Journal of Cellular Biochemistry 97:735–743 (2006)

Atheroma Development in Apolipoprotein E-Null Mice Is Not Regulated by Phosphorylation of $p27^{Kip1}$ on Threonine 187

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Abstract Excessive cellular proliferation is thought to contribute to neointimal lesion development during atherosclerosis and restenosis after angioplasty. Inhibition of cyclin-dependent kinase (CDK) activity by p27 inhibits mammalian cell growth. Mounting evidence indicates that p27 negatively regulates neointimal thickening in animal models of restenosis and atherosclerosis, and its expression in human neointimal lesions is consistent with such a protective role. Cell cycle progression is facilitated by cyclinE/CDK2-dependent phosphorylation of p27 on threonine 187 (T187) during late G1. The purpose of this study was to assess whether this phosphorylation event plays a role during atherosclerosis. To this end, we generated apolipoprotein E-null mice with both p27 alleles replaced by a mutated form non-phosphorylatable at T187 (apoE−/−p27T187A mice) and investigated the kinetics of atheroma development in these animals compared to apoE−/− controls with an intact *p27* gene. Fat feeding resulted in comparable level of hypercholesterolemia in both groups of mice. Surprisingly, aortic p27 expression was not increased in fat-fed apoE−/−p27T187A mice compared with apoE−/− controls. Moreover, atheroma size, lesion cellularity, proliferation, and apoptotic rates were undistinguishable in both groups of fat-fed mice. Thus, in contrast to previous studies that highlight the importance of p27 phosphorylation at T187 on the control of p27 expression and function in different tissues and pathophysiological scenarios, our findings demonstrate that this phosphorylation event is not implicated in the control of aortic p27 expression and atheroma progression in hypercholesterolemic mice. J. Cell. Biochem. 97: 735–743, 2006.

Key words: atherosclerosis; transgenic animal models; protein phosphorylation; p27; cell cycle control

Progression through the mammalian cell cycle requires the sequential activation of holoenzymes composed of a catalytic cyclin-dependent protein kinase (CDK) and a regulatory subunit named cyclin [Morgan, 1995]. Members of the CDK family of inhibitory proteins (CKIs) interact with and inhibit the activity of CDKs/cyclins [Sherr and Roberts, 1999; Vidal and Koff, 2000]. Accumulation of the CKI p27 causes growth arrest, and abnormally low level of p27 protein is associated with pathological states of excessive cell proliferation, including cancer [Sherr and Roberts, 1999; Vidal and Koff, 2000] and vascular obstructive disease [Andrés, 2004] (see below).

The amount of p27 in the cell is regulated primarily at the level of translation [Pagano et al., 1995; Hengst and Reed, 1996; Millard et al., 1997; Servant et al., 2000; Miskimins et al., 2001] and protein turnover [Malek et al., 2001; Kamura et al., 2004]. Studies with cultured cells and genetically modified mice have shown that phosphorylation of p27 on serine 10 (S10) and threonine 187

Grant sponsor: Instituto de Salud Carlos III (Red de Centros RECAVA, C03/01); Grant sponsor: Regional Government of Valencia; Grant number: GV04B-288; Grant sponsor: Spanish Ministry of Education and Science and Fondo Europeo de Desarrollo Regional; Grant number: SAF2004-03057; Grant sponsor: BEFI Fellowship (from the Spanish Ministry of Health to S.M.S.-G.).

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Received 27 July 2005; Accepted 9 September 2005

DOI 10.1002/jcb.20680

(T187) play a major role in two sequential proteolytic pathways that regulate p27 concentration in G1 and S phase, respectively. Although phosphorylation at S10 seems to account for approximately 70% of the total phosphorylation of p27 [Ishida et al., 2000], the bulk of p27 degradation appears to be mediated by phosphorylation on T187. Nuclear accumulation of p27 during G0/early G1 phase appears to depend on its stabilization via S10 phosphorylation by the Mirk/dyrk1B kinase [Deng et al., 2004]. In contrast, in mitogen-stimulated cells, KIS-dependent phosphorylation of p27 on S10 during G1 leads to p27 binding to CRM1 and its subsequent nuclear export and cytoplasmic proteasomal degradation by a Skp2-independent Kip1 ubiquitination-promoting complex (KPC)-mediated pathway [Hara et al., 2001; Boehm et al., 2002; Ishida et al., 2002; Connor et al., 2003; Kamura et al., 2004], although evidence also exists suggesting the requirement of Skp2 for the degradation of p27 during early G1 [Malek et al., 2001; Kossatz et al., 2004]. Moreover, recent studies have demonstrated that phosphorylation of S10 is dispensable for p27 nuclear export [Kotake et al., 2004]. Jab1/CSN5 and the COP9 signalosome complex have been shown to contribute to the cytoplasmic shuttling and subsequent degradation of p27 in G1 [Tomoda et al., 1999, 2002; Chopra et al., 2002]. Nuclear export and cytoplasmic proteolysis of p27 may serve to lower the nuclear concentration of p27 below a critical threshold, thus, allowing the activation of cyclinE-CDK2 complexes and S-phase progression. Phosphorylation of p27 on T187 by CDK2 is thought to trigger the nuclear pathway for p27 proteolysis, which occurs during the S and G2 phases of the cell cycle via Skp2-dependent multiubiquitylation [Morisaki et al., 1997; Vlach et al., 1997; Carrano et al., 1999; Montagnoli et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999; Nakayama et al., 2000; Malek et al., 2001; Spruck et al., 2001].

Progressive accumulation of cellular and non-cellular material within the subendothelial space of the artery wall to form the so-called neointimal lesion is a hallmark of atherosclerosis. Factors contributing to the accumulation of neointimal cells are the recruitment of circulating leukocytes and excessive macrophage and vascular smooth muscle cell (VSMC) proliferation [Ross, 1999; Lusis, 2000; Libby, 2002; Andrés, 2004]. Activation of neointimal leukocytes triggers an inflammatory response characterized by abundant production of cytokines and chemokines, which exacerbates leukocyte recruitment and proliferation, and induces VSMCs to undergo hyperplastic growth and migration from the tunica media towards the growing neointimal lesion [Libby et al., 1995; Burke-Gaffney et al., 2002]. Evidence implicating p27 as key regulator of vascular re-modeling include the following: (a) p27 protein level in rat and porcine arteries is upregulated at late time points after balloon angioplasty when VSMCs return to a quiescent state, suggesting that induction of p27 may be a protective mechanism that contributes to the cessation of cellular proliferation in mechanically-injured vessels [Chen et al., 1997; Tanner et al., 1998]. Indeed, overexpression of p27 efficiently blocked mitogen- and c-fos-dependent induction of cyclin A promoter activity in cultured VSMCs [Chen et al., 1997; Sylvester et al., 1998], and adenovirus-mediated overexpression of p27 inhibited neointimal thickening in balloon-injured arteries [Chen et al., 1997; Tanner et al., 2000]; (b) p27 may serve as a molecular switch that determines whether VSMCs undergo hypertrophic or hyperplastic growth [Braun-Dullaeus et al., 1999; Servant et al., 2000]; (c) p27 global inactivation increases arterial VSMC and macrophage proliferation and accelerates diet-induced atherosclerosis in fat-fed apolipoprotein E-null mice (apoE−/−) [Díez-Juan and Andrés, 2001]; (d) changes in p27 expression might regulate human vascular cell proliferation within atherosclerotic lesions [Tanner et al., 1998; Ihling et al., 1999]; (e) high level of p27 expression coordinately inhibits VSMC proliferation and migration [Díez-Juan and Andrés, 2003]; and (f) differential regulation of p27 might contribute to establishing differences in proliferative and migratory properties of VSMCs, both when comparing neointimal versus medial cells from the same vessel [Olson et al., 2000] or VSMCs from distinct vascular beds [Yang et al., 1998; Castro et al., 2003]. It is also noteworthy that p27 suppression enhances hematopoietic progenitor cell proliferation and facilitates early development of promyeloid cells into macrophages [Liu et al., 1999; Cheng et al., 2000]. In agreement with these findings, transplant of irradiated apoE-null mice with p27-deficient bone marrow (BM) cells enhanced neointimal macrophage content and proliferation and accelerated atherosclerosis in fat-fed

apoE-null mice [Díez-Juan et al., 2004]. Likewise, vascular occlusion was substantially augmented when BM-derived cells from p27-null mice re-populated vascular lesions induced by mechanical injury in recipients with intact p27, in contrast to wild-type BM donors [Boehm et al., 2004].

To further elucidate the molecular mechanisms underlying p27-dependent control of vascular re-modeling, the present study was designed to critically test the importance of p27 phosphorylation on T187 during atherosclerotic plaque progression induced by hypercholesterolemia. To this end, we took advantage of the availability of the p27T187A knock-in mouse, which expresses a mutant form of p27 that cannot be phosphorylated at T187 [Malek et al., 2001], and atherosclerosis-prone apoE−/− mice, which develop hypercholesterolemia and atherosclerosis, and re-capitulate important aspects of the human disease [Plump et al., 1992; Zhang et al., 1992].

MATERIALS AND METHODS

Animals and Atherogenic Diet

Care of animals was in accordance with the institutional guidelines. Mice deficient in apoE (apoE−/−) (C57BL6/J, Charles River) and p27T187A knock-in mice (129sv) [Malek et al., 2001] were mated. The resulting F1 was intercrossed and apoE−/− mice heterozygous for p27 (one allele wild-type and the other T187A) from F2 were selected and crossed for five generations to generate the two experimental groups (apoE−/− and apoE−/−p27T187A).

Mice genotyping was done by PCR using the following primers: apoE-OIMR180 (5′-GCCTAGCCGAGGGAGAGCCG-3′), apoE-OIMR181 (5′-TGTGACTTGGGAGCTCTGCAGC-3′), apoE-OIMR182 (5′-GCCGCCCCGACTGCATCT-3′), p27T187A-H3 (5′-CCAATATGGCGGTGGAAGGGAGGCTGA-3′), and p27T187A-Y1 (5′-GAGCAGGTTTGTTGGCAGTCGTACACCTCC-3′) as previously described [Malek et al., 2001; Tan et al., 2004].

At 2 months of age, mice received an atherogenic diet containing 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate (Ssniff, SM R/M-H S4892-S010, Germany) for 28 days. Blood was withdrawn from the retro-orbital plexus before and after the high diet administration to measure the plasma cholesterol levels using the Infinity Cholesterol liquid stable reagent (Iberdiagnóstica). Three apoE−/− (two females, one male) and five apoE−/−p27T187A (three females, two males) mice died during the period of fat feeding.

Quantification of Atherosclerosis, Proliferation, and Apoptosis

Fat-fed mice were killed and their aortas were first washed in situ with PBS and then fixed with freshly prepared 4% paraformaldehyde/PBS. After removal of the heart and ascending aorta, tissue fixation continued for 16–24 h at 4°C. Specimens were paraffin-embedded and 3-μm cross-sections were obtained at the level of the aortic valves and aortic arch.

All analyses were performed by an investigator who was blinded to genotype. To determine the extent of atherosclerosis and lesion cellularity in the aortic valve region, cross-sections were immunostained with rat monoclonal antibody raised against Mac3 (Santa Cruz Biotechnology, sc-19991, 1/200), a macrophage-specific surface glycoprotein [Ho and Springer, 1983] that is expressed within the aortic wall of fat-fed apoE−/− mice [Díez-Juan et al., 2004]. Specimens were counterstained with hematoxylin and images were captured with an Olympus CAMEDIA C5060 wide zoom digital camera mounted on a Zeiss Axiolab stereomicroscope. Digital images were analyzed by computer-assisted quantitative planimetry using the Sigma Scan Pro v5.0 software (Jandel Scientific, San Rafael, CA). For each animal, the reported lesion area is the average from nine independent cross-sections (corresponding to three consecutives sections of three different zones of the aortic valve region). Given that no gender differences in lesion size were observed, data from both sexes were included in our analysis. Lesion cellularity (determined as the number of cells per mm^2 of atheroma) was quantified in cross-sections stained with hematoxylin/eosin.

Cell proliferation in atheromatous lesions from the aortic valves was estimated by Ki67 immunohistochemistry. To achieve antigen retrieval, slides were boiled for 10 min in a stainless steel pressure cooker ensuring that slides were fully immersed in 10 mM sodium citrate buffer (pH = 6). Slides were washed with tap water and endogenous peroxidase was blocked for 30 min with 0.3% H_2O_2 in methanol. After extensive washes with PBS, slides were blocked for 30 min at room temperature with 5% FBS. After incubation with monoclonal

anti-Ki67 antibody for 40 min at room temperature (clon SP6, prediluted, Master Diagnostica) and extensive washes with PBS, specimens were incubated with biotin-conjugated anti-rabbit secondary antibody for 30 min at room temperature. Immunocomplexes were detected with the ABC system (Vectastin) and DAB peroxidase substrate kit (Vector laboratories). Slides were counterstained with hematoxylin to visualize all nuclei. For each mouse, the number of Ki67-immunoreactive cells per mm^2 of atheroma was averaged from three consecutive sections from the aortic valves.

Apoptosis in the aortic valve region was determined using oligo B from the Apoptag peroxidase in situ oligo ligation kit according to the manufacturer's instructions (Chemikon). For each mouse, three consecutive sections were counterstained with hematoxylin and apoptosis within the atheroma was estimated as the number of apoptotic cells/total number of cells (average from three sections).

Western Blot Analysis

Expression of p27 in the aorta of fat-fed mice was determined by Western blot analysis. For each condition, snap-frozen arteries (aortic arch and thoracic aorta) from two animals were pooled and lysed in ice-cold 50 mM HEPES (pH 7.5) containing 150 mM NaCl, 2.5 mM EGTA, 10 mM β-glycerolphosphate, 10% glycerol, 0.1% Tween 20, 0.1 mM $NaVO_3$, 1 mM DTT, supplemented with protease inhibitor Complete Mini cocktail (Roche Diagnostics) using a Ultraturrax T25 basic homogenizer. Cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C and the protein concentration in the supernatants was determined by the Bradford assay (BioRad Laboratories). Protein samples (40 μg) were separated onto 12% SDS–polyacrylamide gels for Western blot analysis using the following primary antibodies from Santa Cruz Biotechnology: anti-p27 (sc-1641, diluted 1/100) and anti-tubulin (sc-8035, diluted 1/200). Relative protein intensity was determined by densitometry using the Multi Gauge V2.01 software (Fujifilm).

Statistical Analysis

Results are reported as mean ± SE. In experiments with two groups, differences were evaluated using a two-tail, unpaired *t*-test. For more than two groups, differences were evaluated using ANOVA and Fisher's PLSD post-hoc test (Statview, SAS Institute, Cary, NC).

RESULTS

Phosphorylation of p27 in T187 Is Not Involved in the Control of Aortic p27 Expression and Atheroma Size in Hypercholesterolemic apoE−/− Mice

To assess whether phosphorylation of p27 on T187 participates in the control of atherosclerotic plaque progression induced by hypercholesterolemia, we intercrossed p27T187A and atherosclerosis-prone apoE−/− mice to generate the two experimental groups: apoE−/− (deficient for apoE and intact p27) and apoE−/−p27T187A (deficient for apoE and harboring a mutated *p27* gene non-phosphorylatable at T187). At 2 months of age, both groups of mice were subjected to 4 weeks of fat feeding. In agreement with the initial characterization of p27T187A mice [Malek et al., 2001], apoE−/−p27T187A mice displayed higher body weight than did apoE−/− controls at all time points examined (Fig. 1A). As expected, exposure to the atherogenic diet for 4 weeks significantly increased plasma cholesterol and no differences were observed when comparing apoE−/− and apoE−/−p27T187A mice (Fig. 1B).

We next examined the level of p27 protein in aortic tissue from fat-fed mice. To this end, we pooled the aortic arch and thoracic aorta from two to three animals of each genotype and subjected the lysates to Western blot analysis. Surprisingly, we found no differences in p27 expression when comparing apoE−/−p27T187A and apoE−/− mice (Fig. 2). Likewise, atherosclerotic lesion size in the aortic root, as determined by computerized planimetry of cross-sections immunostained with macrophage-specific anti-Mac3 antibody [Ho and Springer, 1983], was undistinguishable when both groups of mice were compared (Fig. 3).

Histological and Immunohistochemical Characterization of Atherosclerotic Lesions

Given that both cellular proliferation and apoptosis have been demonstrated during atherosclerosis [Ross, 1999; Lusis, 2000; Walsh and Isner, 2000; Libby, 2002; Andrés, 2004] and that p27 dysregulation can affect both processes, we sought to examine whether fat-fed apoE−/−p27T187A mice exhibit reduced cell proliferation compensated for by an attenuated

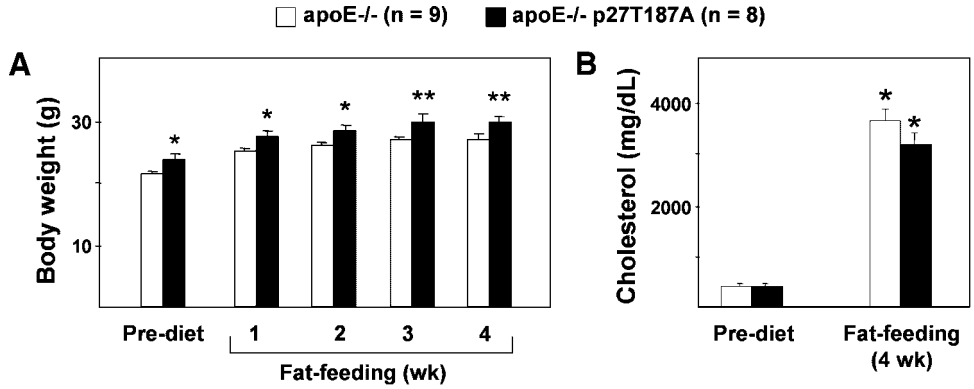

Fig. 1. Body weight and plasma cholesterol level in fat-fed mice. Gender distribution was two females/seven males for apoE−/− and three females/five males for apoE−/−p27T187A. **A**: Body weight before the onset of the atherogenic diet (pre-diet) and at different times of fat feeding in apoE−/− (white bars) and apoE−/−p27T187A (black bars) mice. Results were analyzed by AVOVA. For simplicity, only comparisons between genotypes at each time are shown (*, $P<0.05$, **, $P<0.01$). For both genotypes, body weight increased significantly during the first 2 weeks of fat feeding versus pre-diet values. **B**: Plasma cholesterol level increased significantly in fat-fed mice regardless of genotype (*, $P<0.0001$ vs. pre-diet same genotype), but no differences were seen when comparing apoE−/− and apoE−/−p27T187A mice.

rate of apoptotic cell death, thus, resulting in normal cellularity and atheroma size. Indeed, histomorphometric analysis of aortic root cross-sections stained with hematoxylin–eosin demonstrated no differences in lesion cellularity in apoE−/−p27T187A versus apoE−/− mice ($4{,}425 \pm 438$ and $4{,}149 \pm 334$ cells/mm^2, respectively, $P > 0.05$) (Fig. 4A). Moreover, cell proliferation (Fig. 4B) and apoptosis (Fig. 4C) were undistinguishable when comparing atheromatous cells from mice of both genotypes, as revealed by immunoreactivity for the proliferation marker Ki67 and ApopTag immunostaining, respectively.

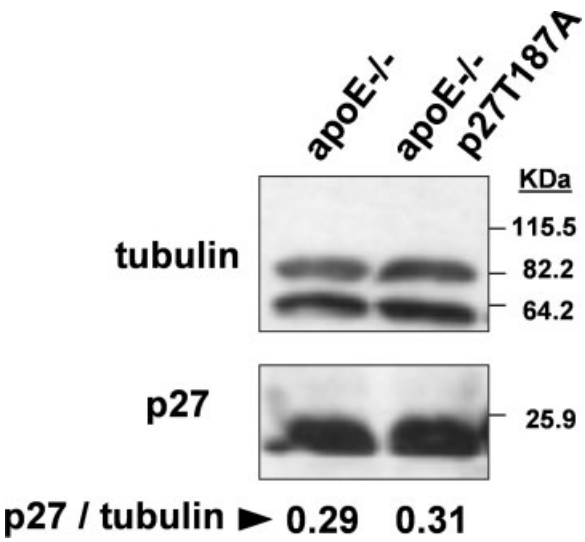

Fig. 2. Loss of p27 phosphorylation on T187 does not affect aortic p27 expression in fat-fed apoE-null mice. For each genotype, Western blot analysis was performed on aortic tissue (aortic arch and thoracic aorta) pooled from two 7-month-old males fed the atherogenic diet for 4 weeks. The intensity of p27 protein and tubulin was determined by densitometry using the Multi Gauge V2.01 software to determine the p27/tubulin ratio.

DISCUSSION

Given that genetic inactivation of p27 leads to augmented arterial cell proliferation and accelerates atherosclerosis in fat-fed apoE−/− mice [Díez-Juan and Andrés, 2001; Díez-Juan et al., 2004], and that ectopic expression of p27T187A leads to p27 stabilization [Morisaki et al., 1997; Malek et al., 2001; Park et al., 2001; Hurteau et al., 2002], we speculated that apoE−/−p27T187A mice would accumulate p27 within the artery wall and display reduced atherosclerosis. However, our studies reveal that both parameters are undistinguishable in fat-fed apoE−/−p27T187A mice compared with apoE−/− counterparts with an intact *p27* gene. Likewise, analysis of atherosclerotic lesions showed that loss of p27 phosphorylation on T187 affects neither cellularity nor the rate of cell proliferation and apoptosis. Thus, physiological level of p27T187A does not affect the course of atherosclerosis in hypercholesterolemic mice. Of note in this regard, Park et al. [2001] have shown that adenovirus-mediated overexpression of a more stable p27T187M/P188I mutant protein in cancer cells induced stronger G1-S arrest and apoptosis than did wild-type p27. Moreover, these authors showed that

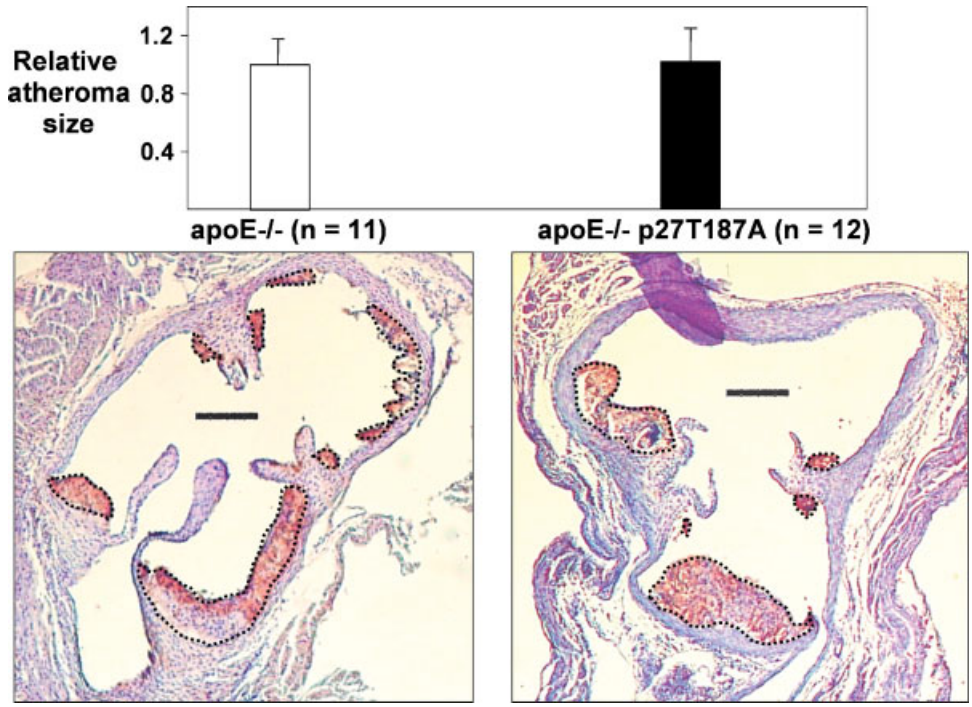

Fig. 3. Loss of p27 phosphorylation on T187 does not affect atherosclerosis in fat-fed apoE-null mice. Animals received the atherogenic diet for 4 weeks. Gender distribution was three females/eight males for apoE−/− (white bars) and five females/seven males for apoE−/−p27T187A (black bars). Atheroma size was quantified in the aortic valves by computerized planimetry of cross-sections immunostained for macrophage-specific Mac3 protein and counterstained with hematoxylin. The photomicrographs show a representative example for each genotype. The discontinuous line marks the boundaries of atherosclerotic lesions and the bar represents 0.2 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

intratumoral injection of adenovirus carrying p27T187M/P188I induced partial regression of established tumors and inhibited the growth of human lung cancer xenografts more strongly than adenovirus encoding for wild-type p27. Whether supraphysiological level of p27T187A or p27T187M/P188I might be effective at inhibiting diet-induced atherosclerosis remains to be investigated.

Previous studies with MEFs isolated from p27T187A mice have shown that p27 is degraded by at least two different proteolytic pathways, which act subsequently during the G1 phase [Malek et al., 2001]. The second pathway, which operates at the G1/S transition, is blocked in p27T187A MEFs resulting in the accumulation of p27T187A to high levels during the remaining phases of the cell cycle. However, it is becoming increasingly evident that the consequences of expressing p27T187A diverge in different cell types, tissues, and pathophysiological conditions. In vitro, DNA replication in stimulated splenic $CD4^+$ T-lymphocytes expressing p27T187A was reduced by 80% compared with control T-cells, however, expression of p27T187A in MEFs caused only a 20–30% reduction in serum-dependent S-phase entry and had no effect on thymocytes [Malek et al., 2001]. In vivo, closure of circular punch wounds by re-growth of epithelial cells was delayed in p27T187A mice, and this correlated with reduced BrdU incorporation in keratinocytes at the wound edge (BrdU positive cells in p27T187A = 13.5% vs. control = 35%); however, the healing of incision wounds, which occurs mostly by epithelial cell migration rather than by proliferation, was similar in p27T187A and control mice [Malek et al., 2001]. On the other hand, induction of liver re-generation following partial hepatectomy in p27T187A mice was not accompanied by perturbations in the onset and duration of S phase in hepatocytes, but caused a delay in the passage through G2 compared with control hepatocytes [Kossatz et al., 2004]. However, in spite of this delayed duration of G2, p27T187A hepatocytes did not arrest in mitosis nor did they display changes in ploidy or cell size.

In summary, our results show that aortic expression of p27 and atherosclerosis in

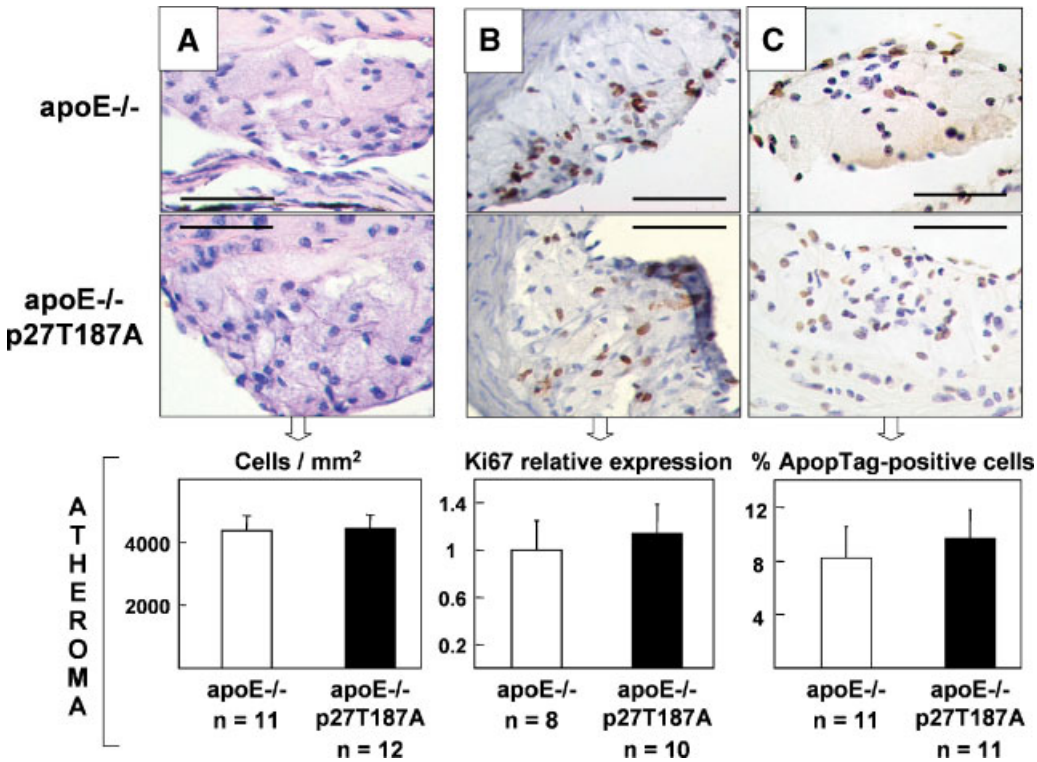

Fig. 4. Loss of p27 phosphorylation on T187 does not affect cellularity, proliferation, and apoptotic rates within the atheroma of fat-fed apoE-null mice. Animals received the atherogenic diet for 4 weeks and cross-sections of the aortic valves region were examined. The top photomicrographs show representative examples (bars represent 50 μm) and the graphs show the average in each group of mice. **A**: Cellularity was quantified in sections stained with hematoxylin/eosin. Gender distribution was three females/eight males for apoE−/− and five females/seven males for apoE−/−p27T187A. **B**: Proliferation was estimated by immunostaining for Ki67. Gender distribution was two females/six males for apoE−/− and four females/eight males for apoE−/−p27T187A. **C**: Apoptosis was determined as the percentage of ApopTag-positive cells. Gender distribution was three females/eight males for apoE−/− and five females/six males for apoE−/−p27T187A. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hypercholesterolemic mice is not regulated by phosphorylation of p27 on T187A, thus, further supporting the notion that the relevance of this phosphorylation event on the level of p27 protein expression and cell cycle regulation varies in different cell types, tissues, and pathophysiological conditions in vitro and in vivo. It remains to be addressed whether alternative mechanisms of p27 degradation may operate to compensate for the loss of phosphorylation on T187 in apoE−/−p27T187A mice. Indeed, it has been suggested that KPC and Spk2 may be functionally redundant [Nakayama et al., 2000]. Given that p27S10A knock-in mice have also revealed striking tissue-specific differences in the level of p27 protein expression (i.e., reduced p27 in the brain, thymus, spleen, testis, and unchanged level in liver, heart, lung, and skeletal muscle) [Kotake et al., 2004], future studies are warranted to analyze the kinetics of atherosclerosis in mice harboring the p27S10A mutation and in double p27S10A-T187A mutants.

ACKNOWLEDGMENTS

We thank J. Roberts for providing p27T187A mice, and M. J. Andrés-Manzano for help with the preparation of figures. Work in the laboratory of V.A. is supported in part by grants from Instituto de Salud Carlos III (Red de Centros RECAVA, C03/01), from Regional Government of Valencia (GV04B-288), and from Spanish Ministry of Education and Science and Fondo Europeo de Desarrollo Regional (SAF2004-03057). S.M.S.-G. is the recipient of a BEFI Predoctoral fellowship from the Spanish Ministry of Health.

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