolate-Elicited Peritoneal Macrophages**

Using quantitative real-time RT-PCR, we studied the expression level of 23 markers by macrophages that expressed p21 and those that did not. We concentrated on genes that were relevant to atherosclerosis development and progression. The transcripts studied include the following: CD36, MSR-A, SR-BI, LDLR, LRP1, phosphatidyl serine receptor (PSR), ABCA1, FcR-yII, TNF-α, macrophage inflammatory proteins 1 and 2 (MIP1, MIP2), IL-1α, IL-1β, TGF-β, MCP-1, peroxisome proliferator-activated receptor-γ a, p16, p27, p57, pRB, and p53 in addition to p21. Genes that showed a difference in expression level involved primarily those that encode inflammation-related molecules. Absence of p21 in macrophages was characterized by significantly reduced expression of the transcripts for the following proinflammatory genes: MIP-1, MIP-2, and IL-1α, and a tendency toward lower expression of IL-1β (Figure 6A). MIPs display mostly proinflammatory actions in vivo and in vitro,24 MIP-1 and 2, both downregulated in the absence of p21, are involved in the recruitment of leukocytes to sites of infection and the activation of these cells as an important component of the local inflammatory response.25 IL-1α acts through its precursor form as an activator of proinflammatory genes via nuclear factor-κB.26 The role of IL-1β in atherosclerosis has been studied recently in apoE−/− mice.27 IL-1β deficiency was shown to protect against atherosclerosis in this mouse model. In contrast to these inflammation-related gene transcripts, we found that macrophages lacking p21 expressed significantly higher amounts of LRP1 and SR-BI (Figure 6, B and C). Both receptors are involved in phagocytosis and in maintaining a protective balance of lipids/lipoproteins and the integrity of the vascular wall.28–30 We next examined the expression by the macrophages of other cell cycle genes, including p16, p27, p57, pRB, and p53 (Figure 6D). The expression of p27 and p57, the other 2 members of the Cip/Kip family (like p21), was not different between p21−/− and p21+/+ macrophages. In the absence of p21, transcripts for p16, pRB, and p53 were significantly upregulated, being the highest for p16 (P<0.0001) and to a lesser extent for pRB and p53 (P<0.05).

**TABLE 1. Survival, Lipid Parameters, and Atherosclerotic Lesions 19 Weeks After Transplantation**

<table>
<thead>
<tr>
<th>Bone Marrow Donors</th>
<th>N</th>
<th>Survival, %</th>
<th>Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
<th>Lesion Size, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21−/−apoE−/−</td>
<td>8</td>
<td>62% (5/8)</td>
<td>582±121</td>
<td>94±7</td>
<td>135 412±25 743*</td>
</tr>
<tr>
<td>p21+/−apoE−/−</td>
<td>7</td>
<td>100% (7/7)</td>
<td>470±105</td>
<td>95±14</td>
<td>200 157±30 817</td>
</tr>
</tbody>
</table>

*Values are expressed as mean±SD.

**TABLE 2. Characterization of Atheromatous Lesions in apoE−/− Bone Marrow–Transplanted Mice**

<table>
<thead>
<tr>
<th>Apoptotic Rates, %</th>
<th>All Cell Types</th>
<th>Macrophages</th>
<th>Smooth Muscle Cells</th>
<th>Leukocyte Content of Lesions, %</th>
<th>Proportion of Animals With Buried Fibrous Cap Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21−/−apoE−/−</td>
<td>1.7±0.61*</td>
<td>1.47±0.67*</td>
<td>0.20±0.13</td>
<td>48.33±7.2</td>
<td>1/5</td>
</tr>
<tr>
<td>p21+/−apoE−/−</td>
<td>0.88±0.38</td>
<td>0.47±0.28</td>
<td>0.39±0.21</td>
<td>46.36±6.67</td>
<td>3/7</td>
</tr>
</tbody>
</table>

*Values are expressed as mean±SD.

*P<0.05.
The prominent upregulation of p16 suggests a compensatory process involving these cell cycle proteins. Unlike p21, which affects the activity of a wide range of cyclin-dependent kinases (CDKs), p16 is a specific potent inhibitor of CDK4. When present at these increased levels, p16 is capable of displacing the Cip/Kip family members (p21, p27, p57) from interacting with CDK4—cyclin D. Consequently, more Cip/Kips would be available to interact with and inhibit CDK2, which action by itself is capable of inhibiting the progression of the cell cycle. We further analyzed the expression of the macrophage cell surface receptors, macrophage scavenger receptor A (MSR-A) and SR-BI, by Western blotting (Figure 7). These receptors are known to be involved in atherosclerosis development in mice. Although the MSR-A level did not differ between cells with and without p21 (Figure 7A), SR-BI protein expression was >3 times higher in macrophages lacking p21 (Figure 7B). This finding corroborates the higher level of SR-BI in the aortic lesions of p21−/−/apoE−/− mice compared with p21+/+/apoE−/− mice as determined by immunohistochemical staining (Figure 7C).

**Discussion**

p21 is a major target of p53, and p53-dependent cell cycle arrest is thought to be mediated primarily by p21. A major rationale for us to investigate p21 in atherosclerosis was that we and others found that p53−/− mice exhibit accelerated atherosclerosis in different genetic and dietary models, implicating p53 as an antiatherogenic molecule. The overall pratherogenic action of p21 revealed by this study was totally unexpected, because p21 had been shown to have potential antiatherogenic effects in several models and was in fact cited as a promising therapeutic gene for the treatment of atherosclerosis.

Indeed, we showed here that global p21 inactivation protects against atherosclerosis development and promotes a stable phenotype in the presence of increased apoptosis but no change in cellular proliferation. Furthermore, transplantation of p21−/− bone marrow cells reproduced the protective effect of global p21 inactivation in terms of atherosclerosis progression and apoptosis rate, suggesting that the absence of p21 function in the macrophages that infiltrate the atherosclerotic lesions was important to the protection. This conclusion was reinforced by the fact that the increased rate of apoptosis in the lesion after bone marrow transplantation affected exclusively macrophages derived from p21−/− donors. The p21−/− macrophages exhibit other important properties, including a higher phagocytic capacity, reduced expression of proinflammatory cytokines such as IL-1β and MIP-1 and 2, but increased expression of putative protective genes such as LRPI and SR-BI. Finally, p16 expression is upregulated as a compensatory response to the loss of p21.

**Why Does the Absence of a CKI (p21) Not Translate Into Proliferation Signals?**

We can envision the scenario outlined below. Cell cycle progression from G1 to S is governed by several classes of CDKs whose activities are in turn regulated by 2 families of CDK inhibitors (CKIs). The INK4 proteins, including p15, p16, p18, and p19, bind only to and inhibit CDK4 and CDK6 (D- cyclin–dependent kinases) but do not bind to other CDKs. The Cip/Kip family proteins (p21, p27, p57) affect the activities of cyclin D–, E–, and A–dependent CDKs. They are potent inhibitors of cyclin E– and A–dependent CKD2 but act as positive regulators of cyclin D–dependent kinases.

The generally accepted view is that cyclin D–dependent kinases initiate Rb phosphorylation in mid-G1 phase, after which cyclin E-CDK2 becomes active and completes this process by phosphorylating Rb on additional sites, annulling its ability to repress E2F family members and activating genes required for entry into S phase (Figure 8). Cyclin A– and B–dependent CDKs activated later during the cell cycle maintain Rb in a hyperphosphorylated form until the cell exits mitosis and Rb is returned to a hypophosphorylated state in the next G1 phase.

Under normal conditions initially, the inhibitory effects of Rb and the Cip/Kips on cell proliferation are overcome through a mitogen-dependent program mediated by the D cyclins. In response to mitogenic stimulation, D cyclins...
accumulate and participate in 2 functions: in one, cyclin D–dependent kinases phosphorylate Rb; in the other, cyclin D–CDK complexes bind to and sequester Cip/Kip proteins. Sequestration of Cip/Kip proteins lowers the inhibitory threshold and facilitates activation of the cyclin E–CDK2 complex and further phosphorylation of Rb. This sequential phosphorylation of Rb ultimately heralds entry into S phase. The decision on how to proceed during the cell cycle is somehow affected by the distribution balance of Cip/Kip family members, whether they are sequestered by the CDK4-cyclin D or they assemble with CDK2–cyclin E inducing their enzymatic inhibition (Figure 8).

The expression of p16 (INK4) increases 3-fold in macrophages that lack p21. Increased amounts of p16 protein would compete more effectively with Cip/Kip (p27, p57) proteins for CDK4 binding and alter the distribution of CDK4 in

---

**Figure 6.** Gene expression profile of thioglycolate-elicited peritoneal macrophages. Real-time PCR quantification of transcripts of proinflammatory genes (A), lipid metabolism–related genes (B), phagocytosis-related genes (C), and cell cycle–related genes (D). Because of multifunctional involvement, some genes are present in more than 1 family group. In addition to RT-PCR, expression of p27 was further assessed by Western blot (D). p16 RT-PCR products were also shown (D, lower set). Three to 6 individual samples were included in each group. *P<0.05, †P<0.0001.
favor of CDK4-p16 complexes (leading to inhibition of CDK4) and thereby induce the release of the sequestered Cip/Kip proteins.\textsuperscript{31,32} p16-induced redistribution of p27 and p57 from CDK4–cyclin D to CDK2–cyclin E complexes increases the amount of available p27 and p57 that can bind to CDK2 (Figure 8). Under these conditions, p27 and p57 contribute more efficiently to the inhibition of CDK2–cyclin E without a need for increased p27 or p57 synthesis. In this way, the absence of p21 is accompanied by a compensatory upregulation of p16, leading indirectly to normalized inhibition of CDK2–cyclin E by the remaining members of the Cip/Kip family.

In contrast to the absence of p21, p53 deficiency leads to an increased rate of cellular proliferation and accelerated atherosclerosis\textsuperscript{4}; probably, cell cycle control is receiving different stimuli in this situation. In the absence of p53, p21 loses one of its major inducers; meanwhile, the balance of other cell cycle genes is not clearly established. However, one possibility remains that in p53\textsuperscript{-/-} cells and, in the absence of any compensatory change, the levels of p21 (and the Cip/Kip family members overall) would not reach the threshold of effective interaction with CDK2 necessary for the inhibition of its activity, and the cell cycle transition signals would be to go forward. Similar consequences (low effective inhibitory Cip/Kip proteins) might occur in the absence of p27. Indeed, inactivation of p27 in apoE\textsuperscript{-/-} mice was shown to lead to accelerated atherosclerosis,\textsuperscript{36} with the p27\textsuperscript{-/-}/apoE\textsuperscript{-/-} mice exhibiting many of the phenotypic effects, eg, increased cellular proliferation, that we previously reported for p53\textsuperscript{-/-}/apoE\textsuperscript{-/-} mice.\textsuperscript{4}

p21 and p53 seem to exhibit contrasting roles in the regulation of apoptosis. During atherosclerosis development, apoptosis occurs in both p53-dependent and p53-independent ways.\textsuperscript{4} There is mounting evidence that p21 may be an inhibitor of p53-dependent apoptosis.\textsuperscript{20} It is important to note that p21 itself is under the control of different signals, such as TGF-\(\beta\), TNF-\(\alpha\), histone deacetylase inhibitors, interferon-\(\gamma\), and other factors that may simultaneously induce p53-independent apoptosis and p53-independent transactivation of p21.\textsuperscript{20} p21 may block apoptosis by interacting with proapoptotic molecules such as procaspase-3,\textsuperscript{37} caspase-8, and the kinase apoptosis signal–regulating kinase 1 (ASK1).\textsuperscript{13}

In the absence of p21, and without the inhibitory influence of p21, apoptosis might be induced through different pathways, which may involve p53 but also many other factors, as mentioned earlier. One mechanism by which p21 might facilitate atherosclerosis progression is by blocking the apoptosis of inflammatory cells. Apoptosis of macrophages itself may retard the accumulation of lipids and the generation of foam cells. Loss of p21 appears to promote apoptosis, limit inflammation in the lesion, and slow down lesion growth in atherosclerosis. Effective clearance of apoptotic cells is an important step in interrupting the inflammatory process and preventing the secondary necrosis that ensues.\textsuperscript{20,38} Efficient phagocytic removal of apoptotic cells by macrophages is a plausible mechanism by which absence of p21 exerts its antiinflammatory effects, because p21\textsuperscript{-/-} macrophages are characterized by enhanced phagocytic capability. The upregulated SR-BI and LR1P1 expression in p21\textsuperscript{-/-} macrophages may mediate the increased phagocytic activity observed. Both receptors have been found to protect against atherosclerosis,\textsuperscript{39–42} and both have been implicated in phagocytosis.\textsuperscript{28–30} For example, SR-BI was shown to recognize apoptotic cells directly; suppression of SR-BI expression by use of antisense oligonucleotides decreases the binding of apoptotic cells by more than 40%.\textsuperscript{29} LR1P1 was found to trigger phagocytosis through its cytoplasmic domain, and mutations in this domain decreased phagocytosis by 70%.\textsuperscript{30} These studies and our
current observations provide an important link between atherosclerosis and macrophage phagocytic activity. In addition to their role in phagocytosis, SR-BI and LRP1 are involved in maintaining the proper balance of lipids/lipoprotein39,40 and in protecting the integrity of the vascular wall by controlling platelet-derived growth factor—dependent signaling pathways and other mechanisms that mediate SMC proliferation and migration.41 In addition to promoting the resolution of inflammation by avidly phagocytosing apoptotic cells, p21−/− macrophages also reduce their production of proinflammatory cytokines. The diminished VCAM-1 immunostaining in p21−/− macrophages also reduce their production of proinflammatory cytokines and protects against cyto-

cytic activity of macrophages, facilitating the safe clearance of apoptotic cells, and preventing secondary necrosis and the uncontrolled release of toxic contents, culminating in a more stable lesion phenotype. Therapies that target p21 for inactivation in the appropriate context might offer protection against atherosclerosis.44

We conclude that, unexpectedly, in contrast to the negative growth suppressors p53 and p27, p21 is a proatherogenic molecule. Inactivation of p21 appears to protect against atherosclerosis, slowing down lesion growth and promoting lesion stability through multiple mechanisms: (1) stimulating apoptosis of immune cells, facilitating inflammation resolution, and retarding lesion growth; (2) limiting the production of proinflammatory cytokines and protecting against cytotoxic inflammatory response; and (3) upregulating the phagocytic activity of macrophages, facilitating the safe clearance of apoptotic cells, and preventing secondary necrosis and the uncontrolled release of toxic contents, culminating in a more stable lesion phenotype. Therapies that target p21 for inactivation in the appropriate context might offer protection against atherosclerosis.

Acknowledgments

This study was supported by Beginning Grant-in-Aid 0465093Y from the American Heart Association, Texas Affiliate (to Dr Mer-

ch) and grant HL-51586 from the National Institutes of Health and the Rutherford Chair in Diabetes Research from St Luke’s Episcopal Hospital and Baylor College of Medicine, Houston, Tex (to Dr Chan). We thank Drs Lawrence Donehower and Pumin Zhang for providing p21-null mice and acknowledge the excellent technical assistance of Ekaterina I. Antonova, Rebecca Montgomery, Elizabeth Williams, and Katherine Tollefson.

References


Absence of p21Waf1/Cip1/Sdi1 Modulates Macrophage Differentiation and Inflammatory Response and Protects Against Atherosclerosis

Aksam J. Merched and Lawrence Chan

Circulation. 2004;110:3830-3841; originally published online December 13, 2004;
doi: 10.1161/01.CIR.0000148681.01282.89
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/25/3830

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/