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Rapamycin attenuates atherosclerosis induced by dietary cholesterol in apolipoprotein-deficient mice through a $p27^{Kip1}$-independent pathway

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Abstract

Activation of immune cells and dysregulated growth and motility of vascular smooth muscle cells contribute to neointimal lesion development during the pathogenesis of vascular obstructive disease. Inhibition of these processes by the immunosuppressant rapamycin is associated with reduced neointimal thickening in the setting of balloon angioplasty and chronic graft vessel disease (CGVD). In this study, we show that rapamycin elicits a marked reduction of aortic atherosclerosis in apolipoprotein E (apoE)-null mice fed a high-fat diet despite sustained hypercholesterolemia. This inhibitory effect of rapamycin coincided with diminished aortic expression of the positive cell cycle regulatory proteins proliferating cell nuclear antigen and cyclin-dependent kinase 2. Moreover, rapamycin prevented the normal upregulation of the proatherogenic monocyte chemoattractant protein-1 (MCP-1, CCL2) seen in the aorta of fat-fed mice. Previous studies have implicated the growth suppressor $p27^{Kip1}$ in the antiproliferative and antimigratory activities of rapamycin in vitro. However, our studies with fat-fed mice doubly deficient for $p27^{Kip1}$ and apoE disclosed an antiatherogenic effect of rapamycin comparable with that found in apoE-null mice with an intact $p27^{Kip1}$ gene. Taken together, these findings extend the therapeutic application of rapamycin from the restenosis and CGVD models to the setting of diet-induced atherosclerosis. Our results suggest that rapamycin-dependent atheroprotection occurs through a $p27^{Kip1}$-independent pathway that involves reduced expression of positive cell cycle regulators and MCP-1 within the arterial wall.

Keywords: Rapamycin; Atherosclerosis; $p27^{Kip1}$; MCP-1/CCL2

1. Introduction

Atherosclerosis and associated cardiovascular disease (e.g. myocardial infarction and stroke) are the major causes of mortality and morbidity in industrialized countries. Neointimal thickening is initiated by transendothelial migration and activation of circulating monocytes and lymphocytes at the sites of vessel injury [1,2]. Recruited leukocytes release inflammatory chemokines and cytokines that promote vascular smooth muscle cell (VSMC) proliferation and migration towards the atherosclerotic lesion, thus further contributing to neointimal hyperplasia [1–4]. It has become increasingly evident that both adaptive and innate immune mechanisms modulate the inflammatory response induced in atherosclerosis, restenosis after angioplasty, and chronic graft vessel disease (CGVD) [1,5,6].

Rapamycin (Rapamune, Sirolimus), a macrolide antibiotic produced by *Streptomyces hygroscopicus* [7], has potent immunosuppressive, antiproliferative, and antimigratory properties (reviewed in [8,9]). Rapamycin exerts these effects by binding to the cytosolic immunophilin FKBP-12 (FK506 binding protein), thus inhibiting the kinase activity of the mammalian target of rapamycin (mTOR). Proposed mechanisms of rapamycin action include dephosphorylation

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and inactivation of p70 ribosomal protein S6 kinase ($p70^{s6k}$) and eukaryotic translation initiation factor 4E-binding protein, accumulation of the growth suppressor $p27^{Kip1}$, inhibition of cyclin-dependent kinase (CDK) activity, accumulation of hypophosphorylated retinoblastoma protein, and inhibition of minichromosome maintenance protein expression [9–21].

By virtue of its potent immunosuppressive activities, rapamycin has been introduced in clinic as a new effective drug for the prevention of allograft rejection [22–24]. Moreover, several animal studies have shown the efficacy of rapamycin in reducing neointimal hyperplasia, both in vessel and cardiac allografts [25–29] and in response to mechanical denudation of the vessel wall [18,26,27,30–33]. These animal studies have led to clinical trials with rapamycin-eluting stents, which have shown a significant reduction in binary restenosis, late lumen loss and repeat revascularization rates as compared with standard coronary stents [34–37].

Cell cycle progression in mammals requires the sequential assembly and activation of different CDK/cyclin holoenzymes at specific phases of the cell cycle [38]. VSMC proliferation in balloon-injured arteries is associated with a temporally and spatially coordinated expression of CDKs and cyclins [20,39]. Importantly, augmented expression of these factors coincides with increased CDK activity [39,40], demonstrating the assembly of functional CDK/cyclin holoenzymes within the injured arterial wall. Moreover, CDK2 and cyclin E expression has been detected in human VSMCs within atherosclerotic and restenotic tissue [39,41,42], suggesting that increased expression (and possibly activation) of positive regulators of cell cycle progression is a characteristic of vascular proliferative disease in humans. CDK activity is negatively regulated by the interaction with specific CDK inhibitory proteins (CKIs) [43]. It has been suggested that the CKI $p27^{Kip1}$ functions as a negative regulator of neointimal thickening during atherosclerosis and at late phases of arterial healing after balloon angioplasty [42,44–48], at least in part via the coordinated suppression of cell proliferation and migration [49]. Exposure of cultured VSMCs and T lymphocytes to rapamycin potently impairs their growth and migratory capacities, and these inhibitory effects correlate with $p27^{Kip1}$ accumulation in vitro and in vivo [10,12,14,15,17,18,46,50]. However, both $p27^{Kip1}$-dependent [51,52] and $p27^{Kip1}$-independent [20,33] mechanisms of rapamycin action have been suggested (see Section 4).

In the present study, we assessed the effect of rapamycin on atherogenesis induced by dietary cholesterol in apolipoprotein E (apoE)-null mice, which develop atherosclerotic lesions that resemble those seen in humans [53,54]. We demonstrate the efficacy of rapamycin in inhibiting atherosclerosis in fat-fed apoE-null mice through a $p27^{Kip1}$-independent pathway associated with reduced expression of positive cell cycle regulatory proteins and attenuated monocyte chemoattractant protein-1 (MCP-1) expression within the injured arterial wall.

2. Materials and methods

2.1. Animals

Mice deficient in apoE (C57BL/6J, Taconic M&B) and doubly deficient for $p27^{Kip1}$ and apoE [47] (backcrossed for more than five generations to a C57BL/6J background) were maintained on a low-fat standard diet (2.8% fat, Panlab, Barcelona, Spain) after weaning. At 2 months of age, mice received an atherogenic diet containing 12% fat, 1.25% cholesterol and 0.5% sodium cholate (S8492-S010, Ssniff) (4 and 6 weeks for apoE-$p27^{Kip1}$ doubly deficient and apoE-deficient mice, respectively). Rapamycin (1 and 4 mg/kg of body weight, s.c., q.o.d.) was suspended in a vehicle solution containing 0.2% sodium carboxymethylcellulose/0.25% polysorbate 80. Control mice received vehicle.

2.2. Lipoprotein isolation and quantification of cholesterol

Blood samples were collected from the orbital sinus under anesthesia. Serum very low-density lipoprotein (VLDL) fraction was obtained by sequential-density ultracentrifugation using a fixed-angle rotor. Serum intermediate (IDL)-, low (LDL)-, and high (HDL)-density lipoprotein fractions were obtained by step-gradient ultracentrifugation using a swing-bucket rotor. The concentration of cholesterol in serum and in lipoprotein fractions was determined using an autoanalyzer Cobas Mira (Roche).

2.3. Histomorphometric studies

Fat-fed mice were euthanized and their aortas were perfusion-fixed in situ with 4% paraformaldehyde to quantify the extent of atherosclerosis using computerized morphometry essentially as previously described [47]. Briefly, one set of animals was used to quantify the area of Oil Red O-stained tissue in the aortic arch region (from the aortic root up to approximately 1–2 mm beyond the left subclavian artery). In another group of animals, the heart and the proximal aorta were fixed with 4% paraformaldehyde, specimens were paraffin-embedded and mounted in a Micron microtome. Once the three valve cusps were reached, sections throughout the first ~2 mm of the ascending aorta were discarded. Then, ~25 consecutive sections (5 μm thickness) were taken from 2 to 3 regions of the aortic arch separated by ~60 μm. Three cross-sections from each region were stained with hematoxylin/eosin. Specimens were examined with a Zeiss Axiolab stereomicroscope to quantify by computerized morphometry the intima-to-media ratio (I/M). For each animal, I/M was calculated by averaging all independent values.

2.4. Western blot analysis

Snap-frozen aortic tissue from fat-fed mice was pooled ($n = 4$ each group) for the preparation of whole cell

extracts in ice-cold lysis buffer (50 mmol/l Hepes [pH 7.5], 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l DTT, 0.1 mM orthovanadate, 10 mM β-glicerophosphate, 10 mM sodium fluoride) supplemented with protease inhibitor Complete Mini cocktail (Roche) using an Ultraturrax T25 basic homogenizer (IKA Labortechnik). Fifty micrograms of protein was separated onto 12% SDS-PAGE and transferred to Immobilon P (Millipore). Blots were incubated at room temperature with blocking solution (4% nonfat dry milk in PBS containing 0.1% Tween-20) for 30–40 min, followed by 1 h incubation with the following primary antibodies from Santa Cruz Biotechnology: anti-tubulin (1/200, sc-3035), anti-CDK2 (1/200, sc-163-G), and anti-proliferating cell nuclear antigen (PCNA) (1/200, sc-7907). After extensive washes with 0.1% Tween-20/PBS, the blots were incubated with species-specific secondary antibodies conjugated to horseradish peroxidase. Blots were washed twice with each 0.1% Tween-20/PBS and PBS, and immunocomplexes were detected using the ECL detection system according to the recommendations of the manufacturer (Amersham). Densitometric analysis of the blots was done using the Labimage version 2.6 software.

2.5. Quantitative RT-PCR

Total RNA was obtained from snap-frozen aortic arch tissue using the Ultraspec RNA isolation system (Biotecx). Two micrograms of DNaseI-treated RNA were reverse transcribed with MuLV reverse transcriptase (Roche). Expression of MCP-1 and GAPDH mRNA was quantified by real-time PCR following the manufacturer's instructions (Lightcycler rapid thermal cycler, Roche) using the following primers specific for exon sequences: 5′-CACCAGCAAGATGATCC-3′ (MCP-1-forward); 5′-ATAAAGTTGTAGGTTCTGATCTC-3′ (MCP-1-reverse); 5′-TGGGTGTGAACCACGA-3′ (GAPDH-forward); and 5′-ACAGCTTTCCAGAGGG-3′ (GAPDH-reverse).

2.6. Statistical analysis

Results are reported as mean ± S.E. In experiments with two groups, differences were evaluated using a two-tailed, unpaired *t*-test. Analyses involving more than two groups were done by ANOVA and Fisher's post-hoc test using the Statview software (SAS institute). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Rapamycin attenuates diet-induced atherosclerosis in apoE-null mice

The apoE-deficient mouse [53,54] has become a valuable tool in elucidating molecular pathways implicated in atherosclerosis and in assessing therapeutic strategies against this disease. As expected, apoE-null mice challenged with a high-fat, cholesterol-rich diet for 4 weeks developed severe hypercholesterolemia compared with pre-diet level ($P < 0.0001$) (Fig. 1A). Importantly, total serum cholesterol level in fat-fed mice was not affected by systemic treatment with rapamycin at 1 and 4 mg/kg (RAPA1 and RAPA4, respectively, $P > 0.05$ versus control fat-fed mice) (Fig. 1A). Likewise, the amount of cholesterol associated with discrete lipoprotein fractions of fat-fed mice was unchanged in rapamycin-treated versus untreated animals (Fig. 1B). Thus, rapamycin does not affect lipid profile in fat-fed apoE-null mice.

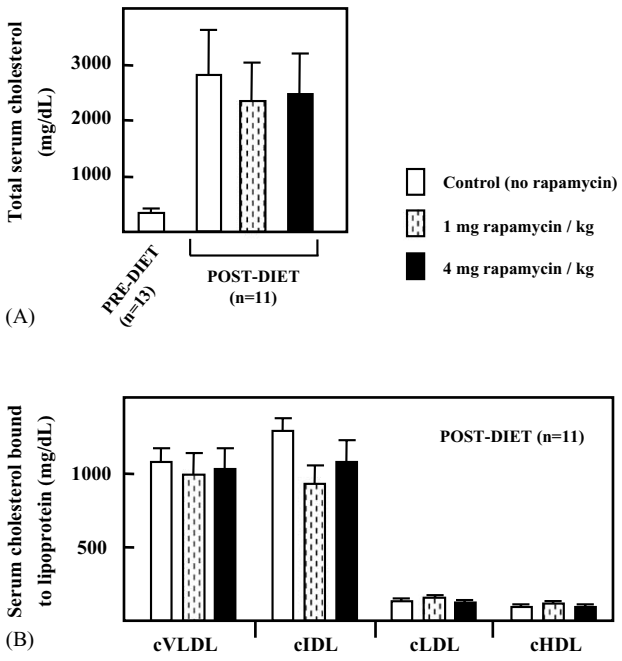

Fig. 1. Rapamycin does not affect lipid profile in fat-fed apoE-null mice. (A) Total serum cholesterol levels were measured in mice fed control chow (pre-diet) or challenged with the atherogenic diet for 6 weeks (post-diet). (B) Cholesterol levels were measured in different lipoprotein fractions isolated from the serum of fat-fed animals. All fat-fed mice displayed similar total cholesterol levels ($P > 0.05$), which were markedly increased compared with pre-diet levels ($P < 0.0001$). Cholesterol content in discrete lipoprotein fractions was also similar in all fat-fed mice ($P > 0.05$ when comparing each lipoprotein fraction among the three groups of mice). Gender distribution in each group of fat-fed mice was six males and five females.

We next examined the extent of diet-induced atherosclerosis in aortic tissue stained with Oil Red O. Consistent with numerous studies in apoE-null mice, atherosclerosis prevailed within the aortic arch in all groups of mice included in our studies (not shown). Thus, we quantified the area of atheroma in the aortic arch region by computerized morphometry using two independent approaches: (1) Oil Red O staining of whole-mounted arteries and (2) quantification of the I/M ratio in arterial cross-sections. As shown in Fig. 2A, both groups of rapamycin-treated mice displayed a significant reduction in the area of Oil Red O-stained atherosclerotic plaques as compared with untreated mice (inhibition

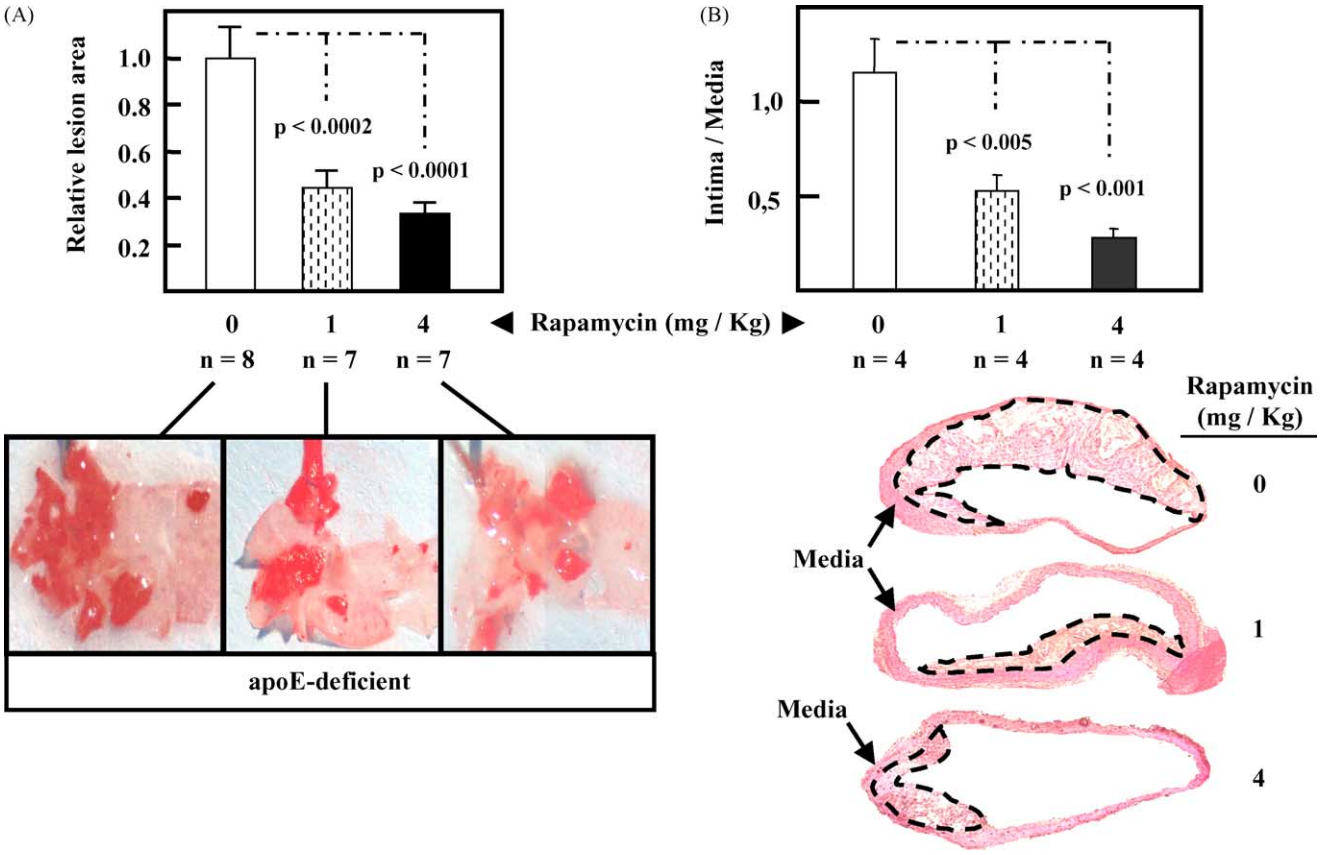

Fig. 2. Rapamycin attenuates diet-induced atherosclerosis. Atheroma development in the aortic arch of apoE-null mice fed the atherogenic diet for 6 weeks was quantified by computerized morphometry. The photomicrographs show representative examples. (A) Arteries were stained with Oil Red O (atherosclerotic lesions are shown in red). Results represent the area of lesion relative to untreated controls (1). Gender distribution was four males/four females (untreated controls), four males/three females (RAPA 1), and three males/four females (RAPA 4). (B) Cross-sections from the aortic arch were stained with hematoxylin and eosin to quantify the average I/M (two males/two females in each group). The edge of the atherosclerotic plaque (intimal lesion) has been drawn with a discontinuous line.

of 56% in RAPA1 and 66% in RAPA4, $P < 0.0002$ and <0.0001 versus control, respectively). Likewise, examination of arterial cross-sections revealed a significant reduction of the I/M in RAPA1 and RAPA4 mice ($P < 0.005$ and <0.001 versus control, respectively) (Fig. 2B). Both studies disclosed a trend towards more protection in RAPA4 versus RAPA1, although the differences between the groups of rapamycin-treated mice did not reach statistical significance. These findings demonstrate a protective effect of rapamycin against atherosclerosis in apoE-null mice challenged with an atherogenic diet in spite of sustained hypercholesterolemia.

3.2. $p27^{Kip1}$ is not required for rapamycin-dependent inhibition of atherogenesis

Arterial cell proliferation is thought to contribute to atheroma development [1–4]. Because of the well-established antiproliferative action of rapamycin, we next performed Western blot analysis to examine the effect of rapamycin on the expression of positive cell cycle regulators in the aorta of fat-fed apoE-null mice. Two independent sets of mice were analyzed in these studies (experiments 1 and 2, Fig. 3). We

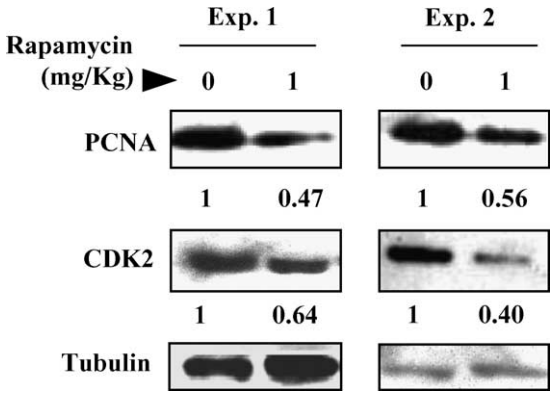

Fig. 3. Effect of rapamycin on aortic expression of cell cycle regulators. Immunoblot analysis of cell lysates prepared from the aorta of control and rapamycin-treated fat-fed apoE-null mice (pool of four arteries in each group). Densitometric analysis was performed and each value was divided by its tubulin loading control. Numbers below the blots indicate the level of expression relative to untreated mice (set as 1). The results of two independent experiments are shown.

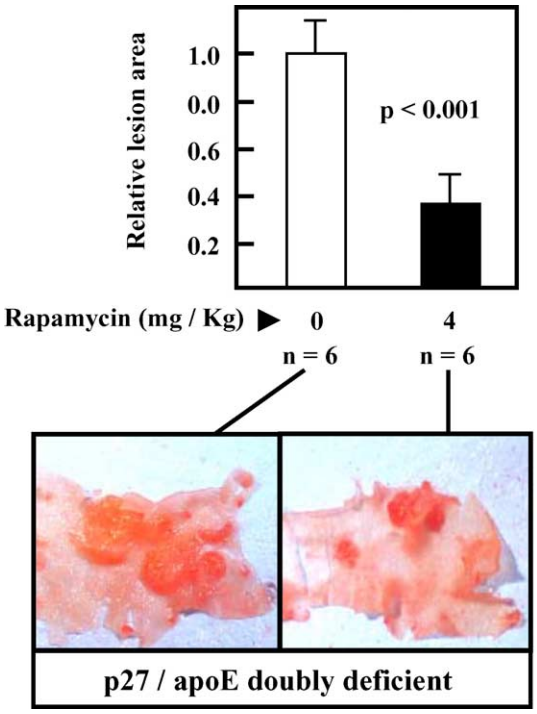

Fig. 4. Genetic disruption of $p27^{Kip1}$ does not impair the atheroprotective effect of rapamycin. Mice doubly deficient for $p27^{Kip1}$ and apoE were fed the atherogenic diet for 4 weeks. Gender distribution in both groups was four males/two females. Atherosclerosis was quantified in Oil Red O-stained arteries (atherosclerotic lesions are shown in red). Results represent the area of lesion relative to untreated controls (1).

found a 50–60% reduction in the expression of the S-phase markers PCNA and CDK2 in rapamycin-treated mice.

The growth suppressor $p27^{Kip1}$ has been implicated in the control of atheroma development [42,45,47,48]. Remarkably, both $p27^{Kip1}$-dependent [51,52] and $p27^{Kip1}$-independent [20,33] mechanisms of rapamycin action have been suggested. Thus, we sought to assess the role of $p27^{Kip1}$ on rapamycin-dependent atheroprotection by examining fat-fed mice doubly deficient for apoE and $p27^{Kip1}$. As shown in Fig. 4, the ability of rapamycin to inhibit atherogenesis in these mice was comparable to that seen in apoE-null mice with an intact $p27^{Kip1}$ gene (63% inhibition, $P < 0.01$ versus control, compare with Fig. 2). These results demonstrate that rapamycin inhibits diet-induced atherosclerosis via a p27-independent mechanism.

3.3. Rapamycin prevents diet-induced aortic MCP-1 upregulation

Chemokines promote the recruitment of immune cells at the sites of vascular injury and the migration of medial VSMC towards the atherosclerotic lesion [1,2]. Gain- and loss-of-function experiments have implicated the chemotactic cytokine MCP-1 and its receptor CCR2 in the development of atherosclerosis [55–60]. Interestingly, rapamycin reportedly inhibits MCP-1 mRNA and protein expression in

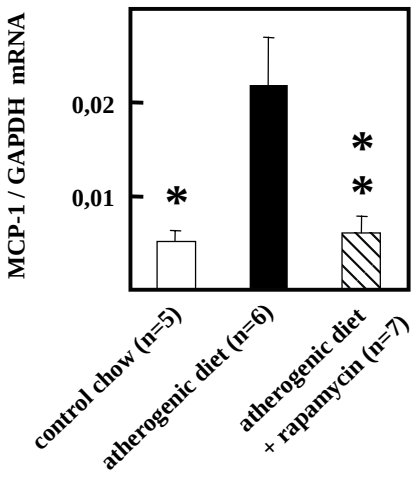

Fig. 5. Rapamycin attenuates diet-induced aortic MCP-1 upregulation. apoE-null mice were fed either control chow or the atherogenic diet for 4 weeks (with or without rapamycin at 1 mg/kg). Total RNA was isolated from the aortic arch for real-time quantitative RT-PCR analysis. The number of animals in each group is indicated (*n*). Results are given as the ratio of MCP-1/GAPDH. Comparisons vs. atherogenic diet (no rapamycin): $^{*}P < 0.005$; $^{**}P < 0.006$.

animal models of cardiac [61] and kidney [62] transplantation. Thus, we examined the effect of rapamycin on aortic MCP-1 expression by quantitative RT-PCR analysis of RNA isolated form the aortic arch of apoE-null mice fed control chow or the atherogenic diet. As shown in Fig. 5, rapamycin blocked diet-induced upregulation of MCP-1 compared with untreated fat-fed mice.

4. Discussion

Activation of immune cells and excessive cellular proliferation and migration within the arterial wall are thought to contribute to neointimal thickening in both experimental animals and humans [1–6]. Rapamycin's immunosuppressive, antiproliferative and antimigratory actions are associated with attenuated neointimal thickening in several animal models of alloimmune and mechanical injury [18,25–33]. Moreover, rapamycin has shown promising results in reducing human coronary in-stent restenosis [34–37]. In this study, we examined the effect of rapamycin on the vessel wall response to dietary cholesterol in the apoE-deficient mouse model of atherosclerosis. We found a significant rapamycin-dependent reduction in the severity of aortic atherosclerosis in spite of sustained hypercholesterolemia compared with untreated controls. This atheroprotective effect of rapamycin coincided with reduced aortic expression of the positive cell cycle regulatory proteins CDK2 and PCNA, consistent with previous studies in the rat carotid artery model of balloon angioplasty [20].

Rapamycin induces $p27^{Kip1}$ accumulation in vitro and in vivo [12,15,17,18,46], suggesting that $p27^{Kip1}$ may mediate

the inhibitory effects of this drug. Consistent with this notion, $p27^{Kip1}$ inactivation impairs rapamycin-mediated growth arrest in fibroblasts and T lymphocytes [51], and migration inhibitory responses in VSMCs [52]. However, evidence of $p27^{Kip1}$-independent mechanisms of rapamycin action have also been provided. First, rapamycin efficiently impaired the growth of $p27^{Kip1}$-null VSMCs in vitro [33]. Second, rapamycin failed to prevent the in vivo down-regulation of $p27^{Kip1}$ seen 24 h after balloon injury of rat carotid arteries [20]. Third, attenuation of neointimal thickening after mechanical injury was similar in wild-type and $p27^{Kip1}$-null mice treated with rapamycin [33]. Although we can not rule out that aortic $p27^{Kip1}$ expression may be induced by rapamycin in the present study, we found that the atheroprotective action elicited by this drug was not impaired in fat-fed mice doubly deficient for apoE and $p27^{Kip1}$ versus apoE-null mice with an intact $p27^{Kip1}$ gene (compare Figs. 2 and 4). Thus, $p27^{Kip1}$ is not essential for the therapeutic effect of rapamycin against neointimal thickening induced by both dietary cholesterol and balloon angioplasty.

Human and animal studies suggest that local production of chemokines within the atherosclerotic plaque plays a critical role in atherogenesis [1,2]. Accumulating evidence has implicated MCP-1 as a proatherogenic factor [58]. For instance, several cell types involved in atheroma formation (i.e. endothelial cells, VSMCs, and macrophages) display abundant expression of MCP-1 and its receptor CCR2 [58], and high level of MCP-1 expression has been observed within the atherosclerotic plaque in both experimental animals and humans [63–65]. Importantly, genetic inactivation of MCP-1 or CCR2 [55–57] and anti-MCP-1 gene therapy [59] reduce murine atherosclerosis. In marked contrast, local infusion of MCP-1 protein increases plaque formation in apoE-null mice [60]. We found that rapamycin abrogates the upregulation of MCP-1 mRNA expression normally seen in the aortic arch of fat-fed apoE-null mice, consistent with recent studies demonstrating rapamycin-dependent inhibition of MCP-1 expression in animal models of cardiac [61] and kidney [62] allografts.

In conclusion, the present study extends previous reports documenting the therapeutic efficacy of rapamycin against neointimal thickening in the setting of CGVD [25–29] and balloon angioplasty [18,26,27,30–37] by demonstrating rapamycin-dependent reduction of atherosclerosis in apoE-null mice challenged with a high-fat, cholesterol-rich diet. Rapamycin's atheroprotective effects occur through a $p27^{Kip1}$-independent pathway that coincides with reduced arterial expression of both positive cell cycle regulatory factors (i.e. CDK2 and PCNA) and proatherogenic MCP-1. Because of this novel therapeutic application of rapamycin, gene expression profiling and proteomic studies comparing untreated and rapamycin-treated fat-fed animals are warranted to identify potential therapeutic targets for the prevention and/or treatment of atherosclerosis.

Note added in proofs

While this manuscript was under review, Elloso et al. reported the protective effect of rapamycin against atheroma development in apoE-null mice (Elloso MM, Azrolan N, Sehgal SN, Hsu PL, Phiel KL, Kopec CA, Basso MD, Adelman SJ. Protective effect of the immunosuppressant sirolimus against aortic atherosclerosis in apo E-deficient mice. Am J Transplant 2003;3:562–9).

Acknowledgements

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References

[1] Ross R. Atherosclerosis: an inflammatory disease. N Engl J Med 1999;340:115–26.
[2] Lusis AJ. Atherosclerosis. Nature 2000;407:233–41.
[3] Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med 2002;8:1249–56.
[4] Andrés V, Castro C. Antiproliferative strategies for the treatment of vascular proliferative disease. Curr Vasc Pharmacol 2003;1:85–98.
[5] Binder CJ, Chang MK, Shaw PX, et al. Innate and acquired immunity in atherogenesis. Nat Med 2002;8:1218–26.
[6] Greaves DR, Channon KM. Inflammation and immune responses in atherosclerosis. Trends Immunol 2002;23:535–41.
[7] Sehgal SN, Baker H, Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J Antibiot (Tokyo) 1975;28:727–32.
[8] Sehgal SN. Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem 1998;31:335–40.
[9] Marx SO, Marks AR. Bench to bedside: the development of rapamycin and its application to stent restenosis. Circulation 2001;104:852–5.
[10] Morice WG, Brunn GJ, Wiederrecht G, Siekierka JJ, Abraham RT. Rapamycin-induced inhibition of p34cdc2 kinase activation is associated with G1/S-phase growth arrest in T lymphocytes. J Biol Chem 1993;268:3734–8.
[11] Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell 1994;78:35–43.
[12] Nourse J, Firpo E, Flanagan WM, et al. Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature 1994;372:570–3.
[13] Brown EJ, Albers MW, Shin TB, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 1994;369:756–8.
[14] Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. Circ Res 1995;76:412–7.

[15] Dumont FJ, Su Q. Mechanism of action of the immunosuppressant rapamycin. Life Sci 1996;58:373–95.
[16] Hara K, Yonezawa K, Kozlowski MT, et al. Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem 1997;272:26457–63.
[17] Kawamata S, Sakaida H, Hori T, Maeda M, Uchiyama T. The up-regulation of p27Kip1 by rapamycin results in G1 arrest in exponentially growing T-cell lines. Blood 1998;91:561–9.
[18] Gallo R, Padurean A, Jayaraman T, et al. Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. Circulation 1999;99:2164–70.
[19] Isotani S, Hara K, Tokunaga C, Inoue H, Avruch J, Yonezawa K. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. J Biol Chem 1999;274:34493–8.
[20] Braun-Dullaeus RC, Mann MJ, Seay U, et al. Cell cycle protein expression in vascular smooth muscle cells in vitro and in vivo is regulated through phosphatidylinositol 3-kinase and mammalian target of rapamycin. Arterioscler Thromb Vasc Biol 2001;21:1152–8.
[21] Bruemmer D, Yin F, Liu J, et al. Rapamycin inhibits E2F-dependent expression of minichromosome maintenance proteins in vascular smooth muscle cells. Biochem Biophys Res Commun 2003;303:251–8.
[22] Sacks SH. Rapamycin on trial. Nephrol Dial Transplant 1999;14:2087–9.
[23] Kahan BD. Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomised multicentre study. The Rapamune US Study Group. Lancet 2000;356:194–202.
[24] Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230–8.
[25] Meiser BM, Billingham ME, Morris RE. Effects of cyclosporin, FK506, and rapamycin on graft-vessel disease. Lancet 1991;338:1297–8.
[26] Gregory CR, Huie P, Billingham ME, Morris RE. Rapamycin inhibits arterial intimal thickening caused by both alloimmune and mechanical injury. Its effect on cellular, growth factor, and cytokine response in injured vessels. Transplantation 1993;55:1409–18.
[27] Morris RE, Cao W, Huang X, et al. Rapamycin (Sirolimus) inhibits vascular smooth muscle DNA synthesis in vitro and suppresses narrowing in arterial allografts and in balloon-injured carotid arteries: evidence that rapamycin antagonizes growth factor action on immune and nonimmune cells. Transplant Proc 1995;27:430–1.
[28] Poston RS, Billingham M, Hoyt EG, et al. Rapamycin reverses chronic graft vascular disease in a novel cardiac allograft model. Circulation 1999;100:67–74.
[29] Ikonen TS, Gummert JF, Hayase M, et al. Sirolimus (rapamycin) halts and reverses progression of allograft vascular disease in non-human primates. Transplantation 2000;70:969–75.
[30] Gregory CR, Huang X, Pratt RE, et al. Treatment with rapamycin and mycophenolic acid reduces arterial intimal thickening produced by mechanical injury and allows endothelial replacement. Transplantation 1995;59:655–61.
[31] Burke SE, Lubbers NL, Chen YW, et al. Neointimal formation after balloon-induced vascular injury in Yucatan minipigs is reduced by oral rapamycin. J Cardiovasc Pharmacol 1999;33:829–35.
[32] Suzuki T, Kopia G, Hayashi S, et al. Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. Circulation 2001;104:1188–93.
[33] Roque M, Reis ED, Cordon-Cardo C, et al. Effect of p27 deficiency and rapamycin on intimal hyperplasia: in vivo and in vitro studies using a p27 knockout mouse model. Lab Invest 2001;81:895–903.
[34] Sousa JE, Costa MA, Abizaid AC, et al. Sustained suppression of neointimal proliferation by sirolimus-eluting stents: one-year angiographic and intravascular ultrasound follow-up. Circulation 2001;104:2007–11.
[35] Serruys PW, Degertekin M, Tanabe K, et al. Intravascular ultrasound findings in the multicenter, randomized, double-blind RAVEL (randomized study with the sirolimus-eluting velocity balloon-expandable stent in the treatment of patients with de novo native coronary artery lesions) trial. Circulation 2002;106:798–803.
[36] Regar E, Serruys PW, Bode C, et al. Angiographic findings of the multicenter randomized study with the sirolimus-eluting Bx velocity balloon-expandable stent (RAVEL): sirolimus-eluting stents inhibit restenosis irrespective of the vessel size. Circulation 2002;106:1949–56.
[37] Morice MC, Serruys PW, Sousa JE, et al. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. N Engl J Med 2002;346:1773–80.
[38] Nurse P. Ordering S phase and M phase in the cell cycle. Cell 1994;79:547–50.
[39] Wei GL, Krasinski K, Kearney M, Isner JM, Walsh K, Andrés V. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. Circ Res 1997;80:418–26.
[40] Abe J, Zhou W, Taguchi J, et al. Suppression of neointimal smooth muscle cell accumulation in vivo by antisense cdc2 and cdk2 oligonucleotides in rat carotid artery. Biochem Biophys Res Commun 1994;198:16–24.
[41] Kearney M, Pieczek A, Haley L, et al. Histopathology of in-stent restenosis in patients with peripheral artery disease. Circulation 1997;95:1998–2002.
[42] Ihling C, Technau K, Gross V, Schulte-Monting J, Zeiher AM, Schaefer HE. Concordant upregulation of type II-TGF-beta-receptor, the cyclin-dependent kinases inhibitor p27Kip1 and cyclin E in human atherosclerotic tissue: implications for lesion cellularity. Atherosclerosis 1999;144:7–14.
[43] Vidal A, Koff A. Cell-cycle inhibitors: three families united by a common cause. Gene 2000;247:1–15.
[44] Chen D, Krasinski K, Sylvester A, Chen J, Nisen PD, Andrés V. Downregulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27Kip1, an inhibitor of neointima formation in the rat carotid artery. J Clin Invest 1997;99:2334–41.
[45] Tanner FC, Yang Z-Y, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. Circ Res 1998;82:396–403.
[46] Roque M, Cordon-Cardo C, Fuster V, Reis ED, Drobnjak M, Badimon JJ. Modulation of apoptosis, proliferation, and p27 expression in a porcine coronary angioplasty model. Atherosclerosis 2000;153:315–22.
[47] Diez-Juan A, Andres V. The growth suppressor p27Kip1 protects against diet-induced atherosclerosis. FASEB J 2001;15:1989–95.
[48] Díez-Juan A, Castro C, Edo MD, Andrés V. Role of the growth suppressor p27Kip1 during vascular remodeling. Curr Vasc Pharmacol 2003;1:99–106.
[49] Diez-Juan A, Andres V. Coordinate control of proliferation and migration by the p27Kip1/cyclin-dependent kinase/retinoblastoma pathway in vascular smooth muscle cells and fibroblasts. Circ Res 2003;92:402–10.
[50] Poon M, Marx SO, Gallo R, Badimon JJ, Taubman MB, Marks AR. Rapamycin inhibits vascular smooth muscle cell migration. J Clin Invest 1996;98:2277–83.
[51] Luo Y, Marx SO, Kiyokawa H, Koff A, Massague J, Marks AR. Rapamycin resistance tied to defective regulation of p27Kip1. Mol Cell Biol 1996;16:6744–51.
[52] Sun J, Marx SO, Chen HJ, Poon M, Marks AR, Rabbani LE. Role for p27Kip1 in vascular smooth muscle cell migration. Circulation 2001;103:2967–72.
[53] Plump AS, Smith JD, Hayek T, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 1992;71:343–53.
[54] Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 1992;258:468–71.

[55] Gu L, Okada Y, Clinton SK, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998;2:275–81.
[56] Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2−/− mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 1998;394:894–7.
[57] Gosling J, Slaymaker S, Gu L, et al. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J Clin Invest 1999;103:773–8.
[58] Peters W, Charo IF. Involvement of chemokine receptor 2 and its ligand, monocyte chemoattractant protein-1, in the development of atherosclerosis: lessons from knockout mice. Curr Opin Lipidol 2001;12:175–80.
[59] Inoue S, Egashira K, Ni W, et al. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. Circulation 2002;106:2700–6.
[60] van Royen N, Hoefer I, Bottinger M, et al. Local monocyte chemoattractant protein-1 therapy increases collateral artery formation in apolipoprotein E-deficient mice but induces systemic monocytic CD11b expression, neointimal formation, and plaque progression. Circ Res 2003;92:218–25.
[61] Wasowska BA, Zheng XX, Strom TB, Kupieck-Weglinski JW. Adjunctive rapamycin and CsA treatment inhibits monocyte/macrophage associated cytokines/chemokines in sensitized cardiac graft recipients. Transplantation 2001;71:1179–83.
[62] Oliveira JG, Xavier P, Sampaio SM, et al. Compared to mycophenolate mofetil, rapamycin induces significant changes on growth factors and growth factor receptors in the early days post-kidney transplantation. Transplantation 2002;73:915–20.
[63] Nelken NA, Coughlin SR, Gordon D, Wilcox JN. Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 1991;88:1121–7.
[64] Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci USA 1991;88:5252–6.
[65] Takeya M, Yoshimura T, Leonard EJ, Takahashi K. Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody. Hum Pathol 1993;24:534–9.